LATROTOXIN-EVOKED BURSTS OF NEUROTRANSMITTER RELEASE AT THE MOUSE NEUROMUSCULAR SYNAPSE

by

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ABSTRACT

Whole cell recordings were made at the mouse neuromuscular junction using sharp glass electrodes. Miniature end-plate potentials (minEPPs) and end-plate potentials (EPPs) were recorded from muscle fibres as measures of quantal neurotransmitter release from motor nerve terminals. Effects of alpha-latrotoxin application on such quantal release were assessed.

Alpha-latrotoxin is a toxin found in black and brown widow spider venoms. The toxin binds irreversibly to a receptor specific to nerve terminal membranes and forms a channel or channels in the neuronal membrane. Latrotoxin channels open to admit excitatory cations into the nerve terminal which causes the release of multiple quanta, recorded as minEPPs, from the terminal.

In the presence of external Ca^{2+}, latrotoxin-evoked release was seen as discrete bursts of quanta. Evidence from previous literature suggests that these bursts originate from single release sites on the nerve terminal called active zones. The incidence of bursts was proportional to the concentration of external Ca^{2+}. Burst intensity, measured as the number of recorded minEPPs per second (fm), varied in a stepwise fashion between 3.5 to 2100. Multimodal conductance states of single latrotoxin channels, previously described in the literature, might underlie the burst intensity sublevels now seen at the whole cell level. Indeed,

single latrotoxin channel openings cooperate into bursts and vary with the external Ca^{2+}, just like whole cell bursts do.

It was also discovered that Ba^{2+} and Sr^{2+}, like Ca^{2+}, can support latrotoxin bursts. However, when latrotoxin was applied in the presence of Mg^{2+} and no other divalent cations, a steady rise in fm was seen without bursts.

Release of quanta during intense and prolonged bursts was seen to decline over time; this decline might reflect depletion of "releasable" vesicles at the single active zone responsible for the burst.

The application of 6% DMSO increased baseline fm 9.53 times, but burst fm was increased only 1.73 times by 6% DMSO. Thus it is probably true that release during the fastest bursts is not far removed from the maximal rate of release possible from a single active zone. This maximal rate of release might reflect the fundamental rate at which vesicles from the "releasable" pool can be induced to fuse with the subsynaptic membrane.

The m, which reflects synchronous release of quanta from multiple active zones, is substantially increased when there is a burst. The burst-associated increase in m can be multiplied by 4% DMSO application to the same extent that baseline m can. Although release from other active zones is clearly increased

during a single active zone burst, the contribution to burst fm made by these other active zones is tiny in proportion to the contribution made by the bursting active zone itself. The response of other active zones to the localized latrotoxin event is most probably due (a) to the entry of excitatory ions like Ca^{2+} and Na^{+} into the nerve terminal or (b) to the triggering of a second messenger cascade.

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Do not follow where the path may lead.
Go instead where there is no path and leave a trail.

-UNKNOWN

Can't never could.

-JIM PAYNE'S MUM

INTRODUCTION

General Introduction

H. H. Dale and colleagues, showed that motor neurons transfer excitatory signals to muscle fibres by releasing a chemical stimulant from the nerve ending[1]. The chemical stimulant, which has been identified as acetylcholine (ACh), crosses the gap between nerve terminal and muscle fibre and binds to receptors on the muscle fibre.

Once bound, the ACh causes an increase of cation permeability--mainly to Na^+ and K^+, but also to Ca^{2+}. As a result, Na^+ and Ca^{2+} diffuse down both electrical and concentration gradients into the muscle, and K^+ diffuses down its concentration gradient out of the muscle. The net entry of cations produces a local depolarization of the muscle fibre, known as an "end-plate potential" (EPP). Normally the reaction is so intense that the resulting end-plate potential rises at a high rate and rapidly exceeds the excitation threshold at which an impulse is initiated. Thus generated, the muscle fibre action potential travels along the whole length of the muscle fibre and activates the contractile mechanism at every point[2].

Spontaneous Release

If one penetrates a muscle fibre with a microelectrode, one finds that the inside is at a negative potential, about -80 mV with respect to the outside bath. The same result is obtained wherever one inserts the microelectrode, in the vicinity of the nerve contacts as well as at all other points of the fibre. However, unlike the rest of the muscle fibre, the junctional region is in a state of subthreshold electric activity even when no stimulus has been applied and when--to all outward appearance--the muscle fibre is completely at rest. One observes a sequence of small, intermittent electric discharges, each of them amounting to a brief depolarization of about 0.5 mV, rising rapidly and declining more slowly, with a total duration of about 2 msec in the rat or mouse. The potential changes are very similar to EPPs, the main difference being their much smaller amplitude (about 0.5 instead of over 40 mV) and their "spontaneous" occurrence. Fatt and Katz called them "miniature end-plate potentials" (minEPPs)[3].

In general, in the absence of an applied stimulus, minEPPs occur at random intervals, with a probability which remains constant over long periods of time and is independent of previous occurrences. There is no refractory period after the occurrence of a minEPP; in fact, two minEPPs can occur simultaneously.

Fatt and Katz suspected that the minEPPs are caused by leakage of acetylcholine from the nerve ending[3]. Indeed, they were able to (somewhat crudely) mimic the miniature potential by closely approaching the end-plate region with a micro-pipette containing a solution of acetylcholine (ACh), and by discharging a small quantity of ACh ionophoretically. Such application produced a local depolarization of the muscle fibre, analogous to a minEPP. Also, by using an assembly of drug-filled micro-pipettes, Fatt and Katz were able to establish that the artificially-induced depolarizations caused by ionophoretic ACh discharge had the same pharmacological sensitivity to curare and anti-cholinesterases as spontaneous minEPPs--the response to the ACh was suppressed by curare, and enhanced by cholinesterase inhibitors such as edrophonium[3,4].

MinEPPs are discrete all-or-none events, of standard size and time course. However, artificial ACh-potentials induced by ACh ionophoresis can be made bigger or smaller in a smoothly graded fashion by increasing or decreasing the amount of ACh dispensed onto the muscle fibre. The time course can also be changed, for example by varying the distance between pipette and end-plate or varying the duration of the ACh pulse. Thus it is clear that minEPPs are not due to one or a few molecules of ACh released from the nerve ending, but are due to the synchronous impact of large multi-molecular packets of ACh[2].

The contents of one ACh packet reach the end-plate almost synchronously (the ionic current rises to a peak within a fraction of a millisecond) which suggests that ACh release is from the nearby nerve ending[2,5]. This hypothesis is very likely, as nerve endings are a rich source of ACh[6,7], and destruction of the nerve terminal stops ACh release without changing the local sensitivity of the muscle fibre to ACh[8]. As well, botulinum toxin, which interferes with ACh release from motor neurons[9], also nearly abolishes minEPPs recorded from muscle fibres[10]. Finally, it has been shown that changing the membrane potential of the nerve terminal, but not muscle fibre, alters both the amount of ACh released and the frequency of minEPPs (fm)[11].

EPPs, which the nerve impulse produces in the muscle fibre, resemble minEPPs, e.g., in sensitivity to curare, anticholinesterase poisons and botulinum toxin. There is little doubt that both are due to local impacts of acetylcholine upon postsynaptic membrane receptors.

Electrical Control of Release

Depolarization of nerve terminals is associated with an fm rise[10]. By increasing the current strength, the rate of discharge can be raised more than a hundredfold until the minEPPs become so frequent that they can no longer be counted individually.

Thus ACh release is electrically controllable. Electrical control of release is exerted physiologically when an action potential wave arrives at a motor nerve terminal; the action potential provides a very intense and very brief depolarization which abruptly and transiently increases release by the same mechanism as direct depolarization. The nerve impulse accelerates the rate of secretion of ACh packets by a factor of a few hundred thousand for a very brief interval of time. As a result, instead of an intermittent discharge at a mean rate of about one per second, a few hundred packets are released within less than one millisecond to produce an EPP big enough to initiate an impulse in the muscle fibre.

End-plate Potentials

It is known that Ca^{2+} is required for electrically evoked transmitter release[12,13], and that Mg^{2+} competes with Ca^{2+} and acts as an inhibitor of Ca^{2+} entry and therefore release[14,15].

Depolarization in the presence of Ca^{2+} is normally required to elicit release. Depolarizing pulses given after transient increases in Ca^{2+} concentration, with the ion no longer present, don't cause release, nor do depolarizing pulses cause release if Ca^{2+} is added only after the stimulus. Also,

Ca^{2+} application by itself only mildly increases release of quanta in physiological K^+, as reflected in a small increase in baseline fm. Under standard conditions, both depolarization of the nerve terminal and the presence of Ca^{2+} in the bath are required to evoke release[16]. The depolarization does not have to be delivered in the form of an action potential; after nerve impulses have been eliminated by tetrodotoxin (TTX) at squid stellate ganglia, local depolarization of the nerve terminal still evokes a postsynaptic potential change, provided Ca^{2+} is present in the bath[15,17].

When Ca^{2+} is reduced to low levels or Mg^{2+} added, the EPP declines in amplitude, at which time it becomes obvious that responses to stimuli fluctuate in a stepwise manner. Some stimuli produce no response at all, some a response of about 0.5 mV in amplitude (identical in size and shape to a minEPP), some stimuli produce responses twice the size of a minEPP, some three times and so on. So random fluctuation of EPP size occurs in discrete steps that are multiples of the minEPP amplitude. It looks as though EPPs are made up of a fluctuating number of quantal components identical to minEPPs[2].

Each unit packet of ACh (whose release produces a minEPP) is pre-formed within a synaptic vesicle in the nerve terminal. The vesicle actively accumulates ACh and maintains it at a high concentration, much higher than exists in the surrounding cytoplasm[18]. Release results from a fusion of vesicular

and nerve terminal membranes, as suggested by electron micrographs[19] of vesicles caught in the act of fusing with the nerve terminal membrane. For further evidence of exocytosis and the vesicular theory of transmitter release, see later sections of this work.

Synaptic delay

When a stimulus is delivered to a motor neuron, the first miniature potential comprising an EPP (measured postsynaptically) may be evoked as early as 0.5 msec, or as late as 2.5 msec, after arrival of the nerve spike at the terminal; the EPP itself generally spans about 2 milliseconds. So there is a delay between the arrival of the presynaptic action potential, and the appearance of a postsynaptic response.

After its release from the terminal, ACh must diffuse across the synaptic cleft and react with receptors on the muscle surface before a postsynaptic depolarization is seen. Yet there is evidence that neither diffusion of ACh across the synapse, nor its reaction with receptors take up much time. With an ionophoretic pulse application of ACh, depolarization of the junctional region of the muscle fibre can be observed as early as 0.1 to 0.2 msec after the start of the pulse[2]. Under natural conditions when ACh is secreted by the nerve terminal

itself, the delay due to local diffusion is presumably much shorter than this. So the major part of the observed synaptic latency must occur presynaptically.

In fact, the opening of voltage sensitive Ca^{2+} channels in the subsynaptic membrane is probably the slow step in the release reaction. Evidence for this view comes from work on the giant synapses of squid stellate ganglia. By applying a large depolarizing current pulse, the researchers were able to abolish the driving force for Ca^{2+} entry and suppress release during the pulse. An "electric potential barrier" was produced, barring the postive Ca^{2+} ions from entering the depolarized cell even though the Ca^{2+} channels were open. However, a great deal of release could be seen as soon as the pulse was taken away--the Ca^{2+} channels remained open for a brief instant after the driving force for Ca^{2+} entry was restored so Ca^{2+} ions were able to enter and cause release. The latency of release upon removal of the pulse was much less than the latency of release upon initiation of the pulse, implying that a significant fraction of the normal synaptic delay is due to the time taken for activation of Ca^{2+} channels by depolarization[16].

Quantal Release is Poisson Distributed

Each nerve terminal has a large population of vesicles beneath the subsynaptic membrane, queuing up for release by the next impulse. However,

the chance that a particular vesicle will fuse at any given time is very low. In a situation of this kind, Poisson's statistical law should apply. To use Poisson's theorem, we must first know the average number (m) of quanta released by an impulse (quantal content). Using the assumption that an EPP is made up of quanta identical in size, variance and origin to minEPPs, we can get m using the relationship:

$$m = (\text{mean size of EPP})/(\text{mean size of spontaneous minEPP})$$

Once we have m, we can use Poisson's theorem to predict the probability of evoking 0, 1, 2, 3 ... x packets with a stimulus:

$$p_x = e^{-m}m^x/x!$$

If m=2.33 (seen in low concentrations of Ca^{2+} or raised concentrations of Mg^{2+}), there will be a certain proportion of failures (EPPs with a quantal content of zero), and a certain proportion of stimuli that yield 1 or more quanta. The proportion of stimuli we'd expect to result in a failure if release is Poisson distributed is:

$$p_0 = (\text{Number of EPP failures})/(\text{Number of impulses}) = e^{-m}m^x/x!$$

Since 2.33^0 and 0! are both =1, $m^x/x!$ cancels out, leaving:

$$p_0 = e^{-m} = e^{-2.33} = 0.097.$$

So with an m of 2.33, there's a 9.7% chance that a given stimulus will evoke no release.

The equation for p_0 also gives us a way of testing the assumption that minEPP quanta are identical to EPP quanta via an alternative way of determining m:

$$\text{(Number of EPP failures)/(Number of impulses)} = e^{-m}$$

can be rewritten as:

$$m = \ln[\text{(Number of impulses)/(Number of EPP failures)}]$$

This method is easy to use--all one need do is count the number of EPP failures (the m must be small so failures are present) and the number of stimuli delivered. As predicted, m values derived from measurements of mean amplitudes agree with those derived from response and failure counts, so

minEPP and EPP quanta do seem to be identical. Furthermore, the variance in size of minEPP quanta is the same as that for EPP quanta[12,20,21].

The proportion of stimuli that are likely to result in release of one quantum if m=2.330 and if release is Poisson distributed is:

$$p_1 = e^{-2.33} 2.33^1 /1! = 0.097 \times 2.33 = 0.23$$

or a 23% chance of a given stimulus evoking the release of one quantum; the same equation can be used to determine the probability that 2, 3 or more quanta will be released with a stimulus.

In fact, a whole distribution of quantal contents can be predicted from Poisson's theorem once m, the average number of quanta released per stimulus, is known. And the experimentally derived distribution of EPP sizes, as plotted on a histogram, agrees with theoretical size distributions that assume release follows a Poisson distribution. Boyd and Martin, for example, compared EPP amplitude histograms plotted from real data, with theoretical amplitude histograms plotted with the assumption of a Poisson distribution. The value of m, determined by the method of failures, was 2.33 and 198 impulses were delivered. Above, it was shown that with an m of 2.33, 9.7% of stimuli (19 out of 198 in this case) are expected to result in failures if Poisson's theorem applies.

In fact, 18 failures were observed by Boyd and Martin, which is very close to expected. Furthermore, the number of stimuli resulting in 1, 2, 3 or more quanta was also close to that predicted from Poisson's theorem[20].

Lowering the temperature of the bath can make assessments of the applicability of Poisson's theorem easier. At low temperature, the release of quanta after a stimulus is greatly dispersed in time; each evoked quantum occurs after a more variable delay. This is convenient, as individual quanta occurring separately and consecutively are easier to identify and count. Katz and Miledi performed such low temperature experiments, bathing their preparation in a low-Ca^{2+} solution to keep m low and failures high[22].

For one experiment, 674 nerve impulses were elicited. The researchers counted 440 failures, 195 single units, 37 "twins", and 2 "triplets" for an m of 0.41. Applying this m value to Poisson's theorem gave them an expected distribution of: 448 failures, 183 singles, 37 "twins", 5 "triplets" and 1 "quadruplet"--very close to the experimentally observed distribution, once again suggesting a Poisson distribution for quantal release[22].

Since release of quanta follows a Poisson distribution, statistical relationships applicable to Poisson distributions also apply to quantal release. It is known that, with a Poisson distribution, the ratio of the variance over the mean is equal to one[23]. Not surprisingly, the variance to mean ratio of the number of

minEPPs in non-overlapping time periods has also been consistently shown to be 1.0. This statistical relationship is useful, as it gives us a sensitive way of quantifying deviations from random--deviations such as bursts (see below). When there are bursts (sudden brief volleys) of minEPPs, spontaneous release occurs in a decidedly clustered, non-random fashion, and the variance is higher than the mean. Thus a variance to mean ratio well above 1.0 denotes that minEPPs are not occurring randomly, but rather in tight clusters, or bursts. The variance to mean ratio of the number of minEPPs in non-overlapping time periods is therefore a quantitative indicator of the amount of "burstiness" in the record.

