ADVANCED METHODS IN ELECTROPHYSIOLOGY

Recording from *Xenopus* Oocytes

The *Xenopus* oocyte expression system was introduced by Gurdon in 1971 as a means to study various aspects of the control of gene expression. Injection of exogenous DNA into the oocyte nucleus or mRNA into the cytoplasm led to the expression of functional proteins by the oocytes. Early studies dealt with the expression of proteins that are of little interest to electrophysiologists, such as globin, interferon and various viral proteins. Consequently, electrophysiologists and biophysicists paid scant attention to studies of protein expression in oocytes. However, beginning in 1982, Miledi and co-workers demonstrated that various types of ion channels and receptors could also be expressed in oocytes after injection of mRNAs that had been isolated from the appropriate tissue. For example, injection of *Torpedo* electric organ mRNA led to the expression of functional nicotinic acetylcholine receptors, while injection of rat brain mRNA resulted in the expression of a large number of different voltage- and ligand-gated ion channels, among them the voltage-gated Na^+ channels, NMDA and non-NMDA subtypes of glutamate receptors, and $GABA_A$ receptors.

Since these initial experiments, a number of workers have adopted this expression system to study various aspects of the structure and function of ion channels and receptors. Some of the more common types of studies that have employed expression of exogenous ion channels and receptors in oocytes include: (1) analyzing the properties of mutated channels as a means to understand structure-function relationships in ion channels; (2) studying the post-translational processing and the assembly of multisubunit channels and receptors; (3) comparing the properties of channels from various tissues expressed in a common environment; (4) examining the modulation of channel and receptor function by various second messenger systems; (5) analyzing various aspects of receptor-effector coupling; and (6) functional screening of cloned genes that encode channels and receptors (expression cloning).

Most of these experiments involve electrophysiological recording from oocytes. This chapter describes the mechanics of recording from oocytes and points out some oocyte features of which one should be aware in order to obtain high-quality recordings.

What is a Xenopus *Oocyte?*

Xenopus oocytes are egg precursors that, upon proper hormonal stimulation, pass through the frog's oviduct and become eggs, which can then be fertilized to make more frogs. Oocytes are stored in the abdominal cavity and can be removed surgically for experimental purposes. They go through six developmental stages (termed stages I to VI). Most researchers use only the large stage V and VI oocytes, which can be used interchangeably in electrophysiological experiments. Oocytes are found in clumps called ovarian lobes, which are made up of oocytes, connective tissue, blood vessels and follicle cells. Figure 5-1 shows an individual stage V or VI oocyte as found in an ovarian lobe.

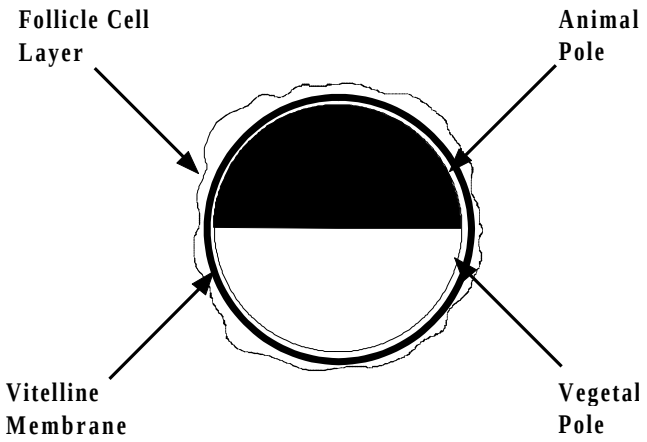

Figure 5-1. Stage V or VI Oocytes as Found in an Ovarian Lobe

The oocyte is a large cell, with a diameter of approximately 1-1.2 mm. It is surrounded by the vitelline membrane, which is a glycoprotein matrix that gives the oocyte some structural rigidity and helps it maintain a spherical shape. The mesh formed by this matrix is rather large; consequently, small molecules and even small proteins such as α-bungarotoxin (molecular weight 8,000 dalton) can interact with proteins on the oocyte surface. Since devitellinized oocytes are extremely fragile, the vitelline membrane is usually removed only for single-channel recording, where the surface of the oocyte must be sufficiently clean to allow the formation of a gigohm seal. A layer of follicle cells surrounds the vitelline membrane. The individual cells in the follicle cell layer are electrically coupled to each other and to the oocyte through gap junctions, so that electrical events taking place in the follicle cells can be detected in the oocyte.

The follicle cell layer is a potential source for many problems. Therefore, most researchers remove it prior to mRNA injection or recording by one of two ways: (1) an extensive collagenase treatment to completely strip off the layer by immersing the oocyte for 1-3 hours in a calcium-free saline containing 2 mg/ml collagenase Type IA (Sigma Chemical Company, St. Louis, MO) until about half of the oocytes have been released from the ovarian lobe; or (2) a less extensive collagenase treatment followed by manual removal of the follicular layer with watchmaker's forceps. The latter is preferred, since oocytes that undergo the extensive collagenase treatment sometimes do not survive as long as those treated less extensively. Some of the problems introduced by the follicle cell layer are rather trivial. For instance, it is more difficult to impale a follicle-encased oocyte with an injection needle or a microelectrode. Other complications, however, can create major problems. The electrical coupling between the oocyte proper and the follicle cells means that one records from both. Numerous examples of oocyte

"endogenous" receptors that turned out to be in the follicle cell layer were published. In fact, the *Xenopus* oocyte is a nearly ideal expression system for ion channels and receptors since it has very few types of endogenous channels and receptors, an advantage that is defeated if the follicle cells are left on the oocyte.

One of the striking features of a *Xenopus* oocyte is its two-toned color scheme: the animal pole hemisphere is dark colored, while the vegetal pole is light colored. This polarity is maintained inside the oocyte: the nucleus is found in the animal pole, and different populations of mRNAs exist in the hemispheres. In addition, a standing Cl^- current flows from one pole to the other, indicating that the distribution of channels in the oocyte membrane is not homogeneous. Published reports showed that ACh receptors expressed from exogenous mRNAs are also unevenly distributed; although receptors were expressed all over the oocyte, more receptors were expressed on the vegetal pole. It is not clear if there is any relation between the site of RNA injection and the location of the expressed channels on the oocyte membrane. This non-homogeneity is not important for whole-cell recording, but could be important for single-channel recording.

Two-Electrode Voltage Clamping of Oocytes

Two factors need to be taken into consideration when voltage clamping oocytes. First, with an apparent surface area on the order of 10^6 μm^2, there is an enormous amount of membrane that must be charged in order to clamp the cell. In fact, the situation is even worse than it may appear since there is considerable invagination of the surface membrane, thereby doubling or even tripling the surface area compared to an "ideal" spherical oocyte. Second, one can encounter currents up to 10 µA or larger after mRNA injection, potentially causing appreciable series resistance errors. In the case of currents from cloned K^+ and Na^+ channels, which activate rapidly and can give rise to very large currents, there may be an inadequate control of the voltage during the initial phases of channel activation.

The response time (τ) of a voltage clamp to a step voltage change is,

$$\tau = \frac{R_I C_m}{A} \qquad (1)$$

where R_I is the resistance of the current-passing electrode, C_m is the membrane capacitance, and A is the gain of the command amplifier. Since not much can be done about C_m (but see below), the only two things one can do in order to achieve fast clamping of the cell is to use the lowest R_I and largest A possible. One "advantage" of the oocyte's large size is that one can use low-resistance electrodes for both the voltage-recording and current-passing electrodes. (This can hardly be regarded as an advantage, since the large size of the oocyte is what caused the problem in the first place). Electrodes that have resistances of 0.5-2 MΩ when filled with 3 M KCl are used. The particular type of glass is unimportant; both regular and fiber-filled electrode glass have been used successfully. Since the electrodes have rather large tip openings, they usually do not clog very often; the same set of electrodes can be used repeatedly during the course of a day. The electrodes' low resistance eliminates the need in a negative-capacitance circuit to correct for the frequency response of the voltage-recording electrode, thereby eliminating this potential source of clamp instability. To achieve the fastest response, the electrodes must be shielded in order to reduce capacitive coupling between them. Two rather simple ways of doing this are (1) placing a grounded sheet of metal between the two electrodes (making sure that it doesn't

make contact with the bath), or (2) wrapping a wire around a piece of tubing that fits over the voltage electrode like a sleeve, and then grounding the wire. In the latter case, the shield should extend as close to the bath as possible.

The other parameter that one can control in order to maximize the speed of the voltage clamp is the command amplifier gain. As with other types of cell-electrode combinations, one must usually introduce a frequency response compensation network to ensure clamp stability at high gains. Therefore, the absolute gain of the clamp and the command voltage of the amplifier are less important than the maximum gain reached before the clamp becomes unstable. The PHASE SHIFT and TIME CONSTANT controls of Axon Instruments' Axoclamp microelectrode amplifier allow one to fine-tune the clamp for operation at full gain. For oocytes, the PHASE SHIFT should be fully counterclockwise (*i.e.*, full lag) and the TIME CONSTANT should be set to 0.02, 0.2 or 2 ms. The lower the setting, the faster the clamp. The STABILITY control of the GeneClamp 500 can be similarly set to operate at full gain.

The goal is to achieve a fast voltage clamp. But, how fast is fast enough? The actual speed required depends on the type of signal being measured. For example, when studying a ligand-activated ion channel that is activated by bath application of an agonist, it makes no difference if the clamp settles in 2 ms or 20 ms, since the exchange time of the chamber is the limiting factor in the rate of activation. On the other hand, when studying voltage-gated channels, one wants the fastest clamp attainable. A well-tuned, two-microelectrode voltage clamp can clamp an oocyte fast enough to study the gating kinetics of most voltage-gated channels. However, it is probably impossible to clamp an oocyte fast enough to study the activation kinetics of fast-gating voltage-gated Na^+ channels in which the rising phase of the current can be on the order of 1 ms or shorter. It is still possible to study slower processes, such as inactivation or pharmacological properties, even if the clamp is not fast enough to study the activation process. A faster clamp can be achieved either by recording from smaller oocytes (stage II or III) whose capacitances are approximately one-fifth those of the larger stage V and VI oocytes, or applying the more common "macropatch" technique. Either of these two approaches provides a sufficiently fast clamp for studying the kinetics of Na^+ channel activation.

Electrode penetration is most easily achieved simply by advancing the electrode into the oocyte until it dimples the membrane and then visibly pops into the cell. Due to the large size of the oocyte, a coarse, inexpensive manipulator, such as the Brinkmann MM-33, provides adequate control of movement. Typical resting potentials in a physiological saline solution (100 mM NaCl, 2 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM HEPES, pH 7.6) are approximately -40 mV or more negative. The voltage clamp is tuned by applying a small (2 mV) square wave at the resting potential and then gradually increasing the gain of the clamp. As the gain increases, the voltage trace "squares up" and the capacitive transient in the current trace gets faster and faster. Instability in the clamp will first appear as small oscillations in the traces; the oscillations will increase as the gain increases. If oscillations do appear, the TIME CONSTANT control should be adjusted until they disappear. This process should be continued until one reaches the highest gain possible without any apparent oscillations. In general, if the electrodes are shielded as described above, full gain can be achieved.

Patch Clamping Xenopus *Oocytes*

While oocytes are obviously too large for the whole-cell mode of patch clamping, they are amenable to all three configurations of single-channel recording, *i.e.*, cell-attached, inside-out

and outside-out. One of the major requirements for achieving the high-resistance seals necessary for single-channel recording is a clean membrane surface. With oocytes, one must remove the vitelline membrane prior to patch clamping by placing the oocyte in a hypertonic stripping solution (usually 200 mM K-aspartate, 20 mM KCl, 1 mM $MgCl_2$, 10 mM EGTA, 10 mM HEPES, pH 7.4) and allowing it to shrink. As the oocyte shrinks, the vitelline membrane detaches from the cell membrane and appears as a transparent sphere around the oocyte. One can then manually remove the vitelline membrane using fine watchmaker's forceps. Since devitellinized oocytes will stick to glass or plastic (but not to agarose) after a few minutes of contact, the shrinking and vitelline membrane removal is usually carried out in small petri dishes with a layer of 2% agarose on the bottom. This allows one to prepare a number of oocytes at once. At this point, the oocytes are extremely fragile. In particular, stripped oocytes have a propensity for disintegrating when they are at an air-water interface; therefore, extreme care must be taken when transferring them to the recording chamber.

Once the vitelline membrane has been removed, gigohm seals can be obtained with high success rates. Oocytes, like most cells, contain stretch-activated channels that can interfere with recording "interesting" channels in the cell-attached mode. Therefore, most researchers prefer to use excised patches for single-channel work, where the incidence of stretch-activated channel currents is much lower. The actual mechanics of obtaining excised patches is the same as for any cell type, with the exception that some researchers prefer to rupture the membrane by applying positive pressure rather than suction, since suction can sometimes cause yolk platelets to clog the pipette tip.

