Perfusion of Patch Pipets

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1. Introduction

The patch-clamp technique allows the measurement of current through a wide variety of channels under reasonably realistic conditions, while controlling ("voltage clamping") one component of the driving force for current, the electrical potential. The other component of the driving force is set by the concentrations of permeant ions on both sides of the membrane, and those need to be controlled as well if the function and mechanism of channels are to be studied. In natural biological settings, current through channels is determined as much by chemical messengers, metabolites, modulators, and drugs as by driving force, and these must be applied to one side of the membrane or another if their action is be understood.

In the patch-clamp, solutions on one side of the membrane easily can be changed because that side is an easily accessible bath. However, solutions on the other side of the membrane are difficult to change. They are in the patch pipet and must be isolated by many gigaohms of resistance from (and coupled by very little capacitance to) surrounding solutions and earth if the voltage clamp is to function at all, let alone with reasonably low noise. Changing solution in a compartment isolated by gigaohms and picofarads is not easy, particularly if the solution-changing apparatus is not to interfere with the other necessities of experimentation. The apparatus must not add too much complexity, inconvenience,

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or cost to the setup. In particular, it must not interfere with the easy changing of pipets. We have developed an apparatus and procedure to change solutions in the pipet that adds little complexity, cost, or noise to the setup for recording single channel currents (Tang et al., 1990, 1992). Here, we modify the apparatus to allow control of the pipet solution during voltage clamp of the whole cell.

The control of pipet solutions permits many kinds of experiments. For example, in nerve cells many types of ionic currents must be identified and separated because multiple voltage-gated channels are activated by depolarization: The current measured in whole cell experiments is the sum of current from many types of channels and of the nonlinear capacity current, called gating current. Isolation of gating current or any one component of membrane current requires the removal of all other components. A pharmacological agent can be applied to selectively remove one type of channel or permeant ions can be removed and replaced by impermeants. For example, substitution of K^+ with Cs^+ eliminates many K^+ currents because many K^+ channels are impermeable to Cs^+. Often impermeants or blockers must be applied to the internal surface of the channel; e.g., in squid axon, tetraethylammonium ion blocks K^+ channels when applied to the internal, but not external side of the membrane (Armstrong and Binstock 1965). Our perfusion apparatus is useful in these cases.

Perfusion of the pipet also helps in studies of the selectivity of ion channels. The permeability ratio of the channel for two ions is usually estimated from measurements of the reversal potential (the potential at which zero current flows through the open channel), if the concentration gradient of the ions is known. Perfusion allows control of the concentration gradient.

2. Methods

2.1. Patch-Clamp of Neuroblastoma Cells

Neuroblastoma cells are grown in tissue culture and are differentiated prior to use in electrophysiological experi-

ments. Following differentiation, the cells have large voltage-gated K currents. Details of the culture conditions have been published previously (Quandt, 1994). Cells are grown in Dulbecco's modified Eagle's medium (DMEM) with 5% fetal bovine serum. To induce differentiation, cells are grown for at least 3 d in DMEM with reduced serum (2.5%) and 1.5% dimethylsulfoxide.

Techniques used to patch-clamp neuroblastoma cells in the whole cell configuration are similar to those published for other preparations (Hamill et al., 1981). Patch pipets have an opening at the tip of 2–3 μm. Typically the seal resistance is >10 GΩ.

Cells were typically bathed in a normal saline composed of (in m*M*): 125 NaCl, 5.5 KCl, 3.0 $CaCl_2$, 0.8 $MgCl_2$, 25 *N*-2-hydroxyethylpiperazine-n'-2-ethanesulfonic acid (HEPES), 25 dextrose. K internal solution consisted of (in m*M*): 150 KCl, 1 NaHEPES, 5 HEPES, 5 ethylene glycol-bis β-aminoethylether N,N,N',N-tetra-acetic acid (EGTA). The pH was adjusted to 7.25 with the addition of KOH. Cs internal solution was identical to the K^+ internal solution, except 150 m*M* CsCl replaced the KCl, and the pH was adjusted with CsOH. The internal solutions were filtered to minimize clogging of the perfusion system with particulate matter. Experiments were performed at room temperature.