Latrotoxin

Introduction and Overview

Alpha-latrotoxin is an acidic protein with a molecular weight of about 130 000 Daltons[27] found in black widow spider venom (BWSV) and brown widow spider venom (BrWSV). The black and brown widow spider venoms are immunologically indistinguishable[24] and have similar toxicological effects [25,26]. In fact, BrWSV, BWSV and alpha-latrotoxin are considered equivalent as far as their actions on vertebrate neuromuscular junctions are concerned; although there are at least seven different toxins in BWSV, only alpha-latrotoxin acts on

vertebrate neuromuscular junctions[27]. However, the other toxins in BWSV act on various insect or crustacean nerve terminals in an analogous fashion [28,29,30].

When the toxin is applied at vertebrate neuromuscular junctions, it produces an increase in "spontaneous" release of quanta, electrophysiologically seen as an increase in frequency of minEPPs. This release, which can be massive, eventually subsides and electron-microscopic examination then shows depletion of the synaptic vesicle population. This stage is associated with block of neuromuscular transmission. The time sequence of events depends on which divalent cations are present, the toxin concentration and the duration of exposure[31]. Latrotoxin also works at other kinds of synapses and seems generally to cause the release of fast-acting neurotransmitter (e.g., dopamine, ACh) mostly via exocytosis, rather than leakage of non-vesicular neurotransmitter from the nerve terminal cytosol.[32] No particular fast-acting neurotransmitter is favoured; latrotoxin makes dopamine-containing neurons release dopamine, and ACh-containing neurons release ACh, etc. Peptide release (via large dense core vesicles) is not promoted by latrotoxin application[33].

The toxin appears to stimulate release (1) by allowing the entry of excitatory ions (see page 29) and (2) by directly stimulating the neuronal release machinery (see page 40). It acts on all types of neurons so far studied, but does

not generally stimulate nonneuronal secretory cells to release transmitter substance (the exception is cultured adrenal chromaffin cells).

Such cell selectivity is attributed to the presence of latrotoxin receptors only on nerve terminal membranes. Since latrotoxin acts on all different types of neurons so far studied, it no doubt targets release machinery that is common to synapses of different types[34].

The initial binding of alpha-latrotoxin to its membrane receptor is voltage-independent. However, insertion of latrotoxin molecules into lipid bilayer to form channels happens much more readily when a positive potential is applied to the bilayer on the same side as the latrotoxin[36]. Binding is influenced by pH, temperature and ionic composition of the medium. Virtually all divalent cations (Ca^{2+}, Mn^{2+}, etc.) increase latrotoxin binding to its receptor except for Mg^{2+} [35,36]. The only known inhibitor of alpha-latrotoxin binding (and therefore alpha-latrotoxin effect) is the plant lectin concanavalin A, which may bind to sugar residues at or near the receptor causing block by steric hindrance [35,36,37,38]. The toxin is irreversible or is only slowly reversible; once bound to its membrane receptor it stays bound for many hours.

Latrotoxin as a Research Tool

Natural toxins are valuable tools in the study of neurotransmitter secretion, and this is particularly true for alpha-latrotoxin. Simply by using alpha-latrotoxin as a secretagogue (a tool for causing release), key support has been gained for (1) Ca^{2+}-dependent endocytosis, and (2) the vesicular theory of neurotransmitter release[39,40]. The following two sections review these important latrotoxin-related discoveries. It should be added that latrotoxin experiments have also resulted in the discovery of key proteins in nerves that affect the release process. These proteins are covered, in the last section entitled, "Proposed Underlying Mechanism of Latrotoxin's Non-Ionophore Action".

The Study of Endocytosis

The secretory products of many secretory cells are released by exocytosis from intracellular storage organelles after the membrane of the organelle has become continuous with the plasmalemma. The factors that control exocytosis have been well studied, and in many systems this process is believed to be triggered by the influx of extracellular Ca^{2+} near the sites of secretion[41].

This exocytotic event is usually followed by an endocytotic event that recovers an equivalent area of membrane from the plasmalemma[41]. At the neuromuscular junction, endocytosis occurs rapidly enough to maintain the

population of secretory organelles, the synaptic vesicles, at near normal levels during long periods of intense secretion[42,43]. The factors that influence endocytosis, on the other hand, have been less studied[44]. However, there is evidence that endocytosis, like exocytosis, requires Ca^{2+}.

When Ceccarelli and Hurlbut applied BWSV to frog muscles bathed in a Ca^{2+}-free solution containing EGTA and 4 mM magnesium ions, massive release of quanta occurred. Within 1 hour, neuromuscular transmission was totally and irreversibly blocked, and the nerve terminals were swollen and depleted of vesicles. Horseradish peroxidase (HRP) was then added to the bath. Normally, HRP is internalized whenever endocytosis occurs and is included inside any new vesicles made. Since HRP catalyzes the formation of an electron-dense reaction product that is visible under an electron microscope, any new vesicles made are also visible. That no HRP reaction product could be seen in the nerve terminals after quantal depletion by latrotoxin implies that vesicle reformation did not occur under these conditions (Mg^{2+} present, no Ca^{2+})[41].

Yet when BWSV was applied to other muscles bathed in a solution with 1.8 mM Ca^{2+} and 4 mM magnesium ions, the rate of secretion rose to high levels and then declined to intermediate levels that were sustained throughout the hour of exposure. Neuromuscular transmission was blocked in fewer than 50% of these fibres. The ultrastructure of these terminals was normal and they

contained large numbers of synaptic vesicles which were labeled with HRP reaction product, indicating that uptake of HRP had occurred via the formation of new vesicles. These observations suggest that Ca^{2+} is required for endocytosis at the frog neuromuscular junction.[41]

Misler and Falke's results with Sr^{2+} -containing Ringer resemble those with Ca^{2+}-containing Ringer. Their results with Mn^{2+} -containing Ringer with 1 mM EGTA and no added Ca^{2+} resemble those with Mg^{2+}-containing Ringer with 1 mM EGTA and no added Ca^{2+}. Thus strontium ions or calcium ions seem able to support synaptic vesicle recycling, but not magnesium or manganese ions at the frog neuromuscular junction. This is probably why latrotoxin-treated terminals release a larger sum of minEPPs with delayed quantal exhaustion if Ca^{2+} or Sr^{2+} versus Mn^{2+} or Mg^{2+} are present.[45]

The Vesicle Hypothesis

Upon alpha-latrotoxin application, the total number of neurotransmitter quanta released from a nerve terminal (about $4x10^5$ as measured electrophysiologically[25]) correlates with the number of synaptic vesicles exocytosed (as measured using electron microscopy) in the cases of Mg^{2+} [25] and Mn^{2+} [45], suggesting that toxin-stimulated neurotransmitter release occurs via exocytosis[46]. Before BWSV, there was no known way of fully depleting synaptic

vesicles--even intense stimulation would not cause depletion. In the cases of Ca^{2+} and Sr^{2+}, quantal exhaustion less clearly translates morphologically into vesicular depletion; this discrepancy probably exists because Ca^{2+} and Sr^{2+} both support endocytosis--vesicular supplies being restocked as they are depleted[45].

Electron micrographs taken after BWSV application in the presence or absence of external Ca^{2+} reveal that the surface area of the subsynaptic membrane has increased a great deal and that there are more infoldings. It is also observed that large particles (presumably proteins) which once appeared on vesicular membranes have moved to the subsynaptic membrane after BWSV treatment. Both observations are consistent with the idea that vesicles have fused with and thereby augmented the subsynaptic membrane, with exocytosis happening more quickly than endocytosis[47]. It also suggests that latrotoxin acts presynaptically.

Latrotoxin-Evoked Release Requires Divalent Cations

At the vertebrate neuromuscular junction micromolar concentrations of a variety of divalent cations (Ca^{2+}, Sr^{2+}, Ba^{+}, Mn^{2+}, Cd^{2+}, Zn^{2+}, and Mg^{2+}) have been shown to support the increase in fm (to as much as 100-200 minEPPs /second from 1 minEPP /second over several minutes) seen after latrotoxin application. The rate of fm rise is proportional to external cation concentration

[45,48]. At the frog cutaneous pectoris neuromuscular junction Ca^{2+}, Sr^{2+} and Ba^{2+} increase fm at less than or equal to 50 μM; and Mg^{2+} at concentrations greater than 1 mM.

Without divalent cations, no affect of alpha-latrotoxin on release is seen, though the toxin can still bind[45,48]. Brain synaptosomes and clonal pheochromocytoma (PC12) cells also require the presence of divalent cations before they will respond to latrotoxin application. Nonetheless, it is clear that the divalent cations do not act by increasing latrotoxin binding to the cells[49,39].

Divalent Cations Other Than Ca^{2+} Do Not Act by Releasing Ca^{2+} from Intracellular Stores

How divalent cations other than Ca^{2+} cause release after latrotoxin application is not known. It was hypothesized that these divalent cations are transported across the plasmalemma via latrotoxin channels much like Ca^{2+}, and that they support release directly[48,39].

It has also been suggested that latrotoxin-induced release in the absence of external Ca^{2+} is due to release of Ca^{2+} from intracellular stores, not due to direct effects of other divalent cations present (rises in intracellular Ca^{2+} are known to activate exocytosis, although the mechanism/s are unknown). To check

for rises in intracellular Ca^{2+} after latrotoxin treatment, PC12 cells and brain synaptosomes were loaded with the Ca^{2+} indicator, quin2. Cells loaded with quin2 emit light whenever the intracellular concentration of free Ca^{2+} increases. One group of cells was bathed in Mg^{2+}-containing, low-Ca^{2+}-EGTA (10^{-8} M Ca^{2+}) Ringer solution, the other group was bathed in a regular Ringer solution containing 1.8 mM Ca^{2+} in addition to Mg^{2+}. When latrotoxin was added to both groups of cells, the intracellular free Ca^{2+} concentration increased in the Ca^{2+}-treated but not Ca^{2+} deficient/ EGTA cells, even though magnesium ions were present under both conditions[50]. Adam-Vizi et al. did equivalent experiments in synaptosomes using fura-2 fluorescence to estimate intracellular free [Ca^{2+}] and found no increase in intracellular Ca^{2+} as a result of applying latrotoxin to the cells in a Ca^{2+}-free medium, but a big increase in intracellular Ca^{2+} in a Ca^{2+}-containing medium plus latrotoxin[51]. These studies indicate that alpha-latrotoxin is unable to cause a redistribution of Ca^{2+} from intracellular stores to the free cytoplasm[50], and implies that divalent cations like Mg^{2+} do not cause release indirectly by raising intracellular Ca^{2+}.

Inhibitory Ions Can Be Excitatory When Given Access to the Nerve Terminal Cytoplasm

Many divalent cations other than Ca^{2+} can support release of quanta from nerve terminals in the absence of latrotoxin; Ba^{2+} and Sr^{2+} are classic examples

of cations which enter through calcium channels and cause release[52]. However, divalent cations that inhibit neurotransmitter release under normal circumstances, such as Mg^{2+}, also have excitatory effects after latrotoxin application. The inhibitory effects of Mg^{2+}, under standard conditions, are probably not due to any direct suppression of the intracellular release process. Rather, Mg^{2+} inhibits release because it hinders the entry of Ca^{2+} through calcium channels while not itself penetrating very well. After latrotoxin application, however, Mg^{2+} can penetrate latrotoxin channels and thereby gain access to the nerve terminal cytoplasm. Having done so, it appears that Mg^{2+} then works from within the nerve terminal[53] to cause release by some mechanism.

In order to support the above idea, it is necessary to show that alternative methods of getting Mg^{2+} into the nerve terminal besides latrotoxin also promote release in the absence of Ca^{2+}. In fact, prolonged high frequency motor nerve stimulation in Mg^{2+}, Mn^{2+}, or Ni^{2+} alone causes a concentration-dependent increase in fm[54,55]. In a similar way, incubation of the muscle with cation-selective ionophores in Ca^{2+}, Sr^{2+}, Ba^{2+}, Mn^{2+} or Co^{2+}[56] all enhance transmitter release from secretory cells. Only micromolar-to-millimolar concentrations of the test divalent cations are required for the release to be observed, as with latrotoxin-treated preparations. We also know that in the virtual absence of extracellular Ca^{2+} and in the presence of high extracellular Mg^{2+}, bursts of

minEPPs can occur through hyperpolarization of frog motor nerve terminals[57], just as hyperpolarization of synaptosomes suspended in media containing various divalent cations sans Ca^{2+} causes cation entry and transmitter release[58]. Overall, it seems likely that Mg^{2+} and other ions can themselves promote release under various conditions, not just after latrotoxin application.

Latrotoxin-Induced Bursts

Del Castillo and Pumplin discovered that BrWSV application resulted in the appearance of well-defined volleys of minEPPs--sudden bursts of elevated minEPP frequency. These volleys, in normal Ca^{2+}-containing Ringer were characterized by a sharp onset and sudden end. Within a volley, the frequency of minEPPs was relatively constant, having a rate of 100-300/s. The occurrence of volleys did not fit a Poisson distribution, so the volleys were not random, or completely independent of one another. If one volley occurred, the probability was above average that another would follow it soon after. The volleys observed by del Castillo and Pumplin occurred at a mean rate of 0.98 per minute. There were both long and short volleys. In normal Ca^{2+} Ringer solution, the volleys observed by del Castillo and Pumplin lasted from 3.6 to 30 seconds. Volley durations were independent of the times at which they originated. The volleys reappeared at intervals over a period of 1-4 hours after which the muscle fibres became electrically silent[59].

The sharply defined, intermittent bursts of release observed after BrWSV application in normal Ca^{2+} Ringer[59] contrast with the progressive, uniform increase in fm evoked by BWSV in low Ca^{2+} EGTA Ringer in the presence of 4 mM Mg^{2+}; intermittent bursts of release are not observed in the absence of external Ca^{2+}, only a sustained fm increase[25].

MinEPP volleys occurring in 0.6 mM Ca^{2+}, 21.4 mM Mg^{2+} Ringer had a more gradual onset and offset than volleys in normal Ca^{2+} Ringer solution--the rise and fall in fm were gradual and unabrupt. The volleys in high-Mg^{2+} also lasted much longer than the normal Ringer volleys did--in some cases the raised fm was sustained until permanent electrical silence occurred[59].

The binding of venom molecules to the membrane is not followed immediately by the occurrence of volleys. Indeed, volleys of minEPPs are observed at times up to 4 hr after the addition of venom to the bath, and occur even though the venom solution has been replaced by normal Ringer solution. Since it is unlikely that such a delay could be due to diffusion, it is reasonable to think that the initiation of a volley requires not only the presence of the venom, but also the intervention of some 'triggering event' having a certain activation energy[59].

Ca^{2+}-Dependence of Bursting

Del Castillo and Pumplin studied bursts in 18 mM Ca^{2+} as well as normal Ringer. The researchers found that in 18 versus 1.8 mM Ca^{2+}, the initial burst fm was higher than normal but declined to intermediate levels within 5 seconds. The burst fms tapered off, rather than abruptly returning to baseline as seen with bursts in 1.8 mM Ca^{2+}[59].

Bursts are Localized

Del Castillo and Pumplin raised the question of the spatial origin of the minEPP bursts. Two extreme possibilities can be considered. Volleys may be made up of quanta released from diverse parts of the motor nerve terminal membrane, or a volley may represent a localized event occurring at a small site on the terminal membrane. Two lines of evidence suggest that bursts are localized to single active zones. The first line of evidence is derived from simultaneous intracellular and extracellular recordings of minEPPs. The second line of evidence is derived from visualizing the nerve terminal using electron microscopy.

<u>Evidence From Simultaneous Intra- and Extracellular Recordings of Bursts</u>

Del Castillo and Pumplin took simultaneous intra- and extracellular recordings of bursts from frog muscle treated with brown widow spider venom. The intracellular recordings were made using a sharp electrode penetrating an individual muscle fibre millimetres from the insertion of the nerve into the muscle fibre. The extracellular recordings, on the other hand, were made using a blunter electrode positioned in the vicinity of the synapse itself. The amplitude of the extracellularly-recorded minEPPs provided a means of distinguishing near from less near events, since the amplitude is inversely proportional to the distance from the recording electrode. Because the amplitude of the extracellular events quickly became insignificant with distance, the extracellular electrode could provide information only on release in the immediate vicinity (e.g., several nearby active zones), while the intracellular electrode saw release events from all areas of the nerve terminal membrane. It follows that all minEPPs that appeared on the extracellular record also appeared on the intracellular record, but not vice versa.

Del Castillo and Pumplin saw minEPPs of different sizes and therefore distances from the extracellular recording electrode. Yet the minEPPs comprising bursts all had the same extracellularly recorded "amplitude" and therefore the distance from electrode to nicotinic acetylcholine receptors (nAChRs) was the same. Since the same set of nAChRs was activated for each

burst minEPP, it was reasonable to suppose a single, corresponding release site for the bursts as well.

