May 1998

Quantal Mechanisms Underlying Stimulation-induced Augmentation and Potentiation

Hong Cheng
East Tennessee State University

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QUANTAL MECHANISMS UNDERLYING STIMULATION-
INDUCED AUGMENTATION AND POTENTIATION

A Dissertation
Presented to
the Faculty of the Department of Pharmacology
James H. Quillen College of Medicine
East Tennessee State University

In Partial Fulfillment
of the Requirements for the Degree
Doctor of Philosophy in Biomedical Sciences

by

Hong Cheng

May 1998
APPROVAL

This is to certify that the Graduate Committee of

HONG CHENG

met on the

30th day of March, 1998.

The committee read and examined her dissertation, supervised her defense of it in an oral examination, and decided to recommend that her study be submitted to the Graduate Council, in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Biomedical Sciences.

Chair, Graduate Committee

Signed on behalf of the Graduate Council

Interim Dean, School of Graduate Studies

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ABSTRACT

QUANTAL MECHANISMS UNDERLYING STIMULATION-INDUCED AUGMENTATION AND POTENTIATION

by

Hong Cheng

Repetitive stimulation of motor nerves causes an increase in the number of packets of transmitter ("quanta") that can be released in the ensuing period. This represents a type of conditioning, in which synaptic transmission may be enhanced by prior activity. Despite many studies of this phenomenon, there have been no investigations of the quantal mechanisms underlying these events, due to the rapid changes in transmitter output and the short time periods involved.

To examine this problem, a method was developed in which estimates of the quantal release parameters could be obtained over very brief periods (3 s). Conventional microelectrode techniques were used to record miniature endplate potentials (MEPPs) from isolated frog (Rana pipiens) cutaneous pectoris muscles, before and after repetitive (40 sec at 80 Hz) nerve stimulation. Estimates were obtained of $m$ (number of quanta released), $n$ (number of functional release sites), $p$ (mean probability of release) and $\text{var}_p$ (spatial variance in $p$) using a method that employs counts of MEPPs per unit time. Fluctuations in the estimates were reduced using a moving bin technique (bin size = 3 s, $\Delta$bin = 1 s). Muscle contraction was prevented using low Ca$^{2+}$, high Mg$^{2+}$ Ringer or normal Ringer to which $\mu$-conotoxin GIIIA was added.

These studies showed that: (1) the post-stimulation increase in transmitter release was dependent on stimulation frequency and not on the total number of stimulus impulses. When the total number of pulses was kept constant, the high frequency pattern produced a higher level of transmitter release than did the lower frequency patterns; (2) augmentation and potentiation were present in both low Ca$^{2+}$, high Mg$^{2+}$ and normal Ringer solutions, but potentiation, $m$, $n$, $p$ and $\text{var}_p$ were greater in normal Ringer solution than in low Ca$^{2+}$, high Mg$^{2+}$ solution. In low Ca$^{2+}$, high Mg$^{2+}$ solution, there was a larger decrease in $n$ compared to $p$; (3) hypertonicity (addition of 100 mM sucrose) produced a marked increase in both basal and stimulation-induced values of $m$, $n$, and $p$. By contrast, there was a marked increase in the stimulation-induced but not the basal values of $\text{var}_p$; (4) hypertonicity produced a decrease in augmentation but had no effect on potentiation; (5) augmentation and potentiation appeared to involve mitochondrial uptake and efflux of cytoplasmic Ca$^{2+}$. Tetraphenylphosphonium (which blocks mitochondrial Ca$^{2+}$ efflux and uptake) decreased augmentation and potentiation in low Ca$^{2+}$, high Mg$^{2+}$ solutions but increased potentiation in the same solution made hypertonic with 100 mM sucrose; (6) the overall findings suggest that this new method may be useful for investigating the subcellular dynamics of transmitter release following nerve stimulation.
DEDICATION

This dissertation is dedicated to my parents, who have always encouraged and supported me to achieve goals in my life, and to my husband Nong Cheng and my son Zhi Alan Cheng, whose great love, sacrifice, continued encouragement, and support made this dream come true.
ACKNOWLEDGMENTS

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I am deeply indebted to the faculty, staff, and graduate students of the Dept. of Pharmacology for their encouragement, support, and friendship. Ms. Doris M. Davis' help and great friendship will be always remembered. I appreciate Ms. Betty Hughes, Lottie Winters, and Beverly Kondas for their secretarial assistance. Finally, I would like to thank my husband, Nong Cheng and my son Zhi Alan Cheng for their great love, endless support, and encouragement.
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ABBREVIATIONS

A augmentation

$[Ca^{2+}]_o$ extracellular calcium concentration

$[Ca^{2+}]_i$ intracellular calcium concentration

CaMKII $Ca^{2+}$ calmodulin-dependent protein kinase II

EPP endplate potential

F1 first facilitation

F2 second facilitation

$m$ number of quanta released per unit time

MEPP miniature endplate potential

$n$ number of functional release sites

$[Na^+]_i$ intracellular sodium concentration

$p$ mean probability of release for quanta at release sites

P potentiation

PTP post-tetanic potentiation

$\tau_{F2}$ decay constant of second facilitation

TPP tetraphenylphosphonium

$var_p$ spatial variance in $p$

Vc control MEPP frequency

Vm normalized MEPP frequency
<table>
<thead>
<tr>
<th>Symbol</th>
<th>Description</th>
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<tr>
<td>$V_t$</td>
<td>MEPP frequency at time $t$</td>
</tr>
<tr>
<td>$\Delta \psi_m$</td>
<td>mitochondrial membrane potential</td>
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CHAPTER 1
INTRODUCTION

Background and Significance

Quantal Transmitter Release

The basic steps involved in chemical synaptic transmission are now well established. When the presynaptic nerve terminal is depolarized by the invading action potential, voltage-sensitive calcium channels open, allowing an influx of $\text{Ca}^{2+}$ into the nerve terminal. The entering $\text{Ca}^{2+}$ triggers the release of neurotransmitter, which diffuses to the postsynaptic membrane and binds to receptors to generate the postsynaptic potential.

Transmitter is released from the nerve terminal in quantal packets, in which each packet represents the contents of one synaptic vesicle. With nerve stimulation, synaptic vesicles are mobilized to distinct release sites in the terminal, then discharge their contents into the synaptic cleft. Quantal packets of neurotransmitter are also released in the absence of nerve impulses, giving rise to the so-called spontaneously-occurring miniature endplate potentials (MEPPs) (reviewed by van der Kloot and Molgo 1994).

Although the nerve terminal is filled with a large number of small synaptic vesicles, only a fraction of them undergo exocytosis with the arrival of an action potential. There are two pools of these vesicles within the nerve terminal: (1) a releasable pool of vesicles, some of which fuse with the plasma membrane upon nerve stimulation, and (2) a reserve
pool of vesicles that are bound to the cytoskeleton and recruited to the releasable pool in response to requirements of the cell (Hirokawa and others 1989; Greengard and others 1993).

**Stimulus-Induced Increase in Transmitter Release**

Virtually all chemical synapses, including the neuromuscular junction, undergo short-term changes in synaptic efficacy as a result of conditioning (synaptic plasticity). When a presynaptic nerve is stimulated tetanically (many stimuli at high frequency), there is a long-lasting enhancement in the number of transmitter quanta subsequently released. At the motor endplate, this can be measured as an increase in the endplate potential (EPP) amplitude or in the frequency of miniature endplate potentials (MEPPs) (Rotshenker et al. 1976; Erulkar and Rahamimoff 1978).

Magleby and his colleagues (reviewed by Magleby 1987) have shown that the increase in transmitter release following tetanic stimulation can be separated into four kinetically distinct components (see Appendix A). These components build up during repetitive stimulation and decay thereafter with characteristic time courses. Semilogarithmic plots of the fractional increase in EPP amplitude (or fractional increase in MEPP frequency) as a function of time are traditionally used to identify each time constant of decay (measured as the time for the increased transmitter release to decay to 1/e (e is the base of the natural system of logarithms, having a numerical value of approximately 2.7183) or 37% of its initial magnitude).
The term "potentiation" (P) or "post-tetanic potentiation" (PTP) has been assigned to the process with the longest time constant (t = 50 s). Strictly speaking, this value is not constant but ranges from tens of seconds to minutes, depending on the number of stimuli in the conditioning tetanus. The term "augmentation" (A) has been given to the process with the next longest decay constant (t = 7 s), while "first facilitation" (F1) and "second facilitation" (F2) are terms assigned to the two remaining processes (time constants of about 50 ms and 500 ms, respectively). FI, F2, A, and P are all defined in terms of the fractional increase in EPP amplitude (or increase in the frequency of MEPPs) over control, after all other components have been set to zero (i.e., values of each component are obtained after subtracting out the contribution of all the others).

Further evidence that the four components are separate and independent is provided by studies showing marked differences in their ionic dependencies (Magleby and Zengel 1976a; Erulkar and Rahamimoff 1978; Zengel and Magleby 1981). For example, replacement of extracellular Ca\(^{2+}\) with Sr\(^{2+}\) leads to an increase in F2 magnitude and time constant of decay but causes little change in the other components. Moreover, Ba\(^{2+}\) produces an increase in the magnitude of A but has little effect on its time constant or on the other components (Zengel and Magleby 1980, 1981). Finally, potentiation can be diminished by 20 \(\mu\)M vesamicol (Maeno and Shibuya 1988) (see Table 1).
TABLE 1. THE FOUR COMPONENTS ARE SEPARATE AND INDEPENDENT

<table>
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<tr>
<th>Ionic Dependency</th>
<th>Decay Constant</th>
<th>Specific Agents</th>
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<tr>
<td>F1</td>
<td>$[\text{Ca}^{2+}]_0$</td>
<td>tens of ms (~50)</td>
</tr>
<tr>
<td>F2</td>
<td>$[\text{Ca}^{2+}]_0$</td>
<td>hundreds of ms (~500)</td>
</tr>
<tr>
<td>A</td>
<td>$[\text{Ca}^{2+}]_0$</td>
<td>seconds (~7)</td>
</tr>
<tr>
<td>P</td>
<td>$[\text{Ca}^{2+}]_0$ dependent and independent parts</td>
<td>tens of seconds to minutes</td>
</tr>
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Alterations in the Stimulus-Induced Increase in Transmitter Release

Several of the post-stimulation components of transmitter release appear to depend on influx of extracellular Ca$^{2+}$ into the terminal. For example, a threshold amount of Ca$^{2+}$ influx is required for facilitation (Rahamimoff and Yaari 1973), but any increases above this threshold amount do not proportionately increase the magnitude of F, i.e., F is about the same at all levels of quantal output. Loading preparations with an intracellular Ca$^{2+}$ chelator, such as BAPTA-AM or EGTA-AM, substantially diminishes facilitation (Molgo and van der Kloot 1991; Bain and Quastel 1992; van der Kloot and Molgo 1993).

Augmentation also appears to depend on influx of extracellular Ca$^{2+}$, because it is
abolished by the removal of Ca\(^{2+}\) (no added Ca\(^{2+}\) plus addition of 1 mM EGTA) (Erukalkar and Rahamimoff 1978; Nussinovitch and Rahamimoff 1988).

