Release properties of isolated neuromuscular boutons of the garter snake

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1. Motor nerve terminals innervating fibres in the transversus abdominis muscle of the garter snake comprise discrete boutons. Using a combination of enzymatic digestion and mechanical manipulation, individual boutons were removed from living terminals for study in isolation.

2. Boutons freed from terminals were usually allowed to remain in their original location on the endplate ('attached' one-bouton synapse). Alternatively, they were removed from the endplate, and then placed on the same or another vacant endplate site to form a 'reconstructed' one-bouton synapse. When removed from the endplate, boutons were 2–4 μm in diameter and nearly spherical in shape, in contrast to the variety of complex shapes seen among boutons still in contact with muscle fibre endplates.

3. Transmitter release was assessed by intracellular recording from the postsynaptic fibre. Boutons produced spontaneous miniature endplate potentials (MEPPs) of nearly normal amplitude; extracellular stimulation elicited endplate potentials (EPPs) which resembled MEPPs. Typical EPP amplitudes fluctuated between zero and five quanta per stimulus. For low-frequency stimulation under normal physiological conditions, mean quantal content, m, averaged 1·4; the binomial number of release sites, n, averaged 2·4; and the binomial probability of release, p, averaged 0·57. Statistics of the quantal fluctuations recorded from single boutons agreed only approximately with predictions of simple binomial theory, the discrepancy being that the apparent number of quanta released exceeded n in 5% of the events.

4. In separate experiments, activity-dependent probes were used to locate rare naturally occurring nerve terminals comprising a single bouton. Activation of these small synapses evoked quantal responses similar to those of attached and reconstructed one-bouton synapses described above.

Katz's theory of synaptic transmission stated that individual quanta are released from discrete presynaptic sites at the frog neuromuscular junction (NMJ) and, by extension, at other fast chemical synapses as well (del Castillo & Katz, 1954; Katz, 1969; reviewed by Kelly, 1993; Van der Kloot & Molgó, 1994). The theory addressed statistics of release from many sites at once rather than release from a single site, in part because the behaviour of a single quantum was inaccessible to direct study.

Despite much progress in the cell and molecular biological properties of nerve terminals, physiological study of release at the level of one or a few putative sites has remained impeded by the lack of a suitable experimental preparation. Observations have been successful primarily in larger non-neuronal cells and in a recent preparation comprising 'giant terminals' of retinal bipolar neurons (von Gersdorff & Matthews, 1994). However, release in these cases is not rapidly triggered by an action potential invading the cell or terminal, which is the case at fast conventional synapses to which the Katz theory specifically applies. Fast synapses include many in the central nervous system (CNS), which would seem ideal for physiological study because they contain only one or a few terminal boutons, the smallest discrete anatomical structure associated with release. However, central synapses occur at varying electrotonic distances from the recording site (the cell body), making postsynaptic currents produced by different release sites non-equivalent. Moreover, central postsynaptic receptors may saturate (Jack, Redman & Wong, 1981), meaning that quantization of synaptic current occurs postsynaptically as well as presynaptically, thereby complicating interpretation of data (Korn & Faber, 1991). Because of these and other considerations, quantal analysis of central synapses, though successful, has been subject to interpretation (e.g. Korn, Mallet, Triller & Faber, 1982; Redman & Walmsley, 1983; Grantyn, Shapovalov & Shiriaev, 1984; reviewed by Korn & Faber, 1991). Problems associated with central synapses are eliminated at the NMJ, where all release sites oppose

clustered (and therefore isopotential) receptors. But most NMJs lack boutons, instead containing hundreds of sites within a large continuous terminal, with hundreds of quanta released per stimulus. While activation of a small portion of the terminal is possible, the number of active zones (putative release sites) involved is not directly known (e.g. Wernig, 1975). Thus neither CNS nor neuromuscular terminals have provided satisfactory experimental access to a small number of conventional release sites.

We describe here a neuromuscular preparation with the potential to combine advantages of both central and nerve–muscle synapses for the physiological study of release. The transversus abdominis muscle of the garter snake comprises a single layer of muscle fibres in which virtually all NMJs are accessible for experimental manipulation (Wilkinson & Lichtman, 1985). Unlike NMJs of mammals or amphibians, those of the snake contain clusters of about twenty (tonic) or about sixty (twitch) discrete boutons, similar to mammalian CNS boutons (see Kuffler & Yoshikami, 1975). By adapting a recent technique for removing and reattaching nerve terminals at snake NMJs (synaptic 'reconstruction'; Wilkinson & Lunin, 1994), we have devised a method to remove all terminal boutons innervating an endplate save one, thus creating a one-bouton NMJ. Electrophysiological study of this small synapse permits analysis of release from sites within a single bouton while maintaining the advantages associated with the study of NMJs. In addition, we have used activity-dependent staining (Lichtman, Wilkinson & Rich, 1985; Betz, Mao & Bewick, 1992) to locate and study rare naturally occurring one-bouton terminals at polyneuronally innervated tonic muscle fibre endplates. The behaviour of reconstructed and naturally occurring one-bouton NMJs was similar, suggesting that the former behave physiologically. Data suggest that release is approximately binomial at the microscopic level, except that there is a bias towards use of fewer than the anatomically demonstrable number of release sites. Single-bouton reconstructed synapses may serve as a useful tool to compare synaptic structure and function at the level of a few release sites. Part of the work described has appeared in abstract form (Wilkinson, 1988; Wilkinson & Lunin, 1992).

METHODS

Garter snakes (*Thamnophis sirtalis*) were submerged in iced water (10 min) and killed by rapid decapitation. The transversus abdominis muscle together with its segmental muscle nerve was dissected from the animal and placed in reptilian saline solution. Details of the dissection procedure and composition of the saline solution appear elsewhere (Wilkinson & Lichtman, 1985; Lichtman & Wilkinson, 1987). The transversus abdominis is a predominantly single-fibre-thick muscle which contains ~100 fibres of three types: fast twitch (F), slow twitch (S) and tonic (T) (Wilkinson & Lichtman, 1985; Wilkinson & Nemeth, 1989). The muscle is innervated on its ventral side; this side was placed facing up in an experimental chamber on the stage of an inverted microscope equipped with Nomarski optics. Thus all NMJs in the muscle were visible and accessible for manipulation. Twitch (F and S) NMJs contain about sixty boutons supplied by a single motor axon, while tonic (T) NMJs contain about twenty boutons supplied by between one and three axons (Wilkinson & Lichtman, 1985). Because nerve terminals were viewed from below (through the muscle fibre), those innervating the more transparent type F twitch fibres (which contain few refractile lipid particles; Wilkinson & Nemeth, 1989) were most visible and were usually chosen for the formation of one-bouton reconstructed NMJs, although type S fibres were occasionally used. Tonic fibres receive five to seven NMJs spaced evenly along their length, about one-half of which are supplied by more than one axon (Lichtman *et al.* 1985; Wilkinson & Lichtman, 1985). Tonic NMJs were also difficult to visualize due to lipid particles. They were not used to form one-bouton NMJs, but were studied in separate experiments as described below.

