Intraterminal \( \text{Ca}^{2+} \) and Spontaneous Transmitter Release at the Frog Neuromuscular Junction

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Received 24 January 2000; accepted in final form 15 September 2000

INTRODUCTION

The cellular and molecular complexities of the processes involved in \( \text{Ca}^{2+} \)-dependent secretion are beginning to be unraveled in detail (reviewed in Angleson and Betz 1998; Neher 1998; Zucker 1996). \( \text{Ca}^{2+} \) plays an essential role in triggering evoked neurotransmitter (Katz 1969). Influx of \( \text{Ca}^{2+} \) during an action potential through \( \text{Ca}^{2+} \)-channels located in the plasma membrane of the presynaptic terminal raises intraterminal \( \text{Ca}^{2+} \) concentration ([Ca\(^{2+}\)])\(\text{a}\)), which then triggers exocytosis and transmitter release. A quantitative understanding of the [Ca\(^{2+}\)]\(\text{a}\) dependence of transmitter release requires knowledge of the [Ca\(^{2+}\)] affinities of \( \text{Ca}^{2+} \) sensors required for transmitter release and the spatial relationships between the site of \( \text{Ca}^{2+} \) influx and \( \text{Ca}^{2+} \) sensors. Different models exist for the spatial relationship between \( \text{Ca}^{2+} \) channels and \( \text{Ca}^{2+} \) sensors for release. The single-channel domain hypothesis places the transmitter release site close (\(~10 \text{ nm})\) to a single \( \text{Ca}^{2+} \) channel where the \( \text{Ca}^{2+} \) sensor for release experiences a restricted, short-lived domain of high [Ca\(^{2+}\)] \(~100 \mu\text{M}\). The overlapping domain hypothesis places the release sites at locations more remote (\(100-200 \text{ nm}\)) from \( \text{Ca}^{2+} \) channels where [Ca\(^{2+}\)] reaches concentrations of \(<10 \mu\text{M}\) and where the \( \text{Ca}^{2+} \) sensor is influenced by \( \text{Ca}^{2+} \) influx from multiple channels (for reviews, see Neher 1998; Stanley 1997).

Neurons that undergo synchronous or evoked release of transmitter in response to an action potential also display asynchronous and spontaneous transmitter release. At the frog neuromuscular junction, there is evidence that the synchronous, evoked end plate potentials (EPPs) may have different \( \text{Ca}^{2+} \) requirements and be subject to distinct regulation from asynchronous release or the spontaneous miniature end plate potentials (MEPPs). For example, there appears to be different divalent cation requirements for the different types of release (Zengel and Magleby 1981). In addition to \( \text{Ca}^{2+} \) stimulation of MEPPs, it has been reported that the frequency of MEPPs can be increased by \( \text{Ca}^{2+} \)-independent mechanisms (e.g., Chen and Grimnell 1997; Kijima and Tanabe 1988).

The ability to simultaneously monitor transmitter release and measure intraterminal [Ca\(^{2+}\)] with fluorescent indicators at the frog neuromuscular junction (Narita et al. 1998; Robitaille et al. 1996; Wu and Betz 1996) can allow for direct assessment of the \( \text{Ca}^{2+} \) dependence of MEPPs. We have used ratiometric imaging of fura-2 in presynaptic terminals together with intracellular recordings of MEPPs to investigate the relationship between presynaptic [Ca\(^{2+}\)] and spontaneous transmitter release at the frog neuromuscular junction. Various treatments that alter both MEPP frequency and [Ca\(^{2+}\)] gave the same [Ca\(^{2+}\)] dependence of transmitter release. The results suggest that MEPP frequency is stimulated by a low-threshold, high-affinity \( \text{Ca}^{2+} \) sensor that senses \( \text{Ca}^{2+} \) from multiple neighboring \( \text{Ca}^{2+} \) channels during mild depolarization. A preliminary report of this study has appeared in abstract form (Angleson and Betz 1997).

METHODS

Preparation and solutions

Frog (\textit{Rana pipiens}) cutaneous pectoris nerve-muscle preparations were dissected and mounted in silicone elastomer (Sylgard)-lined chambers containing normal frog Ringer [which contained (in mM) 115 \text{NaCl}, 1.8 \text{CaCl}_2, 2 \text{KCl}, 5 \text{HEPES-NaOH}, \text{pH} 7.2]. High-KCl solutions were prepared by elevating extracellular KCl concentration.

