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Immunology

Single channel currents induced by complement in antibody-coated cell membranes

(humoral cytolysis/membrane damage)

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ABSTRACT **An extracellular patch electrode was used to record ionic currents from individual complement-induced channels in the membranes of antibody-coated skeletal muscle. The amplitude of the single-channel currents leads to an estimate of 90 pS for the unit conductance. The kinetics of channel opening and closing show marked variability and complexity. Channels flicker open and closed repeatedly, indicating that once these lesions form, they undergo rapid structural transitions between discrete conducting and nonconducting states.**

Activation of complement initiates a cascade of events whereby several protein components combine to damage a cell membrane. Solute molecules pass through the damaged membrane to create an osmotic imbalance which leads to cytolysis (1). Complement-induced damage has been demonstrated in synthetic lipid bilayers, where it is accompanied by discrete conductance increases (2–5). We report here the measurement of electrical current through individual molecular-size lesions produced by complement in the membranes of intact muscle cells grown in tissue culture. These experiments use the recently developed patch-electrode technique for isolating the electrical current passing through a very small area of cell membrane (6). This patch recording method has been used to observe single ionic channel currents in synaptic (7–9) and axonal (10, 11) membranes. Signals observed in this way allow us to estimate the conductance of the channel and to characterize the kinetic behavior of channel opening and closing.

METHODS

Antibody-coated muscle was prepared as described by Stephens and Henkart (12). Cultured rat muscle is first treated with 1 mM trinitrobenzylsulfonic acid in Dulbecco's phosphate-buffered saline (referred to as saline) for 30 min at room temperature. This results in the nonselective attachment of the hapten 2,4,6-trinitrophenol (TNP) to membrane proteins. After washing off the trinitrobenzylsulfonic acid, the cells are bathed in purified antibody against TNP for 45 min.

A patch electrode, filled with rabbit complement diluted up to 1:3 with saline, is pressed against the cell surface as illustrated in Fig. 1*A*. This allows the antibody-hapten conjugates under the tip of the electrode to activate complement in the electrode. The activated complement then attacks the membrane under the electrode tip to produce current fluctuations as shown in Fig. 1*B*.

The patch electrode is fabricated to seal tightly with the cell membrane, confining the complement attack to the 1- to 2-μm^2 patch of membrane under the open tip of the electrode. In addition, the tight seal between the walls of the electrode and the cell membrane lowers the noise level in the current amplifier to approximately 1 pA. The baseline current noise is entirely accounted for by the thermal noise generated in the resistance of the seal between the pipette rim and the membrane. When complement is added directly to the bathing solution of antibody-coated muscle, the cells depolarize and lyse as was demonstrated by Stephens and Henkart (12).

Membrane potential was measured independently with an intracellular microelectrode. A second microelectrode was inserted into the cell in some experiments to hyperpolarize the cell with negative currents. Current records were digitized at 200 μsec.

In one experiment, complement was inactivated by prior activation. A crude preparation of TNP-labeled bovine serum albumin was prepared by mixing 1 mM trinitrobenzylsulfonic acid with 0.15 mM albumin. After standing at room temperature for 4 hr, the mixture was diluted 1:1000 (vol/vol) with saline. One aliquot was mixed with anti-TNP antibody during the dilution so that the final antibody concentration was 0.67 μM. TNP-labeled albumin with and without antibody was mixed 1:1 (vol/vol) with complement and allowed to stand at room temperature for 15 min. This provided two complement samples that contained TNP-labeled albumin and were identical except for the anti-TNP antibody. The 15 min allowed the antibody-containing complement to be activated and, due to the lability of activated complement, inactivated.

Heat-inactivated complement was also used as a control; it was prepared by incubating at 57°C for 30 min.

RESULTS

Records in Figs. 1*B*, 2, and 3 show currents induced by complement in the patch electrode. In these records, the current fluctuates between discrete levels, indicating the abrupt opening and closing of ionic channels.