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Patch-Clamp Recording in Brain Slices

Although it is commonly accepted that patch-clamp techniques offer many technical advantages over conventional intracellular microelectrode recording configurations, one nagging limitation has been the belief that, in order to allow formation of gigohm seals with patch electrodes, cells must be treated in some way that leaves their membrane "clean." Such treatment might entail dissociation of tissue by enzymatic digestion or mechanical disruption, and might or might not be followed by growth in tissue culture. These techniques invariably result in significant damage to the cells or severe alteration of their environment, at least transiently, during the preparation of the cells for recording. Alternatively, tissue explants or slices can be maintained in "organotypic" culture, allowing many of the cell-cell interactions to be maintained and mitigating some of the problems described above. However, with any of these approaches, the possibility remains that significant alteration of cellular properties occurs prior to recording.

Therefore, it is of great interest to be able to apply patch-recording techniques to neurons in acute tissue slices. The marriage of these two approaches offers many of the advantages of both, with few of the limitations:

(1) Cells in tissue slices are likely to be much closer to their original state than cells subjected to the above-mentioned treatments. No disruption of the normal cellular environment need take place until the preparation of slices and disruption is limited to the surface of the slice.

(2) The increased signal-to-noise ratio and improved voltage-clamp quality, compared with conventional intracellular recording using sharp microelectrodes, provide substantial benefits for cellular electrophysiology. These benefits are particularly helpful when measuring small or rapid events.

(3) The ability to control the intracellular environment is greatly enhanced by whole-cell recording, thus facilitating the study of interactions between intracellular biochemistry and electrophysiology.

It should be noted however, that not all problems are solved by applying whole-cell techniques. "Washout" of intracellular biochemical machinery may affect electrophysiological properties, and control of membrane potential in branched or elongated neurons is often inadequate.

Two strategies have been applied in obtaining patch-clamp recordings from tissue slices. One approach, the "cleaning" technique, involves removing overlying neuropil or cellular debris from a visually-identified cell using a relatively large pipette filled with the same solution used to superfuse the slice. Recording is then possible in a manner similar to standard patch recording. In the other approach, called the "blind" technique, the recording pipette is lowered into a tissue slice without high-magnification visual guidance; seal formation is monitored using electrical measurements.

The Cleaning Technique

Tissue preparation for this technique uses procedures slightly modified from those used for standard slices. The slices must be thin enough to allow good cellular visibility (100 - 300 µm, depending on the age of the animal), but thicker slices result in a greater number of undamaged cells. Generally, vibrating microtomes are preferable to tissue choppers because their use results in less dead or damaged tissue at the cut surface, and because they facilitate preparation of thin slices. Special care should be taken when preparing thin slices to ensure that the bathing medium

remains ice cold in order to maintain the necessary firm tissue consistency. Following their preparation and prior to recording, slices are commonly incubated at 37°C for about one hour. This procedure softens the tissue and facilitates subsequent cleaning of cells. Various methods may be used for storing slices, but care is needed to avoid damage to the cut surfaces resulting from procedures such as placing slices on filter paper. Slices may be stored at room temperature or warmed to 30 - 35°C.

The only major piece of equipment required for this technique, in addition to a standard recording setup, is an upright microscope equipped with a water-immersion objective, most commonly 40x magnification. Use of an inverted microscope is not feasible since, even if very thin slices are used, visibility through the tissue to a recording pipette is significantly impaired. Hoffman or Nomarski differential-interference-contrast optics can be helpful, but are not necessary. Unless manipulators are mounted on the stage, it is extremely helpful to use a fixed-stage microscope, so that changing focus does not cause movement of the tissue with respect to cleaning or recording pipettes.

An objective with high numerical aperture is important. A commonly used apparatus is the Zeiss 40x water-immersion objective, which has a numerical aperture of 0.75. This objective offers a working distance of approximately 1.6 mm. The pipettes must, therefore, be placed at a very shallow angle (15 degrees from the horizontal is common). One consideration in mounting micromanipulators is that the movement in true horizontal and vertical directions is preferable to a coordinate system with an axis oriented parallel to the pipette. An advantage of this arrangement is the ability to lower the recording pipette directly onto the target cell in a single motion.

The recording chamber must allow continuous superfusion of the slice; it must also permit immobilization of the tissue despite the fluid flow, and do so using a device that does not interfere with the objective or the pipettes. One solution to this problem is a net consisting of a U-shaped piece of flattened platinum wire with a parallel array of very fine nylon monofilaments, such as stocking fibers, glued across the arms of the U-shaped wire (Edwards *et al.*, 1989; Sakmann *et al.*, 1989). The net can be placed on the slice and is usually heavy enough to prevent movement. Although the filaments may cause some damage to the surface of the tissue, they may be placed sufficiently far apart to leave considerable working space (the precise distance depends on the cellular architecture in the slice). Another solution is to attach the slice to the bottom of the recording chamber using a plasma clot (Blanton *et al.*, 1989).

The pipette used for cleaning should have a tip much larger in diameter than a recording pipette, usually in the range of 5-20 µm. The optimal tip size depends on several factors; in general, larger tips are preferable for larger cells and for deeper cleaning. However, the consistency of the tissue and cell density may also affect the choice of tip diameter. For example, with particularly "sticky" tissue, such as neocortex, small cleaning pipettes may tend to become clogged with membranous debris. For a given preparation, it is best to arrive empirically at an appropriate size. Additionally, since debris tends to accumulate on the jagged edges of broken pipettes, it is usually best to pull unbroken pipettes to the appropriate size. To minimize any damage that the process might inflict on the cell, the cleaning pipette should be filled with the same solution used for the bathing medium.

The first step in establishing a recording is to locate a healthy cell; it is usually possible to distinguish cells in good condition by their appearance of solidity or opacity. In such neurons, it is often possible to follow dendritic processes for some distance. Some healthy cells may be observed whose somata are on the surface of the slice and which display a "clean" membrane suitable for immediate patching with no further cleaning. Such neurons often have dendrites that can be seen projecting into the depths of the slice. It seems that cells that have lost most of their dendrites due to the slicing procedure are often "sick," as characterized by a granular and/or transparent appearance of the soma.

Once a healthy cell has been located, the next step is to remove the overlying neuropil. Mouth control of the pressure applied to the back of the pipette permits rapid, highly precise application of pressure and suction. It is often best to begin by applying positive pressure gently with the pipette placed just at the surface of the slice and directly above the targeted cell. Blowing accomplishes two goals. First, it provides information about the consistency of the tissue, which may be rather variable from slice to slice and which differs dramatically from one brain region to another. Second, application of positive pressure begins the process of disrupting the tissue overlying the desired cell. When tissue has been visibly disrupted, slight suction helps remove the loosened debris (Figure 5-2). Repeated application of this blowing and sucking cycle, with appropriate movement of the pipette to remove specific chunks of tissue, eventually results in a clean cell.

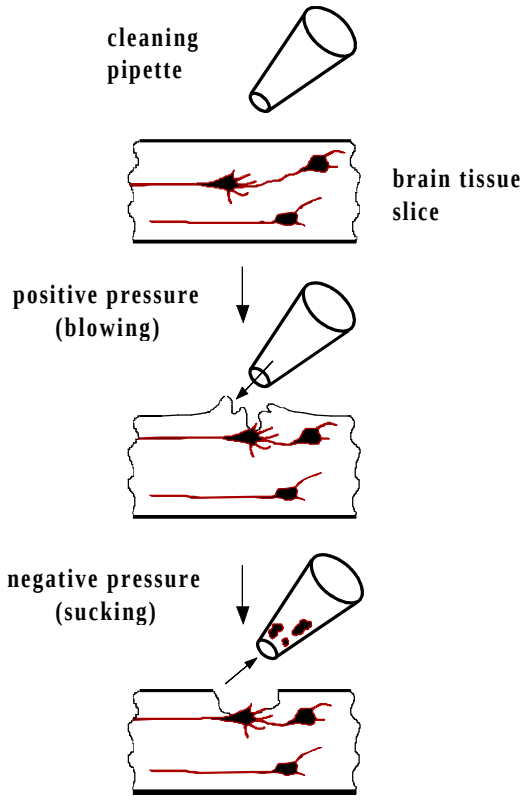

Figure 5-2. The Cleaning Technique

Use of a cleaning pipette to remove tissue overlying a cell in tissue slices. Reproduced with permission according to Edwards *et al.* (1989).

At first, this procedure may take a long time. It is almost inevitable that an inexperienced experimenter, who has not yet developed a feel for the tissue, will kill cells by applying pressure or suction that is too vigorous. With practice, however, one can clean even deep cells quite rapidly. Careful and repeated adjustment of the focus aids greatly in determining the degree of cleaning required at each stage. Depending on the particular type of tissue, a few tricks to facilitate and accelerate the process of producing clean cells may be applied. For example, in regions of densely packed somata, such as the CA1 pyramidal cell layer of the hippocampus, it is possible to remove tissue at the slice surface over a relatively large length of the cell layer by applying continuous suction and moving the pipette along the layer as if using a vacuum cleaner to remove dead tissue. Inspection of the underlying neurons often reveals at least one clean pyramidal cell.

After completing the cleaning procedure, patch-clamp recordings of cleaned cells in slices are carried out following essentially the same procedures that would be applied to cells in culture. All four major patch-recording configurations — cell-attached, inside-out, outside-out and whole-cell — are possible, as well as the nystatin-perforated patch technique (see *Perforated Patches and Vesicles* in this chapter).

The Blind Technique

Apart from a microelectrode holder with the appropriate diameter for patch pipettes, and with a side port for pressure application, no special equipment is necessary to adapt a standard slice setup equipped for intracellular recording to one suitable for blind-patch recording. A dissecting microscope is sufficient. A patch-clamp amplifier (such as the Axopatch) may be preferable to a microelectrode amplifier, such as the Axoclamp (but either type is adequate). The recording pipette should have a resistance in the bath of approximately 3-5 MΩ; the optimal resistance depends on the size and type of cell being recorded.

The procedure for establishing gigohm seals is very simple (Figure 5-3). It is possible to use either the current-clamp or voltage-clamp recording mode. A small repetitive current or voltage pulse is applied to the electrode at relatively high frequency (*e.g.*, 10 Hz) and the voltage or current response is monitored with an oscilloscope. Continuous, slight pressure is applied to the back of the pipette, for example, by blowing gently into the tubing connected to the pipette and closing a stopcock to retain the pressure. The pipette is then slowly lowered into the tissue in the region where the desired cells are found. It is advanced slowly — 1-5 steps per second, each step of 1-2 µm — into the tissue until a sudden increase in resistance is detected at the pipette tip, indicating contact with a cell. Often a further advance of 1-2 µm causes a further increase in the resistance. Release of the positive pressure should result in another approximate doubling of the resistance if the electrode is indeed contacting a cell. Although gigohm seals sometimes form spontaneously at this stage, the next step is to apply light suction to cause the seal to form. This may occur rapidly, in less than one second, or slowly, up to several seconds, requiring constant, gentle suction. The patch is then ruptured by additional suction creating electrical continuity between the pipette and cell interior.

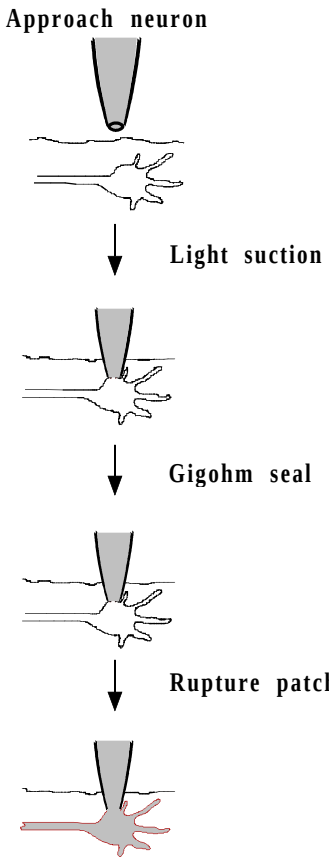

Figure 5-3. The Blind Technique

Obtaining a recording using the blind technique by advancing the patch pipette through a tissue slice. According to the method of Blanton *et al*. (1989).

Although this technique can be applied successfully, achieving a good recording on nearly every attempt, it is almost inevitable that at some point one will encounter some difficulty either in generating seals or establishing whole-cell recordings. Through experience, it is possible to identify the problem-causing aspects of the procedure. Often, changing the pipette size helps. A general rule is to use smaller tips to improve sealing, and larger ones to facilitate the transition to the whole-cell mode. One should be prepared to use a number of pipettes, since the probability of obtaining a recording on the second attempt to apply suction to the same pipette is very small. Another critical variable, which applies to all patch-clamp recording, is the intracellular solution whose osmolarity should be verified routinely, particularly when difficulties are encountered.

