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Initial synaptic transmission at the growth cone in *Xenopus* nerve–muscle cultures

(end-plate potentials/cell culture/filopodia/quantum size/binomial statistics)

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ABSTRACT **The excellent visibility of cultured cells allows the early events during formation of the neuromuscular junction to be suitably studied. It has been shown in various culture systems that synaptic transmission occurs early after nerve–muscle contact. Early synaptic potentials are small in amplitude and slow in time course reflecting a low acetylcholine receptor density at the site of nerve contact. Acetylcholine receptors accumulate later at the contact region. We have examined initial synaptic transmission at the growth cone–muscle contact in *Xenopus* nerve–muscle cultures. The approaching growth cone was observed under a phase-contrast microscope while the membrane potential of its target muscle cell was continuously monitored by using an intracellular microelectrode. The innervating neuron was stimulated extracellularly at the cell body. No synaptic potential was evoked when the growth cone was contacting the muscle only at the tip of filopodia. However, as soon as the main portion of the growth cone contacted the muscle membrane, nerve-evoked synaptic potentials were detected after stimulation of the nerve. This immediate appearance of synaptic potentials raises the possibility that acetylcholine could be released at the growth cone even prior to contact with muscle cells. As the area of contact enlarged during the observation period the amplitude of end-plate potentials also increased. Spontaneous synaptic potentials (miniature end-plate potentials) were rarely observed in these early growth cone–muscle contacts. Although there were several inherent difficulties, quantal analysis of the end-plate potentials was attempted by using binomial statistics. This analysis suggests that nerve-evoked transmitter release at the growth cone occurs in a quantal fashion.**

Functional synaptic transmission occurs early during formation of the neuromuscular junction, shortly after nerve–muscle contact (1–5). For example, in chicken nerve–muscle cultures the growth cones form functional synaptic contacts but the amplitudes of the nerve-evoked synaptic potentials are vanishingly small (50 μV on average) and only detectable after summation of the signal (3). The small amplitudes of these early synaptic potentials could be accounted for by relatively low acetylcholine (AcCho) sensitivities of chicken myotubes at regions of low AcCho receptor density, although one cannot exclude the possibility that the amount of AcCho released at the growth cone is smaller. Studies on *Xenopus* nerve–muscle cultures have shown that early spontaneous synaptic potentials (miniature end-plate potentials, MEPPs) are small in amplitude and slow in time course, probably reflecting a low AcCho receptor density at the contact area. Indeed, staining AcCho receptors with tetramethylrhodamine-conjugated α-bungarotoxin revealed that at this stage the AcCho receptor density is no higher at the site of nerve contact than in the surrounding region (6, 7). Because this indicates that spontaneous release of AcCho can be detected at any part of the muscle membrane in *Xenopus* cultures, in contrast to chicken, we sought to detect directly the earliest synaptic transmission at the growth cone and to test whether transmitter release occurs in a quantal fashion.

We have examined initial synaptic transmission at the growth cone–muscle contact in *Xenopus* nerve–muscle cultures. The approaching growth cone was observed under a phase-contrast microscope while the membrane potential of its target muscle cell was continuously monitored and the innervating neuron was stimulated at the cell body. Nerve-evoked synaptic potentials were measured shortly after a growth cone contacted the muscle membrane. However, spontaneous synaptic potentials were rarely observed at these early growth cone–muscle contacts. Although there were several inherent difficulties, fluctuation of the EPP amplitude was analyzed by using binomial statistics. The analysis showed that the nerve-evoked transmitter release at the growth cone appears to occur in a quantal fashion. Because AcCho release was detected immediately after nerve–muscle contact, it raises the possibility that the growth cone releases AcCho even prior to muscle contact. A preliminary report on this account has been published (8).