Preparation of one-bouton NMJs

We developed previously a technique for removing entire living nerve terminals from endplate sites; the terminals were then replaced onto their original endplate site, or placed onto another vacant endplate site, to form 'reconstructed' NMJs. The method comprised treatment of the entire preparation with bath-applied collagenase, followed by application of protease to a single NMJ via a microperfusion system (details in Wilkinson & Lunin, 1994). Protease solution was perfused until a slight increase in Nomarski contrast of the NMJ was noted. Perfusion was then continued for an additional ~60 s, at which time the terminal was mechanically manipulated (by gentle tugging of its axon branch) from its endplate. The duration of protease perfusion was found to be critical. Too long an exposure resulted in dissociation of the terminal with little need for tugging, but the terminal was no longer functional. Conversely, tugging after too short an exposure would fail to free the terminal. However, tugging after an exposure to protease which was slightly too short (i.e. by 5–15 s) freed most of the terminal but in addition severed some interbouton connectives, leaving one or a few boutons on the endplate site (Fig. 1). These nearly vacant endplates were rejected from our study of reconstructed whole-terminal NMJs, but were exploited in the work presented here as a source of isolated boutons.

The number of boutons remaining on an endplate site after deliberate partial terminal removal was variable, usually in the range from two to six. In some experiments, these 'attached' boutons were studied without further manipulation. In other experiments, attached boutons were manipulated away from the endplate and onto the adjacent muscle fibre surface using a glass probe attached to a micromanipulator. The endplate and probe were viewed on a television monitor at high magnification using Nomarski optics, such that some individual boutons were visible. However, we were unable to reliably visualize all boutons remaining on an endplate in living preparations. This was apparent when all visible boutons were removed from an endplate, yet MEPPs persisted in intracellular recordings from the fibre. Subsequent immunofluorescence staining of the fixed preparation with a synaptic vesicle protein antibody then revealed additional boutons. The protocol adopted to ensure that only a single bouton contributed MEPPs (and therefore that all MEPPs recorded came from the bouton under study) was the following: (1) remove most of the terminal as described above; (2) micromanipulate all remaining visible boutons away from the endplate; (3) record intracellularly to confirm absence of MEPPs; (4) search for and remove additional boutons if MEPPs persisted. To form a 'reconstructed' one-bouton NMJ, one bouton was micromanipulated back onto the endplate from the adjacent muscle fibre surface. Experiments where MEPPs were confirmed to arise exclusively from the bouton under study are

noted below. Otherwise, MEPPs probably came from more than one bouton. Unlike entire nerve terminals (Wilkinson & Lunin, 1994), single boutons stuck to the endplate (seemingly by surface tension) and did not require continued force from a probe to hold them in place. Approximately one in three preparations provided functional one-bouton synapses (either attached or reconstructed). Most non-functional boutons were obviously damaged, either by proteolysis due to excessive enzyme treatment or by the excessive mechanical force required to isolate a bouton when enzyme treatment was insufficient. Some boutons appeared healthy but could not be stimulated, for unknown reasons. The complete isolation procedure required ~3 h. Isolated bouton preparations remained viable for an additional 1–3 h after the start of recording.

Electrophysiological recording

MEPPs and EPPs were recorded with conventional microelectrodes filled with 3 M KCl (30–50 MΩ). Records were digitized and stored on the magnetic disk of a computer for subsequent analysis. Care was taken to set the threshold level of the amplitude discriminator used to detect MEPPs so that small and/or slow-rising MEPPs, if present, were acquired. Details of recording methods are presented elsewhere (Wilkinson, Lunin & Stevermer, 1992). Boutons were stimulated extracellularly using either of two methods. In the first, a glass pipette filled with reptilian saline solution (tip diameter, 2–3 μm) was micromanipulated directly above and in near contact with a bouton. In some cases, the pipette was then lowered so as to partially surround the bouton. Negative-going square stimulus pulses (200 μs duration, 3–10 V amplitude) were applied at rates of from 0·2 to 3 Hz. Care was taken to ensure that the stimulus was supramaximal (see Results). This method was used to activate boutons which were isolated from their axons. The second stimulation method, used to activate single-bouton terminals of axons innervating tonic fibres, has been described in detail previously (Lichtman & Wilkinson, 1987). Briefly, a pipette similar to that described above was micromanipulated just above a nerve terminal belonging to the same axon as the terminal under study, but innervating a different muscle fibre. A suitable terminal was selected by activity-dependent labelling of the entire projection of one axon (Lichtman & Wilkinson, 1987). Stimulation of the terminal activated the axon antidromically, as evidenced by a single all-or-none action potential recorded from the cut end of the muscle nerve, and thus activated all of its terminals, including the one-bouton terminal being studied (Cliff & Ridge, 1973; Lichtman & Wilkinson, 1987).

Anatomical methods

Procedures used to test for uptake of the supravital probes 4-Di-2-ASP and RH 414 (Molecular Probes) are described elsewhere (Wilkinson & Lunin, 1994). Electron microscopy utilized glutaraldehyde fixation and standard techniques which are described elsewhere (Wilkinson & Nemeth, 1989).

Quantal analysis

Amplitudes of MEPPs and EPPs were measured manually from the screen of a computer monitor using cursors, corrected for non-linear summation, and normalized to a resting potential of −80 mV (detailed description in Wilkinson *et al.* 1992). The simple non-linear summation correction of Martin (1955) was used because EPP amplitudes from the small synapses studied were usually within the linear range (<10 mV; McLachlan & Martin, 1981), so that the corrections themselves were very small (~1%). Mean quantal content, m, was taken as the mean corrected EPP amplitude/mean corrected MEPP amplitude. Two methods were employed to estimate the (constant) parameters p (probability of release) and n (number of quanta available for release) of the simple binomial distribution which was the best fit to each data set. The first was analysis of variance using the method described by Wernig (1975; see also McLachlan, 1978). This procedure accounts for the fact that unitary responses are themselves not of equal size by explicitly including the coefficient of variation of MEPP amplitudes into the calculation. The second method was adopted to explore the nature of potential disagreement between data and the binomial theory (see Results). The quantal amplitude of each evoked event was calculated as corrected EPP amplitude/mean corrected MEPP amplitude. Histograms of observed quantal amplitudes (e.g. number of EPPs apparently comprising 0, 1, 2, 3, etc. quanta; see Fig. 5) were constructed using bin widths equal to the mean corrected MEPP amplitude and compared to theoretical histograms containing the same number of binomially distributed events. A computer program (Sigmaplot, Jandel Scientific, Sausalito, CA, USA) was used to generate the binomial histogram

$$N_x = N_{tot}\, n!\, p^x(1-p)^{n-x}/(n-x)!\,x!,$$

where N_{tot} is the number of stimuli delivered and N_x is the number of responses having the amplitude $x = 0, 1, 2, \ldots, n$ quanta. The same program was used to determine a multivariate least-squares fit between the theoretical and the experimental histograms, using an iterative procedure with p and n as the independent variables. Alternatively, the parameters p and n were entered manually to generate histograms for visual inspection, including the one predicted by results from analysis of variance.

