ION CHANNELS

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CHAPTER 8

IONIC CHANNELS IN OCULAR EPITHELIA

J. L. RAE, R. A. LEVIS, and R. S. EISENBERG

1. INTRODUCTION

Membrane phenomena are dominated by channels, integral membrane proteins specialized to allow and control the movement of solutes through aqueous pores spanning the membrane. The tiny aqueous pore (less than 1 nm in diameter) is embedded in a much larger protein cylinder some 10 nm in diameter and length that shields the solute from the low dielectric constant of the lipid membrane, decreasing the electrostatic energy barriers which prevent ion movement across artificial lipid membranes. Proteins play a role in membrane phenomena (whether the proteins form "channels" or other transporters) akin to the role of enzymes in metabolism: they accelerate the rate of chemical reactions or solute translocation so greatly that, to a good approximation, they are the only pathways of biological significance.

The initial hurdle in the study of enzymes or channels is the simple identification of those involved in the system of interest. Tens of thousands of enzymes exist in animals, each with a specialized function, structure, and mechanism, and it has proven impossible to understand the role of each, let alone its structure of mechanism, until it is isolated. Thus, the technology of protein purification determines our knowledge of enzymes, great advances rapidly following the invention of chromatography, gel electrophoresis, monoclonal antibodies, and so on.

The study of membranes has not benefitted so much from this chemical technology because its "enzymes" are embedded in lipid and require specialized technology for purification, identification, and reconstitution. Although much progress has been made (Miller, 1983; Latorre *et al.*, 1985), the

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technology remains frustratingly primitive, particularly when compared to the range of membrane transport systems begging for analysis.

Historically, membrane phenomena have been successfully analyzed only in a few cases distinguished by their homogeneity. Katz (1950) recognized that the function of the mechanoreceptor was determined by a membrane transport system that allowed a *specific* kind of current flow once activated. Subsequently, such "ionic conductances" were studied in axonal membranes (Hodgkin and Katz, 1949; Hodgkin and Huxley, 1952a,b), the postsynaptic membranes of skeletal muscle (Fatt and Katz, 1951), nerve (Coombs *et al.*, 1955), and so on (Hille, 1984). In these tissues, analysis proceeded with surprisingly little controversy and consensus was quickly reached among competitive investigators. The biological phenomena were clearly determined by currents carried by a few types of ions, with dependence on electrical and chemical potential and time, once biologically activated.

In more modern language, we might say that analysis of macroscopic currents has been most successful when the membrane of interest is dominated by one or two channel types, each with a different current carrier, agonist, and/or antagonist: e.g., the squid axon membrane is dominated by just two channel types; indeed, research on that membrane is still limited by its poorly understood "leakage conductance" for which a blocker is sorely sought.

Many membranes are not so dominated by a few channel types; many channel types cannot be so easily identified by agonist or antagonist. And, perhaps not surprisingly, the study of these membranes by macroscopic techniques did not reach consensus.

Logical difficulties can prevent the separation of channel types that can only be studied in parallel, particularly when the properties of the channel types are not known. It is difficult to separate the sum of fluxes through unknown nonlinear channels into components; experiments must be designed that provide enough independent and redundant information to estimate each component, hopefully in more than one way. Such experiments are particularly hard to design because extreme conditions that simplify physical systems (e.g., removal of reactants or use of enzymes) often corrupt the relevant channel (Spalding *et al.*, 1981) and may in fact affect many channels. Even if there is enough information available from independent techniques to identify parallel systems, it is often hard to be convinced of the uniqueness of analysis. Such difficulties bedeviled analysis of macroscopic current in cardiac and smooth muscle, many types of neurons, and most epithelial membranes. Indeed, one might say that macroscopic techniques have not allowed the analysis of cell membrane properties in general—such analysis could only be done in a few fortunately homogeneous membranes.

This situation changed with the development of the gigaohm-seal patch clamp technique (Neher, 1981) which allows the recording of current flow through single ionic channels embedded in their natural membrane. The nearly intractable problem of separation and identification of parallel chan-

nels became manageable: one could record from a small number of channels, if one could record at all.

Applications to Epithelia. Patch voltage clamp is particularly likely to help the study of epithelia, which are characterized by their diversity of transport systems and, presumably, channel types. Macroscopic measurements of epithelia will continue to be valuable, but will be more so once the channel types are established by patch recording. The separation of a sum of fluxes into its components is far easier if the number, types, and properties of the components are known.