METHODS

Dissociation and culture procedures for *Xenopus* nerve and muscle cells have been described (7). Muscle cells were grown for 3–4 days prior to addition of nerve cells. During this period yolk granules in the muscle cells were consumed and cross-striations developed. One day after addition of nerve cells electrophysiological recordings were carried out. The saline used during recording had the following composition: 67 mM NaCl/1.6 mM KCl/2 mM glucose/0.1% (wt/vol) bovine serum albumin (Sigma)/8 mM Hepes/NaOH, pH 7.4. Divalent cations in the saline were either 4 mM $CaCl_2$ and 6 mM $MgCl_2$ or 10 mM $CaCl_2$ and 0 mM $MgCl_2$. The higher concentration of calcium ions was used to facilitate the detection of early EPPs and to obtain stable microelectrode penetration of the muscle cell. The muscle membrane potential was measured with a conventional intracellular microelectrode filled with 4 M potassium acetate (80–100 MΩ) and was recorded continuously on moving film while the neuron was stimulated. The recording noise was usually 200–300 μV peak-to-peak. Neurons were stimulated extracellularly at the cell body with a rectangular pulse, 0.1 msec in duration and 0.1–1.4 μA in intensity, through a saline-filled electrode that had an inner tip diameter of 2–3 μm. Stimuli were delivered approximately once every 5 sec. Cells were visualized with a ×40 water-immersion phase-contrast objective and ×10 ocular lenses (Zeiss). All experiments were carried out at room temperature (21–24°C).

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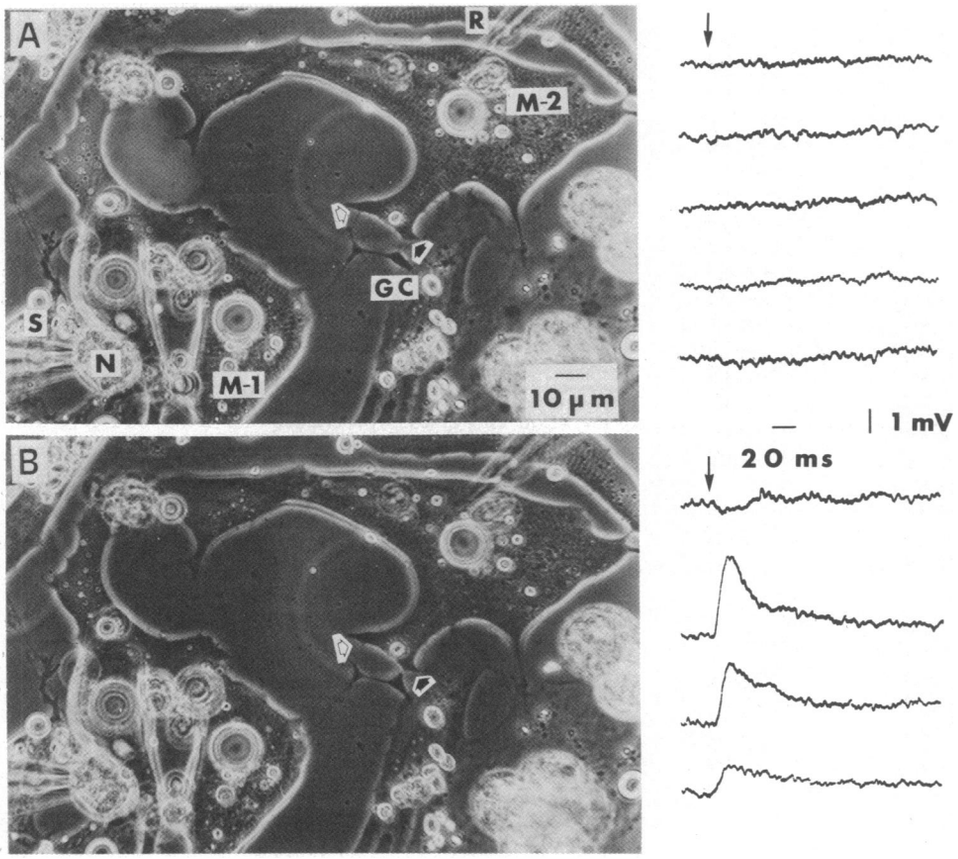
Abbreviations: AcCho, acetylcholine; EPP, end-plate potential; MEPP, miniature end-plate potential.

