INSTRUMENTATION FOR MEASURING BIOELECTRIC SIGNALS FROM CELLS[1]

There are several recording techniques that are used to measure bioelectric signals. These techniques range from simple voltage amplification (extracellular recording) to sophisticated closed-loop control using negative feedback (voltage clamping). The biggest challenges facing designers of recording instruments are to minimize the noise and to maximize the speed of response. These tasks are made difficult by the high electrode resistances and the presence of *stray* capacitances[2]. Today, most electrophysiological equipment sport a bevy of complex controls to compensate electrode and preparation capacitance and resistance, to eliminate offsets, to inject control currents and to modify the circuit characteristics in order to produce low-noise, fast and accurate recordings.

Extracellular Recording

The most straight-forward electrophysiological recording situation is extracellular recording. In this mode, field potentials outside cells are amplified by an AC-coupled amplifier to levels that are suitable for recording on a chart recorder or computer. The extracellular signals are very small, arising from the flow of ionic current through extracellular fluid (see **Chapter 1**). Since this saline fluid has low resistivity, and the currents are small, the signals recorded in the vicinity of the recording electrode are themselves very small, typically on the order of 10 - 500 µV.

[1] In this chapter *Pipette* has been used for patch-clamp electrodes. *Micropipette* has been used for intracellular electrodes, except where it is unconventional, such as single-electrode voltage clamp. *Electrode* has been used for bath electrodes. *Microelectrode* has been used for extracellular electrodes.

[2] Some level of capacitance exists between all conductive elements in a circuit. Where this capacitance is unintended in the circuit design, it is referred to as *stray* capacitance. A typical example is the capacitance between the micropipette and its connecting wire to the headstage enclosure and other proximal metal objects, such as the microscope objective. Stray capacitances are often of the order of a few picofarads, but much larger or smaller values are possible. When the stray capacitances couple into high impedance points of the circuit, such as the micropipette input, they can severely affect the circuit operation.

The most important design criterion for extracellular amplifiers is low instrumentation noise. Noise of less than 10 µV peak-to-peak (μV_{p-p}) is desirable in the 10 kHz bandwidth. At the same time, the *input bias current*[3] of the amplifier should be low (< 1 nA) so that electrodes do not polarize. Ideally, the amplifier will have built-in high-pass and low-pass filters so that the experimenter can focus on the useful signal bandwidth.

Single-Cell Recording

In single-cell extracellular recording, a fine-tipped microelectrode is advanced into the preparation until a dominant extracellular signal is detected. This will generally be due to the activity of one cell. The microelectrode may be made of metal, *e.g.*, glass-insulated platinum, or it may be a saline-filled glass micropipette.

While not specifically targeted at extracellular recording, the Axoclamp and the Axoprobe micropipette amplifiers are particularly suitable for single-cell extracellular recording if the extracellular electrode is a microelectrode of several megohms or more. The input leakage current of these amplifiers is very low and headstages are designed to directly accommodate the micropipette holder. If necessary, capacitance compensation can be used to speed up the response. The x100 AC-coupled outputs of the Axoprobe generate signals that can be further amplified by external amplifiers without the risk that the external amplifiers will introduce measurable noise.

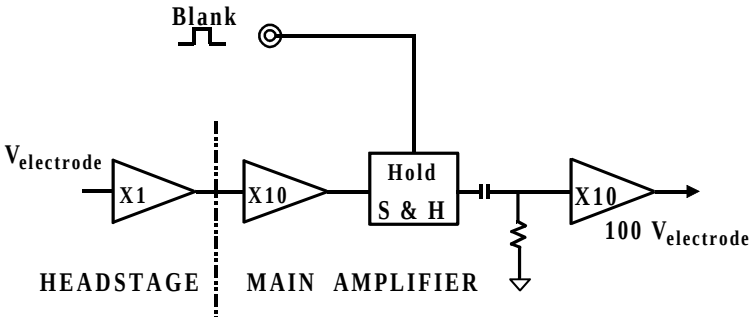

Figure 3-1. Blanking Circuit

The electrode is connected to the unity-gain buffer inside the headstage. In the main amplifier, the signal is given an initial tenfold amplification. This initial low-noise amplification increases the signal size so that noise contributed by the sample-and-hold (S & H) amplifier is irrelevant. During normal operation, the signal passes straight through the S & H, through the AC-coupling circuit and into the final x10 amplifier, for a total gain of x100. When a logic-level signal is applied to the BLANK input, the S & H holds the signal that was present at its input at the start of the BLANK pulse. Whatever transients appear on $V_{electrode}$ for the duration of the BLANK pulse are blocked. Thus the transients are not seen, nor do they saturate the AC-coupled amplifier.

The "blanking" circuit (Figure 3-1) that is part of the output pathway of the Axoclamp and the Axoprobe amplifiers is of particular usefulness. Blanking is used to prevent the capacitive artifact caused by a nearby stimulator from entering the recording pathway. A large artifact can

[3] In the ideal operational amplifier (op amp), no current flows into the inputs. Similarly, in the ideal transistor, no current flows into the gate. In practice, however, amplifying devices always have an input current. This current is commonly known as the *input bias current.*

cause the AC coupling circuit to saturate for several seconds. The blanking circuit consists of a sample-and-hold amplifier that is activated just before the stimulus. The sample-and-hold amplifier is held in the hold mode until the stimulus is finished. Thus, the stimulus artifact is prevented from entering the amplifier circuit, and recording can commence immediately after the stimulus terminates.

The Axon Instruments CyberAmp programmable signal conditioner and transducer amplifiers (CyberAmp 380 and CyberAmp 320) have special ultra low-noise probes suitable for extracellular recording. These probes do not have the special features of the Axoclamp and the Axoprobe, but for low-resistance electrodes, from tens of ohms up to a few hundred kilohms, these ultra low-noise probes have superior noise characteristics. The AI 402, x50 probe for the CyberAmp amplifiers contributes less noise than the thermal noise of a 250 Ω resistor. Electrodes can be connected directly to the CyberAmp amplifiers without using a separate low-noise probe. In this case, the additional noise due to the amplifier will still be very low — less than the thermal noise of a 5 kΩ resistor. If electrodes are directly connected to the main instrument, there is always a risk of picking up line-frequency noise or noise from other equipment. Using a probe located very close to the electrode greatly reduces the probability that it will pick up extraneous noise.

Multiple-Cell Recording

In multiple-cell extracellular recording, the goal is to record from many neurons simultaneously to study their concerted activity. Several microelectrodes are inserted into one region of the preparation. Each electrode in the array must have its own amplifier and filters. If tens or hundreds of microelectrodes are used, special fabrication techniques are required to produce integrated pre-amplifiers. With the large number of recording channels, the demands on the A/D system and programmable amplifiers are overwhelming. Systems for recording from hundreds of cells are not yet commercially available; however, such systems have been developed in research laboratories. If recording is required from up to 32 sites, four CyberAmp 380 amplifiers can conveniently be used with one 32-channel A/D system such as the Digidata 1200.

Intracellular Recording — Current Clamp

Voltage Follower

The traditional method for recording the cell interior potential is the current-clamp technique, also commonly known as "Bridge" recording, and occasionally as "voltage-follower" recording. The essence of the technique is the connection of a micropipette to a unity gain buffer amplifier that has an input resistance many orders of magnitude greater than that of the micropipette and the input resistance of the cell (Figure 3-2). The output of the buffer amplifier follows the voltage at the tip of the electrode. The ideal buffer amplifier draws no input bias current, therefore the current through the micropipette is "clamped" at zero.

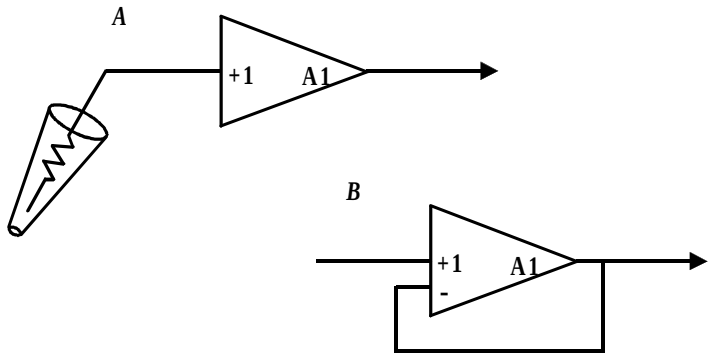

Figure 3-2. An Ideal Micropipette Buffer Amplifier

In ***A*** the buffer (A1) is represented in block-diagram form as a unity-gain amplifier. Note: The symbol +1 indicates a non-inverting input of gain x1. ***B*** shows how A1 is built from an operational amplifier with unity feedback.

If a high-quality current injection circuit (current source) is connected to the input node, all of the injected current flows down the micropipette and into the cell (see Figure 3-3). The current source can be used to inject a pulse of current to stimulate the cell, a DC current to depolarize or hyperpolarize the cell, or any variable waveform that the user introduces into the control input of the current source.

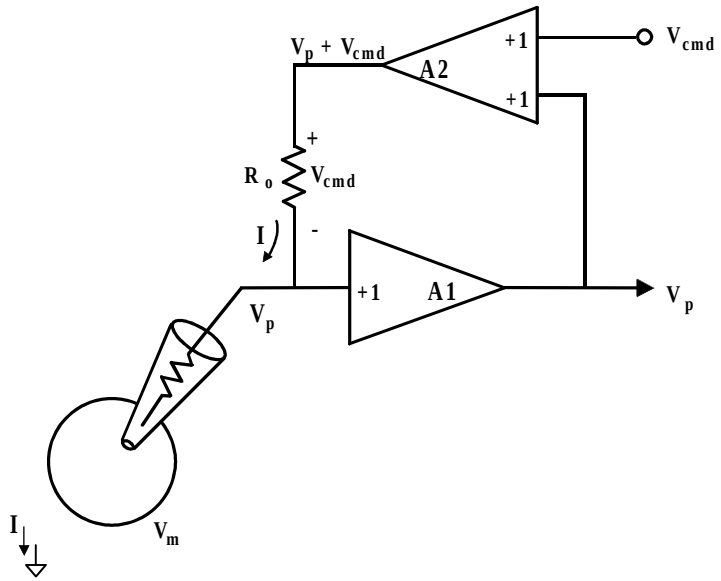

Figure 3-3. A High-Quality Current Source

A high-quality current source can be made by adding a second amplifier to the buffer amplifier circuit. The inputs to A2 are a command voltage, V_{cmd}, and the pipette voltage (V_p) buffered by A1. The voltage across the output resistor, R_o, is equal to V_{cmd} regardless of V_p. Thus the current through R_o is given exactly by $I = V_{cmd}/R_o$. If stray capacitances are ignored, all of this current flows through the pipette into the cell, then out through the cell membrane into the bath grounding electrode.

Bridge Balance

Can the intracellular potential be measured if the current injected down the micropipette is a variable waveform? Without special compensation circuitry, the answer is no. The variable current waveform causes a corresponding voltage drop across the micropipette. It is too difficult to distinguish the intracellular potential from the changing voltage drop across the micropipette. However, special compensation circuitry can be used to eliminate the micropipette voltage drop from the recording. The essence of the technique is to generate a signal that is proportional to the product of the micropipette current and the micropipette resistance. This signal is then subtracted from the buffer amplifier output (Figure 3-4). This subtraction technique is commonly known as "Bridge Balance" because in the early days of micropipette recording, a resistive circuit known as a "Wheatstone Bridge" was used to achieve the subtraction. In all modern micropipette amplifiers, operational amplifier circuits are used to generate the subtraction, but the name has persisted.

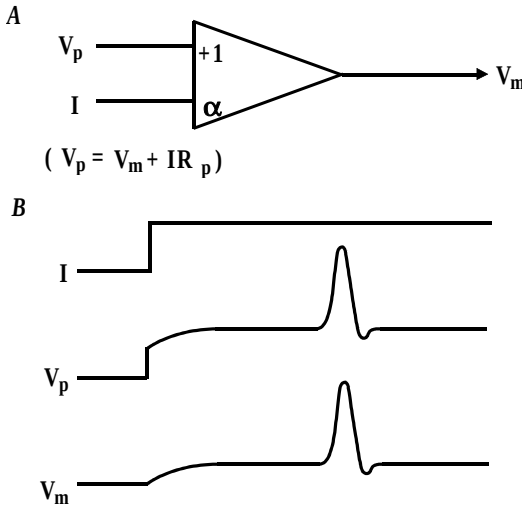

Figure 3-4. The "Bridge Balance" Technique

This technique is used to separate the membrane potential (V_m) from the total potential (V_p) recorded by the micropipette. The technique is schematically represented in ***A***. A differential amplifier is used to subtract a scaled fraction of the current (I) from V_p. The scaling factor is the micropipette resistance (R_p). The traces in ***B*** illustrate the operation of the bridge circuit. When the current is stepped to a new value, there is a rapid voltage step on V_p due to the ohmic voltage drop across the micropipette. Since the micropipette is intracellular, changes in V_m are included in V_p. Thus the V_p trace shows an exponential rise to a new potential followed by some membrane potential activity. The bridge amplifier removes the instantaneous voltage step, leaving the V_m trace shown.

There are several ways to set the bridge balance. A commonly used technique is to apply brief repetitive pulses of current to the micropipette while it is immersed in the preparation bath. The Bridge Balance control is advanced until the steady-state pulse response is eliminated (Figure 3-5). At this point, the circuit is balanced and the micropipette resistance can be read from the calibrated readout of the Bridge Balance control. The same technique can even be used after the micropipette has penetrated the cell. Details of how to achieve this are described in the Axoclamp and Axoprobe manuals.

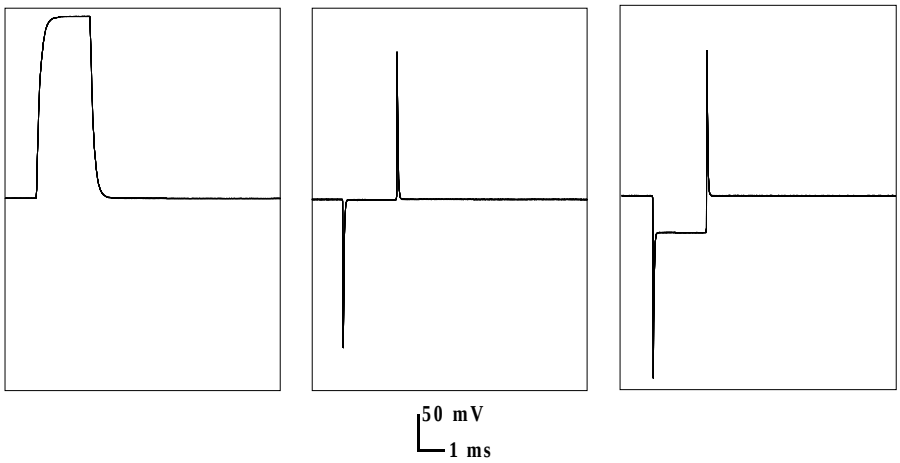

Figure 3-5. Illustration of Bridge Balancing While Micropipette is Extracellular

All traces show the V_m output from the bridge-balance circuit. A 5 nA, 2 ms pulse is applied to a 50 MΩ electrode. No bridge balance is used in the left trace, revealing the full voltage drop across the micropipette. In the middle trace, optimum bridge balance is used and the voltage drop across the micropipette is eliminated from the record. The transients at the onset and finish of the step result from the finite bandwidth of the headstage amplifier. In the right trace, the bridge-balance control is advanced too far; the voltage drop across the micropipette is overcompensated and a reverse-polarity step appears.

It is important that the micropipette resistance be constant. Clearly, if the micropipette resistance varies as the current flows there will be no unique setting of the Bridge Balance control and the measurement will include an artifact due to the variable voltage drop across the micropipette.

Junction Potentials

A second extraneous contributor to the measured voltage at the output of the micropipette buffer amplifier is the sum of the junction potentials. Junction potentials occur wherever dissimilar conductors are in contact. The largest junction potentials occur at the liquid-metal junction formed where the wire from the amplifier input contacts the electrolyte in the micropipette and at the liquid-liquid junction formed at the tip of the micropipette. The sum of all of the junction potentials can be eliminated by introducing a single DC potential of the opposite polarity. In micropipette experiments, the junction potentials can often sum to a few hundred millivolts. The ±500 mV range of the offset controls in the Axoclamp and the Axoprobe is more than sufficient to compensate for even the worst-case junction potentials.

Track

A special form of automatic offset compensation is available in many patch clamp amplifiers, including the Axopatch-1 series and the Axopatch 200 series. In Axopatch amplifiers this technique is called "Track." In some instruments of other manufacturers it is called "Search." The Track circuit is used only during seal formation. During seal formation the tip comes in and out of contact with the membrane and rapid shifts in the tip potential of the headstage sometimes occur. These potential shifts lead to equivalent rapid shifts in the current record, which may exceed the dynamic range of the headstage. Since the headstage itself saturates, AC coupling at

the oscilloscope or any other form of external DC offset removal will not be useful. Instead, the Track circuit dynamically adjusts the offset potential of the micropipette itself, so that the current is forced to be zero on average. It is important that the Track circuit be switched off before recording of ionic currents commences since the Track circuit introduces the same type of distortion produced by AC coupling.

Current Monitor

Typical current-clamp circuits include a monitor output proportional to the current waveform. In some current-clamp amplifiers, the monitor output is merely a scaled version of the control voltage driving the current-clamp circuit. As long as the micropipette resistance is moderate and the current-clamp circuit output does not exceed its linear operating range, this technique is accurate. However, if the micropipette resistance is very high and the current-clamp circuitry saturates, the actual current through the micropipette will be less than the expected current. This simple monitor output will not indicate the failure to pass the expected current. A better technique is to actually measure the current by monitoring the voltage drop across a resistor in series with the micropipette (Figure 3-6). This is a superior technique introduced commercially by Axon Instruments and used in all of Axon's micropipette current-clamp amplifiers.

