Depolarization-induced \( \text{Ca}^{2+} \) entry preferentially evokes release of large quanta in the developing *Xenopus* neuromuscular junction

Abbreviated title; Depolarization Preferentially Releases Large Quanta

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Abstract

The amplitude histogram of spontaneously-occurring miniature synaptic currents (mSCs) is skewed positively at developing Xenopus neuromuscular synapses formed in culture. To test whether the quantal size of nerve-evoked quanta (eSCs) distributes similarly, we compared the amplitude histogram of single quantum eSCs in low external Ca$^{2+}$ with that of mSCs and found that nerve stimulation preferentially released large quanta. Depolarization of presynaptic terminals by elevating [K$^+$] in the external solution or by direct injection of current through a patch pipette increased the mSC frequency and preferentially, but not exclusively, evoked the release of large quanta, resulting in a second broad peak in the amplitude histogram. Formation of the second peak under these conditions was blocked by the N-type Ca$^{2+}$ channel blocker, ω-conotoxin GVIA. In contrast, when the mSC frequency was elevated by thapsigargin- or caffeine-induced mobilization of internal Ca$^{2+}$, formation of the second peak did not occur. We conclude that the second peak in the amplitude histogram is generated by Ca$^{2+}$ influx through N-type Ca$^{2+}$ channels causing a local elevation of internal Ca$^{2+}$. The mSC amplitude in the positively skewed portion of the histogram varied over a wide range. A competitive blocker of acetylcholine (ACh) receptors, d-tubocurarine, reduced the amplitude of smaller mSCs in this range relatively more than that of larger mSCs, suggesting that this variation in the mSC amplitude is due to variable amounts of ACh released from synaptic vesicles. We suggest that Ca$^{2+}$ influx through N-type Ca$^{2+}$ channels preferentially induces release of vesicles with large ACh content.
INTRODUCTION

A commonly used method of calculating the number of quanta contributing to an evoked synaptic potential (eSP) is to divide the mean amplitude of the eSP by the mean amplitude of spontaneously-occurring “miniature” synaptic potentials (mSPs). This is based on the observation that the amplitude distribution of mSPs is roughly Gaussian and the eSP corresponds to integral multiples of the mSP amplitude (Katz 1969; Kuno 1964; Martin and Pilar 1964; Martin 1966; Wernig 1975). However, at a number of synapses, the amplitude distribution of mSPs has been found to be extremely variable, typically positively skewed with a large proportion of small events and a long “tail” of larger events. These preparations include regenerating frog (Dennis and Miledi 1974) and mammalian (Muniak et al. 1982) neuromuscular junctions (NMJs), newly formed NMJs in *Xenopus* (Kidokoro et al. 1980; Evers et al. 1989), larval *Drosophila* NMJs (Kidokoro and Nishikawa 1994), autonomic junctions (Bornstein 1981), many CNS synapses (reviewed by Bennett 1995), and ribbon synapses in rat hair cells (Glowatzki and Fuchs 2002). In these cases, the question arises: do quanta of vastly different amplitude have the same probability of occurrence during evoked release as they do in spontaneous release? Dennis and Miledi (1974) showed that in regenerating NMJs, the mean amplitude of nerve-evoked quanta in low external [Ca\(^{2+}\)] is much larger than that of mSPs, although the distributions overlapped. Muniak et al (1982) concluded that the skew class of quanta is not available for evoked release. The reason for this discrepancy, however, remains unknown.

Attempts have been made to accommodate the skewed distribution of mSP amplitudes into the quantal hypothesis. In synapses formed in culture between *Xenopus* embryonic motoneurons and muscle cells, Kidokoro and Yeh (1982) compared the amplitude distribution of evoked synaptic potentials (eSPs) with that of mSPs. Both were positively skewed. In these synapses, synaptic transmission often failed to respond to stimuli. Thus, the quantal content was estimated by the failure method. They concluded that the amplitude histogram of eSPs can be fitted by convolution of the mSP distribution, implying that mSPs with various amplitudes have an equal probability of release upon stimulation. In the same preparation, however, Evers et al. (1989) demonstrated a clear discrepancy between the quantal size distribution deduced from analysis of multi-quantal evoked synaptic currents (eSCs) and the amplitude distribution of
miniature synaptic currents (mSCs), suggesting that a subpopulation of mSCs is preferentially released upon nerve stimulation. Nevertheless, they found that if they tested various Gaussian-distributed sizes, they could find one that fit the observed multi-peaked eSP histogram reasonably well. Thus the degree of selectivity of evoked release for quanta of different sizes is still not clear.

A second unresolved question is how to account for the wide range of quantal sizes in the skew distributions. Theoretically, this may be due to differences in release locations, postsynaptic receptor density, vesicle volume, or concentration of transmitter within vesicles. Transient fusion pore formation or non-vesicular release may also result in large variability in quantal size. It is well-documented that variations in quantal size can be due to differences in vesicle contents, and that many factors interact in the regulation of vesicle filling (see reviews by van der Kloot 2003; Edwards 2007). Which factors might account for the large quantal variability in the skew distributions at NMJs, and their gradual development into the nearly Gaussian mature distribution, remain to be explained.

Synapses in *Xenopus* nerve-muscle cultures are ideal for addressing some of these problems. Motoneurons form large varicosity synapses on muscle cells, permitting simultaneous pre- and postsynaptic voltage clamp (Yazejian et al. 1997, 2000; Sand et al. 2001; Sun et al. 2004). The high input resistances of isolated myoballs enable high-resolution recording of synaptic currents, and a single presynaptic neuron can be stimulated to evoke eSCs. The amplitude distributions of mSCs are skewed positively. We have used this preparation to compare the amplitude distribution of mSCs with that of eSCs in low external \([	ext{Ca}^{2+}]\) and observed preferential release of large quanta by nerve stimulation. We have further shown that differences in quantal size over a considerable range reflect differences in ACh content.
MATERIALS AND METHODS

Xenopus nerve-muscle cultures

Procedures for culturing Xenopus nerve and muscle cells have been described elsewhere (Young and Grinnell 1994). Mainly 2–3-day-old cultures were used in this study. In two- to three-day-old Xenopus cultures, many nerve-muscle contacts have well developed synapses with ACh receptors accumulated in the postsynaptic membrane. Some muscle cells twitch spontaneously. We examined synaptic transmission in isolated and innervated myoballs with a patch electrode in the whole-cell configuration. Although myoballs sometime receive innervation from more than one neuron, this is rare in our cultures, and preparations were selected in which myoballs appeared to be singly innervated by a single neuron.

