Single Channel Properties of Newly Synthesized Acetylcholine Receptors Following Denervation of Mammalian Skeletal Muscle

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ABSTRACT We have examined the single channel properties of newly synthesized acetylcholine (ACh) receptors in denervated adult mouse muscle. Patch-clamp recordings were made on freshly isolated fibers from flexor digitorum brevis (fdb) muscles that had been denervated in vivo for periods up to 5 wk. Muscles were treated with α-bungarotoxin (α-BTX), immediately before denervation, in order to block pre-existing receptors. Denervated fibers exhibited two types of ACh receptor channels, which differed in terms of single channel conductance (45 and 70 pS) and mean channel open time (~7 and 2.5 ms, respectively). In contrast to innervated muscle, where only 3% of the total openings were contributed by the low-conductance channel type, >80% of the openings in the nonsynaptic membrane of denervated muscle were of this type. Importantly, a similar increase in the proportion of low-conductance channels was observed for recordings from synaptic membrane after denervation. These data argue against the proposal that, in denervated muscle, the low-conductance channels undergo continued conversion to the high-conductance type focally at the site of former synaptic contact. Rather, our findings provide additional support for the idea that the functional properties of ACh receptors are governed uniformly by the state of innervation of the fiber and not by proximity to the site of synaptic contact.

INTRODUCTION

Two functionally distinct classes of acetylcholine (ACh) receptors are present in vertebrate skeletal muscle. The principal class of ACh receptors located at the synapse of innervated adult muscle fibers is characterized by a high single channel conductance and fast kinetics (Katz and Miledi, 1972; Anderson and Stevens, 1973; Dreyer et al., 1976; Cull-Candy et al., 1982; Dionne and Leibowitz, 1982; Brenner and Sakmann, 1983; Colquhoun and Sakmann, 1985). In contrast to adult innervated fibers, ACh receptors with a lower conductance and slower kinetics are present in the nonsynaptic membrane of both embryonic and adult...
denervated muscle (Katz and Miledi, 1972; Neher and Sakmann, 1976a; Dryer et al., 1976; Fischbach and Schuetze, 1980; Michalet and Sakmann, 1980; Miledi and Uchitel, 1981; Cull-Candy et al., 1982; Brehm et al., 1984a; Steele and Steinbach, 1986). The single channel properties of this lower-conductance channel type have been studied extensively in embryonic muscle (Hamil and Sakmann, 1981; Auerbach and Sachs, 1984; Brehm et al., 1984a, b; Leonard et al., 1984; Siegelbaum et al., 1984), but detailed single channel studies on denervated muscle have not been performed. The first aim of the present study was therefore to characterize, at the single channel level, the properties of ACh receptors at both the synaptic and nonsynaptic regions of denervated adult muscle.

Denervation causes a dramatic increase in the sensitivity to ACh (Ginetzinsky and Shamarina, 1942; Axelsson and Thesleff, 1959; Miledi, 1960) and in the density of ACh receptors (Lee et al., 1967) in nonsynaptic membrane. Noise analysis studies indicated that these newly appearing channels were the low-conductance type of receptor (Dreyer et al., 1976; Neher and Sakmann, 1976a). Despite the overall increase in the number of low-conductance ACh receptors, it has been reported that at least half of the channels at the former synapse were of the high-conductance type (Brenner and Sakmann, 1983). On the basis of this observation, it was proposed that high-conductance channels, which are characteristic of synapses of innervated muscle, continue to be localized at the synapse after denervation. This continued expression of high-conductance channels has been attributed to an “imprint” left by the nerve on the muscle cell, so that even in the absence of innervation, low-conductance channels present at the synapse are converted over time to the high-conductance class (Brenner et al., 1983; Brenner, 1985). However, no single channel studies have examined the properties of ACh receptor channels at the site of former synaptic contact. The second aim of this study was therefore to test this hypothesis, at the single channel level, by studying the time-dependent changes in the types of newly synthesized ACh receptors present at the synapse under conditions in which pre-existing receptors did not contribute significantly to the analysis.