Advantages and Disadvantages of the Two Methods of Patch Clamping Brain Slices

It is impossible to give a complete discussion of the relative merits of the cleaning and blind techniques here. However, some of the important considerations are outlined in the table below.

Cleaning Technique	Blind Technique
• Need upright microscope and water-immersion objective	• Need no special equipment beyond that for intracellular recording in slices
• Vibrating microtome almost essential	• Vibrating microtome strongly recommended, essential for some tissues
• Maximum slice thickness limited by visibility	• Maximum slice thickness limited by viability
• Once a cell is located, additional time is invested in cleaning	• Less preparation time for each penetration, but no certainty of cell health
• Cell is visually identified	• Need post-hoc identification of cell type unless electrophysiological identification is possible or a homogeneous cell population is used
• Can see neighboring cells and apply local stimuli	• Visually-guided local stimulation is not possible
• Recording quality similar to standard patch clamp techniques	• High or unstable access resistance is often a problem with whole-cell mode (possibly due to debris in the pipette tip)
• All four standard patch-clamp configurations possible, plus the perforated patch technique	• All four standard patch-clamp configurations possible, plus the perforated patch technique

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Macropatch and Loose-Patch Recording

Measuring the local density of a specific type of ion channel in different regions of a cell provides valuable information. This kind of information is helpful for understanding the specialized functions of axons, cell bodies and dendrites in neurons, as well as for studying mechanisms of sensory transduction, the spatial distribution of ion channels near the muscle end-plate, and localized changes in channel density or channel properties resulting from single-transduction events. To efficiently address these areas of research, one needs a method that is intermediate in scale between single-channel patch clamp and whole-cell voltage clamp. Macropatch recording methods fulfill this need.

Two approaches are used to record ionic currents from patches of membrane containing tens to hundreds of ion channels. The first applies standard gigohm-seal (gigaseal) patch-clamp methods employing large-diameter patch pipettes (5-10 µm tip diameter) and is used to sample currents from much larger areas than are commonly used in single-channel recording (which is done with 0.5-2 µm tip diameter pipettes). The second method of macropatch recording has been termed *loose-patch recording*. This method, which enables one to gather data that could not be obtained with the gigaseal method, differs from single-channel recording in three ways: (1) the tip of the loose-patch pipette is usually much larger than the conventional single-channel patch pipette; (2) the loose-patch pipette does not form a gigaseal with the membrane, whereas gigohm seals are required for single-channel recording; and (3) the loose-patch pipette can be reused and repositioned to map the spatial distribution of channel subtypes on individual cells (Thompson and Coombs, 1988).

Gigaseal-Macropatch Voltage Clamp

Macroscopic patch currents can be recorded using standard patch-clamp instrumentation and a large patch pipette having a resistance of a few hundred kilohms. A gigaseal is formed using suction. This method can be applied to cell-attached and excised patches. Data analysis uses the same methods that are applied in whole-cell clamp experiments. Consequently, data concerning the kinetic behavior and local amplitude of ionic currents can be rapidly acquired. This method has been used to record macroscopic currents in *Xenopus* oocytes (Hoshi *et al*., 1990), which are particularly amenable to this method of recording because channel expression can be controlled, to a certain degree, by the experimental conditions.

There are a few problems associated with this method that have to be considered in the experimental design. The patch-clamp amplifier must be able to fully compensate for the greater capacitance of larger patches. When voltage pulses are applied to macropatches in the cell-attached configuration, current flowing in the patch will change the cell voltage; this change can be significant. For example, with a macropatch current of 100 pA and a cell input resistance of 100 MΩ, the cell voltage will change by 10 mV. Dynamic current-dependent errors of this type are difficult to correct and are most severe in small cells. In some preparations it may be impossible to use cell-attached macropatches to obtain accurate I/V curves, especially for rapidly changing currents. One can eliminate this source of error by studying excised patches.

Milton & Caldwell (l990) identified a more subtle error associated with macropatch recording. They observed that the suction used to form the seal between the patch pipette and the membrane can cause a change in ion channel density in the patch. With large patch pipettes, a noticeable membrane bleb may form in the orifice of the pipette during suction. This membrane bleb can be

visualized under a microscope. It appears that the ion-channel density decreases during bleb formation, possibly because of a flow of lipid into the bleb causing membrane rearrangement and changes in channel distribution. Therefore, the gigaseal-macropatch method may be unsuitable for mapping ion-channel densities because the procedure modifies the channel density before the measurement is made.

Loose-Patch Voltage Clamp

In loose-patch recording it is not necessary to form a tight seal between the patch pipette and the plasma membrane; therefore, strong suction is not needed. This has at least two advantages: (1) the pipette can be repositioned to sample currents from a number of patches on the same cell; and (2) the loose-patch method can be used to measure local currents in voltage-clamped preparations where establishing gigaseals is impossible due to the presence of adherent connective tissue, basement membrane or glial cells. Note that loose-patch recording is done only in the cell-attached configuration.

The low seal resistance between the pipette and the membrane presents the principal technical difficulty one must overcome in applying loose-patch methods. Low seal resistance creates two types of problems. First, the current flowing through the seal resistance and capacitance introduces noise in the current record, thus limiting the resolution (see *The Importance of a Good Seal* in **Chapter 3**). A more significant problem arises from the fact that membrane current flowing through the seal resistance is lost.

The following discussion will focus on the errors introduced by low seal resistance and the procedures used to limit errors to acceptable levels. Three designs are available to combat the problems associated with low seal resistance in loose-patch recording. The choice of the most appropriate method depends on the nature of the preparation. The original literature should be consulted for further details.

Combining Whole-Cell Clamp with Focal Current Recording

The first method to be introduced combines whole-cell voltage clamping with loose-patch current recording. This is the preferred method for many applications. The cell is voltage clamped by a microelectrode or a whole-cell patch pipette using standard microelectrode voltage-clamp or patch-clamp instrumentation, while current is recorded from a restricted area of membrane with a loose-patch pipette using patch-clamp instrumentation. Voltage-clamp pulses are applied via the whole-cell clamp while the loose-patch pipette is maintained at the bath potential to ensure that no current flows across the seal resistance. This method has been used to record local currents in large molluscan neurons (Neher and Lux, l969; Johnson and Thompson, l989; Thompson and Coombs l989; Premack *et al*. l990) and in muscle cells (Almers *et al*., l983). The method is particularly well suited for measuring the kinetics and current/voltage relationships of macroscopic currents expressed in *Xenopus* oocytes where a whole-cell voltage clamp is severely limited by the large capacitance of the cell and high intracellular series resistance. The electrode arrangement is shown in Figure 5-4.

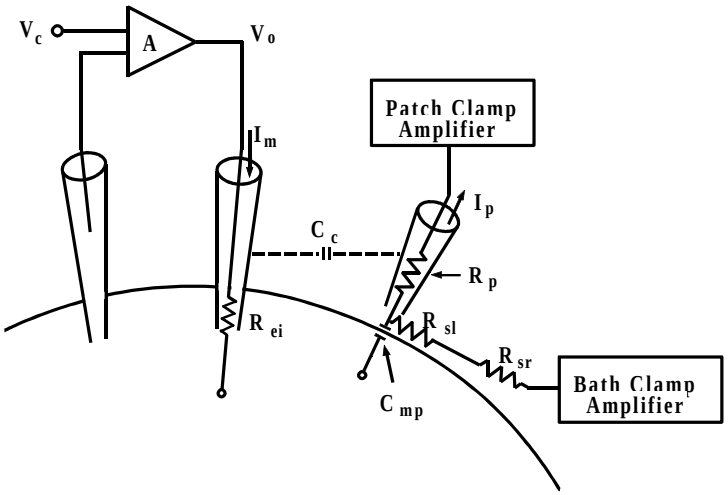

Figure 5-4. Combining Whole-Cell Voltage Clamping with Loose-Patch Current Recording

Schematic diagram of a cell with voltage clamp electrodes and the large patch pipette in place. The symbols used in the figure are as follows: **A**, voltage clamp open loop gain; $\mathbf{C_c}$, coupling capacitance between voltage clamp current electrode and patch electrode; $\mathbf{C_{mp}}$, capacitance of membrane patch; $\mathbf{I_c}$, current error; $\mathbf{I_m}$, membrane current supplied by voltage clamp; $\mathbf{I_p}$, patch membrane current supplied by patch clamp amplifier; $\mathbf{R_{ei}}$, resistance of voltage clamp current electrode; $\mathbf{R_p}$, axial resistance of patch electrode; $\mathbf{R_{sl}}$, resistance of seal between patch pipette and cell membrane; $\mathbf{R_{sr}}$, series resistance between the outside of the patch electrode and the bath clamp amplifier; $\mathbf{V_c}$, voltage clamp command voltage; $\mathbf{V_o}$, voltage clamp output voltage. Reproduced with permission according to Johnson and Thompson (1989).

A reusable loose-patch pipette (5-20 µm in diameter; axial resistance (R_p) of 100-500 KΩ) is used in cell-attached configuration. The resistance of the seal is monitored by applying test voltage steps via the patch amplifier and measuring the amplitude of the resulting current steps. After contacting the cell, gentle suction is applied until the sum of the seal resistance (R_{sl}) and the axial resistance of the pipette (R_p) increases by at least 10 fold; increases greater than 100-fold are common. The suction is released to avoid pulling a "finger" of membrane and cytoplasm into the pipette since this would increase the series resistance and lead to errors in voltage control and current measurement. Since the lumen of the loose-patch pipette is held at the bath potential and R_p is low compared to R_{sl}, there is no need to compensate for the finite value of R_{sl}.

This method allows one to simultaneously measure whole-cell and patch currents as well as whole-cell and patch capacitances. The areas of the cell and of the patch can be estimated by measuring the whole-cell and patch capacitances. A convenient way to do this is to apply a triangle-wave voltage command to the whole-cell voltage clamp and record the resulting whole-cell and patch current waveforms. The patch capacitance (c) is calculated from the change in the time derivative of the voltage (dV/dt) at a vertex of the triangle wave and the corresponding jump in patch current (I), using the following equation (Neher, 1971; Palti, 1971):

$$c = \frac{I}{dV/dt} \qquad (2)$$

Because the voltage of the patch pipette is equal to the bath voltage and only the voltage across the membrane patch covered by the pipette is changing, the capacitance of the patch pipette itself is rejected. The capacitance of the whole cell is measured in a similar fashion. However, the interpretation is complicated by current contributed from poorly clamped regions (see Johnson and Thompson (l989) for further discussion).

There are several issues to consider when applying this combined voltage-clamp method. The most important requirement is that the patch current must be measured from a well-clamped region of membrane, *i.e.*, from a region that is "space clamped" by the whole-cell voltage clamp amplifier. In order to accurately measure current-voltage relationships and limit the capacitive current flowing across the patch during voltage steps, the loose-patch pipette should be positioned close to the point where the whole-cell voltage is controlled. Cell geometry and electrode positioning should be considered when designing the physical layout of the experiment. These considerations limit the kinds of preparations that can be studied with the method. It is reasonable to use the loose-patch pipette to record local current densities at different points on a well-clamped spherical cell body. However, it would be inappropriate to record patch currents from a thin dendrite when the voltage clamp is applied to the soma.

The most significant error results from current flowing in the seal resistance between the loose-patch pipette and the bath. This error current (I_e), which is not measured by the patch-clamp amplifier, is given by:

$$I_e = \frac{I_p R_p}{R_p + R_{sl}} \qquad (3)$$

where R_p is the resistance of the loose-patch pipette and R_{sl} is the seal resistance. If $(R_p + R_{sl})$ is ten times larger than R_p the error in the measured current is 10%. To minimize the error, one can reduce R_p by using a patch pipette with a blunt taper and increase R_{sl} by using suction to improve the seal with the membrane. Enzyme treatment with dispase, pronase or trypsin can be used to clean the cell surface and improve the seal resistance.

Two kinds of errors caused by series resistance should be considered:

(1) The voltage drop that results from macroscopic patch currents flowing through the axial resistance of the patch pipette (R_p) will produce an insignificant error in most cases. For example, if the value of R_p is 300 KΩ and the maximal patch current is 500 pA, the resulting voltage error is 0.15 mV.

(2) A more troublesome error occurs when the bath voltage outside the patch pipette changes due to large whole-cell currents flowing in the series resistance (R_{sr}) between the cell membrane and the bath ground or virtual ground.

The error current is given by:

$$I_e = \frac{I_m R_{sr}}{R_{sl}} \qquad (4)$$

where R_{sr} is the sum of the series resistances of the bath, the agar bridges and the liquid-silver chloride junction at the bath electrode; it typically has a value of several kilohms.