RESULTS

One-bouton NMJ preparations were of three types, referred to below as reconstructed, attached and small tonic. Reconstructed NMJs were those in which a single isolated bouton was placed on an endplate site which had been confirmed vacant. Attached preparations were NMJs from which all but one or a few (~5) boutons were removed. The remaining boutons were well separated from one another and could be activated individually, but MEPPs recorded from the endplate could not be attributed exclusively to the bouton under study. The third type of preparation (small tonic) consisted of rarely occurring one-bouton synaptic contacts at some polyneuronally innervated tonic muscle fibre NMJs. These small synapses underwent no experimental manipulation (except activity-dependent labelling), and could be activated via their intact axon. Results from attached and reconstructed NMJs are presented below first, followed by results from small tonic NMJs.

Characteristics of reconstructed and attached one-bouton NMJs

Boutons remaining on twitch fibre endplate sites after partial terminal removal by our method (attached preparations) retained approximately the same irregular shape that they exhibited before enzyme treatment. However, the boutons immediately assumed a near-spherical shape once they were freed from the postsynaptic site by nudging them with a glass probe (Fig. 1). Further manipulation of a bouton, for example onto another vacant endplate, was also possible, but required that the bouton be drawn into a saline-filled pipette for transport. The technique

used for partial removal of terminals required 2–4 min of localized protease treatment (Methods). In previous experiments, whole terminals, together with their axons, were removed by a similar method and found to function normally according to several criteria (Wilkinson & Lunin, 1994). In the present study, isolated boutons were not only exposed to proteolytic enzymes but were severed from their connectives with the axon and with other boutons. To assess the effect of these additional manipulations, isolated boutons were examined by transmission electron microscopy (EM) and tested for their ability to take up supravital mitochondrial and activity-dependent probes.

EM studies suggested that most boutons remained intact despite disruption of the thin processes which interconnect them. Evidently, the bouton membranes 'healed', as did those of synaptosome preparations (Blaustein, Johnson & Needleman, 1972). EM also provided an explanation for the observed increase in Nomarski contrast of protease-treated NMJs (see Methods): the synaptic cleft of boutons in attached preparations appeared wider than that of boutons in untreated NMJs (3 EM preparations; compare Fig. 2, panels a and c). Presumably, the slight (~100 nm) apparent separation between pre- and postsynaptic membranes of attached bouton preparations (and perhaps partial removal of cleft material; see Wilkinson & Lunin, 1994) was sufficient to create the observed contrast increase. Despite widening of the cleft, electron-dense regions (putative active zones) in the presynaptic membrane of attached boutons retained their normal alignment with postsynaptic secondary folds (Fig. 2, panel c). In contrast, boutons of reconstructed NMJs (2 EM preparations; Fig. 2, panel b) showed no evidence of active zone-to-secondary fold alignment, as might be expected because such boutons were manually placed on the endplate site. In addition, the cleft width (200–300 nm) was greater in reconstructed than attached preparations. Moreover, the spherical shape of isolated boutons meant that they no longer registered with the more gently curved postsynaptic membrane, making a uniform cleft impossible. The 'polarity' exhibited by boutons of normal snake NMJs (compartmentalization of mitochondria into the region of the bouton farthest from the synaptic cleft and vesicles into the region of the bouton nearest the synaptic cleft; Lichtman, Sunderland & Wilkinson, 1989) also remained after reconstruction, at least in the two EM

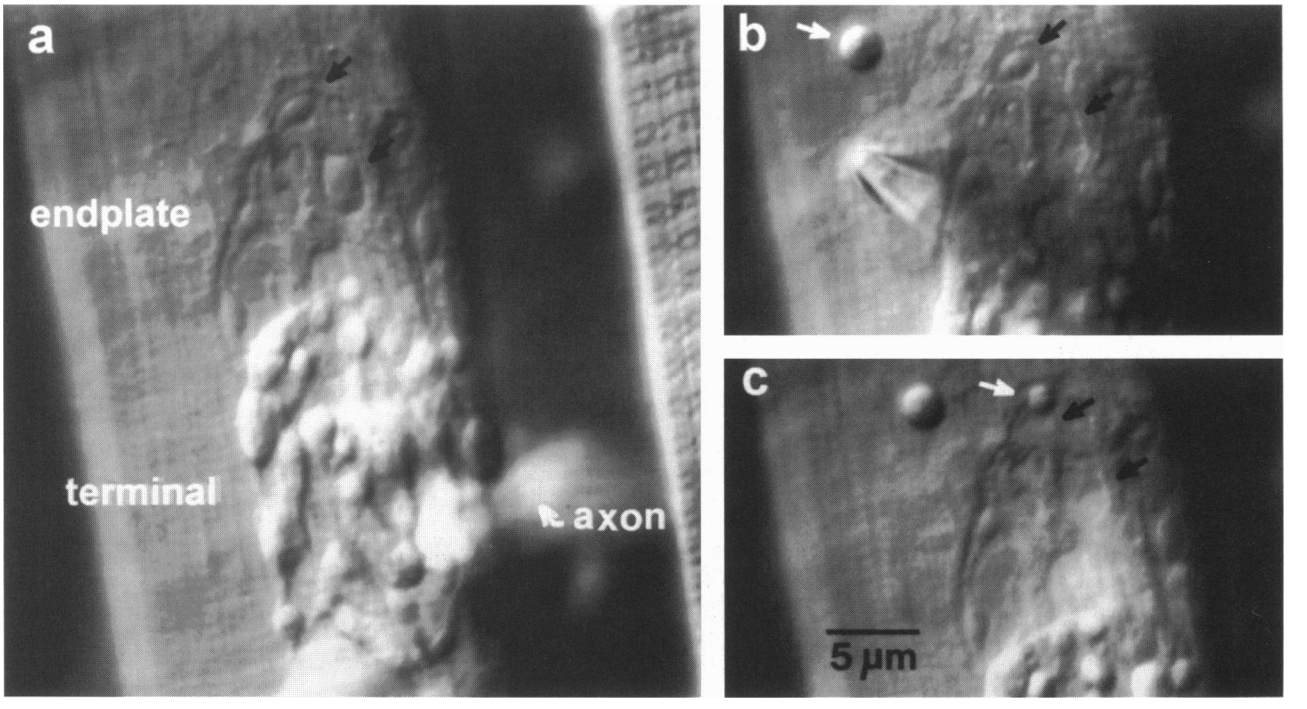

Figure 1. Method for isolating neuromuscular boutons in the snake transversus abdominis muscle

Panel a, most of the axon terminal has been dissociated from its endplate site and retracted downward. Several boutons are severed from the terminal and remain attached to the endplate (above). Arrows point to large and small attached boutons, which have irregular and varied shapes. Panel b, the larger bouton in panel a (white arrow) has been micromanipulated away from the endplate and onto the adjacent muscle fibre surface (glass probe is visible just below bouton). The bouton has become spherical. The smaller bouton in panel a remains on the endplate (upper black arrow), as does a recess formerly occupied by the larger bouton (lower black arrow). Panel c, the smaller bouton in panel a has also been removed (white arrow) and placed near its former location on the endplate (upper black arrow). The bouton has become spherical. Nomarski video micrographs.

preparations studied (Fig. 2, panel b). As shown in this figure, vesicles and active zones remained oriented towards the cleft, while mitochondria were found predominantly near the back of the bouton.