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(KCl) to the indicated level with an offsetting reduction in [NaCl] to keep all solutions isotonic. Ionomycin (Calbiochem), prepared as a 5 mM stock in dimethyl sulfoxide (DMSO) was added to normal frog Ringer at a concentration of 0.1–10 μM immediately before use. The phorbol esters phorbol 12-myristate 13-acetate (PMA) and 4a-phorbol 12-myristate 13-acetate (4a-PMA; Biomol, Plymouth Meeting, PA) and the protein kinase inhibitor staurosporine (Sigma) were prepared as concentrated stock solutions in DMSO. Neostigmine (1 μg/ml; Marsam Pharmaceuticals, Cherry Hill, NJ) was added to some experiments to increase MEPP amplitude. It did not effect MEPP frequency. All solutions used for electrophysiology or Ca²⁺ imaging contained 1 μM tetrodotoxin (Sigma) to block Na⁺-dependent action potentials.

The membrane permeable acetoxymethyl ester form of the Ca²⁺ chelators EGTA-AM and bis-(o-aminophenoxy)-N,N,N',N'-tetraacetic acid-AM (BAPTA-AM, Molecular Probes, Eugene, OR) were stored in stock solutions (5 mM) in DMSO. The standard conditions for loading neuromuscular preparations for our studies were based on those described by Robitaille et al. (1993) and consisted of incubation in normal frog Ringer with 25 μM EGTA-AM or BAPTA-AM for 60 min at room temperature (20–23°C). The preparation was then washed with normal frog Ringer before use. Increasing the concentration to 100 μM, the incubation time to 2 h or the temperature to ~30°C did not improve the apparent effectiveness of the loading. Control incubation with 0.5% DMSO did not significantly alter MEPP frequency in our experiments.

Ca²⁺ imaging

The method for loading nerve terminals with membrane impermeant pentapotassium salt of fura-2 (Molecular Probes) was similar to that first described for loading bullfrog sympathetic nerve terminals (Peng and Zucker 1993) and has been described in detail for this preparation (Wu and Betz 1996). A short piece of freshly cut nerve trunk was placed into a drop of 50 mM of fura-2 salt. It was important to place the nerve into the fura-2 solution within ~5 min of cutting to allow exchange into the nerve, longer time intervals prevented efficient loading, presumably due to resealing of the nerve. Fura-2 was then allowed to diffuse down the cut nerve for 2–3 h at room temperature (20–23°C) in normal Ringer. The preparation was rinsed and incubated overnight in normal frog Ringer at 4°C and used the following day for imaging and electrophysiology. The loading and incubation procedures had no detectable effect on MEPP frequency or amplitude either at rest or in elevated KCl solutions and the loading conditions were previously found not to have an effect on EPP amplitude (Wu and Betz 1996).

Fura-2-loaded terminals were imaged with a Sensys-cooled CCD camera (Photometrics, Tucson, AZ) on a Nikon Optiphot upright microscope using a Zeiss long working distance ×40 water-immersion lens (0.75 N.A.). Excitation was alternated between 360- and 380-nm band-pass filters (Chroma Technology) using a Sutter Lambda-10 filter wheel. Emitted light was collected through 510/40 dichroic and 400-nm long pass filter. Images (16 bit) were collected on a PC running program written in Vwindows (Photometrics) and analyzed off-line on a Silicon Graphics O2 workstation running software from G. W. Hannaway (Boulder, CO). The outline of a terminal from one image was drawn by hand and the integral fluorescence intensity for the pair of images was determined. No noticeable or consistent “hot spots” of elevated [Ca²⁺], were detected. Care was taken to exclude regions of preterminal axon that do not demonstrate depolarization-dependent changes in fura-2 signal (Wu and Betz 1996). Background fluorescence from a region without nerve terminal was subtracted from each image before ratio fluorescence calculations. Intraterminal Ca²⁺ concentration ([Ca²⁺]i) was estimated from the fura-2 ratios (Gryzienksi et al. 1985) using the procedures and in vitro calibrations described in Wu and Betz (1996). The fura-2 concentration in the terminals was estimated to be 5–50 μM using methods detailed in Wu and Betz (1996).

Electrophysiology

Intracellular recordings of MEPPs from surface muscle fibers were performed with micropipettes (15–20 mΩ) filled with 3 M potassium acetate using standard techniques (Wu and Betz 1996). Sweeps of between 25- and 2,024-ms duration were collected with an acquisition time of between 0.1 and 0.25 ms and stored and analyzed on a PC running software written in Axobasic. For conditions with stable and low MEPP frequencies (2 or 10 mM [KCl]), MEPPs were acquired intermittently over a ~120-s period. For high-MEPP frequency conditions (16 or 20 mM [KCl], or ionomycin), MEPPs were acquired during a sufficient number of sweeps of 25- to 250-ms duration to yield 200–300 MEPPs to be counted to determine a frequency. When recordings were made in 2 or 10 mM [KCl]o, the MEPP frequency was constant over the entire period sampled (~30 min). MEPPs in 16 or 20 mM [KCl]o were acquired at points between 20 and 40 s after switch to the indicated solution. This time interval was chosen to avoid muscle contractions that often occurred in 20 mM [KCl], during the first ~15 s after switch to this solution and to avoid recording at after prolonged exposure to high [KCl], something that has been reported to cause rundown of MEPP frequency.