Electrophysiology

We selected relatively mature neuromuscular junctions (NMJs) for study by recording from isolated round muscle cells (myoballs) that were seen to twitch in the culture medium. Synaptic currents were recorded in the myoball with a patch-clamp electrode in the conventional whole cell configuration (Axopatch 200B, Axon Instruments, Molecular Devices, Sunnyvale, CA). The current was filtered at 5 Hz and digitized at 50 KHz. The series resistance was compensated at the 80% level. The recording noise was 4–9 pA (root-mean-square) after compensation. The patch-electrode was filled with an internal solution containing (in mM): K gluconate, 116; NaCl, 4; MgCl2, 2; Na-ATP, 2; EGTA-KOH, 10; HEPES-NaOH, 10 (pH 7.4). The external solution had the following composition (in mM): NaCl, 116; MgCl2, 1; CaCl2, 2; KCl, 2; HEPES-NaOH, 5; glucose 5 (pH 7.4).

The input resistance of isolated muscle cells was high, up to 4.5 GΩ, while the access resistance was between 9 and 20 MΩ. Consequently the membrane potential was well-clamped with a patch electrode in the conventional whole cell configuration, judging from the fast rise-time and single exponential decay of fast synaptic currents. The holding membrane potential was set at -70 mV throughout the experiments of this study, and the liquid junction potential was -3 mV in this combination of external and internal solutions. Thus, the true holding potential was -
Three main types of electrophysiological experiment were performed: 1) Recording of nerve-evoked SCs in extracellular solution with low [Ca\(^{2+}\)] by stimulating the cell body of an innervating neuron through a perforated patch electrode. Perforation was achieved by adding 0.9 mg/ml amphotericin in the internal solution described above. During the first few minutes after forming a giga-seal, the patch membrane was gradually perforated. The neuronal cell body was voltage-clamped at a holding potential of -73 mV. To stimulate the neuron, a rectangular pulse of 30-40 mV in amplitude and 10 ms in duration was delivered at 0.2 Hz. Membrane currents in the presynaptic neuron were recorded simultaneously with those in the postsynaptic muscle cell. The extracellular solutions with low [Ca\(^{2+}\)] were made by substituting Mg\(^{2+}\) for Ca\(^{2+}\), with [Ca\(^{2+}\)] ranging between 0.1 and 0.75 mM. The Ca\(^{2+}\) concentration was adjusted in each case by varying [Ca\(^{2+}\)] in the perfusing solutions until the failure rate was 70-95%. 2) Recordings of spontaneous mSCs in normal extracellular solution, in extracellular solution containing thapsigargin or caffeine, and in extracellular solution with elevated [K\(^{+}\)]. The composition of the latter solution, which caused presynaptic depolarization, was: NaCl, 98; MgCl\(_2\), 1, CaCl\(_2\), 2; KCl 20, HEPES-NaOH, 5; glucose 5 (pH 7.4). 3) Recording of mSCs from a muscle cell during depolarization of a voltage clamped presynaptic varicosity in contact with the cell using the perforated patch configuration. All experiments were carried out at room temperature (21-24 °C).

**Chemicals**

Amphotericin B (Sigma-Aldrich, St Louis, MO) was dissolved in DMSO (4-5 mg/100 μl) as a stock solution, which was then diluted to 0.8-1.0 mg/ml in the internal solution before use. Neostigmine, d-tubocurarine (d-TC), α-bungarotoxin (α-BT), tetrodotoxin (TTX), thapsigargin, and caffeine were also purchased from Sigma-Aldrich. ω-Conotoxin GVIA (ω-CgTX) was purchased from Calbiochem (San Diego, CA).

**Definition of terms**

The rise-time of synaptic currents was defined as the time from 10% to 90% of the peak amplitude. Miniature synaptic currents (mSCs) were defined as spontaneous synaptic currents in
the presence of 1 µM TTX. Fast synaptic currents are defined as those having a rise-time equal
to or less than 1.0 ms and slow synaptic currents as those having a rise-time longer than 1.0 ms.

Data analyses and statistics

Measurements and statistical analyses of synaptic currents were carried out using
MiniAnalysis (Synaptosoft, NJ) and Origin 7 (OriginLab, MA). For comparison of two groups,
the paired student’s t test was used, while for comparison of three or more groups, ANOVA was
used with the Tuky test. When a parameter was not distributed normally, such as the amplitude
of mSCs, the non-parametric test was used. Data are expressed as mean ± SE throughout.
RESULTS

Fast and slow miniature synaptic currents with variable amplitudes in an isolated myoball

Spontaneous miniature synaptic currents (mSCs) were recorded in the presence of 1 µM tetrodotoxin (TTX). The amplitude as well as the time course of mSCs varied over a wide range (sample traces shown in Figure 1A). In the cell producing the data in Fig. 1A, the amplitude ranged between 20 pA and 3.2 nA at the holding potential of -73 mV. The low end of this amplitude range is most likely limited by the recording noise, which was typically about 4-9 pA (root-mean-square) when the series resistance compensation was set to 80%. In fact, mSCs with amplitudes less than 10 pA have been recorded in this preparation at a -90 mV holding potential (Young and Grinnell, 1994). Fig. 1B shows the average percentage of mSCs of different amplitudes (mean +/- se) in 11 junctions. The rise time of the mSCs was also surprisingly variable. Fig 1C shows average data from the same 11 myoballs. The majority of events had a fast rise-time (< 1 ms to reach from 10 to 90% of the peak amplitude, with a modal value of ~0.5 ms). A ~0.5 ms rise-time is close to that reported earlier for extracellularly recorded synaptic currents in the same preparation (~0.3 ms, Kidokoro 1984), indicating that the muscle membrane was reasonably well-clamped, at least at the sites where these fast events were generated.

However, mixed with those fast events were mSCs with slower and more variable time course, with rise-times up to 8 ms and decay $\tau$ of 4-14 ms. In 6 of the 11 junctions, there was a clear distinction between a large, relatively symmetrical population of events with a 10-90% rise time centered around 0.5 ms and a positively skewed population declining slowly from values of 1 ms to 8 ms. The other 5 junctions showed a more prominent tail of slower events, but still with a clear peak at around 0.5 ms RT. Therefore, in further consideration of fast and slow rise time events, we will arbitrarily subdivide these populations into those with 10-90% rise times of 1 ms or less and those with rise times greater than 1 ms. Given these definitions, the proportion of slow mSCs in these 11 preparations ranged from 16% to 66% (42.9±16.2, mean ± S.D.). The majority were small, but some were large (Fig. 1A) While it is difficult to assess how well the postsynaptic cell is space-clamped, there was no correlation between the input resistance or capacitance of the cell and the percentage of slow mSCs. Furthermore, the variable time course
of slow mSCs suggests that they are not generated in unclamped compartments within the cell. The origin of those slow mSCs is unknown.