Isolated boutons were subjected to tests used previously to demonstrate the integrity of entire isolated terminals (Wilkinson & Lunin, 1994). Both attached and reconstructed boutons took up the supravital fluorescent dye 4-Di-2-ASP (4/4 preparations). This mitochondrial probe requires a physiological voltage gradient across mitochondrial membranes and thus stains only healthy nerve terminals in a variety of species (Margrassi, Purves & Lichtman, 1987; Herrera & Banner, 1990). Similarly, both classes of boutons took up the activity-dependent probes RH 414 and FM 1-43 (Molecular Probes) (5/7 preparations; Betz *et al.* 1992). Uptake of these probes by stimulated nerve terminals is taken as evidence that they process vesicles and release transmitter normally. Direct stimulation at 25–40 Hz for 5 min was sufficient to label individual boutons with either probe, as was bath application of 50 mM KCl for 1–10 min.

Spontaneous events

As expected, the average intervals between MEPPs increased substantially when most terminal boutons were removed in both attached (from 155 ± 45 to 2300 ± 1100 ms;

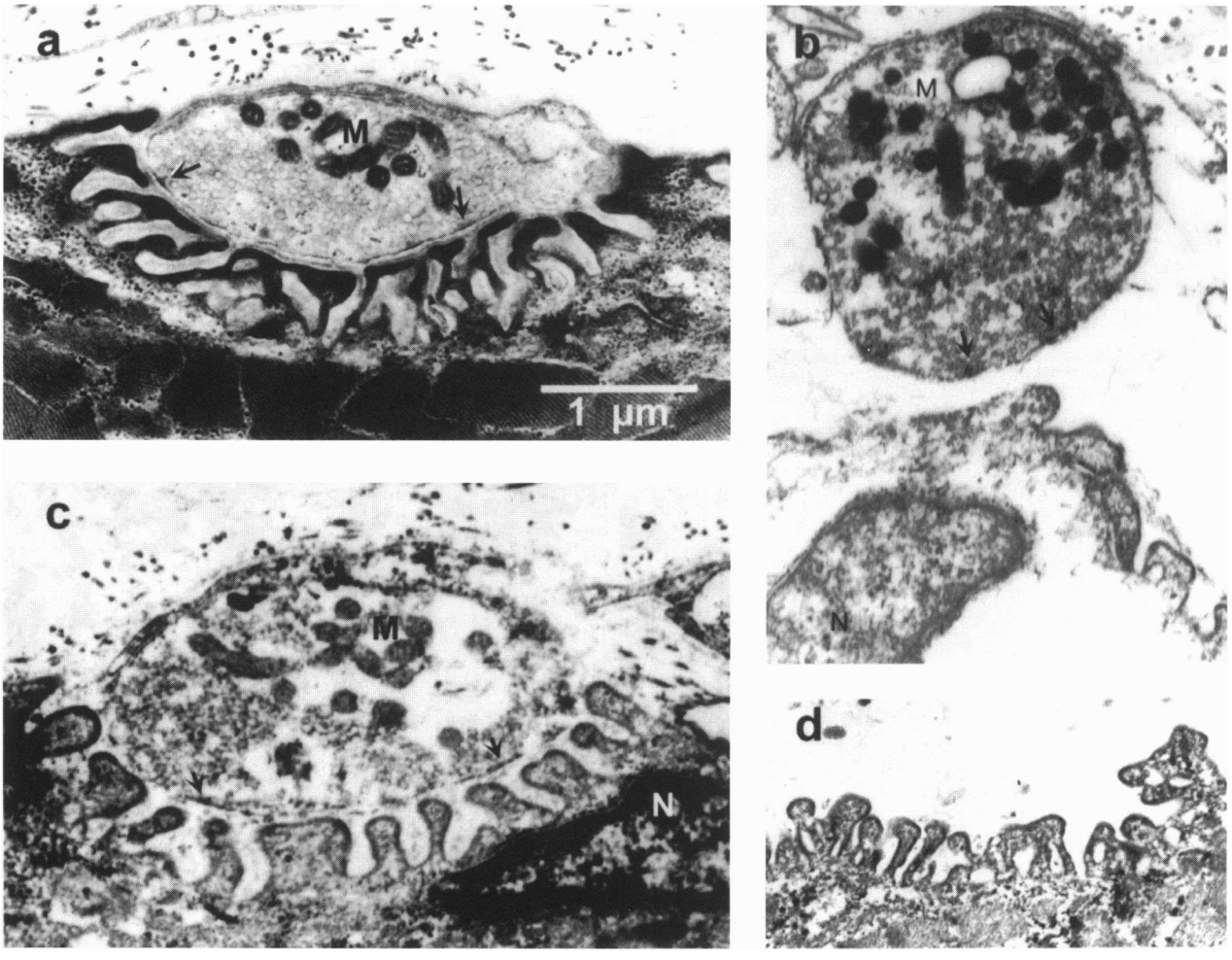

Figure. 2. Electron micrographs of snake neuromuscular boutons

Panel a, typical bouton at intact NMJ. Mitochondria (M) are located at the back of the bouton, away from the presynaptic membrane. Arrows point to electron-dense active zones which precisely oppose postjunctional folds. Panel b, bouton at reconstructed NMJ; the bouton was removed from its original location and placed in a different location on the same endplate. Mitochondria (M) and vesicles are located away from and near the presynaptic membrane, respectively, maintaining the normal 'polarity' of the bouton. Synaptic cleft is widened, and the spherical shape of the bouton no longer corresponds to that of the postsynaptic membrane. Active zones (arrows) no longer oppose folds. N is muscle nucleus. Panel c, bouton remaining on an endplate from which most other boutons were removed (attached bouton). Alignment of bouton to endplate is unaltered, but the cleft appears disrupted and widened; arrows indicate active zones. Panel d, endplate region from which a bouton has been removed.

mean ± S.D.; $n = 5$ NMJs) and reconstructed (from 180 ± 80 to 6400 ± 2200 ms; mean ± S.D.; $n = 5$ NMJs) preparations. Because the average snake twitch terminal contains about fifty-eight boutons, one would expect the mean ~180 ms interval to become ~10 s when the terminal was reduced to a single bouton. Thus MEPPs occurred at slightly higher frequencies than expected at reconstructed NMJs which were confirmed to contain a single bouton, perhaps owing to damage of the bouton by enzyme treatment or other manipulations. Even higher MEPP frequency at attached NMJs (all but one visible bouton removed) probably reflected spontaneous release from a few boutons which were not visualized and removed, in addition to possible bouton damage.