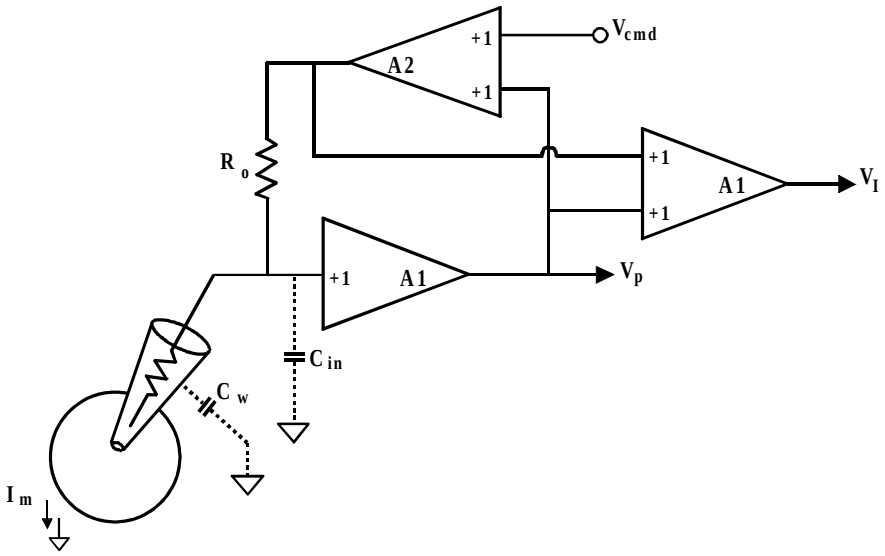

Figure 3-6. Series Current Measurement

The circuit in Figure 3-3 is extended by the addition of a differential amplifier (A3) to measure the voltage drop across the current setting resistor, R_o. This voltage drop is proportional to the total current (I) flowing through R_o. For high-frequency currents (*e.g.*, the transient current during a step change in the command potential), the current through R_o is not quite identical to the membrane current (I_m). There is an additional current that flows through the capacitance (C_w) of the wall of the micropipette and another current that flows into the input capacitance (C_{in}) of the amplifier. Nevertheless, if precautions are taken to minimize C_w (*e.g.*, shallow bath immersion, Sylgard or mineral oil coating) and C_{in} (*e.g.*, bootstrapped buffer amplifier, capacitance neutralization), the error current in these stray capacitances can be minimized and the output signal, V_I, is a good representation of the membrane current.

Another way to measure the true micropipette current is to use a separate circuit called a "virtual ground" (Figure 3-7). Instead of connecting the bath ground electrode directly to ground, it is connected to the virtual ground formed at the negative input of an inverting operational amplifier. The negative input in this configuration is called a "virtual" ground because the operational amplifier endeavors to keep the negative input equal to the ground potential at the positive input. To achieve this goal, the operational amplifier output must continuously pass a current through the feedback resistor (into the node at the negative input) that is equal and opposite to the bath ground current. Thus the voltage at the output of the operational amplifier is directly proportional to the bath current.

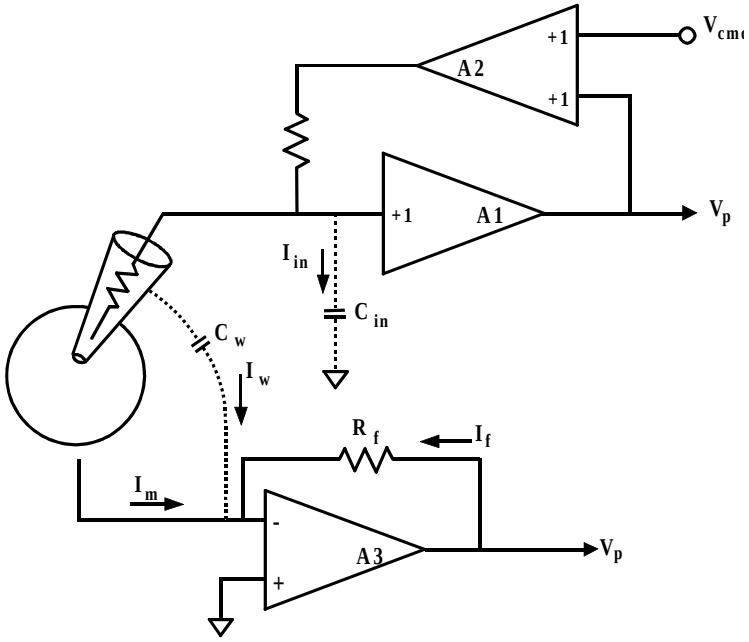

Figure 3-7. Virtual-Ground Current Measurement

The bath grounding electrode is connected to the negative input of operational amplifier A3. The output (V_I) continually adjusts to pass current through the feedback resistor (R_f) equal and opposite to the total current flowing into the junction at the negative input of A3. The potential at the negative input is "virtually" equal to ground at all times. Most of the current measured by A3 is the membrane current, I_m. However, during rapid potential changes, transient current flows through the capacitance (C_w) of the wall of the micropipette and the input capacitance (C_{in}) of the amplifier. The current (I_w) through C_w flows into the bath and is recorded by the virtual-ground circuit. The current (I_{in}) through C_{in} flows into the system ground and is not recorded by the virtual-ground circuit.

Since the series current-measurement technique is much more convenient, it is routinely used in preference to the virtual-ground current-measurement technique. The virtual-ground technique is inconvenient for several reasons:

(1) The input lead to the virtual ground is very sensitive and easily picks up line-frequency and other interference.

(2) It is extremely difficult to use two microelectrode amplifiers in the same preparation bath if one or both of them uses a virtual-ground current-measurement circuit. If one amplifier uses a virtual ground, the individual micropipette currents will be difficult to identify because the virtual-ground current monitor measures the *summed* current from all sources. If two amplifiers use a virtual ground, large DC-error currents will flow between the virtual-ground circuits because of the small differences in offset voltages between the two circuits. This is equivalent to the problem that would exist if two voltage sources were connected in parallel.
(3) The virtual-ground probe is just one more box of electronics that has to be mounted in the crowded space around the preparation bath.

Nevertheless, the virtual ground technique is still used in some circumstances, most commonly with high-voltage two-electrode voltage-clamp amplifiers, because of the technical difficulty of making a series current-measurement circuit to operate at high voltages.

The *Bath Error Potentials* section below describes an important variation on the virtual-ground design to eliminate the voltage error due to current flow through the grounding electrode.

Headstage Current Gain

The HS-2 headstages used in Axon Instruments micropipette amplifiers (Axoclamp, Axoprobe) all have unity voltage gain but come in a variety of current-passing capabilities. The current-passing range and resolution are determined by the size of a single resistor in the headstage. This resistor (R_0) has two functions. In addition to determining the size and resolution of the current that can be passed, it also determines the sensitivity with which current is measured. In the standard HS-2 headstage, R_0 is 10 MΩ; this is arbitrarily assigned a current-passing gain of H = 1. To pass more current, a headstage with a *smaller* value of R_0 is required. The headstage with R_0 = 1 MΩ can pass ten times as much current as the standard headstage; it is assigned a current-passing gain of H = 10. The headstage designed for ion-sensitive micropipettes can only pass very tiny currents. The value of R_0 in this headstage is 100 GΩ; the current-passing gain is H = 0.0001.

Capacitance Compensation

The high-frequency performance of the micropipette amplifier is compromised by the presence of capacitance at the amplifier input. This capacitance comes from several sources: the capacitance across the glass wall (transmural capacitance) of the immersed part of the micropipette; the stray capacitance from the rest of the micropipette to nearby grounded surfaces; the stray capacitance from the micropipette holder; and the capacitance to ground at the input of the buffer operational amplifier.

The input capacitance and the micropipette form a simple low-pass filter. That is, high-frequency signals at the tip of the micropipette are shunted to ground by the input capacitance. There are several ways to improve the bandwidth of the recording system.

The best is to minimize the *physical* magnitude of the various elements contributing to the input capacitance. The transmural capacitance of the immersed part of the micropipette can be quite large. Typically, it is 1 pF or more per millimeter of immersion depth. An effective way to reduce this capacitance is to thicken the wall of the micropipette. This can be done by coating the micropipette with Sylgard or an equivalent material (see **Chapter 4**). Other materials have

been used, and in extreme cases, researchers have used a wide micropipette to form a jacket over the narrower recording micropipette.

The other obvious way to reduce the transmural capacitance is to reduce the fluid level so that the immersion depth is minimal. This is not always as effective as might be expected, because surface tension causes the bath solution to creep up the surface of the micropipette. This generates significant capacitance between the inside of the micropipette and the film of solution on the outside. Forming this film can be prevented by making the surface of the micropipette hydrophobic by dipping the filled micropipette into mineral oil (or silane) immediately before using it. Note that it is essential to fill the micropipette first with the aqueous electrolyte pipette solution so that the aqueous solution prevents the mineral oil from entering the tip. Finally, one should try not to place the holder or the microelectrode too close to grounded surfaces (such as the microscope objective).

Once the physical magnitude of the stray capacitance has been minimized, electrical techniques can be used to reduce the *effective* magnitude. There are three such techniques:

(1) Some researchers surround the micropipette with a metal shield that is connected to the unity-gain output of the buffer amplifier. The principle here is to make sure that the stray capacitance does not have a signal voltage across it. As long as there is no signal voltage across the capacitance, no high-frequency current is drawn and the bandwidth is increased. Unfortunately, this technique can significantly compromise the noise performance of the amplifier because random noise generated in the buffer amplifier is coupled back into its input via the shield capacitance. ***For this reason, Axon Instruments does not recommend the use of a driven shield.***

(2) Unity-gain feedback can be used to reduce the component of stray capacitance that exists between the amplifier input and its power supplies and case (Figure 3-8). In a technique known as "bootstrapping," sophisticated circuitry is used to superimpose the unity-gain output of the buffer amplifier back onto its own power supplies and the headstage case. This eliminates the high-frequency current loss through the power supply capacitance that consequently increases the bandwidth. Since the power supply capacitance is present whether or not the power supply is bootstrapped, there is no noise penalty due to implementing the technique. Bootstrapped power supplies are implemented in the Axoclamp and Axoprobe micropipette amplifiers and contribute to their excellent noise and bandwidth characteristics.

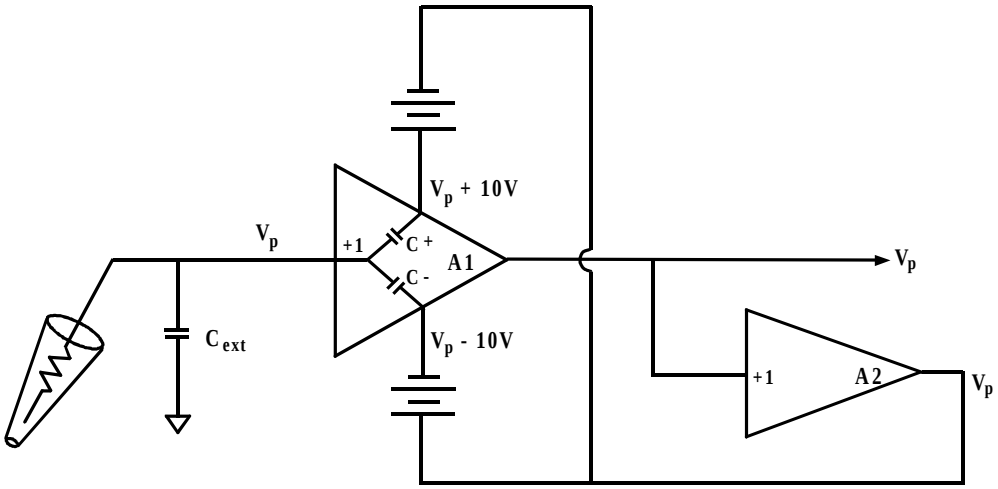

Figure 3-8. Bootstrapped Power Supplies

Bootstrapped power supplies are used to increase the bandwidth by minimizing the high-frequency current loss through the power-supply capacitance. The power-supply capacitance is made up of C^+ and C^- from the positive input of A1 to the positive and negative power supplies, respectively. All of the stray capacitances other than C^+ and C^- are lumped together and called C_{ext} in this figure. The power supplies of A1 are batteries. The center point of the batteries is connected to a buffered version of V_p. If the batteries are 10 V, the power supplies for A1 are (V_p + 10 V) and (V_p - 10 V). The voltages across C^+ and C^- are constant at +10 V and -10 V, respectively. Hence, there are no transient currents in neither C^+ nor C^-, except at very high frequencies where these simple amplifier models are not appropriate. To ensure stability, the bandwidth of A2 must be half or less of the bandwidth of A1. In practical implementations such as the Axoclamp microelectrode amplifier, the batteries are simulated by electronics.

(3) Finally, but not least, the technique known as "capacitance compensation" or "negative capacitance" can be used to reduce the effective value of whatever capacitance remains. An amplifier at the output of the unity-gain buffer drives a current-injection capacitor connected to the input (Figure 3-9). At the ideal setting of the amplifier gain, the current injected by the injection capacitor is exactly equal to the current that passes through the stray capacitance to ground. If the amplifier gain is increased past the ideal setting, the current injected back into the input will cause the input signal to overshoot. As the gain is increased past a certain setting, the circuit will oscillate with potentially disastrous consequences for the cell.

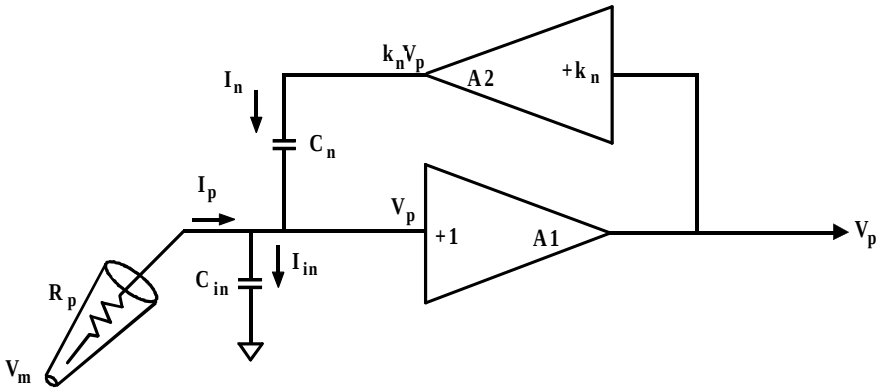

Figure 3-9. Capacitance Neutralization Circuit

Amplifier A2 places a voltage proportional to the pipette potential (V_p) across the capacitance-neutralization capacitor (C_n). At a particular setting of the gain (k_n) of A2, the current (I_n) through C_n is exactly equal to the current (I_{in}) through the stray capacitance (C_{in}) at the input of A1. By conservation of current, the pipette current (I_p) is zero. In the ideal case, the effective capacitance loading the pipette resistance (R_p) is zero and the bandwidth is therefore very high. In practice, limitations due to the finite bandwidth of the amplifiers, the distributed nature of C_{in} and other second-order effects limit the recording bandwidth.

An important consideration in the design of a capacitance neutralization circuit is to avoid introducing much additional noise from the circuitry itself. Low-noise amplifiers must be used and the size of the injection capacitor must be small. Axon Instruments' HS-2 series headstages are available in two varieties. The low-noise "L" series headstages use a 3 pF injection capacitor. The medium-noise "M" series headstages use a 10 pF injection capacitor. Generally, the M series headstages are only used for the output (current-passing) micropipette in a two-micropipette voltage-clamp setup. Noise in this micropipette is less important than noise in the input (voltage recording) micropipette, and it is sometimes useful to have a larger compensation range because in a two-pipette voltage clamp it is common to place a grounded metal shield near the output micropipette to prevent coupling of its signal into the input micropipette.

In standard electronic circuit design, all of the circuitry is supplied by a ±15 V power supply. The normal operating range for signals is ±10 V. If the optimum gain of the capacitance neutralization amplifier is moderately high, (*e.g.*, 2), the amplifier will saturate during large signals. In many experiments, large inputs would be unusual, and even if they occurred the speed penalty incurred would not be disastrous. However, in single-electrode voltage clamping, large signal excursions are common and speed is crucial. To eliminate the saturation of the capacitance compensation circuit, the Axoclamp amplifier uses ±30 V amplifiers in the capacitance neutralization circuit.

If everything were ideal, the bandwidth of the recording system would approach the bandwidth of the buffer amplifier alone (typically a few megahertz). However, in practice the bandwidth is limited by the phase delays in the capacitance compensation pathway, and by the false assumption that the stray capacitance can be represented by a lumped capacitor at the input of the amplifier. The capacitance is distributed along the length of the micropipette. Nevertheless, the techniques described above can be used to substantially improve the bandwidth. It is not unusual

to achieve bandwidths of 30 kHz with micropipettes that would have a bandwidth of just one or two kilohertz if the above techniques were not implemented.

Transient Balance

After the bridge balance and the capacitance compensation circuits have been optimally set, a visible transient remains on the voltage recording at the onset of a current step. It is not reasonable to try to measure any membrane potential changes during this residual transient, since it represents the period of time that the residual effective capacitance at the amplifier input is charging. On some amplifiers, a special "transient balance" control is provided to artificially hide this transient from the output. This control is not provided on Axon Instruments amplifiers because the data recorded during the period that the transient is hidden is of dubious merit. It is better to attempt to minimize this transient by reducing electrode and input capacitance.

On the other hand, a transient balance control can be useful during impedance measurements where a sine wave current is injected down the micropipette. The sine wave in the micropipette response is attenuated by the input micropipette capacitance, even at frequencies well below the -3 dB cutoff frequency. Since, in the absence of a transient balance control, the signal removed by the bridge balance compensation circuitry is not attenuated with frequency, the subtraction is imperfect at the highest bandwidths that the micropipette is capable of measuring. In this case, a transient balance control can extend the useful bandwidth.

Leakage Current

Ideally, a micropipette headstage should pass zero current into the micropipette when the current command is at zero. In practice, there is a leakage current created from two sources. The first is the inherent bias current of the operational amplifier's input. This is usually of the order of a few picoamps or less. The second is the current that flows through the current injection resistor (R_o in Figure 3-3) because of voltage offsets in the current-control circuitry. A trim potentiometer can be used to reduce this offset. In fact, an optimal setting can be found where a small offset is left across R_o so that the resistor current compensates the operational amplifier bias current and leaves a net zero current through the micropipette. However, the leakage current will quickly grow again to unacceptable levels if the offsets in the control circuitry change significantly with temperature or time. To avoid this problem, Axon Instruments uses extremely high-quality, low-drift operational amplifiers in its micropipette amplifiers.

