Chapter 1

A Practical Guide to Patch Clamping

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1. Introduction

The patch-clamp technique is an extremely powerful and versatile method for studying electrophysiological properties of biological membranes. Soon after its development by Erwin Neher and Bert Sakmann, it was adopted by numerous laboratories and subsequently caused a revolutionary advancement of many research areas in both cellular and molecular biology. Not surprisingly, the developers of this technique were awarded the highest scientific recognition. The Nobel Assembly in Stockholm issued the following press release on 7 October 1991:

> The Nobel Assembly at the Karolinska Institute has today decided to award the Nobel Prize in Physiology or Medicine for 1991 jointly to Erwin Neher and Bert Sakmann for their discoveries concerning "The Function of Single Ion Channels in Cells."
> Each living cell is surrounded by a membrane which separates the world within the cell from its exterior. In this membrane there are channels, through which the cell communicates with its surroundings. These channels consist of single molecules or complexes of molecules and have the ability to allow passage of charged atoms, that is, ions. The regulation of ion channels influences the life of the cell and its functions under normal and pathological conditions. The Nobel Prize in Physiology or Medicine for 1991 is awarded for the discoveries of the function of ion channels. The two German cell physiologists Erwin Neher and Bert Sakmann have together developed a technique that allows the registration of the incredibly small electrical currents (amounting to a picoampere—10^{-12} A) that passes through a single ion channel. The technique is unique in that it records how a single channel molecule alters its shape and in that way controls the flow of current within a time frame of a few millionths of a second.
> Neher and Sakmann conclusively established with their technique that ion channels do exist and how they function. They have demonstrated what happens during the opening or closure of an ion channel with a diameter corresponding to that of a single sodium or chloride ion. Several ion channels are regulated by a receptor localized to one part of the channel molecule which upon activation alters its shape. Neher and Sakmann have shown which parts of the molecule constitute the "sensor" and the interior wall of the channel. They also showed how the channel regulates the passage of positively or negatively charged ions. This new knowledge and this new analytical tool has during the past ten years revolutionized modern biology, facilitated research, and contributed to the understanding of the cellular mechanisms underlying several diseases, including diabetes and cystic fibrosis.

Since the first demonstration of single channels in a biological membrane using this methodology (Neher and Sakmann, 1976), several key improvements have refined its use

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Single-Channel Recording, Second Edition, edited by Bert Sakmann and Erwin Neher. Plenum Press, New York, 1995.

and applicability to virtually all biological preparations (including animal and plant cells, bacteria, yeast, and cell organelles). The development of the "gigaseal" (Sigworth and Neher, 1980; Neher, 1982) and the establishment of the various recording configurations ("cell-attached"/"inside-out"/"outside-out"/"whole-cell") allowed patch recording from the cell surface or cell-free membrane patches as well as intracellular recordings (Hamill *et al.*, 1981). In recent years, even more technical and experimental variations of the patch-clamp method have emerged and further expanded its power to address previously unapproachable questions in cell biology. Today, the patch clamp is the method of choice when it comes to investigating cellular and molecular aspects of electrophysiology. At present, more than 1000 scientific articles employing the patch-clamp technique are published each year. Paired with cell and molecular biological approaches (e.g., protein chemistry, cloning and expression techniques, microfluorimetry), the patch-clamp technique constitutes an indispensible pillar of modern cell biology. Comprehensive reviews on the development of the patch-clamp technique and its applications can be found in the Nobel lectures given by Neher and Sakmann (Neher, 1992b; Sakmann, 1992) and other review articles (Sakmann and Neher, 1984; Sigworth, 1986; Neher, 1988; Neher and Sakmann, 1992).

Given the rapid pace at which molecular biology advances and the wealth and complexity of cell proteins interacting with each other to bring about cellular function, the potential use of the patch-clamp technique is still growing and attracting newcomers into either collaborating with patch-clampers or establishing this area of research in their own laboratories. This chapter is designed to be a practical guide to patch-clamping for newcomers and a starting point for students entering a patch-clamp laboratory. It discusses the very basic features of the patch-clamp technique, how a patch-clamp setup functions, what type of equipment is needed, some basic experimental procedures, and what the potential problems with this technique are. This chapter is not meant to discuss these topics fully, as there are numerous well-written and detailed descriptions of each of these points available in the literature. The reader who is interested in introductory reading material relevant to various aspects of modern electrophysiology is directed to some excellent books on the biology of ion channels (Hille, 1992) and on patch-clamp methodology (Kettenmann and Grantyn, 1992; Rudy and Iverson, 1992; Sherman-Gold, 1993). Some of the topics introduced in this chapter are discussed in more detail in individual chapters of this volume.

2. Patch-Clamp Techniques

Originally, the patch-clamp technique referred to voltage-clamp of a small membrane patch, but it now generally refers to both voltage-clamp and current-clamp measurements using "patch-clamp"-type micropipettes. The patch-clamp technique is an electrophysiological method that allows the recording of macroscopic whole-cell or microscopic single-channel currents flowing across biological membranes through ion channels. Active transporters may also be studied in cases where they produce measurable electrical currents, that is, if the transport is not electroneutral (e.g., Na^+–Ca^{2+} exchanger, amino acid transporters). The technique allows one to experimentally control and manipulate the voltage of membrane patches or the whole cell (voltage clamp), thus allowing the study of the voltage dependence of ion channels. Alternatively, one may monitor the changes in membrane potential in response to currents flowing across ion channels (current clamp), which constitute the physiological response of a cell (e.g., action potentials). Thus, the main targets of patch-clamp investigations are membrane-contained ion channels, including voltage-dependent ion channels (e.g., Na^+,

K^+, Ca^{2+}, Cl^- channels), receptor-activated channels (e.g., those activated by neurotransmitters, hormones, mechanical or osmotic stress, exogenous chemical mediators), and second-messenger-activated channels (e.g., those activated by $[Ca^{2+}]_i$, cAMP, cGMP, IP_3, G proteins, or kinases). Indeed, the gating mechanisms or the presence in certain cell types of many of these channels has been discovered as a direct consequence of using the patch-clamp technique in previously inaccessible preparations.

Other electrical parameters may be monitored as well, most notably the cell membrane capacitance, which is indicative of the plasma membrane surface area. The quantification of membrane area not only allows the determination of current densities but the time-resolved monitoring of cell capacitance may also be used to assess exocytotic and endocytotic activity of single secretory cells (Neher and Marty, 1982; Lindau and Neher, 1988; see also Chapter 7, this volume).

With the opportunity afforded by the whole-cell configuration to selectively perfuse and dialyze cells intracellularly with any desired biological or pharmacological probe while monitoring its effects on cell function, the possible applications of the technique are limited only by the ingenuity of the experimental design. In combination with additional techniques (e.g., microfluorimetry, amperometry), as discussed in Chapters 9 and 11 (this volume), one can correlate the measurements to events not amenable to electrophysiological techniques. Furthermore, with the development of powerful molecular biological tools (e.g., polymerase chain reaction, mRNA amplification) it is even feasible to obtain relevant genetic information from the very same cell that was characterized electrophysiologically by extracting the cytosol into the patch pipette and analyzing it later on as described in Chapter 16 (this volume).

2.1. The Patch-Clamp Configurations

The basic approach to measure small ionic currents in the picoampere range through single channels requires a low-noise recording technique. This is achieved by tightly sealing a glass microelectrode onto the plasma membrane of an intact cell, thereby isolating a small patch. The currents flowing through ion channels enclosed by the pipette tip within that patch are measured by means of a connected patch-clamp amplifier. This so-called "cell-attached" configuration is the precursor to all other variants of the patch-clamp technique. The resistance between pipette and plasma membrane is critical for determining the electrical background noise from which the channel currents need to be separated. The seal resistance should typically be in excess of 10^9 Ω ("gigaseal").

The cell-attached configuration may be used (as such) to record single-channel activity, or one may proceed to isolate the patch from its environment by withdrawing the pipette from the cell. This usually retains the integrity of the gigaseal pipette–patch assembly and allows one to study ion channels in the excised patch configuration. This configuration is called "inside-out" because the cytosolic side of the patch now faces the outside bath solution.

As an alternative to excising the patch, one can simply break the patch by applying a pulse of suction through the patch pipette, thereby creating a hole in the plasma membrane and gaining access to the cell interior. Amazingly, this maneuver does not compromise the gigaseal between pipette and plasma membrane. The tightness of the gigaseal both prevents leak currents flowing between the pipette and the reference electrode and prevents flooding of the cell with the constituents of the bath solution. This configuration is characterized by a low-resistance access to the cell interior through the pipette tip (typically a few megohms with tip diameters of about 1 μm and appropriate pipette solutions), allowing one to voltage-clamp the whole cell ("whole-cell" configuration).

