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Single channel currents from excised patches of muscle membrane

(acetylcholine channel/myotubes/mammalian muscle/ionic currents/perfusion)

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ABSTRACT **The currents through single acetylcholine-activated channels were measured on membrane fragments that had been torn from rat muscle myotubes with patch pipettes. The membrane fragments were sealed into the pipette by using the "gigohm-seal" technique of Neher, which also permitted voltage clamp of the membrane via the patch electrode. Membrane patches were excised by sudden withdrawal of the electrode from the cell. Substitution of fluoride for chloride ions in the bathing solution could prevent or reverse the tendency for the membrane at the electrode tip to seal over into a closed vesicle. The single membrane layer at the electrode tip could remain functional for up to 30 min. The apparent single channel conductance was minimally affected by excision. The current–voltage relationships for the single channel currents show that the inside (i.e., cytoplasmic surface) of the membrane fragment was exposed to the bathing solution. In symmetric Na solutions the current–voltage curve was nearly linear and reversed at approximately 0 mV. In other bathing solutions from 40 to 500 mM NaF, the observed zero current potential was close to that predicted by the Nernst equation. We present evidence that internal Na interacts with the channel, causing both saturation of outward current and block of inward current. At +100 mV the apparent dissociation constant for internal Na was 138 mM.**

It has recently become possible to measure the currents flowing through single acetylcholine (AcCho)-activated channels by using a patch electrode (1). With the original Neher and Sakmann method, the resolution, and hence the range of phenomena that could be studied, was limited by the resistance of the seal formed between the cell membrane and the glass recording electrode. A recent modification of this technique, however, has promoted adherence between the membrane and glass, increasing seal resistances into the range of 1–10 GΩ (10^9 Ω; ref. 2). This "gigohm-seal" technique reduces the background noise to a level sufficient to resolve single sodium channels (3), a feat impossible with any previous method.

A limitation of the patch recording method, as presently used, is the inability to control the composition of the solution bathing the cytoplasmic face of the membrane, as in internal perfusion studies (4–7). We wished to develop a method that combined the advantages of the patch recording technique with those of internal perfusion. We took as a starting point Neher's observation that, after gigohm seals had been obtained, the adherence between glass and membrane was maintained after withdrawal of the electrode from the cell (2). The membrane fragment attached to the glass surface normally, as Neher described (2), formed a closed vesicle in the electrode tip. We have found, however, that the closing of this vesicle could be prevented or reversed by substituting fluoride for chloride ions in the bathing solution. The excised patch of membrane behaved like a single bilayer spanning the electrode tip. The patch remained functional for up to 30 min, and survived well, even with salt concentrations up to 0.5 M in contact with the cytoplasmic membrane face. We present observations, made by using this method, that reveal a saturation effect in the ionic conduction through AcCho-activated channels in muscle membrane.

MATERIALS AND METHODS

Tissue-cultured myotubes were prepared from myoblasts of neonatal rat thighs (7). The myoblasts were plated onto glass coverslips in sterile petri dishes. Myotubes were used for experiments from 6 to 14 days after plating.

Experiments were done in a 2-ml chamber whose solution could be completely exchanged in less than 3 min at flow rates of about 15 ml/min. The preparation was visualized with a compound microscope using Nomarski optics. Coverslips bearing myotubes were transferred to the chamber just prior to the experiment. The initial bathing solution was 100 Na Ringer's with tetrodotoxin (see Table 1). The subsequent bathing solutions primarily consisted of NaF (Table 1). The activity coefficients of these salts (8) were used to calculate the Nernst potentials for Na ions (see *Results*). All experiments were done at the room temperature of 23–25°C.

Patch electrodes were prepared by using previously described techniques (2, 9). The inner tip diameter of the pipettes was approximately 0.5 μm, as measured with a scanning electron microscope. The electrodes were coated with a thick layer of varnish to within 100 μm of the tips to reduce capacitive coupling to the bathing solution. The patch electrodes were filled in all experiments with 100 Na Ringer's solution containing 0.2–0.5 μM AcCho. All solutions were passed through a filter with pore size 0.2 μm.