**METHODS**

**Preparation of Tissue**

Individual muscle fibers were obtained from the flexor digitorum brevis (fdb) muscles of adult (1.5–4 mo old) male and female CBA mice (kindly provided by H. Wortis and C. Ellis, Tufts University). Pre-existing ACh receptors were blocked by subcutaneous injections of 5 μM α-bungarotoxin (α-BTX) into the footpads of mice that had been lightly anesthetized with ether. Three injections of 50 μl each were made over a period of 24–30 h. 6–8 h after the final injection, mice were again anesthetized, and the fdb muscle was denervated by cutting either the tibial or the sciatic nerve. To help prevent reinnervation, a piece of thread was tied to the proximal nerve stump, the nerve was pulled outside the incision, and the thread was taped to the animal’s leg. In addition, the nerve stump was recut every 4–5 d to further prevent reinnervation. In some cases, because of the presence of scar tissue, it was not possible to identify the nerve stump or its regenerating processes. In such animals, the scar tissue within the area of the incision was removed in order to sever regrowing processes that may have been present. Although multiple operations were performed in order to maintain fdb muscles in a condition of complete denervation, in several animals denervated for >2 wk, there was evidence that
transient reinnervation may have occurred between the time of the initial denervation and that of the recordings. Indications of reinnervation included: (a) the presence of intact plantar nerves entering the muscle at the time of dissociation, (b) a low frequency of openings in nonsynaptic recordings from the myotendinous region (<1 event/s) when the event frequency at the synapse of the same fiber was high, and (c) a predominance of high-conductance openings in myotendinous recordings, similar to that observed in innervated muscle.

5–23 d after denervation, the fdi muscle was dissected and dissociated according to a modification of the method of Bekoff and Betz (1977). Muscles were incubated in 2–3 mg/ml collagenase (Cooper Biomedical, Freehold, NJ) for 1.5–3 h at 37°C in RPMI medium (Gibco, Grand Island, NY). This was followed by an additional incubation for 30 min in Ca-free minimum essential medium (MEM) containing 0.5 mg/ml trypsin and 0.2 mg/ml EDTA (Gibco). Muscles were then gently triturated into physiological saline to isolate single fibers.

**Physiological Recordings**

All recordings were made in physiological mammalian saline (120 mM NaCl, 1 mM KCl, 1 mM CaCl\_2, 10 mM HEPES; pH 7.2 with NaOH). Patch electrodes were pulled from borosilicate glass (1B150F-4, W-P Instruments, Inc., New Haven, CT) to an outer diameter of 1–2 µm, fire-polished, and filled with 50–500 nM ACh dissolved in the same saline. To reduce background noise, the filled electrodes were dipped quickly into Sigmacote (Sigma Chemical Co., St. Louis, MO).

Single channel activity was recorded in the cell-attached configuration from synaptic and nonsynaptic patches of membrane. The synapse was usually located in the middle portion of each fiber. Irrespective of the time after denervation, the original endplate was morphologically distinguishable at the time of recording (Fig. 1). Nonsynaptic recordings were made from the myotendinous ends of the fibers to minimize any contribution from synaptic receptors. Single channel currents were recorded at 21–23°C with a List L/M-EPC 7 patch-clamp amplifier (Medical Systems Corp., Greenvale, NY) and stored on an FM analog tape recorder (Racal Recorders, Ltd., Hythe Southampton, England). Taped current signals were subsequently filtered with the 3-dB point set at 4 kHz (eight-pole Bessel filter; Frequency Devices, Inc., Haverhill, MA), digitized at 100 µs/point and analyzed with an Indec (Sunnyvale, CA) LSI-11/73 computer system. Using an operator-assisted computer program, records were searched for transitions away from baseline. The threshold for detecting opening and closing transitions was initially set at 50% of the smallest-amplitude events and was automatically reset to the 50% level of each subsequently encountered event. Open durations for each event were measured as the number of sample points that were greater in amplitude than the threshold value. Therefore, events containing brief closures that did not cross this 50% level were counted as a single event. Sections of the record where two or more channels were open simultaneously were excluded from analysis. The minimum duration accepted for analysis was the briefest duration at which current amplitudes were not attenuated (as determined from amplitude vs. duration histograms). Mean channel open times were estimated according to the method of maximum likelihood for a single exponential (Colquhoun and Sigworth, 1983). Both mean channel open times and the percentage of low-conductance channel openings were calculated for patches with >100 openings. These measurements were made at a pipette potential of +60 mV. The records that were analyzed for these measurements were from data collected as soon as possible after seal formation. The same concentration of ACh was used for synaptic and nonsynaptic recordings from the same fiber, unless openings were not evident nonsynthetically, in which case a higher ACh concentration was used in subsequent recordings from that fiber.
Intracellular and iontophoretic pipettes were filled with 3 M KCl and 1 M ACh, respectively. Intracellular electrodes had filled resistances of 50–100 MΩ; iontophoretic pipettes had resistances of 100–300 MΩ. Braking currents of 3–8 nA were applied to the iontophoretic pipettes, and ACh was delivered in pulses of 2–20 nA and 10 ms duration. Iontophoretic pipettes were positioned directly over the endplate. ACh sensitivity was measured by the method of Kuffler and Yoshikami (1975). For each experiment on a given day, uninjected control fiber sensitivity was compared with that of fibers from α-BTX–injected mice using the same iontophoretic pipette and braking current for all fibers. Corrections were not made for differences in input resistances since the average resting potential was not significantly different for α-BTX–treated and nontreated fibers. All values given are means with corresponding standard errors of the mean (SEM). Statistical significance was calculated using a two-tailed Student’s t test.

