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Ca^{2+}-DEPENDENT AND -INDEPENDENT COMPONENTS OF TRANSMITTER RELEASE AT THE FROG NEUROMUSCULAR JUNCTION

BY NORIKO TANABE AND *HIROMASA KIJIMA

*From the Daiichi Hoiku Junior College, Dazaifu, Fukuoka 818-01 and the *Department of Physics, Faculty of Science, Nagoya University, Nagoya 464–01, Japan*

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SUMMARY

1. When a Ca^{2+} chelator, bis (*O*-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid (BAPTA), was loaded into the presynaptic nerve terminal of the frog neuromuscular junction (NMJ), facilitation, measured as an increase in endplate potential (EPP) amplitudes during a train of ten stimulations at 100 Hz, was greatly decreased within 20 min of BAPTA-AM (the acetoxymethyl ester of BAPTA) perfusion, and remained at a constant low level thereafter, suggesting that $[Ca^{2+}]_i$ at the presynaptic nerve terminal was buffered by BAPTA.

2. Detailed examination of the two components of facilitation of EPP amplitude in the BAPTA-loaded NMJs showed that the fast component was lost almost completely, while the slow component was unaffected by loaded BAPTA. Augmentation and potentiation were also unaffected by BAPTA.

3. Under external Ca^{2+}-free conditions (with 1 mM-EGTA), both augmentation and potentiation of miniature endplate potential (MEPP) frequency were clearly observed after tetanic stimulation in the normal NMJ, and were also unaffected by loaded BAPTA.

4. The above findings strongly support the residual Ca^{2+} hypothesis for the fast component of facilitation, and suggest that the three slower processes (the slow component of facilitation, augmentation and potentiation) occur independently of $[Ca^{2+}]_i$. This Ca^{2+} independence was supported by the fact that facilitation and potentiation have multiplicative effects on the amount of release.

5. The quantal content of the first EPP in the train remained unchanged throughout the time course of BAPTA loading for most NMJs. This suggests that $[Ca^{2+}]_i$ immediately adjacent to Ca^{2+} channels at the active zone triggers transmitter release and is little affected by loaded BAPTA.

6. MEPP frequency was almost unchanged during BAPTA loading, suggesting that the basal $[Ca^{2+}]_i$ remained unchanged close to the dissociation constant of BAPTA for Ca^{2+} (108 nM).

7. The slow component of facilitation had a multiplicative relationship with augmentation and potentiation, suggesting that the underlying mechanism for the slow component of facilitation differs from that for augmentation and potentiation.

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INTRODUCTION

It is widely accepted that entry of external Ca^{2+} into the presynaptic nerve terminal triggers transmitter release at chemical synapses (Katz, 1969). Moreover, presynaptic internal Ca^{2+} is known to regulate or modulate many aspects of synaptic plasticity including facilitation (Katz & Miledi, 1968; Charlton, Smith & Zucker, 1982), long-term potentiation (Kuba & Kumamoto, 1986) and associative learning (Abram & Kandel, 1988).

At the frog neuromuscular junction (NMJ), four components of stimulation-induced increases in transmitter release are known. They are clearly discriminated by their different decay time constants after tetanic stimulation, and are classified into the fast component of facilitation (decay time constant, $\tau \sim 50$ ms), the slow component of facilitation, ($\tau \sim 400$ ms), augmentation ($\tau \sim 7$–10 s) and potentiation ($\tau \sim 1$–3 min) (Katz & Miledi, 1968; Magleby & Zengel, 1976; Zengel & Magleby, 1980).

Katz & Miledi (1968) proposed that the fast component of facilitation, measured as the increase in size of the second endplate potential (EPP) with double impulse stimulation, was due to residual Ca^{2+} in the nerve terminal remaining from the first impulse. This residual Ca^{2+} hypothesis has been widely supported (for review cf. Zucker, 1989). The other three components of stimulation-induced increase have also been considered to be due to the increase in the internal Ca^{2+} concentration ($[Ca^{2+}]_i$), (Weinreich, 1971; Rahamimoff, Lev-Tov & Meiri, 1980; Zucker, 1989), but without any conclusive experimental support.

In previous studies, we loaded a calcium chelator, bis-(*O*-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid (BAPTA), into the presynaptic nerve terminal of the frog NMJ, in order to determine whether the enhancement of transmitter release caused by nerve stimulation and certain reagents was due to an increase in $[Ca^{2+}]_i$ (Kijima & Tanabe, 1988; Tanabe & Kijima, 1988, 1989). It was found that facilitation, measured as an increase of EPP amplitude during a train of ten impulses at 100 Hz, was greatly decreased. This suggested that the $[Ca^{2+}]_i$ at the release site was well buffered by BAPTA and that the increase of $[Ca^{2+}]_i$ upon stimulation decreases rapidly to the basal level, leaving little residual $[Ca^{2+}]_i$ at the time of subsequent impulses. This view was further supported by the finding that introduction of external Ca^{2+} using an ionophore, X-537A, (Kita & Van der Kloot, 1976) led to recovery of facilitation (Tanabe & Kijima, 1988).

Using the greatly reduced facilitation as an indicator of Ca^{2+} buffering near the basal level, we suggested that two slower components of stimulation-induced increases in transmitter release, augmentation and potentiation, occurred independently of $[Ca^{2+}]_i$, and that the increase induced by application of 2,4,6-trinitrobenzene-1-sulphonic acid (TNBS), erythrosin B or by hypertonicity, could not be attributed to the increase in $[Ca^{2+}]_i$ (Tanabe & Kijima, 1988, 1989). Qualitatively similar results were also reported recently (Maeno & Hara, 1989).

The present work extends the above studies, and reveals that for the two components of facilitation of EPP amplitude, the fast component of facilitation is abolished almost completely by loaded BAPTA, while the slow component of facilitation remains unaffected. Furthermore, it is shown that under external Ca^{2+}-

free conditions, both augmentation and potentiation of miniature endplate potential (MEPP) frequencies are clearly evident at the normal NMJ, and are unaffected by loaded BAPTA. These results support the residual Ca^{2+} hypothesis for the fast component of facilitation, and suggest that the other three components occur independently of $[Ca^{2+}]_i$. We also report the time course of changes in the characteristics of release during BAPTA loading. The results suggest that the Ca^{2+} immediately adjacent to the calcium channels triggers the evoked release, because EPP amplitudes typically remained constant during BAPTA loading.

METHODS

Nerve–sartorius muscle preparations from the frog, *Rana nigromaculata* were used as reported previously (Kijima & Tanabe, 1988). Each frog was killed by decapitation. For electrophysiological measurements, each preparation was mounted in a recording chamber (about 3·0 ml solution volume) and perfused with the Ringer solution of the following composition at a flow rate of 3–5 ml/min, unless otherwise stated. The temperature of the solution in the chamber was kept at $21 \pm 0{\cdot}5$ °C by circulating water in a constant-temperature bath enclosing the chamber. When the nerve-evoked EPPs were examined, standard low Ca^{2+}–high Mg^{2+} Ringer solution was used, which contained (mM): $MgCl_2$, 4; $CaCl_2$, 0·5–0·6; NaCl, 112; KCl, 2·6; HEPES-Na, 5 (pH 7·5). For Ca^{2+}-free Ringer solution, there was no added Ca^{2+} and 1 mM-EGTA was added. Normal Ringer solution was used for dissection; this contained 1·8 mM-$CaCl_2$, no Mg^{2+}, and the other ionic components were the same as those for the low Ca^{2+}–high Mg^{2+} Ringer solution.