Fura-2 images were recorded at the same time interval as MEPP recording for each experimental condition. For ionomycin experiments, MEPP recording and fura-2 imaging were performed simultaneously from the same neuromuscular junction throughout the first ~60 s of ionomycin treatment. Because both MEPP frequency and [Ca²⁺]i increase constantly during this period (Ravin et al. 1997), a single data point represents the MEPP frequency and fura-2 signal recorded simultaneously during a given 3-s period. Between 1 and 5 such points were acquired from a terminal in a typical ionomycin experiment. The higher concentrations of ionomycin (~10 μM) often resulted in contraction of the muscle fiber after 30–60 s, so data were collected only briefly after addition of high ionomycin concentrations. MEPP frequency was analyzed off-line with the aid of software written in Axobasic. The software employed a peak-detection routine that aided in detection of a MEPP. Each peak was displayed on a time scale that allowed visual inspection to determine whether an event had the characteristic rise and decay times of a MEPP or was a noise artifact. Superimposed MEPPs in which a separate peak could be clearly distinguished riding on another were counted as separate events.

Results

MEPP frequency and intraterminal [Ca²⁺] measured at various extracellular [KCl]

Both the frequency of MEPPs and the average [Ca²⁺], demonstrated a strong dependence on [KCl]o (Fig. 1. A and B) presumably due to the opening of voltage activated Ca²⁺ channels. The relationship between the average values of [Ca²⁺], and MEPP frequency measured between 2 and 16 mM [KCl]o demonstrated greater than a 100-fold increase in neurotransmitter release rates with relatively small increases in [Ca²⁺], (Fig. 1). If the [Ca²⁺], estimated with fura-2 is the same as that present at the release sites, then this result suggests a low threshold for the Ca²⁺ requirement for activation of transmitter release.

Slow Ca²⁺ chelator EGTA reduces the [KCl]o dependence of MEPP frequency

If intracellular microdomains of elevated [Ca²⁺] near the mouth of open Ca²⁺ channels overlapped with a Ca²⁺ sensor
responsible for synaptic vesicle fusion, then the fura-2 estimates of \([\text{Ca}^{2+}]_i\), which reflect global \([\text{Ca}^{2+}]_i\) in the terminal, may not be an appropriate measure for determining the \([\text{Ca}^{2+}]_i\) dependence of MEPP frequency. To test this possibility, we compared the effect of the slow \([\text{Ca}^{2+}]_i\) buffer EGTA with the faster buffer BAPTA on the \([\text{KCl}]_o\) dependence of MEPP frequency. These two \([\text{Ca}^{2+}]_i\) chelators, which have a similar \(K_d\) for \([\text{Ca}^{2+}]_i\), differ greatly in their rates of binding \([\text{Ca}^{2+}]_i\) ions, BAPTA being \(~150\)-fold faster than EGTA under physiological conditions (Naraghi 1997). Therefore BAPTA should reduce \([\text{Ca}^{2+}]_i\) more than EGTA does in transient microdomains. Terminals were loaded with the membrane permeant esters EGTA-AM or BAPTA-AM or with the control solution (0.5% DMSO) under otherwise identical loading conditions (see METHODS). Both \([\text{Ca}^{2+}]_i\) chelators reduced the \([\text{KCl}]_o\) dependence of MEPP frequency by a similar amount (Fig. 2), suggesting that EGTA and BAPTA are equally effective at reducing \([\text{Ca}^{2+}]_i\) at transmitter release sites. This result argues that single-channel domains of high \([\text{Ca}^{2+}]_i\) are not required to overlap the transmitter release sites since, in this case, the faster buffer would more effectively reduce a \([\text{Ca}^{2+}]_i\) microdomain.

While the \([\text{Ca}^{2+}]_i\) chelators EGTA and BAPTA did weakly reduce MEPP frequency under our “resting” conditions (2 mM \([\text{KCl}]_o\)), these chelators did not abolish MEPPs. These residual MEPPs could be due to \([\text{Ca}^{2+}]_i\)-independent events. However, because \([\text{Ca}^{2+}]_i\) measured at rest is \(100 \pm 40\) (SE) nM and the \(K_d\) for \([\text{Ca}^{2+}]_i\) of both chelators (present at unknown concentrations due to AM-ester loading) is on the order of 100 nM, it is not possible to definitively state that these are \([\text{Ca}^{2+}]_i\)-independent events.