Also, where extracellular and intracellular recordings were taken simultaneously from bursting cells, the burst was either visible in its entirety on both electrodes or only on the intracellular record. Yet if bursts are diffuse events, they should be accompanied by an increase in the frequency of the minEPPs recorded externally from any active spot within the end-plate. To the contrary, a dramatic increase in the external fm was only seen when the burst happened to originate at, or in the immediate vicinity of, the extracellular electrode implying that bursts are highly localized discharges[59].

Similar experiments had been performed by del Castillo & Katz, to establish the diffuse nature of normal ACh release. Del Castillo and Katz compared recordings obtained with intracellular- and extracellular micro-electrodes and saw that the site of liberation of ACh packets changed continuously within the nerve terminal and only occasionally was one of the internally recorded minEPPs produced in the vicinity of the extracellular micro-electrode[57]. Del Castillo and Pumplin themselves confirmed that normal release occurs from diffuse areas of the nerve terminal[59].

Bursts do not occur in the absence of Ca^{2+}, as discussed on page 24, even if Mg^{2+} is present. Still, Mg^{2+} application will cause a smooth rise in fm that will continue until quantal exhaustion and vesicular depletion have occurred. This unabrupt increase in fm is the result of increased release from a broad population of active zones, not a single active zone as with Ca^{2+}. This was established by Del Castillo and Pumplin using the above intra-/ extracellular recording technique. Quite simply, BrWSV application in the presence of Mg^{2+} and the absence of Ca^{2+} always caused an increase in both internally and externally recorded fm, so the increased release occurred from diffuse active zones[58]. Why this occurs, given that the latrotoxin channel is probably localized, is unclear--it may be that Mg^{2+} is able to diffuse within the nerve terminal without becoming bound by proteins which complex Ca^{2+}. In this way, Mg^{2+} would be able to spread to multiple active zones from a single entry point to increase release globally.

Electron Micrograph Evidence

Visualizing nerve terminals which are only partially depleted by BrWSV in Ca^{2+} Ringer shows that there is not a uniformly decreased density of vesicles; instead, adjacent regions contain an apparently normal density of vesicles or no vesicles at all[60]. According to Clark et al., the subsynaptic membrane near the depleted regions had infoldings. Such infoldings suggest that a large number of

vesicles fused to augment the subsynaptic membrane at those areas. The well-defined vesicle-poor areas, which exactly correspond to the areas of infolded subsynaptic membrane, are probably caused by depletion of single active zones due to bursts. Nerve stimulation results in depletion from multiple active zones[60].

Other Ways to Cause Bursts

Bursts of minEPPs can also be caused by applying a direct hyperpolarizing current to the nerve terminal membrane. By hyperpolarizing the nerve terminal in this way, the baseline fm stays the same or decreases slightly until, unexpectedly, a burst of minEPPs is seen. Early authors attributed this effect to a "membrane dielectric breakdown", but it is unknown why hyperpolarization of the nerve terminal causes bursts[61].

Various types of ionophores are reported to cause bursts; ionomycin and X-537A (lasalocid) are examples[56].

Mechanisms of Latrotoxin-Evoked Release

It has been suggested that alpha-latrotoxin has a dual action in triggering exocytosis from nerve terminals. It generates a channel in the plasma membrane that leads to an increase in intracellular excitatory cations, including Ca^{2+}, Mg^{2+}

and Na^+ (see below). Secondly, the toxin interacts with the protein secretory machinery of the cell to cause transmitter release. When cells are bathed in physiological media, both mechanisms are active in triggering secretion. In the absence of Ca^{2+} or other stimulatory divalent cation, exocytosis is triggered solely by the direct interaction of the toxin and the channel activity is redundant. The next two sections summarize evidence for both proposed mechanisms of latrotoxin-induced secretion in turn.

Ion Channel Formation

The simplest theory explaining the action of alpha-latrotoxin on nerves is that the toxin works by forming divalent cation-permeable channels. The divalent cations pass through alpha-latrotoxin channels and trigger exocytosis from release sites directly beneath the subsynaptic membrane[62].

Alpha-latrotoxin-induced secretion in Ca^{2+}-containing solutions is accompanied by a very large, sustained increase in intracellular Ca^{2+} in both synaptosomes[63] and PC12 cells[49] after a short delay (probably the time needed for toxin binding). This was evidenced by measuring $^{45}Ca^{2+}$ influx and intracellular Ca^{2+}[49,50,64]. The observed rises in intracellular Ca^{2+} after latrotoxin application occurred even in the presence of calcium channel blockers like verapamil and D600[49,64,38]. Nonetheless, an anti-latrotoxin antibody could prevent the entry of Ca^{2+}; a monoclonal anti-alpha-latrotoxin antibody which impairs the

toxin stimulatory action but does not influence its binding to the receptor, also inhibits toxin-induced channels in artificial membranes[64].

Though alpha-latrotoxin causes release in the absence of Ca^{2+}, no release is seen if other divalent cations (e.g., Mg^{2+}) are also absent[46,48]. Also, latrotoxin's activity can, in some cases, be blocked if the latrotoxin channel is blocked. In PC12 cells and synaptosomes, La^{3+} and Gd^{3+} do not interfere with alpha-latrotoxin binding but block both Mn^{2+} influx and alpha-latrotoxin induced secretion[53]. This provides further support for the idea that latrotoxin acts as a channel to cause an influx of divalent cations that is essential for latrotoxin to trigger neurotransmitter release.

Increasing extracellular K^+ from 2 to 25-40 mM, which should depolarize the resting nerve terminal[53] and hence decrease the electrical driving force for cation entry, reduces the rate of rise in fm resulting from alpha-latrotoxin application or produces an absolute reduction in fm. Hence changes in fm appear to parallel changes in the electrochemical gradient for passive multivalent cation entry into the terminal. This is consistent with the idea that alpha-latrotoxin increases the passive permeability of the plasmalemma to cations.

Since alpha-latrotoxin (and whole venom) appeared to be devoid of any detectable enzymatic (phospholipase, proteinase) activities[27], it was reasonable to propose an "ionophore" (channel forming) mechanism for its action.

Along with synaptic vesicle exocytosis, alpha-latrotoxin depolarizes the presynaptic membrane, an observation consistent with cation entry[64]. The depolarization is probably due to Na^+ influx in the absence of divalent cations, and it still occurred in the presence of the specific Na^+ channel blocker, TTX[48,38,64].

Large or prolonged Na^+ (or other cation) influx, by depolarizing the nerve terminal can result in the inactivation of sodium channels. Without sodium channels to conduct the axonal inward Na^+ current, nerve conduction fails and stimuli never reach the nerve terminal to cause increased divalent cation entry and release of neurotransmitter. This is probably the mechanism whereby large amounts of BWSV eventually cause neuromuscular blockade. Evidence for this view comes from the work of Gorio and Mauro. Frog neuromuscular junctions were pretreated with BWSV in a solution containing no divalent cations, no sodium and 1 mM EGTA. Then, cations were added back in addition to sodium and an antivenin to block the latrotoxin channel. With the antivenin, action potential failure was never seen unlike without the antivenin. Neuromuscular failure that occurred without antivenin could be reversed with the addition of

antivenin. Thus block of neuromuscular transmission probably occurs due to loss of presynaptic membrane potential due to buildup in the nerve terminal of cations like Na^{+}[65].

Surprisingly, there is no homology of alpha-latrotoxin with any known ion channel protein; nonetheless it has large transmembrane domains and might well represent a new class of channel-forming protein[66]. A low-molecular weight protein which may be an ionophore has been found in alpha-latrotoxin preparations. It is therefore possible that this adhering protein, and not latrotoxin itself, forms channels[67].

Patch Clamp Studies

The ultimate evidence that latrotoxin forms channels that allow the entry of excitatory ions comes from patch clamp studies.

XENOPUS OOCYTES

In one patch clamp study, mRNA from rat brain was expressed in Xenopus laevis oocytes[69]. Whole and single-channel patch clamp recordings were taken to check for the formation of latrotoxin ion channels. None were found until alpha-latrotoxin was added to the injected oocytes. The channels that

appeared had single-channel conductances varying from 3 to 200 pS and consistent conductance sublevels were observed. Currents reversed at 0 mV, and the channels were permeable to Ca^{2+}, Na^{+} and K^{+} indicating that non-selective cation channels were produced by addition of the toxin (Mg^{2+} conductances weren't measured). Channel openings came in groups of bursts. Open probability increased slightly with hyperpolarization. Elevation of external Ca^{2+} or toxin concentration promoted the appearance of groups of burst openings. The single-channel conductance, reversal potential and channel open probability did not depend on Ca^{2+} or toxin concentration. This establishes that the combination of latrotoxin, latrotoxin receptor and membrane can result in the formation of novel ion channels[68].

BILAYER

Finkelstein was the first to put forward the hypothesis that alpha-latrotoxin creates ion channels. He added alpha-latrotoxin to pure lipid bilayer membranes (i.e., membrane lacking the alpha-latrotoxin receptor) and observed that long-duration, nonselective cation channels were formed that had highly dispersed single channel conductances ranging from 100-400 pS. The channels were permeant to Na^{+}, K^{+} and Ca^{2+} [69]. Similar experiments showed the channels would conduct alkali earth metals ($Ba^{2+}>Sr^{2+}>Ca^{2+}>Mg^{2+}$)[70]. Their conductance was affected by the lipid composition of the bilayer, and the channels were not highly

voltage sensitive[71]. Presumably, these channels in artificial bilayer were formed by latrotoxin alone, as no other proteins were present. However, the affinity of alpha-latrotoxin for the bilayer was three orders of magnitude less than latrotoxin's known affinity for neuronal membrane (much more latrotoxin was needed). Still, the experiment establishes that alpha-latrotoxin alone (plus an adhering small peptide[67]) has the ability to form ion channels in membrane.

In addition, the idea that latrotoxin can more easily form channels when combined with its receptor neurexin was pursued in further experiments in which latrotoxin, the receptor neurexin, and a complex of the two proteins were prepared in separate liposomes and added to bilayer. Alpha-latrotoxin, by itself, gave a channel with conductances of 60 and 240 pS. The receptor neurexin alone produced no channel activity, whereas the alpha-latrotoxin/ receptor neurexin complex very rapidly produced channels of 65-70 pS[72]. This indicates that the alpha-latrotoxin/ receptor neurexin complex also has channel-forming properties in bilayer, but leaves great uncertainty as to the true conductance of latrotoxin channels. Filippov et al. have suggested that the conductance variability is genuine and due to clusters of cooperatively acting latrotoxin subchannels[68] (see section after next).

PC12 CELLS

Application of alpha-latrotoxin to cultured adrenal chromaffin (PC12) cells also leads to formation of ion channels. Current-clamp and patch-clamp experiments in PC12 cell lines, performed with alpha-latrotoxin in the pipette, have found a noninactivating channel conductance of 15 pS[74]. This conductance is an order of magnitude lower than that seen in bilayers, but cation selectivity for Na^+, K^+, Ca^{2+} [73] and Mn^{2+} was the same[76]. The alpha-latrotoxin channels were insensitive to a broad mixture of native voltage-dependent and receptor-gated channel blockers that were included in the patch pipette[74]. Thus it seems likely that alpha-latrotoxin application results in the formation of non-physiological ion channels.

Latrotoxin Subchannels

One idea that might explain the large variation in single channel conductances is that the latrotoxin channels are clusters composed of a variable number of subchannels. Measurement of nonelectrolyte permeability gives a uniform 10 angstrom pore size irrespective of the size of the apparent unitary conductance estimated from single channel events[75]. So it has been proposed that alpha-latrotoxin channels are clusters of 10 Å subchannels. These clusters graft as a bunch to an active zone's neurexin protein (alias the latrotoxin receptor--discussed later). Channel conductances will vary depending on how many of these constituent subchannels are open at any one time. The

subchannels would have to open cooperatively as the conductance sublevels observed in oocytes are not independent of one another, suggesting the conductance states are functionally tied to one another in some way. Ca^{2+} is known to synchronize channel openings[69].

The Role of Sodium Ions in Latrotoxin-Evoked Release

Na^+, in addition to divalent cations can enter the nerve terminal via latrotoxin channels. This has been affirmed using the fluorescent Na^+ indicator, benzofuran isophthalate[76].

It has been suggested that Na^+ may play a role in promoting neurotransmitter release. There are a number of lines of evidence to support this. Ouabain, a Na^+/K^+ ATPase blocker, increases the concentration of intracellular Na^+ over time[77] by disabling the ability of cells to actively extrude Na^+ in exchange for K^+. This may be the mechanism whereby it stimulates the release of neurotransmitter from the neuromuscular junction[77], cortex slices[78] and synaptosomes[79]. It is also true that disabling the Na^+/K^+ ATPase by withdrawing external K^+ from the solution increases release, perhaps by increasing internal Na^+[80].

Secondly, veratridine, a compound acting on potential-dependent Na^+ channels depolarizes nerve terminals. Veratridine, like ouabain increases intracellular Na^+ concentration[77] and also promotes neurotransmitter release in the absence of Ca^{2+}[81].

Thus, it has been suggested that the divalent cation-independent actions of alpha-latrotoxin may partly be due to an increase in intracellular Na^+. Along those lines, Adam-Vizi et al have determined that there is a good quantitative relationship between the increase in intracellular Na^+ and the release of ACh[51].

It was also reported recently that the effect of latrotoxin has both external sodium-dependent and independent components[82]. Synaptosomes isolated from guinea pig cortex were treated with latrotoxin in Na^+- containing or Na^+-free (sucrose- or N-methylglucamine-containing) solution. Displacement of Na^+ in the medium considerably decreased but did not abolish the elevation of intracellular Ca^{2+} and (carbon-14 labeled) ACh release resulting from latrotoxin. Lack of Na^+ also resulted in the inhibition of high-affinity (carbon-14 labeled) choline uptake. Both Na^+-dependent and -independent components of alpha-latrotoxin-evoked ^{14}C-ACh release partly required the presence of either Mg^{2+} or Ca^{2+}.[83] Likewise, McMahon et al. found a greater alpha-latrotoxin-elicited glutamate release from synaptosomes in Na^+-containing versus Na^+-free medium[83].

Ultrastructural Theory

As an aside, it was initially suggested that the crucial step in latrotoxin action is a redistribution of the molecular components of the nerve terminal membrane, modulated by a microtubular-microfilament array in the cytoplasm[84]. Support for this theory comes from electron micrographs showing that, after prolonged exposure to aggressive amounts of BWSV, vesicular clumping and active zone disorganization occur. However, it is perfectly clear that active zone disorganization and vesicular clumping are not due to latrotoxin per se, they are side effects of excessive cation entry. When latrotoxin is applied in the presence of Mg^{2+} but absence of Na^{+} and Ca^{2+}, cathartic release of neurotransmitter still occurs but with no ultrastructural rearrangement whatsoever[85]. As noted by Smith et al., "Only doses of concentrated venom can sufficiently elevate intracellular Ca^{2+} to a concentration at which synaptic vesicles clump together, thus interrupting the transmitter release process." This is clear from electron micrographs. Vesicular clumping and other ultrastructural effects are not seen with transient, low dose latrotoxin application and cannot explain latrotoxin effects such as bursts seen under these conditions[86].

Latrotoxin Is More Than An Ionophore

The presence of divalent cations, under most conditions, is necessary for latrotoxin to cause release of neurotransmitter. But this is not simply because divalent cations are needed to cause release. Rather, divalent cations are required for the activation of the latrotoxin/ associated protein complex (discussed later), with the opening of a channel being one effect. Thus not only do divalent cations not enter through latrotoxin channels in the absence of extracellular divalent cations, but the channels themselves do not open. If latrotoxin channels opened perfectly well without divalent cation, one would expect latrotoxin to cause depolarizations in the absence of divalent cations due to Na^+ entry. Yet no depolarization of latrotoxin-treated synaptosomes is seen in the absence versus presence of divalent cations, though Na^+ is present and latrotoxin binding preserved[77,87]. Also, raising the concentration of extracellular divalent cations leads to more frequent or more prolonged channel openings. Adding more Ca^{2+} increases channel open probability[68], and extra Mg^{2+} causes more sustained fm increases probably for the same reason[26].

Also, it is not true that the facilitation of divalent cation entry is the sole mechanism whereby alpha-latrotoxin causes release. A few clever ways have been devised to activate dramatic latrotoxin-induced release in the absence of divalent cations. As will be discussed, there is clearly a mechanism other than latrotoxin's ionophore property by which it stimulates release.

Evidence for Non-Ionophore Action

Latrotoxin Fusogenic to Liposomes

Alpha-latrotoxin was reported to induce the fusion of liposomes with artificial lipid bilayer after the toxin was introduced into a solution bathing the opposite side of the bilayer from which a solution of liposomes was applied[88], without any ionic gradients present. This implies that latrotoxin can translocate through membranes to directly induce vesicles on the opposite side to merge with the membrane. However, orders of magnitude higher concentrations of latrotoxin are needed for liposomes to fuse with bilayer than are needed for vesicles to fuse with neuronal membrane.