Headstages for Ion-Sensitive Microelectrodes

The most demanding application in terms of low-leakage current requirements is the measurement of ion concentration using ion-sensitive microelectrodes (ISMs). In spite of the current popularity about ion-sensitive intracellular dyes, ISMs are still the best means available for determining extracellular and, in some cases, intracellular ion concentrations. These electrodes can be very difficult to fabricate because of the efforts required to place the highly hydrophobic ion-sensitive resin at the very tip of the electrode. Due to the hydrophobic nature of the liquid resin, ISMs are also very sensitive to current passing into the electrode. Even the tiniest amount of leakage current from the headstage can cause the resin to move. If the resin leaks out of the electrode it can contaminate the preparation and destroy cells in the vicinity of the electrode. If the resin moves up the barrel of the pipette, the response of the electrode is slowed dramatically or destroyed.

There are two ways to ensure low-leakage currents for these microelectrodes: First, use special operational amplifiers that have bias currents of just a few tens of femtoamps. Second, make the value of R_o very large (100 GΩ) so that the current induced by small offset voltages across R_o is small.

Bath Error Potentials

In most experiments, the bathing solution is grounded by a solid grounding electrode (such as an agar/KCl bridge) and all measurements are made relative to ground, on the assumption that the bath is also at ground. This assumption may not be true in experiments in which the Cl^- concentration or temperature of the bathing solution is significantly changed, or where the membrane current is sufficiently large to cause a significant voltage drop across the resistance of the grounding electrode. The latter circumstance would normally occur only when voltage clamping very large cells such as frog oocytes, in which case the ionic current may be of the order of several microamps or even several tens of microamps.

Depending upon the grounding method, the resistance of the bath grounding electrode (R_b) could be as much as 10 kΩ, although with care it is not difficult to achieve R_b values less than 1 kΩ.

In a simple two-electrode voltage clamp (TEVC) setup, the voltage drop across R_b is indistinguishable from the membrane potential. That is, the potential (V_1) recorded by the voltage-recording micropipette is the sum of the transmembrane potential (V_m) and the bath potential (V_b). Problems arise if the product of the clamp current (I_2) and R_b is significant. For example, for I_2 = 5 μA and R_b = 2 kΩ, the error voltage is 10 mV. In some experiments, a worst-case error of this magnitude might be tolerable; but if the error were to be much greater, the position of the peak of I-V curves and other responses would be seriously affected.

To faithfully record V_m, either V_b must be made equal to or nearly equal to zero, or the value of V_b must be independently measured and subtracted from the potential recorded by ME1. There are several methods to choose from:

(1) The best method is to minimize R_b in the first place.
(2) Electronically predict and subtract the error voltage. This technique is known as "series resistance compensation."
(3) Use an independent electrode to measure V_b and subtract this value from V_1.
(4) Set up an independent clamping circuit to clamp V_b, measured near the surface of the cell, to zero.
(5) Set up an independent virtual-ground circuit to clamp V_b, measured near the surface of the cell, to zero, while simultaneously measuring the bath current.

1. Minimizing R_b

There are three main contributors to R_b:

(1) The cell access resistance from the membrane surface to the bath.
(2) The resistance of the agar bridge, if used.
(3) The resistance of the grounding wire.

Cell Access Resistance

The access resistance to a sphere is given by:

$$R_a = \frac{\rho}{4\pi r} \quad (1)$$

where ρ is the resistivity (typically 80 Ωcm for Ringer's solution at 20°C [Hille, 1984]) and r is the radius of the sphere in centimeters.

Modeling an oocyte as a 1 mm diameter sphere,

$$R_a = 127\ \Omega$$

Resistance of Agar Bridge

The resistance of a cylindrical agar bridge is given by (Hille, 1984):

$$R_{agar} = \frac{\rho \times \text{length (cm)}}{\text{area (cm}^2\text{)}} \quad (2)$$

where length refers to the length of the agar bridge and area is the cross-sectional area of the agar bridge.

For a 1 cm-long agar bridge with a 2 mm internal diameter (ID) filled with Ringer's solution,

$$R_{agar} = 2.6\ k\Omega$$

For an agar bridge 1 cm long x 1 mm ID, filled with Ringer's solution,

$$R_{agar} = 10.2\ k\Omega$$

The resistivity of 3 M KCl is approximately 20 times lower than for Ringer's solution. Thus, for an agar bridge filled with 3 M KCl, having the same length as above,

$$R_{agar} = 130\ \Omega \text{ if the ID is 2 mm}$$
$$R_{agar} = 510\ \Omega \text{ if the ID is 1 mm}$$

Resistance of Grounding Wire

When immersed in 0.9% saline, the impedance of a 1 mm diameter Ag/AgCl pellet is in the range of 300 - 600 Ω, depending on how much of the surface is in contact with the saline.[4]

[4] Measurements made at Axon Instruments, using a 700 Hz signal source.

Conclusion

The access resistance to the cell is quite small and beyond the control of the experimenter. The other major contributors to R_b are the resistance of the agar bridge and the resistance of the grounding electrode.

To minimize R_b, it would be best to eliminate the agar bridge and ground the preparation directly with a Ag/AgCl pellet. The pellet should be as large as practical, and the area of contact between the pellet and the solution should be maximized.

However, if the bathing solution is changed during the experiment, the DC offset of the Ag/AgCl pellet will change with the chloride activity. Thus, in this case it is essential to either use an agar bridge to prevent the DC offset of the bath from changing, or to use a bath-potential recording headstage with a 3 M KCl electrode to follow and remove the shift. Another advantage of an agar bridge is that it prevents metal ions from the grounding electrode from entering the bathing solution.

If an agar bridge is used, it is best to fill it with 3 M KCl instead of Ringer's solution in order to minimize R_b. When the agar bridge is filled with 3 M KCl, the sum of all components of R_b will be approximately 1 - 2 kΩ. If leakage of KCl from the agar bridge is a problem, it may be necessary to fill the agar bridge with Ringer's solution. In this case, R_b will be several kilohms or more.

2. Series Resistance Compensation

This sophisticated technique is used to minimize the effects of the electrode resistance in a whole-cell voltage clamp. It is described later in this chapter and is of paramount importance when there is no alternative available. However, series resistance compensation is never completely effective. The other methods discussed in this section are more reliable and easier to apply when it comes to eliminating the voltage error across R_b.

3. Measure and Subtract V_b

Another technique used to eliminate the effects of the IR voltage drop across the bath resistance is to actively measure the bath potential outside the cell.

In this method, the bath is grounded by any preferred method. The bath potential is allowed to shift because of solution changes, temperature changes or current flow. The changing values of the bath potential (V_b) are continuously monitored by a 3 M KCl-filled electrode placed near the cell. The measured value of V_b is subtracted from the potentials measured by the intracellular microelectrodes. For microelectrodes being used for voltage recording, this subtraction yields the true transmembrane potential (V_m). The signal provided to all subsequent circuits in the amplifier is V_m.

This method operates well. The minor inconveniences are the more complex electronics, the noise of the bath electrode that is added to the noise of the intracellular recording micropipette, and some (minor) potential for instability in a two-electrode voltage clamp if the bandwidth of the bath potential headstage is greater than the bandwidth of the intracellular recording micropipette.

4. Clamp V_b Using a Bath Clamp

Another means to eliminate the effect of the voltage drop across R_b is to actively control the bath potential measured near the outside surface of the cell. This is achieved using a two-electrode virtual-ground circuit (Figure 3-10). One electrode (SENSE) is a voltage-sensing electrode. This electrode is placed in the preparation bath near the cell surface. It is connected to the virtual-ground circuit by an agar bridge or other means, of resistance R_{b2}. Since there is no current flow through this electrode, there is no voltage drop across R_{b2}. The other electrode (I_{BATH}) is placed in the preparation bath. This electrode carries the ionic current. The feedback action of the operational amplifier ensures that the potential at the SENSE electrode is equal to the potential at the negative amplifier input (ignoring the offset voltage at the inputs to the operational amplifier).

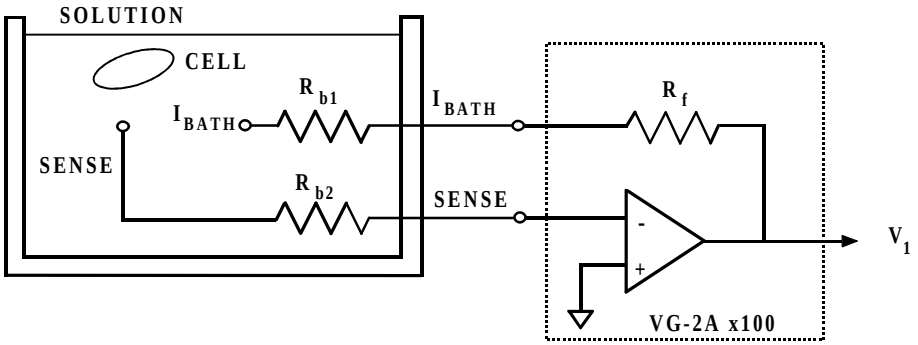

Figure 3-10. Two-Electrode Virtual-Ground Circuit

In this implementation separate electrodes are used to pass the bath current and control the bath potential (unlike Figure 3-7 above). The basic operation is the same as for the virtual-ground circuit described earlier, except that instead of connecting the feedback resistor directly to the negative input of the operational amplifier, it is connected indirectly via the bath solution. The bath potential near the surface of the cell is recorded by the SENSE electrode (resistance R_{b2}) and forced by the feedback action of the operational amplifier to be near ground. The bath current required to achieve this is passed by the operational amplifier through the feedback resistor (R_f) and through the bath current electrode (I_{BATH}; resistance R_{b1})

5. Clamp V_b Using a Virtual-Ground Current Monitor

This technique is identical to the bath clamp described above, except that in this case instead of being ignored, the output of the circuit is used to monitor the current flowing from the micropipettes into the bath. The output (V_I) of this circuit is proportional to the bath current.

$$V_I = (R_{b1} + R_f)\, I_{BATH} \tag{3}$$

There are several problems with this technique. First, if the output is used to record the bath current, it is usual to ignore the R_{b1} term (because its value is unknown) and to assume that

$$V_I = R_f\, I_{BATH} \tag{4}$$

Thus, there is a small error equal to $R_{b1}/(R_{b1} + R_f)$. Second, if there are fluid-filled tubes connected to the bath, and if some of these run outside of the Faraday cage, they will act as antennas and conduct a lot of hum current into the bath that will be recorded by the virtual-ground circuit. Third, if it is desired to use more than one amplifier with the same preparation, it is essential that no more than one of them uses a virtual-ground circuit to clamp the bath potential. This is not a serious problem, since it is easy to make one virtual-ground serve the needs of both amplifiers. The problem is serious, however, if one of the amplifiers introduces command signals via the virtual-ground bath clamp. This is a practice employed by some manufacturers because in some circumstances it is easier to design the electronics for this mode of operation. However, if command potentials are introduced via the bath electrode, it is extremely difficult to perform experiments using two amplifiers, *e.g.*, whole-cell patch clamp of two fused cells.

When used with consideration for the measurement error, and if hum pick up is avoided, a virtual-ground circuit to measure I_{BATH} offers excellent performance.

Summary

As a first priority, we recommend that the value of R_b be minimized by the techniques described above. If it is still considered to be important to compensate for the IR voltage drop, a virtual-ground headstage (such as the VG-2 series) can be used to clamp the bath potential, or a unity-gain voltage follower headstage (such as the HS-2 series) can be used to record and subtract the bath potential.

Cell Penetration: Mechanical Vibration, Buzz and Clear

Often when a micropipette is pressed against a cell, the membrane dimples but the micropipette fails to penetrate. A variety of methods are used to overcome this problem. The oldest and frequently effective trick is to tap the micromanipulator gently. Since this "method" is difficult to calibrate and reproduce, two electronic alternatives are widely used. One is to drive a brief, high-frequency oscillatory current through the micropipette. This is often done by briefly increasing the capacitance compensation such that the compensation circuit oscillates. On many instruments, such as the Axoclamp and Axoprobe, a dedicated button called "Buzz" (or "Tickle") is provided for this purpose. This button may have a duration control associated with it. The duration used should be long enough for penetration, but short enough to prevent damage to the cell. The mechanism of penetration is unknown, but it may involve attraction between the charge at the tip of the micropipette and bound charges on the inside of the membrane.

The second electronic alternative is to drive a large positive or negative current step into the cell. In the Axoclamp and Axoprobe amplifiers, this is achieved by flicking the spring-loaded CLEAR switch. Again, the mechanism of penetration is not known. One cannot even predict *a priori* whether a positive or a negative pulse will be more effective.

None of the three methods described here can be described as "best." For a particular cell type, the researcher must experiment to find the method that works best with those cells.

Command Generation

In general, current and voltage commands arise from a variety of sources; internal oscillators in the instrument, internal DC current or voltage controls, external input from a computer, etc.

These commands are usually summed in the instrument so that, for example, an externally generated step command can be superimposed on the internally generated holding potential.

An important consideration when using externally generated commands is the attenuation provided by the instrument's command input. Manufacturers attenuate the external command so that the effects of noise and offset in the external signal generator are minimized. Noise is particularly common on the analog output of most computer interfaces. To illustrate the problem, imagine a micropipette amplifier used for current injection into cells that has a current passing capacity of ±100 nA maximum. Compare the situation for two different values of the command input attenuation. In the first case, the amplifier passes 1 nA for each millivolt from the computer. If there is a 2 mV of wide-band digital noise on the output of the D/A converter, this will generate 2 nA of noise in the recording — a substantial fraction of the usable range. Further, if the D/A converter had a 3 mV offset, then instead of a command of zero generating 0 nA, it would generate 3 nA. In the second case, the amplifier passes 1 nA for each 100 mV from the computer. This time, the 2 nA of noise from the D/A converter only generates 20 pA of noise in the recording, and the 3 mV offset error only generates a 30 pA current error. The full ±100 nA range of the amplifier can still be controlled, since most D/A converters produce up to ±10 V.

All of the current-clamp and voltage-clamp instruments from Axon Instruments use the maximum possible attenuation so that the ±10 V output from the D/A converter corresponds to the normal maximum operating limits of the instrument.

Another important consideration when using externally generated commands is the presence or absence of *polarity inversion*. To minimize the risk of making mistakes during an experiment, it is best that the instrument does not invert the command signal. Thus the user should always know that a positive command from the computer will generate a positive current or voltage in the instrument.

Intracellular Recording — Voltage Clamp

In the voltage-clamp technique, the membrane potential is held constant (*i.e.*, "clamped") while the current flowing through the membrane is measured. Usually, the investigator has no inherent interest in the membrane current, but is interested in the membrane conductance, since conductance is directly proportional to the ion-channel activity. Current is measured because the investigator has no direct way of measuring conductance. By holding the membrane potential constant (or at the very least, constant after a rapid step), the investigator ensures that the current is linearly proportional to the conductance being studied.

Micropipette voltage-clamp techniques for whole-cell current measurement traditionally assign the role of potential measurement and current passing to two different intracellular micropipettes. This is done using the conventional two-electrode voltage-clamp technique, or it is simulated by time-division multiplexing (time-sharing) using the discontinuous single-electrode voltage-clamp technique. Separation of the micropipette roles by either of these methods avoids the introduction of errors in the measurement due to unknown and unstable voltage drops across the series resistance of the current-passing micropipette.

The so-called "whole-cell patch" voltage clamp is a technique wherein only one micropipette is used full-time for both voltage recording and current passing. A method known as series-resistance compensation attempts to eliminate the aberrations arising when one micropipette is made to perform the work of two. For series-resistance compensation to work, the micropipette resistance must be reasonably low compared to the impedance of the cell. Since the resistance of intracellular micropipettes generally is too high, researchers use very blunt micropipettes that can be tightly sealed to a *patch* of membrane. The patch of membrane is subsequently ruptured so that the micropipette provides a low-resistance access to the *whole cell*, hence the term "whole-cell patch" clamp.

The Ideal Voltage Clamp

The *ideal* voltage clamp simply consists of a battery, a switch, a wire, the cell and an ammeter (Figure 3-11). Since the wire has zero resistance when the switch is closed, the membrane potential steps instantly to the battery voltage. This generates an impulse of current that charges the membrane capacitance, followed by a steady-state current to sustain the voltage across the membrane resistance.

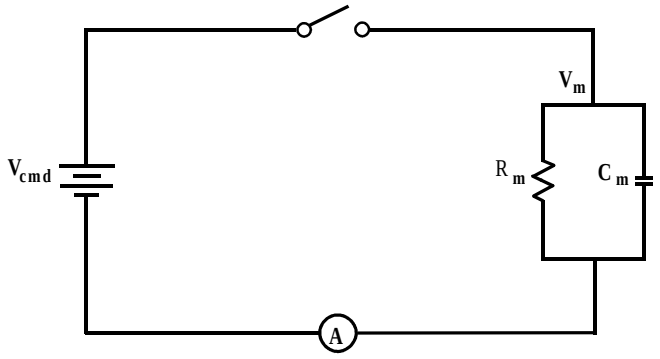

Figure 3-11. The Ideal Voltage Clamp

The *ideal* voltage clamp simply consists of a battery, a switch, a wire, the cell and an ammeter. When the switch closes, the membrane potential steps instantly to the battery voltage. (For simplicity, it is assumed that $V_m = 0$ before the step.) There is an impulse of current injecting a charge $Q = C_m V_{cmd}$; the steady-state current is V_{cmd}/R_m.

In this experiment, the voltage is the independent variable. Its value is controlled and equal to the battery value. The current is the dependent variable; its value is measured by an ammeter. For this reason, the voltage-clamp circuit is sometimes called a "current follower."