Most importantly, the strong inhibition of transmitter release measured at \(\leq 16\) mM \([\text{KCl}]_o\) by these chelators indicates that our fura-2 estimates of \([\text{Ca}^{2+}]_i\) are a reliable measure of \([\text{Ca}^{2+}]_i\) concentration at release sites for the experiments using at least

FIG. 2. Effect of exogenous \([\text{Ca}^{2+}]_i\) chelators on the \([\text{KCl}]_o\) dependence of MEPP frequency. A: MEPP frequency recorded at the indicated \([\text{KCl}]_o\) from preparations loaded with EGTA-AM (○) or bis-(o-aminophenoxy)-N,N,N′,N′-tetraacetic acid-AM (BAPTA-AM: ×) or treated with 0.5% DMSO as a control (●). Data are average ± SE from 6 to 11 terminals for each condition. B: inhibition of MEPP frequency by EGTA-AM (○) or BAPTA-AM (×) expressed as [1-(fMEPPbuffer /fMEPPDMSO)]×100 where fMEPPbuffer is the MEPP frequency measured in either EGTA-AM or BAPTA-AM loaded preparations and fMEPPDMSO is the MEPP frequency measured in 0.5% DMSO.
The weaker inhibition of transmitter release by the two chelators at 20 mM [KCl]o either may reflect a partial contribution of restricted Ca2+ domains under these stronger depolarizing conditions that may activate additional channel types or may reflect Ca2+ loading that surpassed the buffering capacity of the exogenous chelators. The equivalent effect of the two chelators even at 20 mM [KCl]o supports the latter possibility.

These conclusions depend on the amounts of EGTA and BAPTA in the terminals being approximately equal. If the final concentration of free EGTA in the terminals was ~150 times higher than the free BAPTA concentration, then the similar effects of these buffers on the [KCl]o dependence of MEPP frequency (Fig. 2) would not necessarily support the lack of a high-Ca2+ microdomain overlapping release sites. Several observations argue against the observed effects being due to such preferential loading of EGTA over BAPTA. Increasing the time of BAPTA-AM loading (~2 h), the concentration of BAPTA-AM (up to 100 μM), or the temperature of loading (to ~30°C) did not increase the effectiveness of BAPTA reduction in MEPP frequency (see METHODS). In addition, Robitaille et al. (1993) studied the spatial relationship between voltage-gated Ca2+ channels and Ca2+-activated potassium channels in frog motor nerve terminals using EGTA-AM- and BAPTA-AM-loading conditions that were essentially identical to those reported here. They found that loading terminals with BAPTA-AM blocked Ca2+-activated K+ channels while EGTA-AM was completely ineffective (Robitaille et al. 1993). This result indicates that loading of membrane permeant BAPTA and EGTA can be used to detect the presence of processes dependent on spatially restricted domains of high Ca2+ in this preparation.

dependence of MEPP frequency in ionomycin-treated terminals

The Ca2+ ionophore ionomycin offers an additional, independent method to determine the [Ca2+]i dependence of MEPP frequency. This approach precludes both development of Ca2+ microdomains selectively around voltage-gated Ca2+ channels or a substantial change in membrane potential. The fura-2-estimated [Ca2+]i dependence of MEPP frequency from terminals treated with 0.1–10 μM ionomycin (constant extracellular CaCl2 of 1.8 mM) is reported in Fig. 3A. It is evident that for values of [Ca2+]i at least ~1 μM that this relationship is similar to that found when [Ca2+]i was elevated by increasing [KCl]o. Even for values of [Ca2+]i of 1–5 μM (an inherently less accurate value due to the relatively low Kd for Ca2+ of fura-2), the Ca2+-dependence of MEPP frequency is not significantly different for high [KCl]o or ionomycin stimulations. These results with ionomycin further validate the conclusions based on data obtained by altering [KCl]o.