Hypertonicity Effects

Another set of experiments that support a channel-independent action of alpha-latrotoxin looked at the effects of venom in hypertonic solutions in the absence of external divalent cations[48]. Addition of hypertonic solution to neuromuscular junction preparations in the absence of divalent cations normally increases the fm to a stable level; it has been speculated that this is due to an increase in divalent cation concentration intracellularly. When BWSV was added to the muscle in the hypertonic environment, a dramatic, progressive fm increase

characteristic of the venom was seen though no divalent cations were present (no bursts were seen without Ca^{2+}, though). Thus, under these conditions, BWSV can clearly cause release of neurotransmitter independent of the entry of divalent ions.

Temperature Effects

When BWSV is applied, it causes a great deal of release if the temperature of the medium is raised, even though the medium lacks divalent cations and contains 1 mM EGTA[89]. Once again, latrotoxin's action cannot here be ascribed to its ionophore property.

Latrotoxin Release Sans Electrochemical Gradient

Surkova did experiments on cultured bovine adrenal chromaffin cells. Such cells undergo exocytosis in response to various agents[90,91,92] and have the advantage that solution composition can be controlled on both sides of the plasma membrane. Permeabilization of the cells led to rapid abolition of all electrochemical gradients for ions and small molecules across the plasma membrane. Without such electrochemical gradients, channels cannot facilitate net transport of ions. Nonetheless, latrotoxin application still stimulated release of catecholamines, once again showing that latrotoxin causes release not just by

causing ion influx. However, the presence of Ca^{2+} was required for the latrotoxin release[93].

From the above four lines of evidence it is clear that latrotoxin can cause release in ways other than causing increased cation influx. It is also true that at least part of latrotoxin's effect can be activated in the absence of cations. When latrotoxin's effect to evoke quantal release is activated by whatever means, release occurs. However, it is probably important to distinguish between release occurring in the presence and absence of divalent cations. When divalent cations are present, they activate latrotoxin which results in, (1) The opening of an ion channel, so that ions diffuse into the nerve terminal and cause release and, (2) direct stimulation of the secretory machinery (covered in the last section) and thereby further release of neurotransmitter. In the absence of extracellular divalent cations, only the second result is seen after latrotoxin activation. The fact that release occurs but is less dramatic without divalent cations supports this idea. The "secretory machinery" alluded to will be covered in the last section.

Mechanisms of Non-Ionophore Action

Brief Introduction and Preview

At this point, a discussion of neuronal "secretory machinery" is required. This is because latrotoxin is believed to influence this biochemical machinery by influencing the receptor protein it binds to.

The latrotoxin receptor, which belongs to a class of proteins called neurexins, has a cytoplasmic component which is capable of interacting with intracellular proteins. In fact, it is known to bind (1) to the omega- conotoxin-sensitive Ca^{2+}-channel, (2) to syntaxin, and (3) to synaptotagmin, all of which are proteins theoretically involved in exocytosis (discussed below). By interacting with these proteins, the receptor neurexin may itself play a role in release.

In turn, by binding to the receptor neurexin, and presumably changing its conformation and functioning, latrotoxin may influence release. It seems likely that the toxin is changing interactions between the receptor neurexin and the neurexin's associated proteins, in favour of causing release.

The Receptor for Latrotoxin

Some authors suggest that the action of the toxin is due to its spontaneous insertion across the membrane leading to formation of a channel[70,71]. This suggestion leaves unexplained the high potency and strict tissue specificity of alpha-latrotoxin's action[33]. Finkelstein was the first to

propose the existence of discrete, high affinity binding sites, localized at the outer surface of the presynaptic membrane. During the last few years this hypothesis has been borne out by experiments carried out by the use of radiolabelled alpha-latrotoxin. Specific alpha-latrotoxin receptors were detected in dog, rat, guinea-pig, calf, pig and chicken brain cortex synaptosomes, in PC12 cells and more recently, at the frog neuromuscular junction[35,36,37,38]. In contrast, no receptors were found in preparations insensitive to alpha-latrotoxin (e.g., glial cells; fibroblasts; muscle cells)[94,95]. The density (number/ unit of surface area) of alpha-latrotoxin receptors varied considerably from preparation to preparation. Receptor density was especially high (over 1000 per square micrometer) at the neuromuscular junction. Also, the concentration dependence of latrotoxin binding was found to correspond closely to the neurotransmitter release effect[35,36,37].

An immunofluorescence study of the neuromuscular junction indicated that the alpha-latrotoxin receptor is localized exclusively in the presynaptic membrane[96]. There is also ultrastructural evidence; Clark et al. showed that latrotoxin application greatly increased the area and infoldings of the presynaptic membrane, suggestive of presynaptic effects on exocytosis[60]. Also, it was earlier discussed that latrotoxin promotes exocytosis of vesicles, a presynaptic function.

Meldolesi abolished latrotoxin binding by externally applying trypsin, a proteolytic enzyme, to intact synaptosomes; presumably, the trypsin cleaved portions of the exposed receptor neurexin, making it unbindable. This provided evidence that the latrotoxin receptor has an extracellular domain in addition to its cytoplasmic component (mentioned earlier)[35].

Physical Chemistry of the Receptor

The latrotoxin receptor has been successfully purified from bovine brain membranes. Since it can be solubilized only with detergents, it is probably an integral (lipophilic) membrane protein[96]. The purified, solubilized latrotoxin receptor binds latrotoxin with the same high affinity as it does when part of a membrane.[97]

The purified receptor is apparently made up of two structurally similar toxin-binding glycoproteins of molecular weight 200 000 and 160 000 Daltons. The two proteins form a complex with several, much smaller proteins which do not themselves bind the toxin. The two large proteins are specific to nerves, unlike most of the smaller ones. After the purified receptor is reconstituted in liposomes, the addition of alpha-latrotoxin results in the formation of cation channels[73].

The alpha-latrotoxin receptor proteins (160 and 200 kDa) were the first members of a novel class of neuronal cell surface proteins now known as the neurexins. Neurexin mRNAs are found only in nervous tissue[97], perfectly correlating with the distribution of the alpha-latrotoxin receptor[34]. Neurexins are constructed from three genes by differential transcription and alternative splicing, giving over 100 isoforms. According to their primary structure, they consist of three domains: the short, conserved, cytoplasmic C-terminal domain; the extensive N-terminal domain that projects into the extracellular space; and the 20-amino-acid transmembrane region which is too short to itself form a channel.

Possible Receptor Function

Many neurexins are hypothesized to be involved in cell-cell contact, adhesion and recognition. However it is believed that there is an additional role for the alpha-latrotoxin receptor neurexin, a role in vesicular release. This belief is based on the observation that the cytoplasmic tail of the receptor binds a protein specific to vesicle membranes called synaptotagmin[97,98].

Receptor Binds Synaptotagmin

Synaptotagmin is a protein that lies on the external surface of synaptic vesicle membranes (65 000 Da). Synaptotagmin binds to the cytoplasmic tail of the latrotoxin receptor neurexin--this interaction may be a fundamental step in the merging of vesicles with subsynaptic membrane. In any event, neurexin tails have been little changed throughout evolution (as seen by comparing different organisms), which suggests that neurexin/ synaptotagmin binding is important to the organism for something[98,99]. The synaptotagmin/ latrotoxin receptor interaction occurs physiologically, whether or not latrotoxin is present.

As well as the alpha-latrotoxin receptor, synaptotagmin binds calcium ions and membrane phospholipids cooperatively with a concentration dependence and specificity for Ca^{2+} resembling that of the neurotransmitter release reaction[100]. Thus it has been proposed that synaptotagmin is a Ca^{2+} sensor handily situated on vesicular membranes which governs exocytosis.

Synaptotagmin is phosphorylated by casein kinase II in vivo in a Ca^{2+}-dependent manner. It has been proposed that synaptotagmin prevents exocytosis, that is, it is a regulatory component of the secretory machinery[101]; phosphorylation of synaptotagmin decreases its ability (and thus the vesicle's ability) to bind lipids[101]. Synaptotagmin phosphorylation is specifically inhibited by the alpha-latrotoxin receptor in vitro[99], so it may be that the latrotoxin receptor

(with Ca^{2+} present) disinhibits exocytosis by preventing synaptotagmin phosphorylation.

Receptor Binds Syntaxin and Ca^{2+} Channels

The alpha-latrotoxin receptor also binds to the omega-conotoxin-sensitive calcium channel, and to syntaxin[102], a presynaptic membrane protein implicated in synaptic vesicle docking in readiness for exocytosis[103]. Both the Ca^{2+} channel protein and syntaxin also bind synaptotagmin in addition to the latrotoxin receptor, paving the way for a host of putative biochemical interactions.

"Release Machinery" Effects Speculative

It is hypothesized that the latrotoxin receptor, and the latrotoxin molecules that act on it, could be influencing (1) the docking of vesicles by acting on syntaxin[103], (2) the Ca^{2+} sensitivity of exocytosis by interacting with synaptotagmin[101], and (3) the opening of Ca^{2+} channels![104] However, it is only known that latrotoxin binds to receptor proteins called neurexins, and that these proteins in turn bind a complement of other proteins that seem fit to play a role in various aspects of exocytosis. As a result, the biochemistry of latrotoxin's non-ionophore effects, as mediated by the latrotoxin receptor, are exceedingly complex and at this time, highly subject to speculation.

Synthesis

A lot of research has been performed in the past on the electrophysiology of neuromuscular synapses. However, very little of this research exploits the use of alpha-latrotoxin or its whole venom sources as a tool for research. Though many publications on latrotoxin exist, the great majority of these papers are not electrophysiological studies, but ones involving the use of neuronal imaging or biochemical methods. Synaptic physiology studies that have been performed on latrotoxin generally use the toxin in large doses to perforate the nerve terminal membrane, which facilitates the study of the effects of various intracellular ions. It is surprising that the results of del Castillo and Pumplin, which report that low dose BrWSV causes spectacular, punctuated minEPP bursts to occur, have been almost entirely ignored by electrophysiologists. Yet the study of these bursts might be used to deduce fundamental properties of neuronal release. Thus the aim of this thesis is to use the phenomenon of latrotoxin-evoked minEPP bursts to (1) estimate the maximal rate of release from single active zones (2) determine the participation of multiple active zones during latrotoxin bursts using nerve stimulation (3) explain the results of single latrotoxin channel recordings using observations from whole cells (4) evaluate changes in burst intensity over the course of bursts, and to speculate as to their possible

significance and (5) determine the effects and potency of different divalent cations with regards to burst incidence.

METHODS

All experiments were performed on mouse hemidiaphragms, mounted on Sylgard and superperfused as previously described[61] at room temperature. Standard bathing solution contained 145 mM Na^+, 5 mM K^+, 24 mM HCO_3^-, 129 mM Cl^-, 1 mM $H_2PO_4^-$, 11 mM glucose, and was bubbled with 95% 0_2/ 5% CO_2, to which 1 mM Ca^{2+} and 1 mM Mg^{2+} were added, except as otherwise noted.

In all minEPP experiments, tetrodotoxin (Calbiochem $1x10^{-7}$ M) was used to prevent muscle twitches which can dislodge the intracellular electrode.

Mu-Conotoxin

Before nerve stimulation experiments where m was expected to be very large, the preparation was soaked for an hour beforehand in a solution containing 1 μM mu-conotoxin (Sigma). Such a treatment prevented muscle twitching without blocking the presynaptic stimulus or interfering with EPP recording; mu-conotoxin at μM concentrations selectively blocks muscle but not motoneuronal Na^+ channels[104,105].

Latrotoxin

Pure, lyophilized alpha-latrotoxin powder (Alomone labs) was used; 3 nM alpha-latrotoxin was applied transiently at the beginning of each experiment day and then washed away after the appearance of bursts (generally within 20 minutes). BWSV, obtained from ground venom sacs (Sigma) was used for a few experiments; about one-tenth of a sac was added and washed away each time after the appearance of bursts. The applications were transient in the hope that only one or two active zones would bind latrotoxin molecules and produce bursts.

Nerve Stimulation

In all stimulation experiments, the phrenic nerve was stimulated with a suction electrode as previously described[106]. Sharp glass electrodes were used to impale the muscle cells and measure forthcoming electrical signals. Muscle fibre signals were amplified and observed on an oscilloscope, and the oscilloscope signal was converted to a signal recorded on standard VHS tape. VHS tapes were later played back for analysis offline, and a number of computer programmes were used to analyze the raw data.

All experiments involving nerve stimulation were carried out using stimulus frequencies of around 10.4 Hz, to rule out synaptic potentiation, or the

progressive increase in quantal release seen over the course of higher frequency trains of stimuli.

Although efforts were made to reduce selection bias with regards to sampling cells, this was difficult due to the necessity of obtaining a clear record. Also, since bursts of minEPPs were the focus of this work, it was necessary to obtain cells with sufficiently frequent bursts to provide analyzable data. Thus secondary applications of latrotoxin were occasionally necessary, when bursts were not found. It was also necessary to obtain cells that were not over-exposed to latrotoxin, as such over-exposure results in uninterpretably high incidences of bursts, probably due to release from multiple active zones.

Analysis

All records were analyzed "off-line" using a computer programme which obtained the time of occurrence and amplitude of every event (minEPP or quantal component of EPP) within a data sequence[107]. Other programmes used the output of this to obtain minEPP frequencies and quantal contents (m) as they varied in time.

Data Output Files

A programme called "meppseq.ltl" was used to analyze minEPP data. This programme produces an output file called "seq4" which is a list of fm values derived from the wait time for 4 minEPPs. An adjacent column of numbers denotes the time corresponding to each fm value. This output provided an estimate of how fm varied in time, with far less variance than that that would be obtained from individual wait times.

Analyzing m and fm together required a programme written by Dr. Quastel called "timeseq.ltl". This programme produces an output file called "groups", which yields three columns of numbers: one for m values, one for fm values and one for the time scale in seconds. Data points for m and fm are reported at 2 second intervals.

Different Populations of Quanta

Fesce et al. observed that, "Many relatively small and some 'giant' minEPPs occur at the ends of the experiments, and the distribution of minEPP amplitudes broadens"[107]. I have noticed abnormal variability in quantal size after latrotoxin pretreatment in 9 out of 127 junctions--different minEPP populations ranging in size from 0.3 mV to 1.7 mV. Such variation in quantal size did not cause analysis problems, as all but two of the 9 junctions were excluded from consideration. The two cells with abnormal quantal variation that were

considered did not cause analysis problems, as small minEPPs were successfully counted by the computer programme used (see Bain and Quastel 1992)[108] and giant minEPPs were not counted as multiple minEPPs, but were excluded from consideration. Also, stimulation data referred to in this thesis is accurate, as templates establishing quantal size were made from the average of many minEPPs occurring from the same stretch of record as the stimuli. The cause and significance of latrotoxin-associated increased quantal variability are unknown.

RESULTS

Intracellular records showing clearly defined minEPPs and EPPs were obtained (see Fig. 1).

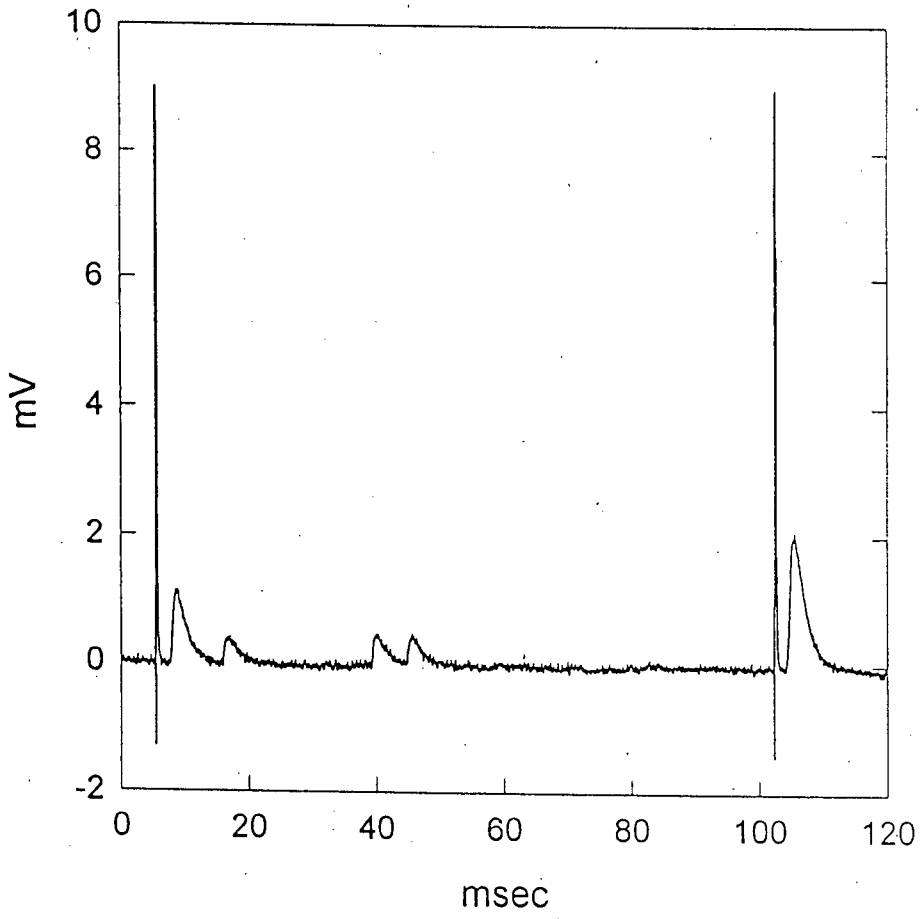

Fig. 1. A short stretch of sample record is shown in the graph. Two stimulus artifacts can be seen denoting the input of an intense, very brief pulse of current. Two EPPs, evoked by the stimuli, can be seen after brief delays. Also, some spontaneous minEPPs can be seen between pulses.

