Patch-Clamp Analysis

Advanced Techniques

Edited by

Wolfgang Walz
Alan A. Boulton
Glen B. Baker

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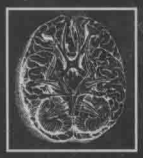

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Series Editors: Alan A. Boulton and Glen B. Baker

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Preface to the Series

When the President of Humana Press first suggested that a series on methods in the neurosciences might be useful, one of us (AAB) was quite skeptical; only after discussions with GBB and some searching both of memory and library shelves did it seem that perhaps the publisher was right. Although some excellent methods books had recently appeared, notably in neuroanatomy, it was a fact that there was a dearth in this particular field, a fact attested to by the alacrity and enthusiasm with which most of the contributors to this series accepted our invitations and suggested additional topics and areas. After a somewhat hesitant start, essentially in the neurochemistry section, the series has grown and will encompass neurochemistry, neuropsychiatry, neurology, neuropathology, neurogenetics, neuroethology, molecular neurobiology, animal models of nervous disease, and no doubt many more "neuros." Although we have tried to include adequate methodological detail and in many cases detailed protocols, we have also tried to include wherever possible a short introductory review of the methods and/or related substances, comparisons with other methods, and the relationship of the substances being analyzed to neurological and psychiatric disorders. Recognizing our own limitations, we have invited a guest editor to join with us on most volumes in order to ensure complete coverage of the field. These editors will add their specialized knowledge and competencies. We anticipate that this series will fill a gap; we can only hope that it will be filled appropriately and with the right amount of expertise with respect to each method, substance or group of substances, and area treated.

Alan A. Boulton
Glen B. Baker

Preface

Neher and Sakmann were the first to monitor the opening and closing of single ion channels in the membranes of cells by conductance measurements. In 1976, they used firepolished micropipets with a tip diameter of 3–5 μm to record currents from a small patch of the membrane of skeletal muscles, thereby decreasing background membrane noise. In order to reduce the dominant source of background noise, the leakage shunt under the pipet rim between membrane and glass, the muscle membrane had to be treated enzymatically. Despite these early limitations, a new technique was born—the patch-clamp. The final breakthrough came in 1981 when the same workers, in collaboration with Hamill, Marty, and Sigworth, developed the gigaohm seal. Not only did this improve the quality of recordings, it was now possible to gently pull the membrane patch with the attached pipet off the cell and study its trapped ion channels in isolation. Another offshoot of the gigaohm seal technique was the whole-cell patch-clamp technique, in which the path is ruptured without breaking the seal. This technique is really a sophisticated voltage-clamp technique and also allows for the altering of cytoplasmic constituents if the experimenter so wishes.

The first part of this treatise on *Patch-Clamp Analysis: Advanced Techniques* presents modern developments associated with the basic patch-clamp techniques outlined above. These chapters are supplemented with information on the newest developments in fast external solution switching to study fast inactivating responses as well as the switching of the pipet solution during recordings. The application of the patch pipet technique not only to clean membrane preparations, but also to brain or other tissue slices, was an important development in the last decade. Other offshoots of the patch pipet technique are the loose patch, the perforated patch, as well as the recording from macropatches and the patch-cram detection technique. These are all introduced and described in detail. Perhaps the recent developments in the patch-clamp field with the biggest impact are the combination of two of the most powerful life science technologies: molecular biology and imaging. This led to the intertwining of the patch pipet with RT-PCR and fluorometric techniques.

The methods associated with the patch pipet are certain to become even more refined in the future, as new applications involving genomics, proteomics, and sophisticated imaging techniques emerge.

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Contents

1

Technology of Patch-Clamp Electrodes

Richard A. Levis and James L. Rae

1. Introduction

The extracellular patch voltage-clamp technique has allowed the currents through single ionic channels to be studied from a wide variety of cells. In its early form (Neher and Sakmann, 1976), the resolution of this technique was limited by the relatively low (~50 MΩ) resistances that isolated the interior of the pipet from the bath. The high resolution that can presently be achieved with the patch-clamp technique originated with the discovery (Neher, 1981) that very high resistance (tens or even hundreds of GΩ) seals can form between the cell membrane and the tip of a clean pipet when gentle suction is applied to the pipet interior. Although the precise mechanisms involved in this membrane-to-glass seal are still not fully understood, the importance of the gigohm seal is obvious. The high resistance of the seal ensures that almost all of the current from the membrane patch flows into the pipet and to the input of the current-sensitive headstage preamplifier. It also allows the small patch of membrane to be voltage-clamped rapidly and accurately via the pipet, and the mechanical stability of the seal is vital to the whole-cell voltage-clamp technique. Of equal importance, the high resistance of the seal greatly reduces the noise it contributes to single-channel measurements. Although the seal can often represent only a small fraction of total patch-clamp noise (particularly as the bandwidth of recording increases), its importance should never be minimized. Without such high-resistance seals, most of the steady progress to reduce background noise levels would not have been possible.