2.2. Internal Perfusion Technology

Internal perfusion requires modifications in the standard patch-clamp apparatus. The electrode holder in the patch-clamp apparatus was modified to make separate ports for inflow and outflow in addition to the usual port for suction. The configuration of the perfusion setup is shown in Fig. 1A. Capsules are used to hold the perfusion solution. For the inflow, one end of a short length (8–10 cm) of Tygon tubing (PE10) is connected to the quartz perfusion capillary within the patch pipet. The tubing leaves the electrode holder through a gasket and pressure fitting and its other end is placed in any one of the several capsules at hand.

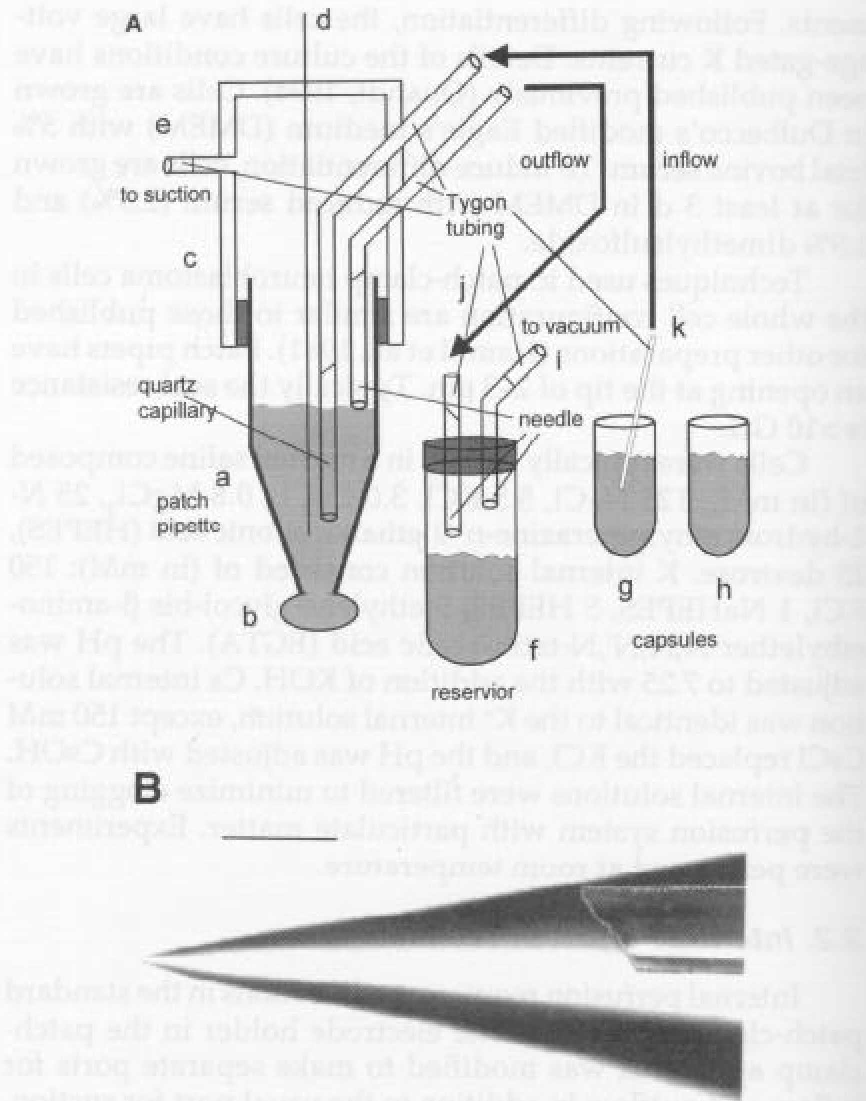

Fig. 1. Configuration of the pipet perfusion system for whole-cell patch-clamp. **(A)** Diagram of the pipet perfusion configuration. Components are: **a**, patch pipet; **b**, cell; **c**, electrode holder; **d**, silver wire; **e**, outflow for suction used to obtain a gigaohm seal; **f**, outflow reservoir **g**, **h**, capsules for the inflow solution; **i**, vacuum line to pressure generator; **j**, perfusate outflow line; **k**, perfusion inflow line. *See text* for explanation. **(B)** The patch pipet and the quartz perfusion capillary are shown retouched to outline the perfusion capillary. The marker is 50 µm.