After the patch electrode was applied to the cell, a highly variable period of time (3–30 min) passed before the current fluctuations appeared. Heat-inactivated complement did not produce events of the type shown in these figures. However, infrequent, small current jumps were seen (unit conductance, 40 pS; mean open time, 2 msec). In experiments with cells lacking TNP or antibody, or both, events were also seen, although on the average the activity was much less vigorous and the lag time until the first jumps appear was longer. This effect was probably caused by activation of complement by heterophil antibodies in the rabbit serum (13).

The complement inactivated by prior activation with TNP-labeled albumin and antibody produced no jumps at all in re-

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Abbreviation: TNP, 2,4,6-trinitrophenol.

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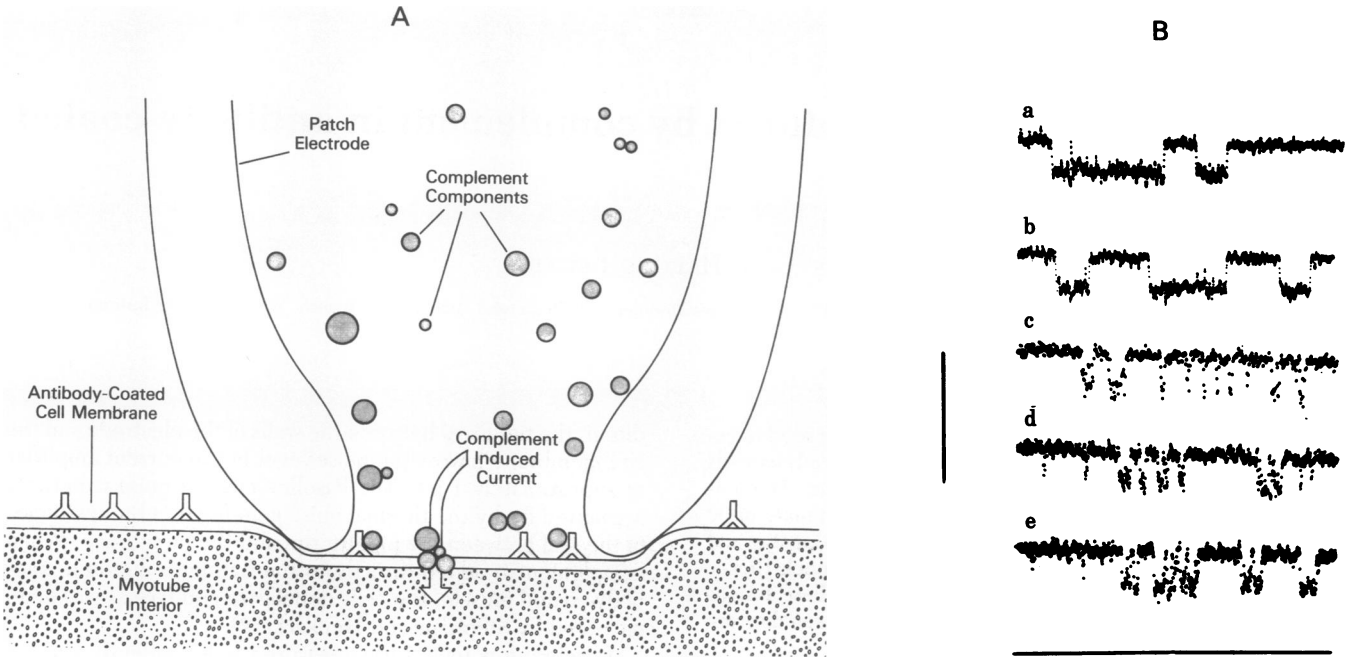

FIG. 1. (*A*) An extracellular patch electrode filled with serum is pushed against the cell surface making a tight seal with the membrane and isolating a small patch under the electrode tip. The antibody coating the cell activates the complement in the sealed patch. As channels open and close, the current flowing through the electrode is recorded by a virtual ground amplifier. This figure is schematic and not drawn to scale. The electrode aperture is ≈1 μm in diameter. (*B*) Records of electrical current through the patch of membrane. Vertical scale, 20 pA; horizontal scale, 410 msec. These are records from cells at their resting membrane potential of −60 mV.

cordings held for 40–50 min. The companion solution, which did not include antibody, was active and produced current fluctuations within 20–25 min.