Fluorescence

Freshly dissected muscles were incubated for 30 min in 0.6 μM rhodamine-conjugated α-BTX (Rh-α-BTX) (kindly provided by Dr. Y. Kidokoro, University of California, Los Angeles) and washed three times for 3 min each time to remove nonspecifically bound toxin. The muscle was subsequently dissociated into single cells (see above). Fluorescently labeled cells were viewed with a 63X Plan-Neofluar water immersion objective and a Zeiss IM-35 microscope. Measurements of fluorescence intensity were obtained using an Ealing side window photomultiplier mounted to the camera port. The diaphragm was stopped down to illuminate an area approximately the size of the endplate, and was not adjusted during any subsequent measurements. The intensity of fluorescence from each fiber was quantitated from the current output of the photomultiplier. Intensity readings were taken from synaptic and nonsynaptic (specifically myotendinous) regions of the fiber and from cell-free regions of the dish (background). Fluorescence caused by specific binding to ACh receptors was estimated by subtracting background readings from the signal generated from the fibers.

RESULTS

ACh Receptors Present at the Time of Denervation Are Blocked by Injection of α-BTX

In order to study receptors that were synthesized after denervation, receptors present at the time of denervation were blocked by injecting the footpads of animals with α-BTX. α-BTX is an essentially irreversible ligand of the ACh receptor, which prevents the binding of ACh and therefore inhibits channel function (Chang and Lee, 1963; Lee, 1972). The extent of block was assessed by both binding of Rh-α-BTX and electrophysiological responses of fdb fibers to ACh. For these experiments, fibers were dissociated 1 d after treatment with unlabeled α-BTX. Rh-α-BTX labeling of dissociated fibers from uninjected mice revealed a bright, highly delineated area of fluorescence that corresponded to the single synapse present on each cell (Fig. 1, inset). Photometric measurements of fluorescence from Rh-α-BTX–labeled synapses from preblocked fibers, which were too dim to photograph, were found to be 7 ± 1% that of control fibers. Similarly, the responses of preblocked fibers to ACh iontophoresed at the synapse indicated that injected animals were 5 ± 8% as sensitive as controls. In addition, synaptic single channel activity from injected fibers was minimal (<10 openings/min) or nonexistent in 17 patches examined.
Two Types of Newly Synthesized ACh Receptors Are Observed in Denervated Fibers

Single channel recordings were made from fibers that had been denervated in vivo for periods of 5–23 d. Two major amplitude classes of single channel ACh-activated currents were evident (Fig. 2, A and B) in both synaptic and nonsynaptic recordings. Slope conductances for the two classes of single channel currents were 45 and 70 pS when measured over a range of pipette potentials between +40 and +140 mV (Fig. 2C). The conductance for both channel classes was independent of the length of time after denervation. Openings by the 45-pS (low conductance) channel were observed in 118 of 119 patches, while openings of the 70-pS (high conductance) channel were evident in 105 of 119 patches. A small-conductance class corresponding to 18 ± 2 pS was also observed in ~40% of the recordings, but the openings were usually infrequent. These openings may correspond to a subconductance state (Hamill and Sakmann, 1981; Auerbach and Sachs, 1984; Colquhoun and Sakmann, 1985), since they were generally associated with an opening to either of the main conductance states (Fig. 2B).

In addition to measurements of conductance, the two channel classes were...
distinguished from one another by an apparent difference in open duration (Fig. 3 and Table I). Individual channel open durations were plotted against their respective amplitudes for a given patch (Fig. 3A). The distribution of open times for the 45-pS channel extended to longer times than did that of the 70-pS channel. This difference was observed in all patches, regardless of the length of time after denervation. The inability to resolve all brief closures, "Nachschlags," from the open state may have led to an overestimation of open duration. In addition, the distributions of open durations for both channel types may be best described by more than one exponential component. This was particularly evident for the open duration distribution for the 45-pS channel, which demonstrated an excess of brief events (Fig. 3C). Despite these uncertainties, mean channel open times for both channel classes were estimated from open duration measurements. The mean channel open time, at a pipette potential of 60 mV, was ~7 ms for the 45-pS channel and 2.5 ms for the 70-pS channel.