Bath series resistance creates an insidious problem when membrane currents are large. However, the error can be reduced by measuring the bath voltage with a separate electrode and using it as the reference voltage at the patch-clamp amplifier. Good practice dictates that the bath voltage be used to correct the membrane voltage signal applied to the whole-cell clamp amplifier as well. Instability can result, however, if the bath voltage is measured with a greater bandwidth than the membrane voltage. To remedy this, one can limit the frequency response of the bath voltage follower, but this degrades performance and causes other errors. A better solution is to eliminate the need to subtract the bath voltage at the other circuits by voltage clamping the bath to ground potential. This can be accomplished using an independent voltage-clamp amplifier operating at a high gain and bandwidth. Two separate agar bridges and silver-chloride electrodes can be used to measure the bath voltage and pass current to the bath. Because of the low resistance of these electrodes, a simple voltage-clamp circuit employing a single operational amplifier is adequate for clamping the bath to ground potential. This arrangement ensures that the bath voltage does not change during clamp steps. The output of the circuit provides the record of whole-cell current.

Other problems can be overcome by a careful design of the experimental layout. Since this method uses several voltage-clamp amplifiers working simultaneously, it is necessary to limit capacitive coupling between electrodes. This can be achieved by inserting a grounded shield between the whole-cell current electrode and the macropatch pipette, and by lowering the volume of saline to the minimum required for covering the cell. To reduce high frequency noise in the patch recording, the submerged tip of the pipette should be coated with Sylgard (Dow Corning, Midland, MI) and only the tip should be filled with saline. Johnson and Thompson (l989) provide a thorough analysis of the problems and the approaches used to reduce errors to acceptable levels.

Voltage Pulses Applied to the Loose-Patch Pipette

Ionic currents can also be studied by applying voltage pulses directly to the loose-patch pipette, thus eliminating the need for an independent whole-cell clamp. Errors resulting from the finite seal resistance and the axial resistance of the pipette are more severe in this case and require more elaborate compensation. The advantage of this method is that membrane currents can be recorded locally without the need to establish an overall space clamp. This loose-patch method has been very successful in studies of acetylcholine (ACh) receptor and Na^+-channel distribution near muscle end-plates (Almers *et al.*, l983a,b).

Although extra effort is required with this method, it may be the best approach for some preparations. It is important to remember that current flowing across a macropatch can cause a significant change in cell voltage. In its simplest form, this method uses a large diameter patch pipette in a cell-attached configuration and a conventional patch-clamp amplifier (Almers *et al.*, 1983a,b; Stühmer *et al.*, 1983). In this configuration, the axial

resistance of the pipette (R_p) and the seal resistance (R_{sl}) act as a voltage divider. When a voltage command pulse is applied to the pipette (V_p), it will be attenuated at the pipette tip. The voltage at the pipette tip (V_t) is given by (Stühmer *et al.*, l983):

$$V_t = V_p \frac{R_{sl}}{R_p + R_{sl}} \qquad (5)$$

When R_{sl} is large, as is the case in gigaseal recordings, the error is insignificant. In loose-patch applications, however, R_p and R_{sl} are approximately equal and a large fraction of the current delivered to the pipette will flow through the seal resistance. It is necessary to correct the amplitude of command-voltage steps by a factor equal to $R_{sl}/(R_p+R_{sl})$. To do so, R_p is measured before the pipette contacts the cell and $R_p + R_{sl}$ is monitored continually throughout the experiment. Almers *et al.* (l983a,b) used a computer to calculate a correction factor $A = R_{sl}/(R_p+R_{sl})$ before each voltage step. The correction factor was used to scale command-voltage steps applied by the computer by 1/A to ensure that the intended voltage appeared at the pipette tip. Recorded currents were divided by A to correct for current (often termed *leakage current*) flowing through the seal resistance. This digital approach to seal resistance compensation is technically sound. To implement it, the user has to develop a software program for continuously updating the parameters needed for scaling the amplitude of the command pulses and correct for leakage current; an extra effort that is well justified in some applications.

Seal resistance compensation can also be achieved with an analog circuit. Stühmer *et al.* (l983) pioneered a method that uses an active voltage divider and potentiometers to compensate for measured values of R_p and R_{sl}. Their circuit allows one to null R_p and limit the series resistance errors to acceptable values. When correctly adjusted, their design ensures that the input impedance of the patch pipette is near zero so that there is minimal loss of membrane current across the seal. A disadvantage of this method is that it requires the user to build a specialized patch-clamp headstage and other circuitry following the example provided by Stühmer *et al.* (l983).

With both digital and analog seal-resistance compensation, accuracy depends on one's ability to measure R_p and (R_{sl} + R_p) and on the assumption that these resistances remain constant during voltage steps that activate conductances in the patch. The error increases as the amplitude of the voltage step and the conductance of the membrane patch increase. It is not certain that R_p and R_{sl} are independent of the current amplitude. In practice, one often observes an unexpected flattening of the current-voltage relationship for inward or outward current activation when the voltage step or the patch current become large. Because most of the uncertainty in current measurement depends on R_{sl}, one should use suction and/or enzyme treatment to maximize the seal. It also helps to limit the diameter of the patch pipette to about 20 µm or less. R_p is minimized by using pipettes with a blunt taper.

Loose-Patch Clamp with Concentric Patch Pipettes

Almers *et al.* (l984) introduced a modification of the loose-patch method that employs a two-chambered concentric patch electrode. The electrode is fashioned from two glass capillaries, one inside the other, with the inner capillary held in place by support rods during the pull. The concentric electrode is pushed against the cell surface where it separates a central membrane patch from an annular surround. The voltage-clamp amplifier is designed

to clamp both the center patch and the surround to the command potential. Membrane current is measured in the central barrel of the electrode while the outer annulus acts as a guard. Since the inner and outer barrels are at the same potential during the command steps, current loss from the central membrane region to ground is minimized and R_{sl} takes a value that is effectively infinite. This approach is historically related to vaseline-gap and sucrose-gap voltage-clamp methods. It has been used successfully to voltage clamp mammalian skeletal muscle. However, the implementation is somewhat difficult since it requires the construction of an elaborate concentric electrode and some specialized instrumentation. It may still be the best approach in some cases and the original literature should be consulted for further details.

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The Giant Excised Membrane Patch Method

Many physiologically important ion pumps, exchangers and co-transporters are electrogenic. The giant excised membrane patch method has been developed to improve electrophysiological studies of such mechanisms. Although whole-cell recording techniques are often employed in transport studies, a method offering free access to the cytoplasmic membrane surface and faster voltage clamping was strongly desirable. While the excised membrane patch method was an attractive alternative, the conventional patch clamp techniques were not useful. First, the single turnover rates of transporters (1-10,000 per second) are far too small to expect resolution of the current generated by a single transporter. Second, the magnitudes of "macroscopic" currents expected in excised patches are difficult to measure because they are much smaller than the patch leak currents.

The giant excised membrane patch technique enables one to achieve 1-10 gigohm seals routinely, using pipette tips with inner diameters of 12-40 µm. The giant membrane patch produced by this method has 2-15 pF membrane capacitance, representing a 100-fold increase of membrane area over the area of conventional, single-channel patch membrane. Pipette diameters of > 50% of the cell diameter can be employed when recording from small, spherical cells.

The giant patch method has proven to be advantageous for fast macroscopic current recording whenever free access to a large surface area of the cytoplasmic membrane is desired. To date, the giant patch method has been applied successfully to cardiac, skeletal and tracheal myocytes, pancreas acinus cells, a Jurkat human T-cell line, Sf9 cells and *Xenopus* oocytes. The method should be useful for a wide range of studies of electrogenic mechanisms involving native proteins and proteins expressed from cloned genes. Promising applications include studies of macroscopic current modulation by intracellular compound (*e.g.*, enzymes or ions acting from the cytoplasmic side of the membrane), studies of charge movement generated during channel gating, and studies of partial reactions of transporters.

Pre-Treatment of Muscle Cells

Cell-surface invaginations of muscle cells limit the ability to form large-diameter seals. To promote sealing, cells can be pretreated to induce separation of the surface membrane from the underlying cell structure or produce large-scale surface membrane "blebbing." In some cases both separation and blebbing occur. To accomplish this, digested pieces of tissue are placed in a "storage solution" consisting of 60 - 150 mM KCl, 10 mM EGTA, 1 - 5 mM $MgCl_2$, 20 mM dextrose, 15 mM HEPES, pH 7, for 1-12 hours. This treatment is similar to the treatment previously used to induce blebbing in skeletal muscle (Standen *et al.*, 1984). Disruption of volume regulation and the Donnan equilibrium is clearly important for the blebbing process. Large-scale (20-50 µm) membrane bleb formation or apparent lifting of sarcolemma away from myofilaments are routinely obtained after 2 h treatment. The treatment has been found effective in inducing blebbing in many different cell types. However, blebbing is not necessarily conducive to large-diameter seal formation in all cell types, and other methods may be required. For instance, giant patches have not been obtained on the blebbed membrane of oocytes, although they are routinely obtained on hypertonically shrunken oocytes after mechanical removal of the vitelline layer.

Pipette Fabrication

Large-diameter (1.5-1.8 mm), thin-walled glass pipettes are used to fabricate pipettes with large-diameter tips and relatively steep descents at their tips. The type of glass is unimportant. A conventional double-pull technique using standard patch-pipette pullers can be employed. For work with myocytes, light fire polishing with barely visible effects on the tip favors the spontaneous formation of inside-out patches upon membrane excision, rather than vesicles that must be disrupted mechanically. Pipette A in Figure 5-5 shows a typical pipette tip with a 17 µm inner diameter.

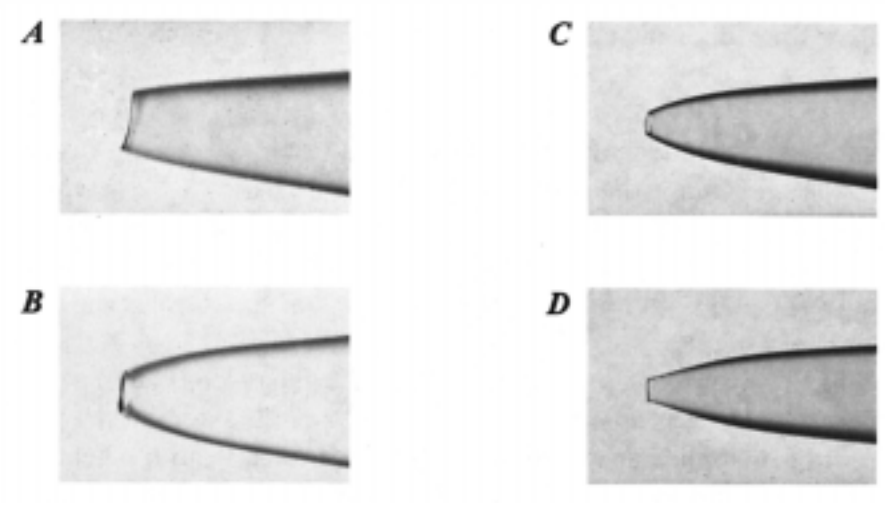

Figure 5-5. Pipette Fabrication for Giant Excised Patch Method

A, oocyte electrode before fire polish; ***B***, oocyte electrode after fire polish; ***C***, myocyte electrode after cutting and fire polish; ***D***, myocyte electrode after fire polish (no cutting).

Alternatively, pipettes can be pulled to smaller diameters and then scratched gently on the edge of a soft glass bead to the desired diameter, leaving a rough edge (pipette B in Figure 5-5, 17 µm diameter). When such electrodes are employed without fire polishing, the membrane usually remains localized at the pipette opening during sealing, and vesicles are obtained in most cases upon membrane excision. For very large cells, particularly for oocytes, a different procedure is employed. Pipettes are first scratched to a 30-50 µm diameter (pipette C in Figure 5-5) and then melted to a diameter of 18-35 µm (pipette D Figure 5-5) close to a glass bead placed on a microforge. The resulting bullet-shaped pipette tips are advantageous for fast voltage clamping, and the oocyte membrane remains localized to the rim of the pipettes. Recently, we have had good success preparing electrodes by melting the tip into a bead of soft glass, followed by cooling and "cutting" by a small pull.

A number of hydrocarbon mixtures consisting of oils, waxes and Parafilm (American National Can Co.) allow large-diameter, high-resistance seal formation. Viscous oils can be used by simply dipping the dry pipette tip into the oil of choice followed by washing with the desired filling solution. The most consistent success has been obtained with acetyltocopherol (Sigma Chemical Co.). Silicone oils, phospholipids and plasticizers are usually not useful, and seals formed with oils alone are generally unstable. Seal stability can be improved with a highly viscous mixture of Parafilm, acetyltocopherol (or light mineral oil) and heavy mineral oil. The

standard mixture is prepared by mixing approximately three parts by weight of finely shredded Parafilm with each of the two oils and vigorously stirring over heat for 30-60 minutes. When a thin mixture is prepared, pipette tips can simply be dipped. With a thick mixture, a peanut-sized amount of the mixture is smeared with a plastic rod on a clean surface, and a thin film of the mixture is drawn into air by lifting the rod. The pipette tip is then passed quickly through the film, and the procedure is repeated once more. In order to reduce the pipette capacitance, a thick mixture is applied as close as possible to the tip. The pipette is then backfilled and a brief, strong, positive-pressure pulse is applied with a syringe to clear the tip. The pipette tip usually appears clean and is visually indistinguishable from that of an untreated electrode.