On the other hand, potentiation of nerve-evoked EPPs or MEPP frequency does not appear to depend on the presence of external Ca\(^{2+}\) (Erukalkar and Rahamimoff 1978; Lev-Tov and Rahamimoff 1980). This raises the possibility that another ion such as Na\(^{+}\) may be involved. Support for this idea comes from the finding that treatments expected to elevate intracellular Na\(^{+}\) produce an increase in potentiation (Rahamimoff and others 1980; Meiri and others 1981; Melinek and others 1982). Specifically, (1) the Na\(^{+}\) ionophore monensin greatly increases potentiation, despite the presence of the Ca\(^{2+}\) chelator EGTA and no added Ca\(^{2+}\) in the extracellular medium, and (2) potentiation in Ca\(^{2+}\)-free solution is increased when extracellular K\(^{+}\) is reduced or when acetylstrophanthidin is present (Lev-Tov and Rahamimoff 1980; Misler and others 1987). Both these treatments inhibit the Na\(^{+}\)-K\(^{+}\) pump and presumably increase [Na\(^{+}\)]\(_i\). This increase in [Na\(^{+}\)]\(_i\) may in turn cause the release of calcium from intracellular stores (mitochondria, endoplasmic reticulum, and Ca\(^{2+}\) binding protein) by a Na\(^{+}\)-Ca\(^{2+}\) exchange process. Thus, potentiation of quantal release in the absence of external Ca\(^{2+}\) may be due to Na\(^{+}\)-mediated Ca\(^{2+}\) release from intracellular stores.

However, potentiation also can be observed in the absence of external Na\(^{+}\), suggesting that entry of Na\(^{+}\) is also not essential for this effect. It may be that both [Na\(^{+}\)]\(_i\) and [Ca\(^{2+}\)]\(_i\) are involved, with the Ca\(^{2+}\)-independent effect due to Na\(^{+}\) and the Na\(^{+}\)-independent effect due to Ca\(^{2+}\). The relative importance of these two cations in the overall process remains
to be determined.

Concept of Residual Calcium and Its Function

One of the best explanations for stimulation-induced increases in transmitter release is that of residual Ca\(^{2+}\) (Katz and Miledi 1968). This hypothesis proposes that the Ca\(^{2+}\) entering with the first impulse is cleared relatively slowly from the terminal and leads to "residual calcium" within the terminal. As a result, Ca\(^{2+}\) entering with the second action potential adds to the Ca\(^{2+}\) still present to enhance quantal release.

Some results, however, appear to be incompatible with the residual Ca\(^{2+}\) hypothesis. Zucker and others (1991) have proposed that Ca\(^{2+}\) entering during stimulation acts at a site other than that directly triggering facilitation. They suggest that the residual Ca\(^{2+}\) hypothesis is unlikely to explain PTP because of the relatively slow decay of this component. On the other hand, it may be argued that influx of Ca\(^{2+}\) during the stimulus train would be expected to diffuse throughout the nerve terminal, so that, averaged over the entire volume, residual Ca\(^{2+}\) might in fact be associated with PTP. Furthermore, measurements of post-tetanic [Ca\(^{2+}\)]\(_i\) seem to correlate well with PTP (Delaney and others 1989), suggesting that this residual [Ca\(^{2+}\)]\(_i\) directly causes PTP or is in equilibrium with some bound Ca\(^{2+}\) which causes PTP. This explanation is supported by the fact that chelation of residual [Ca\(^{2+}\)]\(_i\) with the slow-acting EGTA has no effect on transmitter release to single spikes but blocks both the measured residual Ca\(^{2+}\) and potentiation (Swandulla and others 1991). By contrast, BAPTA (which has a Ca\(^{2+}\) binding affinity
similar to that of EGTA but binds Ca$^{2+}$ more rapidly) is capable of blocking transmitter release to single stimuli (Adler and others 1991). It appears therefore that residual Ca$^{2+}$ does play a role in PTP (and augmentation), but not by way of simply adding to the basal [Ca$^{2+}$]$_i$. Instead, residual Ca$^{2+}$ may be associated with mobilizing synaptic vesicles to transmitter release sites or with "sensitizing" the machinery to Ca$^{2+}$, so that more transmitter can be released in the period following the conditioning stimulus.

Possible Molecular Basis for Post-Tetanic Potentiation

Although neurotransmitter secretion and PTP are both triggered by a rise in [Ca$^{2+}$]$_i$, these two processes differ markedly in their kinetic properties. Neurotransmitter release is triggered by single action potentials, occurs within a millisecond after the arrival of the action potential, and is terminated within a few milliseconds after the end of the action potential. PTP, on the other hand, is evident only after a train of action potentials, builds up over several seconds, and decays over many seconds following the end of the train (Magleby and Zengel 1975). Accordingly, it appears that Ca$^{2+}$ may activate secretion and PTP by different processes because of the differences in the spatiotemporal distribution of these ions during the two responses. Theoretically, there must be separate cellular pathways to process the two types of increases in [Ca$^{2+}$]$_i$ into the signals needed for secretion and PTP. In particular, the Ca$^{2+}$ signal for secretion should be sensed by a receptor that has a low affinity for Ca$^{2+}$ (i.e., one that responds only to the high, localized rises in [Ca$^{2+}$]$_i$, that cause secretion), while the signal for PTP should be sensed by a
receptor that has a relatively high affinity for Ca\textsuperscript{2+}. Recent studies have suggested that synaptotagmin (an integral protein of the synaptic vesicle membrane) may be the mediator of neurotransmitter secretion (Perin and others 1990; Clark and others 1991; Sudhof and Jahn 1991; Brose and others 1992; Bommert and others 1993; DeBello and others 1993; Littleton and others 1993; Nonet and others 1993; Popov and Poo 1993).

Another protein found on synaptic vesicles and elsewhere within presynaptic terminals is the Ca\textsuperscript{2+}/calmodulin-dependent protein kinase type II (CaMKII) (Befenati and others 1992). This enzyme may have a dual function, i.e., to play a structural role as a binding protein for synapsin I, and to play a catalytic role in phosphorylating synapsin I and bringing about its dissociation from the vesicles. Synapsin I (a small synaptic vesicle-associated protein) is found with the reserve pool of synaptic vesicle clustered near transmitter release sites (Pleribone and others 1995). It is believed to regulate neurotransmitter release through phosphorylation-dependent interactions with synaptic vesicles and cytoskeletal elements (Greengard and others 1993). After synapsin I is phosphorylated by CaMKII, it apparently dissociates from synaptic vesicles and cytoskeleton, thereby making the synaptic vesicles available for exocytosis. The functional properties of CaMKII parallel those of the hypothetical Ca\textsuperscript{2+} receptor responsible for PTP (see Table 2). Furthermore, microinjection of this kinase into nerve terminals has been shown to potentiate neurotransmitter secretion (Llinas and others 1991). Accordingly, it is possible that CaMKII may mediate PTP. To further evaluate the involvement of CaMKII in PTP, some workers have injected a peptide inhibitor of CaMKII into the squid.
giant nerve terminal (Malinow and others 1989; Augustine and others 1994). This inhibitor selectively eliminated PTP without affecting the basal level of synaptic transmission. This inhibitory effect could be progressively reversed over a time span of 1 hour. Simultaneous measurements with Fura-2 (a Ca\(^{2+}\) detector) showed that the rise in [Ca\(^{2+}\)], produced by the stimulation that elicited PTP was not affected by injection of the peptide. Thus, the inhibitory effect was not due to alterations in Ca\(^{2+}\) entry or Ca\(^{2+}\) accumulation. Presumably, CaMKII is activated by a rise in [Ca\(^{2+}\)], which allows calmodulin to activate CaMKII and phosphorylate one or more of the many substrate proteins found within the presynaptic terminal. One particularly interesting substrate for CaMKII is synapsin I, a phosphorylation-sensitive link between synaptic vesicles and the presynaptic cytoskeleton (Baines and Bennett 1985; Bahler and Greengard 1987). There is some evidence that phosphorylation of synapsin by CaMKII is associated with enhancement of neurotransmitter release (Llinas and others 1991; Nichols and others 1992); e.g., (1) synapsin antibodies injected into the presynaptic terminal cause a depletion of the distal pool of synaptic vesicles and produce a decrease in transmitter release evoked by high-frequency stimulation (Pleribone and others 1995), and (2) paired-pulse facilitation is blunted in the CA1 hippocampal brain region of mice heterozygous for a targeted mutation of a subunit of CaMKII (Chapman and others 1995). On the other hand, PTP of the MEPP frequency is greatly enhanced in brain slices from heterozygotes and homozygotes, so further work is needed to conclusively determine the role of CaMKII in PTP.
TABLE 2. COMPARISON OF PROPERTIES OF THE Ca\textsuperscript{2+} RECEPTOR FOR PTP AND CAMKII

(after Augustine and others 1994)

<table>
<thead>
<tr>
<th>Ca Receptor for PTP</th>
<th>CaMKII</th>
</tr>
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<tbody>
<tr>
<td>High affinity for Ca\textsuperscript{2+} (μM sufficient)</td>
<td>High affinity for Ca\textsuperscript{2+} (via calmodulin)</td>
</tr>
<tr>
<td>Possible cooperative binding</td>
<td>Cooperative Ca\textsuperscript{2+} binding by calmodulin</td>
</tr>
<tr>
<td>Slow Ca\textsuperscript{2+} binding kinetics (ms to s)</td>
<td>Activates in ms to s time range</td>
</tr>
<tr>
<td>Not clustered at released sites</td>
<td>Found on vesicles and in cytoplasm</td>
</tr>
</tbody>
</table>

It is not entirely clear what controls the rate of decay of residual Ca\textsuperscript{2+} in the presynaptic terminal. Possible routes for removal of Ca\textsuperscript{2+} include (1) uptake and storage by intracellular organelles, (2) active transport across the plasma membrane, and (3) extrusion by the Na\textsuperscript{+}-Ca\textsuperscript{2+} exchanger. All of this these processes are likely to have different rate constants, and therefore any transmitter release mechanism which is
dependent on $[Ca^{2+}]_i$ will be dependent on these rate constants.

**Quantal Hypothesis of Transmitter Release**

The "quantum hypothesis" of del Castillo and Katz (1954) states that transmitter release ($m$) can be described as the product of two factors, the number of quanta available for release ($n$) and the average probability that a given quantum will be released ($p$), i.e., $m = np$. Rosenthal (1969) and Wilson and Skirboll (1974) claimed that potentiation was associated with an apparent increase in the parameter $p$. However, the validity of results such as these are in question, since the equations used to derive these estimates were apparently biased (Brown and others 1976).

A method developed by Miyamoto (1986) allows the calculation of unbiased estimates of $n$ and $p$ using direct counts of MEPPs. One unique feature of this method is the ability to obtain estimates of spatial variance in $p$ (var$_p$), which can be used to monitor $Ca^{2+}$ release from intracellular organelles (Provan and Miyamoto 1993, 1994). This is based on the assumption that release of $Ca^{2+}$ from intracellular organelles would be expected to produce a non-uniform increase in $[Ca^{2+}]_i$ at transmitter release sites (higher at proximal sites and lower at distal sites), which would be seen as an increase in $p$ and var$_p$. By contrast, influx of $Ca^{2+}$ through activated $Ca^{2+}$ channels would be expected to produce a uniform increase in $[Ca^{2+}]_i$ at transmitter release sites (Fogelson and Zucker 1985), due to the large number and even distribution of $Ca^{2+}$ channels there (Cohen and others 1991). This method has the additional advantage of using direct counts of quanta rather than
indirect estimates which rely on the variance in endplate potentials. The latter method has been seriously questioned because of inaccuracy due to noise and deficiencies in the underlying equations (Walmsley 1995).