MEPPs recorded from attached NMJ preparations appeared similar to those at intact NMJs in the same preparation. In contrast, MEPPs at reconstructed NMJs confirmed to contain a single bouton appeared to differ (although not significantly) from those of intact NMJs in two respects. Rise times (time constant of the fit to a single exponential) were slowed from 0·86 ± 0·33 to 1·04 ± 0·33 ms (mean ± S.D., 5 NMJs, 5 MEPPs per NMJ), and amplitude distributions of MEPPs were broadened ($n = 5$ NMJs; Fig. 3; broadening was not quantified). Mean MEPP amplitudes were, on average, diminished with reconstruction (by 15 ± 11%; mean ± S.D., $n = 5$ NMJs, 45–100 MEPPs per NMJ) although in two of the experiments (including that of Fig. 3) they did not differ from controls. These results are consistent with those obtained previously from whole-terminal reconstructed NMJs (Wilkinson & Lunin, 1994).

Evoked EPPs

Figure 4 shows EPPs recorded sequentially from typical reconstructed and attached one-bouton NMJs. Several features common to all isolated boutons examined are illustrated. Quantal fluctuations were evident, as seen when entire nerve terminals are activated in reduced-Ca^{2+} or high-Mg^{2+} bathing solutions. Most EPPs appeared to contain 0, 1, 2 or 3 quanta, although occasional EPPs containing up to 8 quanta were observed. It was anticipated that release could be graded by varying stimulus intensity (or duration), but this proved difficult in most preparations. Once a stimulus intensity was reached which reliably

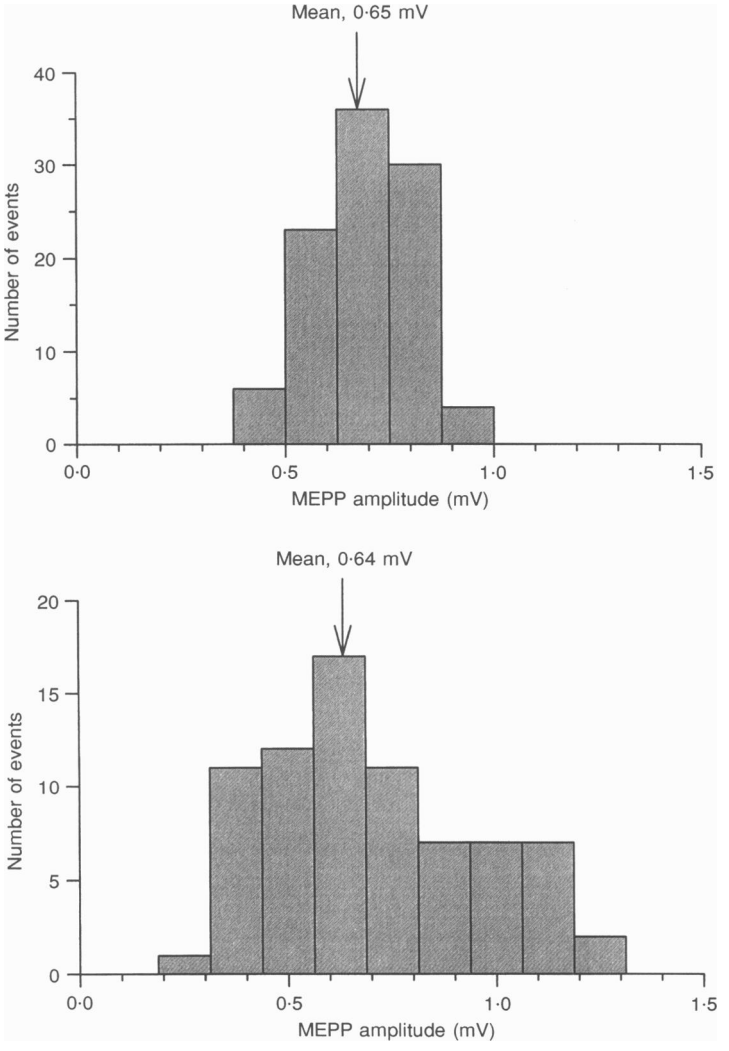

Figure 3. Amplitude histograms of MEPPs
MEPPs recorded intracellularly from a normal twitch fibre endplate (above) and from the same endplate after its terminal was removed and the NMJ reconstructed by application of a single bouton (below). Histograms of MEPPs from one bouton were broadened compared with those recorded from intact NMJ. In this example the mean MEPP amplitude was unchanged (arrows).

activated a bouton, little further increase was needed to reach supramaximal levels. Increase in intensity beyond supramaximal neither increased m nor changed the binomial parameters which best described fluctuations (e.g. bouton 4 in Table 1).

Results from attached (quantal content, $1{\cdot}40 \pm 0{\cdot}53$, mean $\pm$ S.D., $n = 8$ NMJs) and reconstructed (quantal content, $1{\cdot}50 \pm 0{\cdot}65$, mean $\pm$ S.D.; $n = 4$ NMJs) one-bouton NMJs were similar. Boutons in most reconstructed preparations were more difficult to stimulate reliably than those in most attached preparations, for unknown reasons. Increased scatter in event rise times, corresponding to differing diffusion distances between various release sites and receptors, was anticipated, particularly in reconstructed NMJs, but was not observed in our data. Small variations among EPP and MEPP rise times were sometimes seen, as were occasional large, very slow-rising events (example in Fig. 4*B*). Both of these features are also characteristic of intact NMJs. No increase in scatter of event rise times was detectable in a previous study of NMJs reconstructed from entire terminals (Wilkinson & Lunin, 1994).

Sophisticated methods for quantal analysis were unwarranted in the experiments described, due to relatively small data sets and to the fact that some MEPPs probably arose from boutons other than the bouton under study. However, two simple methods, analysis of variance of the EPP amplitude distribution and a least-squares fit of theoretical binomially distributed events to a histogram of actual data (Fig. 5), were consistent with a binomial model in which $p \approx 0{\cdot}5$ and $n \approx 2$–3. A shortcoming of the binomial model was that a number of events appeared to comprise more than n quanta (see Table 1 and Discussion). Table 1 summarizes statistical results from the study of eight single-bouton NMJs, all in the 'attached' configuration. The data were selected from a total of about twenty preparations studied, using the following criteria. To minimize possible damage to the NMJ, the bouton was not deliberately moved from its original endplate site to form a reconstructed NMJ, and effort was made not to move it accidentally by contact with the stimulus pipette. Also, no attempt was made to remove other boutons which remained on the endplate. Thus mechanical manipulations which could potentially damage the NMJ were minimized. Resting potentials were required to be stable (60–90 mV) during acquisition of both MEPPs and EPPs, as was the integrity of boutons as judged by appearance before and after data collection. Both attached and reconstructed NMJs not meeting these criteria gave results consistent with those presented in Table 1, but were excluded because various protocols or additional manipulations were involved, making the direct comparison of results difficult.