Seal Formation

Seals are formed in the usual manner by applying gentle negative pressure at the cell surface. Maintenance of positive pressure up to the time of sealing is essential both to keep the electrode tip clean and to prevent contamination of the pipette tip with bath solution constituents. When working with myocytes, membrane vesicles rather than inside-out patches are often obtained upon membrane excision, as indicated by the absence of the characteristic currents. Although membrane position in the pipette tip can usually be visualized, membrane patches cannot be visually distinguished from vesicles. The method of choice to disrupt vesicles is to touch the tip of the pipette against an air bubble or a bead of hydrocarbon placed on the wall of the patch chamber. Success rate is about 50%.

To date, the best success with oocytes has been obtained by shrinking oocytes with 200 mM K-aspartate rather than sucrose. Patches from oocytes are excised by gently moving the pipette tip from side to side in gradually increasing distances. Dark, granular cytoplasmic material is often excised with the oocyte patches, and vesicles are obtained only rarely. The cytoplasmic material can be washed away by rapid pulses of solution directed at the patch.

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Recording from Perforated Patches and Perforated Vesicles

The study of ion channel function and modulation by neurotransmitters and hormones advanced dramatically with the advent of patch clamping in the mid-1970's. Approximately a decade later, a variation of the single-channel patch-clamp recording method was introduced and termed the *perforated-patch recording* method. Perforated-patch recording techniques allow the measurement of whole-cell currents, single-channel currents and transmembrane voltages much less invasively than do standard patch-clamp or microelectrode approaches.

Properties of Amphotericin B and Nystatin

Perforated-patch recording, as presently implemented, uses either nystatin or amphotericin B to gain electrical access to the cell's interior. These polyene antibiotics form channels in cholesterol- or ergosterol-containing membranes. The channels formed by both perforating compounds are permeable to monovalent cations and Cl^- but exclude multivalent ions such as Ca^{2+} or Mg^{2+}. The monovalent cations are nine times more permeant than Cl^- through the channels formed by either perforating compound. When measured in molar salt concentrations, the single-channel conductance of amphotericin channels is twice that of nystatin channels.

Stock Solutions and Pipette Filling

Because of their limited water solubility, amphotericin and nystatin stock solutions of 30 mg/ml are prepared in DMSO. These stock solutions lose activity upon prolonged storage and freezing. Therefore, it is best to prepare them freshly before use and use within 1 hr. A convenient approach is to weigh 6 mg of the antibiotic powder into each of 40-50 microcentrifuge tubes and store them indefinitely in the freezer without loss of activity. At the time of use, the powder is solubilized by pipetting 100 µl fresh DMSO into the tube. Solubilization is enhanced by sonication or vortex mixing (but this is apparently not required). Stock solution can be added directly to the desired pipette filling solution to a final concentration of 120-350 µg/ml. Since 120 µg/ml appears to be a saturating concentration, it is not clear if higher concentrations offer any advantage. Vortex mixing for a few seconds or sonication for a minute is recommended for maximum solubilization of the antibiotic in the pipette filling solution. When the antibiotic is in excess, some of it settles out of solution and adheres quite well to the wall of a plastic tube or syringe. Therefore, the saturated solution can be used without adding much particulate matter to the pipette interior. The filling solution can be filtered after adding the antibiotics, but care must be taken since some filters might inactivate these compounds. With either antibiotic, the final solution is yellow in color and slightly turbid. After filtration with either a 0.22 or 0.45 micron filter, the final solution is perfectly clear. With nystatin, it is possible to make up larger volumes of the stock solution and adjust the pH to neutrality before use. Under these conditions, no precipitate forms when the final filling solution is made. This approach has not been used with amphotericin B because of its higher cost. The perforating activity of either compound begins to decrease about 3 hr after preparing the stock solution. Since whole-cell recording experiments can easily last for 1-3 hr employing the perforated-patch approach, it is advisable to use stock and antibiotic-containing filling solutions within one hour of preparation.

Both amphotericin and nystatin seem to interfere with seal formation. Although it is possible to seal some cells when either compound is present in the pipette filling solution, the success rate is lower than when an antibiotic-free filling solution is used with the same cells. Therefore, it is best to fill the tip of the pipette with an antibiotic-free solution and then to backfill with the

antibiotic-containing solution. The tip-filling process can be accomplished by simply dipping the tip into the desired solution. With borosilicate glasses, 1-5 s of tip immersion suffices, whereas with the more hydrophobic lead-containing glasses, 30-60 s may be required. With high-lead glasses, gentle suction for a second or so may be beneficial. With any glass, it is important that the antibiotic-free solution *not* exceed 500 µm distance from the tip; otherwise, the time required for the diffusion of the antibiotic from the backfilling solution to the tip will be excessive. For example, if the pipette is filled up to 1.0 mm from the tip with an antibiotic-free solution, more than an hour will be required for the antibiotic to reach a tip-concentration comparable to that in the backfilling solution. Ideally, the tip should be filled to a distance that allows the antibiotic to reach the tip within a few seconds following a gigohm seal formation. Since the time required for seal formation depends on the cells and the setup, tip filling distances should be optimized by each investigator. Consistently optimal tip filling is routinely possible by first overfilling the tip and then forcing the excess solution out by applying positive pressure to the back of the pipette while observing it under a dissecting microscope.

Properties of Antibiotic Partitioning

Following seal formation, the antibiotic slowly diffuses to the tip where it contacts the membrane and begins to partition and form channels. While most of the time delay can be attributed to diffusion, some investigators reported that incorporating a mild detergent like Pluronic F-127 into the filling solution decreases the perforation time. Since it is unlikely that this detergent could affect the diffusion time of the antibiotics, it seems that, at least in some cells, the membrane partitioning (channel formation) time of the antibiotics is appreciable and that mild detergents decrease this time.

In a real experiment, one can observe the partitioning by applying a voltage pulse to the pipette at frequent intervals. As perforation occurs, the size of the capacity transient increases and the time constant of the transient decreases. The time constant to charge the cell capacitance is approximately R_aC_m, where R_a is the access resistance and C_m is the cell capacitance. When R_a is large, a brief voltage pulse will not charge the capacitance fully before the pulse turns off. Therefore, as partitioning continues and R_a decreases, the capacity transient gets larger since an increasing fraction of the cell capacity gets charged during the brief pulse. The decaying phase of the transient gets shorter as R_a decreases. In most cells, a properly filled pipette will produce minimized R_a by 20-30 min after seal formation. R_a of less than 20 MΩ is often achieved in about 5 min. Some investigators begin recording at this time. However, R_a continues to fall and finally becomes stable after 20-30 min. Once R_a stabilizes, it remains stable for 2.5-3 hr before beginning to increase (presumably due to loss of activity of the antibiotic). The remarkable stability of R_a is one of the main virtues of the technique. For example, using amphotericin, one investigator reported R_a of 3.4 MΩ that did not change by more than 100 kΩ for three hours.

The Advantages of the Perforated-Patch Technique

The perforated-patch technique has several advantages over conventional whole-cell patch-clamp approaches:

(1) The channels formed by either amphotericin or nystatin are impermeable to molecules as large as or larger than glucose. Therefore, whole-cell recordings can be done without dialyzing important substances from the cell's cytoplasm. Currents run down significantly

slower; physiologically relevant second-messenger cascades and mechanisms important to cell signaling and channel regulation remain operative.

(2) Intracellular multivalent ions are not affected by the pipette-filling solution since the channels formed by the antibiotics are not permeated by these ions. Therefore, intracellular Ca^{2+}, for example, can be measured by optical techniques simultaneously with recording whole-cell currents.

(3) With carefully fabricated pipettes and the use of appropriate composition of the filling solution, the perforation technique is less damaging to cells than are intracellular microelectrodes or standard whole-cell patch clamping. It is not uncommon to be able to record from a single cell for 3 hr. In some instances, freshly dissociated cells plate out on the bottom of the recording chamber while their whole-cell currents are being measured. The ability to plate out is indicative of the cells' viability.

(4) Unlike the frequently experienced loss of seal when suction or voltage pulses are applied to disrupt the membrane patch (to "go whole cell"), seals are rarely lost with the perforating-patch approach.

(5) The R_a achieved with the perforated-patch technique is as low or lower than that obtained by standard approaches. Moreover, once achieved, the low R_a of the perforated patch is usually considerably more stable than that produced with standard techniques.

(6) In many cells, whole-cell capacitance is easier to compensate when using perforated patches. It appears that the equivalent circuit of the perforated patch is often better approximated by a single RC than is the access resistance and capacitance in a standard whole-cell recording.

The Limitations of the Perforated-Patch Technique

The perforated-patch technique has some disadvantages when compared to the conventional whole-cell patch-clamp approaches:

(1) The perforated-patch approach does not allow one to dialyze the cell's cytoplasm and replace its content with compounds other than the small ions included in the filling solution, as is possible with conventional methods. Therefore, the effects of such compounds on intercellular mechanisms cannot be studied.

(2) The pipette-filling solution and the cell cytoplasm exist in Donnan Equilibrium. Thus, the pipette must contain an impermeant anion and a Cl^- concentration that match the cell interior. Failure to do so results in a Cl^- flux either into or out of the cell (depending on the direction of the mismatch). A charge balancing cation and water will follow, resulting in changes in cell volume. Due to the low Cl^- permeability of the antibiotic channels as compared to their cation permeability, it may take approximately 30 min to reach this equilibrium. It is not yet clear if there is a way to alleviate this problem since it is difficult to find an impermeant anion that matches the effective valence of the intracellular impermeant anions. Recently, it has been reported that including 20 - 25 mM Cl^- and 125 - 130 mM methanesulfonate in the pipette solution appears to keep cell volume reasonably stable.

On the other hand, this disadvantage can be exploited for studies of volume-activated currents by purposefully mismatching the pipette and cytoplasmic Cl^- concentrations causing swelling and shrinking of cells. To date, this approach has not been employed.

In addition to the influx and efflux of ions described above, the cells that are voltage-clamped by the perforated-patch technique are very susceptible to volume changes caused by external perturbations. In a standard whole-cell recording configuration, cell volume is kept reasonably constant by the large volume of the pipette-filling solution and the high hydraulic conductivity of the pipette tip. In contrast, when applying the perforated-patch method, the access to a cell is provided by a million or so tiny parallel channels. While these channels can provide electrical access comparable to that obtained by a standard patch pipette, their composite hydraulic conductivity is significantly lower than that of a single orifice with the same total electrical resistance.

(3) The perforation process requires considerably longer time for achieving access into the cell interior, as determined by the low R_a, than do suction or voltage pulses. When rapid measurements from a large number of cells is important, standard whole-cell patch-clamp approaches would be more productive.

(4) While the relative Na^+, K^+ and Cl^- permeabilities of nystatin and amphotericin channels are known, the permeation by other anions and cations, particularly multivalent ions that are generally considered impermeant, needs further study.

Suggested Ways to Minimize the Access Resistance

Clearly, the access resistance (R_a) cannot be lower than the resistance of the pipette itself. The resistance of the patch of membrane to be perforated is in series with the electrode tip and, therefore, the total resistance is the sum of the two. The final patch resistance depends upon the total number of antibiotic channels formed (partitioning) and the single-channel conductance of each channel. The total number of channels equals the number of channels formed in a unit surface area times the patch area. These simple considerations suggest the following ways to minimize R_a:

(1) The largest pipette tips compatible with seal formation should be used to minimize the pipette resistance.

(2) Pipette geometry should maximize the size of the omega-shaped membrane patch that is drawn into the tip during seal formation. This maximizes the surface area available for channel insertion.

(3) The perforating compound that produces the highest total conductance should be used. It may not be simple to predict the conductance-producing ability of a compound since it requires both the partitioning (number of channels formed) and single-channel conductance to be maximal. To date, amphotericin B has produced the lowest R_a. This can be anticipated since the single-channel conductance of amphotericin channels is twice that of nystatin, at least in measurements made in salts at molar concentration. However, the relative conductance of the two types of channels in lower salt concentrations (*e.g.*, 150 mM) is unknown. In addition, there are no relevant studies of the relative partitioning of the two compounds when presented to the membrane in saturating concentrations. To date, the

lowest patch resistance obtained by perforating with amphotericin is about 2 MΩ. The lowest resistance pipette that is capable of forming gigohm seals is approximately 0.5 MΩ. Thus, at present, an R_a value of approximately 2.5 MΩ appears to be the lowest one can achieve. Lower R_a values will require either perforating compounds with higher single-channel conductance or finding ways to enhance the partitioning of the existing antibiotics. It is unlikely that larger pipettes than those presently used would be successful in forming seals.