From the whole-cell configuration, one may proceed further by withdrawing the pipette from the cell. This will generally result in resealing of both the plasma membrane of the cell and the patch at the pipette tip. This time, however, the geometric orientation of the patch results in the outside of the membrane facing the bath solution ("outside-out" patch).

2.2. Applications, Advantages, Problems

In the very early days of patch clamp, most studies concentrated on the classification of ion channel types in different cells and the characterization of their biophysical properties in terms of conductance, voltage dependence, selectivity, open probability, and pharmacological profile. It was soon realized, however, that cells had a large variety of ion channels (even for the same ion species), and complementary whole-cell measurements were necessary to reveal the relative importance and the physiological role of a given ion channel for the entire cell. Today, whole-cell recordings are the most popular patch-clamp configuration, and single-channel measurements are performed to complement the whole-cell results.

Which patch-clamp configuration is chosen as the experimental paradigm depends on the type of question to be addressed and the kind of ion channel under study. Each of the configurations has its peculiarities, advantages, and disadvantages.

2.2.1. Cell-Attached Recording

This is mainly used when the channel type in question requires unknown cytosolic factors for gating and these would be lost following patch excision. Also, because this configuration is noninvasive, leaving the ion channel in its physiological environment, it may be used to test for possible alterations of channel properties after patch excision. Another important application for cell-attached recording is to determine whether a particular ion channel is gated by a cytosolic diffusible second messenger. In this type of experiment, the ion channel enclosed in the cell-attached patch is isolated from the bath (by the pipette) and cannot be gated directly by the receptor agonist. If ion channel activity in the patch changes subsequent to addition of an agonist to the bath, then it is clear that some intracellular messenger must have been generated and diffused in the cytosol to gate the ion channel in the patch. Generally, the main disadvantages of the cell-attached configuration are the lack of knowledge of the cell membrane resting potential (which adds to the applied pipette potential) and the inability to effectively control and change the ionic composition of the solutions on both sides of the patch during the measurement.

2.2.2. Inside-Out Recording

This configuration enables one easily to change the cytosolic side of the patch. It is therefore the method of choice to study the gating of second-messenger-activated channels at the single-channel level. Because most channels are modulated in one way or another by intracellular processes, the effects of cytosolic signaling molecules or enzymatic activity on channel behavior can be studied using this configuration. Commonly, the main problems with inside-out recordings arise from the loss of key cytosolic factors controlling the behavior of some ion channels. Also, more often than with outside-out patches, there is the chance of obtaining vesicles in the pipette tip rather than planar patches.

2.2.3. Outside-Out Recording

This configuration allows one easily to change the extracellular side of the patch. It is therefore often used to study receptor-operated ion channels. As for the inside-out configuration, the cytosolic environment of the channels is lost on patch excision. Furthermore, high-quality and stable outside-out recordings are more difficult to obtain, because more steps are required to reach the outside-out configuration (from cell-attached to whole-cell to patch excision). In order for the patch to be excised successfully, cells need to adhere very well to the bottom of the recording chamber.

2.2.4. Whole-Cell Recording

This configuration is employed when ion currents of the entire cell are recorded. This method is essentially like an outside out recording with the advantage of recording an average response of all channels in the cell membrane. The whole-cell configuration is also suitable to measure exocytotic activity of secretory cells by measuring cell membrane capacitance. The main disadvantages again are the possible loss of cytosolic factors and the inability to change the cytosolic solution easily without pipette perfusion.

2.3. Special Techniques

Several modifications of the above configurations have been developed in order to overcome some of their limitations or to perform certain experiments that cannot readily be accomplished with the standard patch-clamp configurations.

2.3.1. Perforated Patch Recording

One of the major problems in patch clamp is the washout of cytosolic constituents following patch excision or dialysis of cells during long-lasting whole-cell measurements (Pusch and Neher, 1988). Several methods may be used to alleviate these problems (see Horn and Korn, 1992, for review). The perforated patch technique aims at retaining the cytosolic constituents by selectively perforating the membrane patch by including channel-forming substances in the pipette solution, e.g., ATP (Lindau and Fernandez, 1986), nystatin (Horn and Marty, 1988), and amphotericin B (Rae *et al.*, 1991). Although the channels formed in the patch essentially allow a low-resistance access to the cell comparable to that in the standard whole-cell configuration, the small size of these channels allows passage of only small ions, thus preventing the washout of cytosolic factors. A variation of this technique is provided by the perforated vesicle configuration (Levitan and Kramer, 1990), in which the pipette is withdrawn from the cell to obtain a small vesicle retaining many of the cytosolic constituents. The vesicle membrane facing the pipette interior is perforated and allows recording of single channels from the outside-out patch facing the bath solution. The main tradeoffs of this technique are the long time required to obtain low-resistance access to the cell, the larger noise associated with the recordings, possible osmotic effects, and the inability to effectively control the cytosolic environment.

2.3.2. Double Patch Recording

This method is used to study gap-junction channels in intercellular communication (Neyton and Trautmann, 1985; Veenstra and DeHaan, 1986). The method is basically a paired whole-cell measurement in two connected cells in which synchronously occurring signals correspond to channel activity of junctional channels between the two cells (for review, see Kolb, 1992).

2.3.3. Loose Patch Recording

Focal recordings with large-diameter pipettes (Strickholm, 1961; Neher and Lux, 1969) may be considered a predecessor of the tight-seal patch-clamp technique, comparable to a cell-attached recording with a large-diameter pipette tip (normally 5–20 μm), where the seal resistance is only a few megohms. The so-called loose patch technique is an improved variant of this method in which special electronic enhancements such as leak compensation or the use of special pipettes with two concentric patch tips allow large currents to be measured (for reviews see Roberts and Almers, 1992; Stühmer, 1992). This approach is mainly used to map the distribution of ion channels and current densities of large cells (e.g., muscle cells, giant axons).

2.3.4. Giant Patch Recording

Normally, gigaseal formation becomes increasingly more difficult as the size of the pipette tip is increased. However, when special hydrocarbon mixtures are applied to the rims of large-tipped patch pipettes (tip diameters of 10–40 μm), giant patches with gigohm seal resistances can be obtained (Hilgemann, 1990; see also Chapter 13, this volume). Much like the loose patch technique (yet with high seal resistance), this method is basically a cell-attached configuration and may be used to study macroscopic currents through ion channels or transporters.

2.3.5. Detector-Patch Recording

Ion channels can be exquisitely sensitive detectors of neurotransmitters or second messengers, and this method takes advantage of such ion channels. For example, excising an outside-out patch containing a neurotransmitter detector channel and placing it close to a synaptic structure releasing the appropriate transmitter may be used to monitor synaptic activity (Hume *et al.*, 1983; Young and Poo, 1983; for review see Young and Poo, 1992). Another application is to excise an inside-out patch containing Ca^{2+}-activated K channels and "cramming" the patch into a large cell such as an oocyte to monitor changes in $[Ca^{2+}]_i$ (Kramer, 1990).

2.3.6. Pipette Perfusion

One of the major problems in any patch-clamp configuration is the difficulty of exchanging the pipette solution. Several methods have been designed to achieve an effective pipette perfusion (e.g., Soejima and Noma, 1984; Lapointe and Szabo, 1987; Neher and Eckert,

1988; Tang *et al.,* 1990). They all share the basic principle of having one or more capillary tubes (polyethylene or quartz) inserted into the patch pipette and placed as close as possible to the pipette tip. By means of gentle pressure, the desired solution within the capillaries is expelled and replaces the original pipette solution within seconds to minutes.

2.3.7. Tip-Dip Bilayer Recording

This method is basically a modification of the lipid bilayer technique used to study reconstituted ion channels in a small patch environment. The pipette tip is repetitively immersed in a monolayer lipid film to produce a lipid bilayer at the pipette tip (Coronado and Latorre, 1983; Suarez-Isla *et al.,* 1983). The main advantage consists of minimizing capacitative artifacts of conventional bilayer experiments.

3. The Patch-Clamp Setup

The diversity of experimental preparations and types of experiment that can be studied with the patch-clamp technique is reflected by the variety of patch-clamp setups used in various laboratories. The expansion of the patch-clamp methodology has been paralleled by a growth of the commercial supply industry and availability of patch-clamp equipment. Although there are some basic features of instrumentation common to all functional patch-clamp workstations, in practice, there is no such thing as the "standard" patch-clamp setup. Every laboratory has its own equipment preferences, quite often supplemented by custom-built devices and/or modified commercial instruments.

As desirable as it might seem to the newcomer, it would be impossible to list, describe, or even evaluate all of the available instrumentation for patch clamp in this chapter. Also, the pace at which introduction and modification of available hardware and software occurs would make this attempt useless at the time of publication of this volume. However, the author is willing to compile a fairly complete and continuously updated list of the available patch-clamp equipment. This list is currently being compiled and will be made accessible in electronic form on a fileserver on the internet. The equipment list may be retrieved via anonymous FTP from "ftp.gwdg.de" in the directory "pub/patchclamp." The files will contain information in the form of data sheets grouped into categories corresponding to the subheadings below.