RESULTS

Movement of the Growth Cone in *Xenopus* Cultures. The growing tip of the neurite, the growth cone, had several thin spikes (filopodia) at the leading edge that were incessantly undergoing movement. The length of filopodia was variable and averaged 9 μm in these *Xenopus* cultures. Some of the growth cones had a ruffle extending about 8 μm from the edge. About one-fourth (7/25) of the observed growth cones was advancing at a speed of 1.2 μm/min while others (14/25) were stationary or had swinging extensions. The rest (4/25) showed a retreating movement. This behavior and the shape of growth cones are similar to those reported (9).

Initial Synaptic Transmission at the Growth Cone. Fig. 1*A* shows a typical nerve–muscle contact studied in these experiments. A neuron (N) had an established contact (not visible in this plane of focus) with one muscle cell (M-1) and its neurite extended beyond this cell. The growth cone (GC) was approaching or contacting at its tip a second muscle cell (M-2) that had no other visible nerve contact. When the nerve cell body was stimulated with a saline-filled electrode (S), the first muscle cell (M-1) twitched synchronously with the stimulus pulses. These twitches disappeared when 3.1 μM tetrodotoxin or 5 μM *d*-tubocurarine was included in saline. Because these *Xenopus* muscle cells in culture twitch with large synaptic potentials even without action potentials in the muscle membrane, this indicated that the twitches were mediated by propagating action potentials along the nerve and that the neuron was cholinergic. This procedure facilitated the efficiency of these experiments because about half of the neurons in these cultures was not cholinergic and it was useless to wait for the growth cone of noncholinergic neurons to contact muscle cells. The target muscle cell (M-2) was then penetrated with a recording microelectrode (R) to measure the nerve-evoked synaptic potentials.

When the growth cone contacted the target muscle cell (M-2) only with its filopodia (arrows in Fig. 1*A*), no synaptic potentials were recorded in the muscle cell (M-2) even if the proximal muscle cell (M-1) twitched when the neuron was stimulated (Fig. 1*A*; oscilloscope traces are shown at the right). This indicated that stimulation was effective and the action potential propagated at least along the nerve contacting the proximal muscle cell (M-1). A similar observation was confirmed in four other cases. As soon as the leading edge of the growth cone



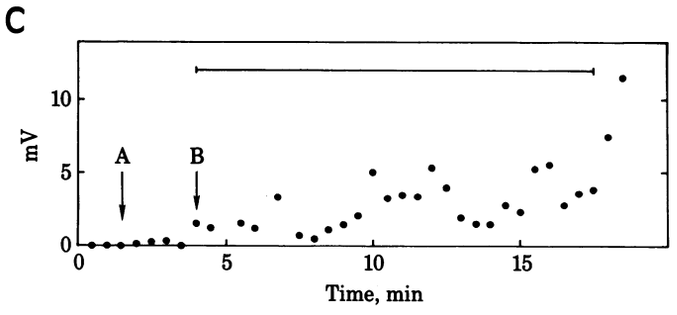


FIG. 1. (*A* and *B*) Phase-contrast micrographs of a *Xenopus* nerve–muscle culture and EPPs in the muscle cell with growth cone contact. *A* shows an experimental situation in which a neuron (N) contacts one muscle cell (M-1) (contacts are not visible in this plane of focus) and extends a neurite beyond it. A growth cone (GC) at the tip of the neurite contacts another muscle cell (M-2) with filopodia (arrows). The neuron was stimulated extracellularly with a glass pipette (S) and the muscle membrane potential was recorded with a microelectrode (R) in the muscle cell (M-2). This picture was taken while oscilloscope traces to the right were recorded. No EPPs were seen during five consecutive traces. The stimulus was delivered every 5 sec (at downward arrows). *B* was taken ≈2½ min later than *A*. The leading edge of the growth cone contacted the muscle cell (M-2) (filled arrow). At the same time EPPs were observed in three of four consecutive stimulations (shown to the right). Calibration bar = 10 μm in *A* and *B* micrographs. (*C*) Sequential changes of EPP amplitude during observation period. Six consecutive EPPs were averaged and plotted. Arrows indicate the times when micrographs and oscilloscope traces in *A* and *B* were taken. The horizontal bar indicates the period during which quantal analysis was performed. All records were taken from cell no. 4 in Table 1. The resting potential was −73 mV.