Both fast and slow mSCs exhibited amplitude histograms skewed toward larger amplitudes, as is typical of synapses in this preparation (Young and Grinnell 1994; Evers et al. 1989). Fig. 2A shows the amplitude distribution for a representative single synapse. Fast mSCs (filled columns in Fig. 2A) tended to be larger in amplitude, but there was a considerable overlap with slow mSCs (open columns). 27% of fast mSCs in this cell had an amplitude greater than 400 pA, while 15% of the population of slow mSCs were larger than 400 pA. In 11 cells, these percentages were 33±20% fast mSCs and 11±9% slow mSCs (significantly different at p=0.01). Thus, fast mSCs tend to be larger than slow mSCs.

The mean amplitude of miniature synaptic potentials (mSPs) increases as ACh receptors accumulate at the subsynaptic membrane during development in culture (Anderson et al. 1979; Kidokoro et al. 1980). Where ACh receptors are densely accumulated, fast synaptic currents can be recorded with an extracellular electrode (Kidokoro 1984). Therefore, large, fast mSCs are likely to be generated at the sites of high receptor density.

The large, slow mSCs are not likely to be due to superposition of multiple fast mSCs, since their rising phase had no discernible inflections (Figure 1A). Could large and slow mSCs be due to superposition of multiple small and slow mSCs? The frequency of mSCs in the cell shown in Fig. 2 was 0.42/s. Thus, it is unlikely that multiple mSCs would be superimposed by chance. Indeed, we have not seen a fast mSC superimposed on a slow one in this cell.

The Xenopus NMJs formed in culture are cholinergic (Cohen 1972). It is possible, however, that slow mSCs are generated by another transmitter than ACh (Borodinsky and Spitzer 2007). To test this possibility, we exposed 14 innervated muscle cells to a high concentration of d-tubocurarine (dTC), 100 μM. All of these cells had fast as well as slow mSCs before application of dTC. In 13 cases, mSCs were completely abolished in the presence of dTC, while small infrequent events, including both slow and fast ones, were observed in one cell. Thus we conclude that both fast and slow mSCs in these nerve-muscle cultures were generated by release of ACh.

Another possibility is that fast mSCs are generated at sites where ACh esterase has accumulated and slow mSCs occur at sites where the enzyme is absent. To test this hypothesis, we added a reversible cholinesterase inhibitor, neostigmine, at 4 μM. In the four cells tested, we
did not observe any consistent effects of neostigmine. This result is in accord with a previous report in which an irreversible cholinesterase blocker, 10 mM methane sulfonylfluoride, had virtually no effect (Kidokoro 1984).

Since at this point we cannot account for the nature of slow mSCs, and both fast and slow quanta can be evoked by terminal depolarization or nerve stimulation, we have not distinguished between slow and fast mSCs in the following analyses.

Quantal release induced by nerve stimulation in low external Ca\(^{2+}\)

Innervated muscle cells usually exhibited large synaptic currents upon nerve stimulation in normal saline with 2 mM Ca\(^{2+}\). For stable and prolonged stimulation, the presynaptic neuron was stimulated at 0.2 Hz in the perforated patch configuration. In the cell yielding the data of Fig. 3, nerve-evoked synaptic currents (eSCs) varied between 6.6 and 10.5 nA (data not shown). The mean amplitude of 5 consecutive eSCs at 0.2 Hz was 8.1±1.7 nA in normal saline. In this experiment, the external Ca\(^{2+}\) concentration was then reduced to 0.5 mM, which resulted in almost complete failure of synaptic transmission. When the Ca\(^{2+}\) concentration was increased to 0.75 mM, nerve stimulation occasionally produced synaptic currents. The upper traces in Figure 3Aa and b show action currents recorded by the perforated patch electrode in the presynaptic neuron in this solution. As the lower traces in Fig. 3A show, one of these action potentials evoked a postsynaptic current in the muscle cell, while the other evoked no response.

The delay between the peak of the presynaptic action current and the onset of synaptic current ranged between 0.4 and 2.3 ms in the cell providing the data in Figure 3A and B, and between 0.8 and 2.1 ms in the cell from which the data in Figure 3C were obtained. This delay includes the conduction time of the presynaptic action potential and the synaptic delay. Synaptic currents following presynaptic action currents within 5 ms were considered to be evoked by the action potential. Since spontaneous synaptic currents were infrequent in these experiments (0.17/s for the case shown in Fig. 3A, B and 0.06/s for Fig. 3C), it is unlikely that spontaneous events fell in this period by chance and were erroneously counted as evoked events.

In the case shown in Fig. 3B, in saline containing 0.75 mM [Ca\(^{2+}\)], 63 of 849 nerve stimuli delivered at 0.2 Hz evoked SCs. The majority of these synaptic currents (56 events, 89%) were fast events with a rise-time less than 1.0 ms, while the others had a slower time course. The
failure rate was 92.6%. With this failure rate, Poisson statistics predict that a great majority of evoked events, 60.3, are single-quantal and 2.3 events are doublets. However, close visual inspection failed to reveal any doublets in this case. Due to the high failure rate and the low stimulus frequency, long recording sessions were required to obtain a sufficient number of eSCs for statistical analysis. To assess the properties of mSCs in these experiments, we therefore measured spontaneous SCs that occurred between stimuli, instead of measuring mSCs separately in the presence of TTX in the same cell. To justify this procedure, we compared spontaneous SCs in the absence of TTX with those in the presence of TTX (defined as mSCs) in a separate group of cells. In 7 cells, the ratio of the mean amplitude in the absence of TTX to that in its presence was 0.99±0.23 and that of the frequency was 0.93±0.47. Thus, the presence of TTX in this recording solution affected neither the amplitude nor the frequency of spontaneous SCs, indicating that spontaneous firing of presynaptic neurons was rare or non-existent in this recording condition. We thus assume that the properties of spontaneous SCs in the absence of TTX faithfully reflect those of mSCs.