Slight modifications were made to the standard patch electrode recording circuit (10). The feedback resistor of the current to voltage converter was 1 GΩ. Leak current compensation was used at the final amplification stage to prevent saturation of the recording amplifiers. Leak currents usually varied linearly with potential and were appropriate for the 1–10 GΩ seal resistances obtained. The bath was grounded via a Ringer's solution-containing agar bridge with an Ag/AgCl junction. The potential inside the pipette was made equal to the bath potential just before contact with the cell. The bath potential (V_{ref}) was measured with a reference electrode and added to the pipette voltage, in order to correct for changes in junction potential in the bath ground system (see Fig. 1).

Membrane–glass adherence was obtained by gentle suction through the patch electrode with intermittent intervals of neutral or slightly positive pressure. The transition from "normal" seals of 50–100 MΩ to gigohm-seals was almost always sudden. After establishment of the gigohm-seal the potential inside the pipette could be changed to the desired holding potential via the positive input of the current-to-voltage converter (see Fig. 1). The series resistance of the electrode (5–10 MΩ) was

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Abbreviation: AcCho, acetylcholine.

Table 1. Composition of solutions (mM)

Solution	Na^+	Cl^-	F^-	Ca^{2+}	Sucrose	Hepes*
100 Na Ringer's†	100	103.6	0	1.8	120	5
40 NaF	40	5	35	0	240	5
100 NaF	100	5	95	0	120	5
160 NaF	160	5	155	0	0	5
500 NaF	500	5	495	0	0	5

* Hepes (Sigma) was titrated to pH 7.35 with NaOH.
† Tetrodotoxin (Calbiochem) was added to the Ringer's solution on the day of the experiment at 0.5 μM.

negligible compared to the sealed membrane. The control recordings were made on large myotubes whose input resistances were also much less than the patch resistance.

Single channel currents were amplified, low-pass filtered at 500 Hz, and sampled at intervals of 100–400 μsec with a small computer. Individual events, which were greater than a preestablished deflection from the baseline, were detected and normalized to a specified starting time and baseline, then stored for later analysis. Mean amplitudes of single channel currents were determined by averaging 20–30 events, eliminating those with overlapping or artifactual currents. Standard errors of measurement were usually less than 0.1 pA.

RESULTS

Myotube Membrane Adheres to the Glass Electrode. After the establishment of gigohm-seals on myotubes, the patch electrode can be pulled away from the cell without disrupting the membrane fragment sealed within the electrode. Fig. 1 illustrates this process. Fig. 1*A* shows the result of pressing the patch electrode against the cell membrane, thereby establishing about a 50-MΩ seal. The lower trace shows a single channel opening and closing. This event is difficult to resolve amidst the background noise, which is inversely proportional to the seal resistance (2). The inside of the pipette is held at the bath potential ($V_h = 0$). The currents through this channel normally reverse at about 0 mV (11, 12). The driving force for currents through the channel is therefore primarily the cell's resting potential. The single channel current was about 2 pA.

Fig. 1*B* shows data after establishment of a gigohm-seal. Neher has postulated that membrane is pulled into the pipette upon suction (2), as illustrated in the figure. The background noise is greatly reduced by the increased seal resistance. Fig. 1*B* shows single channel currents at $V_h = 0$, again driven primarily by the cell's resting potential. Polarizing the patch electrode by 50 mV adds to the driving force, and the currents are markedly increased, as shown in Fig. 1*C*.

After sudden withdrawal of the patch electrode from the cell surface (Fig. 1*D*) the channels continue to function. The driving force of the cell's resting potential is removed, however, so that at the same $V_h = +50$ mV, the single channel events are much smaller.