Estimation of Kd

While lack of clear saturation of the Ca2+ dependence of MEPP frequency prohibits a reliable determination of the Kd for Ca2+ required for transmitter release, estimates of the value of Kd can be made from these data based on rearrangement of the standard Hill equation and an estimate of the Hill coefficient (Ravin et al. 1996). The standard Hill equation

\[ f_{\text{MEPP}} = \frac{f_{\text{MEPP max}} [\text{Ca}^{2+}]}{[\text{Ca}^{2+}] + K_d} \]

where \( f_{\text{MEPP}} \) is the MEPP frequency, \( f_{\text{MEPP max}} \) is the maximal rate of MEPPs, \( n \) is the Hill coefficient, and \( K_d \) is the

\[ \frac{1}{f_{\text{MEPP}}} = \frac{1}{f_{\text{MEPP max}}} \left( \frac{[\text{Ca}^{2+}]}{K_d} + 1 \right) \]

can be rearranged to give

\[ 1/(f_{\text{MEPP}})^n = \left( K_d/(f_{\text{MEPP max}}) \right)^n \left( [\text{Ca}^{2+}] \right) + (1/(f_{\text{MEPP max}})) \]

where \( f_{\text{MEPP}} \) is the MEPP frequency, \( f_{\text{MEPP max}} \) is the maximal rate of MEPPs, \( n \) is the Hill coefficient, and \( K_d \) is the...
half-saturation value for \( n = 1 \). Figure 3B is a plot of \( 1/(f_{\text{MEPP}})^{1/n} \) as a function of \( 1/[\text{Ca}^{2+}] \), for \( n = 2 \). The linear regression yields a line with a slope of \( K_d/(f_{\text{MEPP}})^{1/n} \) and a y intercept of \( 1/(f_{\text{MEPP}})^{1/n} \). From this analysis of our data, we estimate \( K_d \) to be 1.2 \( \mu \text{M} \) for \( n = 2 \). Higher values of \( n \) result in lower values of \( K_d \).

Another method of estimating \( K_d \) is by using Eq. 1 and assumed values of \( f_{\text{MEPP}} \). The highest MEPP frequency we measured was 300 s\(^{-1}\), which can serve as a lower limit for \( f_{\text{MEPP}} \). Quantal release rates of 1,000’s s\(^{-1}\) have been reported based on noise analysis of end plate potentials of black widow spider venom-treated frog neuromuscular junctions (Fesce et al. 1986). While the release events from venom-treated nerve terminals do not reflect the Ca\(^{2+}\)-dependent transmitter release under investigation here, they do provide for a reasonable upper limit for estimates of \( f_{\text{MEPP}} \). Fig. 3C and Table 1 demonstrate that regardless of the \( f_{\text{MEPP}} \) used in Eq. 1, these data are consistent with a \( K_d \leq 5 \) \( \mu \text{M} \). The analysis described in Fig. 3C indicates the data are best described with a Hill coefficient of 1–2, while there is abundant evidence that evoked release from this preparation is described with a Hill coefficient of 3–4 (see VanderKloot and Molgó 1994). We repeated these analyses with Hill coefficients fixed at values of 1, 2, 3, and 4 and with \( f_{\text{MEPP}} \) fixed at values of 350, 500, and 1,000 s\(^{-1}\) (Table 1). The relative \( \chi^2 \) values from these analyses indicate that the data are best described with a Hill coefficient of 1–2. This finding regarding the value of the Hill coefficient is consistent with other studies in which the apparent Hill coefficient for MEPPs could be described by a value less than that of EPPs (Andreu and Barrett 1980) but is different from studies at the crayfish neuromuscular junction that indicated MEPP frequency followed a fourth power Ca\(^{2+}\) relationship (Ravin et al. 1997). Our main conclusion from the analysis of our data is that the Ca\(^{2+}\)-dependent increase in MEPP frequency is consistent with a \( K_d \) for [Ca\(^{2+}\)]

Changes in MEPP frequency due to phorbol esters could be attributed to elevated [Ca\(^{2+}\)].

Compounds that can affect the activity of protein kinases, including protein kinase C (PKC), have been reported to alter various types of neurotransmitter release at neuromuscular junctions (e.g., Redman et al. 1997; Shapira et al. 1987; for review, see VanderKloot and Molgó 1994). Potentiating effects of PKC on evoked transmitter release under certain conditions has been reported to be Ca\(^{2+}\)-independent (Redman et al. 1997). Since we were in position to directly test for possible Ca\(^{2+}\)-independence of one type of transmitter release, we asked the question: can pharmacological manipulation of PKC activity cause Ca\(^{2+}\)-independent increase in MEPP frequency under our experimental conditions?