The frequency of the spontaneous SCs that occurred during the period of nerve stimulation was 0.17/s, and there appears to be a broad second peak in the amplitude distribution at about 2 nA (Figure 3B, upper histogram). The percentage of spontaneously occurring quanta larger than 400 pA was 69% in this cell. The amplitude histogram of the 63 events is broad (Figure 3B lower histogram), overlapping with essentially the whole range of spontaneous SCs (Figure 3B upper histogram). However, in contrast to the spontaneous amplitude distribution, 89% of the evoked events were larger than 400 pA. Since amplitudes of eSCs as well as spontaneous SCs were not normally distributed, this parameter (percentage of events larger than 400 pA), instead of the mean, was used to compare the amplitude distribution of eSCs with that of spontaneous SCs. Fig. 3C presents data from another preparation in which the amplitude of spontaneous SCs was small (mean amplitude, 71 pA) and the frequency was low (0.06/s), indicating that this synapse was developmentally immature (Kidokoro et al. 1980). In normal saline, the amplitude of nerve-evoked SCs varied between 110 and 253 pA, with a mean of 186±51 pA (five consecutive eSCs at 0.2 Hz were averaged.), which was considerably smaller than the example shown in Figure 3A and B. In 0.75 mM Ca²⁺, 68 events were evoked by 406 stimuli, i.e., a failure rate of 0.83. Six events among 68 would be expected to be doublets. Five events were identified as doublets judging from their time courses and eliminated from the
histogram. This was the only cell, of the 9 examined closely, that had no eSCs or spontaneous SCs larger than 400pA. Thus, we cannot use the parameter defined above for comparison of the amplitude distributions. Instead we compared the medians with the Mann-Whitney non-parametric method and found a significant difference. Both fast and slow events were observed among the eSCs even in this case.

The results obtained from 9 nerve-muscle pairs included in this series of experiments are summarized in Figure 3D. In 8 cases, the percentage of quanta having an amplitude larger than 400 pA was greater in nerve-evoked SCs than in spontaneous SCs (significant at p<0.05). Also, in the one case shown in Fig. 3C where neither spontaneous nor evoked SCs had amplitudes larger than 400 pA, the median amplitude of SCs was significantly larger than that of spontaneous SCs.

Taken together, we conclude that nerve stimulation preferentially releases large quanta within the amplitude distribution seen for spontaneous release. This result is similar to that reported at regenerating frog NMJs (Dennis and Miledi 1974).

Large quanta are preferentially released upon depolarization of the presynaptic nerve terminal with high [K⁺]

Depolarization of the presynaptic nerve terminal by bath application of a saline containing high [K⁺] also favored release of large quanta. The left histogram in Fig. 4A shows the mSC amplitude histogram of a preparation in normal saline containing 0.5 μM TTX. As is typical of mSCs at the NMJ in 2- to 3-day-old *Xenopus* nerve-muscle cultures, the amplitude distribution was positively skewed. Upon application of 20 mM [K⁺] in the bath, the mSC frequency increased from 4.2/s to 10.2/s, and the amplitude histogram now showed a prominent broad second peak (middle histogram in Fig. 4A, coefficient of variance, CV, ~42.). The percentage of large mSCs, defined as those with the amplitude larger than 400 pA, increased from 21% in normal saline to 58% in the 20 mM [K⁺] solution. Linearized cumulative plots of the same data (graph to the right in Fig. 4A) show clearly the large increase in the proportion of mSCs above 1 nA in amplitude in this experiment, and ANOVA statistical determination confirmed that the two distributions differ at a high significance level (p<0.01).
In 19 similar experiments, the mSC frequency increased from 1.06±1.05/s in normal saline to 11.8±9.0 in the 20 mM [K⁺] solution. The amplitude histogram before application of high [K⁺] had a skewed distribution in all cases, although in one case a second peak was already evident. In 11 of the 19 cells tested in this way, a second peak appeared in the 20 mM K⁺ solution. Including the 7 cases in which a second peak did not appear in the high [K⁺] solution, the relative number of large mSCs as defined above, increased from 18±13% (n=23) in normal saline to 40±18% (n=23) in the high [K⁺] solution (significant at p<0.05). Thus, treatment with high [K⁺], which causes depolarization and Ca²⁺ influx through presynaptic voltage-gated Ca²⁺ channels, favors the release of large quanta.

The N-type Ca²⁺ channel blocker, ω-conotoxin (ω-CgTX), at 1 µM depresses the amplitude of voltage-gated Ca²⁺ currents to ~9% of the control in the presynaptic varicosities and abolishes synaptic transmission at nerve-muscle synapses in *Xenopus* cultures (Yazejian et al. 1997; Sand et al. 2001). When 1 µM ω-CgTX was added to the 20 mM [K⁺] solution in the present experiments, the increase in mSC frequency and the appearance of a second peak were blocked (Fig. 4A, right histogram). As the lognormal plots in Fig. 4A show, the distribution of mSC amplitudes in high K⁺ after ω-CgTX treatment did not differ from controls in normal K⁺. In 5 cases, 1 µM ω-CgTX reduced the mSC frequency in high [K⁺] from a mean of 8.0±5.2/s to 1.8±1.4/s (significant at p<0.05). In all cases in which a second peak was observed during application of high [K⁺], 0.4-2 µM ω-CgTX abolished it. The percentage of large mSCs was 43±16% in high K⁺ and 26±11% in high [K⁺] plus ω-CgTX (significant at p<0.05). We conclude that ω-CgTX blocked the preferential release of large quanta by depressing Ca²⁺ influx through presynaptic N type Ca²⁺ channels.

Large quanta are preferentially released upon direct depolarization of the presynaptic varicosity by a patch electrode.

To confirm the effect of depolarization on the mSC amplitude distribution, we directly depolarized the presynaptic terminal with a patch electrode. To depolarize the presynaptic terminal, the presynaptic varicosity was voltage-clamped with a patch electrode in the perforated patch configuration. Simultaneously, synaptic currents were recorded in the postsynaptic muscle cell voltage-clamped with another electrode in the conventional whole cell configuration.
Spontaneous mSCs were recorded in the presence of 1 μM TTX while the presynaptic membrane potential was held at -73 mV. In the representative case shown in the left histogram in Fig. 4B, the frequency of mSCs was 0.61/s and the amplitude distribution was, as usual, skewed toward larger values. Then, the terminal was depolarized to -33 mV by passing current through the electrode. The mSC frequency increased to 2.9/s and a second peak appeared in the larger amplitude range (Fig. 4B, right histogram). The percentage of large mSCs, as defined above, was 7% at the resting state and 34% during depolarization. The linearized cumulative plots of the same data (Fig. 4B, graph to the right) clearly show that the distributions are different (p<0.01, ANOVA). On the other hand, it should be noted that the frequency of release of small quanta is also increased, in this experiment to approximately 4.5X the resting level, compared with more than a 9-fold increase in large quanta. In the experiment of Fig. 4A, high K+ increased the frequency of small mSCs by ~50%, compared with an 11-fold increase in large quanta.

