Role of Intracellular Ca\(^{2+}\) in Stimulation-induced Increases in Transmitter Release at the Frog Neuromuscular Junction

JANET E. ZENGEL, MARIA A. SOSA, ROBERT E. POAGE, and DENNIS R. MOSIER

From the Research Service, Department of Veterans Affairs Medical Center and Departments of Neuroscience and Neurosurgery, University of Florida College of Medicine, Gainesville, Florida 32610

ABSTRACT Under conditions of reduced quantal content, repetitive stimulation of a presynaptic nerve can result in a progressive increase in the amount of transmitter released by that nerve in response to stimulation. At the frog neuromuscular junction, this increase in release has been attributed to four different processes: first and second components of facilitation, augmentation, and potentiation (e.g., Zengel, J. E., and K. L. Magleby. 1982. Journal of General Physiology. 80:583–611). It has been suggested that an increased entry of Ca\(^{2+}\) or an accumulation of intraterminal Ca\(^{2+}\) may be responsible for one or more of these processes. To test this hypothesis, we have examined the role of intracellular Ca\(^{2+}\) in mediating changes in end-plate potential (EPP) amplitude during and after repetitive stimulation at the frog neuromuscular junction. We found that increasing the extracellular Ca\(^{2+}\) concentration or exposing the preparation to carbonyl cyanide \(m\)-chlorophenylhydrazone, ionomycin, or cyclopiazonic acid all led to a greater increase in EPP amplitude during conditioning trains of 10–200 impulses applied at a frequency of 20 impulses/s. These experimental manipulations, all of which have been shown to increase intracellular levels of Ca\(^{2+}\), appeared to act by increasing primarily the augmentation component of increased release. The results of this study are consistent with previous suggestions that the different components of increased release represent different mechanisms, and that Ca\(^{2+}\) may be acting at more than one site in the nerve terminal.

Address correspondence to Janet E. Zengel, Ph.D., Department of Neuroscience, University of Florida College of Medicine, Box 100244, Gainesville, FL 32610.

Dr. Sosa’s current address is Institute of Neurobiology, University of Puerto Rico, Medical Sciences Campus, San Juan, Puerto Rico 00901.

Dr. Mosier’s current address is Department of Neurology, Baylor College of Medicine, Houston, TX 77030.
INTRODUCTION

The amount of transmitter released from a synapse by each nerve impulse varies, depending on previous synaptic activity (e.g., Feng, 1941; del Castillo and Katz, 1954b; Liley, 1956). Under conditions of reduced quantal content, repetitive stimulation can lead to a progressive increase in evoked transmitter release. At the frog neuromuscular junction, at least four different processes appear to be responsible for this increase in release: first and second components of facilitation, which decay with time constants of ~60 and 500 ms, respectively (Mallart and Martin, 1967; Magleby, 1973; Zengel and Magleby, 1982); augmentation, which decays with a time constant of ~7 s (Magleby and Zengel, 1976a; Erulkar and Rahamimoff, 1978); and potentiation, which decays with a time constant of tens of seconds to minutes (Rosenthal, 1969; Magleby and Zengel, 1975). Components of increased release with similar properties have also been observed at a number of other synapses, including rabbit sympathetic ganglia (Zengel, Magleby, Horn, McAfee, and Yarowsky, 1980), chick ciliary ganglia (Martin and Pilar, 1964; Poage and Zengel, 1993), cat spinal cord (Curtis and Eccles, 1960), and rat hippocampus (McNaughton, 1982).

Although the mechanisms underlying the various components of increased release are not known, it has long been thought that Ca\(^{2+}\) may play a critical role in stimulation-induced changes in release (e.g., Katz and Miledi, 1968; Rosenthal, 1969; Miledi and Thies, 1971; Weinreich, 1971; Erulkar and Rahamimoff, 1978). In support of this, we recently reported that blocking Ca\(^{2+}\) influx into frog motor nerve terminals with Cd\(^{2+}\) (Zengel, Lee, Sosa, and Mosier, 1993) or the cone snail peptide \(\omega\)-conotoxin (Zengel, Sosa, and Poage, 1993) resulted in a reduction of both facilitation and augmentation, suggesting that Ca\(^{2+}\) entry may be involved in the generation of these two components of increased release.

The simplest model to account for stimulation-induced changes in release, the so-called "residual calcium hypothesis" of Katz and Miledi (Katz and Miledi, 1968; Miledi and Thies, 1971; see review by Zucker, 1989), proposes that each nerve impulse contributes an increment of Ca\(^{2+}\) or some Ca-activated factor that can add to the Ca\(^{2+}\) or Ca factor remaining from previous stimulation, thereby increasing the amount of transmitter released. However, this model in its simplest form fails to account for all of the properties of stimulation-induced increases in release (Zengel and Magleby, 1980, 1981, 1982; Bain and Quastel, 1992), leading to the suggestion that if Ca\(^{2+}\) is in fact involved in increases in release, then Ca\(^{2+}\) must be acting at more than one site in the nerve terminal.