Significance of the Project

The nervous system typically conveys information by a series of closely-spaced rather than isolated action potentials (Magleby, 1987). To understand the operation of this system, it is necessary to determine how synaptic efficacy changes as a function of the stimulation pattern. While there have been several studies describing these phenomena, there have been no investigations of the subcellular basis of these events. The proposed studies will attempt to elucidate these events by correlating the changes in augmentation and potentiation with the quantal release parameters $m$, $n$, $p$ and $\text{var}, p$. The importance of mitochondria in these phenomena will be examined by the use of specific inhibitors, such as tetraphenylphosphonium ions (TPP$^+$).

Changes in synaptic efficacy as a result of tetanic stimulation may be related to storage and manipulation of neuronal information on a short-term basis. Investigation of these changes is only one aspect of exploring the overall subcellular mechanisms involved in the release of neurotransmitter.
Aims of This Study

1. To devise a system for computing the quantal release parameters following tetanic nerve stimulation, when the baseline MEPP frequency is rapidly changing with time.

2. To look for associations between the different components of the stimulation-induced increase in transmitter release (augmentation and potentiation) and the quantal release parameters \( m \) (number of quanta released per unit of time), \( n \) (number of functional release sites), \( p \) (probability of release), and \( \text{vars} p \) (spatial variance in \( p \)).

3. To study the effect of different stimulation patterns on augmentation and potentiation and on the quantal release parameters. Early investigators of stimulation-induced transmitter release (Weinreich 1971; Erulkar and Rahamimoff 1978; Magleby and Zengel 1976b) employed a number of different stimulation protocols (varying between 10-100 Hz and 10-100 s), which made it difficult to compare results. Lev-Tov and Rahamimoff (1980) later did a more rigorous study in which they examined different stimulation paradigms. Despite their detailed investigation, Lev-Tov and Rahamimoff (1980) were not able to study the underlying quantal mechanisms because of the lack of a suitable method for quantal analysis. The present approach was intended to fill this gap.

4. To compare stimulation-induced transmitter release in low Ca\(^{2+}\), high Mg\(^{2+}\) Ringer solution with that in normal Ringer solution containing \( \mu \)-conotoxin. Investigators have traditionally studied stimulation-induced transmitter release under conditions that depress transmitter release (low Ca\(^{2+}\), high Mg\(^{2+}\)), because of the lack of a way of blocking muscle contraction in normal Ringer solution. Accordingly, the physiology involved in non-
depressed conditions was not known. More recently, it has been possible to block muscle
contractions with a 22-amino acid peptide, μ-conotoxin, from the snail Conus
geographus. The toxin specifically blocks the voltage-dependent Na⁺ channels of the
muscle fibers (Cruz and others 1985; Moczydlowski and others 1986). With this toxin it
is possible to carry out quantal analysis of stimulation-induced transmitter release under
normal physiologic conditions.

5. To examine the effect of hypertonicity on the quantal parameters of stimulation-
induced transmitter release. It has long been known that increases in tonicity of the Ringer
solution produce increases in MEPP frequency (Fatt and Katz 1952). The present studies
on the influence of moderate hypertonicity (319 mOsm) on tetanus-induced augmentation
and potentiation and its underlying quantal release parameters were undertaken because
these effects have not been reported.

6. To examine the influence of mitochondria on the quantal parameters of stimulation-
induced transmitter release. The explanation for facilitation, augmentation, and
potentiation appears to be residual Ca²⁺ within the nerve terminal. The source of this
Ca²⁺, however, remains unestablished. Possible mechanisms for removing or decreasing
[Ca²⁺], include (1) uptake and storage by intracellular organelles, primarily mitochondria
and endoplasmic reticulum (Liano and others 1994; Herrington and others 1996; Tang and
Zucker 1997). Mitochondria are abundant in nerve terminals, occupying about 6% of the
total volume, and there is evidence to suggest that they play a role in transmitter release.
It is well established that the mitochondrion is one location for Ca²⁺ storage and release.
TPP+ was used to block mitochondrial uptake and efflux of cytoplasmic Ca2+ to test whether these processes were involved in augmentation and potentiation.
CHAPTER 2
MATERIALS AND METHODS

Experimental Animals

All experiments employed small to medium 2-1/2" frogs (*Rana pipiens*) obtained from J.M. Hazen & Co., Alburg, VT. Small, frequency shipments were used to ensure a fresh supply of healthy animals. Upon arrival, they were washed and kept in stainless steel tanks. Chloramphenicol (0.25gm/L) was added to the tank water, and animals incubated in the antibiotic for at least three days at room temperature. Tanks and animals were then completely washed and kept in the cold room to decrease metabolic requirements. Tanks were washed at least twice a week to remove skin sheddings and excrement.

Neuromuscular Preparation

The cutaneous pectoris nerve-muscle preparation was used for all experiments. Animals were killed by decapitation followed by rapid double pithing (standard procedure for experiments in which anesthetics such as MS-222 cannot be used). This protocol was approved by the University Committee for Animal Care (UCAC). The cutaneous pectoris muscle with its attached nerve was dissected free, mounted in a Sylgard-lined Petri dish (about 1 ml), and continuously perfused with Ringer solution (see below) using a Holter roller pump (Extracorporeal Corp.) and a flow rate of 1.5-2 ml/min.
Solutions

The normal Ringer solution used during dissection contained (mM): NaCl 110, KCl 2.5, CaCl₂ 1.8, Tris 2.0, glucose 5.6. Tris was prepared as a 0.05 M stock solution, using 6.35 g/L of Tris-HCl and 1.18 g/L of Tris-base to give a pH of approximately 7.2 in solution. Sucrose (100 mM) was added to the control Ringer to make hypertonic Ringer solution. In the majority of experiments, low Ca²⁺ (0.3-0.7 mM), high Mg²⁺ (5-6 mM) solution was used to prevent muscle contraction by reducing the amount of transmitter released, although in some experiments 5 μM μ-conotoxin GIIM was used to study the effects under normal physiological conditions.

Stimulation and Recording

The nerve stump was drawn into a commercially-available fluid suction electrode (Transidyne General Corp.) and stimulated with 0.10 to 0.15-ms pulses delivered by a Grass S-88 dual channel stimulator with SIU5 isolation unit (Warwick, RI). The viability of the preparation was tested by first determining the threshold voltage needed to produce muscle contraction, then visually ensuring that the muscle twitched vigorously in response to supramaximal stimuli (3-5 times threshold, usually 10 V). Muscle contraction was then prevented by using either low Ca²⁺, high Mg²⁺ solution or normal solution to which μ-conotoxin was added.

Standard intracellular recording techniques (3 M KCl-filled microelectrodes with 5-20 MΩ resistance) were used to record MEPPs before and after different patterns of tetanic
stimulation. Junctions were selected for focal recording, MEPP rise times <2 ms, large MEPP size, and high and stable muscle resting potential. Selection was made for junctions located near the center of the longitudinal length of the muscle, along a small branch coming off the main nerve trunk. Each experiment consisted of continuous single cell recording during control situation, exposure to drug, and washout of the specific agent. The ability to maintain impalements was greatly enhanced by use of a Micro-G air suspension table (Technical Manufacturing Corp., Peabody, MA) and a Narashige hydraulic microdrive (Medical Systems Corp., Greenvale, NY), which allowed the impalement to be adjusted during the experiment. All experiments were carried out at ambient room temperature (22-24°C).

**Electrical Instrumentation**

Equipment was housed in a Radio Frequency Shielded Room (Keene Corp., Norwalk, CT). Glass microelectrodes for recording (5-15 megohms) were made from 1.0 mm O.D., 0.5 mm I.D. capillary tubing (W-P Instrument, Inc., New Haven, CT), formed with a Model P-87 horizontal micropipette puller (Sutter Instrument Co., Novato, CA). Bioelectric signals were fed into a Neuroprobe 1600 high impedance preamplifier (A-M Systems, Everett, WA) and then to one channel of a Tektronix oscilloscope (Beaverton, OR). Precise indications of time intervals before, during, and after tetanic stimulation were provided by a Tektronix time mark generator connected to the second channel of the oscilloscope. Both MEPP and time mark signals were recorded with a 200T PCM VCR.
modified video recorder (A.R. Vetter & Co., Rebersburg, PA) for off-line analysis. Data on magnetic tape then were fed into a high-frequency (1 MHz) Data Acquisition and Analysis System (R.C. Electronics, Santa Barbara, CA) and stored in digitized form on the hard drive of a Zenith 486 Dx computer.

Reference electrodes consisted of improvised 3M KCl "salt bridges." Wire leads were made from 26 gauge silver wire which had been "chlorided" by electroplating in 0.1 M HCl for 10 min. The leads were inserted into Pasteur pipettes and immobilized using agar (4g/100ml of 3M KCl) to surround all but the unchlorided part of the wire. The electrodes were then stored in 3M KCl under refrigeration.

Analysis of Data

Calculation of the Quantal Parameters \( m, n, p \) and \( \text{var}, p \)

The number of MEPPs in a prescribed time interval (10 msec) were counted, and 300 sequential counts used for each quantal estimate. These numbers provided the input for a computer program which calculated the quantal parameters according to previously described equations (Miyamoto 1986; Provan and Miyamoto 1993). The program was modified to use a "moving bin" technique (Lev-Tov and Rahamimoff 1980) to obtain data points over very short time intervals (bin size = 3s, \( \Delta \text{bin} = 1 \text{s} \); see Appendix C). Control MEPP frequencies were determined by averaging MEPP frequency over a duration of 4-5 min prior to the onset of train stimulation. In plots of MEPP frequency vs. time, data
points from the center of the time bins used were plotted. Thus, the first point measured occurred 1.5 seconds after the end of stimulation. Each protocol was repeated at least six times, and the data were normalized to allow the results to be pooled.

**Calculation of Augmentation and Potentiation**

The first component of facilitation, the second component of facilitation, augmentation, and potentiation are all defined in a similar manner: each is given by the fractional increase in a test MEPP frequency over control when the other processes equal zero.

To determine augmentation and potentiation, MEPP frequencies were plotted against time using semilogarithmic axes (Zengel and Magleby 1982; Zengel and Sosa 1994), i.e.,

\[ V_m(t) = \frac{V_t}{V_c} - 1 \]

where \( V_t \) was the MEPP frequency at time \( t \), \( V_c \) was the control MEPP frequency, and \( V_m \) was the fractional change in MEPP frequency at time \( t \) compared to the resting MEPP frequency \( V_c \). The time constant of potentiation was derived from a least-squares fit to the data points that encompassed the last linear decay phase (on the semilogarithmic plot) as MEPP frequencies returned to their pretetanus values. The slowest component of the MEPP frequency before it settled back to pretetanic levels was assumed to represent the time constant of potentiation. The next slowest component of decay of MEPP frequency was assumed to represent augmentation.
Inhibition of Muscle Contraction

Tetanic stimulation of the motor nerve required a means of preventing the resulting muscle contraction, which would otherwise dislodge or break the recording electrode. There were a number of ways of accomplishing this: (1) disrupting the transverse tubules by incubating the muscle for 1 hour in 400 mM glycerol or formamide Ringer solution, then washing with control Ringer; (2) preventing muscle contraction with tetrodotoxin (which blocks voltage-dependent Na channels of both nerve and muscle) and evoking transmitter release with focal microelectrodes near the nerve terminal; (3) preventing muscle contraction with μ-conotoxin (which specifically blocks voltage-dependent Na channels of muscle); and (4) reducing the amount of transmitter released with low Ca\(^{2+}\) and/or high Mg\(^{2+}\), such that the postsynaptic endplate potential was below threshold for generating a muscle action potential.

