QUANTIFICATION AND TIMING OF PROCESSES INVOLVED IN STIMULUS-SECRETION COUPLING AT THE MOUSE NEUROMUSCULAR JUNCTION.

by

ALLEN IAN BAIN

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Department of Pharmacology and Therapeutics

The University of British Columbia
Vancouver, Canada

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Abstract

At the mammalian neuromuscular junction, perhaps the most studied synapse, many aspects of neurotransmitter release and stimulation-induced enhancement of release are still poorly understood. Central hypotheses include: about release, the Ca^{2+} hypothesis (del Castillo and Katz, 1954), and the Ca^{2+}-voltage hypothesis (Parnas and Parnas, 1988), and about enhancement, the residual Ca^{2+} hypothesis (Katz & Miledi, 1968). In the present work, these hypotheses were tested by analysis of the magnitude and timing of release with the technical advantages of computer-assisted analysis of data for large numbers of stimuli and responses and an emphasis on the relative magnitude of phasic and non-phasic release components.

In mouse nerve-diaphragm *in vitro*, phasic neurotransmitter release evoked by action potentials grew with $\tau \approx 0.1$ ms and decayed with $\tau \approx 0.3$ ms, consistent for Ca^{2+}, Sr^{2+} and Ba^{2+}. Non-phasic release decayed, with a polyphasic time course that varied with the divalent cation.

The time course of the opening of voltage-dependent presynaptic divalent cation channels underlying the release process was assessed using "tails" of raised MEPP frequency induced by trains of "direct" pulses (TTX present) in Ba^{2+}-containing solution. Pulses exceeding 50 ms duration were nearly equi-effective (by integral) to more brief pulses,

indicating that this Ca^{2+} channel undergoes little inactivation.

In the presence of Sr^{2+} or Ba^{2+}, short term stimulation-induced enhancement of release was consistent with a simple "residual ion" model, with 'cooperativity' of 4, and decay of putative intracellular ion with $\tau \approx 200$ ms or $\tau \approx 3$ to 5s, respectively.

In Ca^{2+}, facilitation (short term enhancement) was inconsistent with a residual ion model but could be resolved into two components: a multiplicative component seen as an about two-fold parallel increase in m and f_m for short trains (decay $\tau \approx 80$ ms), dependent on intracellular Ca^{2+} and Ca^{2+} influx, plus an additive component (decay $\tau \approx 200$ ms) consistent with the residual ion model.

Potentiation (long term enhancement) was found to consist primarily in parallel of a parallel multiplication of phasic and non-phasic release with $\tau \leq 20$ s. It was absent when tetrodotoxin was present ('direct' stimulation), suggesting dependence upon Na^{+} influx and accumulation. With prolonged tetani, non-phasic release increased further, in a manner consistent with gradually accumulating Ca^{2+}.

Table of Contents

III. Results

List of Tables

List of Figures

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Dedication

To Dr. David Quastel, whose trail-breaking led to places both curious and delightful, but whose gait I was rarely able to match;

and to Maria and Jason, who waited.

I. Introduction

A. Quantal neurotransmitter release

1. Quantal nature of release

Since Dale presented evidence for chemical synaptic transmission at the junction of nerve and muscle using bioassay (Dale et al, 1936), much descriptive work has been carried out on the nature of chemical neurotransmission.

The quantal hypothesis of synaptic neurotransmitter release (del Castillo & Katz, 1954) held that a miniature end plate potential (m.e.p.p.) recorded post-synaptically with an intracellular microelectrode represented the nearly simultaneous action of thousands of neurotransmitter molecules, a so-called quantum, presumptively reflecting exocytosis of a single presynaptic vesicle filled with acetylcholine. The end-plate potential produced by a nerve impulse, being one to two orders of magnitude larger than a m.e.p.p., was postulated to be due to the synchronized release of many quanta. The primary evidence for this was the observation that with Ca^{2+} concentration in the bath lowered to give an e.p.p. of about 5 mV or less the distribution of e.p.p. amplitudes became multimodal, with modes corresponding quite closely to multiples of the average m.e.p.p. amplitude.

2. Phasic and non-phasic release

Phasic neurotransmitter release includes quantal release that occurs 'in phase' with, and just after, an action potential. The definition of phasic release must always be arbitrary, since release occurs stochastically and

there is a time course of decay of the depolarization-evoked Ca^{2+} (calcium ion) transient and any other process which may be initiated by the stimulus. Thus, arbitrary binning (in time bins of various widths) of release following a stimulus into phasic and non-phasic components may become a significant source of inaccuracy which should be accounted for in testing of any particular model. Mean quantal content, *m*, is the average number of quanta released per stimulus. Phasic release can alternatively be quantified as *m* or as an instantaneous release rate.

Non-phasic release, or spontaneous release, is defined as that quantal release which occurs in the absence of an obvious stimulus to the terminal. Most workers extend this definition to include all release, after a stimulus, that is not within the time window of intensely enhanced release that closely follows a particular stimulus. Thus, the distinction between phasic and non-phasic release is entirely arbitrary and has historically depended greatly on the resolution of the method of measurement and of binning in time. Non-phasic release can be quantified as the rate of m.e.p.p. occurrence, f_m, in defined time bins.