The Ca^{2+} chelator, BAPTA, was loaded into the presynaptic nerve terminal of the NMJ as follows. The nerve–muscle preparation was incubated with a 0·25 mM suspension of the acetoxymethyl ester of BAPTA (BAPTA-AM) in normal Ringer solution for 2·5 h at 29 °C with stirring, in order to ensure sufficient BAPTA loading. After washing for 40 min with normal Ringer solution, the preparation was used for the experiment. The time course of BAPTA loading was examined by perfusing the freshly mounted nerve–muscle preparation with 0·1 mM-BAPTA-AM, while continuously recording EPPs and MEPPs with an intracellular microelectrode at the endplate region, at $23 \pm 0{\cdot}5$ °C.

Two kinds of EPP recording methods were adopted in this work. One was the intracellular recording method described previously (Kijima & Tanabe, 1988). This was adopted for examining MEPP frequency under external Ca^{2+}-free conditions and for studying the time course of BAPTA loading. EPP amplitudes were corrected for the change in membrane potential of the muscle fibre, assuming a reversal potential of −5 mV (Magleby & Stevens, 1972). The quantum content, m, of EPPs was estimated directly by dividing the EPP amplitude by the average of MEPP amplitudes observed for 1 min before EPP measurement. The corrected EPP amplitude was proportional to the quantal content during the time-course study, indicating a constant quantal size during BAPTA loading. The other method used was the surface recording method, similar to that adopted by Magleby (1973*a*). This was used in all other studies. For this, a fire-polished glass electrode with an opening diameter of about 0·2 mm was placed tightly at the endplate region on the surface of the muscle. The reliability of this method has been confirmed (fig. 1 in Magleby, 1973*a*), but we also checked the proportionality between the surface-recorded and intracellularly recorded EPPs in a train of ten stimulations at 100 Hz. Figure 1 shows the average of normalized EPP amplitudes (±S.E.M.) for trains obtained from eight different endplate regions. Coincidence between EPPs obtained by the two recording methods was very good, indicating their proportionality. The surface recording method produced less deviation and allowed more stable and long-term recording (Fig. 1), but could not be used for experiments involving drug application (including BAPTA loading), since access of drugs to NMJs may be difficult when covered by the surface electrode. Only EPPs showing a monophasic change in potential in a negative direction were used in this work, although similar proportionality was observed for biphasic changes in potential (cf. Magleby, 1973*a*).

All the data were A/D-converted, fed into a personal computer (NEC PC-9801) and processed. A complex stimulation pattern was generated in the personal computer and delivered through a

D/A converter. The nerve trunk was stimulated by an isolated pulse of (5–50 V) × 0·1 ms for impulse generation.

Synaptic facilitation was estimated as the increase in EPP amplitude during a train of ten impulses at 100 Hz. The train stimulation was repeated fifteen times with an interval of 10 s and then averaged. As before, the facilitation index, I_f, was defined as the ratio of the mean amplitudes

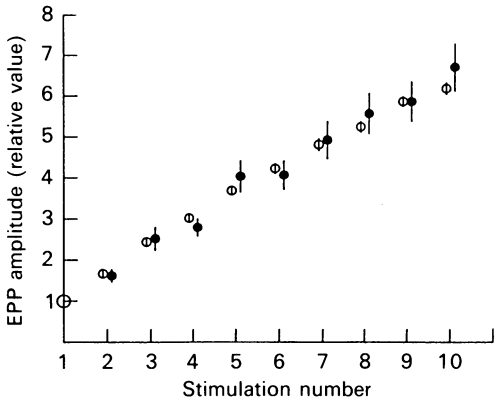

Fig. 1. Proportionality of facilitation during a train of ten impulses at 100 Hz, between EPPs recorded intracellularly (●) and EPPs recorded with a surface electrode (○). Trains of EPPs were surface-recorded 15 times at 10 s intervals at an endplate region containing several NMJs located on different muscle fibres. The surface electrode was then withdrawn and a glass microelectrode (3–5 MΩ) was inserted near the endplate of a muscle fibre, located at the centre of the surface electrode, and trains of EPPs were recorded intracellularly 15 times at 10 s intervals. The EPP trains obtained with each method were averaged. These measurements were then carried out for eight different endplate regions on different muscles, and the averages (±S.E.M.) plotted.

of the 9th and 10th EPPs to the amplitude of the first EPP (Kijima & Tanabe, 1988). The increment of I_f consists mostly of a fast component of facilitation (about 80%) and a small amount of slow component (about 20%) at the normal NMJ, as described later.

The fast and slow components of facilitation of EPP amplitudes, were measured as follows. Test impulses were applied every 4 s 60 times to record the control EPPs. Then a train of fifty impulses was delivered at 33·3 Hz (30 ms interval) for stimulation, and a test impulse was applied once at 0·1, 0·15, 0·2, 0·3, 0·5, 0·7 or 0·9 s after the end of stimulation. Thereafter, test impulses were applied once every 1·5 s 6 times, and then once every 4 s 59 times (cf. Zengel & Magleby, 1982). The stimulation pattern using a selected time was repeated 2–3 times (for a total of fourteen to twenty-one stimulations) every 10 min and averaged. In this stimulation pattern, the decay of the fast component of facilitation was not resolved, and its amplitude was obtained as a gap between the EPP amplitude at the last stimulation impulse and the point extrapolated to zero time (the time of the last stimulation impulse) of the best-fit decay curve consisting of the product of the three slower components (see below and Fig. 3).

For examining augmentation and potentiation of MEPP frequency under external Ca^{2+}-free conditions, the nerve–muscle preparation was perfused with the Ca^{2+}-free Ringer solution and a tetanic stimulation of 100 Hz was delivered for 50 s (5000 impulses). MEPP frequencies were counted using the moving-bin method (Rahamimoff & Yaari, 1973). The bin size was 9 s and the step size was 3 s.

We analysed the decay of all four components of the stimulation-induced change, assuming the following multiplicative relationship among them:

$$\mathrm{EPP}(t)/\mathrm{EPP}_0 = (F_1(t)+1)\,(F_2(t)+1)\,(A(t)+1)\,(P(t)+1), \quad (1)$$

where EPP(t) is the EPP amplitude at time t after the end of stimulation, and EPP_0 is the control

EPP amplitude before stimulation. F_1, F_2, A, and P are the magnitudes of the fast and slow components of facilitation, augmentation and potentiation respectively, which can be expressed as,

$$F_1(t) = F_1(0)\,e^{-t/\tau_1}, \tag{2}$$

$$F_2(t) = F_2(0)\,e^{-t/\tau_2}, \tag{3}$$

$$A(t) = A(0)\,e^{-t/\tau_a}, \tag{4}$$

$$P(t) = P(0)\,e^{-t/\tau_p}, \tag{5}$$

where the τ values are the decay time constants of the four components.

It has been shown that the fast component of facilitation has a multiplicative effect (Magleby, 1973*b*; Landau, Smolinsky & Lass, 1973) on potentiation and augmentation (Magleby & Zengel, 1982), but other relationships among the components have not yet been definitely determined. Thus, relation (1) is a tentative assumption (see Results).

