Correlations Between Active Zone Ultrastructure and Synaptic Function Studied with Freeze-Fracture of Physiologically Identified Neuromuscular Junctions

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The active zone is a unique presynaptic membrane specialization that is believed to be the site of neurotransmitter release. To examine directly the relationship between active zone ultrastructure and synaptic efficacy, frog neuromuscular junctions were studied with a new technique combining electrophysiology, light microscopy, and freeze-fracture of *identified single* muscle fibers. This technique allows correlations to be made between quantal content (measured in low Ca^{2+} and high Mg^{2+} Ringer solution), endplate size, and active zone structure at the same neuromuscular junctions. By measuring physiological and morphological variables at the same junctions, the validity of structure–function correlations is significantly improved. Synaptic quantal content in 91 physiologically identified muscle fibers varied considerably and was only poorly correlated with endplate size, as shown in previous studies. To measure the total length of endplate branches, either a modified cholinesterase stain or rhodamine-labeled peanut agglutinin stain was used. When the same identified muscle fibers were freeze-fractured, active zones were exposed in 17 junctions. In a replica that contained a large part of one nerve terminal, there was no detectable gradient in active zone structure along the length of 3 different nerve terminal branches identifiable with both light and electron microscopy. The results from these 17 identified junctions indicate that quantal content per unit terminal length is positively correlated with the amount of active zone per unit terminal length. The estimated total active zone length and total number of active zone particles per junction are also positively correlated with the quantal content in these identified junctions. This study suggests that active zone size and spacing are better indicators of transmitter release than is endplate size and that the active zone may play an important role in regulating synaptic efficacy at the neuromuscular junction.

An important approach in elucidating the behavior of the nervous system is to study the relationship between structure and function of synapses. One of the most important components of synapses is the active zone (AZ), which is a specialization of the presynaptic membrane thought to be the site of synaptic vesicle exocytosis (Couteaux and Pecot-Dechavassine, 1970; Heuser et al., 1974, 1979). At the frog neuromuscular junction, AZs seen with freeze-fracture techniques are characterized by 2 double rows of large (~ 10 nm) intramembrane particles crossing the protoplasmic (P) face of the nerve terminal membrane in register with the postjunctional folds (Dreyer et al., 1973; Peper et al., 1974). AZ particles are believed to be the membrane channels through which calcium enters nerve terminals and initiates the release of neurotransmitter (Heuser et al., 1974; Pumpkin et al., 1981). Measurements of the number of synaptic vesicle openings along AZs agree well with estimates of the number of quanta released at frog neuromuscular junctions (Heuser et al., 1979; Heuser and Reese, 1981). Therefore, it is possible that differences in the length or spacing of AZs may be a structural basis for differences in synaptic efficacy observed among different synapses. The purpose of the present work was to study whether AZ morphometry is correlated with levels of transmitter release at the frog neuromuscular junction.

The efficacy of synaptic transmission at vertebrate neuromuscular junctions varies considerably, with some junctions releasing 40 times more transmitter than others in the same muscle (Grinnell and Herrera, 1980, 1981). It has been shown that the amount of transmitter release is generally related to the size of endplates (Kuno et al., 1971; Angaut-Petit and Mallart, 1979; Harris and Ribchester, 1979; Grinnell and Herrera, 1981; Bennett and Lavidis, 1982; Nudell and Grinnell, 1982; Trussell and Grinnell, 1985). However, the correlation is not very precise, with a wide scatter of data. Also, the density of synaptic vesicles near the presynaptic membrane is not correlated with transmitter release levels (Rose et al., 1978; Banker et al., 1983; Herrera et al., 1985b). Since the AZ is believed to be the site of neurotransmitter release, attention has been focused on the AZ as the ultrastructural basis of the observed differences in synaptic efficacy. Thin-section electron microscopy of frog neuromuscular junctions has shown that synaptic contact width, an indirect estimate of AZ length, is correlated with synaptic strength (Herrera et al., 1985a, b). However, each thin-section shows only a small portion of an AZ, and it is therefore difficult to measure AZ size directly. Freeze-fracture techniques provide *en face* views of presynaptic membranes and are therefore a more

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direct and accurate way of studying the length, spacing, and intramembrane particles of AZs.