Non-phasic release rates are often not measured or reported for two reasons: firstly, m.e.p.p.s are not measurable in curarized preparations or without the aid of a reliable computer counting method under conditions of very low f_m and, secondly, evidence that *m* and f_m arise from the

same release system is still quite recent (Guan et al, 1988).

In the present studies, it is assumed that non-phasic release in some way indicates a status of release probability for which spatial differences within the nerve terminal are minimal and decay or growth in time is slow relative to the time course of phasic release. Thus, f_m (or, more generally, release rate R) measured at any time after a stimulus is an indication of the presence of intracellular Ca^{2+} and/or any other modulator upon which the events leading to phasic release are then superimposed. Based on this assumption, measurement of non-phasic release and its enhancement by stimulation is essential to the testing of any hypothesis regarding phasic release and its enhancement, including the Ca^{2+} hypothesis, residual Ca^{2+} hypothesis, Ca-voltage hypothesis, as well as the multiplier hypothesis presented below (see DISCUSSION).

3. Ca hypothesis

The importance of Ca^{2+} to neuromuscular function was well established when del Castillo & Katz (1954) proposed an overall scheme for the mechanism of neurotransmitter release, the "calcium hypothesis".

Neurotransmitter release, as observed by postsynaptic intracellular recording at the rat neuromuscular junction, was closely coupled in time to an evoked axonal action potential and was strongly dependent on extracellular Ca^{2+}. Katz and co-workers (del Castillo and Katz, 1954, Katz and

Miledi, 1965a) postulated that the mechanism of Ca^{2+}-dependent neurotransmitter release began with invasion of the nerve terminal by the axonal action potential. Upon depolarization of the nerve terminal, a voltage dependent increase in Ca^{2+} permeability allowed a sudden influx of Ca^{2+} down its approximately 10^3-fold concentration gradient into the terminal. Ultimately, intracellular Ca^{2+} acted to promote fusion of a discrete number of neurotransmitter vesicles with the neuronal membrane releasing neurotransmitter into the synaptic cleft. Katz and Miledi (1967) showed that the same effect could be initiated with depolarizing pulses substituting for action potentials. In their experiments, an iontophoretic pulse of Ca^{2+} from a focally placed micropipette was shown to elicit release only if extracellular Ca^{2+} was present at the nerve terminal at the time of the nerve terminal action potential or direct depolarization. Evoked neurotransmitter release appeared to be a consequence of depolarization and presence of extracellular Ca^{2+} at the same time, suggesting an absolute dependence on Ca^{2+} entry into the nerve terminal.

More recently, it has been emphasized that this influx occurs through a suddenly opened 'channel', a water filled transmembrane pore, as opposed to another carrier mechanism. This is indicated by ion influx rate - in the order of 10^6 ions/sec/channel (Hille, 1984). According to measurements of intracellular Ca^{2+} activity (for example, using Ca^{2+} sensitive fluorochromes before and after excitation of

chromaffin cells; review: Plattner, 1989), opening of presynaptic Ca^{2+} channels results in a 1 to 2 order increase in intracellular Ca^{2+} concentration, from about 10^{-8} M at rest to about 10^{-7} M. Upon channel opening, the actual local concentration of Ca^{2+} at the intracellular mouth of the Ca^{2+} channel, probably close to the active sites for transmitter release (Robitaille et al, 1990), could be much higher than estimated from fluorochrome studies which have limited spatial and temporal resolution.

4. Fourth power model

Quantal content, release within the period, after the stimulus, defined for phasic release (see later), is found to be proportional to the 4th power of extracellular Ca^{2+} concentration (Dodge & Rahamimoff, 1967), over a wide range of $[Ca^{2+}]$. This result supports three hypotheses:

1 The relation between intracellular Ca^{2+} and phasic release is 4th power. It is observed that changing absolute entry (by addition of competitive blockers of the Ca^{2+} channel or by large changes in extracellular Ca^{2+}) does not appear to change the power relationship (Guan et al, 1988).

2 Intracellular Ca^{2+} does not approach saturation at its receptor site when quantal content is low because of low extracellular $[Ca^{2+}]$. The degree of saturation is unknown for *m*'s larger than about 4 primarily due to the difficulties in data interpretation because of the

competing process of depletion, the rundown of quantal content seen at high quantal output rates.

3 The entry of Ca^{2+} during an action potential does not apparently saturate the conductance of the channel. (This assuming that consistency of the 4th power relationship indicates a linear entry process.) Instead, entry appears to be directly proportional to extracellular Ca^{2+}, over a wide range of below-normal Ca^{2+} concentrations. In fact, the apparent power relation between Ca^{2+} entry and transmitter release is observed to be less than 4 under conditions where the Ca^{2+} influx per channel (determined by extracellular Ca^{2+} concentration and thus transmembrane Ca^{2+} gradient) is very great, consistent with a model which accounts for stochastic heterogeneity among release sites (Quastel et al, 1992).