Curve fitting of the time course of EPP decay after stimulation was done according to Zengel & Magleby (1982). First, we determined $P(0)$ and τ_p using data for times exceeding 20 s after the end of stimulation, where components other than potentiation were negligible. The non-linear least-squares method was adopted for exponential curve fitting, in order to take into account the data points below the control level. Then, we obtained the data points for $(F_1(t)+1)(F_2(t)+1)(A(t)+1)$ by dividing the experimental points with the best-fit values of $(P(t)+1)$. Next, we determined $A(0)$ and τ_a using the data points at $1{\cdot}5 < t < 20$ s in a similar way to that above. $F_2(0)$ and τ_2 were obtained similarly.

RESULTS

Effect of loaded BAPTA on fast and slow components of facilitation

We have shown previously that both augmentation and potentiation of EPP amplitudes were unaffected by loaded BAPTA, in contrast to the great reduction of facilitation (Tanabe & Kijima, 1989). This observation was confirmed here for a large number of NMJs, by repeated tetanic stimulation experiments with a stimulation pattern of 450 impulses at 20 Hz, on the normal ($n = 11$) and BAPTA-loaded NMJs ($n = 16$).

It has also been shown that the facilitation index, I_f, remained decreased at the end of long-term experiments, usually for about 4–5 h after BAPTA loading (Fig. 1*Ab* in Tanabe & Kijima, 1989). We further checked whether I_f was decreased just at the end of 450 stimulations at 20 Hz (specifically, the 450th impulse was replaced by a train of ten impulses at 100 Hz) and 1·5 s after the end of stimulation, where both augmentation and potentiation remained at full magnitude. The results, shown in Fig. 2 (compare Figs 1 and 3), indicated that I_f remained decreased both just at the end (*B*) and 1·5 s after the end of stimulation (*C*), as well as at the start (*A*) and at the end of the experiment (*D*) (4·5 min after the end of stimulation). Thus these results showed no recovery of facilitation throughout the tetanic stimulation experiment.

There was a very rapid decrease of EPP amplitude after the end of 450 stimulations at 20 Hz, which was observed as a gap between the EPP amplitude at the end of stimulation (EPP_{450}/EPP_0) and the point extrapolated to zero time of the best-fit decay curve of augmentation and potentiation, even in the BAPTA-loaded NMJ, as well as in the normal one (Fig. 1*B* in Tanabe & Kijima, 1989). This gap corresponded to the product of the fast and slow components of facilitation, $(F_1+1)(F_2+1)$, which decay within 1·5 s. We examined which of the two components remained after stimulation in the BAPTA-loaded NMJs, and to what degree. Nerve

stimulation was done at 33·3 Hz for fifty impulses, and the subsequent decay process after the end of stimulation was resolved (see Methods).

Typical results for the normal and BAPTA-loaded NMJs are shown in Fig. 3. There was a notable difference in the profiles of the increase in EPP amplitude during

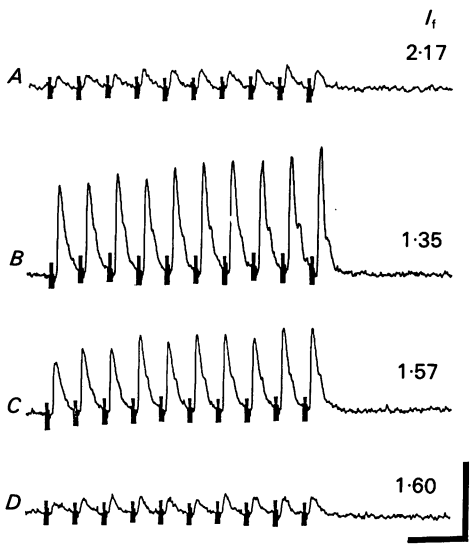

Fig. 2. Facilitation of surface-recorded EPPs by ten impulses at 100 Hz at a NMJ loaded with BAPTA, during and after 450 tetanic impulses at 20 Hz. *A*, control: before tetanic stimulation. *B*, just at the end of tetanus: the 450th impulse was replaced by a train of ten impulses at 100 Hz. *C*, 1·5 s after the end of tetanus. *D*, 4·5 min after the end of tetanus. Upward is the negative direction of the change in surface-recorded potential. Small subpeak or shoulders are surface recorded MEPPs. The trace in *B* is an average of six trains recorded at 20 min intervals and other traces are averages of eight trains (see Methods). Scale bars: 20 ms and 0·4 mV.

stimulation. In the normal NMJ (Fig. 3*Aa*), very rapid increase of EPP amplitude during the first ten impulses was observed, but the increase slowed down thereafter. In the BAPTA-loaded NMJ (Fig. 3*Ba*), the rapid phase of the increase disappeared, and the increase became almost linear during repetitive stimulation, suggesting loss of the fast component of facilitation. This was confirmed by analysis of the decay process within 1·5 s after the end of the stimulation. Two slower components, augmentation and potentiation, were determined using data points from 1·5 to 60 s after the end of stimulation, and removed by successive divisions, as described in Methods. The remaining points (△ in Fig. 3) from 0·1 to 0·9 s after the end of stimulation were curve fitted by an exponential term of the slow component of facilitation, and the amplitude of the fast component of facilitation was given by the gap between the EPP amplitude at the last stimulation and the point extrapolated to zero time of the best-fit decay curve.

There was a marked difference in the decay profile between the normal and BAPTA-loaded NMJs. In the normal NMJ, there was an evident gap between the EPP amplitude at the last stimulation and the point extrapolated to zero time,

which corresponds to the fast component of facilitation. In marked contrast to this, there was no gap between the two points, i.e. no fast component of facilitation in the BAPTA-loaded NMJ, and the decay process was expressed by the product of the three slower components: the slow component of facilitation, augmentation and