Excised Patch Behaves Like a Single Bilayer Covering the Electrode Aperture. It has been suggested that after "tear-off" (i.e., excision) the membrane reseals at the pipette tip to form a closed vesicle (2). This sealed-over membrane would increase the series resistance at the electrode tip, reducing single channel currents by splitting the driving force between two membranes in series. The reduction of currents would be above and beyond the reduction due to the loss of resting potential. It is our intention here to demonstrate that (*i*) the reduction of current amplitude after tear-off is due primarily to the reduction of driving force, specifically the cell's resting potential, (*ii*) under appropriate conditions the excised membrane fragment behaves like a single intact layer spanning the tip of the patch pipette, rather than "sealing over" into a vesicle, and (*iii*) the solution bathing the intracellular surface of the membrane

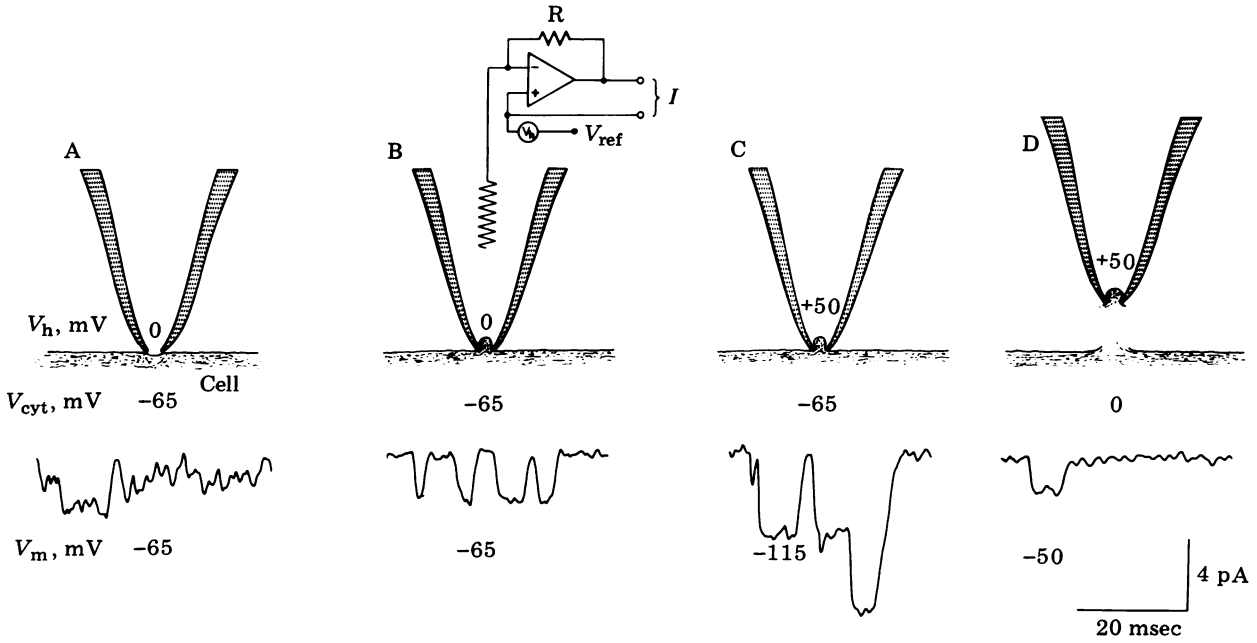

FIG. 1. Single AcCho-activated current events during excision. The top part of the figure shows a representation of the patch electrode and electrical arrangement. The feedback resistor, R, was 1 GΩ. V_{ref} is the signal from a reference electrode (approximately 7 M CsCl, <100 kΩ) in the bath. The lower traces are recordings of single channel currents. Inward currents are indicated by downward-going deflections. The pipette holding potential, V_h, the approximate potential at the cytoplasmic face of the membrane, V_{cyt}, and the transmembrane potential, V_m ($V_{cyt} - V_h$), are indicated for each current trace. These potentials are defined with respect to V_{ref}. (*A*) Patch recording of a single channel current with an approximately 50 MΩ seal. (*B*) Effect of applying a gentle suction to the patch electrode, thereby causing the adherence of the membrane to the glass. The seal was >5 GΩ. *B* and *C* show single channel currents at two holding potentials. Note the summation of two channel currents in *C*. (*D*) A single channel event immediately after tear-off of the membrane fragment into the bathing solution at a transmembrane potential of −50 mV.

fragment is continuous with the bath solution and may be varied at will.