Eight varicosities were depolarized to between -33 and -28 mV in this series of experiments. This treatment increased the mSC frequency from a mean of 1.1±.08/s at -73 mV to 18.8±14.5/s at the depolarized state. In 5 cases, an obvious second peak in the amplitude histogram appeared during depolarization. In the remaining 3 cases, the proportion of large mSCs increased. For all 8 cases, the percentage of large mSCs increased from 6.9±7.5% to 28.7±23.5% (p<0.05). In two of the 8 junctions, 1 μM ω-CgTX was applied in the bath while the varicosity was depolarized to -33 mV. In both cases, the second peak in the amplitude distribution disappeared and the mSC frequency decreased to the control level. We conclude that presynaptic Ca\(^{2+}\) influx through the N-type Ca\(^{2+}\) channels induced by depolarization preferentially facilitates release of quanta generating large mSCs.

Release of internal Ca\(^{2+}\) in the presynaptic terminal does not induce preferential release of large quanta

The results presented above lead to the following question: does an elevation of [Ca\(^{2+}\)] in the varicosities due to release from internal stores have the same effect on the amplitude histogram of mSCs as enhanced Ca\(^{2+}\) influx through voltage-gated channels? An increase in the mSC frequency per se might have induced recruitment of synaptic vesicles from the reserve pool, which might be comprised of vesicles with relatively high ACh content, as is the case for
glutamate-containing vesicles at the *Drosophila* NMJ (Steinert et al. 2006). We tested this possibility by applying drugs that increase the mSC frequency by mobilizing internal Ca\(^{2+}\).

Thapsigargin, an inhibitor of Ca\(^{2+}\) uptake by the smooth endoplasmic reticulum, increases the cytoplasmic Ca\(^{2+}\) concentration by causing uncompensated Ca\(^{2+}\) leakage from ER (Thastrup et al. 1990). In the case shown in Fig. 4C, application of 10 \(\mu\)M thapsigargin increased the mSC frequency by four-fold. The percentage of large quanta was unchanged, 24% before drug application and 22% in the saline containing thapsigargin. In 6 such experiments, application of thapsigargin increased the mSC frequency \(6.3\pm9.4\)-fold. The percentage of large mSCs changed from 17\(\pm16\) to 31\(\pm24\)%, which is not significant at \(p<0.05\).

Caffeine, a ryanodine receptor activator, also elevates cytosolic [Ca\(^{2+}\)] (Rousseau et al. 1988). We applied 5 mM caffeine in two cases, one of which is shown in Fig. 4D. The mSC frequency increased from 0.6/s to 4.6/s in one case and from 0.15/s to 5.8/s in the other. However, the percentage of large mSCs did not change, and was 6% both before and after caffeine application in one case, and stable at 8% in the other. Thus, we conclude that increased spontaneous release due to release of [Ca\(^{2+}\)] from internal stores does not induce preferential release of large quanta.

*mSCs with large amplitudes are more resistant to 10 \(\mu\)M d-tubocurarine than smaller ones*

Still unanswered is the question of what causes the wide variability in mSC amplitudes. Is it due to differences in receptor density, amount of ACh released in different quanta, or does it have some other explanation? The large variation of mSC amplitude within the second peak in the patch-clamp recording could be due either to variation in the amounts of ACh released or to a variation in the postsynaptic receptor density. Since dTC is a competitive blocker of ACh receptors (Jenkinson 1960), its effect will decrease with increasing ACh concentration. Therefore, at submaximal concentrations of dTC, larger quanta will be less completely blocked than smaller ones. On the other hand, if the same amount of ACh is released at postsynaptic areas differing in ACh receptor density, dTC should block mSCs equally efficiently regardless of their amplitudes.

Following this experimental approach, for 5 to 10 minutes we recorded mSCs in mature synapses in which large mSCs were abundant to obtain a representative control amplitude
distribution. Then, in the presence of 10 μM dTC, we recorded mSCs for exactly the same period. In 10 μM dTC, the frequency of mSCs decreased to 29~56% (50±12%, n=5) of the control value, and the mean amplitude declined to 53.8±12.1%. Assuming that the population of synaptic vesicles that had been released during the control and dTC treatment periods did not change, the decrease in the mSC frequency can be attributed to occlusion of small mSCs in the recording noise. To analyze the effect of dTC on the amplitude of remaining mSCs, the 50 largest of these were divided into 5 groups of 10 events sequenced in the order of size. The 1st group of the largest ten events in the control situation should then correspond to the ten largest events in the presence of dTC, the 2nd group in the control should correspond to the 2nd group in dTC, and so on. The amplitude was averaged within each group and the corresponding groups in the control and dTC groups were compared. Since there were more than 50 clearly discernible events even after treatment with dTC, our analysis was not affected by the fact that the smallest events were lost in noise after treatment. In Figure 5, the percentage block by dTC is plotted against the group number (filled circles). The percentage block was defined as \(\{1-(\text{mean amplitude in d-TC}/\text{mean amplitude in control})\}\times100. In the 5 cases in this series of experiments, the largest mSCs (1st group) were blocked by 55.2±10.7%, while those in the 4th group were blocked by 74.8±6.9% and those in the 5th group were blocked by 76.4±7.4% (both are significantly larger than control at p<0.05). Thus the effect of dTC was stronger in the 4th and 5th groups than in the 1st group.

In contrast, we performed similar experiments applying α-bungarotoxin, an irreversible blocker of ACh receptors (Chang and Lee 1963), on 6 cells. The effect of a non-competitive blocker is expected to be independent of the amount of released ACh. In agreement with this notion, all 5 size groups of mSCs were similarly affected by the blocker (Figure 5, open circles) in this series of experiments.