In its simplest form, a patch-clamp setup may consist of a microscope (for cell visualization) placed on a vibration isolation table within a Faraday cage, a patch-clamp amplifier and pulse generator for voltage-clamping the cells, a micromanipulator holding the amplifier probe for positioning the attached patch pipette, and data-recording devices (e.g., ocilloscope, computer, chart recorder). In addition, some instruments for pipette fabrication are required (i.e., a pipette puller and a microforge). The addition of other instruments to the setup can extend the range of possible applications, increase the efficiency, or simply make certain tedious tasks more convenient. An example of a patch-clamp setup (as used in our laboratory) is shown in Fig. 1. There are many variants for all of the devices described below, and there is no strict objective criterion that may be applied to make the right choice. The only advice that can be given here is to collect from colleagues as much information as possible about experiences with the equipment in question. Some detailed discussion of advantages and disadvantages of certain types of equipment and instrumentation are also found in the literature

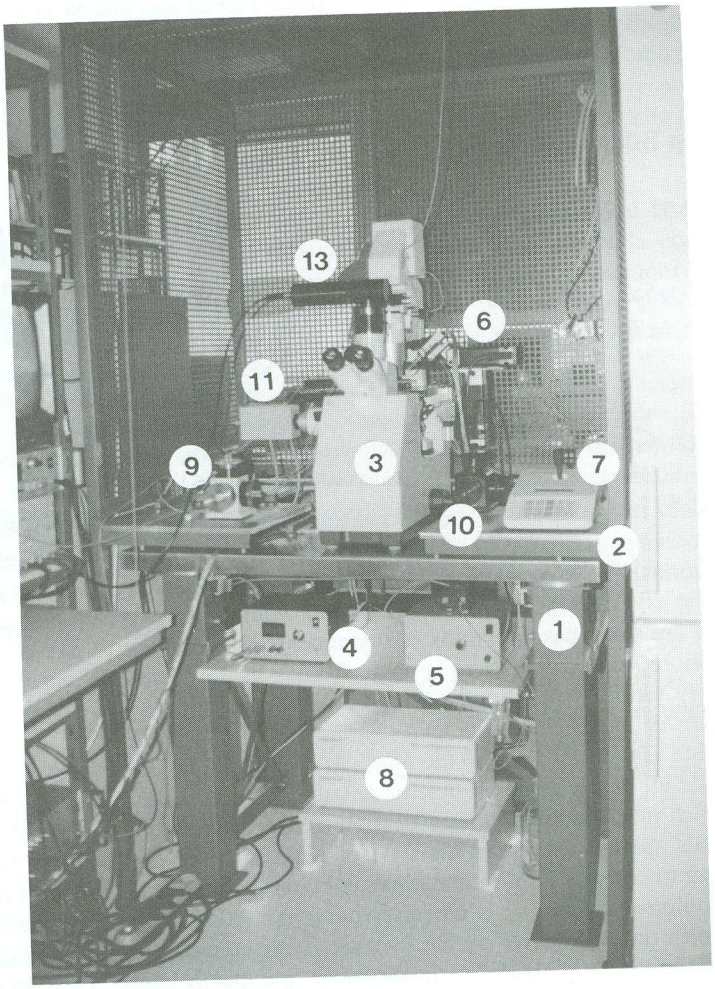

Figure 1. An example of a patch-clamp setup. The two panels show the instrument rack (left panel) and the patch-clamp workstation inside a Faraday cage (right panel). This particular setup is used in our laboratory and is equipped with the following instruments: (1) vibration isolation table (Physik Instrumente T-250); (2) supertable with Faraday cage (custom made); (3) inverted microscope (Zeiss Axiovert 100, fluorescence equipped) and lamp power supply (4); (5) hydraulic pump for bath perfusion; (6) motorized micromanipulator for recording pipette (Eppendorf 5171) with joystick (7) and controller units for manipulator and motorized microscope stage (8); (9) hydraulic micromanipulator for drug application pipette (Narishige WR 88); (10) hydraulic micromanipulator for second patch pipette (Newport MX630); (11) video camera (Kappa CF-6) and video monitor (12); (13) photomultiplier tube (Seefelder Messtechnik SMT ME 930); (14) patch-clamp amplifier (HEKA EPC-9); (15) oscilloscope (Philips PM 3335); (16) computer (Macintosh Quadra 800) with two monitors (17), magnetooptical disk drives (18), keyboard (19), and laser printer (20); (21) multipressure control unit (custom made); (22) function generator (Wavetek Model 19); and (23) various control instruments for dual-wavelength fluorescence excitation of fura-2.

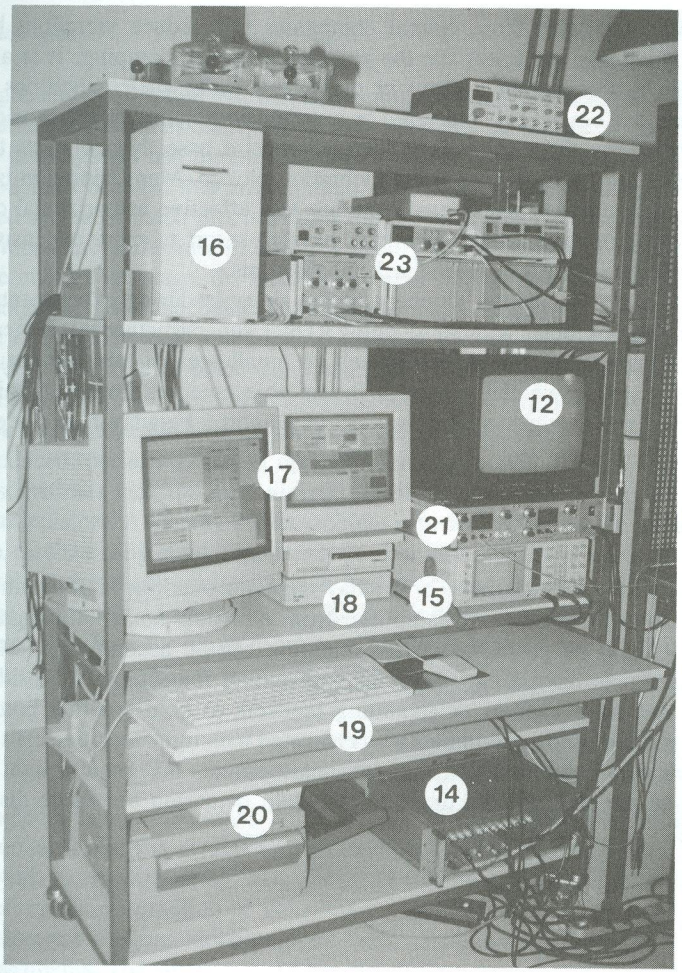

Figure 1. *Continued.*

(see, e.g., Levis and Rae, 1992; Sherman-Gold, 1993). The following section deals in short with the instrumentation of basic and advanced patch-clamp setups.

3.1. Mechanics

In most patch-clamp experiments (particularly whole-cell or cell-attached recordings from small cells), mechanical stability of all setup components must be considered crucial, as even the slightest vibrations or relative movements of the pipette/cell assembly are detrimental to stable recordings.

3.1.1. Vibration Isolation Table

Microscopic movements and vibrations are present to different degrees in all buildings and must be damped out by an appropriate vibration isolation table. Most low-end air-

suspension tables available from optical companies will reduce vibrations beyond a few Hertz, which is usually sufficient for the purpose of patch clamping. It is a good idea to place this table in a corner of a room or close to a wall, where vibrations are generally smaller. In some bad cases (e.g., plastered or wooden floors), one might consider supporting the table by replacing the elastic floor with a concrete base that connects to the support structure of the building to further reduce vibration pickup. Many vibration isolation tables preferentially damp vertical movements and are less effective at horizontal displacements. If horizontal vibrations are severe, one may have to resort to more expensive tables with isotropic properties and/or active feedback mechanisms.

In order to avoid accidental touching of the air table during experiments, it is a good idea to surround the air table (without touching it) by a superstructure that basically represents a table slightly larger than the air table itself. A small area of tabletop should be cut out such that the microscope, which is placed on the air table, emerges through it. This "supertable" could be made of galvanized steel and be used to support a Faraday cage placed on top of it.

3.1.2. Faraday Cage

Most patch-clamp setups have a Faraday cage surrounding it. Its main purpose is to shield the sensitive patch-clamp preamplifier from electrical noise. Although this is not an essential requirement (if proper electrical shielding can be accomplished otherwise), it is nevertheless useful to have a cage of some sort around the setup. The inside walls of the Faraday cage can have shelves or mounting brackets to hold solution bottles, peristaltic pumps, etc. If patch-clamp experiments are performed with light-sensitive cells or substances, or if patch clamp is combined with optical measurements that require light shielding, the outside walls of the cage can be draped with cloth or carton to reduce disturbance from ambient light.

