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Instruments and techniques

Perfusing pipettes

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Abstract. A simple modification of a patch-clamp set-up allows the fluid in a patch pipette to be changed. The time course of the solution exchange is estimated from the time course of change of the reversal potential of the current through an open Ca^{2+} activated K^+channel: solution exchange takes less than 1 min. Measurements of the power spectrum of noise show that the modified set-up introduces little excess noise below 1 kHz.

Key words: Patch clamp – Pipette perfusion – Solution exchange

Introduction

We describe a simple method for changing the fluid in a patch pipette. The method introduces little excess noise and requires only minor modification of equipment and procedures; thus it may be widely used.

Study of current flow through a macroscopic piece of membrane is aided if the independent variables of voltage and concentration are under experimental control. Patch voltage clamp (Sakmann and Neher 1983) allows easy control (i.e., clamp) of voltage. The laboratories of Miledi (Cull-Candy et al. 1980), Noma (Soejima and Noma 1984), Neher (Neher and Eckert 1988), and Gadsby (Gadsby et al. 1985; Bahinski et al. 1988) have perfused the lumen of patch pipettes, but their methods have not been adopted by many laboratories recording single channels. LaPointe and Szabo (1987) perfused pipettes without adding much excess noise, using a clever apparatus requiring modification of a standard set-up and a fairly complex pipette holder. We duplicated their apparatus and procedures; but we found their method restrictive for our preparation (Tang et al. 1989) because changing pipettes took us considerable effort and time (15–30 min), even after much well-tutored practice.

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Materials and methods

Modification of the pipette holder. The pipette perfusion system was built from a polycarbonate patch pipette holder (model EPC-PHP: Medical Systems Corporation, Greenvale, NY, USA: see Fig. 1 of Hamill et al. 1981) simply by drilling a hole (≃ 610 μm) at an angle of approximately 45° between the outlet to the suction line and the BNC pin. About 5 cm of polyethylene (PE) tubing [type PE-10: outside diameter (o.d.) 0.61 mm and inside diameter (i.d.) 0.28 mm: Clay Adams, Parsippany, NJ, USA] was threaded through the hole drilled in the holder (Fig. 1A) and the hole was sealed with grease (Leitz grease 465: Leitz, Rockleigh, NJ, USA) to prevent air leakage during seal formation and perfusion. Using grease (instead of an *o*-ring seal) simplified construction dramatically and allowed easy positioning of the perfusion pipette, as described below.

Quartz tubing. The key to the perfusion system is the remarkably flexible and durable coated quartz capillary used to enter and perfuse the patch pipette (LaPointe and Szabo 1987). With just a little care we have been able to use one perfusion capillary (o.d. ≃ 45 μm and i.d. ≃ 35 μm at the tip of the capillary) for several days. The capillary is made from quartz (i.e., synthetic fused silica) tubing (product no. TSP 100/245: Polymicro Technologies, Phoenix, Ariz. USA) that is coated with a polyimide of enormous volume resistivity (10^{16} Ω-cm) and surface resistance (10^{15} Ω). We use quartz tubing of o.d. 245 μm (i.d. 100 μm) that fits snugly into PE tubing.

Making the perfusion capillary. Weights (alligator clips) were attached to (vertically mounted) quartz tubing so that gravity could draw out the tubing once it softened (at 1590 °C) in the hot microflame of the MicroWelder (model A$^+$: Johnson Matthey, Wayne, Pa., USA). It is also possible to make a hot enough flame with the ordinary methane gas available in many laboratories. Debris – chiefly polymer burnt when the coated quartz capillary was heated – was removed from the capillary with a microknife under a stereomicroscope. The cut tubing, now called a perfusion capillary, was cleaned by sonication. The drawn-out tubing was cut so that its inner diameter was ≃ 35 μm as checked with a microscope. The diameter of the perfusion capillary is fairly important: 35 μm i.d. is large enough to allow good perfusion of the patch pipette but small enough (producing enough resistance to flow) so that the perfusion capillary does not permit too much flow when suction is applied to the patch pipette to form a gigaseal. (If the capillary and flow were too large, suction would be reduced and gigaseals would be hard to make.)