The mean channel currents and the standard deviations at four voltages are given in Table 1. Representative records of channel events at three voltages are shown in Fig. 2. The behavior at −90 and −100 mV was qualitatively the same. The mean channel current increased linearly with voltage from −47 to −60 mV. The increase in current was relatively small when the cell was hyperpolarized to −90 mV and decreased slightly as the cell was hyperpolarized further to −100 mV. The standard deviations in current-jump amplitude increased as membrane potential was made more negative. The spread of amplitude values was much greater than we would expect from uncertainties in the current measurement. By comparison, acetylcholine channel current distributions measured with the same system often had standard deviations of less than 0.5 pA.

In records of individual channel events such as those presented here, the rates of channel opening and closing can be determined from the duration of intervals between events and the duration of openings, respectively. A preliminary analysis indicated that the kinetics are very complicated and variable.

The mean jump duration varied considerably, even with experiments done on the same day under identical conditions. At −60 mV, the average open-state lifetimes ranged from 3.0 to 35.3 msec, with a mean from all experiments of 11.4 msec. At −90 or −100 mV, the mean open time ranged from 6.14 to 20.7 msec with a mean of 16.0 msec. This variability is clearly illustrated by comparing records a and b with records c, d, and e of Fig. 1*B*. Both experiments were done under identical conditions, but the channel jumps in records c, d, and e of Fig. 1*B* have much shorter durations than the channel jumps in records a and b of the same figure.

Table 1. Mean channel currents

Membrane potential, mV	Current, pA	SD	No. of channels
−47	−3.94	1.27	54
−60	−5.38	1.54	508
−90	−5.93	3.64	228
−100	−5.67	2.55	373

The frequent occurrence of well-separated bursts of single channel activity suggests that we are observing the transient histories of distinct individual channels within the isolated membrane patch. An individual long burst of repeated openings and closings that is followed by long periods of time during which no activity occurs is likely to be caused by one distinct structure fluctuating between two states. The long periods of inactivity probably mean that the channel either disassembled or diffused within the membrane out of the patch under the electrode tip. The recovery of membrane potentials after complement-induced depolarization (12), as well as studies suggesting the repair of the complement lesion (14–16), supports the explanation that the channel can disassemble or close permanently.

There appear to be three rates associated with the opening of a channel. These rates are distinguished by large differences in the time intervals separating channel openings. The fastest rate has a characteristic time of roughly 1 msec. Closures of this duration are shown in the bottom records of Fig. 1*B*. Similar rapid transient channel closures also have been seen with axonal K^+ channels (10). Closures lasting on the order of 0.1–0.5 seconds are evident in most of the records in Figs. 1*B* and 2 and are indicative of an intermediate rate of opening. The slowest rate is seen for the intervals between bursts of channel activity; both the bursts and the intervals separating them can last as long as several minutes.

An additional feature that reflects kinetic variation is the excess noise often seen during the open state of a channel (Fig. 3*A*). The noise intensity is far too great to be transport noise through the open pore (17); hence, it must be caused by conductance fluctuations of the open channel. These fast conduc-