In order to determine if a significant resistance existed in series with the excised patch of membrane, we examined the single AcCho-activated channel conductance before and after excision of a membrane fragment. A sealed-over vesicle would be expected to have a lower apparent single channel conductance, due to the reduction in driving force caused by the presence of a series resistance.

Fig. 2A plots the *I–V* (current–voltage) relationship for single channel currents after the gigohm-seal had been obtained but before removal from the cell surface. This constitutes the control case. The potential was varied by changing V_h of the pipette. The *I–V* relationship has been corrected for the resting potential of approximately −65 mV, which was measured on two plates of cells by impaling myotubes with standard 3 M KCl microelectrodes having resistances of 30–90 MΩ. The single channel conductance in seven myotubes of 34.6 pS was nearly independent of membrane potential over the range of −140 to +35 mV (Fig. 2A).

After tearing the membrane fragment off into the normal 100 Na Ringer's solution, we observed that the single channel conductance, measured as the slope of the *I–V* relationship, remained nearly constant for 1–4 min. Then the conductance decreased to about half that of the control. This suggests that the membrane had indeed sealed over. We discovered that this sealing process could often be prevented or reversed by perfusing the bath with solutions containing fluoride anions. Membrane fragments could remain patent and functional in fluoride solutions for up to 30 min. Sealed-over patches could occasionally be reopened in fluoride solutions by small sudden alterations of the hydrostatic pressure within the pipette. The subsequent shift back to the control level of conductance was often sudden and dramatic.

Fig. 2B shows that the apparent single channel conductance remained high in the excised membrane fragment. The bath solution was 160 NaF, which produced a slightly negative reversal potential, as in the control case. The single channel conductance of 33.5 pS was close to the control value, indicating that the sealing-over process was minimal.

Because the bath and the patch pipette contained solutions

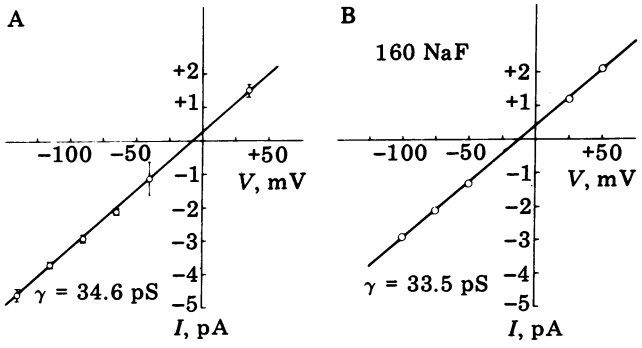

FIG. 2. Current-voltage (*I–V*) relationship before (*A*) and after (*B*) tear-off. The *I–V* curve was obtained by measuring single channel currents while varying V_h in 25-mV increments. The *I–V* curve in *A* has been corrected for a resting potential of −65 mV measured in other cells. Each data point shows mean ± SEM from five to seven cells. The linear regression line has a slope of 34.6 pS. The coefficient of determination $r^2 = 0.98$. *B* shows the *I–V* curve in 160 NaF (Table 1) after tear-off. Each data point is the mean from two to five membrane fragments. The SEM was <0.2 pA for each potential. The linear regression line has a slope of 33.5 pS; $r^2 = 0.996$. The reversal potential was approximately −12.5 mV, compared to the Nernst value of −10.7 mV.