To examine whether the channel open time depended on proximity to the synapse, the mean channel open times for all patches were compared in synaptic

**Figure 2.** (A) Amplitude distribution of single channel current events recorded in the on-cell configuration at a pipette potential of +60 mV from the synapse of the fiber shown in Fig. 1. The muscle had been treated with α-BTX and denervated in vivo 14 d before recording. The minimum duration accepted for analysis was 300 μs. (B) Representative single channel current records demonstrating the presence of two distinct classes of channels. Pipette potentials are indicated to the right of each trace. Partial closure to a subconductance level from the 46-pS main state is also evident in the bottom trace (arrowhead). [ACh] = 100 nM. These and all subsequent current records were filtered at 4 kHz. Scale bars: 10 pA, 20 ms. (C) Current vs. voltage plot for the two amplitude classes. Conductances were estimated by linear regression analysis for pipette potentials between +40 and +140 mV.
FIGURE 3. (A) Amplitude vs. duration plot of single channel current events recorded from the synapse of a fiber preblocked with α-BTX and denervated in vivo 14 d before recording. The lower-amplitude channel class was composed of events of appreciably longer duration. $V_{\text{pipette}} = +60$ mV, $[ACh] = 250$ nM. (B) Noncumulative distribution of open durations plotted for the larger-amplitude events shown in A. The distribution was fitted to an exponential curve determined by the time constant corresponding to the mean channel open time of 2.5 ms. (C) Noncumulative distribution of open durations for the low-amplitude events shown in A. The mean channel open time was estimated to be 6.8 ms. The distribution demonstrated an excess of brief events and was not well fitted with a single exponential curve. The minimum duration of events accepted for B and C was 300 μs.

|                  | Conductance | Mean channel open time |
|------------------|-------------|------------------------|
|                  | Low         | High                   |
| Synaptic         |             |                        |
|                  | 45.2±0.8    | 70.0±1.4               |
| (n = 24)         | (n = 25)    |                        |
| Nonsynaptic      | 44.6±1.0    | 68.4±3.1               |
| (n = 10)         | (n = 6)     |                        |

Averaged conductances and mean channel open times are given for ACh-activated single channel currents from receptors synthesized in denervated fib fibers. Channel properties did not change significantly with the length of time after denervation. The slope conductances were estimated by linear regression analysis of current-voltage relations ($V_{\text{pipette}} = +40$ to +140 mV). The mean channel open times were estimated at $V_{\text{pipette}} = +60$ mV by the method of maximum likelihood.
and nonsynaptic recordings. To do this, patches at all times after denervation were included in the same distribution (Fig. 4). The observed distribution of mean open times for synaptic recordings was similar to that of nonsynaptic recordings for each channel class. Moreover, the average mean channel open time for either the 45-pS or the 70-pS channel did not differ significantly between synaptic and nonsynaptic recordings. These data demonstrate that the kinetic properties of the two conductance classes were independent of their location on the muscle cell.

**Openings of the Low-Conductance Channel Predominate Both Synaptically and Nonsynaptically in Denervated Fibers**

Openings by the low-conductance channels predominated at all locations in denervated muscle, including the former synapse (Figs. 2A and 5B). This
contrasts with innervated fibers in which low-conductance events constituted <3% of the openings at both synaptic and nonsynaptic regions (Brehm and Kullberg, 1987) (Fig. 5A). When fibers were examined between 5 and 23 d after denervation, openings of low-conductance channels constituted >50% of all openings in all but four synaptic patches (64 out of 68) and all but three patches from nonsynaptic regions (36 out of 39). In some patches from both synaptic and nonsynaptic membrane, 100% of the openings were of this class. The percentage of openings by the low-conductance channel type increased up to 17 d after denervation (Table II). Recordings at 19–23 d showed a slight reduction in the average percentage of openings by the low-conductance channel in both synaptic and nonsynaptic membrane. This reduction is likely to be the result of transient reinnervation of some of the fibers (see the criteria outlined

![Figure 5](image-url)
in the Methods). When individual fibers that were judged to have been reinnervated were excluded, the percentage of openings of low-conductance channels was ~90% for both synaptic and nonsynaptic recordings (Table II). These values are not significantly different from the values obtained between 5 and 17 d.