3.1.3. Racks

Depending on the needs, a significant number of electronic instruments must be placed outside the Faraday cage, reasonably close to the recording stage. There are commercially available racks that hold standard 19-inch chassis instruments and have provisions for stacking nonstandard instruments on shelves. However, because most commercial racks do not provide for arrangement of computer keyboards, and, in practice, patch-clamp instruments rarely remain fixed in the rack, it is best to have a custom-made rack to hold the equipment. This can be constructed to provide the best ergonomic placement and allow for easy rearrangement of instruments (see Fig. 1-1).

3.2. Optics

The vast majority of patch-clamp studies are carried out on small single cells. Microscopic observation of the cells during the measurement and, even more importantly, the approach to the cell by the patch pipette for seal formation generally require a good optical visualization of the preparation.

3.2.1. Microscopes

In principle, any microscope that will allow observation of the desired cell at micrometer resolution is suitable for patch clamping as long as it allows access of the cell by a patch pipette. Most investigators use inverted microscopes for studying acutely dissociated cells or cultured cell lines because this arrangement allows both good visualization of the cells and unhindered access of patch pipettes from the top. Inverted microscopes also tend to be mechanically more stable. Because focusing is usually accomplished by moving the objective rather than the microscope stage, the bath chamber as well as the micromanipulators can be fixed to the stage for good mechanical stability. Upright microscopes are mainly used for studying cells in sliced tissues, and for this, one has to use objectives with long working distance in order to be able to place the patch pipette underneath the objective. The main disadvantage of conventional upright microscopes is that focusing is accomplished by moving the microscope stage and leaving the objective fixed (although recently, some optical companies have introduced special versions of upright microscopes with fixed stages). The conventional stages sometimes lack the mechanical stability and rigidity to support the weight of some micromanipulators. A remedy to this problem is to detach the stage from the microscope and fix it to a mechanically rigid superstructure that also holds the micromanipulator. The microscope itself is placed on a mounting plate that can be moved vertically to allow focusing, while the stage movement is controlled by X–Y translators. An alternative is to leave the microscope fixed and have the stage moving in all three axes (including the focus axis), in which case the manipulator has to be fixed to the stage.

3.2.2. Video

Video cameras can be attached to most microscopes (inverted or upright). Since affordable video technology is available these days, it is highly recommended to supplement a microscope with video monitoring. Most low-end video cameras and appropriate monitors are suitable for simple observation of cells. Of course, more dedicated video equipment is needed if fluorescence imaging, video-enhanced microscopy, or time-lapse video is an integral part of the experiments. The advantages of having a video camera to monitor the preparation during seal formation and throughout the experiment are numerous. One can form seals much more easily, as one can simultaneously observe the approach of the patch pipette and the change in pipette resistance on the oscilloscope or computer screen when the pipette touches the cell membrane. During an experiment one can monitor any morphological changes of the cell under investigation (e.g., swelling or shrinking, blebbing, contraction).

3.3. Micromanipulation

In order to place patch pipettes on cells as small as a few micrometers, it is essential to be able to precisely control the movement of the patch pipette in the submicrometer range. Another important requirement is that the position of the pipette be free of drift after seal formation to maintain stable recordings for several minutes. This is accomplished by micromanipulators, of which there exist a large variety.

The types of commercially available manipulators include mechanical, hydraulic, motorized, and piezoelectric drives. In principle, all of these varieties can be used for patch-clamp experiments. The ideal manipulator should be reasonably small and mechanically rigid. It

should allow long travel distances at fast speed and smooth submicron movements in at least three axes; it should be exceptionally stable and drift-free and ideally allow for remote control. Motorized manipulators are probably closest to such ideal manipulators, followed by hydraulic manipulators (although the latter can sometimes drift considerably). Some of the criteria mentioned may also be fulfilled by combining coarse and fine manipulators, e.g., by using long-range mechanical manipulators with attached fine-positioning devices such as piezoelectric drives for the final approach.

In typical setups, the amplifier probe is mounted directly on the micromanipulator. The probes are normally supplied with a plastic mounting plate that can be fixed on a flat surface of the manipulator. Some manipulators have clamps for holding the probe. The headstage should be fixed tightly to the manipulator, but care must be taken that the metal enclosure of the probe should never be in contact with metallic parts of the manipulator. The arrangement of the manipulator and the attached headstage should also allow for easy access of the pipette holder for exchange of pipettes. This may be accomplished by mounting the manipulator or the headstage on a rotatable or tiltable platform that makes it possible to swing out the headstage for pipette exchange and return it to its fixed position for experiments.

3.4. Amplifiers

A number of commercial patch-clamp amplifiers capable of recording single channels as well as whole-cell currents and operating in voltage- or current-clamp mode are available. There is no general advice that can be given in favor of or against a certain model. One might point out that there are two basic modes in which current-to-voltage conversion is implemented: resistive and capacitive feedback. Resistive feedback is the classical mode and suitable for all types of patch-clamp recordings, whereas capacitive feedback is currently superior only for ultra-low-noise single-channel recordings (provided all other noise sources are meticulously eliminated). Some amplifiers allow switching between resistive and capacitive feedback mode. Others provide for total digital control of the amplifier with automatic series resistance and capacitance compensation.

3.5. Stimulators

Patch-clamp amplifiers are usually capable of applying a steady command voltage to the pipette, and sometimes a test-pulse generator is provided. However, in order to apply complex stimulus protocols in the form of square, ramp, or even more complex voltage pulses, there is a need for more sophisticated pulse generators.

There are basically two options to consider: stand-alone analogue or digital stimulators and computer-based stimulators that are integrated into a data acquisition program. The latter option is favored by most investigators because it allows convenient stimulation using complex stimulus patterns (including leak-pulse protocols) and proper processing of acquired data (see Chapter 3, this volume). Analogue stimulators (often digitally controlled) are sometimes required when very fast repetitive or very long-lasting pulses need to be applied. An analogue lock-in amplifier may be considered a special type of stimulator. It is used for measuring membrane capacitance. It features a sine-wave generator (used to stimulate the cell) and special circuitry that analyzes the membrane currents in response to the applied sine-wave stimulus at different phase angles (see Chapter 7, this volume). An alternative to

analogue lock-in amplifiers is provided by the implementation of a software lock-in featured by some commercial or public-domain data acquisition packages.

3.6. Data Acquisition and Analysis

The registration and documentation of patch-clamp recordings requires equipment for data acquisition, storage, and data analysis.

3.6.1. Oscilloscopes

Most computerized data acquisition systems feature both data acquisition and display of acquired data, often in a leak-corrected or otherwise processed form. However, for test purposes, it is still a good idea to have an oscilloscope connected to the amplifier. It is often convenient to observe the voltage and current monitor signals on the oscilloscope rather than from the computer-processed screen display (which might often be quite sluggish and more difficult to scale appropriately). In many situations it is easier to observe the signals on the oscilloscope (maybe at an increased resolution or less heavily filtered), as some fine details may become apparent that might be missed by the data acquisition software. Furthermore, a look at the oscilloscope and comparison with the digitized recordings can increase the experimenter's confidence that the data are being recorded and processed correctly by the computer.

3.6.2. Chart Recorders

Quite often it is of interest to keep track of the entire time course of an experiment, monitoring simultaneously various additional parameters relevant for the experimental results. Multichannel chart recorders offer this possibility by recording any voltage-encoded signal onto a chart paper. These can monitor, e.g., development of the holding current, temperature changes, pH, solution changes, stimulation protocols, or other parameters of interest. Annotations can be made by simply scribbling remarks or marking special experimental procedures on the chart paper. In recent times, classical paper chart recorders must compete with more versatile and convenient computer-based charting programs, which offer more flexibility and certainly a more effective way of analyzing the acquired data.

3.6.3. Tape Recorders

Continuous high-resolution acquisition of single-channel data from a single patch can easily fill even very large computer hard disks within a few minutes. It is therefore often unavoidable to resort to analogue or digital recordings on tape recorders. Analogue acquisition of electrical signals on frequency-modulated magnetic tape recorders is rarely used these days. Two main systems are currently favored for acquisition of patch-clamp data: VCR/PCM combinations in which analogue signals are converted into pulse-code modulated signals recorded on a video tape, and DAT (digital audio tape) recorders, which use a more convenient recording medium.

3.6.4. Filters

Most patch-clamp amplifiers have built-in filters, often complemented by the capability of offline digital filtering of the acquired data. In some instances, particularly when there is need for accurate filtering of single-channel data or when performing noise analysis studies, a more sophisticated analogue filter with different filter characteristics may be required.