Positioning the capillary. Once the perfusion capillary had been drawn out and cleaned, it was inserted into the end of the PE tubing already threaded through the hole drilled in the pipette holder. The PE tubing

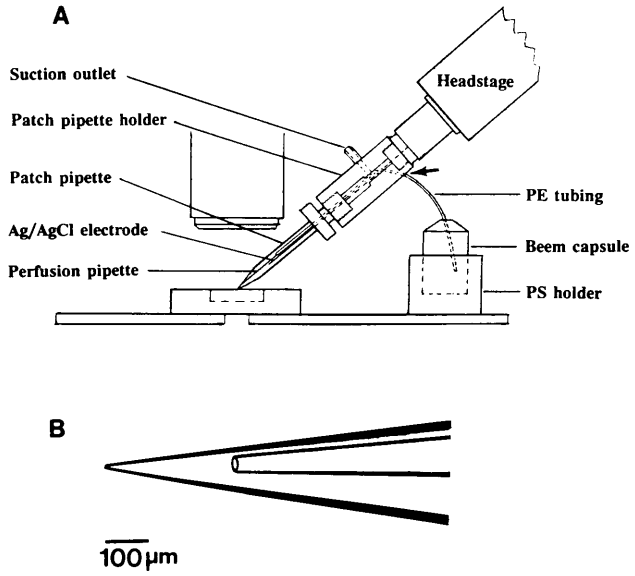

Fig. 1. A The pipette perfusion system. The patch pipette was made from Kimax-51 glass capillary tubing and was connected to the modified patch pipette holder (see text). The perfusion pipette was constructed as described in the text and inserted into the patch pipette so that its tip filled approximately half the cross-section of the patch pipette; here it was ≃ 300 µm from the tip of the pipette (see Fig. 1B). The Ag/AgCl wire electrode was 2–3 mm shorter (further from the tip of the patch pipette) than the perfusion capillary, so it did not interfere with perfusion of the pipette tip. The other end of the perfusion capillary was connected to polyethylene (PE) tubing that had been threaded through a hole drilled in the pipette holder (*arrow*). Grease was used to fill gaps between the PE tubing and the pipette holder. The other end of the PE tubing was placed in one of two solution reservoirs, Beem capsules in a holder made of expanded polystyrene (PS). The suction outlet of the pipette holder was connected with silicone tubing to a pneumatic transducer that monitored and controlled pressure/suction. **B** A tracing from a video screen image of the tip of a patch-clamp pipette containing an internal perfusion capillary. The tip of the perfusion capillary was ≃ 300 µm from the tip of the patch pipette

(now connected to the quartz capillary) was filled with the same solution that was in the patch pipette, using a syringe. The far end of the tubing was immersed in a reservoir of that solution, open to the air, held in a small capsule (Beem capsule: size 00, Ted Pella, Tustin, Calif., USA) mounted in one of the two holes in a block of 1-cm-thick expanded polystyrene to separate the solution from grounded metal (to minimize noise, see below).

A silver/silver chloride wire electrode, 2–3 mm shorter than the perfusion capillary and previously chlorided by dipping it into Clorox solution (sodium hypochlorite), was used. Bullet-shaped patch pipettes ≃ 5 cm long were used to allow solution to be sucked into the pipette, displacing the previous solution back up the pipette, without drawing that solution into the pipette holder and out of the suction line, which would significantly increase noise. The patch pipettes were coated with Sylgard 184 (Dow Corning, Midland, Mich., USA) and heat-polished immediately before use to a final inside tip diameter of ≃ 1 µm and had a resistance in the range of 2–3 MΩ. The initial volume that filled about 5 mm of the tip of the patch pipette was ≃ 2.5 µl. A volume of ≃ 55 µl can be sucked into a pipette without fluid reaching the suction line.