After denervation, this time-dependent increase in the proportion of openings by the low-conductance channel was observed in both synaptic and nonsynaptic regions of the fiber. Although there is a trend toward a slightly lower percentage of low-conductance openings at the synapse, when all recordings were included, differences between synaptic and nonsynaptic recordings were significant only at 8 d (see Table II). Therefore, any differences in the proportion of channel types was minimal when examined at the level of populations of fibers.

Further evidence for a similar proportion of low-conductance channels at synaptic and nonsynaptic regions is reflected at the level of the individual fiber. To quantitate the distribution of low-conductance channels at the level of an individual fiber, the percentage of low-conductance channel openings recorded synaptically was divided by the percentage of those recorded nonsynaptically within the same cell. This ratio was not significantly different from 1.0 for any time tested after denervation, except at 8 d ($p < 0.025$) (Fig. 6). Importantly, the few fibers in which openings of the high-conductance channel predominated at the synapse were the same fibers in which this class of channel predominated nonsynaptically (3 out of 4). This comparison within fibers further indicates that the time-dependent changes in channel function described in Table II are not significantly different for synaptic and nonsynaptic receptor channels.
Openings by the high-conductance channel were observed at all times after denervation. These openings constituted >10% of the total as late as 3 wk after denervation. Thus, it is likely that high-conductance channels continue to be synthesized at long times after denervation. However, it is possible that the high-conductance channels are synthesized only for the 2-3-d period before the onset of denervation supersensitivity (Bekoff and Betz, 1977). To address whether high-conductance channels were made after the onset of supersensitivity, muscles were injected with α-BTX 2 d after denervation and recordings were made 3 d later. The percentage of openings caused by activation of the low-conductance receptor was 95 ± 2% (n = 6). This value was significantly greater (p < 0.01) than that for muscles injected before denervation (see Table II, 5 d). Therefore, high-conductance channels that were synthesized after denervation, but before the onset of supersensitivity, may lead us to overestimate the number of high-conductance channels expressed at late times. Importantly, however, blocking receptors with α-BTX at the onset of supersensitivity did not eliminate all opening by the high-conductance channels, which indicates that denervated fibers must continue to synthesize this type of channel.

**Figure 6.** The ratio of the percentage of openings of the 45-pS channel at the synapse to the percentage from nonsynaptic patches for each individual fiber is plotted as a function of time after in vivo denervation (△). Bars indicate the mean ratio. A ratio of 1.0 results when the percentage of openings of low-conductance channels is the same for synaptic and nonsynaptic regions. The mean values are: 5 d, 0.95 ± 0.03 (n = 7); 8 d, 0.77 ± 0.07 (n = 7); 14 d, 0.95 ± 0.02 (n = 6); 19-21 d, 0.95 ± 0.04 (n = 8); 22-23 d, 1.06 ± 0.13 (n = 9). None of the mean values was significantly different from 1.0, except that for 8 d (p < 0.025).
Previous studies on unblocked denervated diaphragm fibers had suggested that as late as 18–20 d after denervation, at least half of the channels at the former synapse had fast kinetics, characteristic of high-conductance channels in innervated muscle (Brenner and Sakmann, 1983). To test whether the discrepancy between these data and the present study could be accounted for, at least in part, by the presence of original synaptic receptors, recordings were made from fibers that had not been preblocked with α-BTX. For this purpose, nonblocked fibers were examined at 19 d after denervation. In these fibers, the percentage of openings at the synapse caused by activation of the 45-pS channels was 61 ± 6% (n = 6), as opposed to 75% for preblocked fibers (Table II). Although most of the original receptors would have been expected to have been degraded by this time (Levitt and Salpeter, 1981), they still constitute an appreciable fraction of receptors inserted at the synapse of denervated fibers.