Under conditions in which the rate limiting step in the activity of the "divalent agonist" (Ca^{2+}, Sr^{2+} or Ba^{2+}) is one of binding intracellularly to the receptor, non-phasic release also grades with the 4th power of the extracellular concentration, as discussed later.

5. Ca-voltage hypothesis

Parnas challenged the Ca^{2+} hypothesis with a model in which a brief nerve terminal depolarization (action potential or extracellular current electrode) enhances the effectiveness of intracellular Ca^{2+} or is itself directly linked to neurotransmitter release (Parnas et al, 1986;

review: Parnas & Parnas, 1988). In this model, intracellular Ca^{2+} plays a permissive role in a process which is predominantly due to a mechanism other than that described by the Ca^{2+} hypothesis. Simply stated, the Parnas model proposes that the presynaptic depolarization caused by an action potential or current pulse leads to an e.p.p. in two ways: (1) voltage dependent Ca^{2+} channels are opened and Ca^{2+} is admitted from the extracellular medium, and (2) an intracellular Ca^{2+}-binding release site undergoes a transition from an inactive form which does not bind Ca^{2+} and does not promote release to a form which does. Thus, the Ca-voltage model proposes that active release sites which are capable of binding Ca^{2+}, S, are in equilibrium with inactive sites, T, with the equilibrium shifting toward S during a depolarization. S sites are then capable of eliciting release via binding Ca^{2+} to form a complex which can promote exocytosis of a vesicle. One of the attractions of this model is that facilitation of m and f_m, measured in the presence of extracellular Ca^{2+}, can then be ascribed to residual intracellular Ca^{2+}.

In the present work, the Parnas "Ca-voltage" model was tested in two ways:

1 Release under very low extracellular Ca^{2+} and Ca^{2+} channel blocked conditions (Bain & Quastel, 1988); if there were a direct effect of voltage on release which did not require transient high concentration of intracellular Ca^{2+} (or other divalent agonist), phasic

and non-phasic release should not be substantially abolished by these manipulations;

2 Release in the presence of extracellular Sr^{2+} and absence of extracellular Ca^{2+} (Bain & Quastel, 1992a); if the Parnas voltage effect is the primary mechanism underlying release in the presence of Sr^{2+}, phasic and non-phasic release will follow a pattern of facilitation similar to that seen in Ca^{2+}.

B. The divalent agonists

While non-phasic release rate is promoted by a large number of inorganic cations in the presence of nerve terminal depolarization, only three substances have been found to support phasic release, each an alkaline earth divalent cation, Ca^{2+} (Katz & Miledi, 1965), Sr^{2+} (Miledi, 1966) and Ba^{2+} (Blioch et al, 1968). These "divalent agonists" are thus appear to be able to substitute for Ca^{2+}, but with altered kinetics of one or more of the processes involved in coupling of ion entry to neurotransmitter release.

As pharmacological tools, Sr^{2+} and Ba^{2+} may have both pharmacokinetic and pharmacodynamic differences from Ca^{2+}. The former includes non-receptor binding, including non-specific binding to protein and lipid anionic sites, which affects the effective free ion concentration of the divalent agonist either intracellularly or extracellularly. Hydration radius and hydration free energy affect the

diffusion rates through ion-selective membrane channels, as well as binding affinity at a putative intracellular receptor for Ca^{2+}.

By definition, even the characteristics of the Ca^{2+} channel, its voltage dependency, kinetics, and apparent binding specificity, as part of the channel activation process (Quastel et al, 1989), are no more than complexities in the pharmacokinetics of Ca^{2+} (or surrogate) delivery to the hypothetical intracellular active site for promoting exocytosis of neurotransmitter.

Also by definition, pharmacodynamic variables for each of the divalent agonists include: (1) the affinity of the ion for its putative intracellular receptor, (2) the intrinsic activity of the ion-receptor complex in initiating events leading to quantal neurotransmitter release, and (3) the number of intracellular active binding sites for Ca^{2+}, each of which is perhaps made up of two parts - the vesicle and the intracellular surface of the membrane, or docking proteins thereon.

Guan et al (1988) have utilised the residual Ca^{2+} model (see below), generalized to include Ba^{2+} in a study of blockers of the putative Ca^{2+} channel at the neuromuscular junction, to show a consistency between this model and the hypothesis that there is only one voltage dependent Ca^{2+} channel and only one release system involved in both phasic and non-phasic release supported by Ba^{2+} and/or Ca^{2+}, consequent to an action potential or to a 'direct' pulse.

In the present work, the residual ion model as applied to Ba^{2+} is the basis for a study of the activation and inactivation kinetics of the putative Ca^{2+} channel involved in neurotransmitter release at the neuromuscular junction (Bain & Quastel, 1988). For the enhancement of neurotransmitter release produced by stimulation in the presence of Sr^{2+}, the present work demonstrates that the residual ion model is sufficient to account for relative magnitude of the enhancement of phasic and non-phasic release (Bain and Quastel, 1992a), whereas this model cannot account for the relative enhancement of phasic and non-phasic release found with stimulation in the presence of Ca^{2+} (Bain and Quastel, 1992b).