Internal perfusion is much faster if the pipet has a steep final taper to its opening, and so the shape of the pipet must be carefully controlled in perfusion experiments, even though it is not very important in standard setups. We use a Flaming/Brown programmable micropipet puller (model P-80/PC, Sutter Instrument Co., San Rafael, CA) to pull the pipet in multiple steps, with progressively increasing heat. It is rather difficult to pull 3-µm steeply tapered pipets: The glass is liable to break unevenly on the final pull, leaving an opening that is rather large and jagged. Pipets are firepolished to reduce the final opening of the tip to about 3 µm. A typical patch pipet used in this study is shown in Fig. 1B.

A second piece of tubing is used for the outflow of the perfusion solution. This tubing (PE50) is connected to a 27–30-gage needle placed near the back of the pipet. The tubing exits the electrode holder and connects to a reservoir, which collects the perfusate. A vacuum is applied to a port on the reservoir to suck solution from the capsule through the perfusion capillary, then into the patch pipet, out of the pipet, and finally into the reservoir.

Current is collected by a Ag-$AgCl_2$ wire and led out of the holder through the standard connector. Suction is applied to a separate port on the electrode holder to produce gigaohm seals between the glass and cell membrane, or to rupture the membrane under the pipet sealed to its tip.

The quartz perfusion capillary (Polymicro Technologies, Phoenix, AZ) is made as described in Tang et al. (1990, 1992). Briefly, quartz tubing is softened by heat and then drawn out. The capillary is cleaned of debris and the drawn out tubing is cut (near its tip) to the desired opening diameter. The position of the capillary within the pipet is critical. If positioned as shown in Fig. 1B, close to the pipet tip, reasonably rapid exchange of solutions is possible.

Our perfusion procedure starts by flushing the inflow line—the Tygon tubing connected to the quartz perfusion capillary—with the standard (K) internal solution to remove air bubbles and solution left from the previous experiment. The patch pipet is then partially filled with control solution

and mounted in the electrode holder. The quartz perfusion capillary is next positioned close to the tip (of the patch pipet) under a stereomicroscope and the suction and outflow lines are connected. The inflow line is inserted into the capsule containing the standard K^+ internal solution. A valve is used to seal the suction line from atmospheric pressure and a vacuum (typically 60 mm Hg)* is applied to the reservoir on the outflow side to initiate the flow of solution.** The perfusion is then stopped by closing off the line connecting the vacuum generator to the reservoir and the suction line (**e** in Fig. 1) is opened. A gigaseal can then be made between the pipet and cell membrane, using the standard suction procedure of patch-clamp experiments. At this stage the membrane under the pipet must be broken to allow "whole cell" recording. We increased the suction (or applied a large voltage) to break down the membrane and gain diffusion access to the cell interior. Perfusion can be restarted at any time by closing the suction line **e** in the Figure and applying a vacuum to the outflow line (**i** in the Figure). To change the perfusion solution, the vacuum is turned off, the inflow line is carefully moved to the new capsule containing the selected solution, and vacuum is reapplied, monitored, and adjusted if it drifts, presumably because of leakage.

It is important to initiate perfusion with a control solution and to switch to a test solution only after access is gained to the cell interior, so there is a clear start-time of perfusion. Significant flow occurs while establishing a gigaseal, or breaking down the membrane. Flow can also occur in the absence of suction owing to capillary action. Because of this flow the state of the cell and its channels are easier to interpret if control solution is in the perfusion capillary during those preparative procedures.

*Generated by a regulated source, such as the Bio-Tek pneumatic transducer, model DPM-1B (Bio-Tek Instruments, Winooski, VT).

**If the meniscus in the patch pipet is seen to rise, fluid is flowing and perfusion has been established. If the meniscus does not rise, the chances are a perfusion line is blocked or there is a leak somewhere in the vacuum system.

3. Results

3.1. Time Course of Exchange of Internal Solution

The effectiveness of internal perfusion—in particular, the time course of exchange of solutions—can be evaluated by replacing internal K^+ with Cs^+, an ion that does not permeate many voltage-gated K^+ channels. Substitution should completely eliminate outward K current through these channels. Results from a typical experiment are shown in Fig. 2. Every 6 s, the potential was stepped to a more positive value, and the current resulting from this depolarization was recorded. The maximum amplitude (normalized and shown as a fraction of its maximum value) is shown in Fig. 2A. The filled circles show the change of the maximum current (resulting from a 70-mV depolarization) after the solution perfusing the pipet was changed from K^+ internal solution to Cs internal solution at time zero. Following a latency of about 150 s, the current rapidly declined, and reached a much lower steady state (note that the baseline toward the right of the figure is made of filled circles). Figure 2A (filled triangles) also shows the effect of reversal, of changing Cs back to K (note that the baseline toward the left of the figure is made of filled triangles). The maximum current was restored to its original value, following a latency similar to that for the onset, showing that the change of current is produced by the switch in ions, not the perfusion itself or some other artifact.