Here we use the patch clamp method to characterize ionic channels in the membranes of lens and corneal cells, chiefly from the apical membranes of lens epithelium and corneal endothelium, and the basal membrane of the deepest layer of corneal epithelium, in several amphibian and mammalian species. In each of these cell types, the mechanism of ionic movement is not well characterized. The presence of many channel types, along with many active transport systems and carriers and many technical difficulties, has hampered the work. Thus, we proceed with few guidelines, necessarily describing what we find, without being able to ascribe a functional role to many of the channels we study.

This reality is at least as frustrating to us as to readers interested in other properties of ocular tissues. But we must constantly remind ourselves of the analogy with enzymology; as irritating as was and is the simple cataloging and description of all enzymes, so was it necessary if metabolic pathways were to be understood. Indeed, the unglamorous description of enzymes of the 1960–1970s has proven more durable than the more glamorous analyses of enzyme mechanism often made, as we now know, in the absence of sufficient structural (e.g., crystallographic) data.

We hope then the major contribution of this chapter is its approach. We present some simple ways to study cells with unknown macroscopic properties, arising from our experience with the patch voltage clamp and more than 20 different kinds of channels.

2. LOW-NOISE METHODS AND GLASS CONSIDERATIONS

2.1. Noise Performance

2.1.1. Electronics

Electronic noise in patch clamping has been described extensively in the literature (Levis, 1981; Hamill *et al.*, 1981; Sigworth, 1983; Rae and Levis, 1984a) and so we will confine our comments to a general description of the present state of the art.

Electronic noise of the patch clamp "headstage" amplifier is the result of three principal sources. (1) *Thermal and excess noise of the feedback*

resistor. Gigaohm range feedback resistors that are commercially available at this time exhibit excess noise that increases with increasing frequency. For resistors in the range of 10–50 GΩ, this noise rises above the predicted thermal noise levels at frequencies above a few hundred hertz. Resistor noise is a major limitation in the achievable noise performance of a typical headstage amplifier for bandwidths in excess of a few kilohertz. (2) *Noise arising from the voltage noise of the FET input stage*. In conjunction with the input capacitance of the FET plus any additional capacitance at the headstage amplifier input, the input voltage noise of the FET stage produces current noise with a power spectral density that rises rapidly with increasing frequency. With a U430 dual JFET (Siliconix) serving as a differential input stage to the headstage, this noise amounts to roughly 3×10^{-30} A^2/Hz at 10 kHz, which is approximately equivalent to the thermal noise power spectral density of a 5-GΩ resistor, and would account for somewhat more than 0.1-pA rms noise in a bandwidth extending from DC to 10 kHz. FETs selected for the headstage input should have both low-voltage noise and low-input capacitance (gate-to-source plus gate-to-drain capacitance). (3) *Shot noise of the gate leakage current of the FET input stage*. The power spectral density of this noise is given by $2qi_g$, where q is the electron charge (1.6×10^{-19}C) and i_g is the gate current in amperes. Shot noise is independent of frequency—it is white noise. If i_g is 1 pA, then its shot noise spectral density will equal that of the thermal noise of a 50-GΩ resistor. U430s can be selected with gate currents as low as 0.2 pA at 25°C, provided that the device is operated such that its drain-to-source voltage is less than 5 V. The shot noise associated with a gate current is less than 5 V. The shot noise associated with a gate current this small is generally quite insignificant, but it should be realized that i_g is highly temperature dependent, roughly doubling for every 10°C increase in junction temperature. Thus, elevated temperatures (e.g., resulting from high power dissipation in the headstage) should be avoided. Input FET gate current represents one of several practical limitations to achievable low-frequency noise performance of patch clamp electronics. At this time, patch clamp electronics can achieve noise levels as low as 0.02 pA rms with a −3-dB bandwidth (8-pole Bessel filter) of 1 kHz, 0.05 pA rms at a bandwidth of 3 kHz, and somewhat less than 0.2 pA rms at a bandwidth of 10 kHz.