1. Additive properties

Upon stimulation of the nerve terminal in the presence of Ba^{2+}, f_m rises with each pulse and decays for seconds after the stimulation has ended. When trains of various number of pulses are used, the extent of the rise in f_m is related non-linearly to the amount of stimulation. Provided that the stimulus train is much shorter than the apparent time constant of the f_m decline, it is possible to obtain a linear transform of f_m vs the total amount of stimulation (number of stimuli) by taking the fourth root of the raised f_m (Quastel & Saint, 1988).

The model that was suggested is that with each depolarization of the nerve terminal (nerve-evoked or direct), presynaptic channels open and admit an amount of

Ba^{2+} which is the same for constant sized pulses. With continued stimulation, the Ba^{2+} accumulates in the terminal to a steady state level which depends on the frequency of stimulation and the first order decay time constant.

Furthermore, it was shown that if Ca^{2+} is additionally applied in the presence of Ba^{2+}, the instantaneous release rate measured during the approximately one millisecond 'window' of the e.p.p. grows with repetitive stimulation to the same extent as does non-phasic release rate (f_m), when the fourth root transforms are applied. Apparently, Ba^{2+} persists in the nerve terminal for a longer time than Ca^{2+}, and is able to cooperate with incoming and/or residual Ca^{2+} and/or Ba^{2+} (Quastel et al, 1989), in accord with a model for stimulation-induced enhancement of release for Ca^{2+} alone (Katz & Miledi, 1968; see below). The resulting e.p.p. is larger than without the residual Ba^{2+} to an extent which is predicted very closely by the addition of the fourth root of the non-phasic release rate to the fourth root of the release rate during phasic release under conditions where residual Ba^{2+} is absent. The prediction of the additive model holds true for non-phasic release rates elicited in Ba^{2+} up to as high as can be reliably measured (up to 500/s), with or without extracellular Ca^{2+} (Quastel & Saint, 1988; Quastel et al, 1989).

In the present work, similar results obtained in the presence of Sr^{2+} are reported (Bain & Quastel, 1992). With Sr^{2+}, one has the advantage that the major component of the

decay time course is much faster than that for Ba^{2+}. Thus, non-phasic release after each stimulus is apparently a function of the decaying residuum of Sr^{2+} in the nerve terminal and phasic release is the same function of total Sr^{2+} present during evoked openings of presynaptic divalent ion channels.

2. Potency

Sr^{2+} is less potent than Ca^{2+} for eliciting epps (Miledi, 1966) but is approximately equipotent in raising f_m when the preparation is partly depolarized with elevated K^+ (Mellow, 1979). Ba^{2+} is the least potent of the divalent agonists for phasic release (Blioch et al, 1968; Silinsky, 1985).

It is not clear, for each of these 'divalent agonists', what are the relative contributions toward potency of: (a) permeability through the putative channel, and (b) intracellular potency. However, there is evidence for other voltage sensitive Ca^{2+} channels that the permeability of the divalent agonists does not parallel their potency and that they enter through the same channels (Augustine & Eckert, 1984). This implies that the observed potency series is largely dictated by differences in intracellular potency.

It has been suggested that if the maximal response obtainable with the divalent agonists is related to their intracellular potency, then their different abilities to support release possibly reflects a difference among their efficacies at the putative intracellular receptor (Silinsky,

1985). However, this interpretation denies the existence of a Ca-voltage mechanism (see DISCUSSION).

3. Elimination kinetics

While the primary mechanism of removal of the divalent cations Ca^{2+}, Sr^{2+} and Ba^{2+} from the nerve terminal is not known, there is some possibility that the Na/Ca exchanger plays a role. This facilitated diffusion system has an apparent affinity for Ca^{2+} in the region of concentrations similar to that which occurs in the nerve terminal following activation under normal conditions (Philipson, 1985). Sr^{2+} and Ba^{2+} substitute for Ca^{2+} for transport via the exchanger, although poorly (Philipson, 1985), perhaps explaining their prolonged effects on f_m (non-phasic release) after each stimulus.

It must be stressed that there is no method yet available to measure non-invasively nerve terminal concentrations of the divalent agonists. The Ca^{2+}-sensitive dyes (eg. fura, quin, aequorin, and others) cannot be considered to be non-invasive since by virtue of the binding dependency of fluorescence or absorption change, they are Ca^{2+} buffers, raising the complications of altered timing and magnitude of the Ca^{2+} signal in their presence. Even more remote is the ability to carry out such a measurement with temporal and spatial resolution in the order of microseconds and nanometers which would be necessary to adequately describe the pattern of Ca^{2+} entry and distribution in the mammalian motor nerve terminal.