Intact one-bouton synapses

By independently labelling the terminal arbors of two tonic motor axons using two differently coloured activity-dependent probes, we found previously that about one-half of the ~300 tonic fibre endplates in the snake transversus abdominis muscle are polyneuronally innervated by two axons (Lichtman *et al.* 1985; Lichtman & Wilkinson, 1987). At these endplates, both the total number of boutons and the fraction of boutons supplied by either axon are highly variable. In the present study, we took advantage of this variability by searching for tonic endplates to which one of two labelled axons contributed only a single bouton. Only

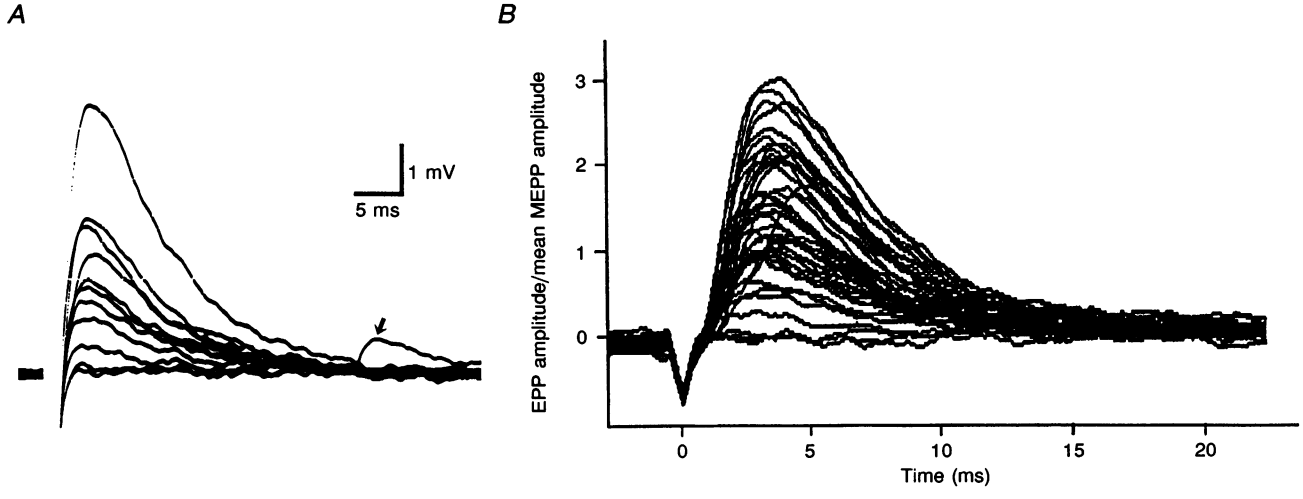

Figure 4. Evoked quantal release from isolated boutons

A, storage oscilloscope traces of EPPs evoked sequentially from a one-bouton NMJ (reconstructed; stimulus frequency, 0·5 Hz). Arrow points to a spontaneous MEPP. *B*, digitized records of EPPs evoked sequentially from a one-bouton NMJ (attached; stimulus frequency, 0·5 Hz). Ordinate is normalized to MEPP amplitude (mean of 64 events) to indicate approximate quantal content. Note that rise times are roughly uniform except for one very slow-rising large event in *B* (see text).

Table 1. Release properties of isolated snake neuromuscular boutons

Bouton	No. of MEPPs	MEPP ampl. (mV)	No. of EPPs	m	Histogram fit p	Histogram fit n	ANOVA p	ANOVA n	EPPs > n (%)	Largest EPP (no. of quanta)
1	96	0·88	61	1·24	0·4	3	0·45	2·8	3	5
2	96	0·88	37	1·34	0·4	3	0·46	2·9	3	4
3	78	0·80	128	1·39	0·3	4	0·66	2·1	7	6
4	63	0·84	64	0·72	0·4	2	0·41	1·7	2	3
(4)	63	0·84	64	0·79	0·4	2	0·41	1·9	0	2
5	63	0·84	34	2·02	0·4	4	0·73	2·8	6	5
6	103	1·22	52	0·65	0·4	2	0·37	1·7	2	3
7	62	0·59	40	1·45	0·3	4	0·58	2·5	0	4
8	59	0·70	64	2·15	0·4	4	0·92	2·4	16	8
Mean	—	—	—	1·40	0·38	3·3	0·57	2·4	4·9	5
A	27	3·8	88	1·20	0·4	4	0·51	3·1	0	4
B	40	4·2	116	1·87	0·35	4	0·38	3·4	3	5

Boutons 1–8 were isolated preparations, boutons A and B were intact tonic preparations. MEPP amplitude (MEPP ampl.) is the mean of corrected amplitudes. Mean quantal content, m, was calculated as described in Methods. Binomial parameters p and n were calculated either by histogram fit or analysis of variance (see Methods). EPPs > n is the percentage of evoked events which appeared to comprise more than n quanta, with n obtained from the histogram fit. The largest EPP recorded is expressed as the number of quanta it apparently contained. Second data set from bouton 4 is at 3 times the normal stimulus intensity and was not included in the means.