These results confirm the previous conclusion that the large variation of mSC amplitude within the second peak is due to variation in the amount of ACh released (Kidokoro 1984).
The quantal content of nerve-evoked synaptic currents cannot be estimated using the skewed mSC amplitude distribution

A principal aim of this study was to determine whether, in a neuromuscular preparation with a positively skewed mSP distribution, evoked single quanta display the same amplitude distribution or conform to a nearly Gaussian distribution. Dennis and Miledi (1974) first described the discrepancy between the nerve-evoked quantal size and the mean mSP amplitude in regenerating frog NMJs, finding that the mSP amplitude histogram was highly skewed while evoked single quantum EPPs formed a roughly Gaussian distribution of larger mean size. They concluded that quantal analysis using the mean amplitude of mSPs would not be expected to produce a meaningful estimate of the quantal content in preparations with a skewed mSC amplitude distribution. Since then, this problem has been largely forgotten and quantal content has regularly been calculated by using the mean of mSPs, even in cases where the mSP amplitude histogram is skewed, such as the Drosophila NMJ (Littleton et al. 1994; Giagtzoglou et al. 2009) and developing synapses in Xenopus nerve-muscle cultures, (Kidokoro et al., 1980; Kidokoro and Yeh 1982; Evers et al 1989). Evers et al (1989), for example, attempted to estimate the quantal content of eSCs independently of the amplitude distribution of mSCs. In a nerve-muscle contact formed within one hour prior to the recording, they observed multiple peaks in the amplitude histogram of eSCs in 2 mM [Ca^{2+}] with a failure rate of 0.08. They did not use the skewed amplitude distribution of mSCs for quantal analysis. Instead, they arbitrarily
assumed a Gaussian distribution of the quantal size with a relatively small CV, 31. By adjusting the mean amplitude of quantal size, they found a reasonable fit of multiple peaks in the eSC amplitude histogram and a quantal content predicted from the failure rate. They concluded that the quantal size of eSCs is normally distributed and different from the mean of mSCs.

We have readdressed this problem in *Xenopus* preparations where we could stimulate a single synaptic input to an isolated small myoball, avoiding complications due to electrical coupling with neighboring muscle cells and ensuring adequate voltage-clamping. In external Ringer with low [Ca$^{2+}$], when the failure rate of nerve stimulation to eSCs was over 0.8, Poisson statistics predict that the majority of eSCs are a single quantum. Under these conditions, we compared the amplitude distribution of quantal size of eSCs with that of spontaneous SCs in the same preparation. We found that nerve stimulation induced preferential release of large quanta compared with the size distribution of spontaneously-occurring mSCs. Moreover, the quantal size of eSCs typically had a broad, non-Gaussian amplitude distribution, e.g., a CV of ~55 pA for the case shown in Figure 3B, which is not compatible with the multiple peaks that Evers et al. (1989) observed in their eSC amplitude histogram. We conclude, therefore, that when the amplitude distribution of mSCs is skewed, the quantal content cannot safely be estimated by dividing the mean amplitude of eSCs with that of mSCs.

As developing or regenerating NMJs mature, the mean mSC amplitude increases and the amplitude distribution becomes nearly Gaussian (Dennis and Miledi 1974; Muniak et al. 1982; Kidokoro et al. 1980; Kidokoro 1984). Evoked release also shows maturational changes (Buchanan e al, 1989; Evers et al. 1989). Kidokoro and Yeh (1982) attempted quantal analysis on the synapses formed by *Xenopus* motoneuron growth cones on muscles cells soon after contact. The growth cones release ACh anywhere they contact muscle cells (Young and Poo 1983; Hume et al. 1983). Since the contact area between the growth cone and muscle cell was small, they used binomial statistics to analyze nerve-evoked synaptic potentials (eSPs). The amplitude histogram of eSPs was reasonably fitted using the amplitude distribution of mSPs, and, unlike our current findings, there was no indication of an excess of large amplitude eSPs.

While the synapses used in the present study were mostly more mature (2~3 days in culture), we encountered one nerve-muscle pair in which the spontaneous SC frequency was low and their amplitudes were small, indicating that this synapse was immature (Fig. 3C). In this synapse, the discrepancy between the amplitude histograms of eSCs and spontaneous SCs was relatively
small compared with that seen in the other 8 cell-pairs that had higher frequency of spontaneous
SCs with larger amplitudes. It seems likely, therefore, that the mechanism that preferentially
releases large quanta upon nerve stimulation develops gradually during synaptogenesis,
associated with formation of specialized presynaptic release sites.

\[ \text{Ca}^{2+} \text{ influx through N-type Ca}^{2+} \text{ channels preferentially releases large quanta} \]

Nerve stimulation activates presynaptic Ca\(^{2+}\) channels and induces localized Ca\(^{2+}\) influx
(DiGregorio et al. 2001). Most of the presynaptic Ca\(^{2+}\) channels in the *Xenopus* developing NMJ
are N-type, blockable by \(\omega\)-CgTX (Sand et al. 2001; Thaler et al. 2001). Because \(\omega\)-CgTX
blocked all release in this preparation, N-type Ca\(^{2+}\) channels are clearly coupled to the
preferential release of larger quanta. Like nerve-evoked release, depolarization of the presynaptic
terminal with high [K\(^+\)] or direct depolarization with a patch electrode induced preferential
release of larger quanta and often produced a second peak in the amplitude histogram (Fig. 4).
Even when the second peak was not observed, the percentage of mSCs larger than 400 pA
increased in most cases when the presynaptic terminal was depolarized. These changes in the
amplitude histogram were blocked by \(\omega\)-CgTX (Fig. 4A). However, it is not simply elevation of
cytosolic [Ca\(^{2+}\)] that induces preferential release of large quanta. An elevation of [Ca\(^{2+}\)] due to
release from internal stores, induced by treatment with thapsigargin or caffeine, caused an
increase in mSC frequency, but not preferential release of large quanta (Fig. 4C, D). At *Xenopus*
NMJs, N-type channels are co-localized with release sites (Cohen et al. 1991; DiGregorio et al.
2001). Similarly, at the *Drosophila* NMJ, the Ca\(^{2+}\) channels responsible for release are clustered
at the release sites (Kawasaki et al. 2004). Thus, localized Ca\(^{2+}\) entry near the release sites seems
to be needed for the preferential release of large quanta, and vesicles that are more “mature”, i.e.,
filled with more ACh, tend to be most tightly associated with Ca\(^{2+}\) channels, probably
predominantly at active zones.

\[ \text{The broad distribution of mSC amplitudes in the second peak is due to variable amounts of ACh in synaptic vesicles} \]
Previously, it has been observed that older *Xenopus* nerve-muscle cultures tend to exhibit a second peak in the mSP amplitude histogram even in normal saline, suggesting that appearance of the second peak reflects a developmental process (Kidokoro and Yeh 1982; Kidokoro 1984). A second peak in the amplitude histogram was also observed using extracellular electrodes, recording mSCs arising at discrete spots a few μm in diameter, where ACh receptors were seen to be accumulated. (ACh receptor clusters were visualized by staining with an antibody that did not block ACh receptor function.) Displacement of the electrode tip by a few μm from the recording site abruptly reduced the current amplitude, reflecting the good spatial resolution of the extracellular recording technique. Since the postsynaptic receptor density is expected to be quite uniform in the small area from which recorded synaptic currents were arising, it was concluded that the large variation in the amplitude of mSCs in the second peak was due to the variation of the amount of ACh released and not to the variation in postsynaptic receptor density (Kidokoro 1984).