3.2. Efficiency of Exchange of Internal Solution

It is not clear that all the current in the pipet and cell can be changed by perfusion: A residual K current can often be measured following substitution of K^+ with Cs^+. Although the measured outward current is normally dominated by flux through K^+ channels, in the blocked situation other components, e.g., nonlinear leakage, may show themselves. Residual current through K^+ channels (following substitution of K^+ with Cs^+) would be expected to be blocked by external tetraethylammonium (TEA) (Quandt and Im, 1992). Figure 3 shows the effects of Cs^+ plus TEA (external). A current volt-

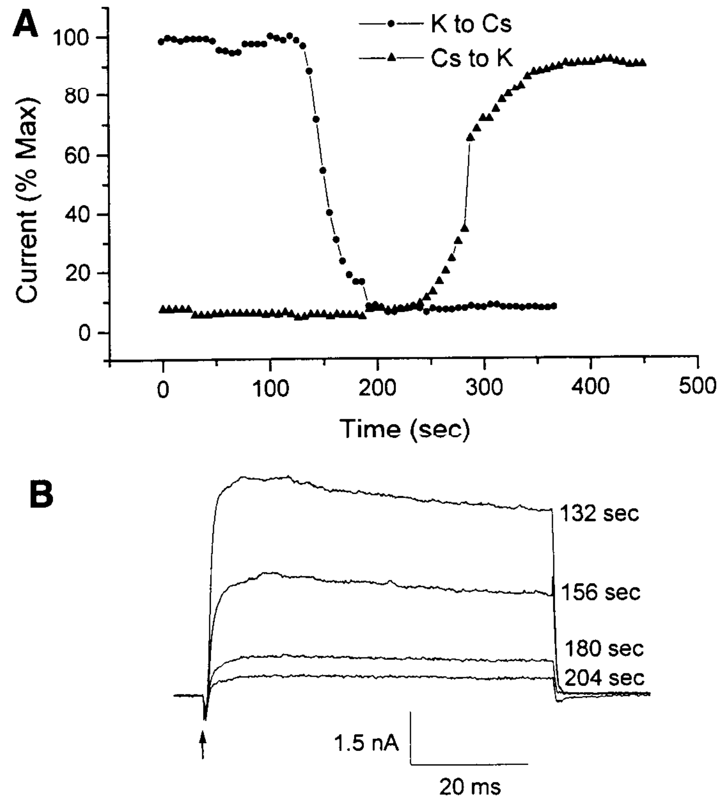

Fig. 2. Time course of exchange of the internal solution. **(A)** The amplitude of outward current in response to a depolarization to 70 mV is plotted, normalized to the maximum. The circles plot the current at 6-s intervals, following a change in the pipet perfusion solution from K internal to Cs internal solution at zero time. The triangles plot the restoration of current following a change back to K internal solution at zero time. The record is continuous: Zero time for the triangles begins immediately after the last time sample given by the circles. Note that the reduction in the outward current is reversible following the reintroduction of K. **(B)** The membrane current traces are shown superimposed and recorded during the period plotted by the circles in (A). The onset of the depolarization is marked by the arrow. The time (following the change from K to Cs internal solution) is given to the right of each trace.

age curve was first measured with K internal solution in the pipet. The residual outward current was next measured. Finally, 20 m*M* TEA was added to the solution perfusing the