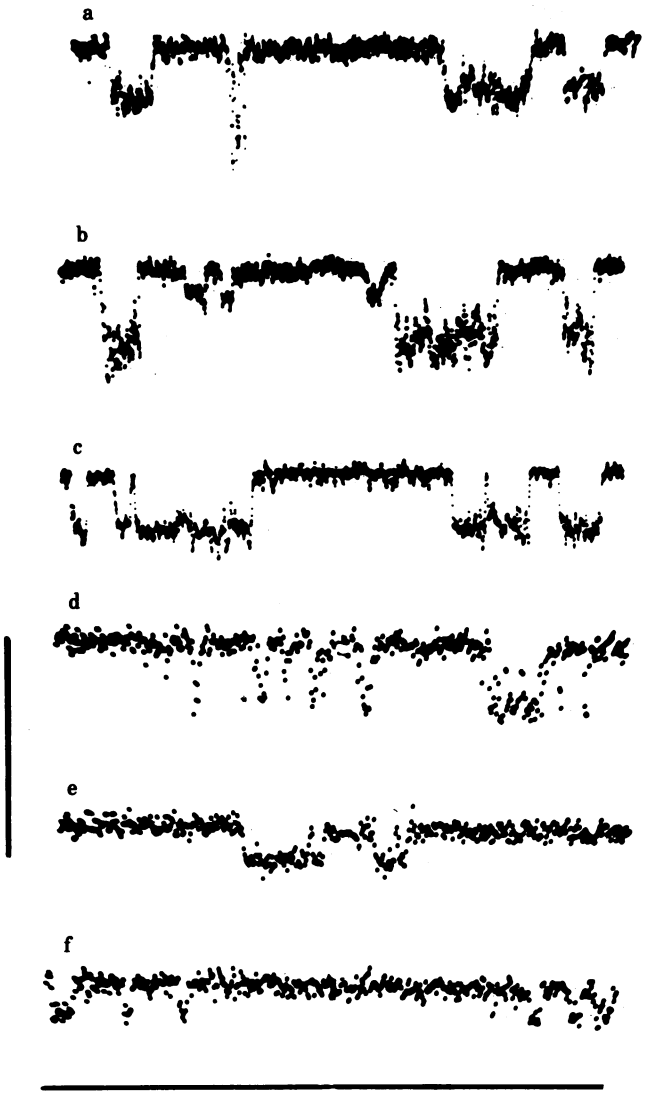

FIG. 2. Conductance jumps at different membrane potentials. Records: a and b, membrane potential of −90 mV; c and d, −60 mV; e and f, −47 mV. Vertical scale, 20 pA; horizontal scale, 410 msec.

tance fluctuations may represent rapid transitions between discrete levels, which appeared as noise because of the limited response time of our electronics (0.5 msec), or, alternatively, the fluctuations could result from more continuous conformational flexibility of the open state. Similar rapid conductance fluctuations have been observed in lipid bilayers treated with hemocyanin (18).

Often, after prolonged treatment and after the events so far described, much larger, very noisy inward currents were seen (Fig. 3*B*). These currents could last as long as several minutes but ended with the signal returning to baseline. Individual jumps could not be resolved in these current records, and we cannot yet determine whether there is one large channel with a noisy open state or many smaller channels contributing noise by independently opening and closing.

DISCUSSION

The currents reported here are seen only when there is serum in the electrode. The absence of these events when serum is heat-inactivated is consistent with the hypothesis that these currents are complement-induced. The omission of antibody only partially reduces the effect, suggesting that it could be only partially dependent on antibody. However, our omission of antibody does not guarantee that no antibody is present. There is antibody in both the rabbit serum that is the source of our complement and in the serum used to supplement the growth of the cells in culture. This antibody could find sites on the muscle cell surface (13), making it more difficult to demonstrate a complete dependence of the currents we are describing on antibody. To overcome this difficulty, we prepared an artificial antigen, TNP-labeled albumin, which would bind to anti-TNP antibody. The complete failure of complement that was preincubated with antibody and antigen, as compared with the vigorous activity induced by complement preincubated only with antigen, demonstrates a strong antibody dependence when the goal is to inactivate the complement prior to its application.

The existence of such variable kinetics reflects the complexity of the complement cascade. The rates of the many steps involved in the formation of the membrane attack complex and the lability of the activated components could all contribute to this complexity.

From −47 mV to −60 mV, the complement-induced channel current increases proportionally with the driving force. However, from −60 mV to −90 or −100 mV, such a linear increase is not found. This kind of behavior in a uniform channel population would indicate either current saturation or a low conductance coupled with an enormous reversal potential. However, the breadth of the distribution of current amplitudes at −90 and −100 mV suggests that there is more than one conductance value and that the distribution is voltage dependent, with lower conductance states favored at increasingly negative membrane potentials.