Other Uses for Perforated Patches

Cellular Voltage Measurements

In addition to whole-cell recording using patch-clamp amplifiers like the Axopatch in the voltage-clamp or current-clamp mode, the perforated-patch method is useful for making cellular voltage measurements, such as for resting and action potentials. These can be done either with patch clamp amplifiers operating in current-clamp mode or with standard microelectrode amplifiers like the Axoprobe or Axoclamp. In any cellular voltage measurement, two main factors affect the accuracy of the measurement: (1) the extent to which the cell's voltage is altered by current flow through the shunt resistance along the electrode's outer surface at either the penetration or seal sites; and (2) the extent to which the pipette-filling solution alters the electrolyte concentrations in the cell cytoplasm. High shunt resistances and minimal alteration of the cell content are desirable.

Patch electrodes, with their tens to hundreds of gigohm seal resistances, clearly have much higher shunt resistances than do intracellular microelectrodes. On the other hand, patch electrodes sealed to cells cause rapid changes in the concentrations of the cellular electrolytes if the pipette-filling solution contains different electrolyte concentrations than the cytoplasm. Intracellular microelectrodes, which are usually filled with molar concentrations of potassium salts, also alter cellular electrolytes but not as rapidly as do patch electrodes. Therefore, the perforated-patch technique provides both the advantage of the higher shunt resistance than intracellular microelectrodes without the significant change in cytoplasmic content caused by conventional patch electrodes.

Single-Channel Recording in Outside-Out Vesicles

The outside-out patch configuration has been particularly useful because it allows direct application of agents to the extracellular side of the membrane while maintaining complete voltage control during single channel recording. These patches have been required for studying ligand-gated ion channels such as the nicotinic acetylcholine, GABAa, glycine and glutamate receptors. Likewise, transmitters that regulate channels through nondiffusible second messengers must be examined with outside-out patches. For example, activation of atrial potassium channels is not seen with cell-attached patches when muscarinic agonists are applied to the bath, but is seen with direct application of transmitter to an outside-out patch.

Unfortunately, conventional outside-out patches have a drawback. The formation of these cell-free patches requires replacement of cytoplasm with artificial pipette solutions. In many cases, this "washout" is accompanied by a loss of ion channel activity and/or modulation. For example, in GH3 pituitary tumor cells, the neuropeptide thyrotropin

releasing hormone (TRH) triggers mobilization of intracellular calcium that in turn regulates calcium and potassium channel activity. With outside-out patches, TRH fails to regulate potassium channels. Furthermore, calcium channel activity runs down within 15 min. These problems had prevented direct detection of transmitter-induced inhibition of single calcium channels until the development of a new patch clamp configuration, termed the perforated vesicle, that results in the formation of outside-out patches that retain cytoplasmic factors and organelles.

A recent development in single-channel recording combines the outside-out patch recording method with the perforated-patch technique. In this application, a perforating antibiotic is used to produce an outside-out patch, termed perforated vesicle, that retains the cytoplasmic content of the cell forming the equivalent to tiny single-channel cells. Thus, the perforated vesicle is an extension of the nystatin-perforated patch technique.

Similar to the protocol described above, nystatin stock solution (25 mg/ml) is prepared by dissolving 2 mg nystatin in 80 µl DMSO. Since this pore-forming agent is susceptible to oxidation, the stock solution can be frozen and used within two days. Simple pipette solutions can be used (*e.g.*, 150 mM KCl, 5 mM $MgCl_2$, 10 mM KHepes, pH 7.1) because nucleotides, proteins and calcium buffers cannot permeate through nystatin perforations. Four (4) µl of nystatin stock solution is pipetted into a glass tube and then 1 ml of filtered pipette solution is added. After covering the tube and vortexing, the slightly cloudy solution is vigorously sonicated for 30 s in a cylindrical bath sonicator to completely dissolve the nystatin, giving a final concentration of 100 µg/ml. This aqueous solution can be used for only 3 hr.

Conventional patch pipettes are fabricated. When filled with pipette solution, their resistance is 2 - 4 MΩ. Smaller pipettes minimize the surface area of the perforated patch and, thus, give larger series resistances (R_s). On the other hand, larger pipette tips were found to form fragile patches that could spontaneously break forming the conventional whole-cell configuration. Pipettes are coated with Sylgard to reduce their capacitance. This not only lowers noise for single-channel recording, but also permits accurate series resistance measurements.

Pipettes are momentarily dipped in nystatin-free pipette solution and then backfilled with nystatin-containing solution. A cell-attached seal is then formed. After compensating, electrode capacitance (*e.g.*, 3 pF), R_s is monitored by examining the unfiltered current response to a 10 mV voltage step from -70 mV (holding potential). The height of the capacitance transient is equal to the voltage step divided by R_s. Series resistance is monitored for 5 - 20 minutes to ensure that it decreases smoothly to a value less than 50 MΩ. Cells that show an abrupt decrease in R_s should be discarded.

The patch-clamp amplifier is switched from voltage-clamp (V-clamp) mode to current-clamp (I-clamp) mode with zero resting current and the pipette is withdrawn from the cell to form a perforated vesicle (Figure 5-6). Switching to I-clamp ensures that no large changes in current could pass through the patch and disrupt the resealing of the membrane that forms the vesicle. The patch-clamp amplifier is then switched to V-clamp mode to record single-channel activity.

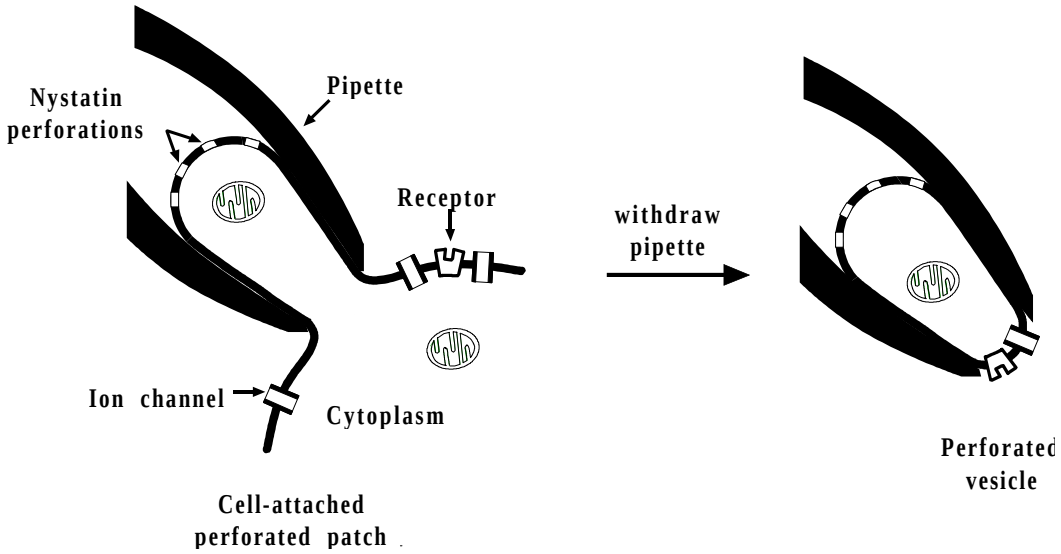

Figure 5-6. Forming a Cell-Attached Perforated Patch and a Perforated Vesicle

The formation of a perforated vesicle can be verified by many methods. In some cases, vesicles can be seen with 400x magnification. The presence of cytoplasm can be confirmed by preloading the cells with a fluorescent dye. For example, cells may be treated with 40 µM carboxyfluorescein diacetate, a membrane-permeant nonfluorescent compound. After crossing the membrane, this agent is hydrolyzed to yield water-soluble carboxyfluorescein. The presence of organelles can also be verified with specific dyes. Rhodamine 123 specifically partitions into functioning mitochondria and produces a characteristic punctate staining that can be detected in GH3 cells and perforated vesicles. Channel activity can also verify the presence of nystatin perforations. With normal vesicles, channel currents appear attenuated and rounded. Furthermore, channels in the inner face of the vesicle are not accessible to bath-applied hydrophilic inhibitors and have opposite voltage dependence to channels in the vesicle surface that is in contact with the bath. In contrast, perforated-vesicle channel activity can be recorded only from the portion of the vesicle in contact with the bath. Through the use of fluorescent dyes and channel recordings, the perforated vesicle can be differentiated from normal excised vesicles and outside-out patches.

The nystatin-perforated vesicle method allows the study of channel activity and modulation that is lost with conventional outside-out patches. Initial studies indicate that cyclic AMP-dependent protein kinase, phosphodiesterase, phosphatase, G-proteins, phospholipase C and intracellular calcium stores can modulate ion channels in perforated vesicles excised from GH3 cells. Two channel types that quickly "wash out" of conventional patches from these cells, L-type calcium channels and voltage-gated potassium channels, survive for extended periods with perforated vesicles. Perforated vesicles were excised successfully from a variety of cell lines and primary cultured pituitary cells. Thus, it appears likely that this patch clamp configuration will aid the study of ion channel modulation in many systems.

Further Reading

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Enhanced Planar Bilayer Techniques for Single-Channel Recording

The planar bilayer recording technique, in which ion channels are incorporated into an artificial, planar lipid bilayer either by fusion of vesicles containing the channels with the bilayer or by direct insertion of water-soluble channels, provides an unique approach for studying single ion channels, enabling experimental designs that are impractical or impossible using standard patch-clamp methods. For example, the effects of membrane composition on channel function can be rigorously studied, changes in solution composition on either side of the membrane can be made easily, and channels can be incorporated from membranes that are normally inaccessible to patch-clamp methods (*e.g.*, cytoplasmic vesicles, sarcoplasmic reticulum) (Wonderlin *et al.*, 1990; Wonderlin *et al.*, 1991). The utility of the planar bilayer method for studying single ion channels, however, is often limited by a low recording bandwidth, primarily due to the high background current noise, and poor resolution of voltage step activation of ion channel activity due to the large, slow capacitive current transient associated with a voltage step applied to a bilayer. These limitations may be especially problematic in investigations of small-conductance, rapidly gating channels, the kinetics of channel-blocking drugs, or voltage-gated ion channels that must be activated by voltage steps because of steady-state inactivation. The purpose of this brief tutorial is to describe a combination of equipment and methods that partially overcome these limitations and improve the quality of planar bilayer recordings. With this approach, it is reasonable to attempt to record single-channel activity with low background noise (< 0.35 pA rms at 5 kHz) and rapid resolution (<< 1 ms) of currents after a voltage step. Several general considerations regarding the equipment and techniques necessary for making high-resolution recordings will be presented first, followed by a more detailed description of the implementation of the approach. For more detailed discussion of the equipment and methods, please see Ref. 1.

I. Solving the Problems of High Resolution and Voltage Steps

Minimizing the Background Current Noise

Bilayer recording systems are prone to high background current noise which can easily obscure single-channel gating and often may require low-pass filtering of the current record to a bandwidth of a few hundred hertz or less. This heavy filtering may obscure rapid gating fluctuations, and it badly distorts the current response to a voltage step. Unlike gigohm-seal (gigaseal) patch-clamp recording, thermal noise currents in either the large value (*e.g.*, 50 GΩ) feedback resistor of a resistive headstage amplifier or in the seal resistance are generally insignificant for bilayer recordings. Rather, the majority of the background noise in a planar bilayer recording system results from "voltage clamping" across the bilayer capacitance (C_b) voltage noise present in the following sources: (1) the FET input of the patch amplifier, (2) the voltage command circuitry, and (3) the access resistance R_a, *i.e.*, the resistance of the solutions and electrodes in series with the bilayer.

The spectral density of the current noise $S^2_{i(f)}$ produced by voltage clamping a noise voltage in either the FET input or the voltage command across the bilayer capacitance is given by Ohm's law,

$$S^2_{i(f)} = \frac{e_n^2}{X_c^2} \qquad (6)$$

where e_n is the rms noise voltage (V/√Hz) and X_c is the capacitive reactance of the bilayer. The capacitive reactance is $1/(2\alpha C_b f)$, where f is the frequency and C_b is the bilayer capacitance. Therefore,

$$S^2_{i(f)} = e^2_n(2\pi C_b f)^2 \quad (7)$$

The spectral density of the current noise produced by voltage clamping the thermal voltage noise in the access resistance R_a across the bilayer capacitance is

$$S^2_{i(f)} = 4kT\,\mathrm{Re}\{Y(f)\} \quad (8)$$

where Re{*Y*(f)} is the real part of the admittance of R_a in series with C_b, and is given by

$$\mathrm{Re}\{Y(f)\} = \frac{\alpha^2}{R_a(1+\alpha^2)} \quad (9)$$

with $\alpha = 2\pi f R_a C_b$. For a constant bandwidth, at the lower limit of R_a (small α), $\mathrm{Re}\{Y(f)\} = R_a(2\pi C_b f)^2$, and equations (7) and (8) have a similar form. At the upper limit of R_a (large α), $\mathrm{Re}\{Y(f)\} = 1/R_a$. The transition from a linear to a reciprocal dependence on R_a produces maxima in plots of this noise component against R_a (see Figure 2A of Ref. 1).