Several studies have compared freeze-fracture ultrastructure in neuromuscular junctions, which are known to differ in their level of transmitter release. For example, AZs are short and segmented during the early stages of regeneration and development of frog neuromuscular junctions (Ko, 1984, 1985) at a time when quantal content is generally lower than in normal adult junctions. The reduced synaptic transmission in Lambert-Eaton myasthenic syndrome is associated with a paucity and disorganization of AZs (Fukunaga et al., 1982, 1983). Also, junctions from phasic muscles, which release more transmitter, have more AZs than the junctions in tonic muscles (Rheuben, 1985; Walrond and Reese, 1985). Recently, we have compared AZs in 2 different frog twitch muscles and found that junctions with a greater average synaptic efficacy have a longer AZ length per unit terminal length (Propst et al., 1986). All these freeze-fracture studies seem to suggest that the amount of AZ is related to synaptic effectiveness (Atwood and Lnenicka, 1986). However, transmitter release in these studies either was not measured at all or was not examined at the same junctions that were freeze-fractured. In addition, the large cell-to-cell variability in synaptic efficacy and randomness of selection of the fracture plane make it necessary to draw qualitative rather than quantitative conclusions from populations that have widely different synaptic efficacies. A much more direct and conclusive approach is to study both physiology and ultrastructure at the same junctions.

Recently, a technique for freeze-fracture of physiologically identified single muscle fibers has been developed in our laboratory (Ko and Propst, 1986). By combining intracellular recording, light microscopy, and freeze-fracture electron microscopy, it is possible to correlate AZ structure and synaptic function in the same endplates. In the present study using this new approach, we found that quantal content per unit terminal length was positively correlated with amount of presynaptic AZ per unit terminal length. In addition, quantal content was proportional to total AZ length and the total number of AZ particles per junction. This suggests that the AZ plays a role in regulating evoked transmitter release at the frog neuromuscular junction. A preliminary report has been presented (Propst and Ko, 1985).

Materials and Methods

Preparation. Experiments were performed on cutaneous pectoris muscles from grass frogs (*Rana pipiens*). Frogs (6–10 cm body length) were kept in running water at 22°C and fed mealworms twice a week. During dissection, the *in situ* muscle length was carefully measured using calipers from the point of skin attachment to the bottom of the sternum because stretch significantly affects transmitter release levels (Turkanis, 1973). Muscle lengths ranged from 6 to 10 mm. Excised muscles with a short piece of the nerve attached were placed in a Sylgard (Dow Corning) lined petri dish and pinned to 115% of resting length. Muscles were bathed in normal frog Ringer (111 mM NaCl, 2 mM KCl, 1.8 mM CaCl₂, 5 mM HEPES at pH 7.2) and kept at room temperature. A more detailed description of the intracellular recording, identification, and single muscle fiber freeze-fracture techniques has been presented elsewhere (Ko and Propst, 1986).

Electrophysiology. Conventional intracellular microelectrode recordings of miniature endplate potentials (MEPPs) and endplate potentials (EPPs) were done with muscles bathed in a Ringer solution containing 0.35 mM Ca²⁺ and 4 mM Mg²⁺. Muscle cells were rejected if their potential was more positive than −80 mV. EPPs were evoked at 0.5 Hz by supramaximal stimulation of the nerve stump via a suction electrode. The usual data collected for each fiber was 150–250 EPPs and 50–100 MEPPs.

A second glass microelectrode, which was filled with aqueous 3% Lucifer yellow CH (Polysciences), was inserted into the muscle fiber within 75 μm of the recording electrode, and the fiber was rapidly filled with dye by iontophoresis (Stewart, 1978). The electrode holder for the injection electrode was filled with 1 M LiCl to improve the electrical connection (Stewart, 1978). Dye intensity was monitored with appropriate epifluorescence optics during the filling procedure (absorbance max = 430 nm, emission max = 550 nm). If any doubt existed about whether the 2 electrodes were in the same fiber, hyperpolarizing pulses were injected through the recording electrode, and the membrane potential change was measured with the injection electrode. The presence of simultaneous MEPPs was also a sign that both electrodes were in the same cell. The recording and injection procedures were then repeated on several other fibers.

Preparation of single muscle fibers for freeze-fracture. Nerve terminal length in the identified junctions was measured in order to calculate transmitter release per unit terminal length. Two different staining methods were developed to be compatible with later freeze-fracture while providing accurate information about terminal shape.