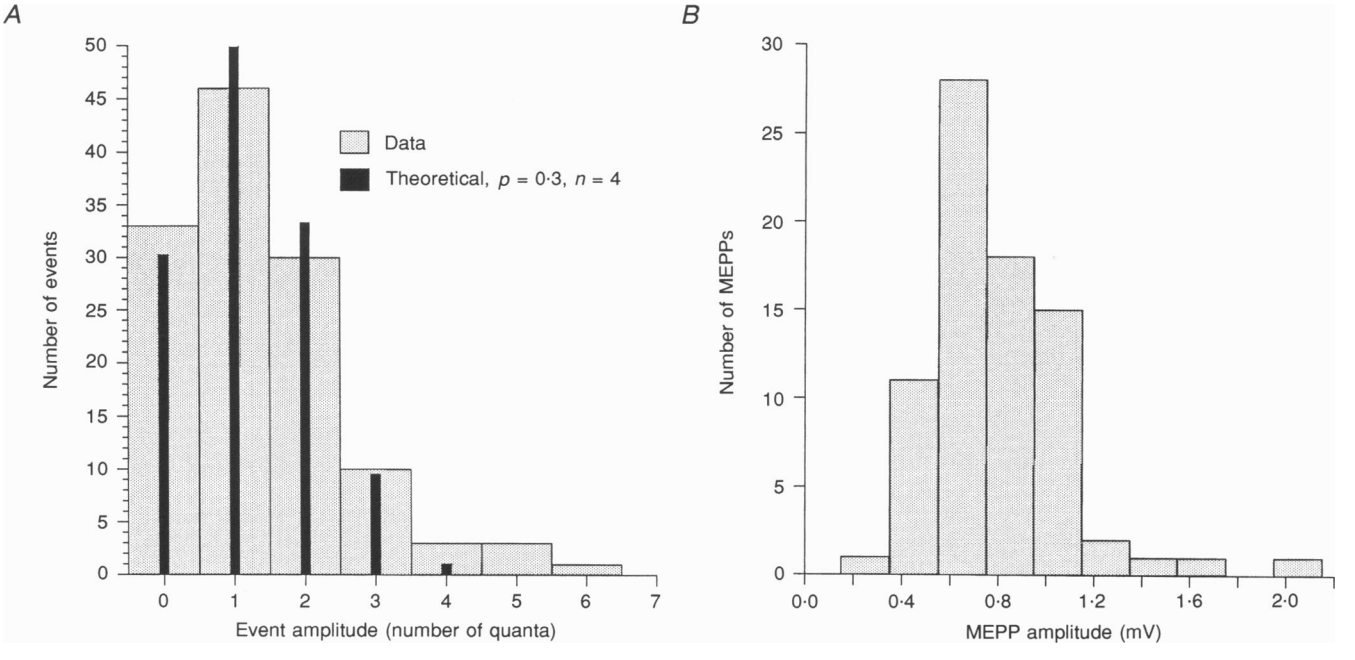

Figure 5. Histograms of release from isolated boutons

A, amplitude histogram of EPPs evoked from an attached one-bouton NMJ (bouton 3 in Table 1). Stimulus frequency, 0·5 Hz. Event amplitudes are normalized to mean MEPP amplitude to indicate quantal content. Theoretical histogram is least-squares best fit to the data; the binomial fitting parameters agree approximately with those obtained from analysis of variance. Several event amplitudes exceeded the theoretical binomial n. *B*, corrected MEPP amplitude distribution (mean, 0·8 mV).

two one-bouton synapses (denoted A and B below and in Table 1) were found and studied among ~3000 doubly innervated tonic NMJs examined (19 preparations). As was the case with attached one-bouton NMJs, it was not possible to isolate MEPPs generated by the individual boutons under study. Instead, most MEPPS presumably arose from nine boutons supplied by the second axon at the endplate innervated by bouton A, or from fourteen boutons supplied by the second axon at the endplate innervated by bouton B. However, in separate experiments ($n = 4$ snakes) we found no difference in mean amplitude of MEPPs at two endplates on the same fibre, each singly innervated by one of two axons labelled with activity-dependent probes. It was therefore unlikely that a significant difference existed between populations of MEPPs supplied by two similar axons at those terminals which co-innervated the same endplate.

Typical records (bouton A) and histograms of EPP and MEPP amplitudes (bouton B) are shown in Fig. 6. Mean quantal content, m, averaged 1·54 for the two boutons tested, a value similar to the means of 1·40 (attached) and

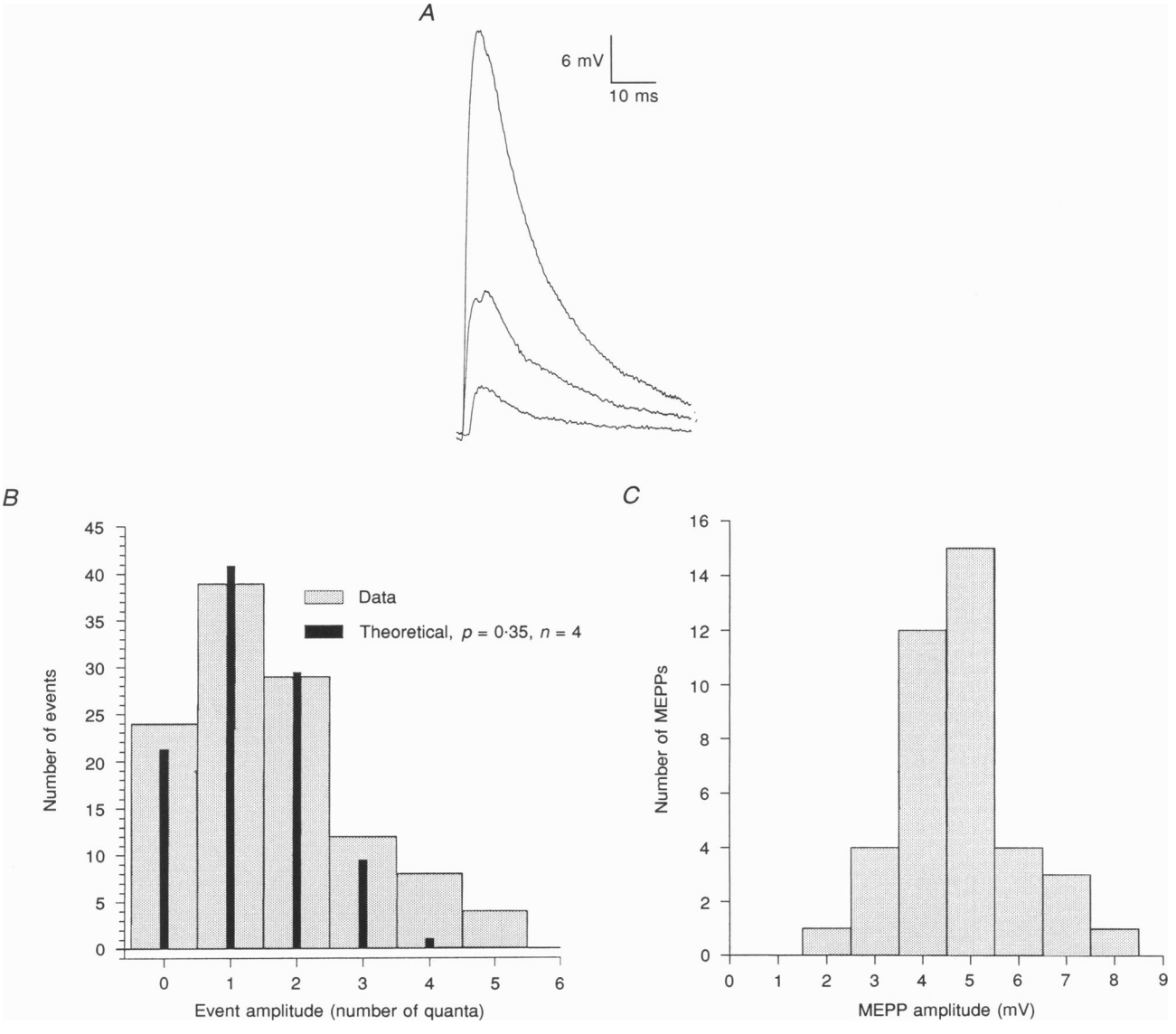

Figure 6. Evoked responses from a normal doubly innervated tonic endplate

A, typical single-quantal and multi-quantal EPPs (two smaller records) from input to endplate which comprised one bouton (bouton A). Larger record, shown for comparison, was obtained by activating second input to the endplate (9 boutons). Stimulus frequency, 0·5 Hz; mean MEPP amplitude, 3·8 mV. Event amplitudes at tonic endplates are large due to high muscle fibre input resistance. *B*, amplitude histogram of EPPs evoked from one bouton (bouton B) at another doubly innervated tonic endplate. Event amplitude is normalized to mean MEPP amplitude to indicate number of quanta. Theoretical histogram is best fit to the data. Quantal content of several events exceeded the best-fit binomial n. Stimulus frequency, 0·25 Hz. *C*, corrected MEPP amplitude distribution (mean, 4·2 mV) from endplate of bouton B.