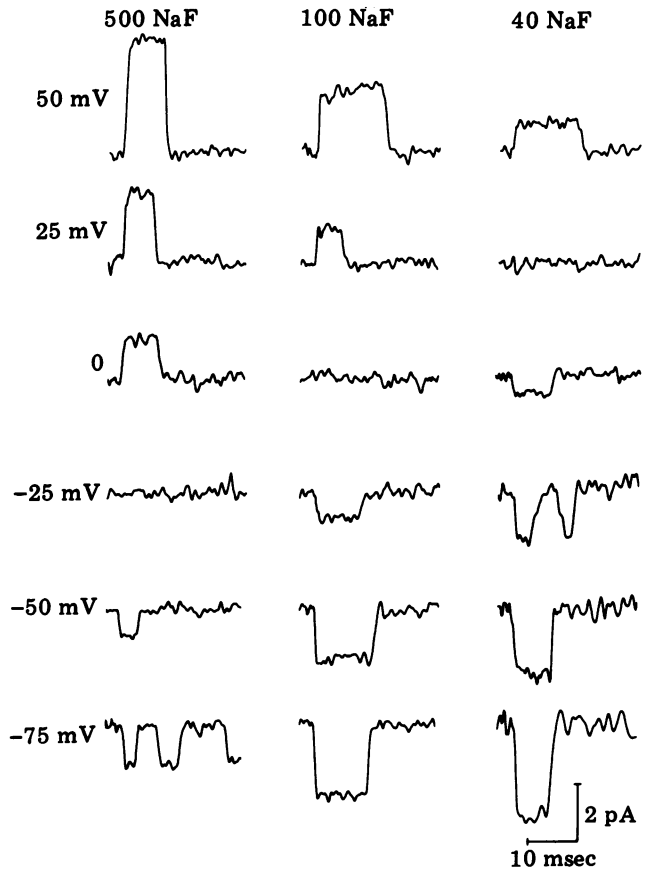

FIG. 3. Single channel currents in excised patches. The single channel currents were recorded at the holding potentials indicated on the left. Three internal solutions were used, as indicated on the top of the columns. The records in 500 NaF and 100 NaF were obtained from the same patch. The records in 40 NaF were obtained from three separate patches. Inward current events are downward-going deflections. Notice that the reversal (i.e., zero-current) potential became more positive as the concentration of internal Na was decreased. The illustrated traces were selected from digitally stored data and reconstructed on a strip-chart recorder. The individual channel open times shown are not indicative of the mean open time at any potential or concentration.

with sodium as the predominant cation (Table 1), the reversal (i.e., zero current) potential of the AcCho-activated current should follow the Nernst potential for sodium. Fig. 3 shows single channel currents measured at various holding potentials in three different sodium concentrations on the cytoplasmic side. Clearly, lowering the sodium concentration caused a shift in the reversal potential to more positive potentials. This is also seen in Fig. 4, which plots the *I–V* relationship for three Na concentrations. Smooth lines drawn through the data were usually within 5 mV of the theoretical reversal potentials, shown by arrows in Fig. 4.

In our experiments the osmolarities of the internal (i.e., NaF) solutions were in some cases much greater than the osmolarity of the Ringer's solution in the pipette. In these experiments we made repeated measurements at the same potentials over a 10-min period. We never observed changes of more than 0.1 pA in the averaged currents at any potential. Apparently water flux across the membrane fragment caused only insignificant changes in the Na concentration near the membrane.

Ionic Conduction Through the AcCho-Activated Channel Shows Saturation. The data in Fig. 4 provide evidence for a saturation of Na flux through the AcCho-activated channel. At a very positive potential, for instance +100 mV, the current is