The inward currents seen here are consistent with the complement-induced depolarizations in muscle seen by Stephens and Henkart (12). Because these depolarizations approach but never overshoot 0 mV, the channel probably has a reversal potential near 0 mV. Assuming this to be true, we can use our current measurements at −60 mV and −47 mV (where the amplitude distribution is more uniform) to obtain an estimate for the channel conductance of 90 ± 26 pS.

Electron micrographs of erythrocytes exposed to complement attack show structures with a diameter of 100 Å and a length of 150 Å (19, 20). Other measurements with molecules of various sizes to block osmotic lysis, under conditions where there is only one channel per cell, indicate a channel diameter of 7–11 Å (21, 22). Unfortunately, there is no direct way to relate channel size to channel conductance. Some insight can be gained, however, by calculating the macroscopic conductance of an equivalent cylinder of electrolyte with a length equal to the thickness of the cell membrane. With an electrolyte resistivity of 100 Ω cm and a membrane thickness of 50 Å, an electrolyte-filled cylinder with a 90-pS conductance would have a diameter of 8 Å. This same value of 8 Å also can be obtained by (*i*) comparing the conductance of the complement-induced channel with the conductance (8) and the diameter (23, 24) of the acetylcholine channel and (*ii*) assuming that conductance scales with area.

One-hit kinetics are seen when the complement is relatively dilute. Higher concentrations are used in the electron microscope studies and in other osmotic blocking experiments where larger sizes are seen. Michaels *et al.* (5, 25) showed that the conductance induced in lipid bilayers by complement components C5b–8 is reversible, whereas C9 stabilizes the lesions. In our experiments, those complement components that are free to diffuse laterally away from the patch may be deficient, and the fluctuating channels may represent a transient precursor of the final attack complex. Thus, the constraints imposed by point-source application of complement mitigate the vigorous