The purpose of the present study was twofold: first, to examine the role of resting levels of intracellular Ca\(^{2+}\) in mediating stimulation-induced changes in end-plate potential (EPP) amplitude at the frog neuromuscular junction, and second, to further examine the possibility that Ca\(^{2+}\) may play a role at more than one step of the release process. We report that increasing the extracellular Ca\(^{2+}\) concentration or exposing the preparation to various agents known to increase intracellular Ca\(^{2+}\) levels resulted in a greater increase in EPP amplitude during repetitive stimulation, primarily as a result of an increase in the magnitude of the augmentation component of increased release. This effect of Ca\(^{2+}\) appears to be mediated by an action distinguishable from
the role of Ca\(^{2+}\) in initiating evoked transmitter release in the absence of repetitive stimulation.

**METHODS**

**Preparation and Solutions**

The frog (*Rana pipiens*) sartorius nerve-muscle preparation was used for these experiments. Animals were killed by pithing and decapitation, and the sartorius muscle with attached nerve was dissected free and pinned to the bottom of a wax-covered recording chamber. The standard bathing solution used during dissection of the preparation had the composition (mM): NaCl, 116; KCl, 2; CaCl\(_2\), 1.8; Hepes, 2; glucose, 5. For data collection, this solution was modified to reduce the level of release by decreasing Ca\(^{2+}\) to 0.3–1 mM and adding 5 mM MgCl\(_2\) (low Ca Ringer). The osmolarity of the solution was maintained by making appropriate changes in NaCl. In the experiments in which we examined the effects on release of ionomycin, carbonyl cyanide m-chlorophenylhydrazone (CCCP), carbonyl cyanide p-trifluoromethoxyphenylhydrazone (FCCP), and cyclopiazonic acid (CPA), concentrated stock solutions (0.5–10 mM) were made up using dimethyl sulfoxide (DMSO) or ethanol as the solvent. Aliquots of these stocks were then added directly to a low Ca Ringer to obtain the desired concentration. Data were also obtained in the presence of the solvent (DMSO or ethanol) alone to control for any effects of these agents. All solutions were adjusted to pH 7.2–7.4 before use. Experiments were carried out at room temperature (22–24\(^\circ\)C).

**Data Collection**

A fluid suction electrode (Dudel and Kuffler, 1961) was used to stimulate the nerve. For most experiments, extracellular recordings of EPPs were obtained with a surface electrode from end-plate regions. Under the conditions of low quantal content used in these experiments, changes in surface-recorded EPP amplitudes give a reliable measure of changes in transmitter release (Magleby, 1973; Magleby and Zengel, 1976a; Mallart and Martin, 1967). Surface recording has the advantage over intracellular recording of summing the response from several end-plates, thus reducing the amount of data required to obtain estimates of the average response. In some experiments, standard intracellular recording techniques were used to record EPPs and miniature end-plate potentials (MEPPs).

In a typical experiment, the nerve was stimulated with conditioning trains of from 10–200 impulses applied at a frequency of 20 impulses/s. In some experiments, testing impulses were applied at various times after the end of the conditioning train to obtain estimates of the different components of increased release. Sufficient time was allowed between trains to ensure that the release level had recovered to the preconditioning level (1–10 min, depending on the number of conditioning impulses; Magleby and Zengel, 1975). Because of the low levels of transmitter release in these experiments and the resulting quantal fluctuation (del Castillo and Katz, 1954a), it was usually necessary to average the response from a number of identical trials to obtain reliable estimates of the stimulation-induced changes in EPP amplitude. For experiments in which short (10-impulse) conditioning trains were used, a stimulator (548; Grass Instruments, Quincy, MA) was used to generate the trains, and the resulting EPPs were averaged using a Nicolet 1170 signal averager (Nicolet Instrument Corp., Madison, WI). Amplitudes of the averaged EPPs were measured directly from the display monitor. In experiments in which we used longer conditioning trains, a MINC-11 computer was used to generate the stimulation patterns, measure and store EPP amplitudes, and analyze data. Details of the stimulating, recording, and data analysis techniques have been published (Zengel, Lee, Sosa, and Mosier, 1993).
Definition of Terms

Estimates of the change in EPP amplitude resulting from repetitive stimulation were expressed as:

\[ V(t) = \frac{EPP_t}{EPP_c} - 1, \quad (1) \]

where \( EPP_t \) is the EPP amplitude at time \( t \), and \( EPP_c \) is the control EPP amplitude in the absence of repetitive stimulation. In experiments in which we applied 10-impulse conditioning trains, \( EPP_c \) was the amplitude of the first EPP of the train. In experiments in which we used longer conditioning trains, \( EPP_c \) was obtained from the average amplitude of 5–10 EPPs obtained before conditioning.

Estimates of the components of stimulation-induced increases in release at the end of a conditioning train (see Fig. 8) were obtained assuming a multiplicative relationship among these components, such that:

\[ V(t) + 1 = (F(t) + 1)(A(t) + 1)(P(t) + 1), \]

where \( F(t) \) is the total facilitation at time \( t \) contributed by the two components of facilitation, \( A(t) \) is augmentation, and \( P(t) \) is potentiation (Zengel and Magleby, 1982; Magleby and Zengel, 1982).

Averaged data are presented as mean ± SE.