1. **Cholinesterase staining.** Conventional cholinesterase stains produce extensive membrane damage that make them incompatible with freeze-fracture (McMahan and Slater, 1984), so we developed an alternative type of stain that produces much less disruption of membranes. Muscles were fixed in the recording chamber for 60 min with frog Ringer containing 4% paraformaldehyde. Cholinesterase was lightly stained for 2–3 min with a modified thiocholine ester technique (Ko and Propst, 1986), which was then quickly washed off with Ringer. However, the use of the cholinesterase stain was discontinued after a few experiments because of the success of an alternate staining method.

2. **Lectin staining.** A recently developed technique for fluorescent staining of frog neuromuscular junctions (Smith and Ko, 1986; Ko, 1987) was used instead of the modified cholinesterase stain because it produced significantly less membrane disruption in freeze-fracture. After pre-fixation for 5 min in Ringer containing 4% paraformaldehyde, tetramethyl-rhodamine-conjugated peanut agglutinin (TMR-PNA; Vector Laboratories) at a concentration of 50 μg/ml was applied to the muscles for 40 min. This lectin selectively labels a glycoconjugate in the extracellular matrix of the frog neuromuscular junction (Smith and Ko, 1986; Ko, 1987) and has a much higher intensity of fluorescence at the frog neuromuscular junction than rhodamine-conjugated α-bungarotoxin. This technique also was completely compatible with later freeze-fracture. After rinsing thoroughly for 15 min, the muscles were postfixed in Ringer containing 4% paraformaldehyde for 60 min.

Muscles were cryoprotected in graded series of aqueous glycerol solutions (Ko, 1981), and the identified fibers were dissected out of the muscle under a combination of bright-field and epifluorescence optics. Muscle fibers filled with Lucifer yellow were highly fluorescent after fixation and could be easily distinguished during the teasing procedure. Isolated single fibers were observed through a compound microscope with a Zeiss 40× water-immersion objective. Those junctions with TMR-PNA staining were photographed under epifluorescent illumination with a 35 mm camera system (Olympus) attached to the microscope. Cholinesterase-stained junctions were drawn by hand with a camera lucida attachment.

Freeze-fracture electron microscopy. Each single fiber was sandwiched between 2 specimen holders with a small drop of aqueous 30% glycerol and frozen rapidly in liquid Freon-22 (DuPont), followed by liquid nitrogen. Freeze-fracture was performed using a complementary replica device (Balzers 400D) at −110°C and <10^{−6} Torr (Ko, 1981). To add structural support to the replicas, a drop of 0.25% Lexan in ethylene dichloride (Steere and Erbe, 1983) was applied to each specimen as it was removed from the vacuum chamber. Replicas were cleaned for 3 hr in 5.25% sodium hypochlorite (Purex), washed in water, and mounted on copper mesh grids. Immersion of mounted replicas for 5 min in a bath of ethylene dichloride removed the Lexan coating (Steere and Erbe, 1983). Freeze-fracture replicas were examined and photographed at 80 kV in a transmission electron microscope (JEOL 100 CXII).

Data analysis. Electron microscopic negatives of freeze-fracture replicas were projected with a photographic enlarger onto a digitizing tablet (Summagraphics Bitpad) with a resolution corresponding to 2 nm on the replicas. The tablet was connected to a microcomputer (Northstar) for calculation, display, and storage of data (Propst et al., 1986). Measurements were made of AZ length, AZ spacing (Propst et al., 1986), the number of AZ segments per junctional fold, and terminal width. Active zone segments at the same junctional fold were considered sep-

Figure 1. Correlation between terminal length and muscle fiber diameter. Larger muscle fibers tend to have larger nerve terminals, possibly to compensate for their reduced input resistance. In this and the following figures, r , p , and n indicate correlation coefficient, significance level, and number of observations, respectively. The line drawn represents the line of best fit.

arate if a gap of 50 nm or more existed between them (Pumplin, 1983; Ko, 1984).

Terminal length, muscle fiber diameter, and sarcomere spacing were measured from light microscopic negatives or camera lucida drawings of the junctions at a final magnification of $675\times$. Quantal content was calculated from filmed oscilloscope records of MEPP and EPP amplitudes. EPPs were adjusted to a resting potential of -90 mV (Martin, 1966) and corrected for nonlinear summation (Martin, 1955). The direct method, $m = \text{mean EPP}/\text{mean MEPP}$ (del Castillo and Katz, 1954; Martin, 1966), was used to estimate quantal content.