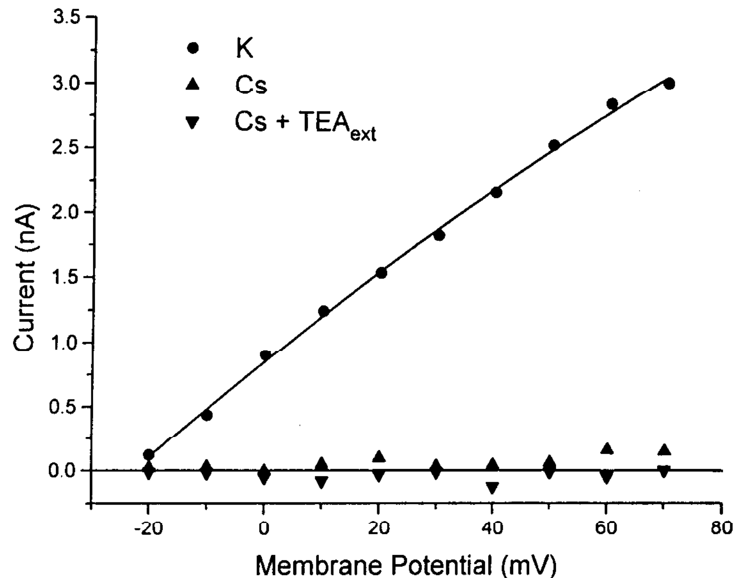

Fig. 3. Measurement of residual current through K channels following substitution of K with Cs. The current-voltage curve was measured with K in the pipet (after substitution of Cs for K by perfusion) and during Cs substitution with 20 m*M* TEA added to the external solution. TEA was found to block a small amount of outward current after perfusion with Cs solution, revealing residual current through the K channel.

outside of the cell and the current-voltage curve was measured again. No significant current flowed in the doubly blocked preparation and one can thus conclude that current through K channels was reduced by 95% following perfusion of the pipet and cell interior by Cs.

3.3. Selectivity of K Channels Measured by Reversal Potentials

The residual current (through K^+ channels after exchange of K^+ with Cs^+) might result from Cs flowing through the K^+ channels. The relative permeability of Cs^+ and K^+ in blocked channels an be gaged by the shift in the reversal potential for K channel "tail current" following a change in the K^+ concentration. A typical experiment is shown in Fig. 4. The

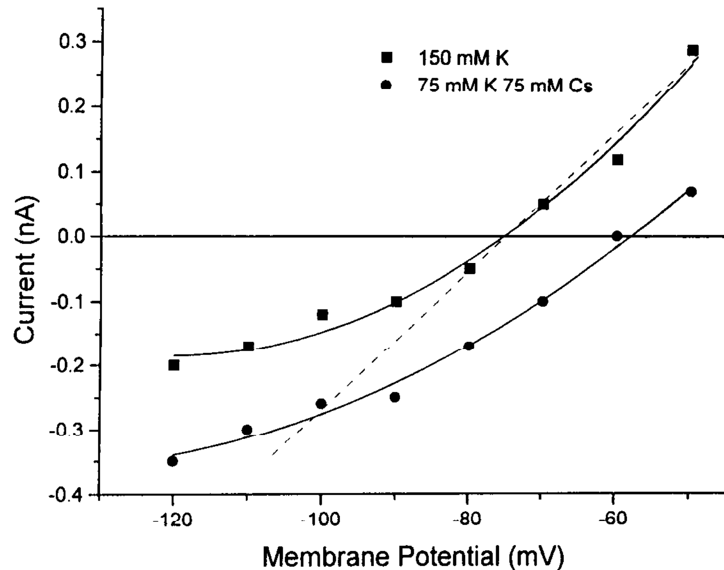

Fig. 4. Measurement of the reversal potential of tail current following Cs replacement. The membrane was depolarized to 60 mV to activate the K channels and repolarized to various potentials to measure the reversal potential. The maximum amplitude of this tail current, measured immediately after repolarization, is plotted. The tail currents were measured first during patch pipet perfusion with K internal solution, and second during perfusion with 50% K and 50% Cs internal solution. Note that the reversal potential shifted to a more depolarized potential in the solution containing Cs. The K inward current was less than that predicted in the absence of rectification (dashed line).

membrane was depolarized to activate K channels, and then repolarized to a variety of potentials (Vj). The amplitude and polarity of the tail current is a function of the potential Vj to which the membrane is repolarized. The initial amplitude of the tail current (immediately following the repolarization to Vj) is plotted in the figure as a function of Vj. This relationship was measured during perfusion of K^+ internal solution (filled squares) and following a change to 50% K^+ and 50% Cs internal solution (filled circles). The reversal potential (Vj) at which current is zero) was found to be –78 mV for K^+ internal solution and –64 mV for the mixed K^+/Cs^+ solution:

The reversal potential shifted 14 mV to a more depolarized potential following the change to the solution with reduced K^+. The amplitude of this shift is the same as that which is predicted by the Nernst equation for a channel permeable only to K^+, suggesting that the channel is impermeable to Cs^+. Although Cs^+ permeability was not measurable, it may be greater at a large depolarization (*see* Section 4.).