Inasmuch as non-phasic release rates in some way reflect the persistence of the activity of the divalent agonists in the nerve terminal, in accord with the 'residual Ca' model (see Enhancement processes, below), these ions appear to have elimination rates that parallel their potencies or efficacies. However, the measurement of time course of decay of the divalent agonists and estimation of a time constant (assuming first order kinetics) depends greatly on the adopted model for the effector pathway between intracellular divalent agonist and quantal release. In particular, the time constant for decay of divalent agonist activity derived from the time course of decay of non-phasic release rate should be highly dependent on the intracellular cooperativity (n) of the divalent agonist. It has been demonstrated that the cooperativity for Ba^{2+} is about 4 and that at this n the time constant (τ) for apparent Ba^{2+} decay is about 5 seconds (Quastel & Saint, 1988).

In the present work (Bain & Quastel, 1992a) the n and time constant for intracellular Sr^{2+} were determined. Release evoked in Ca^{2+} contrasts with that evoked in Sr^{2+} or Ba^{2+} in that release terminates largely upon completion of the phasic component, with some indication of one or more small components of Ca^{2+} persisting with time constants varying from milliseconds to seconds (see Enhancement processes, below).

C. Time course of evoked release

Evoked release includes phasic release, as defined above, as well as that component of non-phasic release which results from stimulation. The non-phasic component which is evoked appears to have several underlying mechanisms and time courses.

1. Phasic release

In the adult mammalian neuromuscular junction, phasic neurotransmitter release is highly synchronized, following a delay after a stimulus given to the nerve terminal. Most of the delay between a nerve terminal stimulus (action potential or 'direct', see METHODS) and post-synaptic potentials, the synaptic delay or latency, is of presynaptic origin (Katz & Miledi, 1965c). With stimulation of the nerve, part of the latency is attributable to action potential conduction to the terminal. Katz & Miledi (1965c) postulated that of the remaining presynaptic processes which are involved in stimulus-secretion coupling, the processes involved in actual exocytosis of neurotransmitter from the presynaptic cell are likely to contribute most to the minimum synaptic delay. While there is considerable variability, among responses, of the latency of release following the nerve terminal stimulus, the underlying activation process resulting from a given stimulus appears relatively stereotyped both in time course and magnitude. Variability in both delay and magnitude of the response presumptively reflects the stochastic character of release -

there occurs a high probability of release for a finite brief time (Barrett & Stevens, 1972a).

In the present work, measurements of latency and time of growth and of decay of the phasic release period were made for large numbers of quanta under a variety of stimulus parameters and superfusate constitutions. These data confirm and extend the finding of other workers (e.g., Barrett & Stevens, 1972a; Parnas & Parnas, 1988) that the time course of phasic release is independent of most manipulations (including substitution of other divalent agonists for Ca^{2+}) and is tightly coupled, with a fixed minimum latency, to the presynaptic depolarization.

In mammals, temperature sensitivity of phasic release time course, which appears to be parallel for latency, growth and decay, exhibits Q_{10}'s of about 4 for 10C to 20C. Furthermore, that the Van't Hoff plot for f_m is non-linear with a Q_{10} of nearly 1 from 37C down to about 20C is suggestive of a lipid phase change, according to some workers (eg. Datyner & Gage, 1980). Barrett and Stevens (1972b) showed that, in the frog, phasic release during their "ERP" (early release period, defined by the authors as a post stimulus period of heightened release probability) had a falling phase fitted by a single exponential over 2 orders of magnitude fall, with a τ at 11°C of approximately 0.5 ms.

During repetitive nerve stimulation, there are differing reports on whether the time course of phasic

release is altered. After a conditioning train of 4 pulses at 10 Hz, 11°C in frog, Barrett & Stevens (1972b) showed that the decay τ of the ERP was slightly prolonged with each successive stimulus. This contrasts with the result of Datyner & Gage (1980), at 18°C in mouse, that after a high frequency train of three pulses at 65 Hz there was no change in the time course of phasic release. Datyner and Gage postulate that the prolongation in time course observed by Barrett and Stevens was due to prolongation in the action potential time course at the nerve terminal, since the effect could not be duplicated except under conditions which promoted failure of the action potential. Using clamped current pulses delivered focally to the nerve terminal, such a change in time course of decay was not found (Datyner & Gage, 1980).

2. Techniques of measurement

Various types of time course measurement techniques have been utilised by other workers: the method of first latencies, using a computer to record the time of first threshold crossing after a stimulus (Barrett and Stevens, 1972a, 1972b; Baldo et al, 1986), the method of latency measurement of each quantum (Katz and Miledi, 1965b), and the method of deconvoluting the average e.p.p. by the average unitary e.p.p. (van der Kloot, 1988a).

The method of first latencies does not allow an accurate measure of the decay time course of phasic release, except with very large numbers of stimuli at very low

quantal content, since the probability of no quanta appearing until late in the phasic release period is very low (Barrett and Stevens, 1972b). On the other hand, spotting every quantum and its latency will lead to an underestimate of quantal release probability at any latency if e.p.p.s of multiple quanta are counted as unit quanta (Katz and Miledi, 1965b), which may occur if the quanta making up the e.p.p. are of a small height within the normal distribution of quantal heights. Without prior knowledge of the relative contributions to latency variance of the various known or postulated events in stimulus-release coupling, this latter method is accurate only at release rates low enough that multiple quantum e.p.p.s are rare, such as at low quantal contents or late in the phasic release period.