Nerve Terminal Hyperpolarization Causes Bursts

Cooke and Quastel achieved spontaneous bursts of minEPPs[61] by placing a blunt NaCl-agar extracellular electrode with a tip of diameter 25 to 50 μm in the vicinity of the nerve terminal and passing a current through the extracellular electrode in such a way that the nerve terminal was hyperpolarized. Using the same methodology, I hyperpolarized 6 nerve terminals from 2 mice and confirmed the presence of minEPP bursts in all 6 cells. However, I found that bursts caused by hyperpolarization, once initiated, often failed to die down for many minutes, even upon removal of the hyperpolarizing field. Further study of this form of bursting was not undertaken as a result, and will not be included in this thesis.

Baseline Versus Burst fm

As discussed on page 13, quantal release normally follows a Poisson distribution, and thus the variance to mean ratio of the number of minEPPs in non-overlapping time periods is equal to 1.0. However, when bursts of minEPPs are observed, release is non-Poisson, and the variance is greater than the mean. Thus, a burst is theoretically present if the variance to mean ratio of the number of minEPPs in non-overlapping time bins is greater than 1.0. However,

for purposes of analysis I defined a burst as having occurred in a 30 second stretch of record when the variance to mean ratio of the number of minEPPs in non-overlapping time bins is greater than 1.3. This provided me with a quantitative means for detecting the presence of bursts in record. Variance to mean ratios can also provide a quantitative estimate of the incidence of bursts occurring during the course of a treatment; a record with an overall variance to mean ratio of 9.6 is said to contain a higher incidence of bursts than a record with a variance to mean ratio of 2.2. Burst intensity is measured as the highest fm value occurring within the burst. Baseline fm refers to an average of all fm values during stretches of record where the variance to mean ratios are less than 1.3. Thus baseline fm reflects the average rate of release in the absence of bursts.

However, the key difference between baseline and burst fm, as discussed in the introduction, is origin of release. Baseline fm results from release of packets of neurotransmitter from many areas of the subsynaptic membrane, analogous to drops of rain falling from scattered clouds. Burst fm, however, is mostly (but not entirely, as discussed later) the result of a *single* active zone suddenly releasing a large proportion of the vesicular pool immediately available to it.

Incidence of Bursts

Del Castillo and Pumplin were the first to observe that the application of latrotoxin (in the form of BrWSV) causes rapid, sharply- defined volleys or bursts of minEPPs to appear. The minEPP volleys they observed at the frog neuromuscular junction occurred at a mean rate of 0.98 per minute[59].

It should be noted that burst incidence is extremely low without alpha-latrotoxin treatment; so low that it is essentially absent in the 1 mM Ca^{2+} solution used to bathe the hemidiaphragm preparation in the present series of experiments. I have reviewed data from 57 junctions that were never exposed to latrotoxin, hyperpolarization or ionophore chemicals and none evidenced bursts. Bursts, such as the ones discussed in this thesis, do not occur without latrotoxin pretreatment with any significant incidence.

Burst Incidence is Dependent on Extracellular Ca^{2+}

The effect of lowering the extracellular Ca^{2+} (to 0.05 mM with 0.1 mM EDTA from 1 mM Ca^{2+}) on the incidence of bursts was measured quantitatively by taking the variance to mean ratio of numbers of minEPPs in non-overlapping time periods from each treatment group. Remember from the introduction that a variance to mean ratio equal to one is consistent with a Poisson distribution, whereas a higher variance to mean ratio denotes clustering or bursts of release.

The experimental record was divided into 30-second substretches, and the variance to mean ratio for counts within 400 ms time periods within each substretch was then derived, yielding a collection of variance to mean ratios for each treatment group. Variance to mean ratios from the 1 mM Ca^{2+} group were compared with variance to mean ratios from the low- Ca^{2+} EDTA group using a two-tailed t-test assuming unequal variances. The following table summarizes the results of 8 junctions from 5 mice after latrotoxin;

Table 1

	1 mM Ca^{2+}	Low Ca^{2+} EDTA
Mean variance to mean ratio	3.63	1.80
Standard Error	0.35	0.26
Number of 30-second intervals	165	133
P	3.57×10^{-5}	

As you can see, there is a very significant difference in the overall variance to mean ratio between the two treatment groups. The low- Ca^{2+} EDTA group shows a variance to mean ratio lower (closer to one) than the 1 mM Ca^{2+} group, implying that the reduction in Ca^{2+} concentration led to a reduction in burst incidence (p=0.000036). Furthermore, del Castillo and Pumplin have reported that bursts are altogether abolished when a solution containing Ca^{2+} is replaced by one without Ca^{2+} that contains 1 mM EGTA and 4 mM Mg^{2+}[59].

Although they tested raising the external Ca^{2+} concentration from 1.8 to 18 mM, del Castillo and Pumplin id not mention any increase in the incidence of bursts due to the extra Ca^{2+}, only that there tended to be a wider range of burst

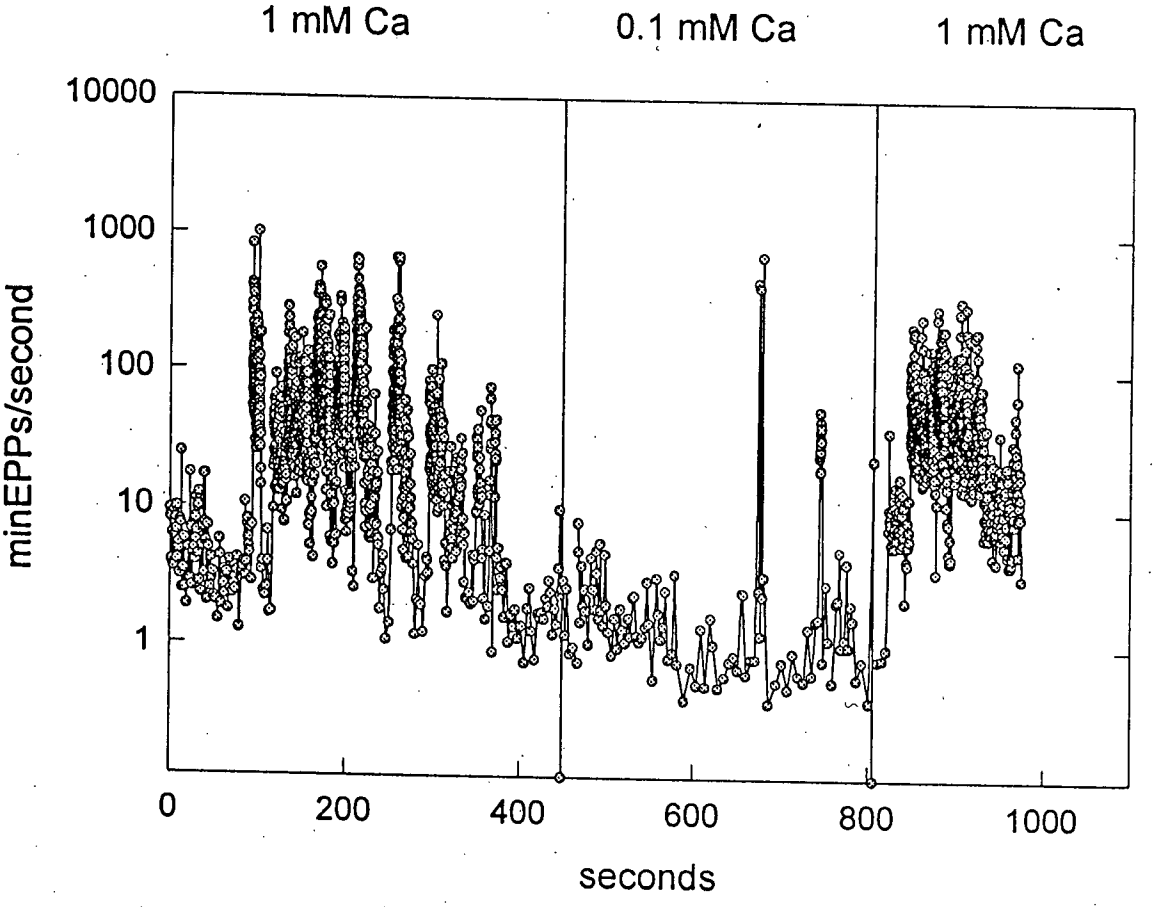

Fig. 2. Bursts occurring in alternating treatments of 0.1 versus 1 mM Ca^{2+}; burst incidence was clearly reduced in 0.1 versus 1 mM Ca^{2+}.

durations (more long, more short and less medium duration bursts). Also, although they stress that bursts require external Ca^{2+}, they do not mention that burst incidence is proportional to the external Ca^{2+} concentration[59]. However, I have observed that the incidence of bursts can be changed by changing the external Ca^{2+} concentration. For example, burst incidence after latrotoxin pretreatment can be increased by raising the extracellular Ca^{2+} concentration to 8 from 1 mM ($p<0.05$). This result was obtained by comparing the variance to mean ratio of the number of minEPPs in non-overlapping time periods in 8 mM Ca^{2+}, which was 7.93 +/- 1.23, with the equivalent ratio in 1 mM Ca^{2+}, which was 3.16 +/- 0.51 (n=4 junctions from 2 mice). Both ratios are much greater than 1.3, so the incidence of bursts was high generally, but it was higher for the 8 mM Ca^{2+} treatment. Also, Table 1 and Fig. 2 clearly show that bursts are less frequent in low Ca^{2+} solutions versus 1 mM Ca^{2+} solutions.

Other Divalent Cations and Bursts

Experiments were also performed with Ba^{2+} and Sr^{2+} added to the external bath in place of Ca^{2+} after latrotoxin pretreatment. These experiments established for the first time that Ba^{2+} and Sr^{2+}, like Ca^{2+}, are able to support latrotoxin bursts. The variance to mean ratio of the number of minEPPs in non-overlapping time periods was 2.54 +/- 0.35 in 1 mM Ca^{2+} solution, and 3.37 +/- 0.45 in 1 mM Sr^{2+} solution. Thus the variance to mean ratios were still elevated,

and bursts still prevalent, even when Ca^{2+} was replaced by Sr^{2+} (n=8 junctions from 1 mouse). Variance to mean ratios in 1 mM Ba^{2+} solutions lacking Ca^{2+} were similarly high at 4.44 +/- 0.52 versus 3.90 +/- 0.28 in 1 mM Ca^{2+} (n=10 cells from 4 mice).

Mg^{2+}, in the absence of extracellular Ca^{2+}, does not support bursts. This result was described by del Castillo and Pumplin, who added BrWSV to frog muscle bathed in a solution containing no divalent cations and 1 mM EGTA. The two researchers saw no bursts after 4 mM Mg^{2+} were introduced, only a smooth rise in fm similar to that observed by Longenecker et al[25,59].

The above results would seem to support the suggestion that Ba^{2+} and Sr^{2+} tend to act more like Ca^{2+} than Mg^{2+}; only the first three can cause bursts when added after latrotoxin. Also, only Ba^{2+}, Sr^{2+} and Ca^{2+}, but not Mg^{2+}, can support endocytosis as mentioned in the introduction, and they can penetrate Ca^{2+} channels unlike Mg^{2+}.

Mg^{2+} Causes Release After Latrotoxin Application

Since Mg^{2+} does not normally have access to the nerve terminal cytoplasm (it does not penetrate Ca^{2+} channels well), it cannot cause release under most conditions[2]. However, Mg^{2+}, like Ba^{2+}, Sr^{2+} and Ca^{2+} can cause

release after latrotoxin treatment[25,59]. Mg^{2+} causes release, however, in a manner dissimilar to that of Ca^{2+},[59] Ba^{2+} or Sr^{2+} (see previous page) in that no bursts have been observed[59] . I have found that, after latrotoxin application, increasing the external Mg^{2+} concentration from 1.1 mM to 2.1 mM caused 5 out of 12 junctions from 4 mice to exhibit a smooth, non-abrupt rise in baseline fm in solutions with 0.1 mM EDTA and no external Ca^{2+}. Other authors have stated that such a progressive rise in baseline fm is accomplished at 1 mM Mg^{2+} or less[45,48], no doubt because they used much higher concentrations of venom for longer time intervals. But what is important is that, in 5 out of 5 cases when 2.1 mM Mg^{2+} was seen to cause a rise in the fm after latrotoxin, the rise was very smooth, non-abrupt and lasting as illustrated in Fig. 3. As a result, the variance to mean ratio, calculated over 30 second time intervals, was 1.09 /- 0.05 when only 1.1 mM Mg^{2+}, 0.1 mM EDTA and no external Ca^{2+} were present, and was still only 1.12 /- 0.15 when the Mg^{2+} concentration was raised to 2.1 mM even though baseline fm increased (n=5 cells from 5 mice).

The situation changes in the presence of Ca^{2+}, at which time bursts are much more frequently seen[59] with corresponding increases in variance to mean ratios (see Table 1 for Ca^{2+} -dependence of bursting and Fig. 3).

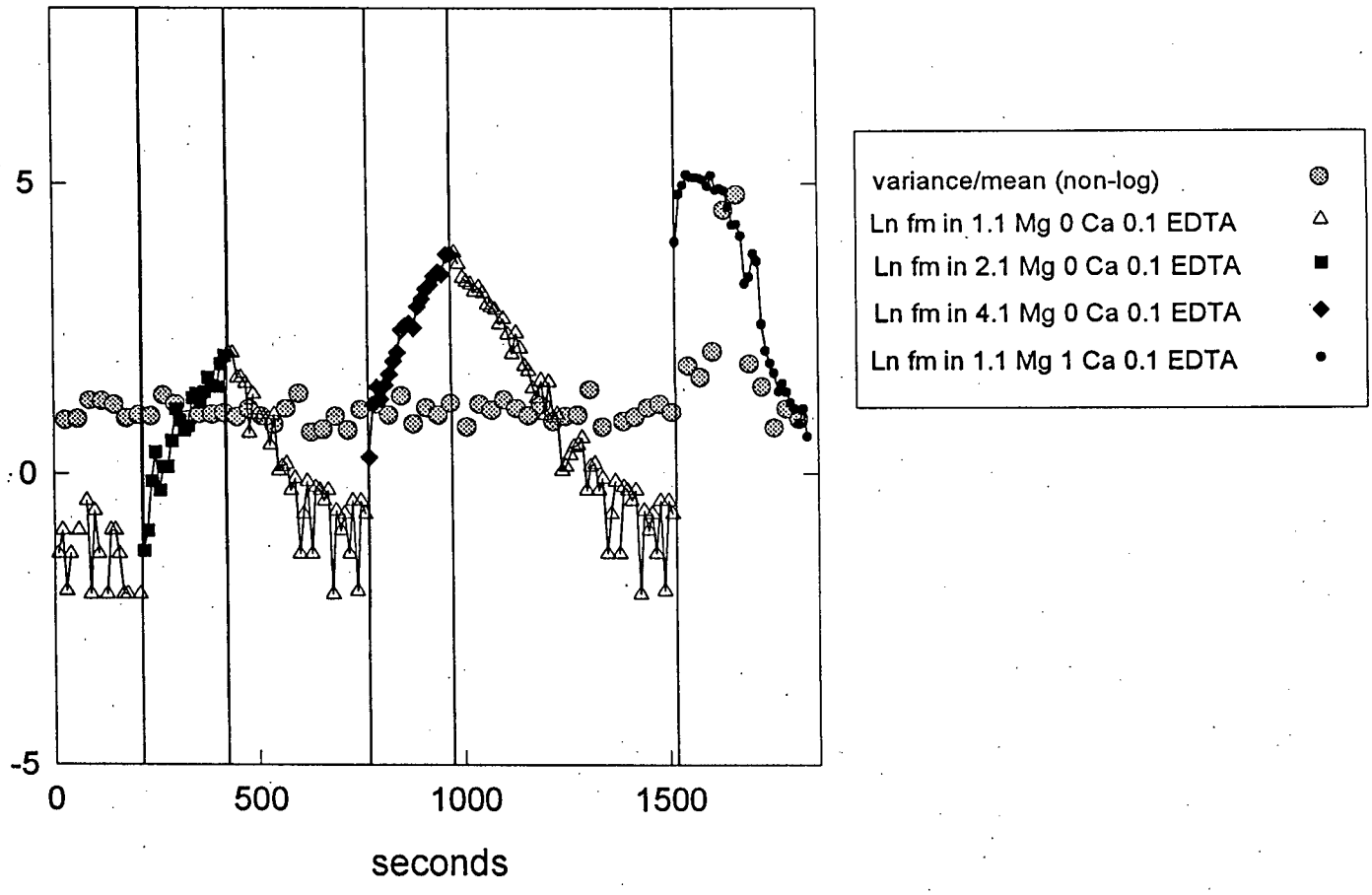

Fig. 3. Effect of a series of treatments with 1.1, 2.1 and 4.1 mM Mg^{2+} in the absence of extracellular Ca^{2+} and presence of 0.1 mM EDTA is expressed as natural log fm (black and white points). Raising the extracellular Mg^{2+} concentration is associated with a smooth rise in ln fm. The last treatment is 1 mM Ca^{2+}, 0 mM EDTA; the rise in ln fm is more abrupt and transient with the Ca^{2+} addition versus the addition of extra Mg^{2+}. A superimposed graph of variance/ mean ratio values (untransformed) is also provided to show that the variance to mean ratio is always about 1 in the absence of Ca^{2+}, even when the ln fm goes up due to raising the external Mg^{2+} concentration, yet when 1 mM Ca^{2+} is introduced, the variance to mean ratio increases substantially implying non-Poisson bursts of release not seen with any of the Mg^{2+} treatments.