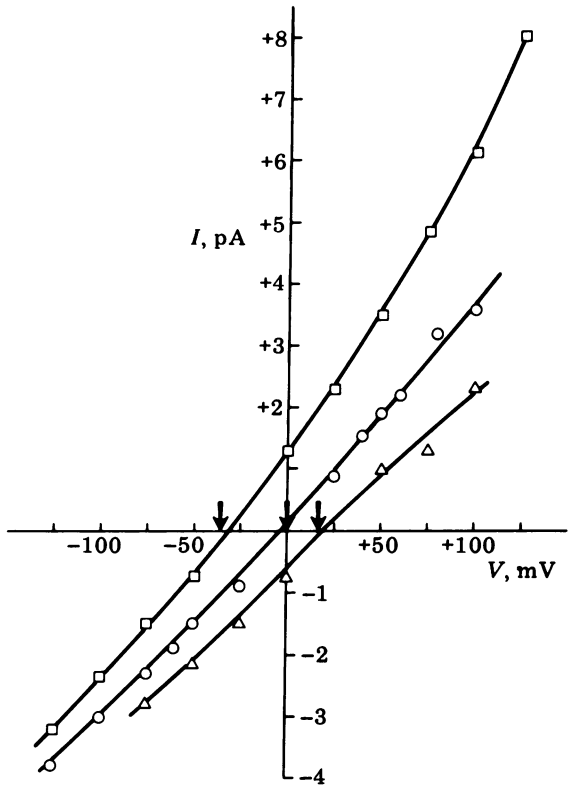

FIG. 4. *I–V* relationship after excision. The internal solutions were 40 (△), 100 (○), and 500 (□) NaF. The theoretical reversal potentials (ignoring a small effect of the Ca ions in the pipette) are shown by arrows for the three concentrations. Each data point is a mean value of single channel recordings from one to six different cells. In most cases the standard error between measurements on different cells was less than 0.1 pA.

approximately equivalent to the efflux of charge through the channel. If Na fluxes obeyed "independence" (13), the outward current would be a linear function of Na activity. For example, the current in 100 NaF is 3.6 pA at +100 mV. After correction

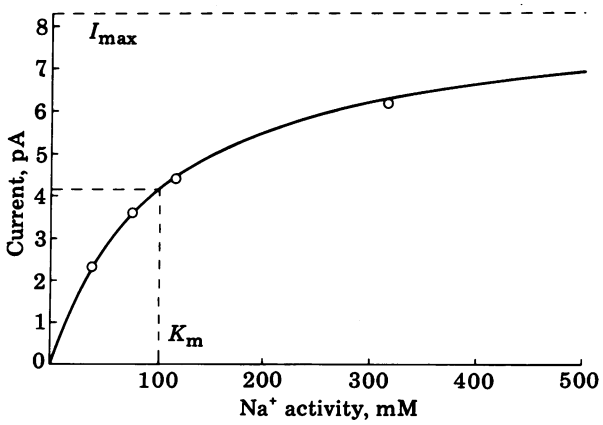

FIG. 5. Saturation of outward currents in the AcCho channel. The single channel currents at +100 mV are plotted against the internal Na activity. The smooth line through the data points has the formula

$$I = \frac{I_{max} \cdot [Na]}{[Na] + K_m},$$

in which I is the measured current, I_{max} is the maximal current at +100 mV, [Na] is the activity of Na ions, and K_m is an apparent dissociation constant. The best fit curve gives the values 8.3 pA for I_{max} and 102 mM for K_m.

for activity coefficients the current in 500 NaF should be 14.9 pA at +100 mV. The measured value was only 6.2 pA. Fig. 5 shows that the outward current at +100 mV has the behavior of a first-order saturation. A plot of current vs. Na activity is well fit by a rectangular hyperbola. The theoretical maximum current at +100 mV was 8.3 pA. K_m, the activity of Na that produces half the maximum current, was 102 mM. This corresponds to a NaF concentration of about 138 mM.

Further evidence for a violation of the independence of Na fluxes can be seen by examining the *I–V* curves at negative potentials (Fig. 4). By using the same argument as above, it can be shown that the total current at a very negative potential (−125 mV) is predominantly influx, with efflux being negligible. The decrease of this current caused by raising internal Na concentration must then be due to a decrease of influx. The simplest explanation for this effect is a blocking action of internal Na ions upon the movement of external Na ions through the channel.