There may be a fraction of the internal K^+ that is not subject to exchange with the patch pipet, for example, owing to an unstirred layer. This unexchanged fraction could produce the residual current following perfusion with Cs^+. We do not have any evidence that this is the case. In the experiment described, if the K^+ concentration was greater than that in the pipet after perfusion with reduced K^+, the magnitude of the shift would have been smaller.

The current-voltage curve for the tail currents of Fig. 4 shows rectification. The outward current is larger than inward current for an equivalent driving force (the absolute value of the difference between the membrane and reversal potential). The dashed line in the figure gives the relationship for the control K^+ solution assuming no outward rectification, plotted using linear extrapolation from the potentials exhibiting outward current. The magnitude of the reduction in inward current increased as the membrane was hyperpolarized. Although not investigated in the present study, the rectification probably arises from a voltage-dependent block by external divalent cations, including Ca, as seen in other monovalent cation channels and preparations (Yamamoto et al., 1985).

3.4. Pharmacology of 4-Aminopyridine

Internal perfusion can be used to apply pharmacological agents—e.g., a K^+ channel blocker 4-aminopyridine (4-AP)—to the inside of a cell. Although 4-AP blocks some species of voltage-gated K^+ channels from the outside, because it is permeable to the membrane, it may act from the inside. Figure 5 shows a direct test of this idea. The current in control K internal solution is shown as well as the outward current (super-

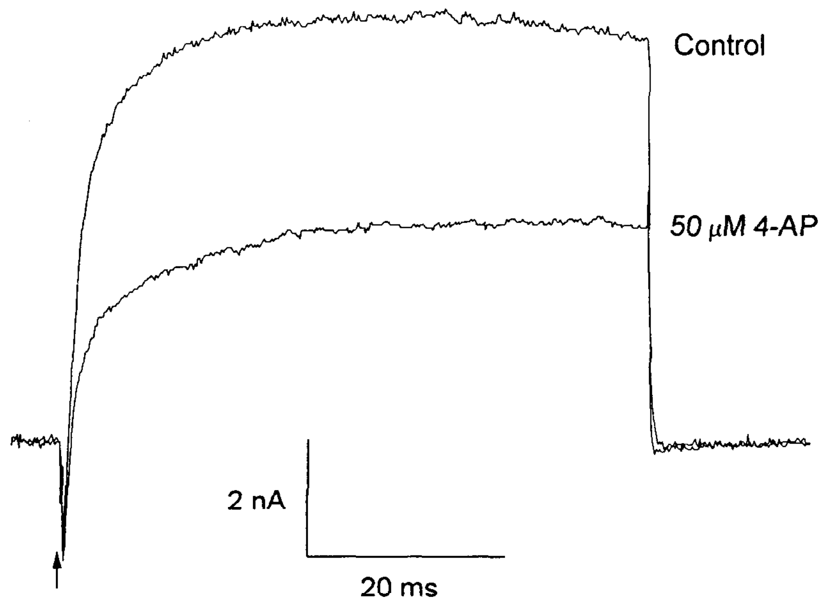

Fig. 5. Block of K current by internal 4-aminopyridine. Membrane current was measured in response to a depolarization to 70 mV. Superimposed records were obtained during perfusion of the patch pipet with K internal solution and following the addition of 50 μM 4-aminopyridine to the pipet perfusion solution. The onset of the depolarization is marked by the arrow.

imposed) in the steady state, following perfusion with solution containing 50 μM 4-AP. The 4-AP blocked the current by 50% and reduced the rate of rise of the current, both typical effects of the drug acting on this preparation (Hirsh and Quandt, 1993). 4-AP clearly can block the K^+ current in this preparation from the inside.

3.5. Parameters Controlling the Rate of Exchange of Solution

Figure 2A shows a delay, followed by a relatively rapid change in the current. We imagine that the delay is the time to exchange the solution within the lumen of the patch pipet. In this case, the delay should be dependent on the length of external tubing and the rate of perfusion. Indeed, we found

that varying the rate of perfusion (by changing perfusion pressure) varied the delay.