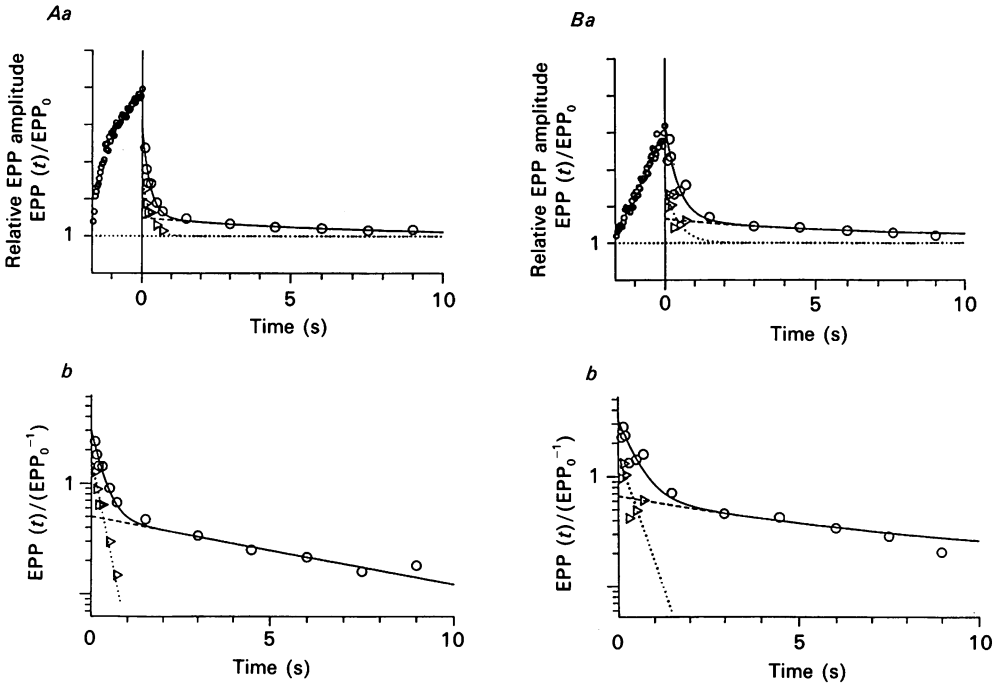

Fig. 3. Two components of facilitation estimated from the decay of EPPs after a train of fifty impulses at 33·3 Hz, for normal (*A*) and BAPTA-loaded NMJs (*B*). *a*, plots of relative EPP amplitudes during (○ at $t < 0$) and after ($t > 0$) the train. At $t > 0$, measured EPP amplitudes (○) and the best-fit curves of $(F_2+1)(A+1)(P+1)$ (——), the best-fit curves of $(A+1)(P+1)$ (– – –), experimental points of the slow component of facilitation obtained by dividing measured EPP amplitudes by the estimated values of $(A+1)(P+1)$ (△), and the best-fit exponential curves to the slow component of facilitation (· · · ·) are shown. The best-fit values of F_1, F_2, and τ_2 are 1·019, 1·225 and 0·325 ms, respectively, in *A*, and −0·101, 1·570 and 0·451 ms in *B*. *b*, semilogarithmic plots of the decay of EPP amplitudes after the train. Increments above the control level are plotted. Symbols are the same as in *a*.

potentiation. In the summary of the results given in Table 1, the values of the amplitude of slow facilitation, $F_2(0)$s, in the normal and BAPTA-loaded NMJs were almost the same. The decay time constant, τ_2, of the BAPTA-loaded NMJ was slightly larger than that in the normal NMJ, and the difference was statistically significant ($P < 0·05$ by Student's *t* test). The amplitude of fast facilitation, $F_1(0)$, was observed unambiguously as a gap at the end of stimulation in the normal NMJ ($F_1(0) > 0$, statistically significant at $P < 0·01$), but no significant gap ($P > 0·5$) was observed in the BAPTA-loaded NMJ.

In conclusion, the fast component of facilitation was lost almost completely after BAPTA loading, while the magnitude of the slow component of facilitation remained little affected.

The facilitation index, I_f, never decreased to unity and had an average value of $1{\cdot}83 \pm 0{\cdot}09$ ($n = 15$), irrespective of the degree of BAPTA loading into the nerve terminal. We can now account for this: the increment of the facilitation index, $I_f - 1$, contains mostly the fast component of facilitation (I_f is reduced from 4·91 to 1·83 on average, giving a fast component content of about 80%), with only a small proportion of the slow component (20%). After BAPTA loading, the fast component was lost almost completely, but the slow component remained intact. Thus, I_f remained above unity, and corresponded to the residual slow component.

TABLE 1. Parameters of fast and slow components of facilitation after fifty tetanic stimulations at 33 Hz

	τ_2*	$F_2(0)$	$F_1(0)$**	$(P(0)+1)\times(A(0)+1)-1$	n
Normal	$0{\cdot}29 \pm 0{\cdot}03$	$1{\cdot}44 \pm 0{\cdot}12$	$0{\cdot}91 \pm 0{\cdot}17$	$0{\cdot}47 \pm 0{\cdot}06$	7
BAPTA-loaded	$0{\cdot}49 \pm 0{\cdot}08$	$1{\cdot}40 \pm 0{\cdot}26$	$0{\cdot}04 \pm 0{\cdot}09$	$0{\cdot}71 \pm 0{\cdot}15$	5

Means ± S.E.M. Differences in the values between normal and BAPTA-loaded preparations were statistically significant at 5% (*) and 1% (**), respectively.

The contribution of augmentation and potentiation to I_f was also checked on the normal NMJ by delivering the 11th test stimulation 1·5 s after the 10th stimulation. The ratio of the 11th EPP amplitude to the first one was $1{\cdot}05 \pm 0{\cdot}04$ ($n = 13$), indicating that their content was negligibly small.

Multiplicative relationship between the slow component of facilitation and augmentation and/or potentiation

The experiment shown in Fig. 2 shows that the value of I_f 1·5 s after the end of 450 impulses of stimulation at 20 Hz (1·57, Fig. 2*C*) was similar to the control values before and 4·5 min after stimulation (2·17 and 1·60, Fig. 2*A* and *D*). On average, for six similar experiments, I_f was $1{\cdot}79 \pm 0{\cdot}16$, 1·5 s after the end of stimulation, and was $1{\cdot}98 \pm 0{\cdot}22$ and $1{\cdot}64 \pm 0{\cdot}23$ before and 4·5 min after stimulation, respectively, showing also the relative constancy of I_f for the three cases. Taking into account the above finding that only the slow component of the two components of facilitation remained at the BAPTA-loaded NMJ, the invariance of I_f at 1·5 s after the end of 450 impulses, when augmentation and potentiation have not yet decayed but the two components of facilitation have, shows the following multiplicative relation between the slow component of facilitation and augmentation and/or potentiation:

$$\mathrm{EPP}(t)/\mathrm{EPP}_0 = (F_2+1)\,(\text{terms containing } A \text{ and } P). \tag{6}$$

Thus, part of eqn (1) is shown to be valid.

Values of I_f just at the end of stimulation in Fig. 2 (1·35), and on average ($1{\cdot}35 \pm 0{\cdot}06$), were smaller than the above three values of I_f. This is quite natural, since at the end of stimulation, slow facilitation, F_2, caused by 450 impulses remained undecayed and I_f was expressed as $(F_2+F_2'+1)/(F_2+1)$, which is smaller than the I_f

value for the above three cases, $(F_2'+1)$, where F_2' is the increment in the slow component of facilitation caused by ten impulses at 100 Hz.