Normally distributed variables were described by their mean and SDs, and other variables by their median and interquartile ranges (iqr). The relationship between 2 variables was studied with linear-regression analysis and was indicated by a line drawn by the least-squares method (Snedecor and Cochran, 1967). In addition, correlation coefficients, r , and p values were shown.

Results

Terminal length and physiology in low Ca^{2+} /high Mg^{2+}

Although not all of the identified junctions could be successfully freeze-fractured, the physiology and light microscopic data from them were still useful. Electrophysiological recordings of 91 junctions from 21 cutaneous pectoris muscles displayed a 20-fold variability in quantal content (median, 2.80; iqr, 0.95–4.25). It is known that some of this variability can be accounted for by differences in nerve terminal size (Kuno et al., 1971). Nerve terminal size was taken as the summed lengths of all individual branches outlined by either cholinesterase or TMR-PNA stains (Smith and Ko, 1986; Ko, 1987) and ranged from 144 to 2302 μm (median, 675 μm ; iqr, 400–875 μm). Terminal length was correlated linearly with muscle fiber diameter (Fig. 1) as previously reported by others (Kuno et al., 1971; Banner

Figure 2. Relation between quantal content (measured in Ringer solution containing 0.35 mM Ca^{2+} and 4 mM Mg^{2+}) and terminal length. The correlation coefficient is quite low. This confirms the results of earlier studies that found a similar wide scatter in the relationship between release and terminal size.

and Herrera, 1986). For the measurement of terminal length, the lectin staining technique seemed to be optimal in terms of membrane preservation and staining intensity of the several methods we tried (Ko and Propst, 1986). In agreement with previous studies (Kuno et al., 1971; Angaut-Petit and Mallart, 1979; Harris and Ribchester, 1979; Grinnell and Herrera, 1980, 1981; Bennett and Lavidis, 1982; Nudell and Grinnell, 1982; Trussell and Grinnell, 1985), positive correlations found between terminal length and quantal content were not very precise (Fig. 2). As shown by the low correlation coefficient, there were often large differences in quantal content among some junctions of very similar size. To compare the intrinsic strength of the different junctions without including the effect of terminal length, transmitter release per unit terminal length was used. To calculate transmitter release per unit terminal length, quantal content was divided by the terminal length measured with light microscopy. There was a 35-fold range of quantal content per 100 μm of terminal length among the identified junctions we studied, although most values were less than 0.8 quanta/100 μm (median, 0.43; iqr, 0.26–0.65 quanta/ μm).

Single-fiber freeze-fracture

Out of the 91 physiologically identified and freeze-fractured junctions, 17 revealed AZs. In the remaining 74 muscle fibers,

Figure 3. Example of freeze-fracture of physiologically identified single muscle fiber. *a*, Drawing of a junction stained with TMR-PNA. Several fluorescence micrographs of different parts of the junction were projected onto a sheet of paper and traced. Before junctional staining, synaptic potentials of this muscle fiber were recorded intracellularly and the fiber was identified with injection of Lucifer yellow. The dotted rectangle indicates the same terminal branch shown in panels *c–e*. *b*, Drawing made from freeze-fracture negatives of the same junction as *a*. Grid bars are indicated where they occurred under the replica. Dotted outlines of portions of the terminal indicate where either the fracture plane did not pass through the

presynaptic membrane or the replica was hidden by grid bars. Calibration bar applies to both *a* and *b*. *c*, Fluorescence micrograph of a portion of the same junction in *a* (dotted rectangle) and *b*. The junction was stained with TMR-PNA and photographed under epifluorescent illumination. *d* and *e*, Low-magnification P face (*d*) and complementary E face (*e*) electron micrographs of the same junction after freeze-fracture. The branches of the nerve terminal (arrowheads) and the edges of the muscle fiber (arrows) can be seen clearly. Calibration bar in *c* also applies to *d* and *e*.