3.6.5. Computerized Systems

Most current recordings, be they single-channel or whole-cell data, published in patch-clamp studies rely on powerful data acquisition and analysis software available on different computer platforms (see Chapter 3, this volume). The data acquisition and analysis of the early days of patch clamp was largely performed on DEC (Digital Equipment Corporation) computers running self-programmed software developed in the leading laboratories. The rapid pace at which personal computers grew in performance and affordability promoted the development of dedicated software for these computers on a commercial basis. Today most of the commercial data acquisition packages are running either on personal computers under the DOS operating system or on the Macintosh line of computers. Some laboratories still use software tailored to their specific needs and often are willing to share it with colleagues who ask for it.

With the rate at which computer hardware turns over these days, it is not so much a question of which computer platform to use but rather which software to obtain. As for any of the other components of a patch-clamp setup, this is a difficult question that cannot be answered by naming a particular software package. In general, most of the commercial acquisition software is quite adequate to perform basic patch-clamp experiments by providing stimulation output and acquisition of data at high speed. However, there are differences in performance in terms of user interface, ease of operation, flexibility, and special features between different software packages. It is worth looking into the idiosyncrasies of the data acquisition software, as software performance will often be the limiting factor in what type of experiments can be done and how effective or time-consuming data acquisition and analysis will be.

3.7. Grounding the Setup

Because of the extreme sensitivity of the headstage, special care must be taken in grounding all surfaces that will be near the probe input in order to minimize line-frequency interference. Even 1 mV of AC on a nearby surface, which can easily arise from a ground loop, can result in significant 50- or 60-Hz noise. A high-quality ground is available at the terminal of the probe; this is internally connected through the probe's cable directly to the signal ground in the main amplifier unit. The ground terminal on the probe is best used for the bath electrode and perhaps for grounding nearby objects such as the microscope.

All other metallic surfaces (e.g., the air table, manipulators, Faraday cage) should be grounded by low-resistance ground cables at a central point, usually on the amplifier's signal ground. It is a good idea to have a brass or copper rod inside the Faraday cage to which all grounds are connected. This grounding rod is then connected by a high quality ground wire to the signal ground on the amplifier. It is best to have this ground wire run parallel to the probe's cable in order to avoid magnetic pickup and ground loop effects. Besides 50- or 60-

Hz magnetic pickup, there may be some 35-kHz pickup from the magnetic deflection of the computer monitor. This pickup becomes visible only when the filters are set to high frequencies; it can usually be nulled by changing the orientation or spacing of the ground wire from the probe cable.

In most cases, the patch clamp is used in conjunction with a microscope; it and its stage typically constitute the conducting surfaces nearest the pipette and holder. In a well-grounded setup, the microscope can provide most of the shielding. It should be made sure that there is electrical continuity between the various parts of the microscope, especially between the microscope frame and the stage and condenser, which are usually the large parts nearest the pipette.

Electrically floating surfaces can act as "antennas," picking up line-frequency signals and coupling them to the pipette. It is important that the lamp housing also be well grounded. It is usually not necessary to supply DC power to the lamp provided that the cable to the lamp is shielded and that this shield is grounded at the microscope.

4. Pipette Fabrication

Procedures for fabricating pipettes are presented in some detail elsewhere (Cavalíe *et al.*, 1992; Rae and Levis, 1992a), and the shapes and properties of such patch pipettes are described in Chapter 21 (this volume). The basic equipment required, a summary of the procedures, and some tips that might be helpful are presented in this section. Depending on which patch-clamp configuration is used and the abundance of ion channels in a given preparation, an optimal adjustment of the size, shape, glass type, and coating of the patch pipette is required. The main steps in pipette fabrication involve pulling of appropriately shaped pipettes from glass capillary tubes, coating the pipette with a suitable insulation to reduce the background noise, and fire-polishing the tip of the pipette to allow gigaseals to be formed without damaging the cell membrane. Various instruments can contribute to obtaining the best possible results when fabricating patch pipettes.

4.1. Pipette Pullers

These are used to pull patch pipettes from glass capillary tubes. In its simplest form, a patch-pipette puller passes large currents through a metal filament made of tungsten or platinum and uses gravitation to pull the glass apart as the heat starts to melt the glass. Such vertical pullers usually employ a two-step pull mechanism in which the first pull softens the glass and pulls it a short distance to thin the capillary, after which the second pull (usually with lower heat) separates the capillary, yielding two pipettes with large-diameter tips. Other types of pipette pullers operate in a horizontal arrangement and apply elastic or motorized force to pull the glass in one or multiple steps. These latter pullers are also suitable for fabricating standard intracellular electrodes. They are often microprocessor controlled, and some even use laser technology, thus allowing one to pull quartz glass. Some pullers feature heat polishing while pulling, which is only useful when coating of pipettes is not necessary.

4.2. Pipette Microforges

Once pipettes have been pulled, they are often further processed. One objective is to reduce the pipette capacitance by coating the tapered shank of the pipette up to a few

micrometers of the pipette tip with a hydrophobic material (e.g., Sylgard®), which will prevent liquid films creeping up the pipette. A second purpose is to optimize the success rate of seal formation and to obtain stable seals for longer periods of time by smoothing the pipette tip (fire polishing). Pipette microforges have been designed to ease these manipulations. They basically consist of a microscope with a low-magnification objective for controlling the coating step and a high-magnification objective for monitoring the fire-polishing step. In addition, there must be a means by which a jet of hot air is directed to the pipette tip (used for curing of the Sylgard® coating) and a heated platinum wire for melting the pipette tip.

4.3. Glass Capillaries

Pipettes can be made from many different types of glass (for review, see Rae and Levis, 1992a). It has been found that different types of glass work better on different cell types. Glass capillaries are available from soft (soda glass, flint glass) or hard glasses (borosilicate, aluminosilicate, quartz). Soft-glass pipettes have a lower melting point (800°C vs. 1200°C), are easily polished, and can be pulled to have a resistance of 1–2 MΩ. They are often used for whole-cell recording, where series resistance rather than noise is the limiting criterion. The large dielectric relaxation in soft glass sometimes results in additional capacitive transient components that interfere with good capacitance compensation. Hard-glass pipettes often have a narrow shank after pulling and consequently a higher resistance. Hard glass tends to have better noise and relaxation properties; however, the important parameter here is the dielectric loss parameter, which describes the AC conductivity of the glass. Although the DC conductivity of most types of glass is very low, soft glasses in particular have some conductivity around 1 kHz; that is sufficiently high to become the major source of thermal noise in a patch-clamp recording (see Chapter 5, this volume). Borosilicate and aluminosilicate glasses have lower dielectric loss and produce less noise. Quartz glass may be used for exceptionally low-noise recordings (Rae and Levis, 1992b) but requires a laser-driven puller for pipette fabrication.

4.4. Pulling

Depending on the puller used, pipettes are pulled in two or more stages: the first to thin the glass to 200–400 μm at the narrowest point over a 7- to 10-mm region, and the next to pull the two halves apart, leaving clean, symmetrical breaks. Both halves can be used. The length of the first pull and the heat of the last pull are the main determinants of the tip diameter of the final pipette.

4.5. Coating

The capacitance between the pipette interior and the bath, and also the noise from dielectric loss in the glass, can be reduced by coating the pipette with an insulating agent such as Sylgard.® Sylgard® is precured by mixing the resin and catalyst oil and allowing it to sit at room temperature for several hours (or in an oven at 50°C for 20 min) until it begins to thicken. It can then be stored at −18°C for many weeks until use. The Sylgard®

is applied around the lower few millimeters of the electrode to within 10–20 μm of the tip and then rapidly cured by a hot-air jet or by heat from a coil. Coating should be done before the final heat polishing of the pipette, so that the heat can evaporate or burn off any residue left from the coating process.

4.6. Heat Polishing

Heat polishing is used to smooth the edges of the pipette tip and remove any contaminants left on the tip from coating. It is done in a microforge or similar setup in which the pipette tip can be observed at a magnification of 400–800×. The heat source is typically a platinum or platinum–iridium wire. To avoid metal evaporation onto the pipette, the filament should be coated with glass at the point where the pipette will approach it. This is done by simply pressing a noncoated patch pipette onto the glowing filament until it melts and forms a drop of liquid glass covering the bare metal. To produce a steep temperature gradient near the filament (which helps make the pipette tip sharply convergent), an air stream can be directed at the filament. The amount of current to pass through the filament must be determined empirically for each type of glass, but a good place to start is with sufficient current to get the filament barely glowing. The typical practice is to turn on the filament current and move either the filament or the pipette (whichever is movable) into close proximity of the other until the pipette tip starts to melt and the desired tip size is reached. Because the opening in the pipette tip is usually at the limit of resolution of viewing, one might not see the change in shape at the tip but instead only a darkening of the tip. One can tell whether the tip was melted closed, and also get an idea of the tip diameter, by blowing air bubbles in methanol with air pressure supplied to the back of the pipette by a small syringe.