Burst Intensity Varies

A truly enormous variation in burst intensity is observed; bursts fm varies between 3.5 and 2100 minEPPs/ second. Clear variability in burst intensity was observed in virtually all cells exhibiting bursts; data reviewed from 50 cells exhibiting bursts indicated that 41 of the cells showed variation in burst intensity greater than 20 minEPPs/ second. Those that did not show such variation tended to have extremely few bursts on which to make comparisons. Latrotoxin bursts, therefore, are by no means all-or-none (see Fig. 4).

Del Castillo and Pumplin observed that the frequency within a specific burst is generally quite constant--once a burst has begun at an intensity of 100 minEPPs/ second, it will tend to stay at 100 minEPPs/ second before abruptly ending; it will not fluctuate randomly between 2 and 2000 minEPPs/ second, for example[59]. Using the computer programmes provided by Dr. Quastel, I have been able to add the observation that a burst of 100 minEPPs/ second, instead of abruptly ending, can intensify to 227 minEPPs/ second, for example, or diminish to 20 minEPP/s just as abruptly. However, this is not generally seen as random fluctuation, but more often is seen as distinct steps or modes of burst intensity. "Burst archetypes" can sometimes be observed, where several common burst intensities will be repeatedly reached. The stepwise nature of burst intensity fluctuations can make graphs of fm versus time resemble the

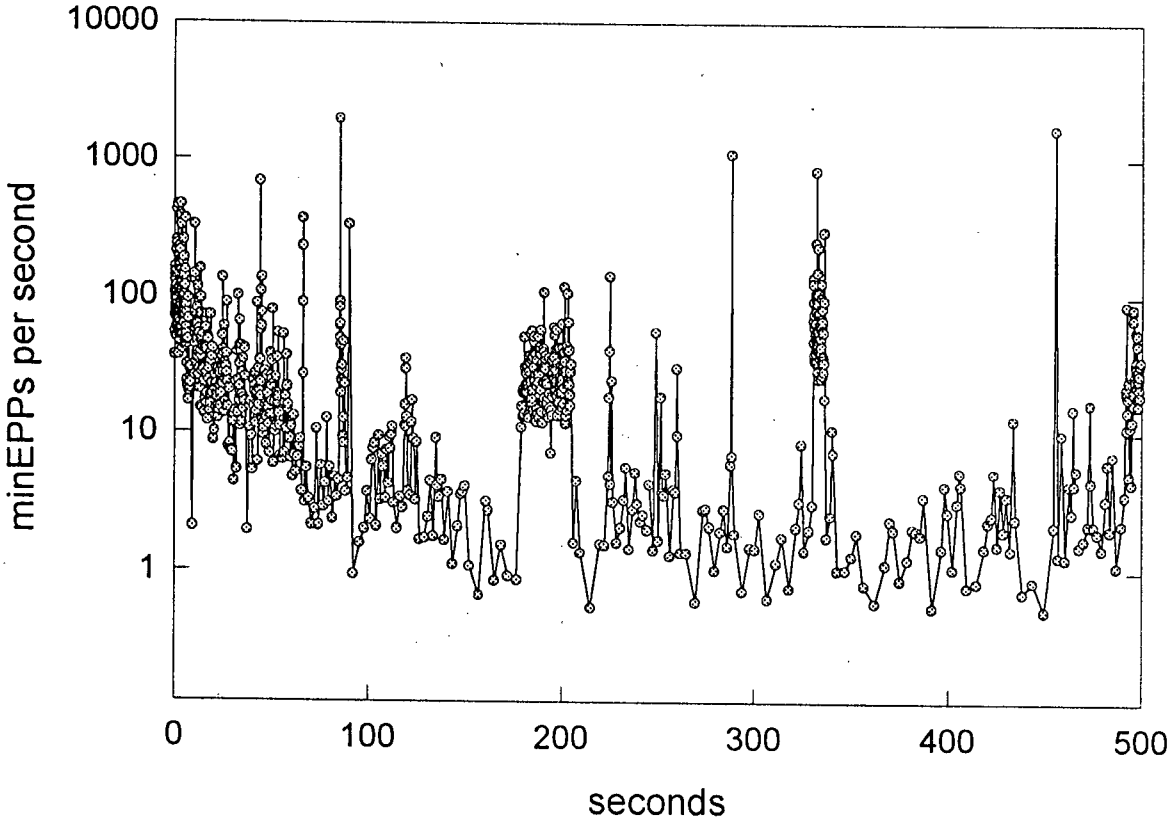

Fig. 4. Bursts of minEPPs following latrotoxin pretreatment are shown here to be dramatic, transient increases in fm of varying intensity. Solution contains 1 mM Ca^{2+} which supports bursts in addition to 1 mM Mg^{2+}. Expressed as minEPPs/ second versus time on a logarithmic scale.

single-channel current recordings of Filippov, et al., who studied latrotoxin channels in Xenopus oocytes using patch clamp methodology[69].

Reduction of Fm During Bursts

The resemblance of burst records to latrotoxin single channel current records[68], though tempting, is not perfect. Unlike latrotoxin single channel current records[68], recordings of particularly intense and prolonged minEPP bursts often show a decline in fm over the course of the burst (n=7 out of 25 junctions from 10 mice). In 4 out of 25 cells, decline in burst fm appeared to be carried over from one burst to the next; bursts occurring shortly after previous, declining bursts began at the same diminished fm and continued to decline at the same rate. At one junction, after decline had occurred for up to 95 seconds, the burst fm stopped declining and leveled off at about 7 minEPPs per second (n=1 out of 25, see Fig. 5).

The Effect of DMSO on Burst Intensity

Dimethyl sulphoxide (DMSO) is an agent that, when applied, multiplies the amount of both spontaneous release (fm) and quantal content (m)[109]. Thus if 6% DMSO multiplies release ten times, a baseline fm of 1 /s will be raised to 10 /s, and a baseline fm of 2 /s will be raised to 20 /s and the same for m.

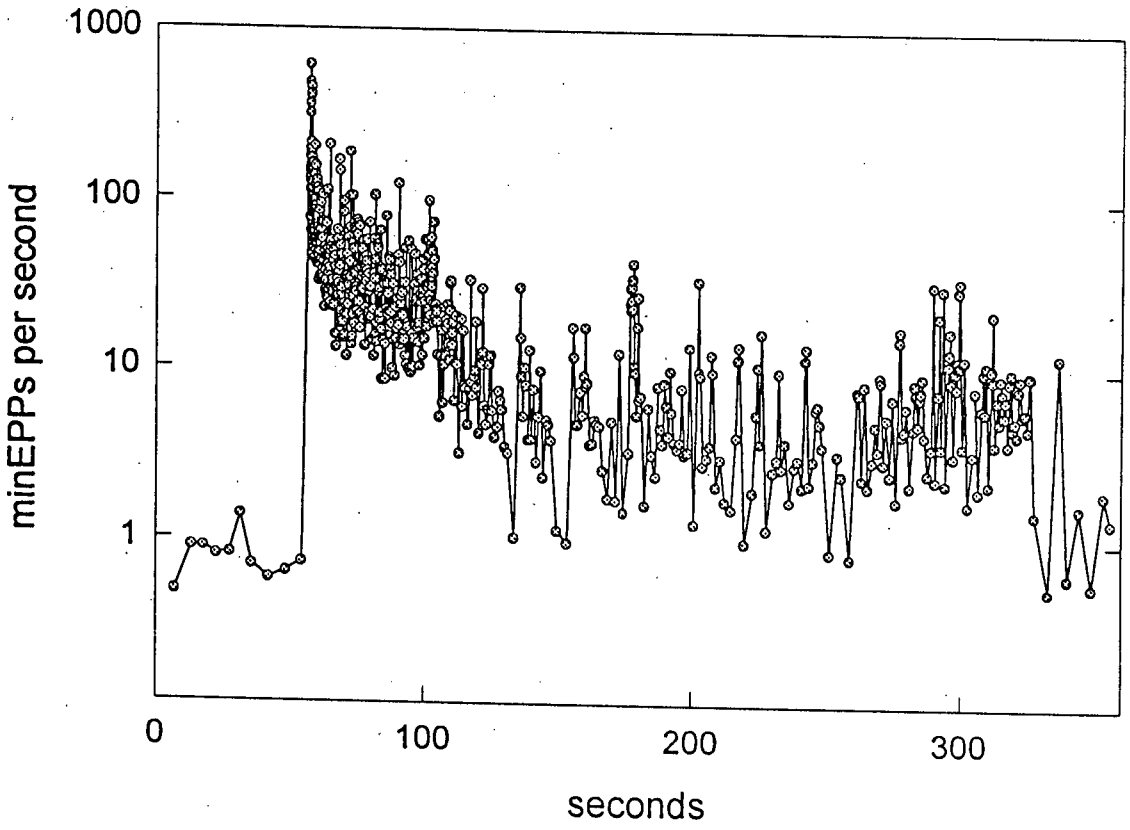

Fig. 5. Decline in the rate of release of quanta as part of a burst; burst fm tapers with time. This phenomenon is only observed during more intense, more prolonged bursts.

As mentioned, a tremendous variety of burst intensities were observed, and the start and finish of these bursts was ambiguous due to their ability to abruptly increase or decrease to different intensity levels, and due to the fm decline seen over the course of more intense bursts. As a result, a simple and reliable method of analysis was devised to evaluate burst intensity, without attempting to define and quantify each burst. Firstly, only the highest three fm values (from seq4 output files) in each 10 minute treatment were chosen. These three maximum fm values, being the highest fm values from 10 minute treatments all of which had bursts (13 out of 13 junctions from 7 mice), could unambiguously be called burst fm values. The baseline fm was determined simply by averaging stretches of record where the variance to mean ratio of the number of minEPPs in non-overlapping time periods was less than 1.3. The following table illustrates the procedure, by showing raw data from an actual junction (fm values are in minEPPs per second):

Table 2

	Top 3 fm values	Baseline fm
6% DMSO	1931.40, 1286.91, 1123.27	4.26
No DMSO	1665.70, 1649.12, 1311.60	0.88
6% DMSO	1312.91, 1036.91, 73.55	4.25
No DMSO	1687.49, 207.26, 186.23	0.72

The first column lists each 10 minute treatment; as shown, this junction was exposed to consecutive 10 minute treatments of 6% DMSO, No DMSO, 6% DMSO, No DMSO. The second column gives the top three fm values from seq4,

for each of the 10 minute treatments. Burst frequencies are very high (over 1000 minEPPs/ second!) because of the seq4 output file's high resolution of fm--short intense bursts are registered without being averaged down by slower record. The third column gives the baseline fm, derived from averaging fm values of substretches of record obviously lacking bursts.

All six peak fm values pertaining to the two 6% DMSO groups were averaged together, as were the six peak fm values pertaining to the two control groups. This procedure yielded two burst fm values to compare, one derived from bursts in 6% DMSO, the other derived from bursts in the absence of DMSO. Averaging of baseline fm values also yielded two numbers, one for each treatment. As an illustration, the following table is provided to summarize the results of this one junction; for a complete analysis summarizing 13 junctions (including standard error values) see the table after this one.

Table 3

	Burst fm	Baseline fm
No DMSO	1117.90 minEPPs/ s	0.80 minEPPs/ s
6% DMSO	1127.49 minEPPs/ s	4.26 minEPPs/ s

The next table is a summary of data from 13 junctions (7 mice):

Table 4

	Burst fm	Std. Error	Baseline Fm	Std. Error
No DMSO	754.50	159.35	0.70	0.08
6% DMSO	1307.27	181.05	6.67	0.97
Increase	**1.73X**		**9.53 X**	

It seems clear from the above table that 6% DMSO multiplies the baseline fm far more than the peak burst fm (9.53 times versus 1.73 times). To confirm this, a two-tailed t-test assuming unequal variances was performed, which compared for each junction the increase in baseline fm due to DMSO with the increase in burst fm due to DMSO. Once again, it was determined that the baseline fm was increased by DMSO far more than the burst fm overall ($p=0.0007$) (see Fig. 6 for illustration).

Although release from a bursting active zone is only weakly enhanced by DMSO, a small increase in burst fm due to DMSO is expected. This is because a burst does provoke some release from non-bursting active zones, as reflected by a greater quantal content (m) during a burst (1.25-12x, see below). This increased release from non-bursting active zones would contribute to what we see as a burst. This would be true for both control and DMSO bursts, but in 6% DMSO the increased release from other active zones would be multiplied almost tenfold (see above table). That no great increase in burst fm is seen in 6% DMSO versus control is likely because non-bursting active zones make a minor contribution compared to the bursting active zone. A burst occurring in 6%

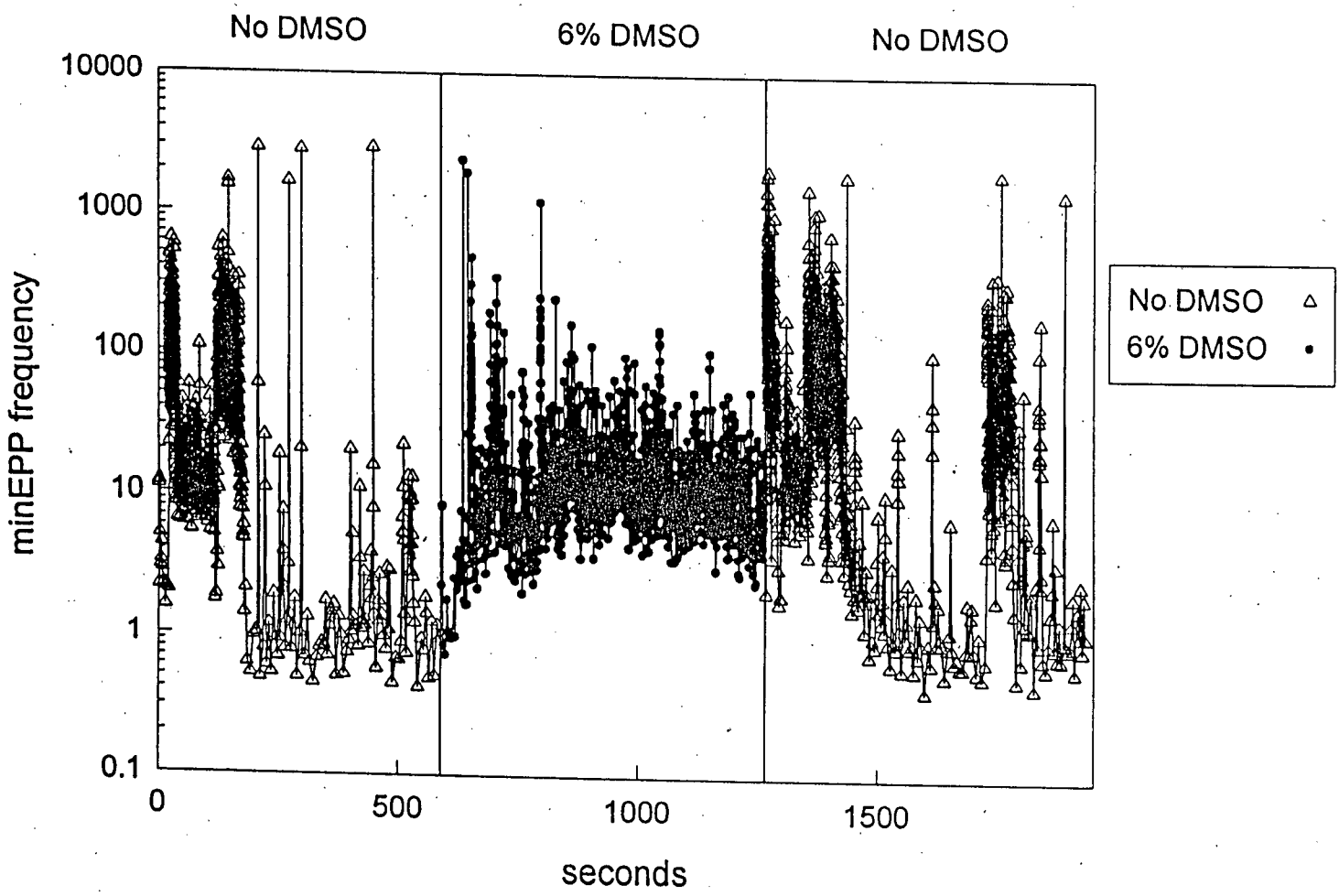

Fig. 6. Increase in baseline but not burst minEPP frequency as a result of 6% DMSO application.