RESULTS

Effect of \([Ca^{2+}]_0\) on Stimulation-induced Increases in EPP Amplitude during 10-Impulse Trains

Previous studies have shown that the resting level of cytoplasmic \( Ca^{2+} \) is dependent on the concentration of extracellular \( Ca^{2+} \) (e.g., Nachshen, 1985; Smith, 1990). Thus, we began our study of the role of intracellular \( Ca^{2+} \) in mediating stimulation-induced increases in transmitter release by first examining the effect of changes in the extracellular \( Ca^{2+} \) concentration. Although small (0.1–0.3 mM) increases in extracellular \( Ca^{2+} \) were reported to have little or no effect on the components of increased release after relatively long (> 200-impulse) conditioning trains (Zengel and Magleby, 1980), it was not clear what effect larger changes in \( Ca^{2+} \) concentration might have on these components. It was also not known if changes in the level of extracellular \( Ca^{2+} \) affected the components of increased release during shorter trains of stimulation, when there is less likelihood that depression might obscure the components of increased release (Magleby and Zengel, 1976b). Therefore, we first examined the effect of changes in extracellular \( Ca^{2+} \) on release during short conditioning trains.

The effect of increasing the extracellular \( Ca^{2+} \) concentration is illustrated in Fig. 1, which presents digitized records of EPPs recorded extracellularly during 10-impulse trains in Ringer containing 0.4 (Fig. 1A) and 0.6 mM (Fig. 1B) Ca. In this experiment, the control EPP amplitude (the amplitude of the first EPP of the train) was increased more than eightfold when the \( Ca^{2+} \) concentration was increased from 0.4 to 0.6 mM (notice different voltage scales for Fig. 1, A and B). This large increase in EPP amplitude is only slightly less than the 10-fold increase in release predicted by the fourth power relationship between extracellular \( Ca^{2+} \) and release that has been demonstrated at the frog neuromuscular junction (Dodge and Rahamimoff, 1967).
The effect of extracellular Ca\(^{2+}\) concentration on another experimental preparation is illustrated in Fig. 2A, which plots changes in EPP amplitude during 10-impulse trains in 0.4 (filled circles), 0.5 (filled squares), and 0.6 mM Ca (filled triangles). Again, the changes in control EPP amplitude were similar to those predicted on the basis of the fourth power relationship between Ca\(^{2+}\) and release.

In addition to affecting control EPP amplitude, changes in extracellular Ca\(^{2+}\) also affected the magnitude of the stimulation-induced increase in EPP amplitude, although to a much smaller degree. This is seen in Fig. 2B, which plots changes in $V(t)$, the fractional increase in EPP amplitude (Eq. 1 in Methods), for the trains plotted in Fig. 2A. Notice the greater increase in $V(t)$ with increased extracellular Ca\(^{2+}\). Similar effects of changes in $[Ca^{2+}]_0$ were seen in the majority of experiments.

**Figure 1.** Effect of extracellular Ca\(^{2+}\) concentration on EPP amplitude during trains of 10 impulses applied at a frequency of 20 impulses/s. Extracellular recordings of EPPs were obtained in low Ca Ringer containing 0.4 mM Ca (A) and 0.6 mM Ca (B). Averages of 128 (0.4 mM Ca) and 32 trials (0.6 mM Ca). Note different voltage scales for A and B.

Within a single experiment, we found it difficult to vary $[Ca^{2+}]_0 > 0.2-0.3$ mM. At too low a concentration of Ca\(^{2+}\), the EPPs became so small in amplitude that it was difficult to obtain reliable measures of $V(t)$; at too high a Ca\(^{2+}\) concentration, we...
observed either a depression of EPP amplitude or the EPPs became large enough in amplitude to lead to the generation of muscle action potentials and muscle contraction. We were, however, able to look at the effects of a wider range of Ca\(^{2+}\) concentrations by pooling data from a large number of experiments carried out as part of other studies conducted in our laboratory over the past several years.

The results of these experiments are presented in Fig. 3, which shows more convincingly the effect of \([\text{Ca}^{2+}]_0\) on stimulation-induced increases in EPP amplitude. Fig. 3 A plots changes in V(t) during 10-impulse trains in 0.3 mM (filled diamonds), 0.4 mM (filled circles), 0.6 mM (filled triangles), and 0.8 mM Ca (filled inverted triangles).

![Figure 2](image)

**Figure 2.** Effect of \([\text{Ca}^{2+}]_0\) on EPP amplitude and stimulation-induced increases in EPP amplitude during trains of 10 impulses applied at a frequency of 20 impulses/s. Data were obtained in 0.4 mM Ca (filled circles; average of 495 trials), 0.5 mM Ca (filled squares; average of 304 trials), and 0.6 mM Ca (filled triangles; average of 342 trials). Data were averaged from both sartorius nerve-muscle preparations from a single experimental animal. (A) EPP amplitude normalized to the amplitude of the first EPP \((t = 0)\) in the 10-impulse trains in 0.4 mM Ca. (B) Plot of \(V(t)\), the fractional increase in EPP amplitude \((\text{Eq. 1 in Methods}), \) during the 10-impulse trains plotted in A. Note the greater increase in \(V(t)\) with increasing extracellular \(\text{Ca}^{2+}\) concentrations. (C) Effect of \([\text{Ca}^{2+}]_0\) on \(V(450)\), the magnitude of \(V(t)\) at the time of the last EPP of the 10-impulse trains. Data were obtained from 29 experimental preparations in which the effect of at least two concentrations of \(\text{Ca}^{2+}\) were examined. The order of exposure to the different \(\text{Ca}^{2+}\) concentrations was randomized to control for any effects of time. Lines connect data obtained from the same preparation.