Effect of loaded BAPTA on augmentation and potentiation of MEPP frequencies under external Ca^{2+}-free conditions

The above and previous results, which suggested Ca^{2+}-independence of the three slower components of stimulation-induced changes in EPP amplitude, were obtained

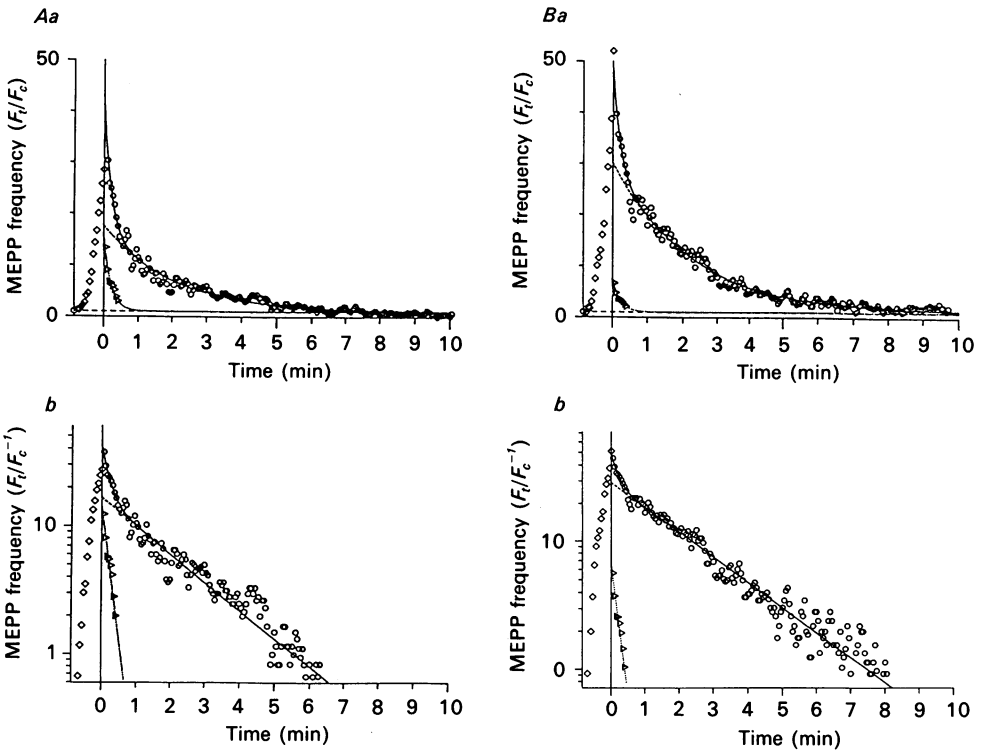

Fig. 4. Augmentation and potentiation of MEPP frequency after tetanic stimulation of 5000 impulses at 100 Hz for a normal NMJ (*A*) and a BAPTA-loaded NMJ (*B*) under external Ca^{2+}-free conditions. *a*, normal plot of relative MEPP frequencies during (◇, $t < 0$) and after (○) tetanic stimulation. The control MEPP frequencies before stimulation (sampled for 1 min) were set at unity. A moving-bin display was used, with a bin size of 9 s and a step size of 3 s. After tetanic stimulation ($t > 0$), the measured MEPP frequency (○) in each bin, the best-fit curve of $(A+1)(P+1)$ (——), the best-fit exponential curve of potentiation, $(P+1)$, (----), experimental points of augmentation obtained by dividing the measured frequencies by the best-fit values of $(P+1)$, (△), the best-fit curve of augmentation (· · · ·) and the control level (– – – –) are shown. *b*, semilogarithmic plot of *a*. The measured increments above the control level or the estimated increments $(P+1)(A+1)-1$, P and A are plotted. Symbols are the same as in *a*. The values of the parameters are given in the text. Tenfold values are plotted for augmentation. F_t and F_c show MEPP frequencies at time after the end of stimulation and the control value before stimulation, respectively.

in the presence of external Ca^{2+}, where every nerve stimulation evoked entry of Ca^{2+} into the nerve terminal. A time-course study of BAPTA loading, described below, also suggested that the peak $[Ca^{2+}]_i$ very near the Ca^{2+} channels at the instant of Ca^{2+} entry might be little affected by loaded BAPTA. Therefore, it is possible that the Ca^{2+} which had entered may have affected the release mechanisms and evoked the three slower components in some indirect way.

TABLE 2. Parameters of augmentation and potentiation of MEPP frequency after tetanic stimulation of 5000 impulses at 100 Hz

	τ_a	τ_p	I_p	$A(0)$	$P(0)$	n
Normal	13·41 ± 3·60	183·02 ± 26·92	35·89 ± 4·71	1·39 ± 0·27	16·26 ± 3·50	9
BAPTA-loaded	14·80 ± 2·43	141·42 ± 10·29	46·16 ± 8·41	1·07 ± 0·23	22·64 ± 4·14	6

Means ± S.E.M. I_p = total relative increment of MEPP frequency ($= (A(0)+1)(P(0)+1)-1$). None of the differences in values between normal and BAPTA loading were statistically significant ($P > 0·1$).

We checked this possibility by examining the spontaneous release under external Ca^{2+}-free conditions. The normal and BAPTA-loaded nerve–muscle preparations were perfused with Ca^{2+}-free Ringer solution containing 4 mM-Mg^{2+} and 1 mM-EGTA. Under these conditions, no phasic transmitter release occurred upon nerve stimulation, but MEPP frequencies were increased by tetanic stimulation (Erulkar & Rahamimoff, 1978). MEPP frequencies before (control), during and after tetanic nerve stimulation (5000 impulses at 100 Hz) were counted, and their relative values were plotted as shown in Fig. 4, using the moving-bin method. MEPP frequencies increased nearly exponentially, and no major differences between the normal and BAPTA-loaded NMJs were observed during the tetanus. The decay processes after tetanus were also similar. They were expressed well by two exponential terms: the fast term corresponded to augmentation and the slow one to potentiation. The two components of facilitation could not be resolved in this analysis using the bin and step sizes of 9 and 3 s, respectively. Under our Ca^{2+}-free conditions in the presence of 4 mM-Mg^{2+}, augmentation of MEPP frequency was consistently observed in both the normal and BAPTA-loaded NMJs, as shown in Fig. 4. This observation differs from that of Erulkar & Rahamimoff (1978), who did not observe augmentation under external Ca^{2+}-free conditions in the presence of 1 mM-Mg^{2+}.

In the experiments shown in Fig. 4, the amplitudes of augmentation and potentiation, $A(0)$ and $P(0)$, were similar in both cases (1·57 and 17·2 in the normal NMJ and 0·49 and 29·2 in the BAPTA-loaded NMJ, respectively). The decay time constants, τ_a and τ_p, were also similar (8·3 and 189 s in the normal and 17·9 and 133 s in the BAPTA-loaded NMJ). In the summary of nine (normal NMJs) and six (BAPTA-loaded NMJs) experiments given in Table 2, there were no significant differences in the four parameters between the normal and BAPTA-loaded NMJs.

In conclusion, both augmentation and potentiation of MEPP frequency were clearly observed under external Ca^{2+}-free conditions, and they were little affected by loaded BAPTA. This result excludes the possibility that only Ca^{2+} entry into the presynaptic nerve terminal might cause augmentation and/or potentiation in some indirect way. It is also unlikely that the Ca^{2+} mobilized from the internal store caused

augmentation and/or potentiation, because loaded BAPTA had little effect on both processes of changes in MEPP frequency.