After trying each of these methods, it was decided to use low Ca\(^{2+}\), high Mg\(^{2+}\) solution to immobilize the muscle, since it was more consistent, caused little damage to the preparation, and was relatively inexpensive.

Drugs and Chemicals

The specific blocker of Na channels in muscle, μ-conotoxin GIIIA (peptide content = 72% ± 3%, purity > 93%), was purchased from RBI (Natick, MA). Vials containing the peptide were warmed to room temperature in a desiccator prior to being opened. The peptide was initially dissolved in distilled water (35 μg pure peptide/13 ml), then further
diluted with Ringer solution and applied to preparations to give a final concentration of 5 μM. Peptide solutions were prepared immediately before use to avoid loss of activity.

Tetraphenylphosphonium bromide (TPP*) was obtained from Aldrich (Milwaukee WI).

All other chemicals used were of analytical grade or better.
CHAPTER 3

RESULTS

Determination of the Quantal Components Underlying Augmentation and Potentiation

To determine the quantal parameters underlying the post-stimulation phenomena, MEPP frequencies were recorded before and after tetanic nerve stimulation. As indicated in "Materials and Methods," the number of MEPPs in a 10 ms time interval were counted, and 300 sequential counts used for each quantal estimate. These values provided the input for a BASIC computer program (Miyamoto 1986; Provan and Miyamoto 1993), which was modified to use a "moving bin" technique (Lev-Tov and Rahamimoff 1980) to obtain data points over very short time intervals (bin size = 3 s, Δbin = 1 s). Using the midpoint of each bin to represent the time of each quantal estimate meant that the first estimate would occur at 1.5 s after the end of stimulation (10 ms per bin times 300 bins equals 3 s, and half of 3 s equals 1.5 s). Thus, it was not possible to capture the first two components (F1 and F2) in these measurements. To obtain estimates of augmentation and potentiation, the normalized MEPP frequency was plotted on semilogarithmic graphs.

In these experiments, low Ca\(^{2+}\), high Mg\(^{2+}\) solution was used to immobilize the muscle. Data were recorded before and after a stimulus train of 40 s at 80 Hz (using a pulse duration of 0.15 ms). The results from six experiments obtained from four preparations are shown in Fig. 1. Fig. 1a shows the changes in MEPP frequency following tetanic
stimulation, while Fig. 1b shows the two-phase decay in MEPP frequency at the end of stimulation. In Fig. 1b, MEPP frequency is expressed as $V_m(t)$, the fractional increase in MEPP frequency over the prestimulation control frequency, such that $V_m(t) = V_t/V_c - 1$ (where $V_t$ is MEPP frequency at time $t$ and $V_c$ is control MEPP frequency). $V_m$ is plotted as a function of time after the end of stimulation. The slowest component of the decay in MEPP frequency is assumed to represent potentiation, and the next slowest component is assumed to represent augmentation (Lev-Tov and Rahamimoff 1980; Zengel and Sosa 1994). Figures 1c, d and e show the effect of tetanic stimulation on the quantal release parameters $n$, $p$, and $\text{var}_p$. There are two-phase decays for $n$, and $p$. For $\text{var}_p$ there is an initial brief increase at the end of stimulation, a drop below baseline, and finally a return to baseline.

**Effect of Stimulus Frequency on Transmitter Release**

To study the effect of stimulus frequency on transmitter release, different stimulus frequencies and a constant train duration were used. For these experiments, three sets of MEPPs were continuously recorded from the same endplate before, during and after tetanic stimulation. As mentioned, muscles were incubated in 0.3 mM Ca$^{2+}$, 6 mM Mg$^{2+}$ Ringer solution, and the motor nerve was stimulated at either 80, 40, or 20 Hz for a fixed duration of 40 s. Stimulus trains were separated from each other by at least a half hour to allow the muscle to fully recover. The results collected from six junctions in five muscles are shown in Figs. 3a, b, c, d. There were obvious increases in $m$, $n$, $p$ and $\text{var}_p$. 

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following stimulation at 80 Hz. By contrast, there were only small increases in these parameters after stimulation at 40 Hz, and finally there were no apparent changes following stimulation at 20 Hz.

**Effect of Number of Stimuli on Transmitter Release**

Another study was undertaken to examine whether the above results were due to stimulus frequency *per se* or to the total number of impulses. As before, three sets of MEPPs were recorded from the same junction before, during, and after tetanic stimulation, and each stimulus train was separated from the other by at least one-half hour. Muscles were incubated in 0.3 mM Ca\(^{2+}\), 6 mM Mg\(^{2+}\) Ringer solution. Stimulus frequency was varied from 80 to 40 to 20 Hz, but the total number of pulses in the train was kept constant at 3200 pulses. Thus, stimulation at 80 Hz was provided for 40 s, that at 40 Hz was maintained for 80 s, and that at 20 Hz was continued for 160 s. Data were collected from six junctions from six different muscles. In half of these trials, the order of stimulation was 80, 40 Hz, and 20 Hz, while in the remaining half, the order was reversed. The results are shown in Figs. 2a, b, c, d. There were obvious increases in \(m, n, p\) and \(\text{var}_p\) following stimulation at 80 Hz for 40 s. Again, there were only small increases in MEPP frequency, \(m, n, p\) and \(\text{var}_p\) after stimulation at 40 Hz for 80 s, and there were no apparent changes following stimulation at 20 Hz for 160 s. From these results, it was concluded that the stimulation paradigm of 80 Hz for 40 s was appropriate for evoking measurable responses without unnecessary fatiguing of the preparation.
Comparison of Stimulation-Induced Transmitter Release in Low Ca\(^{2+}\).

High Mg\(^{2+}\) Solution vs. That in Normal Ringer Solution with \(\mu\)-Conotoxin

To examine the effect of low Ca\(^{2+}\), high Mg\(^{2+}\) on the phenomena under investigation, it was necessary to collect data from the same junction, in the presence of low Ca\(^{2+}\), high Mg\(^{2+}\) and in its absence. MEPP frequencies were recorded before and after tetanic stimulation, first in low Ca\(^{2+}\), high Mg\(^{2+}\) Ringer solution, then after switching to normal Ringer solution containing \(\mu\)-conotoxin (5 \(\mu\)M). This order of solutions was used in three single junction experiments, and the reverse order was used in three additional trials. Each train of stimulation (80 Hz for 40 s) was separated from the others by at least 30 min. In each case, a control run was performed to ensure that the responses were reproducible after the 30 min wait. The results obtained in low Ca\(^{2+}\), high Mg\(^{2+}\) solution from six different muscles are shown in Figs. 4 and 5. The results reveal no difference between the two identical sets of stimulations separated by the 30 min interval. In both low Ca\(^{2+}\), high Mg\(^{2+}\) and normal Ringer solutions, there are the previously mentioned two-phase decays. However, the magnitude of potentiation is greater for results obtained in normal Ringer solution. Similarly, the values for \(m\), \(n\), \(p\) and \(\text{var},p\) obtained in normal Ringer are greater than those obtained in low Ca\(^{2+}\), high Mg\(^{2+}\) solution. One striking feature is that values for \(n\) are much higher in normal Ringer than the same values obtained in low Ca\(^{2+}\), high Mg\(^{2+}\) (Fig. 4b). This implies that low Ca\(^{2+}\), high Mg\(^{2+}\) decreases the number of functional release sites much more than the mean probability of release, even though the curves...
appear to have the same shape. The only phenomenon that was not affected by the
difference in solutions was augmentation, as there appeared to be no significant difference
in low Ca²⁺, high Mg²⁺ vs. normal Ringer solution (Fig. 5).

Effect of Hypertonicity on Stimulation-Induced Transmitter Release.

Augmentation and Potentiation

To examine the effect of hypertonicity on the phenomena under investigation,
preparations were incubated first in low Ca²⁺, high Mg²⁺ Ringer solution with normal
tonicity (211 mOsm), then perfused Ringer solution made hypertonic by adding 100 mM
sucrose (319 mOsm). Muscles were allowed to equilibrate in the hypertonic solution for
20 min before recording. Preparations were then washed with isotonic low Ca²⁺, high
Mg²⁺ Ringer solution. The electrode was kept in the same junction throughout these three
changes in Ringer solution, in which the motor nerves were stimulated at 80 Hz for 40 s in
each solution. The results from six single junction experiments are shown in Fig. 6. Fig.
6a indicates that hypertonicity produced a marked increase in the stimulation-induced
MEPP frequencies over control. Fig. 7 illustrates that hypertonicity produced a marked
decrease in the amount of augmentation but had no effect on potentiation. The time
course of the post-stimulus MEPP frequency returned to the control value within 20 min
after washing with isotonic control Ringer solution.
Quantal Analysis of the Changes in Stimulation-Induced Transmitter Release Produced by Hypertonicity

Compared to the control groups, hypertonicity caused an increase in both the basal values and the tetanus-induced values of $m$, $n$, and $p$ (Figs. 6b, c and d). Of particular interest is that the post-stimulus decays in $m$, $n$ and $p$ are gradual and monotonic. The shapes of the decay curves are not like those observed in isotonic solutions, where there are rapid decays in $m$, $n$, and $p$ in the first 10 s and then a slow decay to the baseline (two-phase decay). As indicated in Fig. 6e, there is a marked increase in var, $p$ following tetanic stimulation in hypertonic solutions, but with time, values return to baseline just slightly higher than the level in isotonic solutions. Although there is a great deal of variation in var, $p$, examination of the mean values during the first 10 s following stimulation reveal the same brief increase, decrease, and subsequent increase that is seen in isotonic conditions (Fig. 1e). It can be seen that the post-stimulus increases in $m$, $n$, $p$ and var, $p$ return to the control values within 20 min after washing with isotonic control Ringer solution.

Mitochondria and Stimulation-Induced Transmitter Release in Isotonic Solution

To examine the role of mitochondria in stimulation-induced transmitter release, Ca$^{2+}$ regulation by mitochondria was blocked by tetrabenylphosphonium (TPP$^-\)$. TPP$^-$ is a lipophilic cation known to block Na$^+$-dependent (K1 = 0.2 μM) and Na$^+$-independent (K1 = 10 μM) Ca$^{2+}$ efflux in mitochondria, with little effect on ATP production. TPP$^-$ is likely to block Ca$^{2+}$ uptake as well (Gunter and Pfeiffer 1990; Gunter and Gunter 1994; Gunter
and others 1994). The effect of this inhibitor on augmentation, potentiation and on the corresponding quantal parameters $m, n, p, \text{var, } p$ in isotonic conditions was examined.

The experimental protocol was the same as before, using low Ca$^{2+}$, high Mg$^{2+}$ Ringer solution for the control group. TPP$^-$ (3 μM) was added to the experimental group 10 min before recording. Recordings were made from the same junction throughout each experiment, and the combined results are shown in Fig. 8.