1·50 (reconstructed) for isolated twitch boutons. The binomial parameters p and n were also similar to those of isolated boutons (Table 1). Thus the directly stimulated isolated boutons described above behaved similarly to boutons at intact NMJs which could be activated via their axons.

DISCUSSION

The mean quantal content for a snake motor bouton at low stimulus frequency was just over one quantum per stimulus, indicating that each bouton represents the anatomical substrate for release (on average) of about one transmitter quantum per invading action potential. This finding is consistent with previous results from other types of boutons within the vertebrate CNS, based on indirect measurements of release from several boutons per synapse (e.g. Korn *et al.* 1982; Triller & Korn, 1982; Redman & Walmsley, 1983; Grantyn *et al.* 1984). Snake neuromuscular boutons are considerably larger than CNS boutons (~3 μm *vs.* ~1 μm diameter, or more than an order of magnitude larger in volume), but evidently the larger size does not translate into more transmitter release under low frequency conditions.

Release statistics

Measurement of release from single boutons extends previous tests of the Katz binomial theory to a small number of anatomically discrete sites. Data were approximately consistent with a simple binomial process except that the binomial n which provided the best overall fit to observed quantal content was smaller than the maximum number of quanta occasionally released. This discrepancy could have been due to any of several factors. For example, relatively small numbers of events were recorded, and some MEPPs used in the analyses probably arose from boutons other than the one under study. These factors decreased the precision of statistical analyses. In addition, isolated bouton preparations were not physiological; events interpreted as having quantal contents larger than n might have resulted from damage to boutons which was not otherwise apparent. On the other hand, release properties of all boutons studied were similar, and release from a bouton (particularly the fraction of events with apparent quantal content in excess of n) did not change over time. These observations argue that the discrepancy with binomial statistics observed in about 5% of trials might have been representative of boutons at native NMJs. If true, it is likely that the discrepancy was detected in the experiments described (and not previously) because single boutons were studied. Synchronous release from only a few boutons would be unlikely to exceed n, and would therefore resemble a simple binomial process. Wernig (1975), for example, found good agreement with the binomial theory when small regions of frog NMJs were stimulated in the presence of TTX, perhaps because more release sites were activated than in the present study. Also, it is possible that the technique used by Wernig (and similar stimulation techniques which rely on electrotonic spread of the stimulus) activates a group of sites whose number varies randomly, thereby biasing experimental results towards randomness. Alternatively, frog (or mammalian) terminals, which lack discrete boutons, might function differently to those of the snake. One interpretation of our results is that a subset of release sites was usually active (the best-fit n), with additional sites available with a lower probability of release, so that single events occasionally exceeded n quanta. Such a model has been proposed, in which n and p are allowed to vary in space or with time (Miyamoto, 1986; see also Provan & Miyamoto, 1993).

Releasable quanta and active zones

The binomial n ranged from 1 to 4 among all boutons studied, averaging 2–3, depending on whether analysis of variance or least-squares fits were used to determine parameters. Although the actual number of anatomical active zones in the boutons studied physiologically was unknown, there is evidence that it was almost certainly greater than n. EM serial sections of two boutons (P. Bridgeman & R. Wilkinson, unpublished observations) revealed at least eight putative active zone densities per bouton; five or more densities are often visible in a single EM section (e.g. Fig. 1, panel a). An even larger number of putative active zones (i.e. ~20 double- or single-row arrays of intramembranous particles) is evident in freeze-fracture replicas of lizard boutons, which resemble those of snake (Walrond & Reese, 1985). Thus, conservatively, n represented fewer than one-half of the anatomical active zones in a bouton. Recent evidence at the frog NMJ suggests that only a fraction of anatomical active zones are used when a nerve terminal is stimulated at low frequency (Zefirov, Benish, Fatkullin, Cheranov & Khazipov, 1995). If this intriguing result holds true at the snake NMJ, a hypothesis consistent with our observations is that a subset of active zones representing the binomial n release quanta with relatively high probability, with additional active zones being capable of occasional release.

Integrity of single-bouton preparations

Both attached and reconstructed NMJs were not physiological in several respects. Some of these, such as the possible enzymatic destruction of part of the synaptic cleft material, a widened cleft, and the probable misalignment between active zones and postjunctional folds, should not have interfered with the ability to record quantal fluctuations. Other possible consequences of enzymatic digestion, for example disruption of active zones, had the potential to affect results by diminishing quantal content. Active zones are disrupted after prolonged treatment with proteolytic enzymes; the required treatment is more severe than that required to destroy acetylcholinesterase (AChE) activity within the synaptic cleft (Nystrom & Ko, 1988). However, enzyme treatment was brief in the present study, and AChE activity was only slightly diminished at whole-terminal reconstructed NMJs, which require longer exposure to protease than do single-bouton preparations

(Wilkinson & Lunin, 1994). Quantal content of those NMJs was about one-half of normal despite probable misalignment of many boutons with receptors, suggesting that most active zones functioned normally. Moreover, every bouton in detached terminals was uniformly stained by activity-dependent probes, indicating that each of about fifty boutons in a terminal endocytosed about the same quantity of the probe and therefore presumably released about the same amount of transmitter. Finally, all isolated boutons in the present study exhibited similar release fluctuations, as did two tonic boutons which were studied in their native state (except for labelling with activity-dependent probes). These observations suggest that active zones in boutons isolated by our method remain viable. The possibility remains, however, that n or p was underestimated because some active zones were damaged or non-functional.

Underestimation of n or p could also result from a different mechanism, namely if some released quanta produced no detectable postsynaptic depolarization. This might occur if the spherical boutons of reconstructed NMJs rotated, positioning some active zones on the side opposite the cleft, 2–3 μm above the receptors. However, in this case, other active zones would be positioned at an intermediate distance from the cleft, thereby producing detectable EPPs with slow rise times. EPP rise times were therefore measured to assess whether different diffusion distances could be detected. Only a few (0–2%) very slow-rising events were seen and no EPP-like events were seen with prolonged rising phases, the same result as that obtained from reconstructed whole-terminal NMJs (Wilkinson & Lunin, 1994). We therefore conclude that evoked release from all functional active zones was detected, a result consistent with EM evidence from two preparations that active zones were oriented towards the cleft.

Because release was characterized exclusively at low stimulus frequencies in the present study, a general description of release fluctuations at the level of single boutons is not yet available. It will be of interest to determine how various phenomena which have been argued to modulate release by changing p, such as potentiation subsequent to tetanic stimulation (e.g. Rosenthal, 1969; Delaney, Zucker & Tank, 1989), will affect the statistics of release from single boutons. The attached configuration of one-bouton NMJs is perhaps more physiological and therefore preferable for most studies. Interestingly, however, reconstructed NMJs also functioned normally, compared with both isolated and native tonic one-bouton NMJs. This behaviour raises questions about the role of precise pre- to postsynaptic alignment seen at intact NMJs, which should be investigated. Moreover, the reconstructed NMJ preparation offers a unique method for manipulation of boutons and postsynaptic sites independently (as, for example, with the application of drugs), and thereby provides a potential means for the direct comparison of different classes of boutons at the same endplate site.

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