These data were obtained from a total of 147 experimental preparations; the numbers in brackets indicate the numbers of preparations from which data were averaged for each \(\text{Ca}^{2+}\) concentration. Standard error bars were also plotted on this figure, but were small enough to be masked by the data points themselves. Notice that the effect of increased \([\text{Ca}^{2+}]_0\) became greater with time during the 10-impulse trains. Fig. 3 B presents a scatter plot of values of \(V(450)\), the magnitude of \(V(t)\) at the
end of 10-impulse trains, as a function of \([\text{Ca}^{2+}]_0\) for data obtained from 215 experimental preparations. Again, although there was considerable variability in values of \(V(450)\) at a given \(\text{Ca}^{2+}\) concentration, the magnitude of \(V(450)\) was clearly a function of extracellular \(\text{Ca}^{2+}\) levels. This relationship is illustrated by the line in this figure, which plots the linear regression of the data points. A t test (using the t ratio of the slope to the standard error) indicated that the slope of this line was significantly different from 0 \((P < 0.0001)\).

![Figure 3](image)

**Figure 3.** Effect of \([\text{Ca}^{2+}]_0\) on stimulation-induced increases in EPP amplitude during 10-impulse trains. (A) Changes in \(V(t)\) during 10-impulse trains in 0.3 mM Ca (filled diamonds), 0.4 mM Ca (filled circles), 0.6 mM Ca (filled triangles), and 0.8 mM Ca (filled inverted triangles). Data were obtained from a total of 147 experimental preparations. Numbers in parentheses indicate numbers of experimental preparations from which data were averaged for each Ca 2+ concentration. Standard errors were calculated and plotted for each data point, but were small enough to be masked by the data points themselves. With the exception of \(V(t)\) at time 0, which by definition equals 0 (Eq. 1), all values of \(V(t)\) were significantly different from the corresponding point obtained in the other three \(\text{Ca}^{2+}\) concentrations \((P < 0.01,\) paired t test). (B) Plot of \(V(450)\) as a function of extracellular \(\text{Ca}^{2+}\) concentration. Data from 215 experimental preparations. The line plots the linear regression of the data points \((R = 0.70)\). The slope of this line was significantly different from 0 \((P < 0.0001)\), as indicated by a t test using the t ratio of the slope (1.202) to the standard error (0.075).

**Effect of CCCP on Stimulation-induced Increases in EPP Amplitude during 10-Impulse Trains**

Because changes in extracellular \(\text{Ca}^{2+}\) levels can result in changes in both the resting level of intracellular \(\text{Ca}^{2+}\) as well as the amount of \(\text{Ca}^{2+}\) that enters the nerve terminal in response to depolarization, we also looked at the effects of several chemical agents known to increase resting intracellular \(\text{Ca}^{2+}\) levels.

We first examined the effects of the metabolic uncouplers carbonyl cyanide \(m\)-chlorophenylhydrazone (CCCP) and carbonyl cyanide \(p\)-trifluoromethoxyphenylhydrazone (FCCP). Metabolic uncouplers have been shown to increase intracellular \(\text{Ca}^{2+}\) levels, presumably by inducing \(\text{Ca}^{2+}\) efflux from mitochondria (Baker and Schlaepfer, 1978; Akerman and Nicholls, 1981; Heinonen, Akerman, and Kaila, 1984; Nachshen, 1985). We found that exposure of the nerve-muscle preparation to
CCCP or FCCP resulted in a dramatic increase in \( V(t) \) during 10-impulse trains. This is illustrated in Fig. 4 A, which plots changes in \( V(t) \) before (filled circles) and at various times after (open circles) the addition of 200 nM CCCP to the 0.6 mM Ca Ringer. Fig. 4 B shows averaged data from three experimental preparations exposed to 100–200 nM CCCP. Like the effect of increased \([Ca^{2+}]_0\), the effect of CCCP on \( V(t) \) clearly increased with time during the 10-impulse train. Similar results were obtained in nine additional experiments in which we examined the effects of 100–2000 nM CCCP, and in two experiments in which we examined the effects of 1,000 nM FCCP.

The increase in \( V(t) \) observed after addition of CCCP or FCCP was always accompanied by a progressive decrease in control EPP amplitude. This is illustrated in Fig. 5 A, which plots \( V(450) \) (filled circles) and EPP amplitude (open circles) as a function of time during the course of the experiment shown in Fig. 4 A. Notice that the effect of 200 nM CCCP on both \( V(450) \) and control EPP amplitude developed slowly, reaching a maximal effect only after about 3 h in CCCP. The time courses of the CCCP-induced changes in EPP amplitude and \( V(t) \) were concentration-dependent, as illustrated in Fig. 5 B. This figure, which shows data from six experiments, plots control EPP amplitude and \( V(450) \) as a function of time after addition of 100 nM (open diamonds), 200 nM (filled circles), 500 nM (open squares), and 1,000 nM CCCP (filled triangles). It can be seen that the CCCP-induced changes in both EPP amplitude and \( V(450) \) developed more rapidly in higher concentrations of CCCP. With continued exposure to CCCP, \( V(t) \) often eventually began to decrease again. The time course for this effect also appeared to be concentration dependent, the decrease in \( V(t) \) occurring more rapidly in higher concentrations of CCCP (data not shown).
Effect of Ionomycin and CPA on Stimulation-induced Increases in EPP Amplitude during 10-Impulse Trains