contacted the muscle cell (Fig. 1*B*; the filled arrow is pointing to the part of the growth cone where a morphological change occurred), synaptic potentials could be recorded (Fig. 1*B*; oscilloscope traces are shown at the right). However, during successive stimulations, stimuli frequently failed to evoke EPPs. These failures were not due to failure of stimulation at the nerve cell body because the proximal muscle cell (M-1) twitched each time a stimulus pulse was delivered. Therefore, these failures result either from the failure of transmitter release at the growth cone or from conduction block distal to the proximal muscle (M-1).

The amplitude histogram of EPPs from the cell identified in Fig. 1 is shown in Fig. 2*A*. The amplitude varied in a wide range. The histogram was skewed toward larger amplitudes and, in spite of numerous failures, did not form clear symmetrical peaks as seen at the adult neuromuscular junction (10, 11).

Spontaneous synaptic potentials were rarely observed during the period between stimuli at these early cone–muscle contacts. However, in two cases where the area of contact was relatively large, sufficient numbers of spontaneous synaptic potentials were recorded to be analyzed, as will be described later.

In three of six cases successfully studied for a long period of time, the amplitude of EPPs increased (Fig. 1*C*), but in the other three cells it remained unchanged during observation periods as long as 27 min. Because the contact area between the growth cone and the muscle often increased during the recording period, this increase in the amplitude of EPPs is likely to be the result of an increase in the contact area.

Estimation of the Quantum Size. We wished to test whether the amplitude fluctuations were due to the quantal nature of EPPs. Because there were practically no MEPPs detected during the observation period, the estimation of the quantum size from the mean MEPP amplitude cannot be done. To circumvent this problem we mechanically agitated the nerve with the stimulus electrode at the end of EPP recording. When the nerve was pulled or pressed with an electrode ≈50 μm away from the growth cone, synaptic potentials occurred in a burst. During this period the proximally located muscle (M-1 in Fig. 1*A*) often twitched, which was probably caused by antidromically propagated action potentials. After the initial burst the synaptic potentials were seen sporadically for a few minutes and then they decreased in frequency to essentially disappear. We measured these synaptic potentials excluding those occurring during the initial burst, which were probably caused by depolarization of the growth cone by the mechanical agitation procedure.

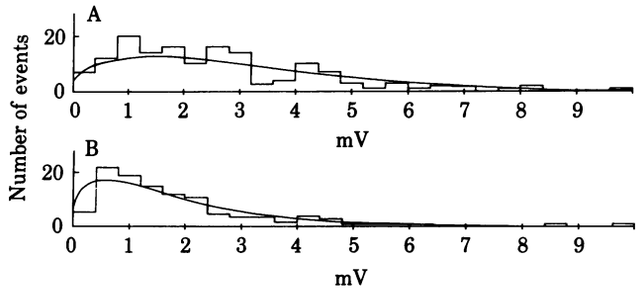


FIG. 2. Amplitude frequency histograms of EPPs (*A*) and synaptic potentials after agitation (*B*). These data were taken from cell no. 3 in Table 1. Solid curves are drawn by using a γ function. For the histogram (*B*), the following γ function was drawn:

$$P(x) = \frac{\lambda^k}{\Gamma(k)} e^{-\lambda x} x^{k-1},$$

in which $P(x)$ is a probability density of the synaptic potential with an amplitude of x mV; $\lambda = v/s^2$; and $k = v^2/s^2$; v and s are defined in the legend to Table 1. In this case, $\lambda = 0.7$ and $k = 1.4$. $\Gamma(k)$ = a γ function with $\lambda = 1$. For the EPP histogram, the following equation was drawn:

$$P(x) = \sum_{r=1}^{n} \binom{n}{r} p^r (1-p)^{n-r} \frac{\lambda^{kr}}{\Gamma(kr)} e^{-\lambda x} x^{kr-1},$$

in which the same notation and the same values as above are used. p and n are defined in the legend to Table 1. In this case, $p = 0.80$ (representing p_1 and p_2) and $n = 2$ (representing n_1 and n_2).