2.1.2. Pipette Holders

Holders can be a substantial source of noise and we have recently learned that the noise depends on the material from which the holder is constructed. We presently use custom-designed and -manufactured input connectors and holders machined from Teflon and polycarbonate, respectively. Our test headstage has 0.21-pA rms noise in a bandwidth extending from DC to 10 kHz, without holder. With holder connected (Ag–AgCl pellet electrode included), the total noise rises to 0.22 pA rms. With a fluid-filled

pipette in the holder and with its tip in air, there is an additional noise increment to about 0.28 pA rms if the glass is soda lime (0080, R6), 0.25–0.26 if the glass is a Kovar sealing glass (7040, 7052, 7056). If a high-lead glass such as 8161, EG-6, or 0120 is used, there is essentially no increment above the 0.22 pA rms. Teflon and Delrin perform quite well when attached to the headstage without the pipette but they perform rather poorly when the pipette is included. Other materials such as Kel-F, Plexiglas, and nylon perform *much* worse with the pipette in air whereas polyethylene and polypropylene perform almost as well as polycarbonate. The noise associated with the holder and pipette is relatively more important at high frequencies (wide bandwidths) than it is at lower frequencies. Finally, it should also be noted that both our connector and holder are completely free from metallic shielding. Such shielding increases capacitance to ground at the headstage input and therefore increases wideband noise. We have found that shielding of connector and holder is quite unnecessary to reduce the pickup of stray electrical fields to negligible levels.

2.1.3. Glass

Noise associated with the pipette is more difficult to quantify when the pipette is immersed in the bath and sealed to Sylgard (or to a cell membrane). We have studied the noise produced by some 20 varieties of glass fabricated into patch pipettes and sealed to Sylgard. These results are included in Fig. 1 where the rms noise in a 10-kHz band (see figure legend) is plotted against the glass loss factor/wall thickness. Every attempt is made to keep the experimental situation constant in tests of this type (e.g., use of the same amplifier and holder, immersion of pipette tip to a constant depth into the bath, and so on). Our general conclusions are relatively simple: noise associated with the pipette is usually inversely correlated with the loss factor of the glass from which it is fabricated. Pipettes with thicker walls are less noisy than those with thinner walls. Coating pipettes with Sylgard is important for all glasses, since this prevents the noise-producing creep of fluid up the wall of the pipette that has been described previously (Hamill *et al*, 1981). With the lowest noise glasses, it is not necessary to bring this coating extremely close to the tip. 8161 is an exceptional glass from a noise standpoint and its advantage extends to low frequencies, its noise at 1 kHz being about the same whether its tip is in air or sealed to Sylgard. Clearly, the glass used to make patch pipettes can be of extreme importance to noise in practical experimental situations.

For pipettes fabricated from Corning 8161 (lead glass) and sealed to Sylgard, total noise in a bandwidth of 10 kHz can be as low as 0.28–0.30 pA rms with the walls Sylgard coated and the pipette tip submerged to a depth of about 2 mm. Minimum noise from Corning 7052 (a Kovar sealing glass) was 0.36–0.37 pA rms, whereas soda lime glasses were routinely 0.50–0.55 pA rms under the same conditions.

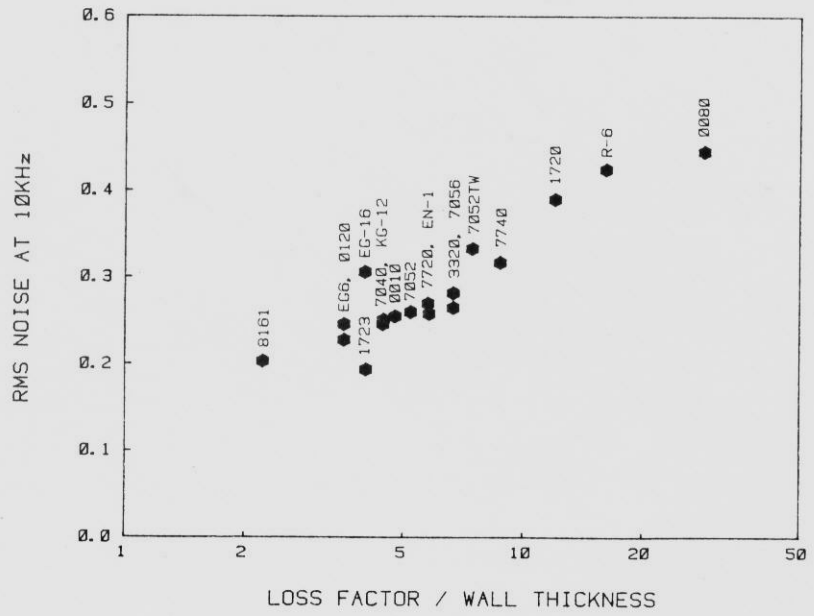

FIGURE 1. rms noise of electrodes constructed from several glass types and sealed to Sylgard plotted against the glass loss factor divided by the wall thickness of the glass. The rms noise plotted is [(noise with pipette sealed to Sylgard)^2 − (noise with pipette tip in air)^2]$^{1/2}$. Fluid depth in measuring chamber was about 2 mm. Loss factor is given as % at 1 MHz, wall thickness is in mm. rms noise is in pA at 10 kHz.