Because the effects on \( V(t) \) of the metabolic uncouplers CCCP and FCCP could result directly from a decrease in intracellular ATP levels, we also looked at the effects of other agents that increase intracellular \( \text{Ca}^{2+} \) levels without affecting ATP production. As illustrated in Fig. 6A, a brief (1-min) exposure of the nerve-muscle preparation to 10 \( \mu \)M ionomycin, a calcium ionophore that has been shown to increase intracellular \( \text{Ca}^{2+} \) levels (Liu and Hermann, 1978), also results in a small increase in \( V(t) \) during 10-impulse trains. A similar ionomycin-induced increase in \( V(t) \) was observed in seven experiments of this type. The results of these experiments are summarized in Fig. 6B, which plots \( V(t) \) before (filled circles) and after (open circles) 1–1.5 min exposure to 10 \( \mu \)M ionomycin. The ionomycin-induced increase in \( V(t) \) was typically seen within 15–20 min after exposure to ionomycin. Interestingly, despite the brief exposure time, the effect of ionomycin on \( V(t) \) was usually maintained for several hours. Unlike
the case with CCCP, we observed no consistent effect of ionomycin exposure on control EPP amplitude.

Ionomycin has been shown to produce increases in intracellular Ca$^{2+}$ levels, even under conditions in which Ca$^{2+}$ is absent from the extracellular bathing solution, suggesting that this ionophore may act by releasing Ca$^{2+}$ from intracellular stores (e.g., Grierson, Petroski, O’Connell, and Geller, 1992; Yoshida and Plant, 1992). To determine the source of the presumed increase in intracellular Ca$^{2+}$ in our experiments, we also conducted experiments in which the nerve-muscle preparation was exposed to ionomycin in a Ringer solution containing no added Ca$^{2+}$. In these experiments, the preparation was washed repeatedly with the no-added Ca Ringer until EPPs could no longer be detected. Ionomycin was then added to the bathing solution and removed after 1–1.5-min exposure by repeated washing with fresh no-added Ca Ringer. After 15–20 min, the no-added Ca Ringer was replaced with a fresh low Ca Ringer. Although it usually took an additional 20–30 min before EPPs could be recorded again, once the EPPs reappeared, we consistently observed a small increase in $V(t)$ over that observed before ionomycin treatment. For example, in seven experiments of this type, $V(450)$ increased from $1.03 \pm 0.09$ to $1.12 \pm 0.07$ after exposure to ionomycin ($P < 0.05$, paired $t$ test). Thus, it appears that the effect of ionomycin is not dependent on the presence of extracellular Ca$^{2+}$.

We also examined the effects of cyclopiazonic acid (CPA), a membrane-permeable agent which increases intracellular Ca$^{2+}$ levels by inhibiting the endoplasmic reticulum Ca-ATPase (Seidler, Jona, Vegh, and Martonosi, 1989; Demaurex, Lew, and Krause, 1992). We found that the addition of 10–20 $\mu$M CPA to the low Ca Ringer resulted in a small increase in $V(t)$ during 10-impulse trains. For example, $V(450)$

**FIGURE 6.** Effect of ionomycin on stimulation-induced increases in EPP amplitude during 10-impulse trains. (A) Changes in $V(t)$ before (filled circles) and at the indicated times after (open circles) a 1-min exposure to 10 $\mu$M ionomycin. Each train represents the average of from four to seven consecutive trials from a single preparation in 0.8 mM Ca Ringer. (B) Averaged data from seven experiments in which the preparation was exposed to 10 $\mu$M ionomycin for 1–1.5 min. The data points plot $V(t)$ obtained in low (0.7–1 mM) Ca Ringer before (filled circles) and from 30–90 min after (open circles) exposure to ionomycin. Standard errors indicated by vertical bars. Asterisks indicate statistically significant differences between values obtained before and after ionomycin exposure ($P < 0.05$, paired $t$ test).
increased from \(0.83 \pm 0.14\) to \(0.89 \pm 0.10\) after exposure to CPA \((P > 0.05, \text{ paired } t \text{ test, } n = 5)\). Although this effect of CPA on \(V(t)\) during 10-impulse trains was not statistically significant, we did observe a much larger, statistically significant increase in \(V(t)\) during longer conditioning trains (see below).

**Effect of Increasing \([Ca^{2+}]_i\) on Stimulation-induced Increases in EPP Amplitude during Long Conditioning Trains**

Increases in \(V(t)\) resulting from increasing the extracellular \(Ca^{2+}\) concentration or exposing the nerve-muscle preparation to CCCP, ionomycin, or CPA were all more clearly seen during longer conditioning trains. This is illustrated in Fig. 7, which plots changes in \(V(t)\) during 100-impulse conditioning trains in the presence of different extracellular \(Ca^{2+}\) concentrations (Fig. 7 A) and after exposure to CCCP (Fig. 7 B), ionomycin (Fig. 7 C), and CPA (Fig. 7 D). Notice that in each case, the increase in \(V(t)\) became progressively greater during the conditioning train. Most of the increase
in EPP amplitude at the start of the conditioning train can be attributed to the two components of facilitation, which, because of their rapid time courses (time constants of ~60 and 500 ms, respectively), would have reached a steady state level during the first 1–2 s of stimulation (Mallart and Martin, 1967; Zengel and Magleby, 1982). Thus, the progressive increase in \( V(t) \) observed in higher \( \text{Ca}^{2+} \) concentrations or after exposure to CCCP, ionomycin, or CPA must arise from an increase in one of the more slowly decaying components of increased release, either augmentation or potentiation.