Figure 4. *a*, High-magnification view of P face of a small portion of the same branch shown in Figure 3*d* (box). Double rows of large particles outline the active zones (arrow). *b*, Complementary (E face) view of the same area shown in Figure 3*e* (box). Depressions in the E face membrane correspond to rows of AZ particles on the P face replica. Calibration bar is the same for both *a* and *b*.

the fracture plane did not go through the nerve terminal membranes and showed only the fracture faces of muscle membranes. The results from one successful experiment showing AZ structures are presented to demonstrate the usefulness of the single-fiber technique. Figure 3*a* is a drawing made from fluorescence micrographs of an identified junction stained with TMR-PNA that has a total terminal length of 378 μm . PNA recognizes synapse-specific carbohydrates in the extracellular matrix at the neuromuscular junction, and the fluorescent binding pattern indicates the endplate size (Smith and Ko, 1986; Ko, 1987). Figure 3*b* is a drawing made from the freeze-fracture replicas of the same junction. It can be seen that a large part of the nerve terminal membrane has been freeze-fractured, and different branches of the terminal can be identified by both light microscopy and freeze-fracture.

The lengths of individual terminal branches measured from light microscopy agreed to within 5% with the lengths of the same branches measured from freeze-fracture replicas. The reliability of the lectin staining for measurement of endplate size was also checked by measuring 25 junctions that had been double-labeled with Karnovsky's cholinesterase stain (Karnovsky, 1964) and with TMR-PNA. The average difference in the measurements of endplate size with the 2 methods was only 2%, and this difference was not statistically significant ($p > 0.50$, paired t test) (Ko, 1987).

A fluorescence light micrograph of a branch of the same junction shown in Figure 3*a* (marked by dotted rectangle) is illustrated in Figure 3*c*. A freeze-fracture view of this branch is presented in low magnification (Fig. 3, *d, e*) for comparison with the light micrograph. In this experiment, both the P face (Fig. 3*d*) and the complementary exoplasmic (E) face (Fig. 3*e*) of the same junction were obtained. To show the individual AZs more clearly, Figure 4, *a* and *b*, presents higher magnification views of the P and E faces of one portion of this terminal branch (marked by boxes in Fig. 3, *d, e*). The rows of AZ particles on the P face correspond to depressions in the E face membrane. Putative synaptic vesicle openings can also be seen along the AZs. From a comparison of Figure 3, *a* and *b*, it is evident that about 70% of the entire nerve terminal length in this small endplate can be found on the freeze-fracture replicas. However, since some of the terminals did not fracture through the side of the presynaptic membrane facing synaptic clefts and some of the replica was hidden by grid bars, only 122 AZs could be measured in this junction. This is about 30% of the number estimated to be contained in the whole terminal. From the average of these 122 AZ length and spacing measurements, AZ length per junctional fold was $1.25 \pm 0.48 \mu\text{m}$, and the spacing between neighboring AZs was $0.93 \pm 0.39 \mu\text{m}$. The AZ length and spacing measurements from all of the exposed presynaptic membranes were used to estimate the total AZ length per 100 μm of terminal length. This is calculated by dividing the AZ length per junctional fold by the spacing to the adjacent consecutive AZ and multiplying the result by 100. The individual values at each AZ were then averaged to estimate the mean for the terminal as a whole. In the identified junction shown above, there was 161 μm of AZ per 100 μm of terminal length. The mean quantal content was 0.82 and quantal content per 100 μm of terminal length was 0.22 in this junction.

Correlations between structure and function

Examinations of the morphometry of AZs revealed in the 17 physiologically identified and freeze-fractured neuromuscular

Figure 5. The correlation between quantal content per 100 μm of terminal length and the amount of AZ per 100 μm in physiologically identified neuromuscular junctions. This correlation illustrates the important role of the AZ in regulating transmitter release.