We investigated the effects of parameters that might alter the time course of the rapid rate of exchange. Figure 6A shows the effect of pipet geometry and cell size on the time course of exchange. K^+ internal solution was changed to Cs^+ internal solution and the peak outward K^+ current in response to a depolarization was measured at 6-s intervals. The dead time of the perfusion system, owing to entry of the new solution into the pipet, is not illustrated. Time zero is the time the current first deviates noticeably from zero. The most rapid exchange, illustrated by two experiments (unfilled squares and unfilled triangles) labeled as "optimal," was obtained under the conditions previously elaborated. A third experiment plotted in the figure (filled circles) compares the effect of perfusion of a large cell. In this case, although the onset of exchange is similar to the optimal conditions, the final rate of exchange is markedly slowed. The cell used in this experiment is shown in Fig. 6B. Note that the cell has a large cell body and long processes. Most of the current is recorded from the cell body and is reduced following exchange with the pipet. However, the long time of exchange is likely to be the time required for diffusion and exchange in the processes. Figure 6 also shows an experiment to examine the effect of the geometry of the pipet (filled triangles). The pipet used in this experiment (shown in Fig. 6C) has a rather gentle final taper, particularly compared to the standard pipet (Fig. 1B), and so the perfusion capillary cannot fit far down the patch pipet but must be placed farther from the pipet opening. Perfusion does not show a rapid phase under this condition, probably because the solution at the tip is only slowly displaced by the new solution in this situation.

4. Discussion

4.1. Applicability of the Technique

The pipet perfusion method used here to control the internal solution in whole-cell patch-clamp of neuroblastoma cells should be easily applicable to a wide variety of prepa-