After the patch pipette had been mounted in the pipette holder and the holders's *o*-ring tightened, the perfusion capillary was positioned (Fig. 1A) simply by sliding the PE tubing back and forth in its grease seal while watching the pipette with a microscope (in the patch-clamp set-up). The perfusion capillary was placed (Fig. 1B) so that its tip filled half the cross-section of the lumen of the patch pipette (as suggested by LaPointe and Szabo 1987), thus maximizing flow up the patch pipette (around the perfusion capillary) as well as flow out of the perfusion capillary itself. With the patch pipettes we used, the perfusion capillary was ≃ 300 µm from the tip of the patch pipette; more blunt patch pipettes would allow closer positioning and thus more rapid exchange of solution. Pipettes could be mounted and perfusion capillaries positioned in about 30 s.

Cell culture, solutions, and procedures. Gigaseals were formed on neuroblastoma cells (5–14 days old; grown on cover slips; cell line N1E-115; Quandt and Narahashi 1984) at room temperature (nominally 22 °C) in a bathing solution containing (in mM): 150 NaCl, 5.5 KCl, 1.8 $CaCl_2$, 0.8 $MgCl_2$, and 10 HEPES (*N*-2-hydroxyethyl piperazine-*N*'-2-ethane sulfonic acid), which we call normal Ringer. Three other solutions were used: high K^+extracellular (i.e., inside pipette perfusion) solution containing 150 KCl, 2 $CaCl_2$, 10 HEPES; low K^+ extracellular (i.e., inside pipette) solution containing 50 KCl, 100 NaCl, 2 $CaCl_2$, with 10 HEPES; high K^+ intracellular (i.e., outside pipette) solution containing 150 KCl, 0 Na [along with 10 HEPES; 5 EGTA (ethylene glycol-bis-(β-aminoethyl ether) *N,N,N',N'*-tetraacetic acid], and 4.76 $CaCl_2$, giving a calculated value of 3 µM for free Ca^{2+}). All solutions were adjusted to pH 7.2 and were passed through a filter with a nominal pore diameter of 0.2 µm just before use.

Perfusion solutions usually contained 1 mM phenol red to allow visual localization of the perfusing solution. The phenol red did not produce obvious changes in channel activity. Suction of 10–20 mmHg (flow rate of 1.6–2.2 µl/s) was applied (by a pneumatic transducer, model DPM-1: from Bio-Tek Instruments, Burlington, Vt., USA) until the phenol red solution could be seen in the PE tubing approaching the perfusion capillary. Suction was then reduced to 2–4 mmHg (flow rate of 0.7–1.0 µl/s) and maintained throughout perfusion. We lost many gigaseals and damaged many channels before we started controlling suction with the pneumatic transducer.

Results

The quality of pipette perfusion was determined by a bioassay. The reversal potential (B. Hille 1984, pp. 234–237) of a Ca^{2+} activated K^+ channel of neuroblastoma (Quandt 1988) was used to estimate the K^+ concentration at the mouth of the pipette. This channel was chosen because it is highly selective, has a high probability of being open, and occurs reasonably often in a convenient preparation available to us. This bioassay seems a good method to estimate the relevant parameters of pipette perfusion. It seems preferable to physical measurements of pipette properties (e.g., resistance) that estimate the bulk property of the solution in the pipette. After all, the important variable in applications is the composition of the solution near the channel, at the mouth of the pipette, not the average composition of the fluid in the pipette.