In the present experiments, the time parameters of phasic release are examined in higher resolution than previously. Latency results were obtained using a computer program (developed by Professor D. M. J. Quastel) which spotted every quantum in time (± 0.025 ms, digitizing rate), including within multiple quantum e.p.p.s, by deconvoluting suprathreshold events by an average quantum, with several checks in the procedure to avoid deconvoluting various voltage or noise artifacts in the record. In this way, both the rising phase and falling phase time courses can be measured accurately and these time parameters can be measured over a wide range of quantal contents, from less

than 0.001 up to 3 or 4 (or until muscle twitching or post-synaptic action potentials are limiting). Non-linear summation of the e.p.p. did not significantly affect deconvolutions of e.p.p.s of quantal contents of up to 3 or 4. This method also gave values for f_m before and after stimuli.

3. Non-phasic release

Non-phasic release time course was studied in the present experiments only in conjunction with measurements of phasic release, since distinction between models to explain any particular time component of increased non-phasic release is not appropriate unless the fitting to a model is consistent for a wide bracket of release rates, especially the relatively high release rates inherent in phasic release. Thus, time courses of raised non-phasic release rates during stimulation in Sr^{2+}, while being well defined by other workers (eg. Zengel & Magleby, 1981), are re-examined concurrently with phasic release in terms of a model which can accommodate both (Bain & Quastel, 1992a). The time course of non-phasic release rates elevated by stimulation in the presence of Ca^{2+}, in association with facilitation of quantal contents, was similarly re-examined (Bain & Quastel, 1992b).

D. Enhancement processes

1. Overview

Even before the Ca^{2+}-dependency of the release process was first shown, a number of additional attributes of neuromuscular transmission were described. One of the most studied attributes is the 'enhancement' (a general term defined here as any stimulation-induced increase) of neurotransmitter release, both phasic (*m*) and non-phasic (f_m), by preceding stimulation. Short term (lasting in the order of 100 ms) enhancement of e.p.p.s was observed by Feng (1940) and Eccles et al (1941), and in mammalian tissue by Liley and North (1953). Many others since have described a short term enhancement of *m* under various conditions and the phenomenon has usually been called 'facilitation'. The frequency of m.e.p.p.s has also been shown to facilitate with a similar time course (del Castillo and Katz, 1954; Liley, 1956; Hubbard, 1963). Facilitation has been carefully described in terms of the time course of its effects on f_m and *m* at the frog neuromuscular junction and has been separated into two time components by peeling exponentials (eg. Mallart and Martin, 1967; Zengel and Magleby, 1980,1981; and see review: Silinsky, 1985).

Tetani appear to induce an enhancement process with a time course of seconds, called 'augmentation' (Zengel and Magleby, 1982a,b). Prolonged tetani induce enhancement lasting tens of seconds to tens of minutes, generally termed

'potentiation' - 'frequency facilitation' or 'tetanic potentiation' for observations during the tetanus, otherwise 'posttetanic potentiation'.

2. Retrospective

A number of problems arise in an analysis of much of the literature, past and current, on enhancement phenomena. First, the terminology is inconsistent, with a wide variety of terms in use including facilitation, potentiation, augmentation, and a number of derivations and abbreviations of these and other descriptions. Usually, the distinction of one enhancement phenomenon from another has depended exclusively upon the time course of the development or the decay of the processes. However, in comparisons between enhancement processes of two tissues or experimental conditions, a time course in common may not be appropriate evidence for a common mechanism since this may arise not only by coincidence but from two different underlying processes with different time courses but with transfer functions which make the observed time courses appear similar. The importance of carrying out the transform inherent in the model used, prior to attempting to determine the time course of a process, has been shown for Ba^{2+} (Quastel & Saint, 1988) and for Sr^{2+} (Bain & Quastel, 1992a and see DISCUSSION). Furthermore, in most of the literature on enhancement phenomena, data are shown either for non-phasic or for phasic release exclusive of each other, thus precluding any analysis which requires a concurrent measure

of both. Finally, in many cases, data have been presented in normalized form (relative to unstated control levels), preventing any analysis of the reported enhancement process in terms of the relation between absolute phasic or non-phasic release rates.

3. Facilitation

There are many indications in the literature and in the present data that facilitation, defined in terms of its short time course, is actually a convolution of several processes with different time courses ranging from a few milliseconds to about 200 ms. Various proposed mechanisms have been matched with particular time course phases of facilitation.

a) Mobilisation

The data of Hubbard (1963) show m and f_m modulating in a parallel multiplicative manner, both for "primary potentiation" (facilitation) and for post-tetanic potentiation. It was suggested by Hubbard (1963) that since the effect was parallel for m and f_m, it could be considered an increase in the probability of release due to mobilisation of neurotransmitter vesicles to positions of closer approximation to the intracellular surface of the nerve terminal membrane, resulting in a higher overall probability of exocytosis, multiplicative of both m and f_m to an equal extent. Braun et al (1966a) further explored the effects of various stimulation frequencies and duration of tetani. Since both facilitation and potentiation (short

and longer term enhancement) affected both f_m and *m* to an equal extent, both were multiplicative over a wide range of stimulation parameters and consequent magnitude of enhancement, they suggested that the enhancement process seen after one pulse (lasting for milliseconds) and that seen after many hundreds or thousands of pulses (lasting for seconds) might have a common origin.