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Of course the patch pipet is not simply a tool in the formation of gigohm seals. The pipet serves as a fluid bridge that connects the current-sensitive headstage amplifier input to the surface or interior of the cell. The insulating properties (both resistive and more importantly capacitive) of the glass that forms the wall of the pipet are also crucial to the ability to measure current originating in the patch and to the background noise levels that can be achieved.

For any patch-clamp measurement, several steps are required to construct a proper glass electrode. First, a glass that has optimal properties is selected. The required properties differ substantially for single-channel recordings and whole-cell-current recordings. For single-channel measurements, low noise is the most important electrical parameter, whereas for whole-cell measurements dynamic performance is more important than the contribution of the electrode to the background noise. This is simply because the background noise in a whole-cell recording is dominated by the noise from the electrode resistance (actually the access resistance) in series with the capacitance of the entire cell. The dynamic bandwidth of a whole-cell recording also depends on the same factors. Therefore, the goal in constructing an electrode for whole-cell recording is simply to make it as blunt and as low in resistance as is compatible with sealing it to the cell. In single-channel recordings, the pipet is a major contributor to the background noise and so requires many subtle considerations to produce an electrode optimal for recording single-channel currents.

As a second step in pipet construction, the electrode glass stock is pulled into a pipet with a tip of optimal geometry. This geometry differs for whole-cell and single-channel recordings. In a third step, the outside wall of the pipet is coated with a hydrophobic elastomer possessing good electrical properties. This procedure is essential for low-noise single-channel recordings but can be done much less carefully for whole-cell recordings. Fourth, the tip is firepolished to round it and clean its surface of any thin film of elastomer coating. This step can also be used to adjust the final tip diameter. Firepolishing promotes seal formation but often is not required. After all these procedures, the electrode can be filled and used.

Several general properties of glasses must be considered when trying to construct optimal electrodes for patch clamping (*see* Table 1).

Table 1
Glass Properties

Glass	LF	Log_{10} vol. R.	Diel. const.	Softening temp. C°	Description
7940	.0038	11.8	3.8	1580	Quartz (fused silica)
1724	.0066	13.8	6.6	926	Aluminosilicate
7070	.25	11.2	4.1	—	Low-loss borosilicate
8161	.50	12.0	8.3	604	High lead
Sylgard	.58	13.0	2.9	—	#184 Coating cmpd.
7059	.584	13.1	5.8	844	Barium-borosilicate
7760	.79	9.4	4.5	780	Borosilicate
EG-6	.80	9.6	7.0	625	High lead
0120	.80	10.1	6.7	630	High lead
EG-16	.90	11.3	9.6	580	High lead
7040	1.00	9.6	4.8	700	Kovar seal borosilicate
KG-12	1.00	9.9	6.7	632	High lead
1723	1.00	13.5	6.3	910	Aluminosilicate
0010	1.07	8.9	6.7	625	High lead
S-8250	1.08	10.0	4.9	720	—
7052	1.30	9.2	4.9	710	Kovar seal borosilicate
EN-1	1.30	9.0	5.1	716	Kovar seal borosilicate
7720	1.30	8.8	4.7	755	Tungsten seal borosilicate
7056	1.50	10.2	5.7	720	Kovar seal borosilicate
3320	1.50	8.6	4.9	780	Tungsten seal borosilicate
7050	1.60	8.8	4.9	705	Series seal borocsilicate
S-8330	1.70	8.0	4.6	820	—
KG-33	2.20	7.9	4.6	827	Kimax borosilicate
7740	2.60	8.1	5.1	820	Pyrex borosilicate
1720	2.70	11.4	7.2	915	Aluminosilicate
N-51A	3.70	7.2	5.9	785	Borosilicate
R-6	5.10	6.6	7.3	700	Soda lime
0080	6.50	6.4	7.2	695	Soda lime

Thermal properties determine the ease with which desired tip shapes can be produced and they determine how easily the tips can be heat-polished. Optical properties often result in a distinct visual endpoint so that tips can be firepolished the same way each time. Electrical properties are important determinants of the noise the glass produces in a recording situation and determine the size and number of components in the capacity transient following a change of potential across the pipet wall. Glasses are complex

substances composed of many compounds and most of their properties are determined to a first order by the composition of the glass used. Glass composition may also influence how easily a glass seals to membranes and whether of not the final electrode will contain compounds leached from the glass into the pipet-filling solution that can activate, inhibit, or block channel currents.