Real Voltage Clamps

There are many reasons for real voltage clamps to be inferior to the ideal voltage clamp. These include finite resistance of the current-passing micropipette, limited bandwidth of the voltage-recording micropipette, capacitive coupling between the two micropipettes, limited bandwidth of the clamp amplifier, and non-ideal phase shifts in the membrane. These phenomena and the minimization of their impact are discussed in the following sections.

Large Cells — Two-Electrode Voltage Clamp

The theory of two-electrode voltage clamping is discussed in detail by Finkel and Gage (1985). Without attempting to duplicate that discussion, some of the most important conclusions will be presented here.

A conventional two-electrode voltage clamp is shown in Figure 3-12. This figure has been simplified by ignoring several frequency-dependent components: the input capacitance at the input of micropipette ME1, the coupling capacitance between the micropipettes, and the special phase lead and lag circuitry that is often introduced deliberately into the electronics. Furthermore, series resistance in the membrane and the bathing solution has been ignored. In the figure, the sole frequency-dependent component is the membrane capacitance.

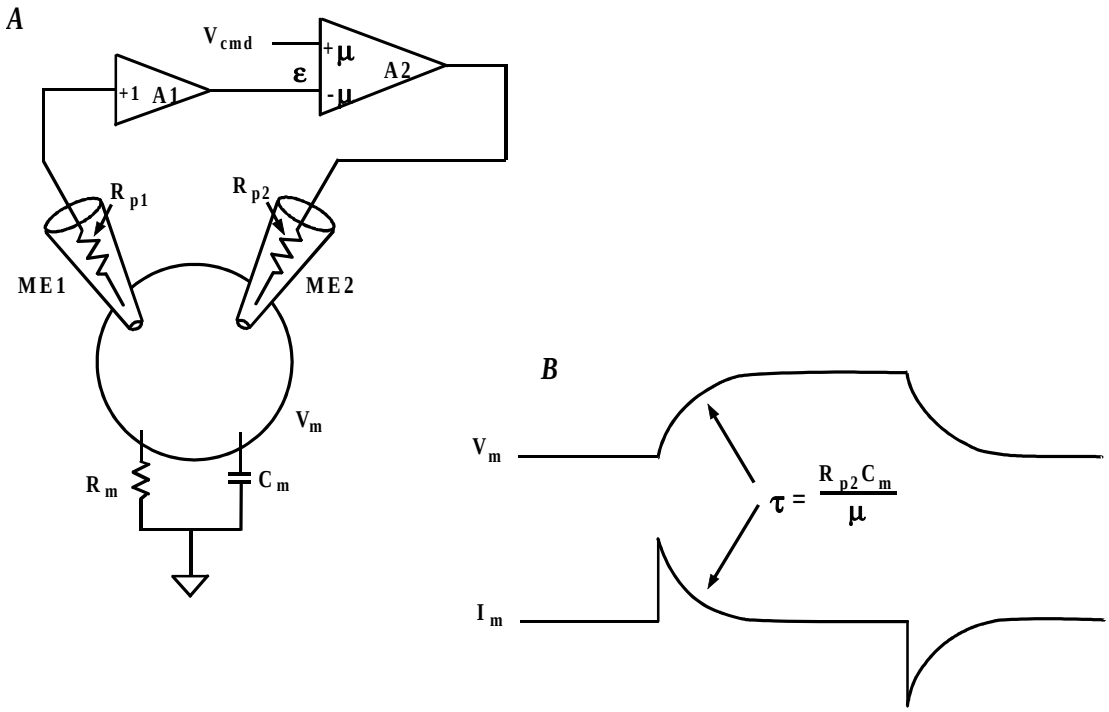

Figure 3-12. Conventional Two-Electrode Voltage Clamp

A. The membrane potential (V_m) is recorded by a unity-gain buffer amplifier (A1) connected to the voltage-recording microelectrode (ME1). V_m is compared to the command potential (V_{cmd}) in a high-gain differential amplifier (A2; gain = μ). The output of A2 is proportional to the difference (ε) between V_m and V_{cmd}. The voltage at the output of A2 forces current to flow through the current-passing microelectrode (ME2) into the cell. The polarity of the gain in A2 is such that the current in ME2 reduces ε. ***B.*** Unlike the ideal case, there is a finite time required to charge the cell capacitance. The time constant for the current and potential transients is $\tau = R_{p2}C_m/\mu$, where R_{p2} is the resistance of ME2. If μ was infinite, or if R_{p2} was zero, the response would approach the ideal case.

Error

The steady-state membrane potential (V_m) after a step change in the command voltage (V_{cmd}) is:

$$V_m = V_{cmd}\frac{\mu K}{\mu K + 1} \tag{5}$$

where μ is the gain of the clamp amplifier and K is the attenuation of the clamp amplifier caused by the cell membrane resistance (R_m) and the resistance (R_{p2}) of the output micropipette (ME2).

$$K = \frac{R_m}{R_m + R_{p2}} \tag{6}$$

As the product μK becomes very large, the difference between V_m and V_{cmd} becomes very small. Ideally, the error will be very low: just a fraction of one percent. The gain of the clamp μ is typically set by a front-panel gain control in the range of about 100 to 10,000. If K were unity, the error would vary from 1 percent down to 0.01 percent. However, if K is less than unity (as it always is), the error will be worse. If the output micropipette resistance is 90 MΩ and the membrane resistance is 10 MΩ, K is 0.1 and the error will be ten times worse than if K were unity. If the two resistances are equal, K will be 0.5. Thus, as a rule of thumb it is desirable to use an output micropipette whose resistance is as low as possible, ideally about the same size or smaller than the membrane resistance.

Step Response and Bandwidth

After a step command, the membrane potential relaxes exponentially towards its new value. For $\mu K >> 1$, the time constant for the relaxation is:

$$\tau = \frac{R_{p2}C_m}{\mu} \tag{7}$$

Increasing the clamp gain decreases the time constant for the step response. For example, if R_{p2} = 10 MΩ, C_m = 1000 pF and μ = 100, the time constant is 100 μs.

Stated differently, increasing the clamp gain also increases the bandwidth with which V_m can follow changes in V_{cmd}. The -3 dB frequency of the bandwidth is:

$$f_{-3} = \frac{\mu}{2\pi R_{p2}C_m} \tag{8}$$

Stability

The voltage clamp circuit shown in Figure 3-12 is unconditionally stable. The membrane capacitance provides a 90° phase shift, which is required for stability in all negative feedback circuits. Unfortunately, other factors combine to make the circuit unstable at high clamp gains.

The coupling capacitance (C_x) between the micropipettes is extremely destabilizing. Values as small as 0.01 pF can lead to oscillation if μ has a magnitude of several hundred or more. There are several ways to minimize this coupling capacitance. The two best ways are by: (1) introducing the two micropipettes into the preparation at a wide angle, preferably greater than 90°. (2) Placing a grounded metal shield between the two micropipettes. This shield should be large enough to block all line-of-sight pathways between the two micropipettes and their holders.

Another destabilizing factor is the non-ideal nature of the membrane. In Figure 3-12, the membrane is simply modeled as a parallel resistor and capacitor. In practice, a distributed model applies. The capacitance elements are themselves non-ideal; they should be modeled by an ideal capacitor with a series-resistance component. For real membranes, the phase shift at high frequencies is less than 90°. In the Axoclamp, a phase-shift control is included to allow the user to empirically introduce a phase lag to the circuit to build the total high-frequency phase shift up to 90°.

The input capacitance of the voltage-recording micropipette (ME1) adds another frequency-dependent variable into the system that also tends to decrease the stability. The effect of this input capacitance is usually minimized by carefully adjusting the capacitance neutralization control to maximize the bandwidth of ME1.

Small instabilities in the voltage clamp usually show up as an overshoot in the current and voltage responses. Full instability shows up as a continuous oscillation that can destroy the cell. In some voltage clamps, special circuits are used to detect oscillations and switch the instrument back to current-clamp mode. Such circuits are difficult to use because they must not respond to legitimate signals such as a series of closely spaced pulse commands. The Axoclamp and the GeneClamp 500 do not include an oscillation guard circuit. Instead, the inherent stability of these instruments has been maximized. If the correct procedures are followed, oscillations are not a problem and the best voltage-clamp responses are achieved.

Membrane Conductance Changes

It can be shown that the current response to a step change in the membrane conductance is identical to the membrane potential response to a step change in the command voltage. This is fortunate, because it means that if the voltage clamp is set up optimally by observing the response to a repetitive command step, it is also optimally set up for measuring conductance changes. In order for this equivalence to be maintained, it is essential that the experimenter not use tricks such as filtering the command voltage to eliminate an overshoot in the response. This is a bad practice because it disguises the dynamic performance of the clamp circuit.

Noise

There are several sources of noise in a two-electrode voltage-clamp circuit, but the most important is the thermal and excess noise of the input micropipette, ME1. To the voltage-clamp circuit, this noise appears as an extra command signal. It is imposed across the membrane and leads to a noise current. Because of the membrane capacitance, this noise current increases directly with frequency in the bandwidths of interest. The magnitude of the noise is worst in cells with large C_m values.

Since the current noise increases with frequency, a single-pole filter is inadequate. An active two-pole filter is required at minimum; while a four-pole filter is preferred.

Micropipette Selection

Ideally, both micropipettes should have very small resistances. The resistance of ME1 should be small to minimize the micropipette noise, and the resistance of ME2 should be small to maximize the product μK. However, low-resistance micropipettes are more blunt than high-resistance micropipettes and do more damage to the cell. In general, it is more important to use a low resistance micropipette for ME2, because if its value is high, the voltage-clamp error will be high and the recorded data will be of dubious merit. The membrane potential could deviate significantly from the command potential. A relatively high-resistance micropipette for ME1 has its problems — increased noise and reduced bandwidth — but at least it does not introduce an error.

Input *vs.* Output Offset Removal

Certain offset-removal circuits act only on the output signal delivered to the oscilloscope or data acquisition system. Examples of these are the AC input of the oscilloscope itself, the DC-offset removal in the CyberAmp, and the "output" offset controls in the Axoclamp, Axoprobe and Axopatch. Adjusting these controls does not affect the membrane currents injected through the micropipette.

On the other hand, some offset-removal circuits act on the "input" signal seen by the voltage-clamp circuit. For example, the junction potential and micropipette-offset controls in the Axopatch amplifiers alter the potential that is clamped by the voltage-clamp circuit. Thus the actual membrane potential is affected when these controls are altered and, consequently, the membrane current is affected. Care should be taken not to alter the setting of an input-offset control once the experiment has begun.

Small Cells — Discontinuous Single-Electrode Voltage Clamp

In discontinuous single-electrode voltage clamp (dSEVC) the tasks of voltage recording and current passing are allocated to the same micropipette. Time-sharing techniques are used to prevent interactions between the two tasks. The principles of operation are discussed in detail by Finkel and Redman (1985).

A block diagram and a timing diagram illustrating the technique are shown in Figure 3-13. A single micropipette (ME1) penetrates the cell and the voltage recorded (V_p) is buffered by a unity-gain headstage (A1). To begin the discussion, assume that V_p is exactly equal to the instantaneous membrane potential (V_m). A sample-and-hold circuit (SH1) samples V_m and holds the recorded value (V_{ms}) for the rest of the cycle.

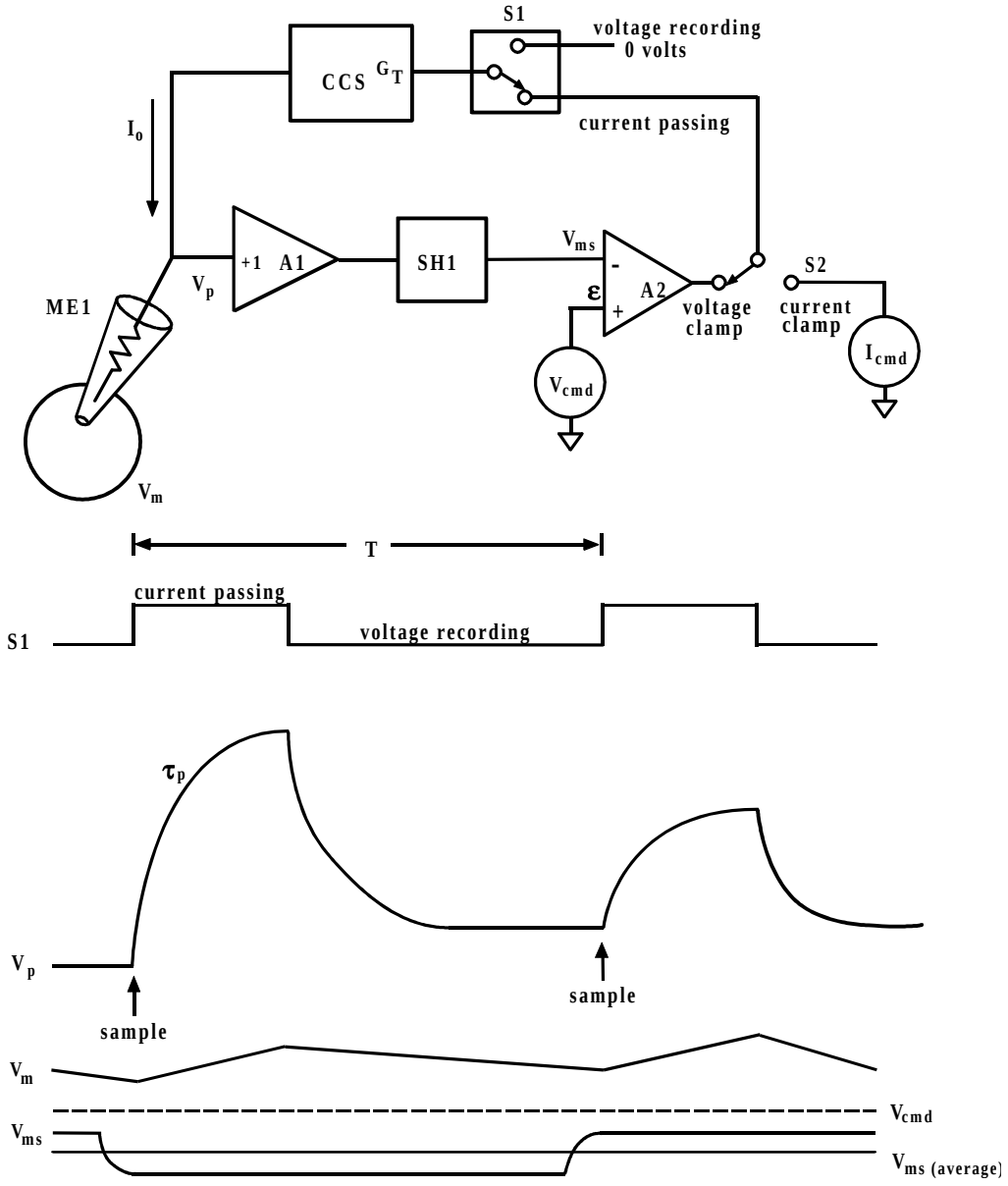

Figure 3-13. Block Diagram and Timing Waveforms
See text for details.

V_{ms} is compared with a command voltage (V_{cmd}) in a differential amplifier (A2). The output of this amplifier becomes the input of a controlled-current source (CCS) if the switch S1 is in the current-passing position.

The CCS injects a current into the micropipette that is directly proportional to the voltage at the input of the CCS irrespective of the resistance of the micropipette. The gain of this transconductance circuit is G_T.

The period of current injection is illustrated at the start of the timing waveform. S1 is shown in the current-passing position during which a square pulse of current is injected into the micropipette, causing a rise in V_p.

The rate of rise is limited by the parasitic effects of the capacitance through the wall of the glass micropipette to the solution and the capacitance at the input of the buffer amplifier. The final value of V_p mostly consists of the IR voltage drop across the micropipette due to the passage of current I_o through the micropipette resistance R_p. Only a tiny fraction of V_p consists of the membrane potential (V_m) recorded at the tip.

S1 then switches to the voltage-recording position. When the input of the CCS is 0 volts, its output current is zero and V_p passively decays. During the voltage-recording period V_p decays asymptotically towards V_m. Sufficient time must be allowed for V_p to reach within a millivolt or less of V_m. This requires a period of up to nine micropipette time constants (τ_p). At the end of the voltage-recording period, a new sample of V_m is taken and a new cycle begins.

The actual voltage used for recording purposes is V_{ms}. As illustrated in the bottom timing waveform, V_{ms} moves in small increments about the average value. The difference between $V_{ms,ave}$ and V_{cmd} is the steady-state error (ε) of the clamp that arises because the gain (G_T) of the CCS is finite. The error becomes progressively smaller as G_T is increased.

Minimum Sampling Rate and Maximum Gain

If the sampling rate (f_s) is too slow, the dSEVC will become unstable. This is because the resultant long current-passing period (T_i) allows the membrane potential to charge right through and past the desired potential before the clamp has had an opportunity to take a new sample of potential and adjust the current accordingly. The larger the cell membrane capacitance (C_m) the larger the value of T_i (and hence the slower the sampling rate) that can be used for a given average gain (G_T). The stability criterion is:

$$0 < \frac{G_T T_i}{C_m} < 2 \tag{9}$$

For optimum response (critical damping) we require:

$$\frac{G_T T_i}{C_m} = 1 \tag{10}$$

Thus for a given G_T, if C_m is small, T_i must be small (*i.e.*, f_s must be large).

For example, if G_T = 1 nA/mV and C_m = 100 pF, then T_i must be 100 µs for optimum response. If T_i is greater than 100 µs, the step response will overshoot and at 200 µs the clamp will oscillate. (Note that in the Axoclamp-2A and 2B, T_i = 100 µs corresponds to a sampling rate of 3 kHz because the duty cycle (discussed later) is 30%.)

If T_i in this example cannot be as short as 100 µs because the micropipette response is too slow, then a lower value of G_T will have to be used to maintain optimum response.

Fastest Step Response

In a critically damped system, that is, where the gain has been turned up to achieve the fastest possible response with no overshoot, the clamp can settle to its final value in as little as one or two periods.

Error Number One — Clamping the Micropipette

If the micropipette voltage does not have sufficient time to decay to zero before a new sample of V_m is taken, the sample will include a micropipette artifact voltage. The clamp circuit will be incapable of distinguishing this micropipette artifact voltage from the membrane potential and thus it will voltage clamp the sum of the two potentials. This leads to inaccurate and bizarre data collection. For example, cells with known rectifiers will generate I-V curves that are almost linear.