In the present study, we re-examined the mechanism of variability in mSC amplitude in a different way. D-tubocurarine (d-TC) is a competitive inhibitor of ACh receptors (Jenkinson 1960). Therefore, a given concentration of d-TC competes more successfully the lower the ACh concentration. If the cleft ACh concentration is higher when large mSCs are generated, d-TC will be less effective in blocking such mSCs than smaller ones. We found this to be the case (Fig. 5, filled circles). In contrast, when the ACh receptor density was reduced by an irreversible ACh receptor blocker, α bungatotoxin, the amplitude of mSC was equally reduced irrespective of their size (Fig. 5, open circles). Therefore, we conclude that the mSC amplitude variation in the second peak is primarily due to a variation of the amount of ACh released, in accord with the previous report (Kidokoro 1984).

How does the ACh content in synaptic vesicles (SVs) vary over such a wide range? It is well known that high frequency stimulation of the frog NMJ can reduce mEPP size by approximately 20%, apparently via a presynaptic mechanism (Doherty et al. 1984; Naves and Van der Kloot 2001), Blocking activity at mammalian NMJs leads to a large increase in quantal size, again via a presynaptic mechanism (Wang et al. 2005). Indeed, in many different synapses, there is evidence that transmitter content of vesicles is subject to wide variability (reviewed by Van der Kloot 1991; Sulzer and Pothos 2000; Edwards 2007). Sometimes this is correlated with differences in vesicle volume (Karunanithi et al. 2002; van der Kloot 2003; Bruns et al. 2000); in
other cases, the difference is in transmitter concentration rather than vesicle size (Colliver et al. 2000; Wu et al. 2007). It is not clear what mechanisms explain the differences either in vesicle size or transmitter concentration, but both may be dependent to a large degree on the number and activity of transmitter transporters (Edwards 2007). Overexpression of vesicular ACh transporters (VAcHt) in the *Xenopus* nerve muscle cell cultures sharply increases quantal size (Song et al. 1997), while reduction in VAcHt expression sharply reduces quantal size (Prado et al., 2006). ACh in SVs is not likely to be in a free form. The number of ACh molecules in a mature SV has been estimated to be approximately 10,000 at the snake NMJ (Kuffler and Yoshikami 1975). If 10,000 molecules are contained in one vesicle of 40 nm diameter, the concentration will be ~0.5 M, similar to that measured for catecholamines in rat chromaffin cells (0.7 M, Albillos et al. 1997). It seems highly unlikely that these high concentrations of ACh and catecholamines could be in a dissociated state, due to the very high osmotic pressures that would result. In the chromaffin cells, catecholamines are bound to chromogranins (Helle et al. 1985). In fact, knock-out of chromogranin A resulted in smaller amounts of catecholamines in each vesicle (Montesinos et al. 2008). If the same applies to ACh-containing SVs, SVs of similar sizes could contain various amounts of ACh depending on the amount of the binding proteins.

Apart from the wide variability in size of mSCs in the second peak of the amplitude histogram, the origin of the much larger number of much smaller events in the first peak of the histogram remains unexplained. Electron micrographs of synapses in the *Xenopus* cultures do not show large numbers of tiny vesicles (Weldon and Cohen 1979; Takahashi et al. 1987; Buchanan et al.1989). The mean amplitude of mSPs has been correlated with the process of ACh receptor accumulation in the postsynaptic membrane during development (Anderson et al. 1979; Kidokoro et al. 1980). During the period of ACh receptor accumulation, the skewed amplitude distribution remains unchanged. Only later does the second peak in the mSC amplitude histogram appear (Kidokoro 1984). It seems likely that the wide amplitude variation within the whole population of mSCs is due, at least in part, to the variations in postsynaptic ACh receptor density. However, by extrapolation of the evidence for variable filling as an explanation for the size differences in the quanta forming the second peak in the amplitude distribution, variable filling with ACh may also contribute to this population. A developmental delay in the mechanism for packaging ACh inside SVs might explain the large variability and gradual disappearance of the population of small events during maturation. The vesicular ACh
transporter exhibits a millimolar $K_m$ and a slow transport rate (Edwards 2007). This implies a high concentration of ACh in the cytoplasm. If, at early stages in development, SVs form with a paucity of VACHTs or of H$^+$ transporters to drive the VACHTs, they may contain only approximately the cytoplasmic concentration of ACh. If filling of vesicles is slow at this developmental stage, vesicles might be only partially filled when they are released. However, one would then expect synapses with lower release rates to have larger mSCs. This was not observed to be the case. An alternative possibility would be a partial release of SV contents through a fusion pore during brief “kiss-and-run” fusion (Ceccarelli et al. 1973; Fesce et al. 1994), which would produce small mSCs. It is not clear, however, whether fusion pore formation could be so brief that only a small fraction of a vesicle’s content of molecules as small as ACh could escape (Stiles et al. 1996).

Our data are consistent with the interpretation that there are two general mechanisms of release, with different internal [Ca$^{2+}$] requirements (Angleson and Betz 2001). When [Ca$^{2+}$] is elevated within the terminal by release from internal stores, the concentration at release sites near the membrane is relatively modest and release of all quantal sizes increases approximately in proportion to their spontaneous release probability at resting levels of [Ca$^{2+}$], presumably via the same mechanism. However, when [Ca$^{2+}$] is locally elevated to high levels by influx through N-type Ca$^{2+}$ channels, there is preferential release of a different category of quanta (or vesicles) that are selectively associated with those channels. In the youngest synapses, there may be few if any quanta that exceed the mean size of those that are spontaneously released at rest; but as development proceeds, an increasing proportion are much larger. These large quanta probably represent vesicles with relatively high concentrations of ACh, although alternative explanations for the large variability in quantal size, like differences in vesicle diameter or contribution of “kiss and run” fusion, have not been ruled out. The increased proportion of large quanta during synapse maturation may reflect the growth of the active zone apparatus, and suggests that it somehow induces associated vesicles to fill more completely than those that are not near the active zone, or that the structures at the active zones associated with Ca$^{2+}$ channels selectively attract the vesicles containing the most ACh.
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**FIGURE LEGENDS**