4.7. Use of Pipettes

Pipettes should be used within 5–8 hr after fabrication, even if stored in a covered container; small dust particles from the air stick readily to the glass and can prevent sealing. However, with some easy-sealing cells, experience has been that pipettes may even be used the next day. It is very important to filter the filling solutions (e.g., using a 0.2-μm syringe filter). Pipettes can be filled by sucking up a small amount of solution through the tip. This can be done by capillary force (simply dipping the tip for a few seconds into a beaker containing the pipette solution) or by applying negative pressure to the back of the pipette (e.g., using a 5-ml syringe). Thereafter, the pipette is back-filled, and any bubbles left in the pipette can be removed by tapping the side of the pipette. Overfilling the pipette has disastrous consequences for background noise because the solution can spill into the holder, wetting its internal surfaces with films that introduce thermal noise. Therefore, the pipette should only be partially filled, just far enough to make reasonable contact with the electrode wire (the pipette holder is not filled with solution but is left dry). However, one might still want to fill the pipette high enough such that hydrostatic pressure outweighs the capillary suction, thus causing outflow of solution when the tip enters the bath. If this produces untolerable noise, one may reduce the filling level and apply slight positive pressure to the pipette to obtain outflow of solution.

4.8. Pipette Holders

Commercial pipette holders come in different varieties for accommodating different sizes of pipettes. Many laboratories with decent workshops have their holders custom made to meet their needs, sometimes modified to allow pipette perfusion. Holders are usually made from Teflon® or polycarbonate, both having low dielectric loss. The pipette electrode is simply a thin silver wire that is soldered onto the pin that plugs into the probe's connector. The chloride coating on the wire gets scratched when pipettes are exchanged, but this does not degrade the stability very much; the wire does need to be rechlorided occasionally, perhaps once per month or whenever a significant drift in pipette potential occurs. A wire for the standard electrode holder should be about 4.5 cm long; after it is chlorided along its entire length, an O-ring is slipped onto it, and the wire is inserted into the holder. A good alternative for a bare silver wire is one that is coated by a Teflon® insulation, where only a few millimeters at the tip of the wire are stripped and chlorided; this reduces the scratching of the Ag–AgCl coating during pipette exchanges and capacitative noise. Chloriding can be done by passing current (e.g., 1 mA) between the wire and another silver or platinum wire in a Cl^- containing solution (e.g., 100 mM KCl, or physiological saline). Current is passed in the direction that attracts Cl^- ions to the electrode wire; this produces a gray coating.

The noise level of a holder can be tested by mounting it (with the electrode wire installed but dry) on the probe input and measuring the noise using the noise test facility of the patch-clamp amplifier. The probe should be in a shielded enclosure so that no line-frequency pickup is visible on an oscilloscope connected to the current monitor output at a bandwidth of 3 kHz or less. A good holder increases the rms noise of the headstage alone by only about 20%. The final noise level relevant for the actual recording, which includes all noise sources other than the cell itself, can be estimated by measuring the noise with a filled pipette just above the bath surface.

For low-noise recording, the electrode holder should be cleaned before each experiment with a methanol flush, followed by drying with a nitrogen jet. Before inserting a pipette into the holder, it is a good idea to touch a metal surface of the setup to discharge any static electricity that one may have picked up. The holder should be tightened firmly enough that the pipette does not move (on a scale of 1 μm) when suction is applied.

4.9. Reference Electrodes

The main requirements for a bath electrode are stability, reversibility, and reproducibility of the electrode potential (for review see Alvarez-Leefmans, 1992). A bare, chlorided silver wire makes a good bath electrode unless the cell type under investigation shows intolerable sensitivity to Ag^+ ions. A good alternative is an electrode incorporating an agar salt bridge, in which case the silver wire is either embedded in an agar-filled tube inserted directly into the bath or, as another option, the chlorided silver wire is immersed in saline kept outside the bath but in contact with it through an agar-filled bridge made of a U-shaped capillary tube. The agar should be made up in a solution that can be a typical bath solution or something similar, such as 150 mM NaCl. More concentrated salt solutions are not necessary, and they can leak out, changing the composition of the bath solution. The technical problems related to reference electrodes are discussed in Chapter 6 (this volume).

5. Experimental Procedures

The technical aspects of patch-clamp experiments are fairly simple, provided some basic precautions are taken. In the following, a brief description of the techniques for establishing a seal and recording from either a membrane patch or from an entire small cell are given. This is complemented by some practical tips as well as notes on possible problems that may be helpful when conducting these experiments. The reader is referred to more detailed reviews on recording techniques and data analysis in this volume (Chapters 2, 3, 5, 18–20) and other publications covering data acquisition in general (French and Wonderlin, 1992) as well as practical and theoretical considerations of single-channel data analysis (Jackson, 1992; Magleby, 1992; Sigworth and Zhou, 1992).

5.1. Preparing Experiments

Usually, the aim is to obtain as many experiments as possible from a given preparation (including test and control experiments). Often, experiments have to be carried out at a rapid pace, as some preparations have a short lifetime. To avoid delays and interruptions, it is therefore a good idea to get the technical aspects organized before an experimental session. It is helpful to have all the required solutions made ready, have a reasonable number of patch pipettes prepared, have the stimulation protocols programmed, have the tools for pipette filling and bath exchange at hand, etc.

Often-used extracellular solutions may be kept in stock (liter quantities) in a refrigerator. Intracellular solutions may be frozen in smaller stocks (5–10 ml) and thawed before experiments. If solutions need be prepared during an experimental session, they can be mixed from appropriately concentrated stock solutions. In any case, the pH and osmolarity of the solutions should be adjusted appropriately. Solutions should always be filtered unless a "sticky" substance is included; such substances should be added after the filtering of the normal saline. Supplements (e.g., ATP, GTP, fluorescent dyes, second messengers) are added from frozen stocks to a small volume (100–500 μl) of the pipette-filling solution in an Eppendorf tube as needed shortly before the experiment. The pipette-filling solutions might be kept in a rack on top of a container filled with ice to prevent degradation of labile ingredients. The patch pipettes are filled as described earlier.

The choice of solutions is probably one of the most crucial determinants for any patch-clamp experiment. The formulation of individual bath and pipette solutions depends on many factors. In general, the bath solution should mimic the natural extracellular environment of the cell, while the pipette solution should substitute for the cytosol. On the other hand, one often wants to study a certain ion current in isolation or increase its amplitude. In such cases one needs to alter the ion composition of external or internal or both solutions in order to abolish the masking effect of currents interfering with the conductance of interest (or increase the latter). Some general guidelines on choosing saline compositions are given elsewhere (Swandulla and Chow, 1992). Furthermore, it is a good idea to screen the available literature on the cell type under study and the particular composition of solutions used to study the ion current in question.

5.2. Forming a Seal

The process of seal formation is monitored by observing the pipette currents on an oscilloscope while applying voltage pulses to the pipette. A convenient pulse amplitude is

2 mV, which can be obtained from a pulse generator. Before the pipette is inserted into the bath, the current trace should be flat except for very small capacitive transients caused by the stray capacitance of the pipette and holder. When the pipette enters the bath, the 2-mV pulses will cause 1 nA to flow in a 2-MΩ pipette. The approach to the cell membrane and the formation of a gigaseal will cause the resistance to increase, reducing the currents. For observation of the current pulses, it is convenient to pick a gain setting and oscilloscope sensitivity such that the current through the open pipette is reasonably sized.

5.2.1. Entering the Bath

The surface of the bath solution is relatively "dirty," even if (as is strongly recommended) one aspirates some solution from the surface to suck off dust and contaminants. For this reason it is important always to have solution flowing out of the pipette until the pipette is in contact with the cell (either by applying a small amount of positive pressure to the pipette or by filling it appropriately). Also, one should avoid going through the air–water interface more than once before forming a seal. When moving the pipette tip into the bath, the current trace may go off scale; in that case, one needs to reduce the amplifier gain until the trace reappears. Then, one needs to cancel any offset potentials between pipette and reference electrode; this is done by setting the holding potential of the patch-clamp amplifier to 0 mV and adjusting the pipette offset control such that the DC pipette currents are close to zero. For a detailed discussion of offset compensation procedures, see Chapter 6 (this volume). From the size of the current response to the test pulses, the pipette resistance can be calculated (good data acquisition software usually provides for this).

5.2.2. Forming a Gigaseal

After the pipette has entered the bath, one should proceed as fast as possible to obtain a gigaseal because the success rate of sealing is inversely proportional to the time the pipette tip is exposed to the bath solution (presumably from an increased probability of picking up floating particles). It is even more important to obtain a seal rapidly when the pipette solution contains peptides or proteins, as they tend to cover the pipette tip and interfere with seal formation. When the pipette is pushed against a cell, the current pulses will become slightly smaller, reflecting an increase in resistance; when the positive pressure is released from the pipette, the resistance usually increases further. Some cell types require more "push" from the pipette than others, but a 50% increase in resistance (i.e., a reduction in the current pulse amplitude by this value) is typical. Application of gentle suction should increase the resistance further and result (sometimes gradually, over maybe 30 sec; sometimes suddenly) in the formation of a gigaseal, which is characterized by the current trace becoming essentially flat again (hyperpolarizing the pipette to −40 to −90 mV often helps to obtain or speed the seal formation). To verify gigaseal formation, one may increase the amplifier gain; the trace should still appear essentially flat except for capacitive spikes at the start and end of the voltage pulse.