We found that treatment of neuromuscular preparations with the protein kinase activator PMA (400 nM) increased the MEPP frequency observed in 10 mM [KCl]\(_o\) (Fig. 4A). Identical treatment with the related phorbol ester 4a-PMA, which does not activate protein kinases, did not alter MEPP frequency (Fig. 4A). Incubation of neuromuscular preparations with 500 nM staurosporine (a nonspecific protein kinase inhibitor at this concentration) reduced the MEPP frequency both at rest and in conditions of elevated [KCl]\(_o\) (Fig. 4), consistent with previous results demonstrating that staurosporine treatment reduced the elevated MEPP frequency observed following tetanic stimulation (Henkel and Betz 1995). The lack of an effect of 4a-PMA and the opposite effect of staurosporine suggest that the elevation of MEPP frequency by PMA is due to protein phosphorylation. These results are consistent with other studies

<table>
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<th>( n )</th>
<th>( f_{\text{MEPP}} ), s(^{-1})</th>
<th>( K_d ), ( \mu \text{M} )</th>
<th>Relative ( \chi^2 )</th>
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Results for \( K_d \) and the normalized \( \chi^2 \) values from fitting of Eq. 1 with \( n \) and \( f_{\text{MEPP}} \) fixed to the indicated values. \( f_{\text{MEPP}} \) is the miniature end plate (MEPP) potential frequency at the maximal rate of MEPPs.
suggesting that active phorbol esters enhance neurotransmitter release at frog neuromuscular junctions (Redman et al. 1997; Shapira et al. 1987).

To test whether the enhancement of neurotransmitter release in PMA is due to an increase in [Ca$^{2+}$], or to a Ca$^{2+}$-independent mechanism, we performed fura-2 measurements before and after PMA treatment. Under conditions identical to those used to measure effects on MEPP frequency, PMA caused an increase in [Ca$^{2+}$]. (Fig. 4B). The relationship between [Ca$^{2+}$]i, and MEPP frequency in PMA-treated terminals was similar to that described for both high [KCl]o and ionomycin experiments (Fig. 3A, △). This result suggests that in frog motor nerve terminals, PMA increases MEPP frequency by elevating [Ca$^{2+}$], and does not have to exert a Ca$^{2+}$-independent action on any component of release machinery associated with the type of release we measured. Our results do not rule out a Ca$^{2+}$-independent enhancement of evoked release (Redman et al. 1997).

**DISCUSSION**

**Low-threshold, high-affinity Ca$^{2+}$ dependence of transmitter release**

We investigated the relationship between intraterminal [Ca$^{2+}$], and the frequency of MEPPs at the frog neuromuscular junction. Raising [Ca$^{2+}$]i, from resting values of ~100 nM to 1 μM markedly increased the frequency of MEPPs. This is in full agreement with studies at crayfish neuromuscular junctions in which [Ca$^{2+}$]i was raised uniformly by either photolysis of caged Ca$^{2+}$ (Mulkey and Zucker 1993) or by application of ionomycin (Ravin et al. 1997). Together these results indicate a low threshold for Ca$^{2+}$ in the activation of asynchronous transmitter release at these synapses. This is comparable to the low-Ca$^{2+}$ requirement for exocytosis revealed by patch-clamp capacitance measurements of synaptic vesicle fusion in rod photoreceptor cells (Rieke and Schwartz 1996) and for secretory granule fusion in chromaffin cells (detected at [Ca$^{2+}$]i <0.2 μM) (Augustine and Neher 1992). These apparent low-threshold Ca$^{2+}$ requirements are in striking contrast to the high threshold of [Ca$^{2+}$]i >20 μM [Ca$^{2+}$]i, required for activation of exocytosis found in retinal bipolar nerve terminals (Heidelberger et al. 1994; von Gersdorff and Matthews 1994).

Direct measurements of the Kd for Ca$^{2+}$ required for transmitter release or vesicle fusion have only been made in a few preparations. Our estimates of a Kd on the order of 1 μM for the Ca$^{2+}$ requirement for MEPP frequency implicate a high-affinity Ca$^{2+}$ binding site for the sensor responsible for this transmitter release. Ravin et al. (1997) found the Kd for Ca$^{2+}$ required for asynchronous transmitter release following evoked release at crayfish neuromuscular junctions to be 2–4 μM. How do these studies of asynchronous release relate to other studies of Ca$^{2+}$-dependent exocytosis? Direct measurements of evoked release at a fast central synapse have demonstrated that an intraterminal [Ca$^{2+}$]i of just a few micromolar is required to trigger release (Bollmann et al. 2000; Schneggenburger and Neher 2000). Studies using membrane capacitance as a measure of secretory granule or synaptic vesicle fusion with the plasma membrane while uniformly raising [Ca$^{2+}$]i, by photolysis of caged Ca$^{2+}$ revealed a Kd in the range of 7–21 μM for bovine chromaffin cells (Heinemann et al. 1994), 27 μM for pituitary melanotrophs (Thomas et al. 1994), and ~200 μM for retinal bipolar nerve terminals (Heidelberger et al. 1994). This wide range in reported Ca$^{2+}$ affinities required for activation of transmitter release likely reflects involvement of distinct Ca$^{2+}$ sensors.