Gigaseals were formed on neuroblastoma cells in normal Ringer. Just before measurement an infusion/withdrawal pump (model 944: Harvard Apparatus, Natick, Mass., USA) was used to introduce high K^+ intracellular (i.e., outside pipette) solution, nearly 150 mM KCl, into the bath. The patch pipette was then pulled off the cell, creating an excised, inside-out recording configuration with nearly 150 mM KCl on the intracellular side (the side bathing the outside of the pipette) and nearly 50 mM KCl, 100 mM NaCl on the other side of the channel. (The pipette and perfusion capillary initially contained low K^+ extracellular solution, as did one of the two Beem capsules.) The

current/voltage (*I-V*) relationship of the excised open channel was then measured with a ramp voltage of 500 ms duration, extending ± 60 mV from the holding potential of zero.

Perfusion of the high K^+ extracellular (i.e., inside pipette perfusion) solution was then started so that eventually the channel should have the same concentration of K^+ and Na^+ on both sides. Perfusion was started by moving the PE tubing to the Beem capsule containing the high K^+ extracellular solution and suction was applied to the suction line on the pipette holder. We decreased the amplifier gain when moving the PE tubing between the two Beem capsules to avoid excessive voltage transients. It took 2–4 s of "dead time" for a bolus of the high K^+ extracellular solution to move through the PE tubing and the perfusion capillary. Figure 2 shows the current recorded after the bolus reached the pipette and the current began to decline, ≃ 20 s after suction was started.

The record in Fig. 2 is the actual measurement of total current through the pipette read from the analog tape at low sweep speed to illustrate the entire time course of the solution change. The current declines to zero as the K^+ concentration near the channel, at the mouth of the pipette, reaches its final value of 150 mM. The exchange time between the first decline in current and its final value was 53 s in this experiment. Six similar experiments were performed and with a mean exchange time of 57 ± 9 s (range 22–84 s).

The value of the reversal potential was measured from each $I - V$ relationship and used to compute the concentration K_0 near the channel in the mouth of the pipette using the Goldman-Hodgkin-Katz equation (B. Hille 1984, pp. 234–237). K_0 was then written as a fraction of the change in potassium concentration, as the percentage exchange of solution (Fig. 3). The P_K was measured but the permeability ratio $\alpha = P_{Na}/P_K$ was assumed to maintain its original value (see below).

Figure 3 demonstrates that perfusion changed all the solution in an average of ≃ 60 s in four different experiments. The nearly linear time course of concentration change suggests that a convective-not diffusive-process determines the change of concentration with time. After the solution change was complete (see Figs. 2, 3) the reversal potential was steady for at least 30 min, showing that back-diffusion from the solution originally in the pipette tip does not have significant effects under these conditions.

Some attempt was made to check the validity of the Goldman-Hodgkin-Katz equation for this channel and conditions by fitting the open channel $I - V$ relationship with its theoretical counterpart (B. Hille 1984, p. 234, Eqs. 10–12), using an equation for the slope conductance γ to determine the K^+ permeability P_K:

$$\gamma = \frac{\delta I}{\delta E}_{E=0} = \frac{F}{RT}\,[FP_KA]\left[\frac{K_o + K_i}{2} + \alpha\,\frac{Na_o + Na_i}{2}\right] \qquad (1)$$

where P_K is the permeability constant for potassium, RT/F = 25 mV, and F is the Faraday constant. K_i and Na_i, and K_0 and Na_0 are (respectively) the concentration of K^+ and Na^+ in the cytoplasmic side (the outside of the pipette) and the extracellular side (the inside of the pipette) in mol/cm^3.