2.1.4. Membrane Seal

Background noise in an actual patch clamp experiment will always exceed the noise measured with a pipette sealed to Sylgard, but in our best experiments with the pipette sealed to membrane, the noise can be almost the same as that with a Sylgard seal. The incremental noise results from the membrane–glass seal and the patch itself. Specifically, new noise components will include thermal and excess noise of the patch membrane and seal and current noise arising from the voltage noise of the pipette in series with the patch/seal admittance. Theoretical predictions for the frequency dependence of Re{Ysh} have been presented (Sachs, 1984; see also Sachs and Auerbach, 1983), but it is not presently possible to actually experimentally measure this presumed frequency dependence or the noise of the seal *per se*. Thus, we simply note here the probability that the seal contributes thermal noise with a power spectral density in excess of that predicted from the thermal noise of the seal resistance at frequencies in the kilohertz range and the possibility that such noise may be a significant limiting factor in the ultimate noise performance achievable with the patch clamp technique. Because the precise nature of the membrane-glass interaction involved in seal formation is not known, we can not rule out the possibility that seal noise may depend on the type of glass used to fabricate the pipette.

An additional source of noise arises from the patch capacitance in series with the thermal voltage noise of the pipette (Levis, 1981; Rae and Levis, 1984b). Patch size and hence patch capacitance can vary a great deal from one experiment to the next. Sakmann and Neher (1983) report patch capacitances in the range of 10 fF to 250 fF, which should correspond to patch areas ranging from 1 to 25 μm^2. It can be estimated that a 5-MΩ pipette in series with 10 fF of patch capacitance should only produce incremental noise of 0.01 pA rms in a 10-kHz bandwidth. However, the same pipette in series with 250 fF would produce about 0.26 pA rms of noise in the same bandwidth; with a 10-mΩ pipette, this value would increase to 0.36 pA rms. These latter values are quite significant in comparison to the noise levels discussed previously for the headstage, holder, and low-noise pipettes and suggest that patch electrodes should be fabricated so as to produce the smallest patch size if the minimal noise is to be achieved.

It should be noted that the noise considered above assumed that no field was imposed across the patch and that symmetrical solutions are used in the pipette and bath. Noise arising from the patch membrane and the seal is expected to exceed the Nyquist prediction (i.e., to exceed thermal noise) when a voltage exists across the patch. This additional noise is difficult to quantify and is rather variable, but it is almost always present. Sachs and Auerbach (1983) report that such noise has a $1/f$ spectral density and suggest that it may be due to unresolved channel openings and/or diffusion across the seal or patch.

Using pipettes fabricated from Corning 8161 with a geometry that minimizes patch area, it is often possible to achieve noise levels of 0.3–0.35 pA rms at a bandwidth (−3 dB of an 8-pole Bessel filter) of 10 kHz when sealed to cells.

Further reductions in noise seem possible, but significant improvements will require reduction of the noise from each of the sources considered. There is still room for improvement in electronic noise, but in the absence of continued efforts to minimize noise from the pipette, seal, and patch itself, greatly improved electronics would only result in modest reductions of overall noise. Ultimately, the noise performance of the patch clamp technique will probably be limited by noise associated with the pipette, seal, and the patch itself, and any serious attempt to improve on the present state of the art must also deal with these aspects of the technique.

2.2. Comments on Glass

There is considerable diversity in the properties of the many glasses we have tested to date and it is useful to exploit that diversity to produce electrodes that are optimal for the particular measurements at hand. Relevant properties of glass fall into three broad classes: sealability, formability, and noise. We have had sufficient experience with more than 20 glass types to make relevant comments and recommendations concerning all three properties.