In low Ca$^{2+}$, high Mg$^{2+}$ solution, 3 μM TPP$^-$ decreased the amplitude of augmentation and potentiation and caused potentiation to decay faster than the control group (Fig. 8a), even though the curves appeared to have the same shape. TPP$^-$ also decreased $m, n, p$ and var,$p$ (Figs. 8b, c, d and e), despite the fact that the curves still appeared to display the two-phase decay.

Mitochondria and Stimulation-Induced Transmitter Release in Hypertonic Solution

Preparations were incubated first in low Ca$^{2+}$, high Mg$^{2+}$ Ringer solution with normal tonicity, then bathed with hypertonic Ringer solution containing 100 mM sucrose. Muscles were allowed to equilibrate in the hypertonic solution for 20 min before recording and in the TPP$^-$-containing hypertonic solution for 10 min before recording. Fig. 9 shows the data obtained from six preparations. In the hypertonic solution with TPP$^-$ (3 μM), there was a substantial decrease in the amount of augmentation, similar to that observed in isotonic solutions. Although the potentiation component was still present, its decay was slower than that in the control group. Compared to the control group, hypertonicity plus
TPP\textsuperscript{*} caused an increase in both the basal values and the tetanus-induced values of $m$, $n$, $p$ and var$_p$ (Figs. 9b, c, d and e). It can be seen that the post-stimulus decays in $m$, $n$ and $p$ are gradual and monotonic, appearing to consist of a one-phase decay, not like that seen in isotonic solution. There was a marked increase in var$_p$ in hypertonic solutions, with values returning to baseline higher than the levels in isotonic solutions. In hypertonic solution, TPP\textsuperscript{*} increased potentiation (Fig. 10), $m$, $n$, and var$_p$ but had no obvious effect on $p$ (Fig. 10d).
Figure 1. The change of transmitter release following tetanic stimulation. Data were collected from six junctions obtained from four muscles. The 80 Hz, 40 sec tetanus was administered in Ca$^{2+}$ 0.3, Mg$^{2+}$ 6 mM Ringer solution. Each point and bar represents the mean ± SEM (n = 6). Moving bin = 3 sec, Δ bin = 1 sec.

Figure 1a: Decay of MEPP frequency after stimulation.
Figure 1. b: Two-phase exponential decay of augmentation and potentiation ($r^2 = 0.9897$). Decay of the fraction increase in MEPP frequency, $V_m$, after the conditioning train in Fig. 1a was plotted semilogarithmically against time. $V_m = V_t/V_c - 1$, where $V_t$ is the MEPP frequency at time $t$ and $V_c$ is the control MEPP frequency. The slowest component of MEPP frequency before it settled back to pretetanic level was assumed to represent potentiation. The next slowest component of decay of MEPP frequency was assumed to represent augmentation. The equation for generating line of augmentation is $A = 4.84\exp^{[-0.045t]}$. The equation for generating line of potentiation is $P = 37.34\exp^{[-0.499t]}$. 

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Figure 1. c: Two-phase decay of $n$ underlying augmentation and potentiation. Data derived from the same data in Fig. 1a. Use $n_t/n_c - 1$ to normalize $n$. $n_t$ is $n$ value at time $t$ after stimulation; while $n_c$ is the value at time before stimulation.
Figure 1. d: Two-phase decay of p underlying augmentation and potentiation. Data derived from the same data in Fig. 1a. Use $p_t/p_c^{-1}$ to normalize p. $P_t$ is p value at time t after stimulation; while $p_c$ is the p value at time before stimulation.
Figure 1. e: The changes in var,p following tetanus-stimulation. Data derived from the same data in Fig. 1a.
Figure 2. The effects of stimulation paradigm on quantal release parameters. Three sets of MEPPs were continuously recorded from the same endplate. Total stimulation number of pulses was kept constant (3200 pulses). Stimulus trains were separated from each other by 30 min. Data were collected from 6 junctions of six muscles in Ca$^{2+}$ 0.3, Mg$^{2+}$ 0.6 mM Ringer solution. Each point and bar represents the mean ± SEM. Moving bin = 3 sec, Δ bin = 1 sec. The same data generated $m$ (2 a), $n$ (2 b), $p$ (2c) and $var, p$ (2 d).

Figure 2a: The effects of stimulation paradigm on $m$. 

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Figure 2. b: The effects of stimulation paradigm on $n$. 

- ▲ 80Hz 40s
- • 40Hz 80s
- ○ 20Hz 160s
Figure 2. c: The effects of stimulation paradigm on $p$. 

- 80 Hz 40s
- 40 Hz 80s
- 20 Hz 160s
Figure 2. d: The effects of stimulation paradigm on var_\rho.

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Figure 3. The effects of stimulation frequency on quantal release parameters. Three sets of MEPPs were continuously recorded from the same endplate. Stimulus trains were separated from each other by 30 min. Data were collected from 6 junctions of five muscles in Ca$^{2+}$ 0.3, Mg$^{2+}$ 0.6 mM Ringer solution. Each point and bar represents the mean ± SEM. Moving bin = 3 sec, Δ bin = 1 sec. The same data generated $m$ (3a), $n$ (3b), $p$ (3c) and $var_p$ (3d).

Figure 3a: The effects of stimulation frequency on $m$. 

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Figure 3. b: The effects of stimulation frequency on $n$. 

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Figure 3. c: The effects of stimulation frequency on $p$. 

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Figure 3. d: The effects of stimulation frequency on $\text{var}_p$. 

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Figure 4. Comparison of stimulation-induced quantal release parameters in low Ca$^{2+}$, high Mg$^{2+}$ vs. that in normal Ringer solution with μ-conotoxin. Two sets of MEPPs were continuously recorded from the same endplate, first in Ca$^{2+}$ 0.3, Mg$^{2+}$ 6 mM Ringer solution, then in normal Ringer solution containing μ-conotoxin GIIIA 5 μM in three single junctions. The reverse order was used in three additional trials. Stimulus trains (80 Hz, 40s) were separated from each other by 30 min. The lower one was control group with two stimulations (80 Hz, 40s) separated by 30 min in low Ca$^{2+}$ high Mg$^{2+}$ solution. Each point and bar represents the mean ± SEM. Moving bin = 3 sec, Δ bin = 1 sec. The same data generated m (4 a), n (4 b), ρ (4 c) and var, p (4 d).

Figure 4a: Comparison of stimulation-induced m in low Ca$^{2+}$, high Mg$^{2+}$ vs. that in normal Ringer solution with μ-conotoxin.

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Figure 4. b: Comparison of stimulation-induced $n$ in low $\text{Ca}^{2+}$, high $\text{Mg}^{2+}$ vs. that in normal Ringer solution with $\mu$-conotoxin.
Figure 4. c: Comparison of stimulation-induced $p$ in low Ca$^{2+}$, high Mg$^{2+}$ vs. that in normal Ringer solution with $\mu$-conotoxin.
Figure 4. d: Comparison of stimulation-induced \( \text{var}_p \) in low \( \text{Ca}^{2+} \), high \( \text{Mg}^{2+} \) vs. that in normal Ringer solution with \( \mu \)-conotoxin.
Figure 5. Comparison of augmentation and potentiation in low Ca\(^{2+}\), high Mg\(^{2+}\) vs. that in normal Ringer solution with \(\mu\)-conotoxin. Data derived from the same data in fig. 4a. Equation for generating curve in low Ca\(^{2+}\), high Mg\(^{2+}\) is
\[
V_m = 4.844 \exp^{-0.04584t} + 37.35 \exp^{-0.4994t}, \quad r^2 = 0.9897; \quad \text{Equation for generating curve in normal Ringer solution is} \quad V_m = 7.091 \exp^{-0.03337t} + 31.58 \exp^{-0.4427t}, \quad r^2 = 0.9907.
\]
* Potentiation magnitude in normal Ringer solution shows significant difference from that in control group.
Figure 6. The effect of hypertonicity on stimulation-induced transmitter release. Three sets of MEPPs were continuously recorded from the same endplate. Stimulus trains were separated from each other by 30 min. Stimulation parameters were 80 Hz, 40 sec. Data were collected from 6 junctions of six muscles. The control group was in Ca\(^{2+}\) 0.3, Mg\(^{2+}\) 6 mM Ringer solution with normal tonicity (211 mosM). The experimental group was in Ca\(^{2+}\) 0.3, Mg\(^{2+}\) 6 mM Ringer solution with adding sucrose 100 mM (319 mosM). The muscles were allowed to equilibrate in hypertonic solution for 20 min before recording. Then the muscles were washed with Ca\(^{2+}\) 0.3, Mg\(^{2+}\) 6 mM isotonic Ringer solution. Each point and bar represents mean ± SEM (n = 6). Moving bin = 3 sec, Δ bin = 1 sec. The same data generated m (6. b), n (6. c), p (6. d) and var_, p (6. e).

Figure 6a: The effect of hypertonicity on stimulation-induced MEPP frequency.
Figure 6. b: The effect of hypertonicity on stimulation-induced $m$;
Figure 6. c: The effect of hypertonicity on stimulation-induced $n$. 

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Figure 6. d: The effect of hypertonicity on stimulation-induced $p$.
Figure 6. e: The effect of hypertonicity on stimulation-induced $\text{var}_p$. 

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Figure 7. The effect of high osmotic pressure on augmentation and potentiation. Data derived from the same data in fig. 6.

Figure 7a: Vm was plotted normal scale as a function of time after stimulation. Figure 7b: Vm was plotted semilogarithmically as a function of time after stimulation.
Figure 8. The effect of TPP⁺ on stimulation-induced transmitter release. Two sets of MEPPs were continuously recorded from the same endplate, first in Ca²⁺ 0.3, Mg²⁺ 6 mM Ringer solution, then after switching to Ca²⁺ 0.3, Mg²⁺ 6 mM Ringer solution containing TPP⁺ 3μM. Stimulation parameters: 80 Hz, 40 sec. Stimulus trains were separated from each other by 30 min. Data were from 6 junctions of six muscles. Each point represents the mean value. Moving bin = 3 sec, Δbin = 1 sec. The same data generated m (8 b), n (8 c), p (8 d), and var.p (8 e).

Figure 8a: The effect of TPP⁺ on augmentation and potentiation.
Equation for generating curve in TPP⁺ is \( V_m = 1.928 \exp^{[0.05679t]} + 20.99 \exp^{[-0.7773t]} \), \( r^2 = 0.9970 \); Equation for generating curve in control is \( V_m = 3.295 \exp^{[0.04106t]} + 33 \exp^{[-0.5647t]} \), \( r^2 = 0.9987 \).

**Both augmentation and potentiation magnitude of control group show significant difference from that in TPP⁺ group.**

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Figure 8. b: Effect of TPP$^+$ on stimulation-induced $m$.

Figure 8. c: Effect of TPP$^+$ on stimulation-induced $n$.
Figure 8. d: Effect of TPP⁺ on stimulation-induced $p$;
Figure 8. e: Effect of TPP⁺ on stimulation-induced $\text{var}_s p$. 

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Figure 9. The effect of TPP⁺ and hypertonic solution on stimulation-induced transmitter release. The control group was in Ca²⁺ 0.3, Mg²⁺ 6 mM isotonic solution (211 mOsm). Experimental group was in hypertonic solution produced by adding 100 mM sucrose to give 319 mOsm. TPP⁺ 3 μM was added to hypertonic solution 10 min before recording. Stimulation parameters: 80 Hz, 40 sec. Each point represents mean value (n = 6). The same data generated m (9 b); n (9. c); p (9. d) and var. p (9. e).