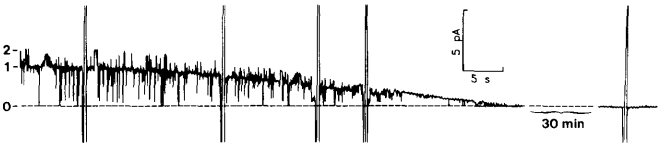

Fig. 2. Continuous recording of the total current through the patch pipette. When suction and thus perfusion were started, the patch pipette was filled with low K extracellular solution, the perfusion capillary was filled with high K extracellular solution and the bath contained high K intracellular solution with 3 μM of free Ca^{2+}. The holding potential was 0 mV. An upward deflection is a positive (outward) current, from cytoplasm to extracellular space, i.e., from outside the pipette to inside the pipette. The record shows the total current in an excised, inside-out patch with two active channels, a Ca^{2+} activated K^+ channel and a delayed rectifier, with upwards deflections indicating current flowing outward across the membrane. The slow sweep speed was needed to show the entire time course of the current change; the vertical deflections were analyzed by examining the tape recording at higher sweep speeds. In particular, *level 0* represents the baseline with all channels closed; *level 1* represents the current through the open Ca^{2+} activated K^+ channel; *level 2* represents that current plus the current through an open delayed rectifier channel. The downwards deflections from level 1 are closings of the channels, not properly resolved at this sweep speed. The upwards deflections from level 1 are openings of the delayed rectifier channel. The five prominent *vertical lines* are the current response to applied ramps of voltage. The records were filtered at 1 kHz

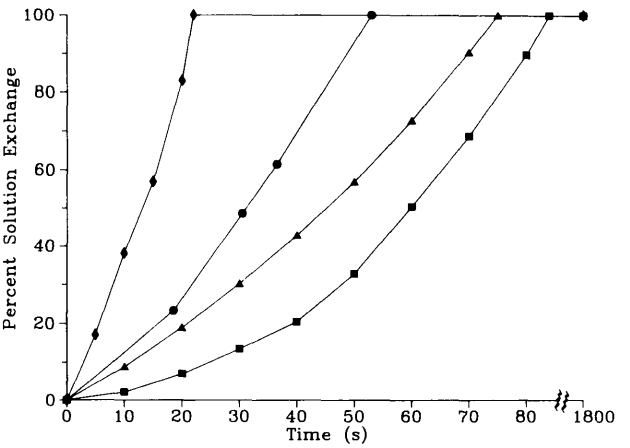

Fig. 3. The time course of solution exchange determined from the reversal potential and current/voltage relationships measured with a ramp voltage (see text). Data are from four separate experiments (different symbols). The Goldman-Hodgkin-Katz equation is used to estimate the concentration of K^+ on the extracellular side of the Ca^{2+} activated K^+ channel, that is, in the lumen of the pipette just next to the membrane. That concentration is expressed as the fraction of solution exchange. α is assumed constant with time. The estimated error in measurement of reversal potential and the standard error of our measurements are both about 1 mV, giving a maximum error of ± 2.5 mM in the 50 mM K concentration (i.e. ± 5% in the ordinate of Fig. 3). The initial reversal potential was −21.3 mV giving α = 0.15. of Fig. 3). The initial reversal potential was −21.3 mV giving α = 0.15. = 2.9×10^{-13} [cm/s]·cm^2. γ = 165 pS and the reversal potential was indistinguishable from zero in the final (nearly) symmetrical 150 mM K solutions. Note that the numerical values of the "permeabilities" P_K and P_{Na} should differ from those in macroscopic treatments because the latter depend on the number of channels per square centimeter of membrane. Initial and final conditions are defined in the text

Equation (1) can be derived by repeated application of L'Hospitals's rule (E. Hille 1964, pp. 381–386) to Eqs. 10–13 of B. Hille (1984, p. 235). It should be noted, however, that the single channel permeabilities P_K and P_{Na} defined here do not depend on either the cross-sectional area A of an open pore or on the number n of open channels in 1 cm^2 of membrane while the usual macroscopic values (B. Hille 1984) depend on both A and n. The slope conductance surprisingly stayed within 3% of its initial value during a perfusion. Theoretical curves computed from the Goldman-Hodgkin-Katz equation fit the observed $I - V$ relationships.