A major difficulty which is readily apparent in the study of neuromuscular facilitation is the relative weakness of data for f_m as a function of time after the last stimulus in a train, compared to the data for *m*. Thus, in the range of f_m seen in healthy preparations, from about 1/s resting to about 30/s during potentiation, a very large number of trials would be required in order to resolve the time course of f_m and its enhancement with any accuracy. For example, under optimum conditions for paired pulse facilitation, f_m might be 2/s falling to 1/s over a time course of a few hundred milliseconds, necessitating time bins no longer than about 20 milliseconds, which in turn means that on average only one m.e.p.p. is counted in a time bin every 25 trials. For a Poisson process, to obtain a standard deviation of 10% of the mean, 100 m.e.p.p.s must be counted in each time bin, requiring about 2,500 trials. This is compounded by the need to carry out the experiment with varying number and frequency of preceding pulses. The very large number of trials (stimuli or stimulus trains) required takes sufficiently long to carry out that sufficient data for

accuracy are either unobtainable due to lack of ability to maintain the intracellular recording, or may be questionable due to significant changes in the preparation over the period of study.

The difficulty of obtaining data for enhancement of f_m, to correlate with *m* enhancement, has limited the ability to devise a model to account for the phenomena. Hubbard (1963) and Braun et al (1966) collected both *m* and f_m data, but for a limited number of stimulation paradigms and with precision sufficient to establish only the general multiplicative nature of facilitation and potentiation. Katz & Miledi (1965a) and others have largely disregarded f_m, creating the following "residual calcium model" solely on the basis of observations of e.p.p. facilitation. In the present work (Bain & Quastel, 1992a,b), a novel method is presented for optimizing the stimulation pattern in order to acquire sufficient f_m data at the desired intervals after the desired antecedent stimulation history (see METHODS, below).

b) Residual Ca^{2+}

A few years after the work of Hubbard (1963), Katz & Miledi (1965a) investigated the Ca^{2+} dependence of facilitation by iontophoresis of the cation onto the nerve terminal. They were able to show that the presence of Ca^{2+} was not necessary for propagation of the presynaptic excitation, but was necessary to facilitation. Since facilitation had earlier been shown to occur in the absence of any change in the presynaptic spike (Hubbard & Schmidt,

1963) and the effect of Ca^{2+} on the e.p.p. also occurred in the absence of any change in the presynaptic spike, Katz & Miledi suggested that "facilitation may be due to a residual change in ionized calcium concentration at some important site of the membrane" (Katz & Miledi, 1965a). Of the Ca^{2+} which entered the nerve terminal following each stimulus, a fraction remained 'residual' in the terminal to add to the effect of Ca^{2+} entering after a subsequent stimulus. It may be noted that the residual Ca^{2+} hypothesis of Katz & Miledi (1965a, 1968) was proposed to account only for the behaviour of one of the earliest components of facilitation of *m*, which has an apparent τ of 35 ms.

Further support for the residual Ca^{2+} hypothesis arose from its apparent success in predicting the facilitation produced by a train of pulses, from the facilitation after one pulse, in accord with a residual Ca^{2+} model (Miledi and Thies, 1971). Similar experiments were carried out by Younkin (1974) for a later component of facilitation (apparent τ of 250 ms). Without considering the effect of the facilitation on non-phasic release in the model, the residual calcium hypothesis of Katz and Miledi was shown to be able to explain e.p.p. facilitation observed with certain stimulation patterns.

Part of the evidence of Katz & Miledi (1965a, 1968) for a residual calcium model for facilitation was its dependence upon the presence of Ca^{2+} at the nerve terminal during the conditioning pulse or train, as shown by timed iontophoretic

pulsing of Ca^{2+}. In the present work (Bain & Quastel, 1992b), it is suggested that Ca^{2+} presence may be required for facilitation, but that this role is only permissive, not rate limiting in terms of experimentally observed facilitation.

Support for the residual Ca^{2+} hypothesis is also given by both Parnas and Zucker, from different perspectives. Zucker's predictions are based on models in which diffusion from discrete channel sites in the presynaptic membrane is taken into account, with the effective cooperativity for intracellular Ca^{2+} (n) changing, depending on the source of the Ca^{2+} (Zucker and Fogelson, 1986). Thus, predictions of facilitation magnitude are based on $n = 3$ for Ca^{2+} entering during a nerve terminal stimulus, and $n = 5$ for Ca^{2+} which is already intracellular, some of which may be a residuum from a previous stimulus. Although this model can account for facilitation of m, it cannot account for the parallel facilitation of m and f_m. On the other hand, the Ca-voltage hypothesis (Parnas & Parnas, 1988; see above) supports the residual Ca^{2+} hypothesis by providing a model which correctly predicts the parallel facilitation of m and f_m which is often observed (see DISCUSSION). However, certain requirements of the Ca^{2+}-voltage model are not consistent with the present data.