5.3. Patch Recording

It is a good idea to start out with the holding potential set to zero (this will leave the patch at the cell's resting potential); if a whole-cell experiment is planned, an alternative is

to start with the desired holding potential (e.g., −70 mV) in order not to depolarize the cell when breaking the patch. When the test pulse is applied, the fast capacitive spikes recorded arise mainly from the pipette itself and the enclosed membrane patch. One should now compensate the capacitance by adjusting the amplitude and time constant of the fast capacitance neutralization controls of the patch-clamp amplifier to minimize the size of these spikes (some amplifiers offer an automatic compensation of this capacitance). Transient cancellation will be essential if one will be giving voltage pulses in the experiment; if not, the test pulse should be discontinued to avoid introducing artifacts.

In single-channel recordings, the gain should be set to at least 50 mV/pA or above for lower noise (most patch-clamp amplifiers use a high feedback resistor only for these high-gain settings); in whole-cell configuration this setting will depend largely on the size of currents to be recorded. The gain setting should be calculated by dividing the output voltage of the current monitor (e.g., ±10 V) by the gain setting (e.g., 10 mV/pA). This will record currents up to ±1 nA without saturation. It should be kept in mind, however, that the output voltage needs to be sampled by an AD converter, which will resolve the input voltage of ±10 V with its intrinsic accuracy (typically 12 or 16 bits, equivalent to 4,096 or 65,536 discrete levels, amounting to a digitized resolution of a ± 10 V signal of 5 and 0.3 mV, respectively). It is therefore important that the expected current amplitudes be recorded with an appropriate gain setting, thus providing for the best possible resolution of currents.

If one is applying voltage pulses to the patch membrane, it is important to try to cancel the capacitive transients as well as possible in order to avoid saturating any amplifiers, the recording medium, or the AD converter. It is a good idea to set the fast capacitance neutralization controls while observing the signal without any filtering beyond the 10-kHz filtering. Then, during the recording, one should observe to see if the clipping indicator of the amplifier flashes. If it does, it means that internal amplifiers are about to saturate and/or that the current monitor output voltage is going above 10–15 V on the peaks of the transients, and one should readjust the transient cancellation controls. Otherwise, it is likely that the recording will be nonlinear, and subtraction will not work correctly.

The fast transient cancellation is not sufficient to cancel all of the capacitive transients in a patch recording. This is partly because the pipette capacitance is distributed along the length of the pipette; therefore, each element of capacitance has a different amount of resistance in series with it, so that a single value of the time constant of the fast capacitance will not provide perfect cancellation. The time course of the transients also reflects dielectric relaxation in the material of the pipette holder and in the pipette glass. These relaxations are not simple exponentials but occur on time scales of about 1 msec or longer. If one is using pipette glass with low dielectric loss (e.g., aluminosilicate glass), or if one is careful to coat the pipette with a thick coating and near to the tip, the relaxations will be smaller. Remaining transients can be canceled by subtracting control traces without channel openings from the traces containing the channels of interest.

For cell-attached or inside-out patch configurations, positive pipette voltages correspond to a hyperpolarization of the patch membrane, and inward membrane currents appear as positive signals at the current monitor outputs. Some data acquisition programs compensate for this by inverting digital stimulus and sampled values in these recording configurations such that the stimulation protocols, holding voltages, and displays of current records in the oscilloscope all follow the standard electrophysiological convention. In this convention, outward currents are positive, and positive voltages are depolarizing. However, even if the data acquisition software conveniently processes and displays the signals in apparently "physiological" polarity, the analogue current and voltage monitor outputs are not inverted in these recording modes. One should always make sure that one really understands what

exact data processing is in effect during data acquisition, and the software should allow the user to reconstruct off line what the experimental settings were during data acquisition.

5.4. Whole-Cell Recording

5.4.1. Breaking the Patch

After a gigaseal is formed, the patch membrane can be broken by additional suction or, in some cells, by high-voltage pulses (these need to be established empirically for the particular cell type; 600–800 mV for 200–500 μsec is a good starting point). Electrical access to the cell's interior is indicated by a sudden increase in the capacitive transients from the test pulse and, depending on the cell's input resistance, a shift in the current level or background noise. Additional suction pulses sometimes lower the access resistance, causing the capacitive transients to become larger in amplitude but shorter in duration. Low values of the access (series) resistance (R_s) are desirable, and when R_s compensation is in use, it is important that the resistance be stable as well. A high level of Ca-buffering capacity in the pipette solution (e.g., with 10 mM EGTA) helps prevent spontaneous increases in the access resistance as a result of partial resealing of the patch membrane, which is favored by high intracellular Ca^{2+} concentrations.

5.4.2. Capacitive Transient Cancellation

If the fast capacitance cancellation was adjusted (as described above) before breaking the patch, then all of the additional capacitance transient will be attributable to the cell capacitance. Canceling this transient using the *C*-slow and *R*-series controls on the amplifier will then give estimates of the membrane capacitance and the series resistance. With small round cells, it should be possible to reduce the transient to only a few percent of its original amplitude. However, if the cell has an unfavorable shape (for example, a long cylindrical cell or one with long processes), the cell capacitance transient will not be a single exponential, and the cancellation will not be as complete.

5.4.3. Series Resistance Compensation

In whole-cell voltage-clamp recording, the membrane potential of the cell is controlled by the potential applied to the pipette electrode. This control of potential is not complete but depends on the size of the access resistance between the pipette and the cell interior and on the size of the currents that must flow through this resistance. This access resistance is called the series resistance (R_s) because it constitutes a resistance in series with the membrane. Part of the series resistance arises from the pipette itself, but normally the major part arises from the residual resistance of the broken patch membrane, which provides the electrical access to the cell interior. In practice, the series resistance usually cannot be reduced below a value about two times the resistance of the pipette alone.

Series resistance has several detrimental effects in practical recording situations. First, it slows the charging of the cell membrane capacitance because it impedes the flow of the capacitive charging currents when a voltage step is applied to the pipette electrode. The time constant of charging is given by $\tau_u = R_s \cdot C_m$, where C_m is the membrane capacitance. For

typical values of R_s = 5 MΩ and C_m = 20 pF, the time constant is 100 μsec. This time constant is excessively long for studying rapid voltage-activated currents such as Na^+ currents in morphologically convoluted cells such as neurons, especially because several time constants are required for the membrane potential to settle at its new value after a step change. Series resistance and cell capacitance also impose limitations on the recording bandwidth of the acquired currents, which are filtered by the combination of R_s and C_m. For an *RC* filter, the corner frequency (−3 dB) is calculated from $f = 1/(2\pi R_s C_m)$, and in the above example we arrive at a limiting bandwidth of 1.6 kHz. This bandwidth would be reduced with larger capacitance and/or series resistance. It should be realized that although series resistance can be compensated for electronically (R_s compensation control of the patch-clamp amplifier), the capacitance cannot (capacitance neutralization controls of patch-clamp amplifiers do not increase the bandwidth of the recording, nor do they speed up the charging of the capacitance). Another detrimental effect of series resistance is that it yields errors in membrane potential when large membrane currents flow. In the case of R_s = 5 MΩ, a current of 2 nA will give rise to a voltage error of 10 mV, which is a fairly large error.

To use R_s compensation in practice, one first has to adjust the transient-cancellation controls (including *C*-fast and τ-fast if necessary) to provide the best cancellation. Then one activates the R_s compensation control by turning it up to provide the desired percentage of compensation. Most patch-clamp amplifiers use this setting to determine the amount of positive feedback being applied for compensation. It should be adjusted with some care, because too high a setting causes overcompensation (the amplifier will think that R_s is larger than it is); this can cause oscillation and damage to the cell under observation.

Optimal settings of the R_s compensation controls depend on the approximate value of the uncompensated membrane-charging time constant τ_u, which can be calculated as the product of the *C*-slow and *R*-series settings (for example, suppose *C*-slow is 20 pF and *R*-series is 10 MΩ; the time constant τ_u is then 20 pF · 10 MΩ = 200 μsec). The speed of the R_s compensation circuitry can also be adjusted on most amplifiers. If τ_u is smaller than about 500 μsec one should use a fast setting of the R_s compensation circuitry to provide the necessary rapid compensation. The slower settings, on the other hand, will provide compensation that is less prone to high-frequency oscillations from misadjustment of the controls. How much compensation one can apply is also determined by τ_u. If τ_u is larger than about 100 μsec, one can use any degree up to the maximum of 90% compensation without serious overshoot or ringing in the voltage-clamp response. For smaller values of τ_u the R_s compensation setting should be kept below the point at which ringing appears in the current trace.