We have yet to find a glass that will not seal to our cells. It appears that all glasses are able to interact with cell membranes to produce the poorly understood situation known as the gigaohm seal. For lens epithelial cells with which we have the most experience, several glasses seal much better than others. This does not mean that they produce higher average seal resistances but only that they produce seals more frequently than other glasses. Most notable in this regard are the Kovar sealing glasses with the Corning designation numbers of 7052, 7056, and 7040. All of these glasses have the useful ability to form more than one gigaohm seal with the same pipette, certainly not a general feature of other glasses in our experience. If noise is very important, we do not recommend using these glasses for more than one seal since each subsequent seal is generally noisier than the one preceding it. The high-lead glasses such as Corning numbers 0010 and 0120, and Kimble numbers KG-12, EG-6, and EG-16 were earlier thought to be noisy and so their ability to seal to membranes has not been adequately tested at present. Corning 8161 has been tested and seals well to our membranes. Corning 0080 and R-6, classical soft soda lime glasses, seal quite easily to our cells but under the best situations are much noisier than Kovar sealing glasses or 8161, and thus we do not use them. Corning's Pyrex and Kimble's Kimax glasses do not seal as well as the Kovar sealing glasses, and so we do not use them either. Corning 7070 (low loss electrical) seals easily to membranes but is very difficult to pull as described later. Corning 1723 (aluminosilicate) seals well to our cells.

Because the various glasses studied have quite different thermal properties, they vary in their ability to be formed into optimally shaped pipettes. The Kovar sealing glasses 7040, 7056, and 7052 cannot easily be pulled into bluntly tapering tips and so they are not very useful if one desires to do whole-cell recordings at wide bandwidth. The difficulty arises from the series resistance present at their tips. They are, however, perfectly acceptable for recording from small membrane patches. Corning 8161 is an ideal glass from the standpoint of thermal properties. Because of the very low melting point of this glass, it is possible to pull tips that range from being extremely sharp with a tiny opening to being blunt with the tip actually broken and jagged. The broken tips can be easily firepolished into a geometry that resembles the tip of a bullet. Tips of this shape are optimal for whole-cell recordings because they minimize series resistance. This thermal advantage is not limited to 8161 since all of the high-lead glasses previously discussed have low melting points and their broken tips can be firepolished into something with low series resistance. Were some of these other glasses like EG-6 or 0120 to seal easily to other cells, they might also be excellent candidates for whole-cell recordings. 1723 (aluminosilicate) is an excellent glass for both whole-cell and patch recordings since both blunt and sharp tips can be drawn from it. Its only disadvantage is that it softens at a temperature above 900°F and so is quite hard on pullers. 7070, one of the more attractive glasses from the standpoint of electrical properties, has not proven useful to date since its basic structure changes when it is pulled. It may be possible to get around this problem by using one of the new computerized

pullers which control both heating and cooling cycles and allow many pull cycles for each pipette pulled.

From the standpoint of noise, only two glasses studied to date are optimal. 8161 and 1723 are capable of producing noise with a gigaohm seal to our cells as low as 0.3 pA rms at 10 kHz with our electronics and electrode holders. With these glasses, it is routinely possible to obtain total noise of 0.35 pA rms or less with a membrane seal. We have also had some low-noise seals with 7040. 7052 and 7056 do somewhat worse. Total noise with a membrane seal is usually about 0.38–0.4 pA rms at 10 kHz. Soda lime glasses give total noise in the range of 0.6 to 0.7 pA rms at 10 kHz.

When all of these factors are considered, it is clear that 8161 is the best glass for all types of patch clamping if one were permitted only one glass. 1723, except for its high softening temperature, is about as good. For recording from only small patches of membrane, 7040 and 7052 are also very useful. In our hands, 7052 still produces the highest seal rate and is useful from that standpoint even if its noise is a little higher than the other glasses discussed. Of course, any glass may contain leachable components which might enter the pipette solution and either activate or block channels. It is therefore useful to measure the currents with pipettes made from several glass types to investigate this possibility.

3. GENERAL APPROACH TO CHANNEL IDENTIFICATION

We chose to study small patches of epithelial membrane, rather than whole cells, for several reasons. The lens and corneal epithelia considered here are not simple membranes. They are functional syncytia, each cell coupled to its neighbors through gap junctions or some similar structure. Attempts to study properties of single cells while they remain electrically part of a syncytium are futile. If one tries to voltage clamp a "single" ocular epithelial cell, using the whole-cell mode of the patch clamp, the currents measured cannot be uniquely interpreted simply because they may not have been driven by the membrane of the cell in question. They may well have flowed from adjacent cells through the junctions. The way to study a cell in isolation is to either block flux through all gap junctions or physically dissociate the cells, disrupting the gap junctions and the tight junctions that make the syncytia.

Dissociated cells are likely to have properties distinctly different from intact cells. For example, many epithelial cells have different channel populations in their apical and basal membranes, for reasons closely connected to their natural function. In any case, one cannot expect such a specialized distribution of channels to survive long in isolated cells. The channels may migrate around the cell or even leave the plasma membrane, creating in that way a cell quite different from those in the natural syncytium. In addition, the dissociation procedure almost invariably involves the use of enzymes which may change the properties of channels.