DISCUSSION

We have developed an excised patch technique that permits simultaneously making high-resolution single channel recordings and controlling the composition of the solution bathing the cytoplasmic face of the membrane. The technique, involving the removal of a small membrane fragment from a myotube, has the following advantages: (*i*) it permits direct control of the transmembrane potential, (*ii*) it allows the concentration of cytoplasmic-face ions to be increased to values well above the usual physiological range, and (*iii*) it allows AcCho-activated currents to be examined at voltages considerably more positive than previously reported. This technique has made it possible for us to demonstrate ionic saturation of Na fluxes in the AcCho channel.

Evaluation of this technique requires demonstration that both electrical and diffusional barriers between the bath and the cytoplasmic membrane surface are absent. The largest possible electrical and diffusional barrier would be the residual membrane of a sealed-over vesicle. We have presented the means to eliminate this residual membrane, using both fluoride and hydrostatic pressure. We have demonstrated that this membrane is electrically invisible by showing that the apparent conductance of single channels was not decreased significantly after excision.

The sensitivity of the electrical measurement sets an upper limit on the magnitude of any diffusional barrier between the cytoplasmic face of the membrane and the bath. Assuming independent ion fluxes across any such barrier, the unidirectional ion flux, M, can be shown (14) to be

$$M = \frac{GRT}{F^2},$$

in which G is the conductance of the barrier, R is the gas constant, T is absolute temperature, and F is the Faraday constant. The electrical measurement suggests that this barrier has a conductance greater than $(1\ G\Omega)^{-1}$. For this conductance the unidirectional flux is 2.6×10^{-16} mol/sec. The "dead space" between the cytoplasmic surface of the patch and the bath is approximately 1 μm^3. For this volume the flux causes a concentration change of approximately 250 mM/sec, thus ensuring rapid and complete exchange with the bath. This correlates well with the observation that, after solution changes, the *I–V* relationship changes rapidly and then remains stable for at least 10 min. A diffusion barrier might be expected to impede the rapid development of a new ionic gradient.

We do not believe that other series resistances cause significant errors in our experiments. A voltage error is contributed

by leakage current across the excised patch in series with the pipette resistance of 5–10 MΩ. The worst case, a pipette resistance of 10 MΩ in series with a GΩ patch resistance, produces only a 1% error in the measured voltage across the membrane patch. Single channel currents are yet an order of magnitude smaller than the holding currents and cause negligible error.

Our data may be compared with previous measurements on the AcCho-activated channels. The single channel conductance between 30 and 40 pS is smaller than the approximately 50-pS conductance previously reported in rat myotubes (15). This discrepancy is well explained by the fact that we used 100 mM Na^+ in the pipette, whereas 145 mM Na^+ and 4.2 mM K^+ was used in the previous study. The *I–V* relationship we observed in the control case and in the presence of nearly symmetrical solutions was approximately linear, and thus also consistent with previous studies (7, 12).

Our results indicate a saturation of Na fluxes within the AcCho-activated channel. A previous study using frog neuromuscular junction did not detect saturation, although evidence for ionic interaction within the channel was found (11). A possible explanation for this discrepancy is that we could measure currents in solutions containing up to 500 mM Na^+, whereas the previous study used a maximum concentration of only 115 mM. Furthermore, we observed a saturation most clearly at +100 mV, whereas the previous study examined currents at −70 mV. The dissociation constant for Na ions is likely to be voltage dependent and may be greater at negative potentials, depending upon the detailed mechanism of permeation.

The excised patch technique described here has much of the flexibility of experiments on artificial bilayer membranes. It has the added advantage that channels can be studied in the presence of their native membrane. It has the potential for use on a wide variety of preparations that have previously been inaccessible to both voltage clamp and internal perfusion. This technique has allowed us to show saturation of Na currents through the AcCho-activated channel.

Note Added in Proof. Hamill and Sakmann (16) have recently developed a similar technique for excision of membrane patches from tissue-cultured embryonic muscle. They opened sealed-over patches by briefly passing the pipette tip through the air-water interface. They have recorded AcCho-activated currents in symmetrical Ringer's solution.

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