**Diffusion of Ca$^{2+}$ from Ca$^{2+}$ channels to transmitter release sites**

The short delay between a presynaptic action potential and a postsynaptic response at the neuromuscular junction suggests a close spatial relationship between the site of Ca$^{2+}$ influx and the Ca$^{2+}$ binding site that triggers evoked transmitter release (reviewed in Vander Kloot and Molgò 1994). Theoretical considerations predict a local domain of high Ca$^{2+}$ concentration around an open Ca$^{2+}$ channel (e.g., Fogelson and Zucker 1985; Simon and Llinás 1985; Yamada and Zucker 1992). Such models predict short-lasting (~100 μs) domains of Ca$^{2+}$ that reach values as high as a few hundred micromolar in a region ≤20 nm from the mouth of a Ca$^{2+}$ channel. The existence of such domains of high Ca$^{2+}$ during an action potential have been demonstrated in the presynaptic terminal of the squid giant synapse (Llinás et al. 1992) although the limits placed by the resolution of light do not allow for the measured domains to be assigned to a single channel. The location of the Ca$^{2+}$ sensor for transmitter release relative to domains of high [Ca$^{2+}$] around Ca$^{2+}$ channels has important implications for the Ca$^{2+}$ dependence of release. If the sensor is <20 nm from the mouth of a Ca$^{2+}$ channel, it would be exposed to very high [Ca$^{2+}$]i (~100 μM) for tens of microseconds and would be dominated by Ca$^{2+}$ from the Ca$^{2+}$ channel, whereas at distances of 100–200 nm from a channel the sensor would be exposed to [Ca$^{2+}$] of <10 μM for up to 10 ms and would sense Ca$^{2+}$ overlapping from several channels (reviewed in Neher 1998; Stanley 1997).

There is good evidence for clustering of Ca$^{2+}$ channels at release sites of the neuromuscular junction. It has been suggested that large intramembranous particles evident at active zones in freeze fracture electronmicroscopy are Ca$^{2+}$ channels (e.g., Heuser and Reese 1981) and fluorescently labeled toxins that specifically bind to Ca$^{2+}$ channels intensely stained active zones of the neuromuscular junction (Robitaille et al. 1990). However, these approaches do not allow for the functional relationship between Ca$^{2+}$ domains and transmitter release to be addressed.

The “single-channel-domain” and “overlapping-domain” hypotheses can be experimentally distinguished by determining the Ca$^{2+}$ dependence of transmitter release and by addition of exogenous Ca$^{2+}$ chelators (Neher 1998). Our finding that EGTA and BAPTA were equally effective at competing with the release sensor for binding Ca$^{2+}$ when [Ca$^{2+}$]i, was elevated by mild depolarizations (Fig. 2) suggests that a single-channel domain of Ca$^{2+}$ was not responsible for increases in spontaneous release at least when low-threshold Ca$^{2+}$ channels were responsible for Ca$^{2+}$ influx. This conclusion is further supported by our finding that the [Ca$^{2+}$]i, dependence of release was similar when Ca$^{2+}$ was elevated by either activation of voltage-activated Ca$^{2+}$ channels or application of ionomycin, which will not form Ca$^{2+}$ domains related to Ca$^{2+}$ channels. Under our depolarizing conditions ([KCl]o of 10–20 mM), we may have activated Ca$^{2+}$-induced Ca$^{2+}$ release as has been
reported to occur during trains of action potentials and found to enhance asynchronous release in this preparation (Narita et al. 1998). Regardless of the source of [Ca\(^{2+}\)], during our high K\(^{+}\) experiments, it is clear that microdomains of Ca\(^{2+}\) are not necessary to enhance MEPP frequency.

The use of exogenous Ca\(^{2+}\) chelators to investigate the spatial relationship between Ca\(^{2+}\) domains and transmitter release was first employed by Adler et al. (1991) at the giant synaptic terminal of the squid. They found that presynaptic injection of BAPTA could inhibit transmitter release, but high concentrations of EGTA were completely ineffective at reducing transmitter release. This was taken as evidence of local domains of high Ca\(^{2+}\) being necessary for transmitter release. Further support for the single-domain hypothesis has been reported at a chick calyx synapse (Stanley 1993). In contrast, simultaneous pre- and postsynaptic recordings at a central calyx-type synapse in the rat auditory system revealed that EGTA and BAPTA were equally effective even at relatively low concentrations in reducing transmitter release, arguing for overlapping domains of Ca\(^{2+}\) from neighboring Ca\(^{2+}\) channels being required for triggering transmitter release (Borst and Sakmann 1996; Borst et al. 1995). Evidence for the presence of both mechanisms in the same cell has been provided by capacitance measurements from mouse adrenal medulla slices (Moser and Neher 1997) and retinal bipolar terminals (Menerick and Matthews 1996) where ultrafast components of exocytosis were more sensitive to BAPTA than EGTA, whereas slower components of exocytosis were equally sensitive to the two chelators.