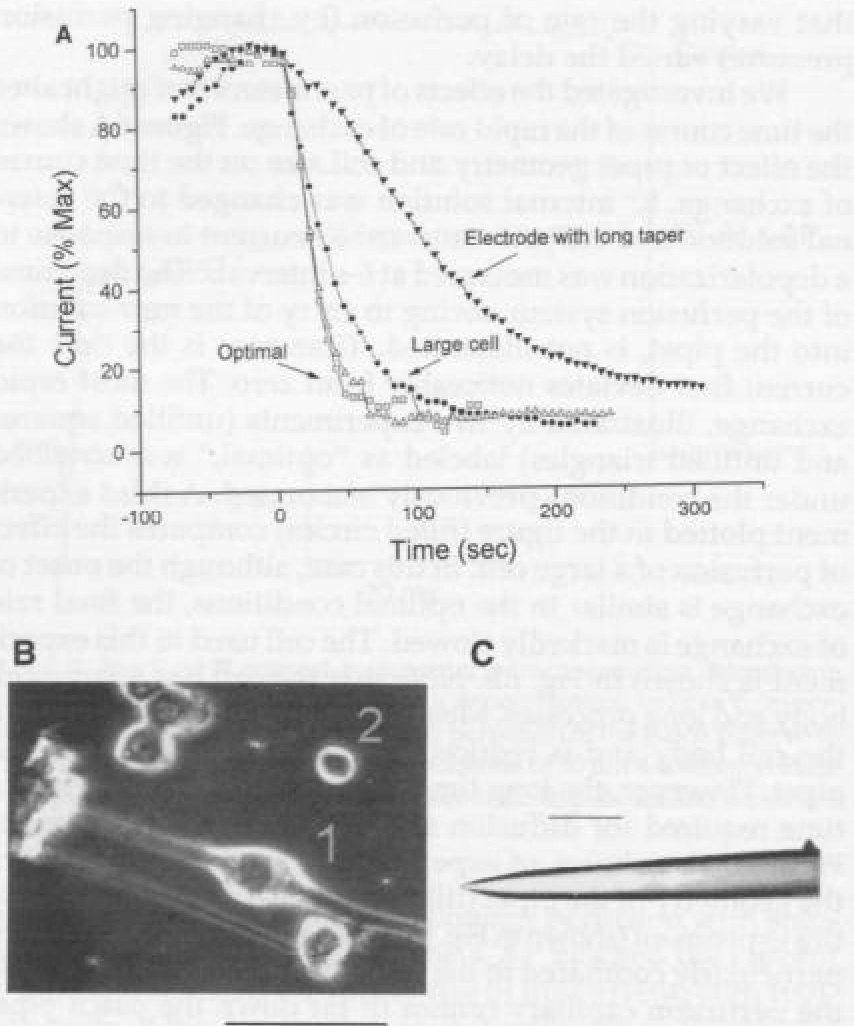

Fig. 6. Variables affecting the time course of exchange of internal solution. **(A)** The amplitude K membrane current in response to a depolarization to 70 mV, measured at 6-s intervals, during pipet perfusion is plotted. Experiments on four separate cells are plotted. Following Cs substitution for K in the patch pipet perfusion solution, the K current declined. To compare the time course of the rapid decay in current, the time at which the current started to decrease was set to zero time. The time course of exchange of K and Cs was slower for large cells, and in the experiment employing a pipet electrode with a long taper, com-

rations; only simple modifications of the existing procedure likely will be required. Although neuroblastoma cells are relatively large (20 μm in diameter), the pipet perfusion should be no less successful for smaller cells, even cells with an irregular or asymmetric geometry, such as cardiac myocytes. The only modification of the recording configuration is the use of an electrode holder to accommodate the inflow and outflow lines and the only special equipment recommended is a regulated vacuum generator. The procedure does add to the pain and duration of recording, because the perfusion capillary must be repositioned and tested each time the patch pipet is changed, but we find the trouble and time (a few minutes) involved to be bearable.

The control of internal solution should aid investigation of the biophysical properties of channels, the pharmacology of internal receptors, and control of membrane phenomena by internal transmitters. The time course of exchange of solution indicates that the effect being studied needs to last some 10–15 min if it is to be recorded faithfully.

4.2. Possible Problems

Occasionally we found that the perfusion would stop even though a constant perfusion pressure was maintained. The solution entering the reservoir can be monitored to determine whether this situation has developed.

In the present studies, the ionic composition of the solution was not varied dramatically. However, it should be noted that the liquid junction potential across the interface between the pipet solution and the Ag^{2+} wire can change dramatically when the Cl^- is replaced. The liquid junction

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pared to the optimal condition using small cells and an electrode with a steep taper. **(B)** The cell marked with a 1 was used in the experiment marked by the filled circles in (A). The cell marked with a 2 is typical of the smaller cells giving an optimal time course. The marker is 100 μm. **(C)** An electrode with a long taper, such as the one used in the experiment marked by filled triangles, is shown. The marker represents 50 μm.

potential can be measured prior to an experiment to compensate for this potential change under this condition or the setup can be modified to include a KCl/agar type bridge.

We observed a 5% residual outward current through K^+ channels following substitution of K^+ with Cs^+. Two possibilities may explain this observation. Following the substitution there may be a residual concentration of K^+ in the cell that cannot be exchanged even in the steady state. However, the shift of the reversal potential we obtained following a change in K^+ concentration is not consistent with this idea. Alternatively, Cs^+ may flow through the K^+ channels. In the latter case, K^+ channels must be permeable to Cs^+. The measurement of the reversal potential under this condition did not reveal a substantial Cs^+ permeability to the membrane. It should be noted, however, that internal Cs^+ may flow through the channel with a large depolarization, but not at a membrane potential closer to the reversal potential. Block of current through K^+ channels by Cs^+ has been observed to escape under some conditions, such as with a large driving force (e.g., Cecchi et al., 1987). This escape could explain the residual current.

We studied the sensitivity to block of the channels to internal 4-AP. Previous studies have found that single K^+ channels are blocked more completely when 50 μM 4-AP is applied to excised inside-out membranes than in the experiments given here. Two possibilities may cause this discrepancy. Multiple types of K^+ channels contribute to the whole-cell current. Some of these types of channels may have a lower sensitivity to 4-AP. A second possibility is that internal 4-AP may diffuse out of the cell so that the steady-state concentration is less than that added to the internal perfusate. A more complete study employing stop flow experiments would be required to distinguish between these possibilities.

4.3. Improvements

The configuration we have used for pipet perfusion can be improved. A positive pressure could be applied to the inflow solution. This negative pressure at the outflow would

then be reduced. The pressure across the cell would then be negligible, so that there would be little tendency for the cell to enter the pipet during perfusion. The disadvantage to this scheme is that the inflow side of the perfusion system then becomes a closed system, making it harder to change solutions. The method used to change perfusate is primitive and mishaps occasionally occur that ruin the experiment. We anticipate that future improvements would increase mechanical stability and reliability without adding too much complexity.

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