The noise introduced by the perfusion apparatus can be as important as the time needed to change the solution. Measurements were made of the power spectral density of background noise (analogous to closed channel noise) with and without the perfusion apparatus. Figure 4 (trace a) shows the instrumentation noise, the noise of the amplifier with the pipette holder and pipette in air (0.07 pA rms with bandwidth of 100–3000 Hz); Fig. 4 (trace b) shows the noise with a pipette sealed to Sylgard (5-GΩ seal: noise 0.25 pA) when the pipette holder does not have the PE tubing inserted. Figure 4 (trace c) shows the noise with a pipette sealed to Sylgard (also a 5-GΩ seal: noise 0.27 pA) when the pipette holder does have the PE tubing inserted and is connected to the solution in the Beem capsule. Care was taken to have the same level of solutions, the same shape and resistance pipette, and the same seal resistance in each case. The noise below 1 kHz is hardly affected by the perfusion apparatus, provided the solution reservoirs are separated from a conducting surface by at least 1 cm thickness of a good insulator, such as expanded polystyrene. The noise above 1 kHz is somewhat increased: the difference between the instrumentation noise and the total noise rises at 6 db/oct above 1 kHz, suggesting that the increment is produced by added capacitance to ground. This capacitance would also be expected to modify the transient response in "whole cell" recording. The noise of the perfusion pipette system sealed to a typical membrane (5-GΩ seal) was 0.38 ± 0.01 pA (range 0.33–0.40 pA, $n = 6$) at 3 kHz bandwidth.

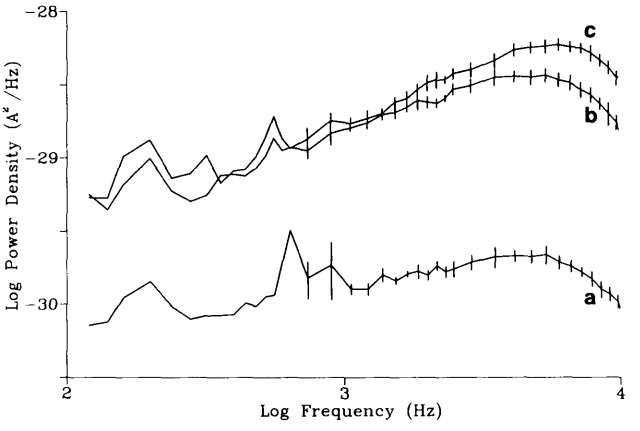

Fig. 4. Power spectra from (*a*) the headstage, (*b*) the patch pipette without perfusion apparatus, (*c*) the patch pipette with perfusion apparatus. All curves were obtained by averaging 96 spectral estimates each containing 2048 data points (= 40 ms of data) padded with zeros to 4096. The *abscissa value 2* means the frequency is $10^2 = 100$. At high frequencies neighboring frequency points have been averaged and data are displayed as mean ± standard error of the mean (*vertical bars*). The droop at high frequencies is caused by a combination of the 10 kHz Bessel filter and roll-off of the patch-clamp amplifier

Discussion

We have changed the solution with this technique many times and have hardly ever destroyed a gigaseal or disturbed channel behavior. The apparatus does not introduce much excess noise and is easy to use. We hope it will allow workers to change solutions inside patch pipettes almost as routinely as they change solutions in the bath.

Acknowledgements. We thank Rick Levis and Atticus Hainsworth for help with the noise measurements and Gabor Szabo for taking much time and care to tutor us in his tricks of the perfusion trade.

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Errata

Perfusing Pipettes J.M. Tang, J. Wang, F.N. Quandt, and R.S. Eisenberg
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Page 349, equation 1 should appear as follows:

$$\gamma \equiv \left.\frac{\partial I}{\partial E}\right|_{E=0} = \frac{F}{RT}\,[FP_K A]\left[\frac{K_o + K_i}{2} + \alpha\frac{Na_o + Na_i}{2}\right]$$

Page 349, figure legend of Fig. 3 (line 13) should read as follows:

$[P_K{\cdot}A]$ = 3.9 x 10^{-13} [cm/s]·cm^2 and γ = 160 pS initially and $[P_K{\cdot}A]$