Strategies for Minimizing the Background Current Noise

Reduce the Bilayer Capacitance. The single most effective approach to reducing the background current noise is to reduce the bilayer diameter and hence its capacitance. This assumes that the access resistance is not increased (see Ref. 1 and below (Assembling a Bilayer Setup for High-Resolution Recordings: Choosing a Recording Chamber) for a discussion of open chamber versus pipette/bilayer configurations). There is, however, a practical limit to the extent of reduction as the bilayer diameter must be large enough both to allow easy visualization and physical manipulation and to ensure an acceptable rate of channel incorporation. Bilayer diameters in the 40-60 µm range (having 10-25 pF C_b at 0.8 µF/cm^2) represent a reasonable compromise between the competing needs of reducing the capacitance versus ease of channel incorporation and manipulation. A very good method for making these small bilayers is the shaved-aperture method, which is described in detail below (*Assembling a Bilayer Setup for High-Resolution Recording: Making a Small Aperture*).

Use an Amplifier with Low Internal Noise Voltage. Noise voltages are present in the FET input of the headstage and in the circuitry with which voltage command inputs are buffered and applied to the bilayer. Reduction of the noise voltage in these internal sources is difficult for the investigator to control without being involved in the design and construction of the amplifier. However, when selecting an amplifier, the magnitude of the internal noise voltage sources can be quickly but qualitatively determined by comparing the noise in the current output before and after connection of the amplifier input through a bilayer-sized capacitor to ground. This test should be done *without* the addition of any resistance in series with the test capacitor to avoid confusing an internal noise voltage source with voltage noise in the access resistance. It should be noted that amplifiers which exhibit low noise when used for patch recording (where the input capacitance is small and the

background noise is dominated by thermal noise currents in the feedback resistor and seal resistance) can perform poorly when the input capacitance is increased from patch to bilayer values, because of high internal voltage noise.

Use Low-Noise Voltage Command Sources. Although many commercially available amplifiers are equipped with internal voltage command generators, voltage-step protocols will usually require the generation of the voltage command from an external source, usually a computer-driven digital-to-analog (D/A) converter. This can present a problem if the D/A source has a significant noise voltage. This is very likely if the board containing the D/A is mounted internally in a computer, using the computer's power supply. The best configuration is an outboard D/A unit, with optically isolated digital connections and its own, isolated power supply. Once again, a D/A source that provides perfectly suitable voltage commands for patch clamping can fail miserably when used to provide voltage commands for bilayer recordings, because of the much greater sensitivity to voltage noise in the voltage command source. One should also be careful about using digital function generators for producing command voltages such as voltage ramps, as they can introduce considerably more noise than analog function generators.

Minimize the Access Resistance. Even with perfect, noise-free electronics, considerable background current noise can be generated when the thermal voltage noise in the access resistance is voltage clamped across the bilayer capacitance. This source can account for as much as one half the noise under many bilayer recording conditions (Wonderlin *et al.*, 1990). The access resistance responsible for generating the voltage noise is composed primarily of the resistance of the solution within the aperture in which the bilayer is formed and the convergence resistances between each end of the aperture and the bulk solutions (see Ref. 1 for more details). Because the resistance of these solutions is inversely related to the salt concentration, it may be possible to reduce the intra-aperture and convergence resistances by increasing the salt concentration, within the limits of the experimental design. Furthermore, the shaved-aperture method can be used to minimize the length of the aperture and, therefore, its resistance. The resistance of the bulk solution should not contribute appreciably to the access resistance when an open chamber design is used; but it can be an important factor if large bilayers are made on the tips of glass pipettes (Wonderlin *et al.*, 1990). Finally, if salt bridges are used to connect the Ag/AgCl pellets to each bath, they should be designed with a low resistance in mind, such as by using relatively large diameter (*e.g.*, 2 mm) glass capillaries filled with a high-salt solution such as 3 M KCl.

Choice of Chamber Construction Materials. When selecting a type of plastic from which to fabricate a recording chamber, one should consider, in addition to factors such as machinability, durability and solvent resistance, the fact that some plastics generate excess current noise when a hole in the plastic is filled with a salt solution and connected to the input of a patch amplifier. For example, when an Axopatch-1B amplifier was connected via a Ag/AgCl electrode to a 0.5 ml well filled with 3 M KCl, the following relative rms noise levels (30 Hz -5 kHz) were measured: high-density polyethylene (1.0), polystyrene (1.0), polycarbonate (1.0), Teflon (1.0), acrylic (1.4) and nylon (1.5) (Wonderlin *et al.*, 1990). Although the absolute amount of this excess noise is small relative to other sources listed above, this source of noise can be eliminated easily by selecting an appropriate plastic.

Type of Solvent in a Painted Bilayer. We have found that the type of solvent present in a painted bilayer can affect the background current noise. If a bilayer is painted from phospholipids dissolved in decane, the background noise can often be reduced by rewiping the bilayer with hexadecane alone. The reason for the improvement is not known and appears somewhat paradoxical, since it is usually associated with a slightly larger bilayer capacitance.

Maximizing the Bandwidth

Minimization of the background noise currents by one or more of the methods described above may help increase the usable bandwidth by decreasing the amount of low-pass filtering required to clearly discern single channel gating. If the low-pass filter cutoff (-3 dB) frequency is increased to several kilohertz or more, a second, less obvious source of bandwidth limitation may come into play. This limitation is the decreased efficiency of the high-frequency boost circuit used in resistive-headstage amplifiers due to loading the headstage input with large bilayer capacitance. The boost circuit is designed to restore the bandwidth that is lost because of low-pass filtering (< 100 Hz) by the parallel combination of the feedback resistor and its stray capacitance. The high-frequency boost circuit adds a scaled derivative of the headstage current to the raw headstage current, thereby increasing the headstage bandwidth. For patch recording, a boost circuit can work well because of the dominant role of fixed electrical elements, such as the feedback resistance and stray feedback capacitance, whose properties can be optimally compensated in the selection of circuit components. When a resistive headstage amplifier is used for bilayer recordings, however, the loss of bandwidth due to the variable, and frequently large, bilayer capacitance must also be restored. This is much more difficult because the optimal amount of high-frequency boost that is required will change in parallel with any change in bilayer capacitance. Therefore, it is difficult to select components in the design of the amplifier that enable optimal boosting across a large range of bilayer capacitance values. Furthermore, because the bilayer capacitance will often vary by 50% within a day's experiments, it may be very difficult to maintain optimal boost adjustment.

Strategies for Maximizing the Bandwidth

Use a Capacitive-Headstage Patch Amplifier. An integrating-headstage patch-clamp amplifier (*e.g.*, the Axopatch 200) measures the patch current as the integral of the current flow across a feedback capacitor, rather than as the voltage drop across a feedback resistor. A boost circuit is not required for an integrating-headstage amplifier because the feedback capacitor does not produce any significant low-pass filtering of the patch current. Integrating headstage amplifiers therefore provide a broader recording bandwidth that is also independent of the size of the bilayer capacitance.

Resolving Voltage Steps Across Bilayers

A change of voltage across a bilayer of capacitance C_b by an amount V_{step} requires the movement across the bilayer of electrical charge Q_b where

$$Q_b = V_{step} C_b \qquad (10)$$

For a resistive-headstage amplifier, the amount of time required to move Q_b is

$$\text{Charging time} = \frac{Q_b R_f}{V_{max}} \qquad (11)$$

where V_{max} is the maximum voltage that can be applied across the feedback resistor R_f. For example, a headstage with a 50 GΩ feedback resistor and a V_{max} of 10 V can inject charge at a maximum rate of 200 pA, requiring 25 ms to change the voltage across a 100 pF bilayer by 50 mV. During this 25 ms period, the output of the amplifier will be saturated (at V_{max}) and the bilayer will not be voltage clamped. This example illustrates that the primary difficulty associated with applying voltage steps to a bilayer is the requirement for rapid movement of a large amount of charge across the bilayer. For traditional resistive-headstage patch amplifiers this is a nearly insurmountable problem because of the very slow rate at which charge can be delivered to the bilayer capacitance through the large feedback resistance.

A secondary problem is that planar bilayers do not behave as ideal capacitors. The application of a voltage step to a planar bilayer compresses the bilayer (*i.e.*, causes electrostriction). This electrostrictive force can change the relative areas of the bilayer and surrounding annulus, producing a concomitant change in the capacitance of the bilayer. During the period of time that the bilayer capacitance is changing, a capacitive current will flow across the bilayer. Electrostrictive changes in bilayer capacitance are relatively slow (milliseconds to seconds) and rarely large enough to produce capacitive currents that obscure single-channel gating. However, the electrostrictive capacitive current tends to be variable in magnitude and kinetics and it can change significantly as a bilayer "ages" during the course of an experiment. This variability can make it difficult to subtract the electrostrictive component of the capacitive current from the current record.

Strategies for Resolving Voltage Steps Across Bilayers

Reduce the Size of the Bilayer and Annulus. An essential first step is to make small bilayers, because reducing the bilayer capacitance proportionally decreases the charging time. Unfortunately, reducing the bilayer diameter into the 40 - 60 µm range (10 -25 pF) may still result in an excessive saturation time (several milliseconds). Because the magnitude of the electrostrictive change in capacitance depends on the area of the annulus, decreasing the bilayer diameter also reduces this component. The width of the annulus (*i.e.*, in the plane of the bilayer) is also roughly proportional to the thickness of the margin of the aperture. A substantial, further reduction in the electrostrictive component can be achieved by using the shaved-aperture method, because it produces a margin of the aperture that is very thin (a few micrometers). Other techniques, such as drilling or punching apertures, produce a margin that is much thicker. Finally, any manipulation of the bilayer that produces a "bulkier" annulus, such as addition of excess solvent or repeated wiping, can greatly increase the electrostrictive component and should be avoided.

Use Capacity Compensation. The capacity compensation circuits in most commercially available patch clamps are only slightly effective in reducing the duration of saturation because they are designed to compensate the relatively small capacitance of a patch pipette rather than the much larger capacitance of a planar bilayer. Special circuits capable of compensating the larger bilayer capacitance can be added to the headstage, but they require the injection of compensating current into the amplifier input through a large capacitor,

which increases the total input capacitance and degrades the noise performance. Sometimes, the electrostrictive capacitive current can be partially subtracted by adjustment of a capacity compensation circuit with a slow time constant.

Decrease the Feedback Resistance. The charging time can be reduced by decreasing the value of R_f, thereby increasing the rate at which charge can be delivered to the bilayer. However, the price for this improvement is a proportional increase in the background thermal noise currents in R_f and, therefore, a concomitant decrease in usable bandwidth. Although this may work for large conductance channels, it is hardly a general solution. A better solution is to include both a large (50 GΩ) and a small (50 MΩ) feedback resistor in the headstage with logic-controlled circuitry that can switch between the two. This approach was taken in the CV-4B headstage from Axon Instruments. During a voltage step, the bilayer capacitance is rapidly charged through the small R_f. Immediately after the bilayer capacitance is charged, the feedback pathway is switched to the large R_f, enabling high resolution recording of single-channel activity. This approach was used successfully to study voltage-dependent activation of K^+ channels from squid (Wonderlin *et al.*, 1990; Wonderlin *et al.*, 1991).

Use a Capacitive-Headstage Patch Amplifier. Capacitive-headstage patch clamps can very rapidly charge the bilayer capacitance with a maximum instantaneously injected charge of

$$\text{Maximum charge} = V_{max} C_f \qquad (12)$$

where C_f is the feedback capacitance. With a V_{max} of 10 V and C_f equal to 1 pF, 10 pC of charge can be delivered nearly instantly. This charge is sufficient to change the voltage across a 100 pF bilayer by 100 mV without saturation of the headstage output. Because integration of a constant patch current will eventually saturate C_f, an integrating headstage requires a reset circuit that periodically discharges the feedback capacitor; this is usually a logic-controlled switch that shunts the feedback capacitor. In some designs, this same reset switch can be activated during a voltage step. Such a "forced reset" allows rapid charging of the bilayer capacitance.