DISCUSSION

Mammalian skeletal muscle is characterized by ACh receptor aggregates at the region of synaptic contact with nerve. This high density of receptors at the neuromuscular junction (>10⁴/μm²) contrasts with the low receptor density in nonsynaptic membrane (<100/μm²) (Fertuck and Salpeter, 1976). This distribution of ACh receptors was demonstrated for innervated fdb fibers by iontophoresis of ACh (Bekoff and Betz, 1977) and by the pattern of Rh-α-BTX fluorescence (Fig. 1). After denervation, the density of ACh receptors in the nonsynaptic membrane increases ~2 orders of magnitude (Fambrough, 1974), resulting in cells that are "supersensitive" to ACh over their entire length (Ginetzinsky and Shamarina, 1942; Axelsson and Thelesleff, 1959; Miledi, 1960). We observed a time-dependent increase in ACh receptor density in denervated fdb fibers, as indicated by an increased frequency of channel openings in nonsynaptic membrane. The increase in the frequency of channel openings was detectable by 2–3 d after denervation. This time corresponded to the onset of ACh supersensitivity reported for this muscle by Bekoff and Betz (1977).

ACh receptors in nonsynaptic membrane of denervated muscle have a lower single channel conductance and longer mean channel open time than those recorded from the synapse of innervated muscle (Katz and Miledi, 1972; Neher and Sakmann, 1976a, b; Dreyer et al., 1976; Sakmann et al., 1980). Our single channel records from fdb fibers also indicated the appearance of low-conductance (45 pS) channels after denervation of this muscle. The low-conductance channel accounted for <3% of all openings in freshly dissociated, innervated muscle, the balance being provided by openings of the high-conductance (70 pS) channel. However, after denervation, this channel type accounted for >80% of the openings. These single channel data provide further evidence that amplified synthesis of the low-conductance channel is responsible for denervation supersensitivity.

It has been reported that at least half of the channels at the synapse of chronically denervated muscle have fast kinetics characteristic of the high-
conductance channel (Brenner and Sakmann, 1983). This observation suggested that, in denervated muscle, a functional distinction could be made between the type of channel localized to the synapse and that expressed in nonsynaptic membrane. We tested whether this distinction is the result of a functional differentiation imposed upon newly synthesized receptors by blocking pre-existing ACh receptors at the time of denervation. Our results, on fibers that had been preblocked with α-BTX and denervated for 3 wk, indicated that openings of the 45-pS channel predominated in both synaptic and nonsynaptic membrane. A significant difference did exist between the proportion of channel types expressed in synaptic and nonsynaptic regions in fibers denervated for 8 d (Table II). However, for all other times tested, including 3 wk after denervation, no significant differences were observed. Additionally, neither the conductance nor the mean open duration for either channel type differed between synaptic and nonsynaptic membrane. These comparisons indicate that topographical separation of receptor types, if it exists at all, is minimal in denervated fdb fibers. The differences between our results and those of the previous study (Brenner and Sakmann, 1983) could be due to differences in the muscle type or species studied. Alternatively, kinetic data from fluctuation analysis may provide different estimates of proportions of channel types than does single channel recording. However, our findings on chronically denervated fdb fibers in which pre-existing receptors were not blocked indicate that the percent of openings by the 45-pS channel was significantly lower than in preblocked fibers. Therefore, the differences between these two studies can also be explained, at least in part, by the persistence in nonblocked fibers of high-conductance channels that were present at the time of denervation.

The finding that the synaptic region of denervated muscle is not significantly enriched in high-conductance channels when compared with nonsynaptic membrane calls into question the notion that channels are converted to a high-conductance form at the synapse after denervation (Brenner et al., 1983; Brenner, 1985). At no time after denervation did we observe a majority of high-conductance channels at the site of former synaptic contact. Even 3 wk after denervation, which corresponded to the longest time previously examined (Brenner and Sakmann, 1983), we observed >80% low-conductance channels at the synapse. Thus, should conversion to the high-conductance channel type occur, it must do so over a slower time course than that reported for either denervated (Brenner and Sakmann, 1983) or developing muscle (Sakmann and Brenner, 1978; Fischbach and Schuetze, 1980; Michler and Sakmann, 1980). Our data are more consistent with the idea that focal conversion at the synapse does not occur. We must consider the possibility that our synaptic recordings were not made directly from the site of highest receptor density and therefore were not truly "synaptic." If this were the case, we might have missed recording from aggregates of high-conductance channels. This possibility seems unlikely. First, channel activity was much higher in most synaptic patches than in nonsynaptic recordings made close to the synapse. The high frequency of openings would be expected if we were recording from the high-density aggregates at the synapse. In addition, it seems unlikely that we would have missed high-density aggregates in all 93 of the recordings made from synaptic regions.
It is evident from these experiments that the increase in sensitivity to ACh in denervated fibers is due primarily to an increase in the synthesis of low-conductance channels. Nevertheless, even as late as 3 wk after denervation, ~10% of the recorded events are by the high-conductance channel. Our estimates of the proportion of high-conductance channels that were synthesized in denervated fibers are inexact. First, at late times, toxin may have dissociated from the receptors and unmasked high-conductance channels that had been synthesized before denervation. However, not all high-conductance events were due to unmasked receptors, since they were evident in recordings made at times <1 wk after denervation, when dissociation of toxin from the receptor would have been minimal (Bevan and Steinbach, 1983). Second, some high-conductance receptors were synthesized before the increase in receptor synthesis that produces denervation supersensitivity. Nonetheless, high-conductance channels were still evident when receptors were blocked after the onset of denervation supersensitivity. Although it was not possible to tell whether the synthesis of high-conductance channels was amplified by denervation or remained constant, we conclude that fdb fibers do continue to synthesize these channels even at late times after the nerve has been cut.