Figure 9a: The effect of TPP⁺ and hypertonic solution on augmentation and potentiation.
Figure 9. b: The effect of TPP⁺ and hypertonic solution on m.

Figure 9. c: The effect of TPP⁺ and hypertonic solution on n.

Each point and bar represents mean ± SEM (n = 6).
Figure 9. d: The effect of TPP⁺ and hypertonic solution on ρ.
Figure 9. e: The effect of TPP⁺ and hypertonic solution on var.ρ.
Each point and bar represents mean ± SEM (n = 6).
Figure 10. The effect of TPP⁺ on stimulation-induced transmitter release in hypertonic solution. Data are taken from Fig. 7 and Fig. 9a. Each point represents mean value (n = 6). Stimulation parameters: 80 Hz, 40 sec.

Figure 10a: The effect of TPP⁻ on potentiation in hypertonic solution.
Figure 10. b: The effect of TPP+ on stimulation-induced $m$ in hypertonic solution. Figure 10. c: The effect of TPP+ on stimulation-induced $n$ in hypertonic solution. Data are taken from Fig. 6b, 6c and Fig. 9b, 9c. Each point and bar represents mean ± SEM (n = 6). Stimulation parameters: 80 Hz, 40 sec.
Figure 10. d: The effect of TPP⁺ on stimulation-induced $p$ in hypertonic solution. Figure 10. e: The effect of TPP⁺ on stimulation-induced var.$p$ in hypertonic solution. Data are taken from Fig. 6d, 6e, and Fig. 9d, 9e. Each point and bar represents mean ± SEM (n = 6). Stimulation parameters: 80 Hz, 40 sec.
CHAPTER 4
DISCUSSION

Determination of the Quantal Components Underlying Augmentation
and Potentiation of Transmitter Release

Although there have been many investigations of the increase and decline in transmitter release arising from tetanic stimulation, there have been no studies of the quantal nature of this phenomenon. The problem stems from the very short time periods involved and the rapid changes in quantal output during this time. A novel approach was devised to undertake this study. The method of Provan and Miyamoto (1993), which requires only the measurement of MEPPs to obtain unbiased quantal estimates, was used in conjunction with the moving bin approach (Lev-Tov and Rahamimoff 1980) to obtain reliable estimates during the post-stimulation period. As indicated by the results from this study, it was possible to observe changes in $m$, $n$, $p$, and $\text{var}_p$ in association with augmentation and potentiation, something never before possible. Unfortunately, the very brief (about 1 s) time courses for first and second facilitation (F1, F2) precluded the study of these phenomena at this time.

The results of this study showed increases in $m$, $n$, and $p$, and two-phase decays for these parameters in the post-stimulation period during which augmentation and potentiation occurred (Figs. 1b, c, and d). The increase in $m$ was due to increases in both $n$ and $p$ (according to the working hypothesis, $p$ reflects the $[\text{Ca}^{2+}]_i$ at the active zones,
and \( n \) represents the number of functional transmitter release sites). At least two separate processes are assumed to play a role in the increase in \( m \), i.e., influx of extracellular calcium and translocation of intracellular calcium. The first process is due to the increase in calcium permeability following depolarization by the action potential (Katz and Miledi 1967a, b, 1969). Influx of \( \text{Ca}^{2+} \) immediately adjacent to transmitter release sites presumably causes an increase in \( p \). The second process probably involves release of \( \text{Ca}^{2+} \) from intracellular organelles. In this regard, Erulkar and Rahamimoff (1978) and Lev-Tov and Rahamimoff (1980) reported an increase in "tetanic potentiation" during conditions of reversed \( \text{Ca}^{2+} \) gradient (zero extracellular \( \text{Ca}^{2+} \)). Since no net influx of \( \text{Ca}^{2+} \) should occur under a reversed gradient, they postulated that the increase in transmitter release was due to \( \text{Ca}^{2+} \) from intracellular sources. One explanation was that \( \text{Na}^+ \) was the link between repetitive stimulation and release of \( \text{Ca}^{2+} \) from mitochondria (via a \( \text{Na}^+-\text{Ca}^{2+} \) exchange mechanism) (Alnaes and Rahamimoff 1975). If so, the ensuing rise in \([\text{Ca}^{2+}]_i\), would activate the cascade of events leading to phosphorylation of synapsin I and dissociation of synaptic vesicles from the cytoskeleton. Accordingly, \( \text{Ca}^{2+} \) from either the extracellular fluid or intracellular organelles could lead to an increase in the number of functional (i.e., occupied by vesicles) release sites \( n \) by making more vesicles available for exocytosis. The result is that increases in \( \text{Ca}^{2+} \) would be expected to lead to increases in \( p \) and \( n \) (and thus in \( m \), since \( m = np \)), which is what was observed in the present study.

With regard to \( \text{var}, p \), there was an initial brief increase at the end of stimulation, a drop below baseline, then a return to baseline (Fig. 1e). Because \( \text{var}, p \) may represent \( \text{Ca}^{2+} \)
release from intracellular organelles (Provan and Miyamoto 1993, 1994, 1995), these findings suggest there may be a brief increase in Ca\(^{2+}\) release from intracellular organelles followed by a decrease, and finally a return to baseline. This is consistent with the idea that PTP is associated with Ca\(^{2+}\) sequestration by intracellular organelles during tetanic stimulation and with Ca\(^{2+}\) efflux into the cytoplasm following termination of stimulation (Tang and Zucker 1997). The results discussed thus far imply that augmentation and potentiation are due to increases in both the number of functional release sites and the probability of release. The findings with \(\text{var}_p\) suggest that Ca\(^{2+}\) released from intracellular organelles may also contribute to these phenomena.

**Effect of Stimulation Protocol on Transmitter Release, Augmentation and Potentiation**

Early investigators of stimulation-induced transmitter release (Weinreich 1971; Erulkar and Rahamimoff 1978; Magleby and Zengel 1976b) employed a number of different stimulation protocols (varying between 10-100 Hz and 10-100 s), which made it difficult to compare results. Lev-Tov and Rahamimoff (1980) later did a more rigorous study in which they examined different stimulation paradigms. They found that post-stimulation MEPP frequency increased with both frequency and duration of stimulation and that the effectiveness increased more than expected from simple algebraic addition of the number of stimuli (indicating there was a cumulative effect). Impulses at the highest rate of stimulation (100 Hz) were about 14 times more effective than those at the lowest frequency (10 Hz) when a constant number of stimuli was employed. Frequencies below
10 Hz were usually ineffective. These results agreed with those of Magleby and Zengel (1976b), who had shown earlier that augmentation and potentiation increased with the number of tetanic stimuli.

In the present study, variable frequency (80, 40, and 20 Hz) and a fixed duration of stimulation (40 sec) was used to test the effectiveness of nerve stimulation. The results (Figs. 3a, b, c, and d) demonstrated obvious increases in MEPP frequency following stimulation at 80 Hz, but only small increases at 40 Hz, and no apparent changes at 20 Hz. The results thus demonstrate a disproportionate increase in MEPP frequency with increasing stimulus frequency, in agreement with Lev-Tov and Rahamimoff (1980).

Despite their detailed investigation, Lev-Tov and Rahamimoff (1980) were not able to study the underlying quantal mechanisms because of the lack of a suitable method for quantal analysis. The present approach was intended to fill this gap. The results (Figs. 3a, b, c, and d) showed marked increases in \( m, n, p \), and \( \text{var}_p \) following stimulation at 80 Hz. There were only small increases in all of these parameters after stimulation at 40 Hz and no apparent changes following stimulation at 20 Hz. Accordingly, the results for the quantal release parameters mimicked those of MEPP frequency in showing a disproportionate increase with increasing stimulus frequency. Augmentation and potentiation increased with the number of stimuli in the tetanus (van der Kloot 1994; Magleby and Zengel 1976b). This study showed that the high frequency paradigm caused more transmitter release when the number of stimuli was kept constant (Figs. 2a, b, c, and d). This result is also consistent with the finding of Lev-Tov and Rahamimoff (1980).
Quantal analysis showed there were obvious increases in \( m \) (number of quanta released per unit time), \( n \) (number of functional release sites), \( p \) (probability of release) and \( \text{var}_p \) (spatial variance in \( p \)) following stimulation at 80 Hz. There were only small increases in \( m, n, p, \) and \( \text{var}_p \), after stimulation at 40 Hz, and finally there were no apparent changes following stimulation at 20 Hz.

The above results may be explained by the residual calcium hypothesis (Katz and Miledi 1968) as follows: when the nerve is stimulated at high frequencies, the influx of \( \text{Ca}^{2+} \) during the train will be cleared from the nerve terminal in relatively less time and \( [\text{Ca}^{2+}] \); would be expected to remain high for some time (Delaney and others 1989; Zucker and others 1991). Alternatively, the elevated \( [\text{Na}^+] \); produced by high frequency stimulation may cause \( \text{Ca}^{2+} \) release from intracellular stores by \( \text{Na}^-\text{Ca}^{2+} \) exchange (Meiri and others 1981; Melinek and others 1982; Lev-Tov and Rahamimoff 1980; Rahamimoff and others 1980). The higher residual \( \text{Ca}^{2+} \) may cause the increases in \( p \) and \( n \), and the release of \( \text{Ca}^{2+} \) from intracellular stores may be responsible for the increase in \( \text{var}_p \).

**Stimulation-Induced Transmitter Release in Low Ca^{2+}, High Mg^{2+} Solution vs. That in Normal Ringer Solution with \( \mu \)-Conotoxin**

Investigators have traditionally studied stimulation-induced transmitter release under conditions that depress transmitter release (low \( \text{Ca}^{2+} \), high \( \text{Mg}^{2+} \)), because of the lack of a way of blocking muscle contraction in normal Ringer solution. Accordingly, the physiology involved in non-depressed conditions was not known. More recently, it has
been possible to block muscle contractions with a 22-amino acid peptide, \( \mu \)-conotoxin, from the snail *Conus geographus*. The toxin specifically blocks the voltage-dependent Na channels of the muscle fibers (Cruz and others 1985; Moczydlowski and others 1986). With this toxin it was possible for the first time to carry out quantal analysis of stimulation-induced transmitter release under normal physiologic conditions.

In the present study, it was found that augmentation and potentiation were present in both low \( \text{Ca}^{2+} \), high \( \text{Mg}^{2+} \) and normal Ringer solutions but that the magnitude of potentiation was greater and its decay slower in normal Ringer solution. Augmentation was essentially constant in both solutions (Fig. 5). In normal Ringer solution, there were larger increases in \( m, n, p \), and \( \text{var}, \text{p} \) in comparison to data obtained in low \( \text{Ca}^{2+} \), high \( \text{Mg}^{2+} \) solution in the same junction (Figs. 4a, b, c, and d). Because \( \text{Mg}^{2+} \) competes for the influx of \( \text{Ca}^{2+} \) through Ca channels (Narita and others 1990), one would expect increases in \( [\text{Ca}^{2+}]_i \) by this route to be minimal in low \( \text{Ca}^{2+} \), high \( \text{Mg}^{2+} \) solution. This in turn should result in a much lower level of quantal release, which is what was observed. One striking finding, however, was the dramatically higher values of \( n \) in normal Ringer solution compared to values obtained in low \( \text{Ca}^{2+} \), high \( \text{Mg}^{2+} \) (Fig. 4b). This meant that low \( \text{Ca}^{2+} \), high \( \text{Mg}^{2+} \) depressed the number of functional release sites much more than the mean probability of release.