This error is easily avoided by carefully observing the monitor output on the Axoclamp amplifier. It is essential that the micropipette voltage trace on this output be given sufficient time to decay to zero before the sample is taken. The user can achieve this by adjusting the sampling frequency and by optimizing the micropipette capacitance compensation.

Error Number Two — Steady-State Clamp Error

Even if the sampling is such that there is negligible voltage drop across the micropipette, there will still be a steady-state error due to the finite gain, G_T, of the clamp. To minimize the error, it is necessary to use as high a gain setting as possible, consistent with stable operation.

Ripple in the Membrane Potential

Due to the switching nature of the dSEVC circuit, the actual membrane potential changes during each cycle. The size of the ripple is proportional to the command voltage; for a clamp at the resting membrane potential, the ripple is zero. Typically, the ripple is no more than one or two percent of the command voltage. The actual error between V_m and V_{cmd} is worst at the end of each cycle, just when the sample is taken. Therefore, the presence of ripple means that the average value of V_m is closer to V_{cmd} than is suggested by the sampled voltages.

Aliased Noise and the Anti-Aliasing Filter

The dSEVC is a sampling system, and thus it suffers from the noise amplification property called "aliasing" (see **Chapter 12**). The only way to prevent aliasing is to sample two or more times more rapidly than the bandwidth of the signal. For example, if the micropipette has a 20 kHz bandwidth, the sample rate should be 40 kHz or more to prevent aliasing. Unfortunately, in the dSEVC there is a competing consideration: the sample period (T) should be at least ten times longer than the micropipette time constant. For a 20 kHz micropipette, the time constant is about 8 µs; therefore the sampling period should not be less than 80 µs, equivalent to a 12.5 kHz sampling rate.

The anti-alias filter is a special filter used in the Axoclamp to reduce the bandwidth of the micropipette. Ordinarily, this is not desirable, because the sampling rate has to be reduced to suit. However, some micropipettes have a biphasic decay after a current pulse (Figure 3-14). There is an initial rapid decay as the micropipette capacitance is discharged, followed by a smaller, slower decay resulting from ion redistribution in the tip. For these micropipettes, the maximum sampling rate is severely restricted by the slow phase of the

micropipette decay. However, the wide-bandwidth noise of the micropipette is related to the initial fast phase. For these micropipettes, it makes sense to slightly filter the micropipette response to try to remove some of the wideband noise as long as this can be achieved without increasing the ultimate settling time (which is limited by the slow decay). This filtering is provided by a variable single-pole filter called the Anti-Alias Filter.

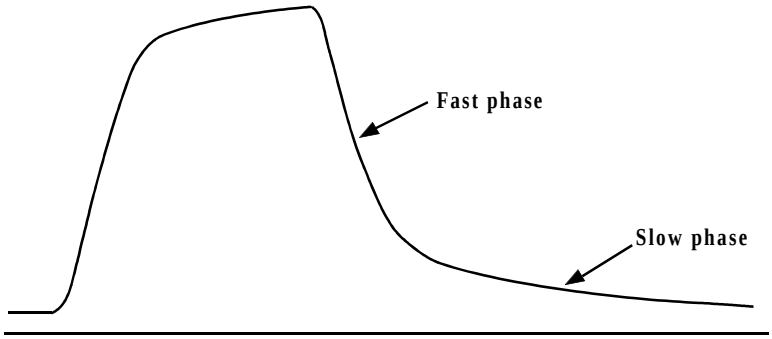

Figure 3-14. Biphasic Voltage Response of a High-Resistance Micropipette

The voltage response of a high-resistance micropipette is often biphasic. The initial fast phase reflects the time constant formed by the micropipette resistance and the stray capacitance. This time constant sets the cutoff frequency for thermal noise. The slow phase is thought to be due to ion redistribution in the tip. The maximum sampling rate during dSEVC is limited by the time taken for the slow phase to settle.

Capacitance Neutralization Noise

Another source of noise in discontinuous single-electrode voltage clamps arises from the capacitance neutralization circuitry. A fundamental property of all capacitance neutralization circuits is that they introduce noise in excess of what is contributed by the thermal noise of the recording micropipette and the input noise of the buffer amplifier. The excess noise becomes progressively larger as the amount of capacitance neutralization is increased to reduce the micropipette time constant. In discontinuous systems the micropipette time constant must be reduced more than in continuous systems because of the need for the micropipette voltage to decay to V_m within the time allotted for passive recording. Compared to a continuous two-electrode voltage clamp, the dSEVC will always be a factor of three or more times noisier at similar gains.

Selecting the Duty Cycle

The duty cycle is the fraction of each period spent in current passing. As the duty cycle approaches unity, the clamp fails to operate properly because insufficient time is left for the micropipette artifact to decay, unless the sampling period is made unreasonably slow. On the other hand, if the duty cycle is made very small, the magnitude of the current pulse becomes very large (to maintain a given average current). Since there are conflicting requirements on the selection of the duty cycle, a compromise must be found. It has been shown that the best compromise is a duty cycle equal to 0.3. This is the value used in the Axoclamp.

Useful Signal Bandwidth

As a general rule, the useful signal bandwidth is about one tenth of the switching rate. If the dSEVC is switching at 20 kHz, it is reasonable to expect to record membrane currents to within a bandwidth of 2 kHz. While this rule is true in the Axoclamp, it is not true of all dSEVC instruments. Some use special techniques to enable higher switching frequencies without increasing the effective signal bandwidth. This means that the useful signal bandwidth may only be one twentieth or less of the switching rate in these instruments.

Current and Voltage Measurement

Since the system is injecting current through the micropipette during every cycle, there is no place that a continuous record of the membrane current and voltage exists. Instead, sample-and-hold amplifiers must be used to store the values measured at the appropriate times.

For the V_m output, the appropriate time to measure the voltage is at the end of the period of passive decay, just before the next current pulse begins. This sampled value is held for the duration of the cycle.

For the I_m output, the current is sampled during the middle of the current pulse. The value is held until the next current pulse. Since the user is interested in the average current entering the cell, the output of the sample and hold is multiplied by the duty cycle before being presented on the output.

Setup Procedure

Two oscilloscopes are required to set up a dSEVC. The better quality oscilloscope should be used to monitor V_m and I_m. The other oscilloscope is used to monitor the signal at the output of the anti-aliasing filter. Initially, the anti-aliasing filter is set at its widest bandwidth so that the monitored signal is basically the micropipette response.

The voltage-clamp instrument is switched to the discontinuous current-clamp (DCC) position (described below) and a current command is established. The user watches the micropipette decay on the monitor oscilloscope and increases the capacitance neutralization control as much as possible without causing any overshoot. Next, the sampling rate is increased, always making sure that sufficient time is allowed for complete decay of the micropipette voltage artifact.

After checking to make sure that the clamp gain is set to a low value, the instrument is switched to the dSEVC mode. A repetitive voltage command is provided and the gain is increased as far as possible without causing instability or overshoot in the step response. Phase lag or lead is introduced if this improves the step response. All the while, an eye is kept on the monitor oscilloscope to make sure that the micropipette artifact decays fully by the end of each cycle. Lastly, the anti-alias control can be carefully introduced. For micropipettes exhibiting a biphasic response, the noise on V_m and I_m might be reduced without compromising the final settling of the micropipette voltage.

If the micropipette resistance changes with current, the capacitance neutralization might require re-adjustment after the command voltage is stepped. It is acceptable to re-adjust the capacitance neutralization while still in dSEVC mode, as long as the monitor oscilloscope is checked to make sure that the micropipette artifact decays fully by the end of each cycle.

Conditions For Good dSEVC

(1) The micropipette resistance should be as small as possible.

(2) The micropipette capacitance should be minimized.

(3) The membrane time constant must be at least ten times the micropipette time constant.

Comparison with Two-Electrode Voltage Clamp

The TEVC is generally superior to the dSEVC. If the gains are adjusted for a similar steady-state error, the TEVC will generally have just one third of the noise of the dSEVC. If the gain of the TEVC is increased so that the noise of the two clamps is similar, the TEVC will generally settle three times faster.

It is clear that a dSEVC should not be used in preference to a TEVC. It should only be used in those cases where it is impractical to implement a TEVC, either because the cells are not individually discernible or because they are too small.

Discontinuous Current Clamp (DCC)

Discontinuous current clamp mode (DCC) uses the same circuitry and principles as the dSEVC mode. The main difference is that in dSEVC the amplitude of each current pulse depends on the difference between the membrane potential and the command potential, whereas in DCC the amplitude is determined by the experimenter. Thus, to inject a constant current into the cell, a DC command is presented and successive current pulses should be identical.

The main use of the discontinuous current clamp technique is to aid in the setup of the instrument before selecting dSEVC. DCC-mode recording has been used in some experiments in place of Bridge-mode recording to eliminate the possibility of a badly balanced Bridge introducing an error in the voltage measurement. However, in general, the advantage of DCC mode is small because in DCC mode the capacitance neutralization control plays a similar role to the Bridge Balance control in continuous current clamping. If the micropipette response is too slow, the transient after the current pulses will not have decayed to baseline before the next sample is taken, and a current-dependent error voltage will be measured. Thus, DCC mode just replaces one problem with another. At the same time, DCC mode has more noise and a lower signal-recording bandwidth, so its use as a substitute for Bridge mode should be undertaken with caution.

Continuous Single-Electrode Voltage Clamp

To implement a continuous single-electrode voltage clamp (cSEVC; also known as a whole-cell patch clamp), a blunt, low-resistance pipette is sealed by suction to the surface of the membrane. The patch of membrane enclosed within the tip of the pipette is ruptured by one of a variety of techniques. The electrolyte solution in the pipette then forms an electrical continuity with the interior of the cell. It is equivalent to an extremely low-resistance (*e.g.*, 1 - 10 MΩ) intracellular micropipette.

The voltage at the *top* of the pipette is controlled by a voltage-clamp circuit. It is important to realize that this is quite different from the situation in TEVC or dSEVC. In both of the latter cases, the voltage at the *tip* of the voltage-recording micropipette is controlled (remember, in

dSEVC, time-division multiplexing effectively yields two micropipettes: the voltage-recording micropipette and the current-passing micropipette). In cSEVC, the same electrode is used simultaneously for voltage recording and for current passing. The voltage recorded at the top of the pipette is the sum of the membrane potential V_m, which the experimenter wishes to control, and the current-induced voltage drop across the pipette.

The current through the series resistance of the pipette and the residual resistance of the ruptured patch is often sufficiently large to introduce significant voltage errors. Techniques exist for compensating these errors, but the compensation is never perfect. To get a feeling for the magnitude of the errors, assume that the maximum compensation is 80%, beyond which the system oscillates and destroys the cell. Further assume that the access resistance (R_a; the sum of the pipette resistance and the residual resistance of the ruptured patch) is 5 MΩ. After compensation, the effective value of R_a ($R_{a,eff}$) would be just 1 MΩ. In this case, a 10 nA current would cause a 50 mV uncompensated voltage error, reduced to 10 mV by the compensation. Clearly, the cSEVC technique cannot be used to record large currents. Even for modest whole-cell currents, care must be taken to compensate for the series resistance and then correctly interpret the residual error. The dSEVC technique should be considered as an alternative to the cSEVC technique when the access resistance is too large.

The temporal resolution of the whole-cell patch clamp is also affected by $R_{a,eff}$. The time constant for resolving currents is the product of $R_{a,eff}$ (assuming that the membrane resistance is much greater) and the membrane capacitance ($\tau = R_{a,eff}C_m$). Thus the technique is also limited to small cells where this product is small enough for the desired time resolution to be achieved. Figure 3-15 illustrates the voltage and temporal errors caused by the presence of R_a.

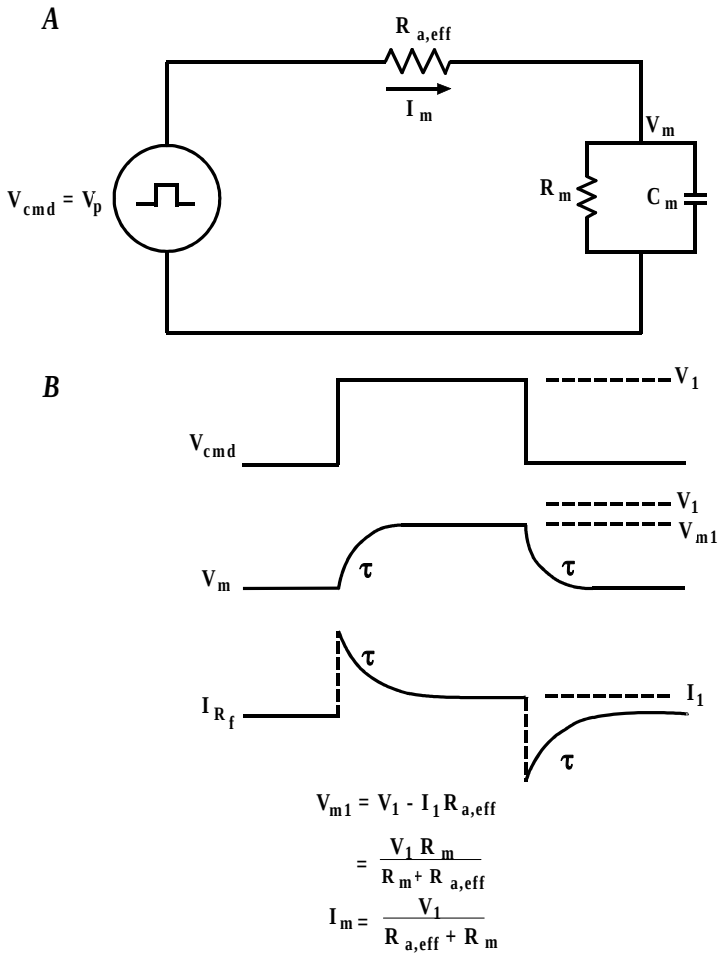

Figure 3-15. Voltage and Temporal Errors Caused by the Presence of R_a

A. The cSEVC circuit is simply illustrated as a voltage source (V_{cmd}) in series with the effective access resistance ($R_{a,eff}$) and the membrane (R_m, C_m). The cSEVC circuit ensures that the pipette potential (V_p) is equal to V_{cmd}. ***B.*** After V_{cmd} steps to V_1, a steady-state current, I_m, flows in the circuit. The membrane potential is equal to $V_{cmd} - I_m R_{a,eff}$. After the step change in the command potential, I_m and V_m settle exponentially to their steady-state values with $\tau = [R_{a,eff}R_m/(R_{a,eff} + R_m)]C_m$, but since in general $R_m >> R_{a,eff}$, a good approximation is $\tau \approx R_{a,eff}C_m$.

Series Resistance Compensation

In the ideal experiment, the resistance of the patch micropipette in whole-cell experiments would be zero. In this case, the time resolution for measuring membrane currents and changing the membrane voltage would be limited only by the speed of the electronics (typically just a few microseconds).

Positive Feedback ("correction")

Series resistance compensation using positive feedback is an attempt to achieve this ideal electronically. This technique is also called "correction." It is the same technique that has been widely used in conventional two-electrode voltage clamps. Basically, a signal

proportional to the measured current is used to increase the command potential. This increased command potential compensates in part for the potential drop across the micropipette (Figure 3-16). The amount of compensation achievable is limited by two considerations. First, as the compensation level (α) approaches 100%, the increase in the command potential hyperbolically approaches infinity. For example, at 90% compensation, the command potential is transiently increased by a factor of ten ($V_{cmd}/(1 - \alpha)$). Thus at large compensation levels the electronic circuits approach saturation. Second, the current feedback is positive; therefore, the stability of the circuit is degraded by the feedback and at 100% compensation the circuit becomes an oscillator. In practice, the oscillation point is much lower than 100% because of non-ideal phase shifts in the micropipette and the cell membrane.

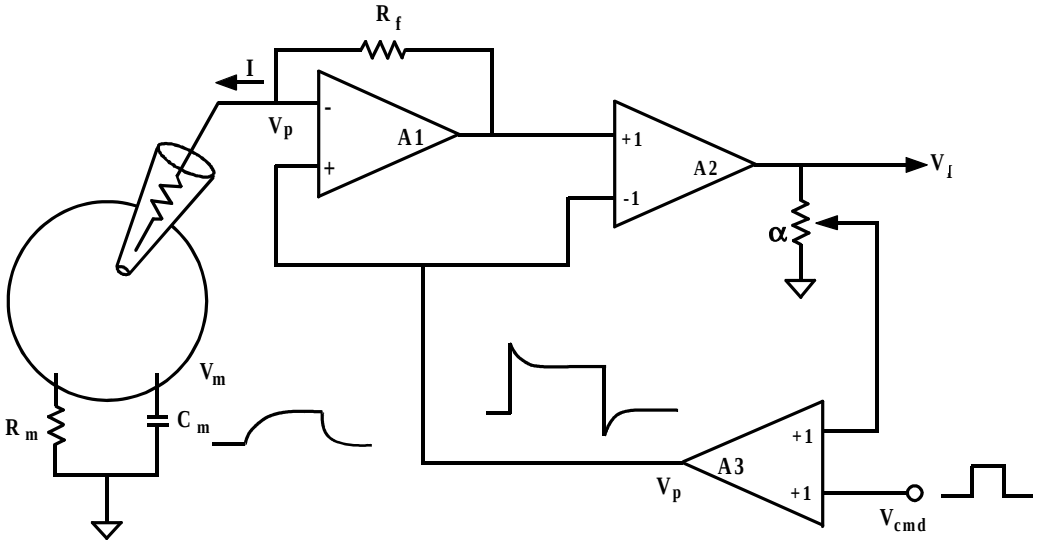

Figure 3-16. Series Resistance Correction

In this figure, a single pipette is used to voltage-clamp the cell. Operational amplifier A1 is configured as a current-to-voltage converter. Differential amplifier A2 subtracts the pipette potential (V_p) to generate the current output (V_I). A fraction (α) of V_I is summed with the command voltage (V_{cmd}) used to control V_p. This causes both a transient and a steady-state increase in V_p compared with V_{cmd}. As a result, the membrane charges faster, the voltage drop across the electrode resistance is compensated and the bandwidth is increased.