junctions provided important new information about the ultrastructural basis of transmitter release variability. In each of these 17 junctions an average of 30 AZs (ranging from 5 to 122 AZs) could be measured. To examine the correlation between AZ structure and transmitter release without including the effect of terminal length, quantal content was divided by the terminal length measured with light microscopy. Quantal content per 100 μm of terminal length could then be compared with the amount of AZ calculated to be contained in 100 μm of terminal length. The result of these 17 identified junctions indicates that active zone length per 100 μm of terminal length is positively correlated with quantal content per 100 μm ($r = 0.53$, $p < 0.03$) (Fig. 5). A 3-fold increase in transmitter release per 100 μm is associated with an approximately 4-fold increase in AZ per 100 μm in Ringer solution containing 0.35 mM Ca^{2+} and 4 mM Mg^{2+} . However, the slope of this relationship may be dependent on calcium concentration in the extracellular medium, as quantal content has a nonlinear relationship to calcium concentration (Dodge and Rahamimoff, 1967; Augustine and Charlton, 1986). We also estimated the total active zone per terminal to see whether it was superior to terminal length in predicting the total transmitter release. To do this, AZ per unit terminal length was multiplied by the measured terminal length. The total amount of active zone per terminal was highly correlated ($r = 0.82$, $p < 0.0001$) to quantal content (Fig. 6), which again may reflect the important role of the AZ in regulating evoked transmitter release. Since AZ particles may be calcium channels (Pumplin et al., 1981), we also examined the relationship between quantal contents and the total numbers of AZ particles in the nerve terminals. To estimate the total numbers of AZ particles in a given terminal, the numbers of large ($> 10 \text{ nm}$) particles per micron of AZ was first counted from at least 10 AZs in that terminal. In general, the number of AZ particles per micron is fairly consistent at different AZs in the same nerve terminal. The average number of large particles per micron of AZ was then multiplied by the estimated size (in μm) of total AZs in

Figure 6. Correlation between quantal content and the estimated total amount of active zone size per terminal in physiologically identified junctions. The correlation is much better than between terminal length and quantal content (Fig. 2).

that given nerve terminal and the result estimated the total number of AZ particles. Of the 17 successfully freeze-fractured, identified junctions, 11 produced P face views of the nerve terminal and thus displayed AZ particles. The results from these 11 junctions (Fig. 7) indicate that the estimated total number of AZ particles per junction is also positively correlated with the quantal content measured in the low Ca^{2+} /high Mg^{2+} .

In addition to evoked EPPs, we also examined whether there was a correlation between the frequency of MEPPs and the AZ structure. As shown in Figure 8, the correlation between MEPP frequency and the total amount of active zone per terminal was

Figure 7. Correlation between quantal content and the estimated total amount of AZ particle per terminal in physiologically identified junctions. Only AZ particles on P face terminals were correlated.

Figure 8. MEPP frequency (number/sec) is not significantly correlated with the estimated total amount of AZ per terminal. This may indicate that MEPP frequency is regulated differently than EPP size.

not statistically significant. This implies that there may be different processes or synaptic structures responsible for the regulation of evoked and spontaneous transmitter releases.

Distribution of AZ sizes

Because the estimates of AZ length and spacing for each junction were usually based on a small (approximately 5%) sample of the total number of AZs, it was important to determine if any gradients or large differences in these variables existed among different branches of the same nerve terminal. A significant gradient could make our estimates of AZ length and spacing unrepresentative of the terminal as a whole. To observe a gradient in AZ structure it was necessary to freeze-fracture large portions of nerve terminals. This proved to be very difficult in most cases due to the uncontrollable nature of the fracture plane, but in one example shown in Figure 3, 70% of a nerve terminal was fractured. In this terminal, as well as in several other examples, each freeze-fractured branch could be identified in both light and electron microscopy. Thus, the individual measurements of AZ length and their position along different terminal branches could be plotted as a function of the distance from the distal tip (Fig. 9). The position of distal tips seen in the replicas was verified from light microscopic photographs of the nerve terminal shape similar to those shown in Figure 3, *a* and *b*. In one branch, 70 μm long, the entire length was fractured and 75 AZs were seen. In this branch, AZ length and spacing varied considerably from one AZ to the next. To detect any gradient in AZ size along terminal branches, a 3-point moving average of consecutive data points was used (Snedecor and Cochran, 1967). In this junction, it does not appear that there are significant gradients in either AZ length or spacing. This implies that randomly sampled AZs can be used to estimate average AZ length and spacing per terminal without introducing a bias into the results. Of course, increasing the number of AZs measured will improve the accuracy of these estimates.

Discussion

This is the first study to directly correlate transmitter release and AZ structure seen with freeze-fracture technique at the same

Figure 9. AZ length as a function of distance along different nerve terminal branches. Small symbols (dot, circle, cross) represent individual measurements of AZ length per junctional fold in 3 different branches. Three-point averages for each branch are also given (large symbols). There does not appear to be a significant gradient in AZ length or spacing along these 3 different branches, and the mean values of AZ length for each branch are also very similar.