For these reasons, we chose to begin our studies by measuring currents from on-cell patches and to leave the measurements of whole-cell currents to

a later date when we have an extensive catalog of the individual channels which might contribute to the total membrane conductance.

Measurements of single channel currents have their problems too. The essential problem in single channel measurements is the extrapolation to the properties of the cell as a whole. It is, for example, usual (1) to assume that the frequency of occurrence of a channel in membrane patches is related to the channel's density on the cell membrane; (2) to assume that the general properties of the channel—its conductance, selectivity, and gating—are not affected by the distortion of the membrane required by the process of gigaohm seal formation; (3) to assume that the kinetics of channel opening and closing, including the voltage dependence of the kinetics, are the same in the patch as in the other membranes of the cell; (4) to assume that the channel currents recorded are stationary in the sense that estimates made from them do not vary substantially during the course of an experiment more than would be expected from the inherently stochastic nature of single channels themselves.

The test of these assumptions requires both macroscopic and patch measurements which is a tiresome task at best. It may be an impossible task (with existing technology) in epithelia or other tissues where sensitive measurements of macroscopic properties of single cells are not available. For example, many tissues consist of small cells of a variety of types not easily separated by dissection or identified in the microscope. Such cells in general could not be studied with microelectrode techniques and so little is known of their properties. What is known is frequently the result of flux measurements on large populations of heterogeneous cells, subject to substantial uncertainties because of extracellular diffusion and the ambiguities inherent in "compartmental" analysis. Thus, present investigators are often faced with the necessity of identifying channels in cells whose channel population is only vaguely known and whose macroscopic electrical properties are largely unknown.

In this situation, progress is determined by one's efficiency as much as one's direction. Techniques need to be as productive as possible, so quick overviews are possible and interesting directions can be rapidly identified and followed. We have spent considerable effort working out procedures to allow rapid identification of the channel types found in most ocular epithelia.

In a general sense, on-cell patches are attractive because their channels continue to interact with cytoplasmic elements involved in their maintenance and control. In addition, several of our channel types quickly disappear from excised patches whether inside-out or outside-out. While work is being done on this phenomenon (Chad and Eckert, 1985), it is not understood and systematic procedures to maintain or reestablish channels are not known. Finally, alternative configurations of the patch clamp involve technical difficulties: excision often results in patches of membrane attached to sealed vesicles, whether we use the inside-out or outside-out variant of the technique. In some cells, these are difficult to disrupt; indeed, the various

tricks which break the vesicle often destroy the gigaseal between pipette and membrane that is required for single channel recording. Given our goal of an efficient technique applicable to unstudied tissues, we clearly need an efficient on-cell protocol for channel identification.

The choice of the particular on-cell protocol is determined in large measure by the need to solve problems inherent to the on-cell configuration. First, the internal contents of the cell are not known. Second, the resting potential of the particular cell being studied is not known during the experiment. The patch clamp amplifier controls the series combination of the potential across the channel and the resting potential of the preparation. While the resting potential can often be measured at the end of an experiment, it is useful to have procedures that allow the experiment to proceed without waiting for estimates of its value.

One procedure minimizes both difficulties and so is used routinely in our labs. Cells are soaked for about an hour in a Na^+-free solution of potassium methane sulfonate ($KMeSO_4$) with the same product K^+ and Cl^- concentrations as normal Ringer. This solution depolarizes cells to near 0 mV resting potential and should both minimize the gradient of K^+ concentration across the membrane and wash out most of the intracellular Na^+, if net fluxes are dominated by passive processes (Boyle and Conway, 1941). In that case, both the cell resting voltage and the intracellular K^+ concentration became known; both of the problems of the on-cell method are removed at once. This bathing solution is also useful when the K^+ concentration in the bath is to be altered. Such solutions of normal $[K^+][Cl^-]$ product minimize passive changes in cell volume when $[K^+]$ is altered.

Because our work relies on this method, it is quite important to check its validity experimentally whatever the strength of one's confidence in the underlying theory. We have, therefore, used two methods to check that the resting potential is within a few millivolts of zero following the incubation in elevated-K^+ solutions.