**Figure 1.** Miniature synaptic currents (mSCs) in an isolated myoball. A, Sample trace showing typical variability in amplitude of spontaneously occurring mSCs. Below are representative single mSCs at higher time resolution grouped into the categories of “fast” (10-90% rise time <1 ms) and “slow” events (10-90% RT >1 ms). Note that the time courses of slow mSCs are extremely variable. B. Averaged amplitude histogram of mSCs in the presence of 1 µM TTX for 11 junctions in which long duration recordings were made under good voltage clamp conditions. The portion of the distribution > 1 nA are plotted on a magnified scale as an inset. C. Histogram of 10-90% rise times of mSCs recorded in the same 11 junctions used for part B. Fast and slow mSCs are defined arbitrarily as explained in the text.

**Figure 2.** The amplitudes of fast and slow mSCs overlap extensively. A. Amplitude histogram of “fast” (dark columns) and “slow” mSCs (open columns) from a single representative synapse. The cell from which the data shown in this figure were obtained had an input resistance of 980 Ω and input capacitance of 56 pF. B, Relationship between amplitude and rise-time. Closed circles; fast mSCs in the same synapse. Open circles; slow mSCs. C, Relationship between amplitude and decay time constant. Closed circles; fast mSCs. Open circles; slow mSCs.

**Figure 3.** Nerve-evoked synaptic currents in saline with low external [Ca²⁺]. Aa and Ab, Sample traces from one cell pair. Upper traces; action currents in the presynaptic neuron. Lower traces; synaptic currents in the postsynaptic myoball. In the upper pair of traces, an action potential in the presynaptic neuron evoked a quantal release, while in the lower traces, no synaptic current was evoked. In this cell pair 93% of stimuli did not evoke synaptic currents. The external Ca²⁺ concentration was 0.75 mM. Stimuli were given at 0.2 Hz. B, amplitude histograms for spontaneous mSCs (upper histogram) and evoked SCs (lower histogram) for the cell pair presented in A. The myoball had an input resistance of 103 MΩ and input capacitance of 91 pF. C, amplitude histograms for another cell pair. In this myoball, in 0.75 mM Ca²⁺, the spontaneous SC frequency was 0.06/s and the mean amplitude was 71 pA, indicating that the synapse was immature. Amplitude histograms for spontaneous SCs (upper histogram) and
evoked SCs (lower histogram). This myoball had an input resistance of 220 MΩ and input capacitance of 83 pF. D, Percentage of large events among nerve-evoked quanta (right) and spontaneous SCs (left) in 10 cell pairs. Events with amplitudes larger than 400 pA were defined as large. Two points connected by a line present paired data from the same cell.

Figure 4. Effects of high [K⁺], direct presynaptic depolarization, thapsigargin and caffeine on the amplitude histogram of mSCs. A, Effects of depolarization induced by a solution with 20 mM [K⁺] on the amplitude distribution of mSCs. Left histogram; control in normal saline. The frequency of mSCs was 4.2/s. Note the skewed distribution of mSCs toward larger amplitudes. Middle histogram; in the 20 mM K⁺ solution, the frequency of mSCs increased to 10.2 Hz and the distribution of mSCs displayed a pronounced second peak. Right histogram; in the presence of 0.4 μM ω-CgTX in 20 mM [K⁺], f = 3.8 Hz. Note that ω-CgTX abolished the effect of depolarization on the mSC amplitude histogram. The graph to the right shows a lognormal plot of the cumulative amplitudes of mSCs in all three conditions. Linear fits were estimated separately for events <1 nA and >1 nA for data obtained in NFR and in high K⁺. The data for high K⁺ + ω-CgTX clearly does not differ significantly from that in NFR. The 95% confidence limits are shown by dashed lines bordering the linear fit lines. (The confidence interval lines are obscured by the data symbols for the fits >1 nA.) B, Effects of direct depolarization of the presynaptic varicosity by a patch-electrode. Left histogram; control with the varicosity clamped at -73 mV. Right histogram; the varicosity was clamped at -33 mV. The mSC frequency increased from 0.6/s in the control to 2.9/s during depolarization. Note that direct, presynaptic depolarization also induced a second peak in the histogram. The graph to the right, similar to that in 4A, shows a lognormal plot of cumulative amplitudes in control and depolarized states, with linear fits and the he 95% confidence limits for events <0.3 nA and >0.3 nA. C, Effects of thapsigargin. Left histogram; control in normal saline. Right histogram; in the presence of 2 μM thapsigargin in normal saline. The mSC frequency increased from 0.27/s in the control to 1.2/s in the presence of thapsigargin, but no second peak appeared in the histogram. There was no significant difference between the two sets of data. D, Effects of caffeine. Left histogram; control in normal saline. Right histogram; in the presence of 5 mM caffeine in normal saline. The mSC frequency increased from 0.6/s in the control to 4.6/s in the presence of caffeine. Caffeine also failed to
induce a second peak in the histogram and there was no significant difference between the amplitude distributions under the two conditions.

Figure 5. Effects of d-TC and α-bungarotoxin on the amplitude of mSCs. mSCs were initially recorded for a sufficient period to collect more than 200 mSCs in normal saline with 1 µM TTX. Then the preparation was exposed to a competitive blocker, d-TC at 10 µM, or an irreversible blocker, α-bungarotoxin (α-BTX) at 10 nM in normal saline and mSCs were recorded for exactly the same period as in control solution. In the case of α-BTX, the preparation was exposed for approximately 5 min, and the blocker was washed out before the recording started. The recorded mSCs were first arranged in descending order of amplitude. The average of ten events was calculated consecutively and grouped from 1 to 5 in descending order, i.e., group1 comprises the 10 largest mSCs. The percentage of blockade, i.e., \(\{1-(\text{mean amplitude in d-TC}/\text{mean amplitude in control})\} \times 100\), is plotted on the y-axis against the group number on the x axis. For the d-TC-exposed preparations (filled circles), the differences between the 1st and the 4th and 5th groups were statistically different at p<0.05 and are indicated by *. Thus, the blocking effect of d-TC increases as the mSC amplitude decreases. For the α-BTX-exposed preparations (open circles), none of the groups were statistically different from any other groups. N=6 for both series of experiments.