identified synapses. With a new technique for freeze-fracture of physiologically identified single muscle fibers, we found a positive correlation between quantal content (measured in low concentration of calcium and high magnesium) per unit terminal length and average AZ per unit length. In addition, quantal content was proportional to estimates of the total amount of AZ and the total number of AZ particles per terminal. These results suggest that the AZ is the structural basis for variability in synaptic efficacy at the frog neuromuscular junction and further support the idea that AZ particles may be calcium channels (Heuser et al., 1974; Pumplin et al., 1981). In addition, the present study indicates that the AZ is superior to endplate size in predicting the level of transmitter release. As reported in previous studies (see reviews in Grinnell and Herrera, 1981; Wernig and Herrera, 1986), there was a poor correlation between quantal content and endplate size. Others have explained some of this variability by an inverse relationship between transmitter release per unit terminal length and endplate length (Nudell and Grinnell, 1982, 1983) or by a difference in Ca^{2+} influx (Pawson and Grinnell, 1984). The present results can explain this low correlation by the variability in total AZ length among different neuromuscular junctions. This is shown by the significantly better correlation between total AZ length and quantal content (Fig.

6) over that between endplate length and quantal content (Fig. 2).

The use of identified single-fiber freeze-fracture techniques significantly improves the validity of structure-function correlations. Previous studies compared synapses that were not physiologically identified but were known to differ in average synaptic efficacy. Neuromuscular junctions in 2 different fast muscles of the frog (*Rana pipiens*) were found to differ by 20% in the total length of AZ per 100 μm of terminal length (Propst et al., 1986), although average quantal content per 100 μm , measured in low calcium, differed by 3.5-fold (Banner and Herrera, 1986). In addition, a comparison of tonic and phasic junctions in the lizard showed a 1.5-fold difference in the numbers of AZs per terminal and total numbers of AZ particles (Walrond and Reese, 1985), which cannot by itself explain the 10-fold difference in transmitter release observed in other studies. While there was not a significant difference in individual AZ size between slow and fast neuromuscular junctions of the moth, there was an approximately 4-fold difference in total AZ length per junction and a 6-fold difference in AZ particles that might explain the difference in the amplitude of excitatory junctional potentials (Rheuben, 1985). However, the quantal content of the junctions in this study was not reported.

The present approach permits exact quantitative comparisons to be made between different junctions, and we found that linear relationships exist between quantal content and both AZ size and particle number. A 2-fold increase in total AZ size or particle number correlates with an approximately 1.7-fold increase in quantal content measured in Ringer solution containing 0.35 mM Ca^{2+} and 4 mM Mg^{2+} . In a recent study of 9 physiologically identified frog neuromuscular junctions examined with serial thin-sections, Herrera et al. (1985b) reported that a 5-fold increase in synaptic contact width (which estimates AZ length) was correlated with a 15-fold increase in quantal content in 0.3 mM Ca^{2+} and 1 mM Mg^{2+} . The difference in the results of thin-section and freeze-fracture experiments may be due to one of several factors, including the uncertainty in estimating AZ length and spacing with thin-sections and the different calcium and magnesium concentrations used. This last point is particularly important, because it has been shown that there is a nonlinear relationship between quantal content and external calcium concentration (Dodge and Rahamimoff, 1967; Augustine and Charlton, 1986). Therefore, it is unclear whether correlations between AZ size and transmitter release obtained in low calcium can be extrapolated to normal calcium levels. Unfortunately, this question cannot be resolved until the accuracy of various methods of measuring transmitter release in low and normal calcium concentrations has been verified (reviewed in Wernig and Herrera, 1986).

Although a positive correlation was found between the total AZ length and quantal content, we did not find a similar correlation between MEPP frequency and the total amount of AZ per terminal. MEPP frequency is more variable than quantal content at a given frog neuromuscular junction, with large fluctuations that can occur over several seconds (Erulkar and Rahamimoff, 1976). These changes in MEPP frequency would not have been detected by our relatively small samples. Alternatively, the factors that control the release of MEPPs may be different from those that control EPPs. Synaptic vesicle exocytosis is seen between AZs during MEPP release in high potassium (Ceccarelli et al., 1979), and MEPPs can be released in the absence of external calcium (Miledi and Thies, 1971), so it is very possible that MEPP release may not normally require AZs (Ko, 1981).