DMSO might reach 1050 minEPPs per second instead of 1000 minEPPs per second, but certainly wouldn't approach 10 000 minEPPs per second. The following table illustrates the point, assuming that a burst increases fm from other active zones 5x, and 6% DMSO increases release by a factor of ten (note: data in this table are hypothetical).

Before Burst:

Table 5

	CONTROL	6% DMSO
Release from bursty active zone	0 /s	0 /s
Release from all other active zones	1 /s	10 /s
Total fm	**1 /s**	**10 /s**

During Burst:

Table 6

	CONTROL	6% DMSO
Release from bursty active zone	1000 /s	1730 /s
Release from all other active zones	5 /s	50 /s
Total fm	**1005 /s**	**1780 /s**

So the 6% DMSO would be expected to increase release from all non-bursting active zones by only an additional 45 minEPPs/ second (discussed below) which would be dwarfed by release from the bursting active zone.

Quantal Content During Bursts Is Elevated

Corrected m (defined below) goes up dramatically when there is a burst of minEPPs (see Fig. 7).

All m values discussed in this thesis are corrected m values. The m requires correction because bursts cause an unusually high number of spontaneous quanta to appear in the interval after each stimulus in which Dr. Quastel's computer programme looks for an EPP[107]. Thus there is a danger that the m will falsely appear to go up during a burst due to burst quanta being counted as EPP. The interval after each stimulus in which the computer programme registers EPPs is 0.0043 seconds, so the m correction was made by multiplying 0.0043 seconds by the corresponding fm value and subtracting the product (in quanta) from each m value, thus: m - 0.0043 seconds X fm = corrected m. Even after the m correction was made, the rise in m during a burst was obvious in all 79 junctions from 34 mice analyzed, and the burst-associated m rise was between 1.25 to over 12 times.

Also, it is clear that the m tracks the fm very closely in time; when a burst occurs, the m is very quick to rise with the fm (see Fig. 8).

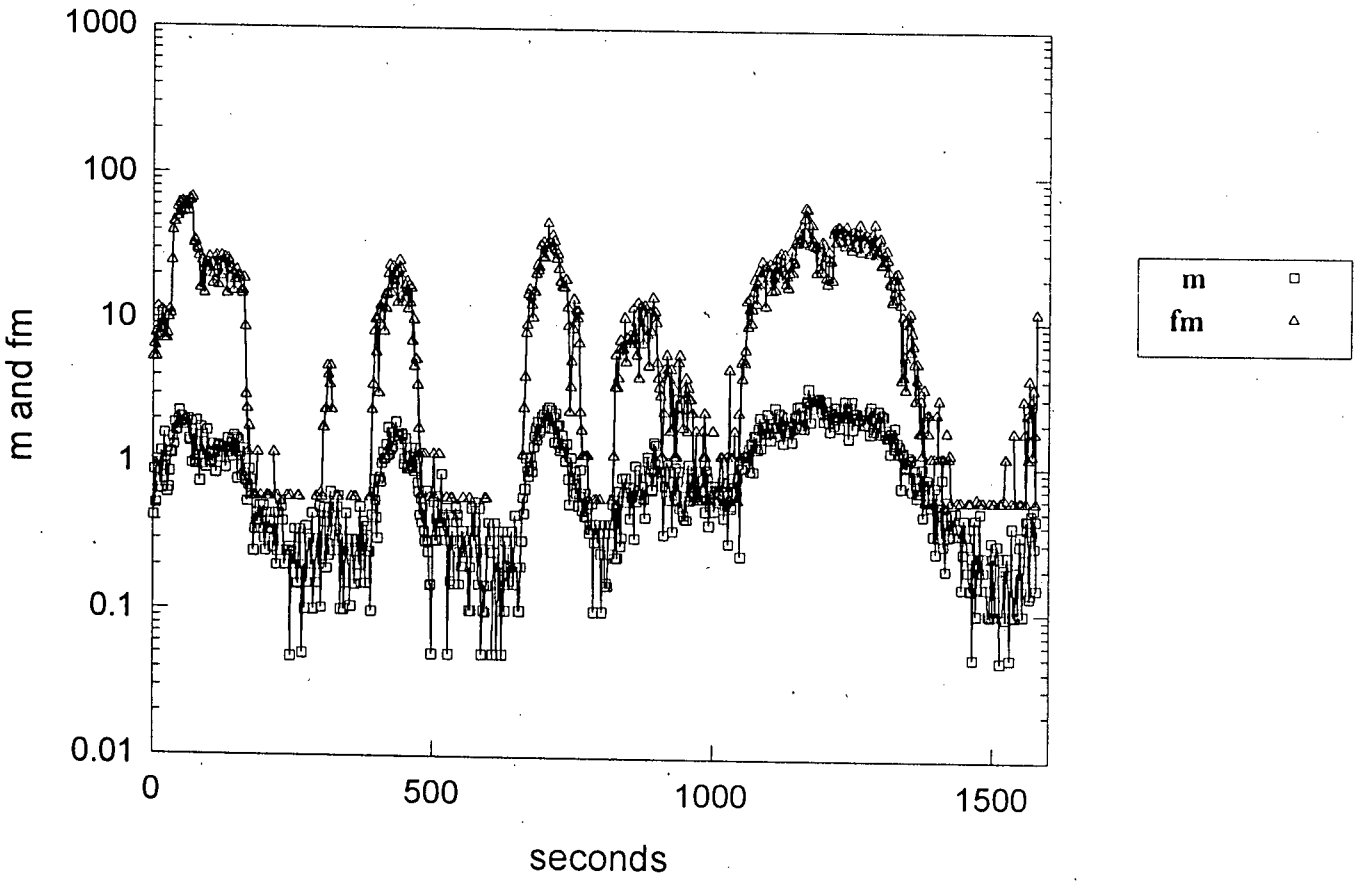

Fig. 7. m and fm values are plotted. Each point represents average release over a 2 second interval. Note that there is a dramatic tendency for the corrected m to rise whenever there is a burst.

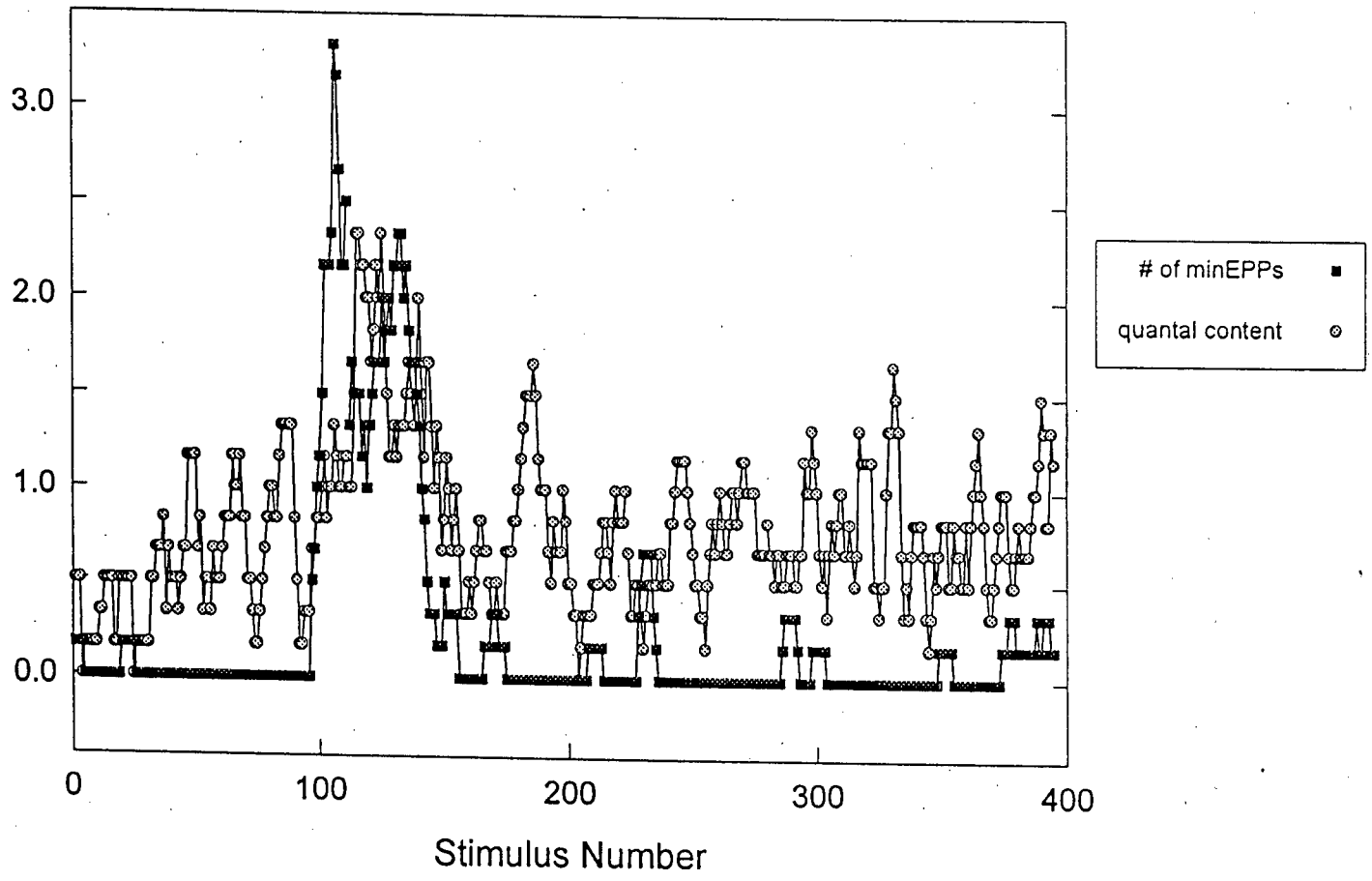

Fig. 8. Postsynaptic responses to each stimulus (as corrected number of quanta liberated by each stimulus) are shown alongside the number of minEPPs occurring between every two stimuli. Such optimally high resolution, where the results of every stimulus are given, makes it clear that there is an extremely tight temporal relationship between minEPP bursts, and concomitant rises in evoked release.

The possibility exists that the rise in m was due to the entry of large quantities of Mg^{2+} through the latrotoxin channel, which, as described by del Castillo and Pumplin (see introduction) tends to cause release from multiple active zones[59]. To eliminate this possibility, nerve stimulation was carried out in the absence of external Mg^{2+} and presence of 250 μM neomycin; since neomycin, like Mg^{2+}, blocks presynaptic Ca^{2+} channels (and was found not to itself cause release after latrotoxin), it was used to replace Mg^{2+} in order to keep the m low. A mu-conotoxin pretreatment was also used to prevent twitching (see Methods). Fig. 9 demonstrates that the corrected m still rises during a burst even in the absence of external Mg^{2+}, thus Mg^{2+} entry through latrotoxin channels cannot alone be responsible for the m rise (n=12 cells from 4 mice). To determine whether or not Mg^{2+} entry made any contribution to burst-associated m increases, burst-associated m increases in 0 mM Mg^{2+}/ 1 mM Ca^{2+}/ 250 μM neomycin solution were compared with burst-associated m increases in 6 mM Mg^{2+}/ 1 mM Ca^{2+} solution. Two junctions from two mice were given three consecutive 10 minute treatments in the following order: (1) zero Mg^{2+} solution (2) 6 mM Mg^{2+} solution and (3) zero Mg^{2+} solution. A total of 11 bursts were measured in all four zero Mg^{2+} treatments from both cells, and the ratio of burst/ baseline m was 2.17 /- 0.27 for this treatment. A total of 6 bursts were recorded in both 6 mM Mg^{2+} treatments, and the ratio of burst/ baseline m was 2.19 /- 0.05 for this treatment. Apparently, m is multiplied by the same extent in the absence or presence of 6 mM Mg^{2+} ($p=0.93$).

It should also be observed from Fig. 9 that the absolute burst-associated increase in m is large (1.5 to 4.5 or so). Examples from other junctions show the m increasing from 5.5 to 11.5, 4 to 7, 7.4 to 11, and 5.5 to 9 during bursts. Thus, substantial relative increases in m associated with bursts can be observed even when the baseline m is high to start with. This makes less likely the hypothesis that the increase in m is caused by the bursting active zone alone contributing more quanta with each stimuli; the idea that one bursting active zone could release up to six additional quanta each stimulus, within roughly a 1 msec interval, while sustaining its high spontaneous release seems an unlikely one. It seems probable that non-bursting active zones are becoming excited during bursts to release more quanta.

DMSO's Effect on Burst m

A good way of determining whether or not the non-bursting active zones are responsible for release during a burst is to compare two additional variables in the presence and absence of 4% DMSO. The variables are (1) the baseline m (the m recorded in the absence of bursts) and (2) the m measured at the peak of a burst.

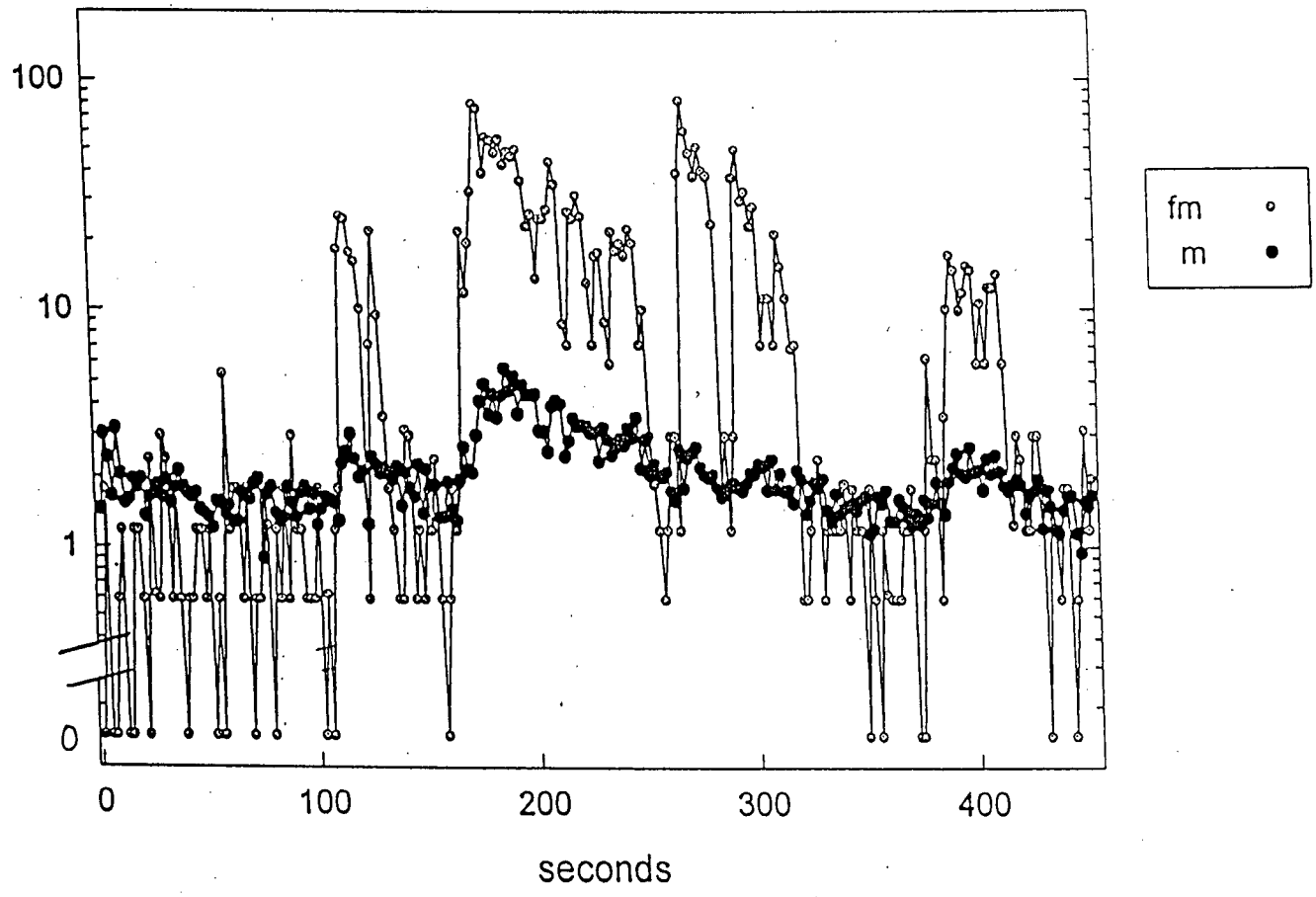

Fig.9. Large rises in the corrected m during bursts in the absence of external Mg^{2+}, and presence of 250 μM neomycin, 1 mM Ca^{2+}.