In these studies it was found that 5-7 \( \mu \text{M} \) \( \mu \)-conotoxin GIIIA paralyzed skeletal muscle without affecting the motor nerve or neuromuscular junction, in agreement with the observations of Sosa and Zengel (1993). However, with slightly higher concentrations

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(10 μM), EPPs and MEPPs were abolished in some muscles. During washout of the toxin, EPPs recovered first (20 min), whereas MEPPs recovered later (50-60 min). The effect of μ-conotoxin GIIIA was always reversible, with muscle contraction returning between 5-20 min after toxin removal. However, 30 min after washing out μ-conotoxin GIIIA and restoration of contractions, only 2-4 μM of the toxin was needed to reinstitute the block of contraction. This second dose varied between different muscles, which made it difficult to do control groups in the same junction.

An analog called μ-conotoxin GIIIB (Sigma Chem. Co.) required 15-20 μM to immobilize the muscle and caused marked inhibition of MEPP amplitude in some muscles. Selectivity of μ-conotoxin GIIIB for muscle sodium channels over nerve sodium channels was even less than with μ-conotoxin GIIIA. The findings with the GIIIB toxin were similar to those reported by Hong and Chang (1989). They found that the selectivity of μ-conotoxin GIIIB for muscle Na channels over nerve Na channels was only about threefold, much less than originally reported (Nakamura and others 1983; Cruz and others 1985; Ohizumi and others 1986). Given these drawbacks and the fact that μ-conotoxin was very expensive, it was decided to use low Ca²⁺, high Mg²⁺ to immobilize muscles for the majority of the experiments.

One explanation for the lower than reported selectivity of both μ-conotoxins may be that EPPs and MEPPs were monitored rather than muscle action potentials or axonal conduction. EPPs and MEPPs are dependent on ACh release which is dependent on
voltage-dependent \( \text{Ca}^{2+} \) channel opening in the nerve terminal. The \( \mu \)-conotoxins are from \( C. \text{geographus} \) venom which also contains \( \omega \)-conotoxin GVIA (27 amino acid residues) and \( \alpha \)-conotoxin GI (13 amino acid residues) (Kerr and Yoshikami 1984; Olivera and others 1990). \( \omega \)-Conotoxin GVIA blocks voltage-dependent \( \text{Ca}^{2+} \) channels of presynaptic terminals and inhibits release of transmitter. \( \alpha \)-conotoxin GI blocks nicotinic acetylcholine receptors in skeletal muscle, as do \( \alpha \)-neurotoxins from snake venom. All three peptides have either the same cysteine residue grouping as \( \mu \)-conotoxin (CC...C...C...CC) and \( \omega \)-conotoxin (C...C...CC...CC) or the same pattern of cysteine residue groupings as \( \alpha \)-conotoxins (CC...C...C...). These cysteine residue groupings play a very important role in maintaining the three dimensional structure for the active conformation. This might be why \( \mu \)-conotoxin (especially at higher concentrations) displayed the properties of \( \omega \)-conotoxin (inhibition of transmitter release) and \( \alpha \)-conotoxin (depression of MEPP amplitude) in the present experiments. The lower than reported selectivity of \( \mu \)-conotoxin for muscle Na channels over nerve Na channels was confirmed by Dr. Doju Yoshikami at the 1997 Society for Neuroscience Meeting. He indicated plans to publish a correction concerning these results (personal communication).

**Effect of Hypertonicity on Stimulation-Induced Transmitter Release**

It has long been known that increases in tonicity of the Ringer solution produce increases in MEPP frequency (Fatt and Katz 1952). The effects of hypertonicity are transient in the mammal (Hubbard and others 1968) but more sustained in the frog (Kita
and van der Kloot 1977). MEPP frequency rises along a line relating log (MEPP frequency) to (osmolarity)\(^{0.5}\) up to about 420 mOsm, with a plateau in this relationship for >460 mOsm (Kita and van der Kloot 1977). Washing out the hypertonic solution returns MEPP frequency to the resting level. The increase in transmitter release is not due to additional entry of Na\(^+\), because the effect can be seen with non-permeating sucrose as well as with NaCl. Moderate increases in tonicity also increase nerve-evoked quantal release (Barton and others 1983), but very hypertonic solutions produce a decrease in nerve-evoked quantal output (Kita and van der Kloot 1977).

The present studies on the influence of moderate hypertonicity (319 mOsm) on tetanus-induced augmentation and potentiation were undertaken because these effects have not been reported. The results showed that hypertonicity produced a marked increase in the stimulation-induced MEPP frequencies over control. The time course of decay of the post-stimulus increase in MEPP frequency returned to control values within 20 min after washing with isotonic Ringer solution. The reversible nature of this effect was consistent with previous reports.

Compared to the control groups, hypertonicity caused an increase in both the basal (before tetanic stimulation) and the tetanus-induced values of \(m\), \(n\), and \(p\) (Fig. 6b, c, d, and e). The surprising new finding was that hypertonicity produced a marked decrease in the amount of augmentation but had no effect on potentiation (Fig. 7). In essence, there was a conversion of the normal two-phase decay (with rapid decays in \(m\), \(n\), and \(p\) in the first 10 s and then a slow decay to the baseline) into a one-phase decay.
There was a marked increase in var, p following tetanic stimulation in hypertonic solution, but with time, values returned to baseline just slightly higher than the level in isotonic solutions. The mean values during the first 10 s following stimulation showed the same brief increase, decrease, and subsequent increase seen in isotonic conditions (Fig. 1e). These changes may reflect release of Ca^{2+} from mitochondria (Provan and Miyamoto 1994, 1995) or a decrease in the rate of Ca^{2+} uptake by these organelles (Alnaes and Rahamimoff 1975). An intracellular source of Ca^{2+} is indicated, since hypertonicity enhances the increase in MEPP frequency following tetanus in Mg^{2+}/EGTA solution (Kita and others 1982; Narita and others 1983).

The present results indicate that hypertonicity caused an increase in stimulation-induced quantal output due to increases in n, p, and var, p. This supports the idea that hypertonicity increases transmitter release by elevating [Ca^{2+}]_i (Alnaes and Rahamimoff 1975). On the other hand, Tanabe and Kijima (1988) found that pretreatment with BAPTA-AM (a membrane-permeable Ca^{2+} chelator) greatly reduces facilitation but does not alter the increase in MEPP frequency or evoked release produced by hypertonic solutions. Thus, the question of whether elevated [Ca^{2+}]_i is the basis for increased transmitter output with hypertonicity remains problematic.

Other explanations for the effect of hypertonic solutions on spontaneous and nerve-evoked MEPP frequency include an overall increase in the ionic strength in the axoplasm (van der Kloot and Kita 1973; Silinsky 1985) and possibly a nonspecific physical
mechanism. However, none of the proposed hypotheses predicts the observed decrease in augmentation. It may be that hypertonic solutions in some unknown fashion decrease the number of intraterminal Ca\(^{2+}\) binding sites that are responsible for augmentation.

**Mitochondria and Stimulation-Induced Augmentation and Potentiation**

Despite the uncertainty, the most plausible explanation for facilitation, augmentation, and potentiation appears to be residual Ca\(^{2+}\) within the nerve terminal. The source of this Ca\(^{2+}\), however, remains unestablished. Possible mechanisms for removing or decreasing [Ca\(^{2+}\)]\(_i\) include (1) uptake and storage by intracellular organelles, primarily mitochondria and endoplasmic reticulum (Liano and others 1994; Herrington and others 1996; Tang and Zucker 1997), (2) extrusion by plasma membrane pumps, and (3) buffering by cytoplasmic constituents (Pozzan et al. 1994).

TPP\(^+\) was used to block mitochondrial uptake and efflux of cytoplasmic Ca\(^{2+}\) to test whether these processes were involved in augmentation and potentiation. It is well established that mitochondrial Ca\(^{2+}\) transport is mediated by at least three separate mechanisms:

**Ca\(^{2+}\) Uniporter**

The Ca\(^{2+}\) uniporter is activated by Ca\(^{2+}\), ADP (Gunter and Pfeiffer 1990; Saris and Kroner 1990; Rottenberg and Marbach 1990), and certain drugs (Kroner 1990). It is one of the fastest gated pores known (Gunter and others 1994), with calcium uptake occurring within tens of milliseconds after exposure to Ca\(^{2+}\) (Chance 1965; Chance and others 1972).
The resting value of Ca\(^{2+}\) in the mitochondrial matrix is between 80-200 nM. Thus, there is virtually no Ca\(^{2+}\) gradient across the resting mitochondrial membrane, despite a large, inside negative $\Delta \psi_m$. This may indicate that the uniporter is shut down at rest.

The present study showed that TPP\(^+\) had no effect on resting MEPP frequency in both isotonic and hypertonic solutions, in agreement with the idea that the uniporter is at rest under these conditions. The uniporter is activated only at Ca\(^{2+}\) concentrations above 500 nM (Gunter and Pfeiffer 1990; Gunter and others 1994). The only time mitochondria are exposed to Ca\(^{2+}\) in these micromolar ranges is during Ca\(^{2+}\) pulses evoked by nerve depolarization. This supports the idea that mitochondria may participate in removing the residual Ca\(^{2+}\) responsible for augmentation and potentiation. It is possible that the Ca\(^{2+}\) uniporter may also be involved in the effect of osmotic pressure on transmitter release, since increased osmotic pressure causes a decrease in calcium uptake by mitochondria (Scarpa and Azzone 1968). In agreement with this, hypertonic solutions produced an increase in resting MEPP frequency as well as $m$, $n$, $p$, and $\text{var}, p$.

**Na\(^+\)-Dependent and Na\(^+\)-Independent Calcium Efflux**

Calcium efflux occurs by both Na\(^+\)-dependent and Na\(^+\)-independent mechanisms (Gunter and Pfeiffer 1990). Na\(^+\)-dependent transport dominates in heart, skeletal muscle, and brain, whereas Na-independent transport dominates in liver and kidney. The Na\(^+\)-dependent efflux mechanism has been identified as a Ca\(^{2+}\)/2Na\(^+\) exchanger. Accordingly, it is possible that an increase in [Na\(^+\)], following tetanic stimulation may trigger Ca\(^{2+}\) release.
from mitochondria. Consistent with this, the present results showed that TPP* (which blocks Na+-dependent and Na+-independent Ca2+ efflux) decreased augmentation, potentiation, m, n, and p (Figs. 8a, b, c, and d). This indicated that at lower quantal output (low Ca2+, high Mg2+), Ca2+ efflux from mitochondria plays an important role in augmentation and potentiation. However, in the same solution made hypertonic with 100 mM sucrose, TPP* produced an increase in potentiation, m, n and var, p, but not p (Figs. 10a, b, c, d, and e).