2. General Properties of Pipet Glass

Before proceeding to the details of electrode fabrication, it is useful to consider in more detail glass properties that are important for patch-clamp pipet construction. We will begin with thermal properties. It is important that glasses soften at a temperature that is easily and reliably achieved. This formerly was a stringent constraint because glasses like aluminosilicates, which melt at a temperature in excess of 900°C, would shorten the lifetime of a puller-heating filament so much that their use was unattractive. Quartz, which melts above 1600°C, could not even be pulled in commercially available pullers and so was not used at all. Today, at least one puller exists that will do these jobs easily (P-2000, Sutter Instruments) and so virtually any kind of glass can be used routinely. It is generally true that the lower the melting temperature of the glass, the more easily it can be firepolished. Low melting-temperature glasses such as those with high lead content can be pulled to have tip diameters in excess of 100 microns and still be firepolished to a small enough tip diameter that the pipet can be sealed to a 7–10 micron diameter cell. With such glasses, one has greater control over the final shape of the tip than is possible with higher melting-temperature borosilicate glasses. Quartz pipets cannot be firepolished with a usual firepolishing apparatus, although with care they can be firepolished in a temperature-controlled flame.

Electrical properties are most important for providing low noise as well as low amplitude, simple time-course capacity transients. As will be discussed later, it is not possible to achieve low background noise without an elastomer coating the outside of the pipet. In general, glasses with the lowest dissipation factors have minimal dielectric loss and produce the lowest noise. There is a wide variety of glasses to choose from that will produce acceptable single-channel recordings although quartz is clearly the best material to date. Good electrical glasses are also necessary for whole-cell recordings not because of noise properties but because they result in the simplest and most voltage and time-stable capacity transients.

Major chemical constituents in glass are important since they determine the overall properties of the glass and because they are potential candidates to leach from the glass into the pipet-filling solution where they can interact with the channels being studied. No glass can be deemed to be chemically inert since even tiny amounts of materials leached in the vicinity of the channels may produce sufficient local concentrations to interact with channels and other cellular processes. Again, quartz would be expected to have fewer chemical impurities than other glasses but every kind of glass should be suspected of having an effect on the channels being measured.

3. Whole-Cell Pipet Properties: Practical Aspects

3.1. Choice of Glass

Modern computerized pipet pullers are capable of pulling glass with almost any thermal properties (with the exception of quartz) into the proper blunt-tipped geometry that is ideal for whole-cell recording. Therefore, almost any glass can be used to form whole-cell pipets. Nevertheless, we feel that some types of glass should usually be avoided, while others have some particularly useful properties for this application.

Soda lime glasses like Kimble R-6 and Corning 080 should not generally be used because of their high dielectric loss. When a voltage step is applied across a patch pipet fabricated from one of these glasses, there will be a large slow component in the resulting capacity transient (Rae and Levis, 1992a). For a 2 mm depth of immersion with a moderate coating of Sylgard 184 to within ~200 μm of the tip we have found following a 200 mV voltage step that the slow component for a soda-lime pipet can be as large as 50 pA 1 msec after the beginning of the step. The slow tail of capacity current can still be as much as 10 pA 10 msec after the step and may require as much as 200 msec to decay to below 1 pA. The time course of this slow tail is not exponential, but more closely approaches a logarithmic function of time. In addition, we have observed that for soda-lime pipets the magnitude of the slow component of capacity current is not always constant during a series of pulses, which occur at rates faster than about 1–2/s. Instead, the magnitude of this component is sometimes observed to decrease with successive pulses. Because of these characteristics, these capacitive currents can possibly be mistaken for whole-cell currents. Heavy Sylgard

coating can reduce the amplitude of the slow component of capacity current for soda-lime glasses, but is generally better (and certainly more convenient) simply to use glasses with lower loss factors (for further discussion *see* Rae and Levis, 1992a).

High lead glasses such as 8161, EG-6, EG-16, 0010, 0120, and KG-12 possess much lower dissipation factors than soda-lime glasses and are particularly useful due to their low melting point. This property allows the construction of initially very large-tipped pipets, which can be subsequently fire-polished to blunt bullet-shaped tips offering the lowest possible access resistance. This, of course, minimizes series resistance. In addition, pipets of this shape also draw in the largest surface-area patch of membrane when suction is applied. This is useful in perforated-patch recordings since the larger area of membrane available for partitioning by amphotericin or nystatin results in the maximum incorporation of perforation channels and thus the lowest access resistance. KG-12 (Friedrich and Dimmock) is a good choice for glasses of this class since it seals well, has good electrical properties, and is readily available.