The amplitude distribution of these synaptic potentials is shown in Fig. 2*B*. The histogram was skewed and there were many synaptic potentials whose amplitude was close to the recording noise level (200–300 μV). The shape of the histogram was similar to those of MEPPs at early nerve–muscle contacts (6, 7). In two cases where the contact area between the growth cone and the muscle cell was relatively large, a sufficient number of spontaneous synaptic potentials was recorded before agitation of the neurite. These spontaneous synaptic potentials were previously shown to be predominantly MEPPs (7). After recording MEPPs and nerve-evoked EPPs, the nerve was agitated in the manner described above and resulting synaptic potentials were measured. Thus, in these two cases we compared MEPPs and synaptic potentials that occurred after mechanical agitation. In one case, the average MEPP amplitude (mean ± SD) was 1.9 ± 2.1 mV ($n = 56$) before and 3.1 ± 2.2 mV ($n = 95$) after agitation. In the other case, the respective values were 1.3 ± 2.4 mV ($n = 62$) and 1.8 ± 2.2 mV ($n = 23$). In both cases, the average synaptic potential was slightly larger after agitation but the shape of the amplitude histogram was similarly skewed before and after agitation. Although origin of these synaptic potentials is unclear, we used their mean value for the quantum size and the variance in the following analyses.

Quantum Analysis of Nerve-Evoked EPPs at the Growth Cone. We analyzed the amplitude distributions of the evoked EPPs to determine whether the variance in EPP amplitude resulted from fluctuations in the number of quanta released in each EPP and variations in the amplitude of quantum size. The analysis also provided estimates of the probability (p) that a quantum is released after a stimulus and of a parameter (n), which may correspond to the number of available quanta or to the number of release sites (11). The ratio of the mean EPP amplitude to the mean quantal size is the average number of quanta per evoked EPP (quantal content, m). n is then equal to m/p. The release probability (p) was calculated by two approaches (12, 13). The first calculation was based on the number of failures of evoked release after a stimulus by using the following equation (13).

$$\ln(1 - p_1)/\ln(n_0/N) = p_1/m, \quad [1]$$

in which N is the number of stimulus trials and n_0 is the number of failures. Here we assumed that the failures are due to a failure to release transmitter and not due to conduction block. Values for p_1 thus calculated for six cells are listed in Table 1. It should be noted that this estimate of p does not require assumptions about the distribution profile of the quantum size. The large value of p_1 (0.62) is probably due to the high concentration of Ca ions (10 mM or 4 mM) used to facilitate detection of the earliest EPPs. The value for n_1 was small (Table 1).

The second estimate of p (p_2) is calculated from the mean and the variance of EPPs and synaptic potentials that occurred after mechanical agitation.

$$p_2 = 1 - (S^2/vV) + s^2/v^2, \quad [2]$$

in which v and s^2 are the mean and the variance of the quantum

Table 1. Statistical parameters of synaptic potentials recorded in muscle cells with a growth cone contact

	Synaptic potentials after agitation			EPP				Binomial statistics				
Cell	v	s	n	V	S	N	n_0	m	p_1	p_2	n_1	n_2
1*	1.6	0.9	19	1.9	1.6	350	50	1.2	0.65	0.41	1.8	2.9
2*†	0.5	0.3	23	0.7	0.6	322	63	1.3	0.38	0.41	3.4	3.2
3	1.9	1.6	112	2.8	2.2	154	5	1.5	0.90	0.80	1.7	1.9
4†	0.5	0.2	27	0.5	0.4	110	22	1.0	0.65	0.49	1.5	2.0
5	1.8	1.5	151	1.8	2.0	79	11	1.0	0.78	0.44	1.3	2.3
6	0.3	0.1	17	0.3	0.2	168	40	1.2	0.34	0.55	3.5	2.2
Mean								1.2	0.62	0.52	2.2	2.4