The resting potential is measured using the "tracking circuit" of the patch clamp (Sigworth, 1983). This tracking circuit is essentially a current clamp, applying the potential to the top of the pipette that is needed to maintain zero pipette current. If the pipette contains a patch of membrane shunted by a gigaseal, the amplifier supplies the potential necessary to keep the current across both at zero. We estimate the resting potential by disrupting the patch of membrane (usually by applying damaging voltage pulses, but sometimes with pressure) and measuring the zero-current potential, assuming that the pipette contents are identical to the cell interior (or otherwise have no liquid junction potential) and the other DC offsets in current and voltage have not changed during the course of the experiment.

If the pipette contents are not the same as those of the cell interior, two effects must occur. First, on a rapid time scale a liquid junction potential will be set up, customarily estimated by the Planck–Henderson equation (Bockris and Reddy, 1970); second, on a much slower time scale diffusion from the pipette will alter the contents of the cell, with the cell interior

eventually having nearly the same composition as the pipette lumen, since the pipette is so much larger than the cell (Marty and Neher, 1983). However, the resting potential measured this way, in a cell still part of the epithelia, does not depend noticeably on the contents of the pipette (i.e., whether Na^+ or K^+ was the predominant cation) because the resting potential across a membrane of a cell in a syncytium is not just determined by the equilibrium potential across its channels when its neighbors have a different equilibrium potential. The electrical coupling of syncytial cells allows current to flow from cell to cell, driven by the different equilibrium potentials across the membranes in different cells. The resting potential we record from a cell is a measure of the average resting potential across all the cells electrically coupled to the cell being studied. If these cells are reasonably uniform in membrane properties, the resting potential measured would be that produced by the average internal concentration of permeant ions in all cells of the syncytium.

As a simple check on this method, the cell voltage was measured as a function of time after a lens epithelial cell patch was disrupted while the syncytium was bathed in normal Ringer. The bathing solution was then slowly replaced with the $KMeSO_4$ Ringer. The cell voltage, as expected, changed from the usual −60 mV to within a few millivolts of zero in each of these experiments.

Some parenthetical remarks are needed concerning other effects of the syncytial nature of our preparation. When an on-cell patch is destroyed on an isolated, single cell, the impedance between the pipette lumen and cell interior essentially disappears and current applied from the pipette flows uniformly across the cell membrane, limited by the impedance of the membrane. Because the area of cell membrane is small, the resistance of the entire cell membrane is often similar to that of the gigaseal (which remains a constant shunt path between pipette and bath). Thus, the current pulse being monitored by the investigator will change in a characteristic way when the patch breaks down: the DC conductance (in the absence of channel activity) will hardly change and the time course of current recorded will change dramatically, becoming the characteristic series RC response of a spatially uniform voltage-clamped membrane (Hodgkin *et al.*, 1952).

Something quite different occurs in a syncytium. When the on-cell patch is destroyed, the pipette becomes connected to the interior of *all* the cells of the syncytium, instead of just the interior of the single cell to which the pipette is sealed. The total membrane area in the syncytium is so much larger than that in a single cell that the DC conductance observed increases dramatically, and the experimenter may be misled and think the gigaseal has been disrupted. In fact, the dramatic increase in conductance is expected in a syncytium when the patch is broken down even if the gigaseal remains unchanged. The gigaseal is in parallel with the impedance of the syncytium, unnoticed (once the patch is broken down) because the impedance observed is now dominated by the large input conductance of the entire syncytial preparation.

A second way of measuring the resting voltage requires the fortuitous occurrence of a nonselective cation channel in the patch. There are several varieties of these channels in our epithelia, each of which has essentially the same permeability and conductance for Na^+ and K^+ (Fig. 2). At normal ionic strength, with either a Na^+- or K^+-filled pipette sealed to the membrane, no *chemical* driving force exists across the channels. Channel currents are driven entirely by the *electrical* gradient. No current then flows through the channel when the voltage applied to the pipette is the same as the voltage of the cell and the zero-current potential is thus a measure of the resting potential. This method is particularly attractive because it is noninvasive, and is quite useful for the epithelia studied here because they frequently contain the required nonselective cation channels.