The first problem, saturation of the electronics, could in principle be reduced by using high-voltage (*e.g.*, ±120 V) operational amplifiers. However, this approach has not been pursued because these types of operational amplifiers have more noise and worse drift than good conventional operational amplifiers. The second problem, stability, has been partially reduced in recent years by adding a variable low-pass filter in the current-feedback loop (*e.g.*, the "lag" control of the Axopatch-1D and the Axopatch 200 series). By empirically setting the low-pass filter cutoff frequency, large percentage compensations can be used, but these only apply to the currents at bandwidths below that of the filter cutoff. Thus the DC, low- and mid-frequency series-resistance errors can be substantially reduced while the high-frequency errors remain large.

Supercharging ("prediction")

Another technique for speeding up the response to a command step is the "supercharging" technique, also known as "prediction." In contrast to the "correction" method of series-resistance compensation, "prediction" is an open-loop method. That is, there is no feedback and there is little risk of oscillations.

Supercharging is accomplished by adding a brief "charging" pulse at the start and the end of the command voltage pulse. This means that initially the membrane is charging towards a larger final value than expected (Figure 3-17). In its crudest form, the charging-pulse amplitude or duration are adjusted empirically by the investigator so that the membrane potential does not overshoot. Since the membrane potential is not directly observable by the user, this is accomplished by adjusting the controls until the current transient is as brief as possible. Once the optimum setting for one step size has been found, the size of the supercharging pulse for all other step sizes can be calculated by the computer. Since the large, transient supercharging current has to be carried by the feedback resistor in the headstage, the amount of supercharging that can be used is limited by saturation of the current-to-voltage converter in the headstage.

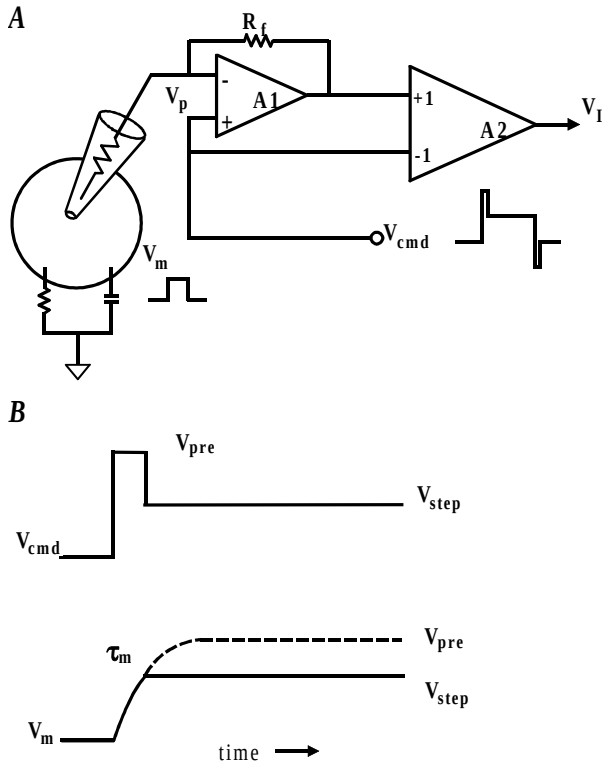

Figure 3-17. Prediction Implemented Empirically by the Computer

A. A brief charging pulse is added to the start and the end of V_{cmd}. The size and duration of the charging pulse is empirically determined to give the fastest charging current. V_m charges rapidly to its final value. V_{cmd} and the supercharging pulses are not part of a positive feedback circuit. ***B.*** An expanded view of V_{cmd} and V_m. Initially, V_m increases towards the amplitude (V_{pre}) of the pre-pulse. However, the pre-pulse is terminated early, just when V_m reaches the desired command voltage, V_{step}.

The Axopatch 200 pioneered an alternative technique for generating the supercharging current. This technique takes advantage of having full knowledge of the R_a and C_m values. Fortunately, this information is generally available on modern whole-cell patch-clamp amplifiers as part of the technique used to unburden the feedback resistor (R_f) from the necessity of passing the charging current into the cell. Once these parameters are determined, it is possible to automatically boost changes in the command voltage to supercharge the cell (Figure 3-18). This technique has the significant advantage that no empirical determination of the charging-pulse amplitude or time is needed and that it works with any command-voltage shape without requiring a computer to calculate the supercharging pulse.

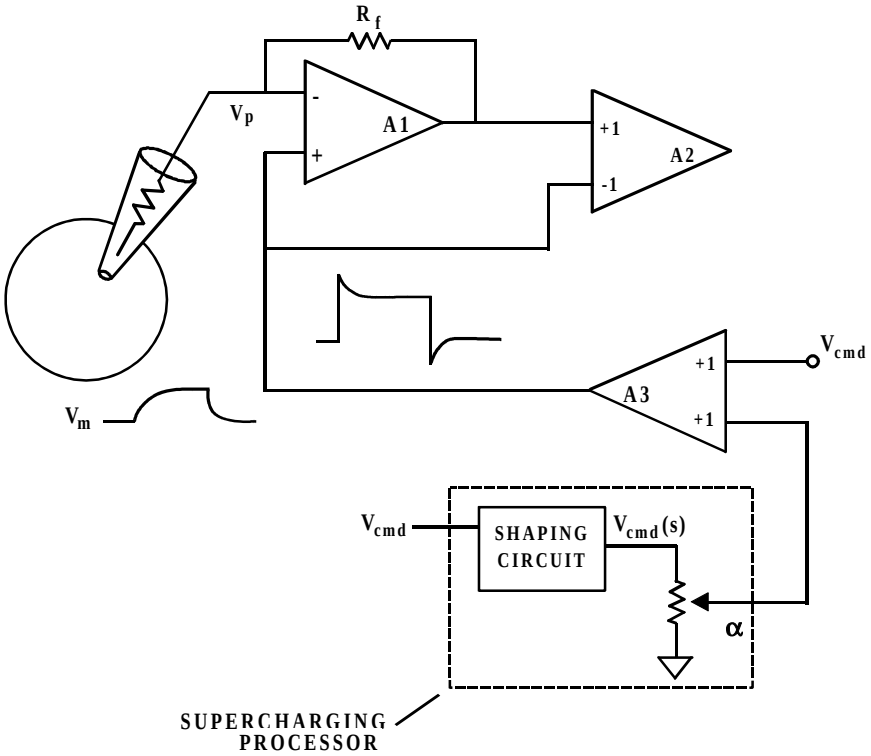

Figure 3-18. Implementation of Prediction Based on the Knowledge of Cell Parameters

The supercharging pulse added to V_{cmd} is a fraction (α) of a shaped version of V_{cmd} itself. The shaped version, $V_{cmd}(s)$, is generated in the whole-cell capacitance compensation circuit described later. Since the calculation of the supercharging pulse is determined by the parameters of the cell and V_{cmd} itself, the system works with all commands (step, triangle, sine, etc.) without the need for an empirical determination of the shape and amplitude for each command shape and size.

Supercharging is appropriate for a specific type of experiment, but it is inappropriate in others and its use should be carefully considered. The appropriate application is the measurement of ionic currents that activate during the time that the membrane potential is normally changing. If supercharging is applied, it might be possible to clamp the potential to its final value before the ionic current substantially activates.

There are two significant shortcomings that the investigator should be aware of when using the supercharging technique:

(1) The technique does not correct for the voltage error that occurs when current flows through the series resistance of the pipette.

(2) The dynamic response of the circuit is not improved. That is, there is no improvement in the speed with which changes in current (and hence, changes in conductance) can be resolved. Membrane current changes are still filtered by the time constant of the access resistance and the membrane capacitance. The investigator should not be misled by the rapid settling of the response to a command step into thinking that this settling time represents the time resolution of the recording system.

Neither of these problems occurs with the correction method of series-resistance compensation. The relative merits of correction versus prediction are shown in Table 3-1.

	Error correction	**Fast response to V_{cmd} change**	**Fast response to R_m change**	**Stability**
Correction	Yes	Yes	Yes	Not guaranteed
Prediction	No	Yes	No	Guaranteed

Table 1. Correction *vs.* Prediction

Usually, the type of series resistance compensation provided in a patch clamp amplifier is a combination of correction and prediction. The Axopatch 200 allows the prediction and correction components of the circuitry to be set independently so that the researcher can optimize the technique that is most appropriate for the experiment. Optimum performance is usually achieved by combining series-resistance "correction" with series-resistance "prediction."

Limitations of Series Resistance Compensation

Series-resistance compensation is an attempt to electronically reduce the effect of the pipette resistance. Because of practical limitations, it is never perfect. Even if 100% compensation could be used with stability, this would only apply to DC and medium-speed currents. Very fast currents cannot be fully corrected.

Pipette-Capacitance Compensation

When the command voltage is stepped, a large amount of current flows into the pipette capacitance during the transition from one potential to the next. In an intracellular micropipette amplifier, such as the Axoclamp or the Axoprobe, this is reduced by setting the capacitance neutralization controls, as discussed in the earlier section titled *Capacitance Compensation*. In a patch-clamp amplifier, such as the Axopatch-1 or the Axopatch 200, a variation of the technique is used for the same purpose — to reduce or even eliminate the transient. This section discusses pipette capacitance compensation in a patch clamp.

Compensating the pipette capacitance in a patch clamp has three purposes. First, many researchers want to remove the transient from the records for "cosmetic" reasons. Second, during the transient the potential at the top of the pipette is changing slowly while the pipette capacitance charges. By rapidly charging the pipette capacitance through the compensation circuitry, the potential at the top of the pipette is stepped more rapidly, reducing the likelihood that rapid-onset ionic currents will be distorted. Third, uncompensated pipette capacitance has a detrimental effect on the stability of the series-resistance correction circuitry. The component of the current that flows into the pipette capacitance is not in series with any resistance. Thus the series resistance correction circuit exceeds 100% compensation for this component of the current as soon as the circuit is switched in.

In the Axopatch amplifiers, two pairs of pipette capacitance compensation controls are available. With these controls, it is often possible to reduce the pipette transients to extremely low levels. The bulk of the transient is reduced by using the *fast* magnitude and time constant (τ) controls. The magnitude control compensates the net charge. The τ control adjusts the time constant of the charge compensation to match the time constant of the command pathway and to compensate for small non-idealities in the frequency response of the pipette and electronics.

The residual slow component seen in many pipettes is reduced by using the *slow* magnitude and τ controls. A simplified circuit of the fast and slow compensation circuitry is shown in Figure 3-19.

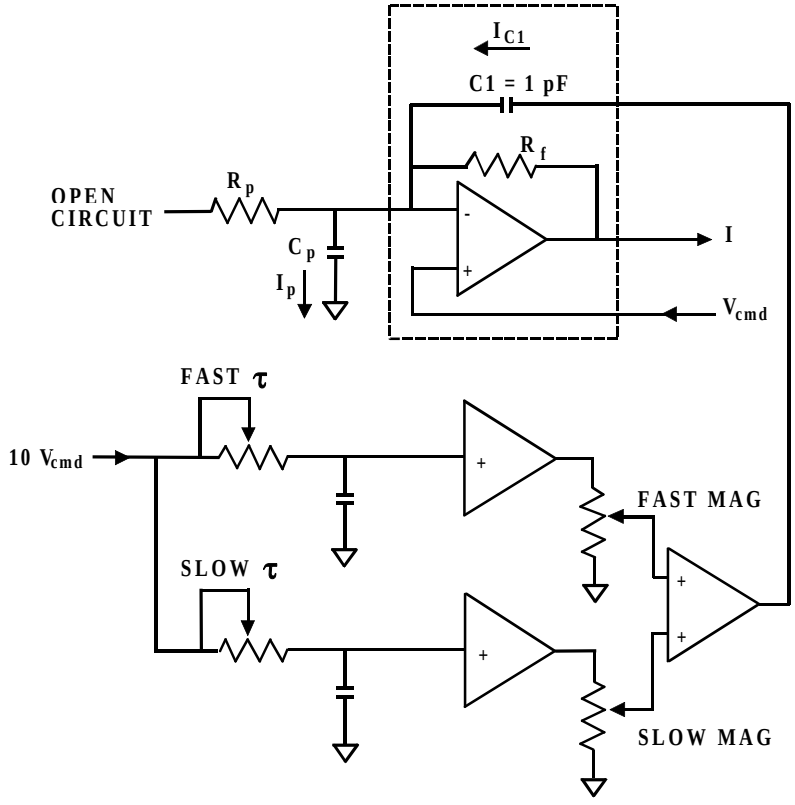

Figure 3-19. Pipette Capacitance Compensation Circuit
See text for details.

When the command potential (V_p) changes, current I_p flows into C_p to charge it to the new potential. If no compensation is used, I_p is supplied by the feedback element (R_f), resulting in a large transient signal on the output (I).

By properly setting the fast and slow magnitude and τ controls, a current (I_{C1}) can be induced in capacitor C1 (connected to the headstage input) to exactly equal I_p. In this case no current needs to be supplied by R_f, and there is no transient on the output.

Whole-Cell Capacitance Compensation

When the membrane potential is stepped, there is a significant current transient required to charge the membrane capacitance. This is true whether the cell is clamped using a two-electrode clamp or a single-electrode clamp. Since it is impossible to record meaningful currents during this transient (the membrane potential has not settled), this transient is ignored in most two-electrode voltage-clamp or discontinuous single-electrode voltage-clamp experiments. However, when whole-cell voltage clamping is performed using a patch-clamp amplifier, it is common to suppress this transient from the recording.

It is important to understand that the transient is not suppressed in the belief that somehow the data during this period is more meaningful in a patch-clamp amplifier than it is in a two-electrode voltage clamp. The transient is suppressed for technological reasons. In a patch-clamp amplifier the value of the feedback resistor for whole-cell clamping is typically 500 MΩ. In an instrument driven from ±15 volt power supplies, the maximum current that can be passed through a 500 MΩ resistor is less than 30 nA. The transient current required to apply a 100 mV step to a cell that is clamped through a 1 MΩ resistor is 100 nA. If series-resistance compensation is used, much larger currents are required. Since the 500 MΩ resistor cannot pass the required current, the system would saturate and the time to effect a step would be prolonged significantly. In order to prevent saturation of the system, the transient current is injected through a capacitor. Since the current monitor output on patch clamps is only proportional to the current through the feedback resistor, the transient current is not seen, even though in reality it is still being passed through the pipette into the cell.

The settings of the whole-cell capacitance controls to eliminate the transient are unique to the cell being clamped. The values of the cell membrane capacitance and the access resistance can be directly read from the controls. Figure 3-20 is a simplified circuit illustrating how the whole-cell capacitance controls operate.

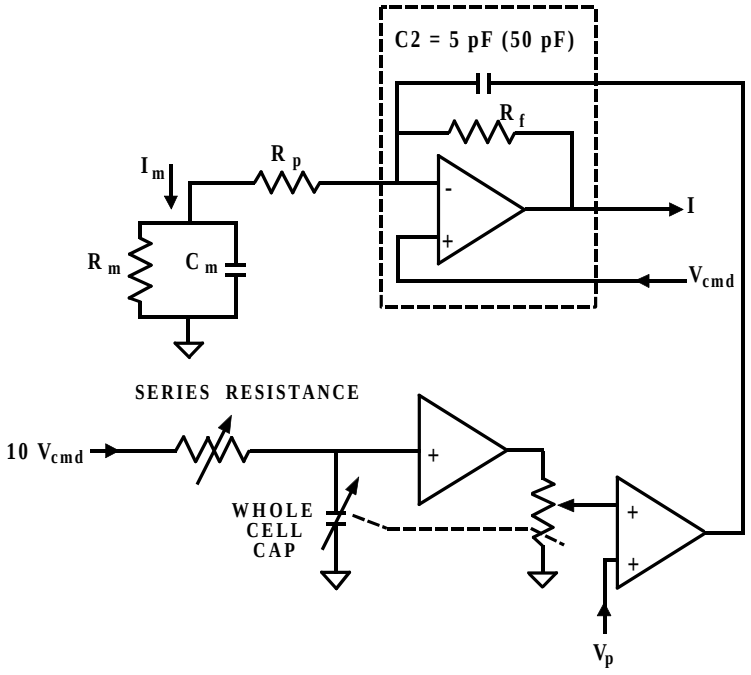

Figure 3-20. Whole-Cell Capacitance Compensation Circuit
See text for details.

Assume that the fast and slow electrode compensation controls have already been set to compensate for C_p. By appropriately adjusting the series-resistance and whole-cell capacitance controls, the current injected through C2 will supply the transient membrane current (I_m). These adjustments do not alter the time constant for charging the membrane. Their function is to offload the burden of this task from the feedback resistor, R_f. In many cells, even a small command voltage of a few tens of millivolts can require such a large current to charge the membrane that it cannot be supplied by R_f. The headstage output saturates for a few hundred microseconds or a few milliseconds, thus extending the total time necessary to charge the membrane. This saturation problem is eliminated by the appropriate adjustment of the series-resistance and whole-cell capacitance controls. This adjustment is particularly important during series-resistance correction since series-resistance correction increases the current-passing demands on R_f. By moving the pathway for charging the membrane capacitance from R_f to C2, the series-resistance circuitry can operate without causing the headstage input to saturate. The effect of transferring the current-passing burden from R_f to C2 is illustrated in Figure 3-21.

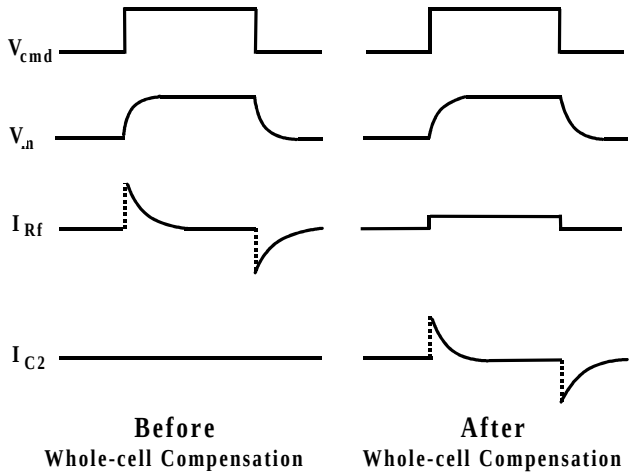

Figure 3-21. Using the Injection Capacitor to Charge the Membrane Capacitance

V_{cmd} is the patch-clamp voltage command; V_m is the voltage across the cell membrane; I_{Rf} is the current across the patch-clamp feedback resistor; I_{C2} is the current injected across the patch-clamp compensation capacitor. See text for details.