Mitochondria are active participants in cellular Ca2+ signaling. Their unique role stems from the ability to rapidly capture a Ca2+ pulse and slowly return it to the cytoplasm where it can prolong the activation of high affinity, Ca2+-dependent processes (Gunter and Pfeiffer 1990; Gunter and others 1994; Babcock and others 1997). Because TPP* and hypertonic solutions both inhibit Ca2+ uptake by mitochondria (Scarpa and Azzone 1968; Tang and Zucker 1997), cytosolic Ca2+ should remain elevated under these conditions. This could account for the increase in potentiation with TPP* in hypertonic solutions (as the effect of TPP* would be masked).

Nonspecific Permeability Transition Pore

Calcium efflux also occurs by a nonspecific Ca2+-induced pore called the permeability transition pore (Evtodienko and others 1994; Ichas and others 1994). This is a Ca2+-, voltage-, pH-, and redox-gated channel of the inner mitochondria membrane (Zoratti and Szabo 1995; Bernardi and Petronilli 1996). It can operate in a high conductance
(persistent pore opening) and a low conductance (transitory pore opening) mode (Zoratti and Szabo 1995). The former mode takes part in the cascade leading to oxidative cell death (Crompton and Costi 1990; Griffiths and Halestrap 1995; Nieminen and others. 1995) and apoptosis (Kroemer and others 1995; Zamzarni and others 1996), while little is known about the latter mode. Ichas and others (1997) reported that Ca²⁺-induced Ca²⁺ release from mitochondria (mCICR) depends on the transitory opening of the permeability transition pore. The Ca²⁺ fluxes taking place during mCICR are a direct consequence of the mitochondria depolarization spike caused by the permeability transition pore. Mitochondria thus appear to be excitable organelles capable of generating and conveying electrical and Ca²⁺ signals.

Tang and Zucker (1997) reported the involvement of mitochondria in post-tetanic potentiation at the crayfish neuromuscular junction. They found that TPP⁺ selectively blocked post-tetanic potentiation while increasing post-tetanic augmentation. The present study shows that TPP⁺ selectively blocked post-tetanic potentiation with no apparent effect on post-tetanic augmentation at the frog neuromuscular junction. One explanation for this may be differences in species. It may be that at frog nerve endings, TPP⁺ does not have a significant effect on mitochondrial uptake of Ca²⁺ in low Ca²⁺, high Mg²⁺ solutions. However, TPP⁺ may inhibit mitochondrial uptake of Ca²⁺ in hypertonic conditions, since there is an increase in potentiation in hypertonic solutions with TTP⁺ relative to hypertonic solutions without TTP⁺. Moffatt and Miyamoto (1988) suggested that the increase in MEPP frequency with dinitrophenol may have been mediated by an effect on the Ca²⁺
uniporter. The principal finding in the present work is that mitochondria can regulate the depolarization-induced Ca\(^{2+}\) pulses that control neurotransmitter release.
CHAPTER 5

SUMMARY AND CONCLUSIONS

1. A system was devised for computing quantal release parameters during the very brief periods following tetanic stimulation when quantal output is rapidly changing. Results from these studies demonstrated the utility of this procedure for investigating the subcellular dynamics of nerve-evoked transmitter release.

2. The amount of increase in transmitter release following nerve stimulation was dependent on stimulation frequency. When the total number of pulses was kept constant, the high frequency paradigm caused more transmitter release. This was reflected by higher levels for augmentation, potentiation, and all the quantal release parameters at the higher stimulus frequencies. Control experiments using identical stimulation patterns separated by one-half hour showed no significant differences in the results. This indicated there was no deterioration or drifting in the system with time or use.

3. Augmentation and potentiation could be observed in both low Ca\textsuperscript{2+}, high Mg\textsuperscript{2+} and normal Ringer solution. The amount of potentiation was greater in normal (non-depressed transmitter release) Ringer solution than in low Ca\textsuperscript{2+}, high Mg\textsuperscript{2+} Ringer solution. Similarly, the increases in $m$, $n$, $p$, and var, $p$ were greater in normal Ringer solution than in low Ca\textsuperscript{2+}, high Mg\textsuperscript{2+} Ringer solution. However, the values for $n$ were much greater in normal Ringer, indicating that the primary effect of low Ca\textsuperscript{2+}, high Mg\textsuperscript{2+} Ringer solution
was to decrease the number of functional (occupied by synaptic vesicles or "docked")
transmitter release sites.

4. Moderate increases in hypertonicity produced a marked increase in both the basal
(pre-stimulation) and the stimulation-induced values of MEPP frequency, \( m \), \( n \), and \( p \).
Hypertonicity produced a marked increase in only the stimulation-induced values of \( \var \), \( p \)
and not the basal values of \( \var \), \( p \).

5. Moderate increases in hypertonicity produced a substantial decrease in the amount
of augmentation but had no effect on potentiation.

6. In low \( \text{Ca}^{2+} \), high \( \text{Mg}^{2+} \) solution, the mitochondrial inhibitor \( \text{TPP}^+ \) (3 \( \mu \text{M} \)) produced
a decrease in augmentation and potentiation. In the same solution made hypertonic with
100 mM sucrose, \( \text{TPP}^+ \) produced an increase in potentiation. Accordingly, mitochondrial
uptake and efflux of cytoplasmic \( \text{Ca}^{2+} \) appear to be involved in both augmentation and
potentiation.


Zengel JE, Magleby KL. 1980. Differential effects of Ba²⁺, Sr²⁺ and Ca²⁺ on stimulation-induced changes in transmitter release at the frog neuromuscular junction. J Gen Physiol 76:175-211.


APPENDIX A

Determination of Facilitation, Augmentation, and Potentiation
Appendix A

Determination of facilitation, augmentation and potentiation.

Figure 11. Stimulation-induced changes in MEPP frequency (From Zengel and Magleby, 198). See next page for description.

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The data were averaged from 12 cell in six preparations. A: Rise and decay of MEPP frequency during (horizontal bar) and after repetitive stimulation of 200 impulses at 20 impulses/s. B: decay of the fraction increase in MEPP frequency, $V_M(t)$, after the conditioning train in A was plotted semilogarithmically against time after the train. The decay of potentiation of MEPP frequency is indicated by $P_M(t)$. C: Decay of augmentation of MEPP frequency, $A_M(t)$, after correcting the data in B for potentiation. D: Decay of the second component of facilitation of MEPP frequency, $F_2M(t)$, after correcting the data in D for the second component.
APPENDIX B

Schematic Diagram of Experimental Set-Up
Appendix B

Schematic diagram of experimental set-up. The cutaneous pectoris nerve muscle preparation was used for all experiments. The nerve stump was drawn into a fluid suction electrode and stimulated with pulses delivered by a stimulator with an isolation unit. MEPPs were recorded with glass microelectrode. Bioelectric signals were fed into an amplifier and then to one channel of an oscilloscope. MEPPs were recorded with VCR for off-line analysis. Data on magnetic tape then were fed into Data Acquisition and Analysis System and stored in digitized form on the hard drive of a computer.
APPENDIX C

Schematic Diagram to Illustrate the “Moving Bin” Procedure
Appendix C

Schematic diagram to illustrate the "moving bin" procedure. This procedure (moving average) was used for smoothing out fluctuations in individual data point (bin = 3 sec. ∆bin = 1 sec.).

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APPENDIX D

Calculation of the Quantal Release Parameters
Appendix D

Calculation of the Quantal Release Parameters

The working model of quantal transmitter release and derivation of the equations for obtaining unbiased estimates of the quantal release parameters are described in detail by Miyamoto (1986). The use of these equations to obtain quantal estimates from miniature endplate potential (MEPP) counts, as well as the advantages and precautions involved, is covered by Provan and Miyamoto (1993).

Briefly, the theory assumes that transmitter release occurs by a Bernoulli process (i.e., in an all-or-none fashion). This is consistent with the idea that quantal release (exocytosis of the contents of a single synaptic vesicle) either occurs or does not occur. The natural distribution for sampling from a Bernoulli population is the binomial distribution, in which the number of “successes” is equal to the probability of success ($p$) times the number of trials ($n$). This distribution is applied to the release process at a single release site, where the number of successes (quantal releases) is equal to the probability of release times the number of trials. For a single release site, $n = 1$. By extending this process over the total number of release sites across the face of the nerve terminal (i.e., sites that are spatially distributed), one gets $x$ number of trials (each independent release site constitutes one trial). One can also increase the number of trials ($n$) by extending the process in time, so that a particular release site may be tested more than once (i.e., there would be a distribution of trials in time, or a temporal distribution). In practice, one must put a time
limit on the process, which is the reason for the 10 ms bin, or one could conceivably end up with an infinite number of trials.

According to this model (Brown and others 1976), there can be variation in the number of release sites (trials) or in the probability of release, either spatially (across the face of the nerve terminal) or temporally (from one time period to another). This leads to the possibility of variation in $n$ and/or $p$, both temporally and spatially. The present model uses a simplification of that of Brown and others (1976), by employing a very short time interval to minimize temporal variation. As indicated by Provan and Miyamoto (1993), temporal variation is not completely eliminated. This results in a small error in the estimates, which is reflected by the small negative estimates for var, $p$. This does not, however, invalidate the fundamental procedure for obtaining the unbiased estimates of $m$, $n$ and $p$ (see discussion of this issue in Provan and Miyamoto (1993)).

The approach for obtaining the unbiased quantal estimates is as follows: continuous recordings of MEPPs are made on magnetic tape for periods up to 70 sec after tetanic stimulation. Data are digitized using the R.C. Electronics data acquisition and analysis system, and the post-stimulation time is divided into 10 ms intervals or bins. The number of MEPPs in each bin is counted and recorded as an integral number. This is defined as $m$, the number of quanta per 10 ms bin. Three hundred consecutive counts are recorded for each sample, so that a typical series of data might read, 3, 5, 2, 0, 0, 2, 1...4, 3, etc.

These 300 numbers provide the input for a computer program called "VARSP97" (written in BASIC computer language), which computes the sample moments for the
mean (first moment), variance (second moment), and skew (third moment) of \( m \). The sample moments are calculated according to equations (22) and (23) in Miyamoto (1986), i.e.,

\[
\begin{align*}
M_1 &= \frac{\sum_{i=1}^{N} m_i}{N} \\
M_k &= \frac{\sum_{i=1}^{N} (m_i - \bar{m})^k}{N - k + 1}
\end{align*}
\]

and where \( N \) is the sample size and \( k = 2, 3, \ldots \).

The sample moments are then used as estimators of the population moments in equation (21) from Miyamoto (1986):

\[
\beta^2 = \frac{3\mu_3 \beta}{2\mu_1} - \frac{3\beta^2}{2} + \frac{1}{2} \frac{3\mu_3}{4\mu_1} \frac{\mu_5}{4\mu_1} = 0.
\]

This is a quadratic equation with two solutions for \( \beta \) (denoted as \( p_1 \) and \( p_2 \)). Computer simulation shows that the \( p_1 \) or positive square root value is the appropriate solution. On infrequent occasions when the root value is very small, the computer program employs an iterative procedure to solve for \( p_1 \). Estimates of var, \( \beta \) are then obtained from

\[
\text{var}, \beta = \bar{\beta} - \bar{\beta}^2 - 1 \mu_2(\mu_4 - \mu_2^2) \bar{\beta}
\]
(equation (25) in Miyamoto (1986)). The estimate of $m$ is obtained as the first moment of
$m$, or simply the mean $m$ for the 300 samples. Since $m$ equals $n$ times $p$ for the binomial,
n may then be obtained from the estimates for $m$ and $p$ as simply $n = m/p$. 
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