The measurement of terminal length relied on the assumption that the staining patterns seen after the cholinesterase or lectin stains were accurate reflections of the true terminal shape. α -Bungarotoxin and cholinesterase staining have been shown to correlate with a good degree of precision to the true terminal length (Krause and Wernig, 1985). The good agreement between light and electron microscopic measurements of terminal size and in double-labeling experiments indicates that the TMR-PNA staining is also a reliable measure of nerve terminal length. Unlike cholinesterase staining, which causes membrane disruption due to crystal reaction products (McMahan and Slater, 1984), the TMR-PNA staining is compatible with freeze-fracture technique and is thus superior in preserving membrane structures as shown in the present study. The compatibility of lectin staining and freeze-fracture is probably related to the fact that PNA binds to the extracellular matrix around the nerve terminal-associated Schwann cells but not to the basal lamina in the synaptic cleft and junctional fold regions as examined in whole-mount (Smith and Ko, 1986; Ko, 1987).

We must assume that transmitter release is fairly uniform over the extent of the nerve terminal branches if release per unit

terminal length is to be compared among different junctions. This has been shown to be true in most regions by precise mapping of evoked release at frog nerve terminals (D'Alonzo and Grinnell, 1985). However, transmitter release has been found to be much more heterogeneous in the toad neuromuscular junction, with a pronounced decrease along the length of longer nerve terminal branches but remaining approximately constant for over 60% of the length of short branches (40–80 μm) (Bennett and Lavidis, 1982; Bennett et al., 1986; see review by Robitaille and Tremblay, 1987). Since an average of 30 AZs or approximately 5% of the total number of AZs per terminal could be measured from freeze-fracture replicas, it is possible that the estimates of AZ lengths and spacing could be unrepresentative of the terminal as a whole. However, in the example shown in Figure 9, 70% of one nerve terminal was fractured (with the longest branch being about 70 μm), and it did not exhibit gradients in the size or spacing of AZs among different terminal branches. Similar uniformity in frog nerve terminal AZ size has also been reported with thin-sections (Werle et al., 1984). In addition, measurements of more than 500 AZs in 16 junctions from 6 cutaneous pectoris muscles display a normal distribution in AZ length per junctional fold or per 100 μm of nerve terminal (Propst, 1986). Thus, estimates of AZ variables from randomly sampled AZs seem to be unbiased and valid for comparison. In addition to the gradient problem, the large variability of individual AZ sizes influences the accuracy of the estimate of AZ variables. In a previous study we have shown that the average of individual AZ size (based on measurements of 535 AZs in 16 junctions) is 1 μm with an SD of $\pm 0.57 \mu\text{m}$ (Propst et al., 1986). Since on the average 30 AZs per terminal were measured in this study, the 0.95 confidence interval estimate of the average AZ size is approximately 0.2 μm ($1.96 \times 0.57/\sqrt{30}$) (Snedecor and Cochran, 1967). In terms of average magnitude, this was probably the major source of experimental uncertainty among the different physiological and morphological variables. While there is no way to freeze-fracture all of the AZs in a given junction, the single-fiber freeze-fracture technique provides useful information about the structure and function of AZs that cannot be found any other way.

Correlations have been found between measured or estimated transmitter release and the number or size of presynaptic densities seen in thin-sections of a variety of different types of synaptic contacts (Sherman and Atwood, 1972; Govind and Chiang, 1979; Govind and Meiss, 1979; Hill and Govind, 1981; Aizu, 1982; Atwood and Marin, 1983; Fields and Ellisman, 1985). Bailey and Chen (1983) have shown with thin-sections that AZ number and size in *Aplysia* are larger after long-term sensitization and smaller after long-term habituation. Our results suggest that evoked transmitter release at the frog neuromuscular junction is also somehow regulated by the amount of presynaptic AZ. This may explain how terminals of a similar size and light microscopic appearance can differ widely in the strength of their synaptic contacts. The present study may also explain the increase in quantal contents observed during regeneration and development of neuromuscular junctions (Letinsky, 1974; De Cino, 1981; Morrison-Graham, 1983) by the increase in AZ sizes during these processes (Ko, 1984, 1985). Likewise, the reduced quantal contents in Lambert-Eaton myasthenic syndrome may be related to a paucity and disorganization of AZs as suggested by Fukunaga et al. (1982). The correlation between AZ structure and synaptic function may also have general implications for understanding the cellular mechanism and ultra-

structural correlates of important processes such as aging, long-term potentiation, learning or memory, which may involve changes in synaptic effectiveness (Banker et al., 1983; Thompson, 1986).

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