The synaptic potentials were evoked by electrical stimulation of the neuron or by mechanical agitation of the neurite. Parameters for binomial statistics were calculated according to the following equations (13, 14): $m = V/v$; Eq. 1; $p_2 = 1 - S^2/(vV) + s^2/v^2$; $n_1 = m/p_1$; $n_2 = m/p_2$. n is the number of synaptic potentials; v and s^2 are the mean and variance of synaptic potentials after mechanical agitation; V and S^2 are the mean and variance of EPPs; n_0 is the number of failures; N is the number of trials. Because the amplitude distribution of synaptic potentials after agitation is skewed toward larger values, these equations, which are derived assuming normal distribution, might not be justified for use in this preparation. However, Robinson (14) showed that even when a skewed γ function was used to simulate the MEPP amplitude distribution, the above equations were still valid. When the EPP amplitudes were increasing as shown in Fig. 1*C*, the middle portion (indicated by a horizontal bar) was chosen for quantal analysis. Cells were measured in saline containing 10 mM $CaCl_2$, unless indicated otherwise.

* Cells were examined in saline containing 4 mM $CaCl_2$ and 6 mM $MgCl_2$.

† Cells were examined after hyperpolarizing the cell membrane by ≈15 mV to increase signal-to-noise ratio. Because all synaptic potentials were <10 mV, correction for nonlinear summation was not done.

size and V and S^2 are the mean and the variance of EPPs. This estimate of p requires knowledge about the distribution of the quantum size. The amplitude histogram of synaptic potentials that occurred after agitation was skewed and non-Gaussian, but it could be described by a γ distribution (see Fig. 2*B*). Robinson (14) has shown that Eq. 2 is applicable when the quantal size distribution is described by a γ function. Values for p_2 thus calculated are listed in Table 1. If transmitter release during EPPs occurs in a binomial fashion and if the above mentioned assumptions are valid, then p_1 should equal p_2. In fact, in the crayfish neuromuscular junction a close correlation between p_1 and p_2 values was shown by Wernig (13). In the case of synaptic contact between the growth cone and the muscle cell the value of p_1 is reasonably close to that of p_2 (Table 1), in spite of various difficulties as mentioned in *Discussion*. The small values of n_1 and n_2 probably reflect the small contact area.

DISCUSSION

Early events during neuromuscular junction formation have been studied by Cohen (3) in chicken cultures. Within 1 hr of nerve–muscle contact, the synaptic potentials were small (≈50 μV on average) and were only detected by using the signal-averaging technique. The smaller synaptic potentials observed in chicken cultures compared to those seen here in *Xenopus* nerve–muscle cultures are probably due to the low AcCho sensitivity of chicken myotubes (280 mV/nC) (15). Due to the high input impedance of *Xenopus* myotomal cells in culture (78 MΩ) (7), the AcCho sensitivity (2,400 mV/nC in nonjunctional area) (16) is high enough to record directly the initial transmitter release. The average amplitude of synaptic potentials from the growth cone that occurred after mechanical agitation of the nerve was 1.1 mV, which is similar to that of MEPPs before AcCho receptor accumulation (6, 7). If we assume that this value represents a single quantum, then the amount of AcCho released per quantum at the growth cone in *Xenopus* nerve–muscle cultures is not smaller compared to that at the later stages. It is still possible that the number of AcCho molecules per quantum might be smaller in the growth cone of cultured chicken neurons. However, even in chicken cultures, on rare occasions spontaneous synaptic potentials were directly observed 22 min after nerve–muscle contact (17). In this case the growth cone might have contacted a preexisting region of high AcCho receptor density, a so-called "hot spot," which has an AcCho sensitivity roughly 10 times higher than the background (18, 19). Therefore, it is possible that the number of AcCho molecules released per quantum at the growth cone is similar in *Xenopus* and chicken cultures and the difference in the amplitude of initial synaptic potentials can be solely explained by the difference in the AcCho sensitivity in the postsynaptic muscle cell.