Pipets for whole-cell recording can be thin-walled by comparison to those for single-channel recording. In whole-cell measurements, other sources of noise far outweigh the contribution from the pipet per se (*see* Subheading 5.8. below). In terms of total background noise, the major consideration in pipet fabrication is simply achieving the lowest possible resistance. Glass with an OD/ID ratio of 1.2–1.4 will have lower resistance for a given outside tip diameter than will thicker-walled glass, and is therefore useful for whole-cell recording. Some precautions are necessary, however, since if the walls become too thin the pipet will more easily penetrate the cell during the attempt to form a seal.

Other glasses that have been successfully used by many laboratories for whole-cell recording include Pyrex (Corning #7740), Kimble's Kimax, and Corning #7056. Schott #8250 or #8330 are also good choices and readily available from Garner Glass (Claremont, CA). Although we usually prefer the high-lead glasses described earlier, these other glasses have produced perfectly acceptable results.

3.2. Pulling Whole-Cell Electrodes

This can be done on any commercially available electrode puller. Here one simply strives for as blunt a taper and as large a tip diameter as is compatible with sealing of the electrode to the cell.

3.3. Elastomer Coating Whole-Cell Electrodes

Elastomer coating of electrodes reduces electrode noise in single-channel recordings. In whole-cell recordings, the noise associated with electrode glass is usually insignificant in comparison to other noise sources and so elastomer coating is not required for noise reduction. Elastomer coating also reduces electrode capacitance. Commercial patch-clamp amplifiers have the ability to compensate about 10 pF of electrode capacitance. For pipets made from glasses with high dielectric constants (e.g., soda-lime and high-lead glasses) immersed deeply into a tissue-bathing solution, the electrode (and holder) capacitance may exceed the compensation range of the electronics. Elastomer coating will help to keep the total electrode capacitance within the compensation range. For whole-cell recordings, it is not usually necessary to paint the elastomer close to the tip. Coating that extends from the top of the shank to 1mm from the tip, is sufficient for whole-cell recordings. Many investigators do not use elastomer coating for whole-cell recordings.

3.4. Firepolishing Whole-Cell Electrodes

Finally to promote gigohm seals and to reduce the possibility of tip penetration into the cell during seal formation, electrode tips should be firepolished. In some cells, fire polishing has proven unnecessary but we have found that sealing is generally promoted by firepolishing the electrode tip, particularly for cells where seal formation is difficult. Whole-cell and single-channel electrodes are firepolished with the same basic apparatus. Fire polishing can be done either using an upright or an inverted microscope. In fact, many investigators have chosen to coat their pipets and firepolish them using an inverted microscope with a 40× or so long working-distance objective. Another very useful approach is to utilize a standard upright microscope equipped with an objective for metallurgical microscopes. Several microscope companies, (Nikon, Olympus, Zeiss) make extra long working-distance high-magnification metallurgical objectives. Most noteworthy are the 100× objectives that have 3–3.5 mm working distances and numerical apertures of 0.8. With these objectives and 15× eyepieces and with the electrode mounted on a slide held in the mechanical stage of the microscope, it is possible to move the electrode tip into the optical field and visualize directly the electrode tip at 1500× magnification. At such high magnifications, it is possible to fire-

polish the tip to a very distinct optical endpoint under direct visualization. This approach ensures very repeatable results from one electrode to the next. The only drawback is that the objectives are quite expensive. The firepolishing itself is accomplished by connecting to a micromanipulator a rod of inert material to which has been fastened a short loop of platinum iridium wire. The ends of this wire must be soldered to two other pieces of wire that can be connected to a voltage or current source to allow current to be passed through the platinum wire. The platinum loop is generally bent into a very fine hairpin so that it can be brought to within a few microns of the electrode tip under direct observation.Because of early reports that platinum can be sputtered from the wire onto the electrode tip and prevent sealing, the platinum wire is generally coated with a glass like Pyrex (Corning #7740) or Corning #7056 to prevent such sputtering. This is done by overheating the platinum wire and pushing against it a piece of electrode glass that has been pulled into an electrode tip. At high temperatures, the glass melts and flows over the platinum wire and ends up thoroughly coating it and forming a distinct bead of glass. If the elastomer has been coated too near the tip, firepolishing causes the tip to droop downward at the juncture where the coating ends. If one desires to paint elastomer extremely close to the tip, it may be necessary to do the majority of the firepolishing before coating and then firepolish lightly again afterwards. As a general rule, firepolishing with the electrode tip close to the heating wire at low temperature produces a tip whose inner walls the parallel and relatively close together. With a hotter heating element and the tip farther away, the tip tends to round more and end up quite blunt.

4. Patch Electrode Fabrication for Single-Channel Recording

4.1. Choice of Glass

There is a limited number of glasses available for single-channel patch clamping. Perhaps the most important feature to consider is the amount of noise in the recording that is due to the pipet itself. This subject is sufficiently important that we include an entire section dealing with noise sources in pipets in hopes that the reader will be able to use the principles to make optimal