Conflicting results from different preparations suggest that the spatial relationship between Ca\(^{2+}\) channels and the Ca\(^{2+}\) sensor for release as well as the apparent affinity of the Ca\(^{2+}\) sensor may be different in different secretory cells. Moreover, it seems that in at least some cell types both the single Ca\(^{2+}\) domain and overlapping Ca\(^{2+}\) domain models may apply under different conditions. Freeze-fracture electron microscopy of frog neuromuscular junctions stimulated with 20 mM KCl demonstrated that most fusion events observed in the first minute of KCl treatment occurred near the active zone, whereas fusion events after 5 min of stimulation occurred uniformly over the presynaptic membrane (Cecarelli et al. 1988), consistent with distinct populations of vesicles based on cellular localization. This also suggests that fusion events measured in our KCl experiments occurred primarily near the active zone, although the channels opened by elevated [KCl]o are not necessarily the same Ca\(^{2+}\) channels as those used for evoked release (Katz et al. 1995; Robitaille et al. 1993), as evident from a report that distinct channel types are coupled to release from sympathetic ganglia depending on whether stimulation was by a brief pulse or by sustained depolarization (Gonzalez Burgos et al. 1995).

Are the vesicles involved in synchronous and asynchronous release distinct? Several studies are suggestive of distinct mechanisms for spontaneous and evoked release. For example, the finding that EPSPs and MEPPs have different temperature dependencies at the frog neuromuscular junction (Barrett et al. 1978) is suggestive of different exocytic mechanisms. Another experimental manipulation that points to distinct triggers for exocytosis is the ability of Sr\(^{2+}\) or Ba\(^{2+}\) to trigger exocytosis to varying extents, a phenomena that may be due to the presence of different synaptotagmin isoforms (Li et al. 1995).

The different divalent cation requirements for synchronous and asynchronous release at frog neuromuscular junctions (Zengel and Magleby 1981) and at hippocampal synapses (Goda and Stevens 1994) as well as the reduction in synchronous but not asynchronous release in synaptotagmin I knockout mice (Geppert et al. 1994) are suggestive of two populations of vesicles with different Ca\(^{2+}\) sensors. Other studies using either mutations or toxins to target SNARE complexes are also suggestive of biochemically distinct fusion events. Synaptobrevin mutant Drosophila show complete loss of evoked release but retain 25% of MEPPs (Deitcher et al. 1998). These residual MEPPs, which were still sensitive to [Ca\(^{2+}\)], may have been due to other synaptobrevin isoforms. More clear evidence for different SNARE complex involvement in evoked and asynchronous release comes from studies of the crayfish neuromuscular junction in which tetanus toxin blocked evoked but not spontaneous release whereas botulinum toxin D blocked both forms of release (Hua et al. 1998).

How does the Ca\(^{2+}\) dependence of spontaneous or asynchronous release relate to the Ca\(^{2+}\) dependence of evoked release at the frog neuromuscular junction? We do not assume that the [Ca\(^{2+}\)] dependence of MEPP frequency that we measured has a direct relationship to the Ca\(^{2+}\) dependence of synchronous release at frog motor nerve terminals. Incorporation of EGTA into motor nerve terminals caused a reduction in both spontaneous release (this study, Fig. 2) and evoked release (Robitaille et al. 1993). In the study of evoked release by Robitaille et al. (1993) BAPTA was more effective than EGTA at reducing evoked release, whereas in our study both chelators [loaded in an identical manner to that of Robitaille et al. (1993)] had the same inhibitory effect on MEPP frequency. This strongly suggests the Ca\(^{2+}\) dependence (Kd and/or microdomains) differs for evoked and synchronous release at the frog neuromuscular junction.

We thank Dr. Ling-Gang Wu (Washington University, St. Louis, MO) for advice on fura-2 measurements. S. Fadul provided expert technical assistance on all aspects of this work.

This work was supported by National Institutes of Health grants to J. K. Angleson and W. J. Betz and a grant from the Muscular Dystrophy Association to W. J. Betz.

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