The traces in this figure were recorded using an Axopatch 200 amplifier and a model cell. After perfect whole-cell compensation is applied, the current to charge the membrane capacitor is removed from the I_{Rf} trace and only the steady-state current remains. All of the transient current appears in the I_{C2} trace. (The I_{C2} trace in the figure was recorded using an oscilloscope probe connected to the internal circuitry). The I and V_m outputs on the Axopatch 200 show the I_{Rf} and V_{cmd} trace illustrated in Figure 3-21. It is easy to mistakenly think that the time course for charging the membrane is very fast, but this is clearly not the case. Use of an independent electrode in the cell would show that the cell charging rate is not affected by these adjustments.

Absolute Value

The absolute value of the membrane capacitance is displayed on the whole-cell capacitance dial after the whole-cell current transient has been eliminated. This value may be used to estimate the surface area of the cell assuming that the membrane capacitance per unit area is 1 $\mu F/cm^2$.

Rupturing the Patch

To go from the patch to the whole-cell mode, the usual method used to rupture the membrane under the pipette is to apply a pulse of gentle suction. This can be done by mouth or by syringe.

"Zap" is another alternative for going whole-cell. In this technique, a large hyperpolarizing voltage pulse (about 1.5 V) is applied to the cell for 0.5-50 ms in the Axopatch 200A (0.1-10 ms in the Axopatch-1D). This pulse initiates dielectric breakdown of the membrane patch and allows access to the interior of the cell. It is important to use the briefest pulse consistent with low-access resistance. If the pulse is too long, the seal might be lost.

With some cells it is difficult to go whole cell without losing the seal. An alternative is the perforated patch technique, which is discussed in **Chapter 5**.

Which One Should You Use: dSEVC or cSEVC?

Two methods can be used to implement a whole-cell patch voltage clamp. The first is the *discontinuous* single-electrode voltage clamp (dSEVC) method. The second is the *continuous* single-electrode voltage clamp (cSEVC) method. The cSEVC method is more commonly known as the "whole-cell patch clamp"; but formally, the term cSEVC is more descriptive of the method. Both methods have their pros and cons. The dSEVC is generally superior when the currents being clamped are modest or large in size (> 5 nA), leading to a concern that the error due to the uncompensated series resistance might be significant. In dSEVC mode, if the sampling rate is correctly chosen and the capacitance compensation correctly set, there is *no* error due to series resistance. For small currents, the dSEVC mode is less attractive because it is more difficult to set up and because it is noisier. Moreover, the error due to the uncompensated series resistance can generally be made negligible for small currents, making the cSEVC mode very attractive.

The cSEVC mode is available on patch-clamp amplifiers (Axopatch) as the "whole-cell patch" mode and on the Axoclamp amplifiers as the "cSEVC" mode. In a patch-clamp amplifier, the voltage-clamp circuit is a current-to-voltage converter located in the headstage (described in detail below; see Figure 3-22). In the Axoclamp, the voltage-clamp circuit is located in the main unit. While electrically the circuitry works better when the current-to-voltage converter is located in the headstage, in practice it turns out that similar noise and step responses can be achieved in many cells using the Axoclamp. However, there are fewer ancillary controls in the Axoclamp amplifiers pertaining to whole-cell patch clamp as compared to the Axopatch. For example, the Axoclamp does not have a whole-cell capacitance compensation control.

Space Clamp Considerations

There is one limitation to the performance of the voltage clamp that cannot be electrically compensated. This is the deviation of the cell from a sphere centered on the tip of the voltage-recording micropipette. The voltage clamp is maintained at the tip of the voltage-recording micropipette. If all portions of the cell membrane are separated from this tip by equal access resistance, then the membrane will be uniformly voltage clamped. However, many cells have processes such as axons, dendrites and filopodia attached to the cell body (where the micropipettes are located). The membranes of these processes are separated from the cell body by an axial access resistance whose value depends on the distance to each portion of the membrane and the cross section in that region of the cell. Thus there is a voltage drop across the access resistance that becomes substantial for distal components of the membrane. Even though the somatic membrane potential may be well controlled, the axonal or dendritic membrane potential may be very poorly controlled. In these cases, the time course of synaptic currents, regenerative currents and measurements of reversal potentials may be grossly distorted.

As a general rule, the voltage clamp is considered to be acceptable if the length of the attached axon or dendrites is no more than 1/10 of the length constants. Even this short length will cause significant distortion of fast currents (see Figure 7 in Rall and Segev, 1985). Calculation of the length constant for a cell is complicated since it depends on the geometry of the particular cell under investigation. Some of the common ways to avoid the problems of poor space clamping are as follows:

(1) Restrict investigations to spherical cells. Many cultured cells are convenient.

(2) Ligate attached axons. For example, the axon of large molluscan neurons can be tied off with nylon thread.

(3) Use short segments. For example, short segments (100 µm) of arteriolar syncytia can be separated from the arteriole by careful cutting with a razor blade.

(4) Restrict the range of the clamp to a short segment of the cell. This is the essence of the "sucrose gap" technique sometimes used on axons.

(5) Restrict the measurement to currents that are generated close to the micropipettes. For example, the end plate currents in muscle fibers can be well clamped, even though the bulk membrane current is very poorly clamped.

(6) Restrict the measurement to the current flowing through a large patch of membrane instead of the whole cell. The "macro-patch" technique is a special case of the single-channel patch-clamp technique described in **Chapter 5**, in which there are sufficient channels for an ensemble current to be recorded.

Single-Channel Patch Clamp[5]

The single-channel patch clamp is a special case of the voltage-clamp technique. This technique permits the direct measurement of the dynamic activity of individual membrane-bound channel proteins.

Like the whole-cell patch technique, a blunt pipette is sealed onto a patch of membrane. If single-channel recording is intended, the membrane at the tip of the pipette is preserved (*i.e.*, not ruptured). The current recorded is then simply the current that flows through the membrane at the tip of the pipette. Since this membrane area is very small, there is a good chance that just one or a a few ion channels are located in the patched membrane. Individual ion-channel currents can thus be recorded. Previously, the only way to estimate kinetics or conductance of these channels was the technique of "noise" or "fluctuation" analysis. Noise analysis yields an estimate of the mean lifetimes of the channels and the mean amplitudes, but no information about actual opening and closing transitions nor about the shape of the conductance changes.

In single-channel recording, the current through the series resistance of the pipette is negligible, perhaps only a few picoamps flowing through a series resistance of just 10 MΩ. The resulting voltage error is just a few tens of microvolts and is always ignored.

[5] Parts of this section have been reprinted with permission from Finkel, A. S., Progress in Instrumentation Technology for Recording from Single Channels and Small Cells, in *Cellular and Molecular Neurobiology: A Practical Approach*, Oxford University Press, 1991.

The Importance of a Good Seal

When a heat-polished pipette is pressed against a cell membrane it may adhere tightly. The interior of the pipette is then isolated from the extracellular solution by the seal that is formed. If the resistance of this seal is infinite, no current can leak across it.

Current leakage though the seal resistance has a crucial bearing on the quality of the patch current recording. Firstly, depending on the size of the seal resistance, a fraction of the current passing through the membrane patch will leak out through the seal and will not be measured. The lower the seal resistance the larger the fraction of undetected current.

Secondly, thermal movement of the charges in the conducting pathways of the seal constitute the major source of noise in the recording unless the seal resistance is very high (several gigohms or more). A high seal resistance is a prerequisite of low-noise recordings.

High-resistance seals, often called "gigaseals," require that very clean pipettes be used and that they only be used once. If this is the case, gigaseals will routinely form after the application of gentle suction. When good gigaseals are formed, the noise due to the leakage current is virtually eliminated and other sources of noise remain the dominant limitations in the resolution of the recording technique. These are the noise of the electronics, the pipette glass, the input capacitance and the feedback resistor. Commercial patch clamps such as the Axopatch-1 and particularly the Axopatch 200 and 200A have significantly minimized the noise of the electronics and the feedback element in the headstage. **Chapter 4** describes the attributes of desirable glasses and the ways to fabricate low-noise pipettes. It also discusses the use of Sylgard coatings to minimize pipette capacitance.

Resistor Feedback Technology

Current-to-Voltage Converter

The basic concept behind the design of patch-clamp electronics is very simple (Figure 3-22). A sensitive current-to-voltage converter is fabricated using a high-megohm resistor (R_f) and an operational amplifier (A1). The pipette is connected to the negative input and the command voltage is connected to the positive input. Since the operational amplifier has extremely high gain, the potential at the negative input is forced to follow the potential at the positive input. All current flowing in the micropipette also flows through R_f. This current is proportional to the voltage across R_f which is measured at the output of the differential amplifier (A2).

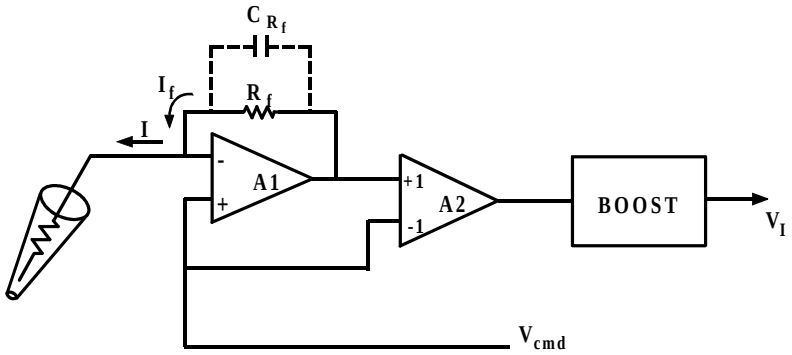

Figure 3-22. Resistive Headstage

Operational amplifier A1 is configured as a current-to-voltage converter. Differential amplifier A2 subtracts V_{cmd} from the output of A1 to generate a voltage that is purely proportional to the voltage across R_f and hence the feedback current, I_f. The boost circuit increases the high-frequency gain to compensate for the narrow bandwidth of the feedback resistor.

In principle, the patch clamp is equivalent to a conventional two-electrode voltage clamp in which the output circuit is connected back to the input pipette. In practice, the patch clamp is better behaved. The patch-clamp system is simpler since all of the gain is in a single operational amplifier stage. The stray capacitance across the feedback resistor guarantees stability. Furthermore, because the gain of this operational amplifier is so high, the difference between the command potential and the potential of the micropipette is negligible. Remember that the potential that is controlled is the potential at the top of the micropipette, not the potential at its tip.

Problems

The special demands of patch clamping lead to complicating factors in the design of a good headstage.

(1) Integrated circuit operational amplifiers do not have the required combination of ultra-low noise and sub-picoamp bias currents. Thus the operational amplifier has to be made from a composite of low-noise FETs[6], such as the U430 type, and conventional operational amplifiers. The voltage noise of the input FETs leads to noise current being injected into the input capacitance. If this input capacitance is large, this source of noise becomes dominant; it is proportional to the product of the input capacitance and the noise of the input FETs.

(2) The minimum theoretically achievable current noise is lower for larger R_f values; therefore it is important to choose large R_f values. The largest value typically used is 50 GΩ, since it allows a reasonable maximum current of more than 200 pA to be measured. Unfortunately, for reasons that are not well understood, high-value resistors are several times noisier than predicted by thermal-noise theory. Since the noise of these resistors cannot be predicted, various brands of resistors must be tested until the best one is found.

[6]Field Effect Transistor

(3) The inherent bandwidth of a 50 GΩ resistor is limited by the stray capacitance across the resistor (C_{Rf} in Figure 3-22). For example, a 50 GΩ resistor with 0.1 pF stray capacitance has a 5 ms time constant, corresponding to a bandwidth of only 32 Hz. This poor time resolution is unacceptable for measuring ionic currents. Therefore, the high-frequency components of the headstage output signal must be boosted. This is typically achieved by an analog frequency compensation circuit. This circuit is made complicated by the fact that R_f cannot be considered as an ideal capacitor in parallel with an ideal resistor; thus a simple high-pass filter cannot be used. Complex circuits consisting of up to four poles and three zeros in the transfer function are commonly used. The placement of the poles and zeros must be carefully set for the particular resistor.

(4) The current required to charge the input capacitance during a step voltage command can easily exceed the maximum current that can be passed by R_f from the typical ±13 V swing of the operational amplifier. For example, to linearly charge 5 pF of input capacitance to 100 mV in 10 µs would require a charging current of 50 nA. This is well beyond the 260 pA that can be passed by a 50 GΩ resistor driven from 13 V. Thus, special circuits are typically added to inject the required charging current through a capacitor.

(5) The best high-value resistors seem to be available only in a miniature "surface mount" form. This means that the headstage electronics have to be manufactured in a hybrid. This manufacturing technique has some advantages. First, the U430 input FETs and other FETs typically used to switch between different values of R_f can be used in an unpackaged form. This is to be preferred, since the sealing glasses used in packaged transistors typically have a leakage resistance that increases the noise. Second, it means that the sensitive input components can be maintained in a hermetically sealed environment that reduces the rate at which they age due to environmental contamination.

If the Probe Output is Slow, How Can Voltage Clamping be Fast?

In resistive-feedback headstages (but not capacitive-feedback headstages, discussed below) the current output of the current-to-voltage (I-V) converter in the probe is slow. The high-frequency boost occurs afterwards and cannot influence the events at the pipette. Thus, one might conclude that the voltage clamp of the pipette must also be slow.

In fact, despite the slow current output of the I-V converter, the voltage clamp of the pipette is rapid. The pipette is connected to the negative input (summing junction) of the op amp. The command potential is connected to the positive input of the op amp. The operation of the op amp in this configuration is to force the potential at the summing junction to rapidly follow the potential at the positive input. If the command potential is a step, the potential at the summing junction (and hence the pipette) is also a step. The current required to achieve this step is passed through the feedback resistor (R_f) and the associated stray feedback capacitance (C_{Rf}) of the I-V converter. The output of the I-V converter settles to the final value with time constant R_fC_{Rf}. This relatively slow settling occurs despite the fact that the step at the summing junction is fast.

In this discussion, we have carefully referred to the fact that it is the *pipette* that is rapidly voltage clamped. The membrane potential is voltage clamped to its final value much more slowly. To a reasonable approximation, the time constant for voltage clamping the membrane is R_pC_m, where R_p is the pipette resistance and C_m is the membrane capacitance.

Capacitor Feedback Technology

Rationale

A new technology in single-channel recording is the capacitor-feedback technique, also known as the integrating headstage technique. While this technique was talked about for many years, it was only first attempted and described in 1985 by Offner and Clark. Practical implementations did not become commercially available until 1989.

In this technique, the feedback resistor in the headstage is replaced by a capacitor (C_f in Figure 3-23). When a capacitor is used instead of a resistor, the headstage acts as an integrator. That is, for a constant input current, the output of the headstage is a ramp. The slope of the ramp is proportional to the current. To recover a voltage that is simply proportional to the input current, the integrator must be followed by a differentiator (A2).

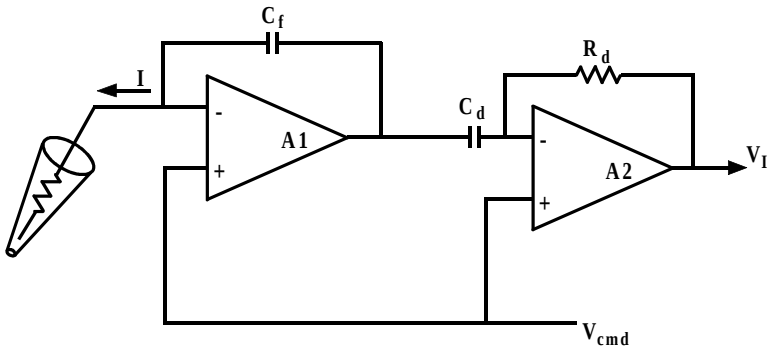

Figure 3-23. Capacitive Feedback Headstage

The feedback element around operational amplifier A1 is capacitor (C_f). Thus the output of A1 is the integral of the pipette current. The actual current is recovered from the integral by differentiating it in the differentiator formed by C_d, R_d and A2.

Compared with resistor-feedback picoamp current-to-voltage converters (RIV), capacitor feedback current-to-voltage converters (CIV) exhibit less noise, increased dynamic range, wider bandwidth and improved linearity.

Noise, Dynamic Range, Linearity and Bandwidth

(1) The noise of the CIV is lower for two reasons. First, the capacitors do not generate as much thermal noise as resistors do. (There is an equivalent resistor noise source due to the differentiator, but with careful design this can be made negligible.) Second, capacitors are commercially available that are free of the excess noise sources that plague high-gigohm resistors.

The noise benefit of the CIV is eliminated if C_f is large (10 pF or more). This is because from the noise point of view, C_f sums with the other sources of input capacitance. The voltage noise of the input FETs leads to a current injection into the total input capacitance. If this input capacitance is large, this source of noise becomes dominant.

The lower noise of the CIV can be realized only in situations where all other noise sources are minimized. That is, the experimenter should use a low-noise glass and pipette holder, Sylgard coating, and a high-resistance seal. By carefully removing other sources of noise, the reduced noise of the CIV can be realized in real patches, not just in theory.

(2) The capacitor-feedback headstage has an equivalent transfer resistance (R_T) given by

$$R_T = R_d \frac{C_d}{C_f} \tag{11}$$

Because the noise is theoretically independent of the C_f value and the gain of the differentiator, R_T, may be kept quite low, *e.g.,* 100 MΩ. At this gain, the maximum current that can be recorded is 500 times greater than for an RIV using a 50 GΩ feedback resistor. Thus the CIV potentially has vastly improved dynamic range.