The baseline m was easily calculated by averaging stretches of record in each treatment group where bursts were absent. The burst m calculation was also simple. First, it was important to identify, in each 10 minute treatment group, the three consecutive fm points with the highest sum. These three consecutive numbers were averaged to represent the fm at the peak of the largest burst. The three corresponding m values, which refer to exactly the same 6 second stretch of record as the peak fm value, were then averaged as well to yield a burst m value.

Four variables to analyze emerge from the above procedure, burst m and fm and baseline m and fm. The following table summarizes the data from five junctions of four mice, each given multiple 10 minute treatments of 4% DMSO alternating with no DMSO.

Table 7

	Base fm	S.E.	Burst fm	S.E.	Base m	S.E.	Burst m	S.E.
No DMSO	0.51	0.08	16.11	2.87	0.74	0.08	1.43	0.23
4% DMSO	2.55	0.38	37.42	9.80	3.10	0.58	5.77	1.28
Increase	**4.98X**		**2.32X**		**4.18X**		**4.03X**	

It can be observed once again that the burst fm is increased less by DMSO application than the baseline (or "base") fm (p 0.05). However, this assertion has already been supported earlier in the thesis by experiments with more junctions (13 versus 5) and more DMSO (6% versus 4%), so the direction of the results is what would be anticipated.

What may seem surprising is the fact that burst intensity numbers for these junctions are roughly 50 times lower than they were for the fm only (6% DMSO) experiments. It's important to realize that the difference is not physiological, but methodological. The minEPP data, first of all, were analyzed as "seq4" output files, which are high resolution reports of fm from the wait time for 4 minEPPs. "Groups" output files report m and fm values each 2 second interval, thus very short and fast bursts of 4 or so minEPPs represent only a few milliseconds of record out of 2 000 milliseconds considered for each data point by "groups" and are thus averaged down. Thus peak burst fms are apparently much smaller in "groups" then "seq4", merely due to differences in burst resolution.

From the above table, it can also be seen that DMSO multiplies the burst m by the same extent that it multiplies the baseline m ($p=0.83$). It seems that the burst-associated m increase can be multiplied by DMSO to a much greater extent than the burst fm can be. Since the burst-associated m increase responds fully to DMSO application, it cannot be supported solely by release from the bursting active zone, as the bursting active zone has been shown incapable of providing a full response to DMSO application. The fact that burst m can be elevated by DMSO to the same extent that baseline m can implies that the burst m, like the baseline m and fm, is made up of release from multiple active zones--

from a vesicular pool large enough to support the elevated release demands of DMSO.

As a result, it can no longer be asserted that bursts of minEPPs are the result of release from a single, hyperactive release site; from the above information other release sites are clearly involved as well. However, release from non-bursting active zones is very small compared to release from the single, bursting active zone. Even a tenfold increase in release from non-bursting active zones does not compare to the increase in release seen at the bursting active zone which suddenly increases its release from low levels (less than 0.1 minEPPs per second) to extremely high levels (1000 minEPPs per second or more), an increase in the order of 10 000 times.

DISCUSSION

Ca^{2+} Dependence of Bursts

This work has revealed that latrotoxin burst incidence is Ca^{2+} dependent; burst incidence is greatly reduced when the external Ca^{2+} concentration is lowered, significantly increased when it is raised.

It seems likely that the increased incidence of bursts seen in raised Ca^{2+} solution results from an increased incidence of latrotoxin channel openings. The latrotoxin single channel patch clamp experiments of Filippov et al. have shown that the open probability of alpha-latrotoxin channels increases with the extracellular calcium ion concentration, just like minEPP burst incidence does[69]. More frequent latrotoxin channel openings in raised Ca^{2+} would result in more frequent inward surges of excitatory cations, and subsequently more frequent bursts of release from the underlying active zone. The tendency for the latrotoxin single-channel openings to cooperate into groups of burst-openings was also increased when the external Ca^{2+} was increased. Thus there is correspondence between latrotoxin single-channel currents and recordings of minEPPs from whole cells--bursts of activity from both are positively correlated with external calcium concentration.

Bursts Have Intensity Sublevels and so do Latrotoxin Single-Channel Conductances

As pointed out in the introduction, latrotoxin application is known to result in the formation of channels in liposomes[70], rat brain mRNA-injected oocytes[69], and PC12 cells[74]. In liposomes and oocytes, the channels show a broad range of conductances, whereas in PC12 cells, a single, uniform conductance of 15 pS is observed.

This has caused a minor controversy among researchers. On the one hand, Filippov et al. postulates that the variability in latrotoxin single channel conductances (3 to 200 pS) he observes in oocytes is due to a property of the toxin to form cooperative clusters of mini-channels in membrane--not only oocyte membrane, but probably nerve terminal membrane as well[69]. Irena Surkova, however, claims that the variability in latrotoxin channel conductances seen in oocytes and liposomes occurs simply because these preparations lack membrane components present in "real" nerve terminals. She suggests that, "...in a differentiated neuronal cell type, the association of the alpha-latrotoxin receptor with other proteins in a membrane complex constrains the alpha-latrotoxin-induced channel to a single conductance state, whereas, in the frog oocytes, the relatively chaotic expression of many rat brain membrane proteins from the injected mRNA leads to the absence or misassembly of the alpha-

latrotoxin receptor protein complex, the constraint on the channel thus being absent. The implication of this idea is that steric constraints in neurons produce a 15-pS alpha-latrotoxin channel, whereas in bilayer, for example, the absence of a specific protein framework leads to channels of very different conductance."[91]

The present results contain evidence that would seem to contradict the idea that neuronal latrotoxin channels have only one conductance state. If, each time a latrotoxin channel opens, cations stream in at the same rate one would expect all bursts to be similarly intense, without huge diversity of burst fm. A burst of 157 minEPPs/ second would be expected to be followed by a burst of about 157 minEPPs/second. Yet what is observed is an incredibly diverse array of burst intensities. This supports the idea that latrotoxin channels in real neurons show variable single channel conductances (Filippov's idea versus Surkova's) which give rise to variable burst intensities. It also hints that perhaps latrotoxin's ionophore property plays an important role in promoting burst release--whole cell behaviour seems related to single channel conductance behaviour.

It is fair to point out that the diversity of minEPP burst intensities does not conclusively prove multiple conductances of latrotoxin channels in real nerves. The diversity may occur due to variation in the so-called direct action of

latrotoxin on the secretory machinery, discussed in the introduction; in the interest of rigour, such a possibility cannot be ruled out. Still, it is very tempting to infer that variable sublevels (or steps) of burst intensity are caused by the variable sublevels of latrotoxin single-channel conductances observed by Filippov et al..

The work of del Castillo and Pumplin is the only paper ever to discuss *bursts* related to latrotoxin application[59], though many other researchers have studied other aspects of latrotoxin-induced release (especially ultrastructural effects). So it should come as no surprise that the incredible variation in intensity of latrotoxin bursts has been completely overlooked until now.

Vesicular Pools

Takashi and Enomoto write that there are different pools of vesicles within nerve terminals. The pools are distinguished by their location within the terminal and by how close they have come to releasing their contents. There are vesicles that exist right beneath the subsynaptic membrane, attached to release sites at active zones; if Ca^{2+} enters the nerve terminal and activates one of these vesicles, it is released with little delay. Vesicles in this group are called "releasable vesicles"[110]. It is believed possible for an active zone to deplete its "releasable vesicle" pool; nerve activity can cause a depression in EPP

amplitude that can be attributed to a reduction in the size of the "releasable pool" due to depletion of quanta by release[111].

In addition to the "releasable pool", it has been speculated that there is also an "available pool" of vesicles. The "available pool" of vesicles does not lie as close to the subsynaptic membrane as the "releasable pool". As a result, vesicles in the "available pool" are not in an immediate position to fuse with the subsynaptic membrane and expel their contents in response to Ca^{2+} entry. Nonetheless they can soon be mobilized; as an active zone exhausts vesicles from its "releasable pool", more vesicles are brought in from the "available pool" to replace them. This process of vesicular mobilization (from the "available" to the "releasable" pool) probably takes more time than the time it takes for a vesicle from the "releasable pool" to expel its contents in response to Ca^{2+} entry.

The Observation of Burst Decline

As stated in the Results section, it seems to the eye as though single active zones cannot always sustain the high release demands of latrotoxin bursts. During fast, prolonged bursts, burst fm steadily declines. Such decline in burst fm probably reflects depletion of the "releasable" pool of vesicles supplying the single, bursting active zone; the latrotoxin channel may remain open, and

excitatory cations may continue to flood in, but there is a dearth of immediately releasable vesicles.

Still, release continues at a reduced rate. Release at this point is probably dominated by the rate at which quanta become available for release, rather than the rate at which available quanta are released. Thus after the "releasable" pool is depleted, the remaining rate of release might largely reflect the rate at which a single active zone (under particular conditions!) is able to mobilize quanta from the "available" vesicular pool to the "releasable" pool.

The decline may alternatively reflect a progressive shift on the part of the latrotoxin channel to a lower conductance state. Though this seems intuitively less likely, it must be considered a valid alternative interpretation.

Burst Release Cannot be Enhanced as Much as Baseline Release

It was hypothesized that the burst fm would not be increased by DMSO application nearly as much as the baseline fm. This hypothesis can be considered reasonable because the mechanism by which each activated vesicle (defined below) fuses with the subsynaptic membrane presumably takes time[69,60], and during a burst, release is already occurring at a very fast rate. Thus it was

considered that perhaps the release of quanta from an active zone, even one fully stocked with vesicles, has a maximum possible rate.

Limitations in the size of the immediate vesicular pool probably does not explain the observation that burst fm cannot be increased by DMSO as much as baseline fm. Peak burst fms (reflecting pre-depletion release) were what were analyzed in this work, and they more likely reflect the *maximal rate at which releasable vesicles can be exocytotically discharged from a single active zone*, not the size of the releasable pool. Thus it is most likely that peak burst fm can little be increased because a single active zone's vesicles can only go through the stages of (1) activation by Ca^{2+} (2) fusion with the subsynaptic membrane and (3) discharge of contents, at a certain maximum rate.

Thus, it is not too unreasonable to surmise that the rate of release during a latrotoxin burst can approach the maximum rate of release possible for a single active zone. We might speculate from the data that a single active zone can release quantal packets at a rate not exceeding about 2000 packets per second.

Burst-Associated Increase In Multi- Active Zone Release

As discussed in the introduction, del Castillo and Pumplin used extracellular recordings to show that BrWSV bursts are the result of release from a very small portion of the subsynaptic membrane. In general, extracellular recordings from non-bursting parts of the terminal showed no increase in release. So for the frog neuromuscular junction, little or no increase in release from active zones other than the bursting one was found.[59]

However, at the mouse neuromuscular junction, the present results indicate that release from multiple active zones is enhanced during a burst. Much of the evidence involves analysis of EPPs resulting from nerve stimulation during latrotoxin-induced bursts. Quantal content (m) provides a method of assessing release from many active zones, since release of quanta is normally derived from diverse sites[57]. Thus, a change in m during a burst implies a change in release from other active zones, although the possibility that the bursting active zone alone is causing the increase in evoked release must be ruled out.

As covered in Results, the elevation in m associated with a burst of minEPPs was fully sensitive to 4% DMSO, as sensitive as the baseline m. Thus other active zones clearly contribute to release during a burst--the bursting active zone could not itself be responsible for the increase in evoked release as it cannot fully respond to DMSO.

Theories Pertaining to the Burst-Associated Rise in m

There are two obvious hypotheses to explain the burst associated m rise, or why non-bursting active zones release more quanta during a burst. The simplest explanation is that excitatory ions like Ca^{2+} and Na^{+} are streaming in through the open latrotoxin channel and are quickly spreading to distant release sites. Although there are proteins in the nerve terminal cytosol which bind free Ca^{2+}, their action may be insufficient to maintain a low concentration of cytosolic Ca^{2+} in the face of such a large influx of Ca^{2+}; spreading of Ca^{2+} to other active zones in the relatively small mouse nerve terminal cannot be ruled out[112]. Also, it was mentioned in the Introduction that Na^{+} can play an excitatory role, and it has been shown using intracellular Na^{+} imaging that Na^{+} penetrates latrotoxin channels[77]; the ion might be diffusing into the nerve terminal after opening of latrotoxin channels to increase release.

The result might also be analogous to a recent "spatial facilitation" discovery by Dudel, Parnas and Parnas. They placed two whole cell patch electrodes 50 micrometers apart on the same (large relative to mouse) frog nerve terminal. They stimulated and recorded from one and then the other terminal in a "twin pulse protocol" for facilitation, the unique aspect being the strictly localized and separate nature of the stimuli. Facilitation was indeed seen

(a doubling of m), thus release from one discrete area (analogous to a burst) rapidly upregulates release from others some distance away. The researchers ruled out both electrotonic input from the first electrode and calcium diffusion. The authors agreed with Bain & Quastel[107] and said that a second messenger was a likely cause for the m increase.[113] Along similar lines, an intense nerve terminal stimulus from a discrete site of the mouse nerve terminal in the form of a burst could be facilitating to release from other active zones in a similar way.

CONCLUSION

There is good evidence from the literature that bursts are single active zone events. Also, bursts require extracellular Ca^{2+}, and the incidence of bursts correlates well with the external Ca^{2+} concentration. Interestingly enough, the incidence of latrotoxin single channel openings also correlates well with the external Ca^{2+} concentration. Ba^{2+}, Sr^{2+} and Ca^{2+} but not Mg^{2+} can support latrotoxin bursts. Mg^{2+} application after latrotoxin causes a smooth and predictable rise in the baseline fm not associated with increased variance to mean ratios.

The intensity of bursts varies greatly, and burst fm tends to increase and decrease in a stepwise fashion analogous to different conductance modes of single latrotoxin channels. Multiple conductance states of latrotoxin channels may give rise to the wide variations in burst intensity observed with whole cell recordings.

Burst intensity, expressed as burst fm, declines during particularly intense and prolonged bursts; this decline may reflect depletion of the "releasable" pool of vesicles at the bursting active zone.

Since peak burst fm responds relatively weakly to 6% DMSO application, it probably reflects a rate of release not far removed from the maximal rate of release possible for a single active zone. This maximal rate of release might reflect the fundamental rate at which vesicles from the "releasable" pool can be induced to fuse with the subsynaptic membrane.

The m, which normally reflects synchronous release of quanta from multiple active zones, is substantially increased when there is a burst; m and fm track each other closely in time.

The burst-associated increase in m can be multiplied by 4% DMSO application to the same extent that baseline m can, implying that active zones other than the bursting active zone are releasing elevated amounts of quanta during a burst.

Although release from other active zones is clearly increased during a single active zone burst, the contribution to burst fm made by these other active zones is tiny in proportion to the contribution made by the bursting active zone itself.

The response of other active zones to the localized latrotoxin event is most probably due (a) to the entry of excitatory ions like Ca^{2+} and Na^{+} into the

nerve terminal or (b) to the triggering of a second messenger cascade in some form analagous to the "spatial facilitation" discovery of Dudel, Parnas and Parnas.

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