The comparison of on-cell and excised patches containing K^+-selective channels is a useful check for equilibration of cell and bath K^+. Consider an experiment in which the pipette and the bath contain the same $KMeSO_4$ solution. In this situation a K^+ channel must have a reversal potential of precisely zero if the cell $[K^+]$ is the same as that in the bath and the cell voltage is 0 mV as we have just determined. The reversal potential should remain zero after the patch is excised. In fact, when care is taken to properly adjust spurious offset voltages, 0 mV reversal potentials are found in both circumstances. While this check is useful, it is not sufficient to determine

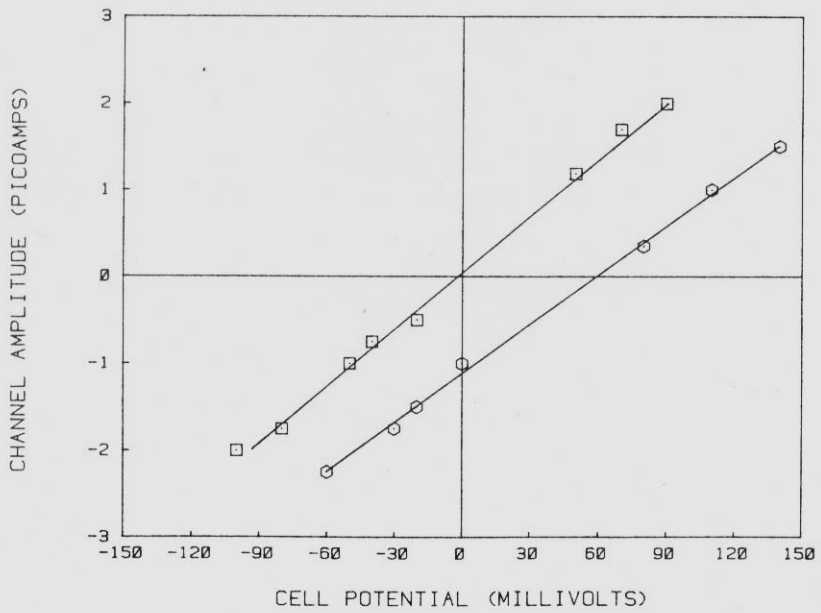

FIGURE 2. Current–voltage plots from a nonselective cation channel of frog lens epithelium. □, an inside-out patch with 120 mM KCl Ringer in the pipette and 120 mM NaCl Ringer in the bath. ○, An on-cell patch from cells bathed in normal Ringer with 120 mM KCl Ringer in the pipette. Resting potential is apparently about −64 mV.

the internal $[K^+]$ with great accuracy since the voltage is related to the logarithm of the concentration ratio. Under these conditions, a 3-mV error in the estimation of the reversal potential corresponds to about a 10% error in the estimate of the internal $[K^+]$.

Thus, we validate the two key assumptions of our procedure: the resting voltage in K^+ depolarized cells is near 0 mV and the $[K^+]$ in the cell is essentially equal to that in the bath.

A note of caution is in order: keeping preparations depolarized in $KMeSO_4$ solutions might be expected to have systematic deleterious effects on the preparation or the channels. However, the cells show every sign of remaining normally coupled one to another and show no visible sign of swelling or damage. We notice no substantial drift in their properties as they remain longer and longer in this solution. Some sign of a very slow effect (taking several hours) is visible when recording from cells containing Ca^{2+}-activated K^+ channels. The curve relating probability of opening to voltage shifts slowly to the left (i.e., the probability at a given voltage slowly increases) as one would expect if the $[Ca^{2+}]$ inside the cell slowly increased.

We are aware, of course, of the phenomena of desensitization and slow inactivation in acetylcholine- and other agonist-activated channels and the phenomenon of slow inactivation in voltage-activated channels. Invariable use of depolarizing solutions would hinder observation of such channels; but we trust that no one would slavishly use bathing solutions of $KMeSO_4$ to the complete exclusion of more physiological bathing solutions.

As we shall now see, the protocol of bathing in $KMeSO_4$ has many advantages. It makes it possible to quite rapidly identify the numerous channel types in these ocular epithelia (even when they have several similar properties), and to assess the conductance, "selectivity," and voltage dependence in on-call patches with reasonable precision and speed.

4. SELECTIVITY

The property of selectivity is fundamental to all biological membranes and has long been recognized as one of their distinguishing features: biological membranes distinguish between different ions and produce much of their interesting behavior in this way.

Fundamental ideas are often difficult to define. "Selectivity" is no exception; loosely, it means any phenomenon that distinguishes between ions, particularly ions of the same column on the periodic chart. Selectivity is often described quantitatively by the relative (slope) conductance for current carried by different ionic species x evaluated near the reversal potential, namely $\partial i_x/\partial V$ at $I = 0$. This relative slope conductance is usually called the permeability ratio because of its close connection to the ratio defined by the constant field equation (Hille, 1984). Unfortunately, selectivity is much too complex a phenomenon (Eisenman and Horn, 1983) to be characterized by a single number; no single number embedded in one theory can predict the