### The Effects of Increasing \([\text{Ca}^{2+}]_0\), Appear to Result from an Increase in Augmentation

To determine which component of increased release was affected by the experimental manipulations used to increase intracellular \( \text{Ca}^{2+} \), we carried out experiments in which we stimulated the nerve with long conditioning trains, then applied testing impulses at various times after the train to obtain estimates of augmentation and potentiation. Results of these experiments are summarized in Fig. 8, which plots \( V(t) \) during and after long conditioning trains in different extracellular \( \text{Ca}^{2+} \) concentrations (Fig. 8A), and before and after exposure to CCCP (Fig. 8B), ionomycin (Fig. 8C), and CPA (Fig. 8D). Notice in each case, that the effects of the various experimental manipulations used to increase intracellular \( \text{Ca}^{2+} \) levels were apparent during the train and for the first 20–30 s of the decay phase after the conditioning trains, the time during which augmentation would be decaying (Zengel and Magleby, 1982). The decay of \( V(t) \) after this time, which results from the decay of potentiation (Zengel and Magleby, 1982), was essentially unaffected by the \( \text{Ca}^{2+} \) concentration or the other experimental manipulations. These results suggest that increasing \([\text{Ca}^{2+}]_0\) and exposure to CCCP, ionomycin, or CPA affect primarily the augmentation component of increased release.

This conclusion is further supported by the predicted decays of \( V(t) \) described by the continuous lines through the decay points in Fig. 8. The immediate drop in \( V(t) \) between the end of each conditioning train and the time of the first testing EPP 2 s after the train results primarily from the rapid decay of the two components of facilitation, which would have essentially decayed to 0 within that 2-s time period (Mallart and Martin, 1967; Zengel and Magleby, 1982). The remainder of the decay of \( V(t) \) results from the combined effects of the more slowly decaying augmentation and potentiation. Estimates of the decays of these two components were used to predict the decay of \( V(t) \) (continuous lines), assuming the multiplicative relationship described by Eq. 2 in Methods. This involved finding values of the magnitudes and time constants of decay of augmentation and potentiation that together would adequately describe the decay of \( V(t) \). These values are indicated in the figure legend.

We found that the increase in \( V(t) \) after the conditioning trains in the higher \( \text{Ca}^{2+} \) concentration or after exposure to CCCP, ionomycin or CPA could best be accounted for by assuming an increase in augmentation. For example, the greater increase in \( V(t) \) observed during the decay period when \( \text{Ca}^{2+} \) was increased from 0.6 to 0.8 mM was accounted for by assuming an increase from 0.6 to 1.1 in the magnitude of augmentation \( [A(T)] \) at the end of the conditioning stimulation. Similarly, the increase in \( V(t) \) after CCCP treatment was accounted for by assuming an increase in
Figure 8. Simulation-induced changes in EPP amplitude during and after long conditioning trains. The nerve was first conditioned with trains of 100-200 impulses applied at 20 impulses/s, then testing pulses were applied at 2-s intervals for three impulses and once every 10 s to follow the change in EPP amplitude back to control. The horizontal bars along the x axes indicate the time of the conditioning trains. The lines through the decay points plot the predicted decay of $V(t)$ obtained with the indicated values of the magnitudes, as well as time constants of decay of augmentation and potentiation (see text). (A) $V(t)$ during and after 100-impulse conditioning trains in 0.6 mM Ca (filled triangles; averaged from three experiments) and 0.8 mM Ca (open circles; four experiments). Values used to describe the decay of $V(t)$ after the train in 0.6 mM Ca: $P(T) = 0.35, \tau_p = 63$ s; $A(T) = 0.60, \tau_A = 5.5$ s. Values used to describe the decay in 0.8 mM Ca: $P(T) = 0.35, \tau_p = 63$ s; $A(T) = 1.10, \tau_A = 6.5$ s. (B) $V(t)$ during and after 200-impulse conditioning trains before (filled triangles) and after (open circles) the addition of 1,000 nM CCCP to the 0.8 mM Ca Ringer. Data obtained from a single preparation. Values used to describe the decay of $V(t)$ before CCCP exposure: $P(T) = 0.75, \tau_p = 50$ s, $A(T) = 0.20, \tau_A = 6.5$ s. Values used to describe the decay in the presence of CCCP: $P(T) = 0.75, \tau_p = 50$ s, $A(T) = 1.70, \tau_A = 6.5$ s. (C) $V(t)$ during and after 100-impulse conditioning trains before (filled triangles) and after (open circles) 1-1.5-min exposure to 10 mM ionomycin. Data averaged from six experimental preparations in low (0.7-1 mM) Ca Ringer. Values used to describe the decay of $V(t)$ before exposure to ionomycin: $P(T) = 0.43, \tau_p = 40$ s, $A(T) = 0.26, \tau_A = 10$ s. Values used to describe the decay after ionomycin exposure: $P(T) = 0.43, \tau_p = 40$ s, $A(T) = 0.46, \tau_A = 10$ s. (D) $V(t)$ during and after 100-impulse conditioning trains before (filled triangles) and after (open circles) addition of 10 mM CPA to the low (0.5-0.6 mM) Ca Ringer. Data averaged from two experimental preparations. Values used to describe the decay of $V(t)$ before exposure to CPA: $P(T) = 0.35, \tau_p = 70$ s, $A(T) = 0.14, \tau_A = 9$ s. Values used to describe the decay after CPA exposure: $P(T) = 0.35, \tau_p = 70$ s, $A(T) = 0.32, \tau_A = 9$ s. The increases in $V(t)$ after ionomycin and CPA treatment were accounted for by assuming increases in $A(T)$ from 0.26 to 0.46 and from 0.14 to 0.32, respectively. In no case could we describe the data by assuming an increase in potentiation.
We also obtained estimates of \( F(T) \), the magnitude of facilitation at the end of the conditioning trains, using Eq. 2 and the values of \( A(T) \) and \( P(T) \) that were found to fit the decay of \( V(t) \) after the trains. Although we found that increasing extracellular \( \text{Ca}^{2+} \) or exposure to CCCP, ionomycin or CPA resulted in small increases (3–31%) in the estimated magnitude of facilitation, these increases were considerably smaller than the nearly two- to eightfold increases observed in the magnitude of augmentation. Thus, we conclude that the greater stimulation-induced increase in EPP amplitude after these various experimental manipulations results primarily from an increase in augmentation.