Change in the features of transmitter release during the time course of BAPTA loading

In order to clarify more precisely the effect of BAPTA on the features of transmitter release, we examined the time course of BAPTA loading by perfusing

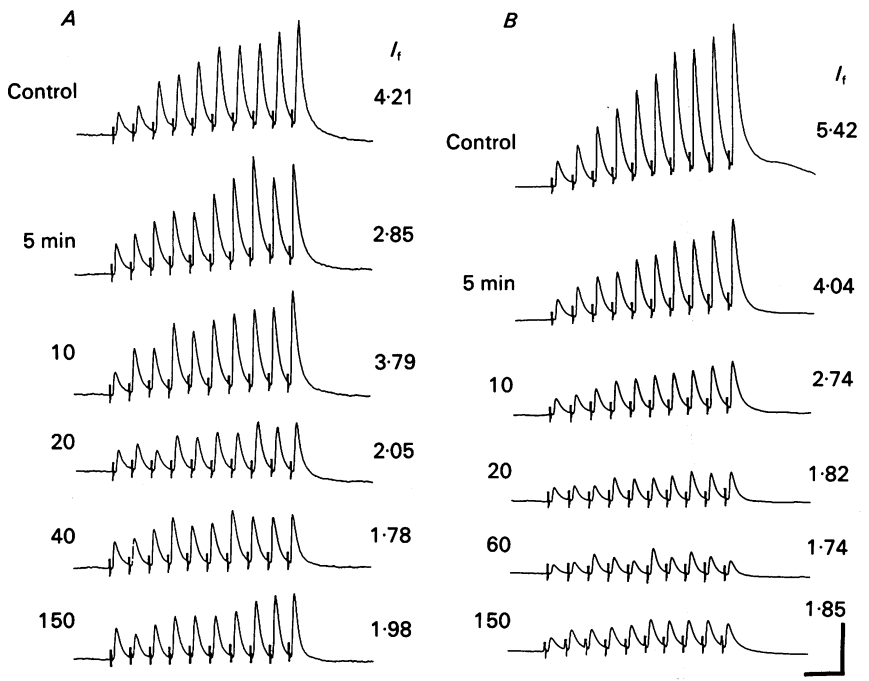

Fig. 5. Two typical examples of trains of EPPs during the time course of BAPTA loading. EPPs were recorded intracellularly before (control) and during perfusion with 0·1 mM-BAPTA-AM at 23 °C. Stimulation was ten impulses at 100 Hz. Numbers on the left show recorded time (in minutes) after onset of BAPTA-AM perfusion. The facilitation index, I_f, is shown on the right. Scale bars: 20 ms and 5 mV. For NMJ in *A*, amplitudes of the first EPPs remained almost constant, whereas in *B*, they decreased with the decrease in I_f. The downward shift after the end of stimulation in the control record of *B* is due to movement of the muscle, which began near the end of the stimulus and had little effect on the estimation of I_f.

BAPTA-AM in the external medium, and observed the quantal contents of EPP, MEPP frequencies and facilitation indices, I_fs, by the intracellular recording method. The concentration of BAPTA-AM in the external solution was 0·1 mM (1/2·5 of that used for usual loading conditions) and also the temperature was lower ($23 \pm 0{\cdot}5$ °C). Figure 5 shows two typical examples of EPP trains during BAPTA loading. In both cases, the facilitation index decreased to a value of less than 2·0 within 20 min, and remained constant thereafter, In some NMJs located at the external surface of the muscle (Fig. 6*A* and *B*), the time required for facilitation to decrease to a constant low

level was less than 20 min. In other cases (Fig. 6*C*), however, it took 40–60 min, probably because the NMJs were located on the inner face of the fibre at the surface of the sartorius muscle. The facilitation index decreased to a constant low level approximately exponentially, its average value being 1·99±0·12 ($n = 19$) 40 min

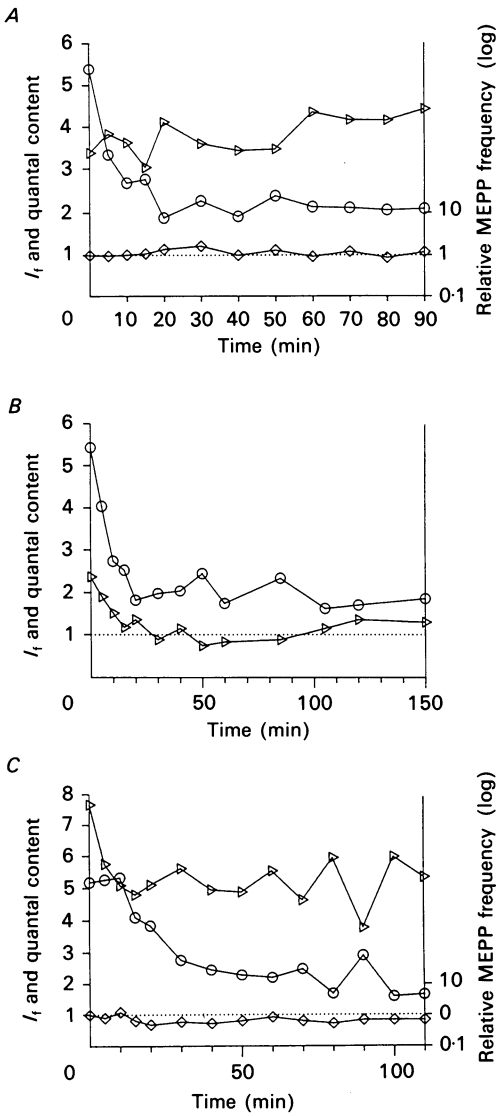

Fig. 6. Changes in the features of transmitter release during the time course of BAPTA loading at three selected NMJs: *A*, *B* and *C*. The NMJ in *B* is the same as that in Fig. 5*B*. In each figure, quantal contents of the first EPP in a train (△——△), the facilitation index, I_f (○——○), and relative MEPP frequencies measured for 1 min before the train stimulations (◇——◇) are plotted on a logarithmic scale against time after onset of BAPTA-AM perfusion. Control MEPP frequencies before BAPTA-AM perfusion were 1·0/s in *A*, and 0·63/s in *C*.

after the start of loading, and the average of its time constants was $6{\cdot}70 \pm 1{\cdot}02$ min ($n = 19$).

The quantal content of the first EPP in a train showed very interesting features during the time course of BAPTA loading. In most NMJs (13/19), the EPP amplitude was almost unchanged, although it fluctuated due to a low quantal content, as shown in Figs 5*A* and 6*A*. In other NMJs (six NMJs from nineteen examined), the EPP amplitude initially decreased, and remained nearly constant after I_f had decreased to a constant low level, as shown in Figs 5*B* and 6*B* and *C*. We suggest that these constant EPP amplitudes during the continuous BAPTA loading arise because the internal Ca^{2+} concentration very near the calcium channels triggers the release. This Ca^{2+} concentration would be little affected by the presence of a Ca^{2+} buffer like BAPTA (see Discussion).

The MEPP frequency measured during the resting period for 1 min before the train stimulation was almost unchanged or slightly decreased (Kijima & Tanabe, 1988) during BAPTA loading. This suggests that the resting level of $[Ca^{2+}]_i$, which may be near the dissociation constant between BAPTA and Ca^{2+} (108 nM), may remain constant throughout the time course, due to cellular homeostatic mechanisms that keep the basal $[Ca^{2+}]_i$ constant in spite of continuous chelation of Ca^{2+} by the loaded BAPTA.

DISCUSSION

Implication of the main finding

The finding that the fast component of facilitation was diminished upon BAPTA loading supports the residual Ca^{2+} hypothesis proposed by Katz & Miledi (1968). They suggested that the entry of Ca^{2+} at the time of the first impulse is indispensable for the 'early' or fast component of facilitation to a following impulse. There is considerable support for this result (Rahamimoff, Meiri, Erulkar & Bavenholz, 1978; Charlton *et al.* 1982; Delaney, Zucker & Tank, 1989).