II. Assembling a Bilayer Setup for High-Resolution Recordings

Choosing an Amplifier

Traditionally, resistive headstage patch amplifiers have been used in bilayer recording systems. These amplifiers are widely commercially available or they can be built rather inexpensively because of the simple design of the circuitry. Recently, integrating-headstage amplifiers have become commercially available. Their sophisticated headstage circuitry will probably prevent most researchers from building their own amplifiers. How should one choose among these amplifiers? The ideal amplifier for high-resolution bilayer recordings should have: (1) low intrinsic current and voltage noise, (2) a wide bandwidth, and (3) the ability to rapidly charge the bilayer capacitance. Although integrating headstages have less intrinsic current noise than resistive headstages, there may be little difference in noise performance during actual bilayer recordings due to the dominance in bilayer recordings of current noise produced by the interaction of the bilayer capacitance with various noise voltage sources. With regard to

bandwidth, the integrating headstage is a clear favorite because it does not require a high-frequency boost circuit. Adjustment of the high-frequency boost of a resistive headstage with a large, variable bilayer capacitance at the input can be very frustrating. On the other hand, within the 5-10 kHz bandwidth that one might hope to achieve with bilayer recordings, the bandwidth of an integrating headstage should not be affected by the bilayer capacitance. Finally, the integrating headstage is the better choice for rapid charging of the bilayer capacitance during voltage steps, although a switching-feedback resistive headstage can perform nearly as well (Wonderlin *et al.*, 1990; Wonderlin *et al.*, 1991), since it also provides a low-impedance pathway for charging the bilayer capacitance. It should be emphasized that the rapid charging by either an integrating headstage amplifier or a switching-resistive headstage amplifier does not decrease the slow, electrostrictive component of the capacitive current, whose time course is determined only by the dynamic properties of the bilayer/annulus structure.

When all of the requirements for an amplifier are considered, it is clear that anyone attempting high-resolution recording from bilayers with voltage steps should seriously consider using an capacitive-headstage amplifier.

Choosing a Recording Configuration

There are two primary recording configurations for planar bilayers: (1) a bilayer formed over an aperture in a partition separating two open bath chambers (open-chamber configuration); and (2) a bilayer formed on the tip of a polished glass pipette (pipette/bilayer configuration), either by "punching" a preformed planar bilayer or by dipping the tip through a lipid monolayer. The relative merits of these configurations have been discussed in (Wonderlin *et al.*, 1990). Briefly, for bilayer capacitance values larger than a few picofarads, the open-chamber configuration with a shaved aperture will generate less background current noise than the bilayer/pipette configuration, due to the much higher access resistance of the interior of the pipette compared to the open chamber. Therefore, if bilayers must be made large enough to permit incorporation of channels by fusion of vesicles with the bilayer, the open chamber configuration should be used. If channels can be incorporated during the formation of the bilayer (*e.g.*, "tip-dip") so that bilayers with a very small area (less than a few picofarads capacitance) can be used, then the pipette/bilayer configuration can be used. Finally, the open chamber configuration offers greater flexibility with regard to manipulation of the bilayer and bath solutions.

Making Small Apertures

It is fundamentally important to be able to work with small bilayers, usually with diameters in the range of about 40-60 µm. This size range represents a trade-off between the need to reduce the area (and capacitance) while maintaining a large enough area to ensure adequate incorporation by fusion of vesicles. Among the many techniques available for making apertures in plastic partitions, the shaved-aperture method is especially well suited for making small apertures. This method has been previously described in detail for making small apertures in plastic cups (Wonderlin *et al.*, 1990).

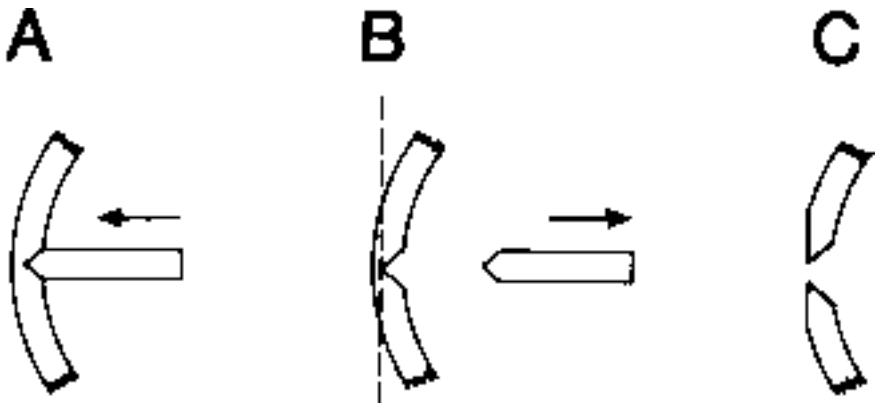

Figure 5-7. Aperture Formation in a Plastic Cup *(not drawn to scale)*

(A) A metal stylus is warmed and pressed against the inner surface of the cup, producing a cone-shaped depression. (B) After cooling, the stylus is retracted and the plastic shaved from the outer surface, leaving (C) a thin-edged aperture where the cut intersects the depression in the wall of the cup. See text for additional details. Reproduced with permission from Wonderlin *et al.*, 1990.

Briefly, a conical metal stylus is warmed and pressed against the inner surface of a plastic cup, forming a cone-shaped depression extending part way across the cup (Figure 5-7). The stylus is then removed and the plastic shaved away from the outer surface, using a disposable microtome blade, until the depression is intersected. The size of the aperture is controlled by varying the depth of shaving. Apertures made using this method have a thin margin beyond which the plastic rapidly increases in thickness, thereby decreasing the stray capacitance across the partition and providing good mechanical strength. The thin margin of the shaved aperture is very important in reducing the size of the annulus and, therefore, the electrostrictive change in capacitance following voltage steps. Following are the essential steps in implementing the method:

(1) **Making the stylus.** The key to successfully using the shaved-aperture method is the manufacture of a high-quality stylus. Using a lathe and dissecting microscope, stainless steel darning needles can be ground to a very fine tip (< 5 µm diameter) and polished to a very smooth finish using ultra-fine abrasive paper (Thomas Scientific, Swedesboro, NJ). Softer metals can be substituted, but it is more difficult to produce the highly polished tip. The stylus should be examined under high magnification (400x) to ensure that the tip is *very smooth*.

(2) **Selecting the plastic.** The original description of the shaved-aperture technique (Wonderlin *et al.*, 1990) recommended polystyrene as a good plastic for recording cups. Since that time, repeated difficulties have been encountered with crazing of the margin of apertures made in polystyrene cups, which sometimes appears to make the cups electrically leaky. More

recently, shaved apertures in cups were made from Ultra-Clear centrifuge tubes (Beckman, Colombia, MD). Also, if a flat partition rather than a cup is preferred, shaved apertures have been formed in plastic coverslips (Fisher Scientific, Pittsburgh, PA). The margin of these apertures does not craze and bilayers formed on apertures shaved in these plastics are very stable, lasting several hours. Other plastics can probably be substituted, with the basic requirement being that they cut cleanly so that the margin of the aperture is very smooth.

(3) **Melting the plastic.** There are many choices of mechanical apparatus for manipulating and heating the stylus. A rather simple method is to mount the stylus in a hole in the tip of a variable temperature soldering iron, and then to mount the soldering iron on a manipulator so that the tip can be carefully pushed into the plastic. Control of the heat is important to ensure replicability and to avoid overheating, which may damage the plastic and, perhaps, the stylus.

(4) **Shaving the aperture.** It is easiest to shave the apertures while observing with a dissecting microscope. The shaved apertures should be examined under high magnification (400x) before use to ensure they are free from deformations that might interfere with bilayer stability.

Viewing with a Microscope

It is not practical to attempt to work with small bilayers without observing them with a microscope. Bilayers are usually observed under reflected light because the disappearance of reflected light provides a good monitor of bilayer thinning. Quite often, the bilayer is viewed through one eyepiece of a dissecting microscope, while the bilayer is illuminated by light focused from a fiber bundle through the second eyepiece. This method is not useful for small bilayers because of the lack of detail visible with reflected light and the relatively low magnification (40-80x) of most dissecting microscopes. Using a horizontally mounted compound microscope (Nikon Inc., Garden City, NY) with a long working-distance objective (Nikon, E-Plan 10x) and a bilayer recording chamber designed for transillumination provides very good resolution of detail in the bilayer and annulus at a magnification of 100x.

Testing the System

The performance of the recording system should be tested using a dummy bilayer, especially if voltage steps are to be used. The dummy bilayer should use a capacitor with a value close to that expected for the bilayer capacitance. It may also be useful to add a resistor in series with the capacitor to mimic the access resistance. In choosing a capacitor, polystyrene capacitors are preferred. Some other types of capacitors, such as ceramic capacitors, can exhibit very non-ideal properties, with odd relaxations similar to the electrostrictive relaxation of planar bilayers. By simulating a bilayer recording using the dummy bilayer, it is possible to determine the dependence of the background noise and voltage-step response on electrical components with ideal electrical properties, as opposed to the non-ideal electrical properties of components used during experiments, such as the bilayer composition and salt solutions. It also permits optimization of the timing of voltage steps relative to logic-controlled switching between different feedback resistors in a resistive headstage amplifier or the timing of a forced reset in a capacitive headstage amplifier.

Making the Recordings

An example of the voltage-step activation of a delayed rectifier K^+ channel from squid is shown in Figure 5-8. The ultimate success of high-resolution recording in planar bilayers depends not only on the various details described above, but also on the ease of fusion of certain populations of membrane vesicles. Rates of incorporation are likely to be lower for small bilayers than are typical for larger bilayers, but the difference may be reduced by methods such as pressure application of vesicles from a pipette onto the bilayer. Although the high-resolution recording method may increase the difficulty beyond that of traditional bilayer recording techniques, it opens exciting opportunities for new experimental designs and the investigation of a broader range of ion channels.

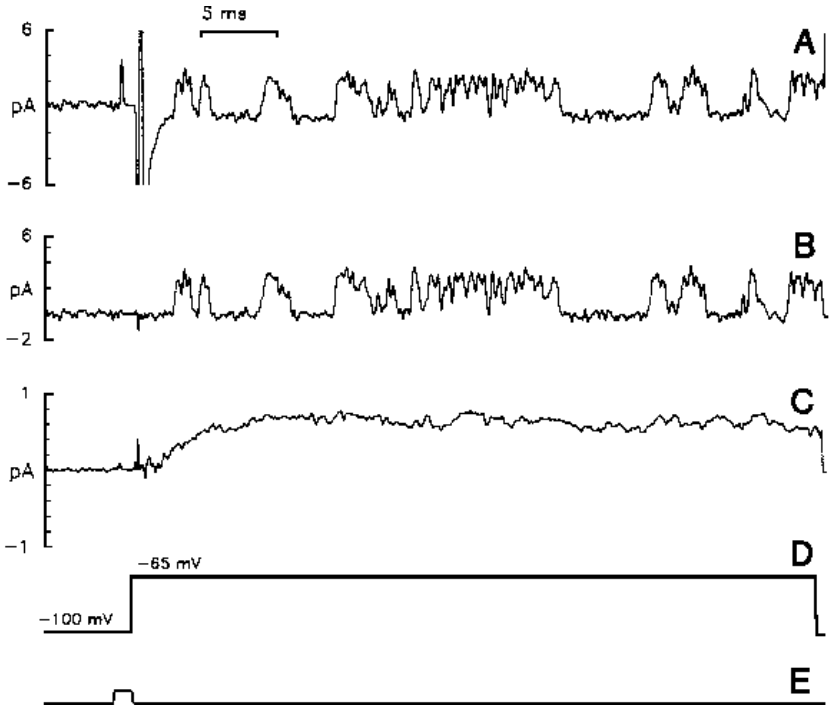

Figure 5-8. Voltage-Step Activation of a K-Channel from Squid Giant Axon Axoplasm

(A) A single, unsubtracted current record showing the response to a voltage step. In A-C, the current is actually inward, but is shown inverted to be consistent with the usual orientation of K-current records. The gain was switched to low 1 ms before the voltage step, producing a small current transient (logic pulse is shown in trace E). The gain was switched back to high 25 µs after the voltage step. (B) Current record from which the capacitive current and switching artifact have been digitally subtracted. Blank traces (7 traces in which the channel was not active) were averaged and subtracted from the active trace. Subtraction is very effective in removing the step artifact, and high-resolution recording is established within 1 ms after the voltage step. (C) An average current record simulating a macroscopic response. 240 current traces were averaged, from which the average of the blank records was subtracted, as in B. (D) Voltage command trace. (E) Logic pulse used to switch the headstage gain. The headstage was switched to low gain during the rectangular pulse. Reproduced with permission from Wonderlin *et al.*, 1990.

Further Reading

Wonderlin, W.F., Finkel, A., French, R.J. *Optimizing planar lipid bilayer single-channel recordings for high resolution with rapid voltage steps.* Biophys. J., 58: 289-297, 1990.

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