As in the case for patch recording, there is rarely need to use the full bandwidth of the amplifier in whole-cell recording. This is because typical membrane charging time constants (even after R_s compensation) are considerably longer than 16 μsec, which is the time constant corresponding to a 10-kHz bandwidth. Thus, the current monitor signal is expected to contain no useful information beyond this bandwidth.

In whole-cell recording, the voltage and current monitor signals follow the usual convention, with outward currents being positive. This is because the pipette has electrical access to the cell interior.

6. Caveats and Sources of Artifacts

For newcomers to the patch-clamp technique it is usually not so difficult to master the technical aspects of the methodology. It is rather easy to gather a large amount of data, but

the problems emerge in analyzing and evaluating the acquired data. As in any other method there are pitfalls and artifacts, too many to address here. Nevertheless, some problems in using the technique and how to avoid them are briefly discussed in the following paragraphs.

6.1. Solutions

Many problems arise from the composition of extracellular and intracellular solutions. The following are some points to be considered.

Differences in osmolarity and pH between bath and pipette solutions can seriously affect all sorts of ion currents and should be avoided (unless one specifically wants to study their effects). For example, hypo- or hyperosmotic solutions cause cell swelling or shrinking accompanied by modulation of volume-regulatory conductances such as Cl^-, K^+, and cation channels (Sarkadi and Parker, 1991; Hoffmann, 1992), and protons are known to modify the properties of many ion channels (for review see Moody, 1984; Chesler and Kaila, 1992).

Divalent ions in the bath can screen surface membrane charges, thereby affecting the voltage dependence of ion channels. Shifts in the activation and inactivation curves of virtually all voltage-gated ion channels by divalents have been described and are reviewed in more detail elsewhere (Green and Andersen, 1991; Latorre *et al.,* 1992). Complete removal of certain ions can alter the properties of ion channels. The most dramatic effects are observed following removal of Ca^{2+} from the bath. Among other effects, this may cause Ca^{2+} channels to lose selectivity and become permeant to monovalent ions (Almers and McCleskey, 1984; Hess and Tsien, 1984), it will shift the activation curve of Na^+ channels to the left (Campbell and Hille, 1976), enhance currents through inward rectifying K^+ channels (Biermans *et al.,* 1987), and long exposure to Ca^{2+}-free bath solutions will eventually cause nonspecific leaks in the plasma membrane.

Another problem might arise from the precipitation of divalent ions in the saline when sulfate or carbonate ions are present, yielding erroneous estimates of the effective concentration of these ions.

Many organic compounds are not easily soluble in aqueous solutions and need to be dissolved in organic solvents such as ethanol or dimethylsulfoxide, whose final concentration should not exceed 0.1%. In any case, appropriate control experiments of the vehicle should be carried out.

The chloride ions are the primary charge transfer ions between the aqueous phases and the silver wires serving as electrodes. Therefore, these electrodes must be immersed in a solution that contains at least some Cl^- ions (at least 10 mM). A complete removal of Cl^- ions in the bath or pipette-filling solution is not feasible unless an agar bridge is used.

In all patch-clamp configurations a number of offsets have to be taken into account. These include amplifier offsets (±30 mV), electrode potentials (±200 mV, depending on Cl^- concentration of pipette and reference electrode), liquid junction potentials, and potentials of membrane(s) in series with the membrane under study. Some of these offsets are fixed during an experiment (such as amplifier and electrode offsets); some are variable. It is standard practice to take care of voltage offsets by performing a reference measurement at the beginning of an experiment. An adjustable amplifier offset is then set for zero pipette current. Thereafter, the command potential of the amplifier will be equal in magnitude to the membrane potential if no changes in offset potentials occur.

The polarity of the command potential will be that of the membrane for whole-cell and outside-out configurations but will be inverted in the cell-attached and inside-out configurations. In cell-attached configurations an additional offset is present because of the resting

potential of the cell under study. Liquid-junction potentials may appear or disappear during the measurement when solution changes are performed or in cases in which the pipette solution is different from the bath solution (Barry and Lynch, 1991; Neher, 1992a). A detailed discussion of liquid junction potentials and how to correct for them is found in Chapter 6 (this volume).

In order to maintain cells viable to receptor-activated signal transduction or to avoid rundown of certain ion currents, it is a good idea and common practice to include ATP, GTP, and other nucleotides in the pipette-filling solution. Thus, the cell is unavoidably exposed to the pipette solution during the approach for seal formation. At least for ATP a note of caution is appropriate, because many cells possess purinergic receptors. Activation of these receptors and the resulting signal transduction events may take place before a recording is started. A similar problem might arise when glutamate is used as the internal anion when studying cells that are sensitive to this neurotransmitter. In order to realize and consider the effects caused by the intracellular solution prior to starting an actual recording, it might be useful to let a cell recover by waiting some time after seal formation (during which the bath solution should be exchanged), then establish the whole-cell recording configuration and apply the internal solution extracellularly.

Contaminations of the solutions with foreign substances that might affect ion channels are very difficult to eliminate completely, because containers, syringes, tubings, needles, or filters may release small amounts of leachable substances or detergents into the solution. Some ion channels are extremely sensitive to such contaminations. Therefore, solutions should always be prepared from chemicals of the highest purity, and the possible sources of contaminants should be thoroughly cleaned and rinsed.

6.2. Electrodes

The reference electrode in the bath and the test electrode in the pipette holder are usually silver wires coated with AgCl. This coating gets scratched during multiple exchanges of pipettes and may also degrade with time when large currents are passed (effectively dissolving the AgCl coating as the Cl^- ions are released into the saline). If the electrodes are not regularly chlorided, shifts in the electrode potential may become so severe that voltage drifts become noticeable in the course of an experiment, making the measurements inaccurate. Stability of the electrodes should be verified occasionally by monitoring the currents of an open pipette in the bath at zero-current potential over a few minutes or by comparing the zero-current potential before and after an experiment. If the current is not stable, and one needs to adjust the holding potential by more than 1–2 mV to return to the zero-current potential, then rechloriding is necessary.

Another potential problem arises from shunts or high resistances in the current path between saline and electrodes, which usually becomes apparent on entering the bath with a patch pipette. If there should be no or only a small current flow in response to a test pulse, there might be an open circuit, for example, (1) a bubble in the pipette, (2) a faulty connection to the probe input, or (3) a missing connection to the bath electrode. If large currents or erratic noise appears, there might be problems with the pipette or bath electrode, for example, (1) the pipette tip is broken, (2) the reference electrode is short-circuited to grounded parts of the chamber holder or microscope (e.g., through spilled bath solution), (3) the pipette holder has spilled, or (4) the setup is not well grounded.

6.3. Data Acquisition

The main problems in this respect originate from leak subtraction procedures, choice of holding potential and stimulation protocols, or inappropriate sampling and filtering.

Leak subtraction is in common use for voltage-activated ion currents to compensate and cancel linear leak and capacitive currents. Typically, a variable number of small voltage pulses is applied in a voltage range that does not recruit voltage-dependent ion currents (leak pulses). The size of each of the leak pulses is calculated from the test-pulse amplitude (*P*) divided by number of leak pulses (*n*), hence the term *P/n* leak correction (Bezanilla and Armstrong, 1977). The currents recorded during the leak pulses are summed, and the resulting leak current is subtracted from the actual test pulse. There are many variations of the *P/n* protocol, including scaling procedures, leak pulses with alternating polarity, etc. (see Chapter 3, this volume). Considerable artifacts may be introduced inadvertedly by using an inappropriate leak-subtraction protocol. It is advisable to inspect the leak pulses to ascertain that no nonlinear current components are subtracted.

Voltage-activated currents are often subject to steady-state inactivation. This can be exploited to dissect currents, as has been shown for Ca^{2+} channels (Tsien *et al.,* 1988). However, problems may arise, e.g., when long-lasting leak protocols at more negative potentials are applied, since some channels (that were inactivated at the holding potential) might recover from inactivation. In addition, frequency-dependent phenomena (e.g., rundown or facilitation of ion currents) should be taken into account when designing pulse protocols.

Sampling frequency and filtering of data should be appropriate for the signals to be recorded. Otherwise, aliasing effects might occur, or the kinetic information is not accurate. Chapter 3 (this volume) addresses the details of sampling and filtering procedures.

The list of problems mentioned above is certainly not complete; there are many more possible complications when conducting patch-clamp experiments, and some have to be actually experienced in order to be fully appreciated. However, this should not deter anybody from moving into the field, since the reward of being able to observe biological processes at the cellular and molecular level in real time as well as the esthetically pleasing records of ion currents offer more than sufficient reward to make the effort worthwhile.

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