This can be interpreted in two ways. One is the residual calcium hypothesis, which maintains that Ca^{2+} entry in response to the first impulse increases $[Ca^{2+}]_i$ almost instantaneously by a definite amount, Ca_I, as shown schematically in Fig. 7*A*. The Ca_I decays rapidly at first, and then gradually. Upon arrival of the second impulse its value is equal to Ca_r, representing the residual calcium. This residual calcium is added to the Ca_I which enters at the second impulse, causing an increase in neurotransmitter release, according to the nth power relationship between release and $[Ca^{2+}]_i$ ($n = 4$–5; Dodge & Rahamimoff, 1967; Barton, Cohen & Van der Kloot, 1983). The basal $[Ca^{2+}]_i$ in the resting state, Ca_b, must be added to the amount of Ca^{2+} that entered. The same inference is possible when the 'active' Ca^{2+} (which is a rapidly reversible and unsaturable complex, XCa_n, between the triggering machinery, X, and nCa^{2+}) is considered instead of $[Ca^{2+}]_i$, on the assumption that the release is proportional to the complex. In the presence of BAPTA, the peak value, Ca_I, may be little affected as discussed below, but it may decay more rapidly as shown in Fig. 7*B*. There may be no residual Ca^{2+}, Ca_r, at the instant of the second impulse, causing no fast component of facilitation.

The other interpretation is that the role of Ca^{2+} is somewhat indirect. The Ca^{2+}

entry may exert a lasting effect on one or several of the unknown intermediate reaction steps between Ca^{2+} entry and release, the residual effect causing an increase in the release in response to the second impulse.

The present results show that the latter interpretation is unlikely. This is because the effect of Ca^{2+} may be strongest at its peak, whereas the peak, Ca_I, seems to be

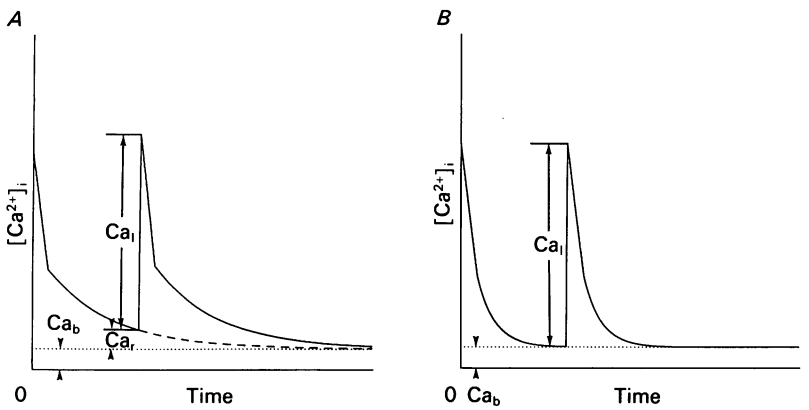

Fig. 7. Schematic diagram of $[Ca^{2+}]_i$ change at the presynaptic nerve terminal during the course of two successive nerve impulses for the residual Ca^{2+} hypothesis. *A*, normal NMJ; *B*, BAPTA-loaded NMJ. Ca_I is the rise in $[Ca^{2+}]_i$ resulting from Ca^{2+} influx by an impulse, Ca_b the basal $[Ca^{2+}]_i$ and Ca_r the residual $[Ca^{2+}]_i$ from the former impulse (modified from Fig. 4 in Kuba *et al.* 1988). At the BAPTA-loaded NMJ (*B*), the magnitude of Ca_I may be unaffected by BAPTA, but its decay may be more rapid, so that there is no Ca_r, and consequently the magnitude of the fast component of facilitation is zero upon arrival of the second impulse.

little affected by the loaded BAPTA, as discussed below. Thus, the lasting indirect effect of Ca_I may be similar in both the normal and BAPTA-loaded nerve terminals and would cause a similar degree of fast facilitation.

The finding that the slow component of facilitation of EPP amplitudes and augmentation and potentiation of MEPP frequency in the absence of external Ca^{2+} remained almost unaffected by the loaded BAPTA, as well as the augmentation and potentiation of EPP amplitudes reported previously (Tanabe & Kijima, 1989), is in striking contrast to the almost complete loss of the fast component of facilitation. The simplest interpretation is that the slow component of facilitation, augmentation and potentiation occur independently of $[Ca^{2+}]_i$. If any of these three components are caused by the increase in $[Ca^{2+}]_i$ at the release site, then the buffering action of BAPTA should decrease the amplitude of that component and increase the decay time constant, depending on the degree of BAPTA loading. No such effects were observed on any of the three components, except for a slightly larger decay time constant for the slow component of facilitation (Table 1).

Computer simulation of $[Ca^{2+}]_i$ distribution around a Ca^{2+} channel in the presence and absence of BAPTA has shown that the slower the rate of $[Ca^{2+}]_i$ change, the

greater the likelihood that it will be affected by BAPTA (Kijima & Kijima, 1990). It is therefore unlikely that all four kinds of stimulation-induced change would occur due to changes in $[Ca^{2+}]_i$, with only the fastest process, the fast component of facilitation, being diminished by BAPTA.

It has been reported that potentiation (and/or augmentation) and facilitation (including the fast and slow components) have a multiplicative relationship (Landau *et al.* 1973; Magleby, 1973*b*; Zengel & Magleby, 1982). Combining this with the finding that the slow component has a multiplicative relationship with potentiation, we can derive the following relation between the fast component of facilitation and potentiation:

$$EPP(t)/EPP_0 \propto (F_1+1)(P+1). \quad (7)$$

If we assume that potentiation occurs due to an increase in $[Ca^{2+}]_i$, the existence of potentiation means an increase in the basal $[Ca^{2+}]_i$, Ca_b, in Fig. 7*A*. When Ca_b is increased, fast facilitation (F_1) caused by the residual Ca^{2+} must decrease, because the ratio $(Ca_I+Ca_b+Ca_r)/(Ca_I+Ca_b)$, whose nth power is the magnitude of the fast component of facilitation (F_1+1), decreases with the increase in Ca_b (Kuba, Minota, Koyano, Tanaka & Tsuji, 1988). Thus, the relation (7) is incompatible with the hypothesis that potentiation occurs due to an increase in $[Ca^{2+}]_i$ (Weinreich, 1971; Delaney *et al.* 1989). In agreement with the above conclusion, Zengel & Magleby (1981) showed that the relationship between MEPP frequency and EPP amplitude during and following repetitive stimulation is inconsistent with a simple fourth power model.

The multiplicative relation, eqn (1) or (7), indicates that $[Ca^{2+}]_i$ dependence of transmitter release always exists and works through the term (F_1+1). Apparently, Ca^{2+}-independent regulation systems change the Ca^{2+} sensitivity of the whole release mechanisms. The term (F_1+1) should be taken to express the following general dependence of release on $[Ca^{2+}]_i$:

$$(F_1+1) \propto [Ca^{2+}]_i^n.$$

Thus, change in $[Ca^{2+}]_i$ at the release site always affects the apparent size of potentiation (and/or other two processes). For example, when stimulation is done under external Ca^{2+}-free conditions, the basal Ca^{2+} may decrease and cause reduction of MEPP frequency at the early stage of stimulation (Erulkar & Rahamimoff, 1978). This may also cause an apparent reduction of potentiation, which may account for, at least in part, the reduced degree of potentiation under Ca^{2+}-free conditions.