(3) In the section on *Resistor Feedback Technology*, it was pointed out that in order to achieve an acceptable bandwidth of, *e.g.,* 20 kHz for single-channel recording, a complex boost circuit has to be used to correct the frequency response. Even if the boost box has as many as four poles and three zeros, the frequency response of R_f is not perfectly corrected. The imperfections of the correction are most easily seen by observing the step response of the headstage. Frequently, there will be overshoots and ripples that can amount to as much as 2% of the response. However, because excellent capacitors are available, the step response is inherently square with a CIV.

(4) The bandwidth of a CIV can be very wide. It is maximized by using a small value of C_f and a small value of R_d. For practical values (C_f = 1 pF and R_d = 150 kΩ), the bandwidth is of the order of 70 kHz or more. This is considerably better than the 20 kHz of a good RIV using a 50 GΩ feedback resistor.

Whether it is practical to use this increased bandwidth or not is another matter. The noise of both the RIV and the CIV headstages increases dramatically with frequency. While the noise of the CV201A headstage is 0.16 pA rms (about 1.3 pA peak-to-peak) at 10 kHz bandwidth, the noise in a 50 kHz bandwidth is about 1.5 pA rms (about 12 pA peak-to-peak). Thus the single-channel currents would have to be extremely large to be distinguished from the noise.

Problems

There are two major problems that make the capacitor feedback technology difficult to implement. The first problem is that after a sustained net DC input current the integrator voltage ramps towards one of the power-supply limits. When this happens, the integrator must be reset by discharging the capacitor. The frequency of the resets depends on the size of C_f and the average input current. For example, if C_f is 1 pF and the input current is 2 pA, the output of the integrator will ramp at 2 V/s. If the resetting circuitry is triggered when the integrator voltage exceeds 10 V, resets will occur every five seconds.

The reset itself lasts approximately 50 µs. During the reset, sample-and-hold circuits are used to maintain the current output at its value immediately prior to the start of the reset. If it is acceptable to lose 0.1% of the data, resets as frequent as every 50 ms would be acceptable. In the above example, this corresponds to an input current of 200 pA. For single-channel recording, this is more than adequate.

Figure 3-24 shows the signal pathway for the capacitor-feedback headstage. The output current (I) of the capacitor-feedback headstage is normally connected through a switch to the output pre-filter amplifier, then to the low-pass filter, and finally to the post-filter amplifier. The signal also goes through a low-pass filter to a sample-and-hold amplifier. During reset, the switch shifts to the RESET position. Simultaneously, the sample-and-hold amplifier is switched to the hold mode so that the signal immediately before the reset transient occurs is presented to the output amplifiers.

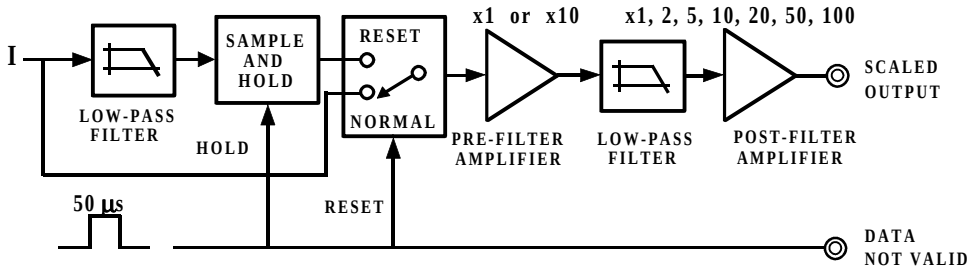

Figure 3-24. Signal Handling During Resets in the Capacitor-Feedback Headstage
See text for details.

A capacitor feedback is not practical for whole-cell recordings. Due to the large whole-cell currents the resets would be too frequent unless the value of C_f was increased. In practice, it is hardly worth it. It is simpler to switch to a modest-sized resistor (*e.g.,* 500 MΩ) and avoid the problem of resets altogether. Reverting to resistor-feedback methods for whole-cell recordings does not represent a setback in terms of noise because the 500 MΩ resistor is not the dominant source of noise.

During the measurement of voltage-activated single-channel currents, the resets can be made irrelevant. This is done by using a control pulse to force a reset immediately prior to the voltage step. This guarantees that C_f is in its discharged state at the beginning of the voltage step and is therefore unlikely to need resetting during the step.

The second problem is that during resets transients are injected into the headstage input by the reset circuitry. If left uncompensated, these would cause unwanted and possibly damaging current pulses to be injected down the micropipette into the patch. Special compensation circuitry needs to be included in the design to exactly balance out these injected currents.

There are other transient problems that occur during and after the reset. These include dielectric absorption in the differentiator and feedback capacitors, and other ill-defined transients that work their way into the system. Compensation circuitry must also be included to compensate for the many transients that occur during reset make the design of an integrating patch clamp challenging. However, in a well-designed system, the reset transients measured at the output will be less than 1 pA measured in a 1 kHz bandwidth.

Special Considerations for Bilayer Experiments

The Advantage of Resets in Bilayer Experiments

While they are normally considered to be a nuisance, there is one application where resets are quite an advantage. This is in voltage-step experiments in bilayers. During a reset, a low resistance of about 10 kΩ is placed in parallel to the feedback capacitor. This resistor allows currents up to 1 mA to pass out of the headstage to rapidly charge the bilayer capacitance. Voltage steps as large as 100 mV are normally achieved with one or two resets separated by less than a millisecond.

Noise *vs.* Access Resistance

In bilayer experiments, the access resistance is quite low, usually ranging from a few kilohms to several tens of kilohms. The access resistance is in series with the membrane capacitance and produces voltage noise just as though the headstage had high intrinsic noise.

Bilayer recording is generally done at bandwidths below 1 kHz. At this low bandwidth, the e_nC_{in} noise has not yet become the major contributor to the overall noise (where e_n is the voltage noise of the probe input FETs and C_{in} is the capacitance at the input of the headstage primarily due to the bilayer membrane capacitance). If the access resistance is large enough, it becomes the dominant noise contributor at low bandwidths.

Figure 3-25 shows the dependence of peak-to-peak noise on access resistance in a 1 kHz bandwidth for a given bilayer membrane capacitance. It can be seen that the lower the access resistance, the lower the noise. It is therefore important that the patch-clamp instrument be capable of operating without oscillation even when extremely small, or zero, access resistances are used. The CV-4B headstage for the Axopatch-1D, and both of the headstages for the Axopatch 200 and 200A, have been designed for complete stability even with extremely low access resistances.

Figure 3-25. Typical Current Noise in Bilayer Experiments
See text for details.

How Fast is "Fast"?

The speed of the observed transitions of single-channel currents is the same as the response time of the electronics. Whenever recordings are made at wider bandwidths, the observed transition rates become shorter. It is not clear that we will ever be able to resolve the actual transition time using electrical measurements (perhaps optical techniques will emerge to do the job); but the discovery of new lower limits to the transition time will be made possible by the low-noise wide-bandwidth patch clamps such as the Axopatch 200 series.

Measurement of Changes in Membrane Capacitance

The measurement of minute changes in membrane capacitance, such as the changes that occur during exocytosis, was made practical by the whole-cell patch-clamp technique. Two methods can be used. The simplest and the most traditional way is to use a fitting technique to find the time constant of the current response to a voltage step. By assuming a simple cell model, the membrane capacitance can be easily deduced. This technique is relatively easy to apply, but it has a resolution no better than 100-200 fF, which is insufficient to resolve the 10-20 fF capacitance increases occurring during fusion of single granules with the membrane.

A much more sensitive technique involves the use of a lock-in amplifier (also known as a phase detector). In this technique, a sinusoidal command voltage is applied to the cell. The magnitude of this voltage must be small enough so that the cell's properties are essentially linear. One measures two outputs that are proportional to the sinusoidal current response at two orthogonal frequencies. It can be shown that the magnitudes of these two responses are a function of the access resistance, the membrane resistance and the cell capacitance. With a third measurement of the DC current, and assuming that the reversal potential of the DC currents is known, there is sufficient information to calculate the value of all three parameters. The resolution of this

technique can be as good as 1 fF, with measurements being made up to one hundred times per second.

The lock-in amplifier technique requires special equipment that is not widely available. Joshi and Fernandez (1988) have described a similar technique where all of the phase-sensitive measurements and sinusoidal stimulations are performed by software. This has the great advantage of not requiring any special equipment other than the patch clamp. An enhanced version of this technique is implemented in AxoData and CLAMPEX (part of pCLAMP), two programs from Axon Instruments.

Seal and Pipette Resistance Measurement

The micropipette resistance is easily measured with micropipette amplifiers by passing a pulse of current through the micropipette. Generally, a convenient pulse size such as 0.1, 1 or 10 nA is used. If the pulse size is 1 nA, the micropipette potential without any Bridge Balance is 1 mV/MΩ of micropipette resistance. For example, if the pulse response is 55 mV, the micropipette resistance is 55 MΩ. Alternatively, the Bridge Balance control can be adjusted to null the pulse response and the micropipette resistance can be measured directly from the Bridge Balance dial.

In patch clamps, the measurement of the combined pipette and seal resistance is not quite as straight forward because the patch clamp is normally used in voltage-clamp mode rather than current-clamp mode. In this case, a voltage step is applied to the pipette. The combination of the pipette and seal resistance is calculated from the current response using the formula: R = V/I. For example, if the test pulse is 1 mV and the current response is 67 pA, the resistance is 15 MΩ. A nomogram is included in the Axopatch 200 and 200A manuals so that the pipette resistance can be determined without performing a calculation.

In the Axopatch-1 series amplifiers, the special Step Command Generator switch can be used to automatically determine the combined pipette and seal resistance. The Axopatch-1 manual should be consulted for details.

In the GeneClamp 500, the on-board microprocessor performs the required calculations. The voltage-step amplitude is automatically adjusted to optimize the sensitivity of the measurement. The dynamic range is from 100 kΩ to 200 GΩ.

Micropipette Holders

High-quality micropipette holders are crucial to successful single-channel recording. Two characteristics of the holder are important. First, the holder must be mechanically stable. When working with a cell-attached patch, drift of the micropipette is unacceptable. Clearly, a low-drift micromanipulator is required (as discussed in **Chapter 2**). But if the holder is loose in the headstage connector or if the pipette does not seat properly, drift will occur despite the high quality of the micromanipulator. One common, exasperating problem associated with loose holders is movement of the pipette when suction is applied, which either prevents seal formation or damages the cell. The HL-1 and HL-2 series holders from Axon Instruments have been designed to fit snugly into the headstage connector and to firmly grip the pipette to prevent drift. Second, the holder must not introduce noise. To assure this:

(1) Select the right holder material. When a holder is connected to the headstage, even before a pipette is connected, the open-circuit noise goes up slightly. The reasons are not understood. Empirical tests have shown that when used with glass micropipettes, polycarbonate adds the least noise. The HL-1 and HL-2 holders use polycarbonate for the pipette cap and the body of the holder. The portion that plugs into the headstage is made of Teflon. Teflon in this position does not add noise, and it facilitates smooth insertion and removal.

(2) Use a small pin for the electrical connection. Large pins have more capacitance contributed by the surrounding grounded surfaces.

(3) Do not use a shield. Surrounding the holder with a driven or grounded shield adds to the input capacitance and thus increases the noise.

Current Conventions and Voltage Conventions

The terminology used in this discussion applies to all amplifiers manufactured by Axon Instruments.

Definitions

Positive Current

The flow of positive ions *out* of the headstage into the microelectrode and out of the microelectrode tip into the preparation is termed positive current.

Inward Current

Current that flows across the membrane, from the outside surface to the inside surface, is termed inward current.

Outward Current

Current that flows across the membrane, from the inside surface to the outside surface, is termed outward current.

Positive Potential

The term *positive potential* means a *positive* voltage at the headstage input with respect to ground.

Transmembrane Potential

The *transmembrane potential* (V_m) is the potential at the inside of the cell minus the potential at the outside. This term is applied equally to the whole-cell membrane and to membrane patches.

Depolarizing / Hyperpolarizing

The resting V_m value of most cells is negative. If a positive current flows into the cell, V_m initially becomes less negative. For example, V_m might shift from an initial resting value of -70 mV to a new value of -20 mV. Since the absolute magnitude of V_m is smaller, the current is said to *depolarize* the cell (*i.e.*, it reduces the "polarizing" voltage across the

membrane). This convention is adhered to even if the current is so large that the absolute magnitude of V_m becomes larger. For example, a current that causes V_m to shift from -70 mV to +90 mV is still said to depolarize the cell. Stated simply, *depolarization* is a *positive* shift in V_m. Conversely, *hyperpolarization* is a *negative* shift in V_m.

Whole-Cell Voltage and Current Clamp

Depolarizing / Hyperpolarizing Commands

In whole-cell voltage clamping, whether it is performed by TEVC, dSEVC, cSEVC or whole-cell patch clamp, a *positive* shift in the command voltage causes a positive shift in V_m and is said to be *depolarizing*. A *negative* shift in the command voltage causes a negative shift in V_m and is said to be *hyperpolarizing*.

Transmembrane Potential *vs.* Command Potential

In whole-cell voltage clamping, the command potential controls the voltage at the tip of the intracellular voltage-recording microelectrode. The transmembrane potential is thus equal to the command potential.

Inward / Outward Current

In a cell generating an action potential, depolarization is caused by a flow of positive sodium or calcium ions *into* the cell. That is, *depolarization* in this case is caused by an *inward* current.

During intracellular current clamping, a depolarizing current is a *positive* current out of the micropipette tip into the interior of the cell. This current then passes through the membrane *out* of the cell into the bathing solution. Thus, in intracellular current clamping, a *depolarizing (positive)* current is an *outward* current.

An *inward* sodium current flows in some cells after a depolarizing voltage step. When the cell is voltage clamped, the sodium current is canceled by an equal and opposite current flowing into the headstage via the microelectrode. Thus it is a *negative* current. When two-electrode voltage clamping was first used in the early 1950's, the investigators chose to call the *negative* current that they measured a *depolarizing* current because it corresponded to the depolarizing sodium current. This choice, while based on sound logic, was unfortunate because it means that from the recording instrument's point of view, a negative current is *hyperpolarizing* in intracellular current-clamp experiments but *depolarizing* in voltage-clamp experiments.

To prevent confusion, Axon Instruments has decided to always use current and voltage conventions based on the instrument's perspective. That is, the current is always unambiguously defined with respect to the direction of flow into or out of the headstage. Some instrument designers have put switches into the instruments to reverse the current and even the command voltage polarities so that the researcher can switch the polarities depending on the type of experiment. This approach has been rejected by Axon Instruments because of the real danger that if the researcher forgets to move the switch to the preferred position, the data recorded on the computer could be wrongly interpreted. Axon Instruments believes that the data should be recorded unambiguously.

Patch Clamp

By design, the patch-clamp command voltage is positive if it increases the potential inside the micropipette. Whether it is hyperpolarizing or depolarizing depends upon whether the patch is "cell attached," "inside out" or "outside out." The patch-clamp pipette current is positive if it flows from the headstage through the tip of the micropipette into the patch membrane.

Cell-Attached Patch

The membrane patch is attached to the cell. The pipette is connected to the outside surface of the membrane. A *positive* command voltage causes the transmembrane potential to become more negative, therefore it is *hyperpolarizing.* For example, if the intracellular potential is -70 mV with respect to 0 mV outside, the potential across the patch is also -70 mV. If the potential inside the pipette is then increased from 0 mV to +20 mV, the transmembrane potential of the patch hyperpolarizes from -70 mV to -90 mV.

From the examples it can be seen that the transmembrane patch potential is inversely proportional to the command potential, and shifted by the resting membrane potential (RMP) of the cell. A positive pipette current flows through the pipette across the patch membrane into the cell. Therefore a *positive* current is *inward.*

Inside-Out Patch

The membrane patch is detached from the cell. The surface that was originally the inside surface is exposed to the bath solution. Now the potential on the inside surface is 0 mV (bath potential). The pipette is still connected to the outside surface of the membrane. A *positive* command voltage causes the transmembrane potential to become more negative, therefore it is *hyperpolarizing*. For example, to approximate resting membrane conditions of V_m = -70 mV, the potential inside the pipette must be adjusted to +70 mV. If the potential inside the pipette is increased from +70 mV to +90 mV, the transmembrane potential of the patch hyperpolarizes from -70 mV to -90 mV.

From the example it can be seen that the transmembrane patch potential is inversely proportional to the command potential. A positive pipette current flows through the pipette across the patch membrane from the outside surface to the inside surface. Therefore a *positive* current is *inward*.

Outside-Out Patch

The membrane patch is detached from the cell in such a way that the surface that was originally the outside surface remains exposed to the bath solution. The potential on the outside surface is 0 mV (bath potential). The pipette interior is connected to what was originally the inside surface of the membrane. A *positive* command voltage causes the transmembrane potential to become less negative, therefore it is *depolarizing*. For example, to approximate resting membrane conditions, assuming that V_m = -70 mV, the potential inside the pipette must be adjusted to -70 mV. If the potential inside the pipette is then increased from -70 mV to -50 mV, the transmembrane potential of the patch depolarizes from -70 mV to -50 mV.

The membrane potential is directly proportional to the command potential. A positive pipette current flows through the pipette across the patch membrane from the inside surface to the outside surface. Therefore a *positive* current is *outward.*

Summary

(1) *Positive* current corresponds to:

Cell-attached patch	patch inward current
Inside-out patch	patch inward current
Outside-out patch	patch outward current
Whole-cell voltage clamp	outward membrane current
Whole-cell current clamp	outward membrane current

(2) A *positive* shift in the command potential is:

Cell-attached patch	hyperpolarizing
Inside-out patch	hyperpolarizing
Outside-out patch	depolarizing
Whole-cell voltage clamp	depolarizing

(3) The correspondence between the command potential (V_{cmd}) and the transmembrane potential (V_m) is:

Cell-attached patch	$V_m = RMP - V_{cmd}$
Inside-out patch	$V_m = - V_{cmd}$
Outside-out patch	$V_m = V_{cmd}$
Whole-cell voltage clamp	$V_m = V_{cmd}$

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