In conclusion, our data indicate that segregation of channel types does not exist in denervated fdb muscle. Moreover, it is the low-conductance channel that predominates at the synapse at all times as late as 3 wk after denervation. This lack of segregation of channel types also exists in innervated fdb muscle. However, in the case of innervated fibers, it is the high-conductance channels that predominated in both synaptic and nonsynaptic regions (Brehm and Kullberg, 1987). These findings make it unlikely that a mechanism exists by which specific functional properties are conferred only on the channels at the synapse. Instead, our data make it plausible that the functional properties of ACh receptor channels are determined before insertion. Recent studies showing that distinct mRNAs encode the low- and the high-conductance channels in embryonic muscle suggest a role for transcriptional control of channel properties (Mishina et al., 1986). Irrespective of the molecular mechanisms governing ACh receptor function, our data demonstrate that expression of a particular channel type is dependent not on the location along a fiber, but rather on the state of innervation of that fiber.

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REFERENCES
Anderson, C. R., and C. F. Stevens. 1973. Voltage clamp analysis of acetylcholine produced end-plate current fluctuations at frog neuromuscular junction. Journal of Physiology. 235:655–691.
Auerbach, A., and F. Sachs. 1984. Single-channel currents from acetylcholine receptors in embryonic chick muscle. *Biophysical Journal.* 45:187–198.

Axelson, J., and S. Thesleff. 1959. A study of supersensitivity in denervated mammalian skeletal muscle. *Journal of Physiology.* 147:178–193.

Bekoff, A., and W. J. Betz. 1977. Physiological properties of dissociated fibres obtained from innervated and denervated adult rat muscle. *Journal of Physiology.* 271:25–40.

Bevan, S., and J. H. Steinbach. 1983. Denervation increases the degradation rate of acetylcholine receptors at end-plates in *vivo* and *in vitro.* *Journal of Physiology.* 356:159–177.

Brehm, P., Y. Kidokoro, and F. Moody-Corbett. 1984a. Acetylcholine receptor channel properties during development of *Xenopus* muscle cells in culture. *Journal of Physiology.* 357:205–217.

Brehm, P., R. Kullberg, and F. Moody-Corbett. 1984b. Properties of non-junctional acetylcholine receptor channels on innervated muscle of *Xenopus laevis.* *Journal of Physiology.* 350:631–648.

Brehm, P., and R. Kullberg. 1987. Acetylcholine receptors on adult mouse skeletal muscle are functionally identical in synaptic and nonsynaptic membrane. *Proceedings of the National Academy of Science.* 84:2550–2554.

Brenner, H. R. 1985. Trophic control of channel gating? *Nature.* 317:572–573.

Brenner, H. R., Th. Meier, and B. Widmer. 1983. Early action of nerve determines motor endplate differentiation in rat muscle. *Nature.* 305:536–537.

Brenner, H. R., and B. Sakmann. 1983. Neutrotrophic control of channel properties at neuromuscular synapses of rat muscle. *Journal of Physiology.* 357:159–171.

Chang, C. C., and C. Y. Lee. 1963. Isolation of neurotoxins from the venom of *Bungarus multicinctus* and their mode of neuromuscular blocking action. *Archives Internationales de Pharmacodynamie et Therapie.* 144:241–257.

Colquhoun, D., and B. Sakmann. 1985. Fast events in single-channel currents activated by acetylcholine and its analogs at the frog muscle end-plate. *Journal of Physiology.* 369:501–557.

Colquhoun, D., and F. J. Sigworth. 1983. Fitting and statistical analysis of single-channel records. In *Single Channel Recording.* B. Sakmann and E. Neher, editors. Plenum Publishing Corp., New York. 191–264.