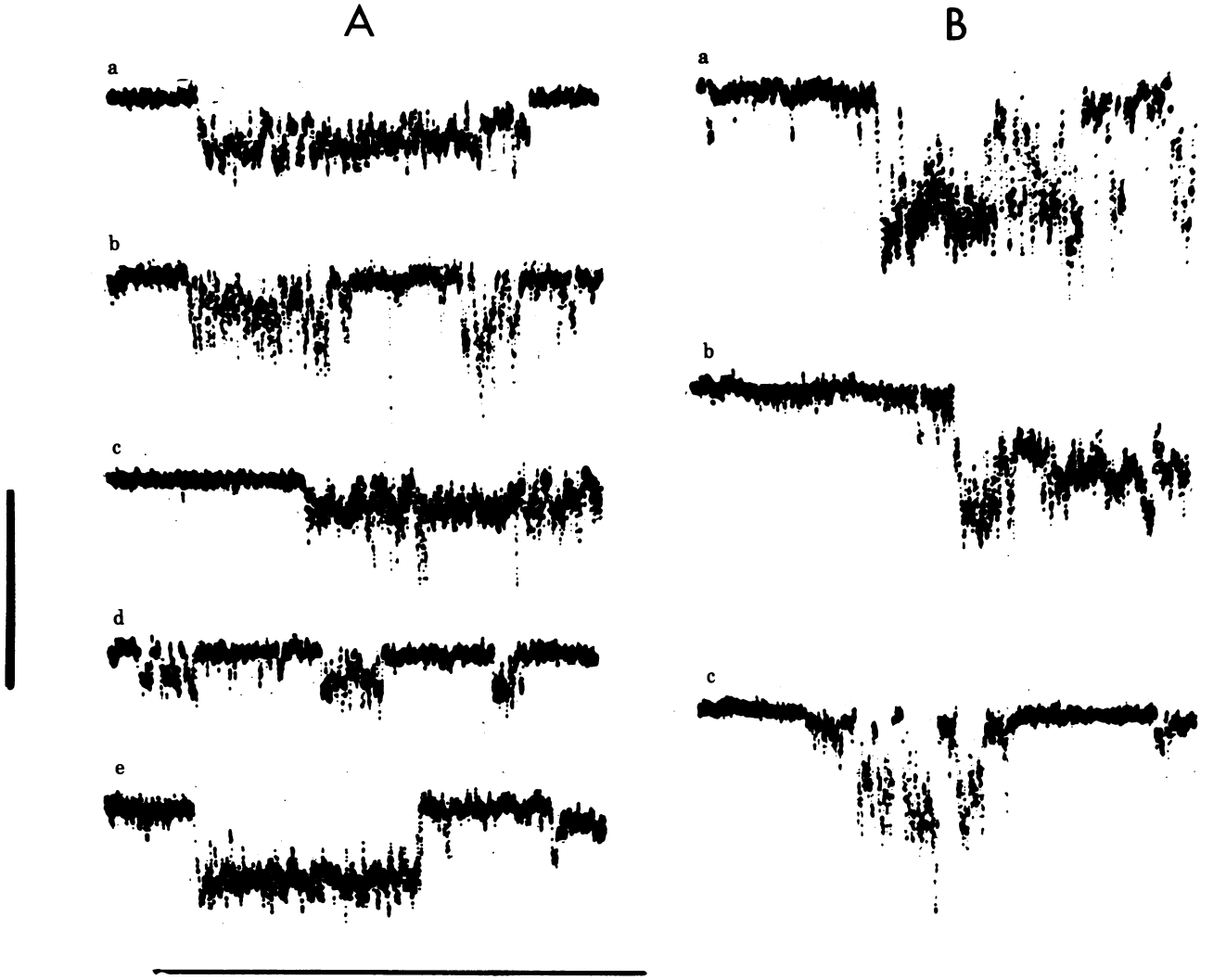

FIG. 3. Noisy, or flickering, conductance jumps. (*A*) Records showing channel currents when the open state produces additional noise. Membrane potentials of records: a, −60 mV; b and c, −90 mV; d and e, −100 mV. (*B*) Records showing events with larger currents and greater excess noise. Membrane potentials of records: a and b, −100 mV; c, −90 mV. Vertical bar, 20 pA; horizontal bar, 820 msec.

attack of the membrane by complement, suggesting that the conditions of our experiment are comparable to experiments using lower concentrations of complement. The very large currents we occasionally see may be much more common under more favorable conditions. Which channel size corresponds to the final membrane attack complex is difficult to say because slow lysis (26) and permeability increases (5) have been induced without C9, the final component of complement.

The change in membrane conductance induced by complement could vary with a number of factors other than the intrinsic conductance, such as duration of channel opening or frequency of opening. Our results suggest that the gross measure of complement-induced permeability increase may not reflect the rate of flux through a continuously open pore but rather the time average of flux through pores that flicker open and closed. Such flickering pore activity might provide a means of resolving the discrepancy, noted by Sims and Lauf (27, 28), between the relatively large pore-size estimates based on size of permeant molecules and the relatively low fluxes through the pores.

In view of the one-hit theory of complement lysis (29, 30), it is of interest to ask whether a single pore with a conductance of the order of 100 pS can indeed lyse an erythrocyte. On the one hand, 100 pS represents a substantial increase in cation conductance because the resting Na^+ permeability of an erythrocyte is 10^{-10} cm/sec (31), which is equivalent to an ionic conductance of 0.03 pS.[§] On the other hand, under an initial potential of −10 mV, the maximum rate of increase of intracellular ion concentration would be 0.1 mM/sec, so that channels would have to persist for several minutes in order to effect lysis. Experiments with varying components should show more clearly what stage of the complement cascade is associated with these flickering ionic channels.

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[§] The expression $g_i = P_i[i]F^2/RT$ is valid for small electric fields and is adequate for the order of magnitude comparisons made here. g_i is the ionic conductance of species i (10^{-10} S). P_i is the permeability of that species. $[i]$ is the concentration (0.1 M). F is Faraday's constant. R is the gas constant. T is the absolute temperature (300 K). The surface area of one erythrocyte was estimated to be 75 μm^2.

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