**DISCUSSION**

The amount of \( \text{Ca}^{2+} \) in a nerve terminal is determined by a balance of several processes, including \( \text{Ca}^{2+} \) entry, intraterminal \( \text{Ca}^{2+} \) buffering and sequestering, and \( \text{Ca}^{2+} \) extrusion. In previous studies (Zengel, Sosa, and Poage, 1993; Zengel, Lee, Sosa, and Mosier, 1993), we found that altering \( \text{Ca}^{2+} \) entry through voltage-dependent calcium channels resulted in changes in the stimulation-induced increases in release that occur at the frog neuromuscular junction under conditions of reduced quantal content. These effects appeared to be mediated by effects on the facilitation and augmentation components of increased release. In the present study, we found that experimental manipulations known to alter resting levels of intracellular \( \text{Ca}^{2+} \) also affect stimulation-induced changes in EPP amplitude, particularly augmentation. The results of these studies suggest that \( \text{Ca}^{2+} \) plays a critical role in the generation of facilitation and augmentation. In none of these studies did we observe any effect on potentiation, consistent with the previous suggestion that this component of increased release may be mediated by a mechanism that does not involve \( \text{Ca}^{2+} \), for example, an accumulation of sodium ions in the nerve terminal (Birks and Cohen, 1968; Atwood, Swenarchuk, and Gruenwald, 1975; Nussinovitch and Rahamimoff, 1988).

While both facilitation and augmentation are dependent in some way on \( \text{Ca}^{2+} \), the augmentation component of increased release seems to be particularly sensitive to the resting \( \text{Ca}^{2+} \) level. It is interesting to note that the timecourse of decay of augmentation, as well as the effects of repetitive stimulation and time on the magnitude of augmentation (Magleby and Zengel, 1976b), are similar to the time course of decay, as well as the effects of repetitive stimulation and time, on changes in ionized \( \text{Ca}^{2+} \) levels in squid axons (Baker, Hodgkin, and Ridgway, 1971). Thus, it is tempting to speculate that perhaps augmentation reflects in some way the \( \text{Ca}^{2+} \) buffering capacity of the nerve terminal. Since both mitochondria (Alnaes and Rahamimoff, 1975) and smooth endoplasmic reticulum (SER) (see review by Blaustein, Ratzlaff, and Schweitzer, 1978) appear to be involved in nerve terminal \( \text{Ca}^{2+} \) buffering, it is tempting to speculate even further that augmentation reflects the \( \text{Ca}^{2+} \)-buffering activity of one or both of these organelles. However, although we have presented evidence that interference with \( \text{Ca}^{2+} \) buffering by mitochondria and the SER results in an increase in the magnitude of augmentation, direct evidence for a physiological role for these organelles in the generation of augmentation must await further studies. (Our observation that increases in intraterminal \( \text{Ca}^{2+} \) levels have little effect on facilitation seems to be contrary to previous reports that facilitation at the
frog neuromuscular junction is blocked after nerve terminal loading with the Ca\(^{2+}\) chelator BAPTA [Tanabe and Kijima, 1992]. However, Bain and Quastel [1992], although observing a reduction of facilitation at the mouse neuromuscular junction after BAPTA loading, reported that, as in the frog, changes in the extracellular Ca\(^{2+}\) concentration had little effect on facilitation. They suggested that facilitation perhaps depends on the presence of a minimal level of Ca\(^{2+}\), but is otherwise unaffected by changes in the level of Ca\(^{2+}\).)