Cull-Candy, S. G., R. Miledi, and O. D. Uchitel. 1982. Properties of junctional and extrajunctional acetylcholine-receptor channels in organ cultured human muscle fibres. *Journal of Physiology.* 355:251–267.

Dionne, V., and M. Leibowitz. 1982. Acetylcholine receptor kinetics. A description from single-channel currents at snake neuromuscular junctions. *Biophysical Journal.* 39:253–261.

Dreyer, F., C. Walther, and K. Peper. 1976. Junctional and extrajunctional acetylcholine receptors in normal and denervated frog muscle fibres. *Pflügers Archiv.* 366:1–9.

Fambrough, D. M. 1974. Acetylcholine receptor: revised estimates of extrajunctional receptor density in denervated rat diaphragm. *Journal of General Physiology.* 64:468–472.

Fertuck, H. C., and M. M. Salpeter. 1976. Quantitation of junctional and extrajunctional acetylcholine receptors by electron microscope autoradiography after 125I-a-bungarotoxin binding at mouse neuromuscular junctions. *Journal of Cell Biology.* 69:144–158.

Fischbach, G. D., and S. M. Schuetze. 1980. A post-natal decrease in acetylcholine channel open time at rat end-plates. *Journal of Physiology.* 503:125–137.

Ginetzinsky, A. G., and N. M. Shamarniy. 1942. The tonomotor phenomenon in denervated muscle. *Uspekhi Sovremennoi Biologii.* 15:283–294.
Hamill, O., and B. Sakmann. 1981. Multiple conductance states of single acetylcholine receptor channels in embryonic muscle cells. Nature. 294:462-464.

Katz, B., and R. Miledi. 1972. The statistical nature of the acetylcholine potential and its molecular components. Journal of Physiology. 224:665-699.

Kuffler, S. W., and D. Yoshikami. 1975. The distribution of acetylcholine sensitivity at the post-synaptic membrane of vertebrate skeletal twitch muscle: iontophoretic mapping in the micron range. Journal of Physiology. 244:703-730.

Lee, C. Y. 1972. Chemistry and pharmacology of polypeptide toxins in snake venoms. Annual Review of Pharmacology. 12:265–286.

Lee, C. Y., L. F. Tsien, and T. H. Chiu. 1967. Influence of denervation on localization of neurotoxins from cladid venoms in rat diaphragm. Nature. 215:1177–1178.

Leonard, R. J., S. Nakajima, Y. Nakajima, and T. Takahashi. 1984. Differential development of two classes of acetylcholine receptors in Xenopus muscle in culture. Science. 226:55-57.

Levitt, T. A., and M. M. Salpeter. 1981. Denervated endplates have a dual population of junctional acetylcholine receptors. Nature. 291:239–241.

Michler, A., and B. Sakmann. 1980. Receptor stability and channel conversion in the subsynaptic membrane of the developing mammalian neuromuscular junction. Developmental Biology. 80:1–17.

Miledi, R. 1960. The acetylcholine sensitivity of frog muscle fibres after complete or partial denervation. Journal of Physiology. 151:1–23.

Miledi, R., and O. D. Uchitel. 1981. Properties of postsynaptic channels induced by acetylcholine in different frog muscle. Nature. 301:162–165.

Mishina, M., T. Takai, K. Imoto, M. Noda, T. Takahashi, S. Numa, C. Methfessel, and B. Sakmann. 1986. Molecular distinction between fetal and adult forms of muscle acetylcholine receptor. Nature. 321:406–411.

Neher, E., and B. Sakmann. 1976a. Noise analysis of drug induced voltage clamp currents in denervated frog muscle fibres. Journal of Physiology. 258:705–729.

Neher, E., and B. Sakmann. 1976b. Single-channel currents recorded from membrane of denervated frog muscle fibres. Nature. 260:799–802.

Sakmann, B., and H. Brenner. 1978. Change in synaptic channel gating during neuromuscular development. Nature. 276:401–402.

Sakmann, B., J. Patlak, and E. Neher. 1980. Single acetylcholine-activated channels show burst kinetics in presence of desensitizing concentrations of agonist. Nature. 286:71–73.

Siegelbaum, S., A. Trautmann, and J. Koenig. 1984. Single acetylcholine-activated channel currents in developing muscle cells. Developmental Biology. 104:366–379.

Steele, J. A., and J. H. Steinbach. 1986. Single channel studies reveal three classes of acetylcholine-activated channels in mouse skeletal muscle. Biophysical Journal. 49:361a. (Abstr.).