We could not accurately determine the interval between the initial contact of filopodia with the muscle membrane and the onset of synaptic transmission because the tip of the filopodium was beyond the resolution of our phase optics at the halo of the muscle cell. Therefore, we estimated the interval in a somewhat indirect manner. We observed that an active growth cone moves at a speed of 1.2 μm/min in our cultures and that the average length of the filopodia is 9 μm. (The tip of the filopodia can be resolved on the substrate but not in the vicinity of cells.) The interval between the initial contact of filopodia and the contact of the leading edge of the growth cone will then be estimated as 7.5 min on average. However, in some cases, the growth cones never reached the muscle cells even after contact by filopodia. We did not observe EPPs when the growth cone contacted the muscle membrane only with filopodia. Because there is a positive correlation between quantum content of EPPs and synaptic contact area in the adult neuromuscular junction (20), it is plausible that contact area of the filopodia is too small to evoke EPPs during the period under observation. In half of the cases shown in Table 1 the amplitude of EPPs became larger while the contact area between the growth cone and the muscle cell probably increased. Thus, the quantum content of EPPs may be proportional to contact area in this case also.

There were several inherent difficulties in applying quantal analysis to the growth cone–muscle contact. Because there were practically no MEPPs during the early contacts we could not estimate the quantum size from the mean MEPP amplitude. Therefore, we used the average size of synaptic potentials that occurred after mechanical agitation of the nerve. These synaptic potentials probably resulted from depolarization of the growth cone but not from action potentials, because similar synaptic potentials were also evoked in the presence of 3.1 μM tetrodotoxin which is known to block sodium-dependent action potentials (21). The average size of these synaptic potentials was

slightly larger than that of MEPPs in two cases in which both MEPPs and synaptic potentials after agitation were compared, and it is also likely that some small synaptic potentials were undetected due to recording noise. Therefore, the average amplitude of these synaptic potentials that occurred after agitation is probably an overestimate of the true quantum size at the growth cone–muscle contact.

The second difficulty came from the fact that the amplitude distribution of both synaptic potentials that occurred after mechanical agitation and MEPPs at early nerve–muscle contacts was skewed and cannot be described by a Gaussian distribution. To simulate the skewed amplitude distribution we used a γ function that has been applied previously to described MEPP amplitude distributions in the guinea pig superior cervical ganglion (22) and for which the mathematical derivation for p and n estimates has been worked out (14). This function roughly simulates the amplitude distribution of synaptic potentials after agitation as shown in Fig. 2*B*.

The third difficulty was that in half the cases there was a progressive increase in the EPP amplitude during the observation period. Because the quantal analysis has to be carried out at the steady state, we chose a section of records where the progressive change was minimal (indicated by a horizontal bar in Fig. 1*C*). However, this procedure limited the sample size available for quantal analysis.

In spite of these difficulties, the estimated values for p_1 and p_2 were reasonably close. This suggests that the amplitude of evoked EPPs at the initial nerve–muscle contact fluctuates stochastically in a quantal fashion.

The morphological correlate of quantal release in the form of clear synaptic vesicles has not been observed in the growth cone of cholinergic neurons (23), but it may be difficult to distinguish synaptic vesicles from other smooth membranous elements in the growth cone. In the early functional synaptic contacts in culture the nerve terminal contains only a few synaptic vesicles of 50 nm in diameter (24). In the case of sympathetic neurons, adrenergic dense-core vesicles have been found in the growth cone (25). Therefore, our results do not contradict the notion that AcCho molecules are contained in synaptic vesicles and are released in a quantal fashion.

We have previously shown that there is no characteristic ultrastructural differentiation at early functional synaptic contacts in rat nerve–muscle cultures (24). It was speculated that close apposition of suitable nerve and muscle cell could be sufficient to initiate synaptic transmission (24). In support of this idea, we have observed nerve-evoked EPPs immediately after growth cones first contact muscle cells. Thus, it is plausible that the nerve also releases AcCho prior to contact with the muscle cell. More specific interactions between nerve and muscle must occur at later stages.

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