Interestingly, although increasing extracellular Ca\(^{2+}\) and exposure to CCCP, ionomycin, and CPA all had the same effect on stimulation-induced changes in EPP amplitude, the effects of these experimental manipulations on control EPP amplitude were quite different. We observed a large decrease in control EPP amplitude after exposure of the nerve-muscle preparation to CCCP. Increasing the concentration of extracellular Ca\(^{2+}\), on the other hand, resulted in a large increase in control EPP amplitude. Exposure of the nerve-muscle preparation to ionomycin and CPA appeared to have no consistent effect on EPP amplitude.

The reduction in control EPP amplitude observed after CCCP treatment, also observed by Molgo and Pecot-Dechavassine (1988), is contrary to the increase in release that one would expect to see in the presence of increased intracellular Ca\(^{2+}\). The CCCP-induced decrease in EPP amplitude cannot be attributed to a depletion of available transmitter quanta or to a reduction in postsynaptic sensitivity to acetylcholine (ACh) because miniature end-plate potentials (MEPPs) can be recorded at the same time that evoked responses are completely blocked (personal observations; see also Molgo and Pecot-Dechavassine, 1988). Adams, Takeda, and Umbach (1985) observed a similar FCCP-induced reduction in evoked release at the squid giant synapse and suggested that perhaps at high enough concentrations, Ca\(^{2+}\) could in some way act to inhibit transmitter release. However, we were able to at least partially overcome the CCCP-induced decrease in EPP amplitude at the frog neuromuscular junction by increasing the concentration of Ca\(^{2+}\) in the bathing Ringer (data not shown). Since this should have resulted in an even greater increase in intraterminal Ca\(^{2+}\) levels, it does not appear that the decrease in EPP amplitude results as a direct consequence of an increase in Ca\(^{2+}\), but rather, from some other effect of CCCP, perhaps for example, a reduction in intracellular ATP levels. We doubt, however, that the effects of CCCP on stimulation-induced increases in release are caused by an effect of ATP reduction because increasing the extracellular Ca\(^{2+}\) concentration resulted in an even greater increase in V(t), consistent with our assumption that the effects of CCCP on stimulation-induced changes in EPP amplitude are mediated by an increase in resting intraterminal Ca\(^{2+}\) levels.

It has long been known that evoked transmitter release at the frog neuromuscular junction, as well as many other synapses, is a steep function of the extracellular Ca\(^{2+}\) concentration (e.g., Jenkinson, 1957; Dodge and Rahamimoff, 1967; see review by Silinsky, 1985). Because depolarization-evoked Ca\(^{2+}\) influx increases in a linear manner with increased extracellular Ca\(^{2+}\), it has been suggested that the nonlinearity in the relationship between extracellular Ca\(^{2+}\) and release must originate at a step (or steps) beyond the influx of Ca\(^{2+}\) into the nerve terminal (Augustine, Charlton, and Smith, 1985; Augustine and Charlton, 1986; Nachshen and Sanchez-Armass, 1987). In this study, we observed the expected steep relationship between extracellular Ca\(^{2+}\)
concentration and control EPP amplitude, but we found that the effect of extracellular Ca\(^{2+}\) on stimulation-induced increases in EPP amplitude was much smaller. For example, we saw < a 10-fold difference in the magnitude of \(V(450)\) at extracellular Ca\(^{2+}\) concentrations ranging from 0.3 to 1 mM (Fig. 3 B), compared to the more than 100-fold increase expected in control levels of release (Dodge and Rahamimoff, 1967).

The differences in sensitivity of evoked release and stimulation-induced changes in release to the various experimental manipulations used in this study are consistent with previous suggestions that these two aspects of transmitter release may involve different mechanisms (Zengel and Magleby, 1981, 1982; Bain and Quastel, 1992). In support of this, there is now good evidence that Ca\(^{2+}\) may play more than one role in the secretory process (e.g., Neher and Zucker, 1993). A multisite action of Ca\(^{2+}\) would explain previously reported differences in the sensitivities of evoked release and stimulation-induced changes in release to Ba\(^{2+}\) and Sr\(^{2+}\) (Zengel and Magleby, 1980) and to calcium channel blockers (Zengel, Lee, Sosa, and Mosier, 1993; Zengel, Sosa, and Poage, 1993).

The actual sites of Ca\(^{2+}\) action are still unknown. Ca\(^{2+}\) clearly appears to be involved in the rapid initiation of the exocytotic process. Because of the great speed of this process, it has been suggested that synaptic vesicles are docked to the plasma membrane by a macromolecular bridge that spans both the terminal and the vesicle membrane; within fractions of a millisecond after Ca\(^{2+}\) appears, this macromolecule is thought to form an aqueous channel connecting the inside of the vesicle with the outside of the cell, resulting in the release of the vesicle's contents into the synaptic cleft (Almers and Tse, 1990; Thomas and Almers, 1992). In addition to this role in the release process, Ca\(^{2+}\) may also play a role in other aspects of release, for example, in the mobilization of releasable pools of transmitter or the membrane retrieval process that recovers vesicular membrane previously fused with the terminal membrane (e.g., Neher and Zucker, 1993). Over the past several years, there have been remarkable advances in the identification and characterization of the proteins involved in the neurosecretory process (see review by Kelly, 1993). With continued advances in our understanding of the molecular components of the release process, we will hopefully soon better understand how the exocytotic machinery is activated and modified as a result of synaptic activity.

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