Augmentation has also been considered to occur due to an increase in $[Ca^{2+}]_i$, because it has been reported that augmentation of MEPP frequency did not occur in Ca^{2+}-free external medium (Erulkar & Rahamimoff, 1978). However, we have consistently observed augmentation of MEPP frequency in Ca^{2+}-free external medium containing 4 mM-Mg^{2+} (Fig. 4).

Differential effects of divalent cations on the four components of stimulation-induced change have been reported (Zengel & Magleby, 1980, 1981). The presence of a small concentration of external Ba^{2+} specifically enhanced augmentation, whereas Sr^{2+} specifically induced an increase in the slow component of facilitation, both in EPP amplitude and MEPP frequency. These differential effects suggest different underlying mechanisms between these components, especially between the fast and

slow components of facilitation, thus supporting our view that these two have different origins. Our observation that the slow component of facilitation had a multiplicative relationship with augmentation and potentiation also suggests a different underlying mechanism between the slow component of facilitation and augmentation and/or potentiation.

Recently, Delaney *et al.* (1989) measured the change in $[Ca^{2+}]_i$ during and after tetanic stimulations at the nerve terminal of the crayfish NMJ using a fluorescent Ca^{2+} indicator, Fura-2. They reported that the decay time course of potentiation of EPP amplitude after the tetanus agreed with that of $[Ca^{2+}]_i$ in the nerve terminal. However, a 13-fold potentiation of EPP amplitude corresponded to a concentration of $[Ca^{2+}]_i$ (700 nM) only 3·5 times higher than the basal $[Ca^{2+}]_i$ (200 nM), which was 20 times smaller than the value of 4 μM estimated by computer simulation, assuming a power law of $n = 5$ (Fogelson & Zucker, 1985). Moreover, when a power law of $n = 4$–5 was assumed, the decay time course of potentiation did not agree with that of $[Ca^{2+}]_i^n$. These findings appear to be inconsistent with the idea that potentiation occurs from an elevation of $[Ca^{2+}]_i$ at the release site. Observed elevation of $[Ca^{2+}]_i$ to about 2 μM during tetanic stimulation seems sufficient to explain the fast component of facilitation on the basis of the residual Ca^{2+} hypothesis, because a higher Ca^{2+} concentration would be expected at the submembrane release site.

Both the augmentation and potentiation of MEPP frequency were observed in the absence of external Ca^{2+} and were unaffected by loaded BAPTA. This observation also supports the above view, and excludes the possibility that only Ca^{2+} entry upon stimulation may cause augmentation and/or potentiation in some indirect way. Entry of Na^+ ions upon stimulation may be responsible for augmentation and/or potentiation (Birkes & Cohen, 1968; Erulkar & Rahamimoff, 1978; Meiri, Erulkar, Lerman & Rahamimoff, 1981).

Changes in the characteristics of transmitter release during the time course of BAPTA loading

The facilitation index, I_f, which is a measure of facilitation, decreased to a steady level of below 2·0 within about 20–40 min after onset of 0·1 mM-BAPTA-AM perfusion (Figs 5 and 6), indicating the time course of BAPTA into the presynaptic nerve terminal.

The EPP amplitude (or quantum content of the EPP) to the first stimulation in a train either remained constant throughout the time course of BAPTA loading (13/19 NMJs), or was initially reduced, remaining constant thereafter (6/19 NMJs). In the latter case, the EPP amplitude usually did not decrease after I_f had been reduced to the steady-state level. These data are surprising, taking into account the fact that the binding reaction between BAPTA and Ca^{2+} is more than 500 times faster than that between EGTA and Ca^{2+}, and is estimated to occur within less than 0·17 ms if the concentration of free BAPTA is 10 μM (Tsien, 1980). The rate of Ca^{2+} binding to BAPTA seems rapid enough to compete with the Ca^{2+} triggering system of transmitter release and to reduce the quantal content of phasic release. However, computer simulations have shown that the submembrane $[Ca^{2+}]_i$ during the inward calcium current is characterized by discrete peaks of Ca^{2+} immediately adjacent to the calcium channels, and that the peak $[Ca^{2+}]_i$ adjacent to the channels is hardly

affected by the presence of a diffusible Ca^{2+} chelator such as BAPTA (Simon & Llinas, 1985; Kijima & Kijima, 1990).

Our finding that EPP amplitudes remained constant during continuous loading of BAPTA can be accounted for if we take into account Ca^{2+} compartmentalization near the calcium channels. Under our low-Ca^{2+} and high-Mg^{2+} conditions, the Ca^{2+} immediately adjacent to the calcium channels may trigger the phasic release, and the concentration of this Ca^{2+} may be unaffected by a fairly high concentration of BAPTA, because reduction of $[Ca^{2+}]_i$ due to chelation by BAPTA may be rapidly compensated by entering Ca^{2+}. Residual Ca^{2+} which remains at the release site for tens of milliseconds after Ca^{2+} channel closure may easily be chelated by BAPTA, bringing about complete loss of the fast component of facilitation.

Dale & Kandel (1990) recently reported a Ca^{2+}-independent modulation of transmitter release by serotonin and a neuropeptide, FMRFamide, for BAPTA-injected sensory neurons. In their study, however, phasic release was completely abolished after intensive injection of BAPTA, suggesting that the distance between the release site and Ca^{2+} channels may be larger than that at the active zone of the frog NMJ (see also Adams, Takeda & Umbach (1985), for the effect of EGTA injected into squid giant synapse).

Possible mechanism of Ca^{2+}-independent regulation of transmitter release

Up to now, there have been no studies on the mechanism of Ca^{2+}-independent regulation of transmitter release, except for findings indicating that Na^+ entry may be responsible for potentiation.

In secretory cells, such as mast cells, pancreatic β cells and adrenal chromaffin cells, there may be two mechanisms for regulation of secretion, one Ca^{2+} dependent and the other Ca^{2+} independent. In the mast cell, whole-cell clamp experiments have shown that secretagogues can trigger exocytosis when $[Ca^{2+}]_i$ is clamped at the resting level or even lower (Penner & Neher, 1988). Intracellular application of a non-hydrolysable GTP analogue, guanosine 5′-*O*-(3-thiotriphosphate) (GTPγS), through a patch-pipette with $[Ca^{2+}]_i$ clamped lower than the resting level also induced full secretion, suggesting the participation of a type of GTP-binding protein. In contrast, application of GTPγS to electropermeabilized chromaffin cells inhibited the secretion of adrenaline (Knight, von Grafenstein & Athayde, 1989).

Our tentative model is that exocytosis in both secretory cells and synapses are regulated by a dual Ca^{2+}-dependent and -independent mechanism, and that both components may regulate the release apparatus independently of each other. Transmitter release at the synapse is triggered by Ca^{2+} entry in order to achieve rapid triggering, and the Ca^{2+}-independent regulation mechanism may be used to modulate the release (Dale & Kandel, 1990), whereas in the mast cell, the Ca^{2+}-independent mechanism is used for secretagogue-dependent triggering and Ca^{2+} is used for the modulation of secretion.

It will be necessary to elucidate the details of both the Ca^{2+}-independent and Ca^{2+}-dependent release mechanisms.

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