It is possible for the residual Ca^{2+} hypothesis to predict facilitation which would appear multiplicative for

phasic release, but the predictiveness is limited, according to the following:

Let facilitation = R_f/R

$R_f^{1/n} = k^{1/n} (c_p + \alpha c_p + c_o)$ and $R^{1/n} = k^{1/n} (c_p + c_o)$

from which:

$$(R_f/R)^{1/n} - 1 = \alpha / [1 + (c_o/c_p)] \quad (1a)$$

$$R_f/R = (1 + \alpha / [1 + (c_o/c_p)])^n \quad (1b)$$

where:

R and R_f are phasic release rates, expressed as quanta per second, for the control and facilitated pulses, respectively.

c_o is the amount of resting intracellular Ca^{2+} contributing to spontaneous release.

c_p is the peak concentration of intracellular Ca^{2+} contributed by Ca^{2+} influx during the period of maximum phasic release.

α is the fraction of the integral of c_p over the period of phasic release which remains at the time of a next pulse; ie. the fraction of the Ca^{2+} (or other divalent agonist) entry which persists in the cytoplasm from one stimulus to the next.

According to equations (1a) and (1b), the facilitation of *m* should reach a maximum equal to $(\alpha+1)^n$ when c_o is very much less than c_p. This is a function of the fraction of Ca^{2+} remaining, independent of Ca^{2+} entry itself and thus of extracellular Ca^{2+} concentration. One would expect that such a condition of $c_p > c_o$ would exist down to very low

quantal contents (very low extracellular Ca^{2+}), as calculated according to this model and shown in Table 1 below. However, this model does not predict that facilitation should be virtually identical over a large range of quantal contents (over a range of extracellular $[Ca^{2+}]$), when c_o (indicated by resting f_m) is virtually unchanged (see RESULTS).

Conditions of larger c_o/c_p would be observed either (a) when c_o rises due to metabolic or other incompetence or (b) when c_p is small due to low extracellular Ca^{2+} concentration or the presence of a competitive Ca^{2+} channel blocker. Under these conditions, it can be seen in Table 1 that it is the ratio of *m* to f_m, not their absolute magnitudes, that leads to a given calculated c_o/c_p. Thus, for a low quantal content 0.01 (a rate of about 10/s based on phasic release in 1 ms) with a f_m of 0.1, c_o/c_p is 0.3, as it is for a much 2higher quantal content of 1 with a f_m of 10, for n=4. The higher the chosen n, the higher the c_o/c_p for any m and f_m combination. At high c_o/c_p ratio, there is virtually no phasic release, i.e., m as a rate approximately equals f_m. Under these conditions, the above model would require a high α for any given level of facilitation (Table 1B).

The residual calcium hypothesis for facilitation, using a cooperativity for intracellular Ca^{2+}, is contradicted by the results of Hubbard (1963) unless the facilitation of f_m

Table 1A: calculated c_o/c_p values for various *m*, f_m and *n*.

n=	2			4			5		
m=	0.01	0.1	1	0.01	0.1	1	0.01	0.1	1
f_o (s^{-1})									
0.1	0.1	0.3	0.01	0.3	0.2	0.1	0.4	0.3	0.2
1	0.3	0.1	0.03	0.6	0.3	0.2	0.6	0.4	0.3
10	1	0.3	0.1	1	0.6	0.3	1	0.6	0.4

Table 1B: α (using *n*=4) for values of facilitation and c_o/c_p.

R_f/R=	1.05	1.2	2
c_o/c_p			
0	0.012	0.047	0.19
0.1	0.014	0.051	0.21
1	0.025	0.093	0.38
10	0.14	0.55	(2.8)

is considered to occur by a different mechanism, unlikely in view of evidence that the facilitation of f_m parallels that of *m* in both magnitude and time course.

Quastel (1974) proposed a simplifying alternative to the residual calcium hypothesis which allows for a single facilitatory mechanism for both e.p.p.s and m.e.p.p.s whereby stimulation induces an increase in the probability of release, evident as a multiplier of one of the final steps in excitation-secretion coupling. A parallel was drawn between the multiplicative effect of ethanol on *m* and f_m and that of facilitation. Other agents, such as DMSO (McLarnon et al, 1986), also multiply both *m* and f_m (see DISCUSSION).

4. Augmentation

Zengel and Magleby (1982a) found, by 'peeling' exponentials by best fitting routines, a component of release enhancement which had a time constant of a few seconds. Although this component was very pronounced in the presence of Ba^{2+}, they also found it to be present, albeit relatively small, in Ca^{2+} without Ba^{2+}. They concluded that Ba^{2+} enhances a process that normally occurs as a result of stimulation in Ca^{2+}, based on the apparent match of the time course of the respective components of enhancement in Ca^{2+} and Ba^{2+} containing solutions. An alternative view is that the increase in f_m produced by stimulation in the presence of Ba^{2+} (Silinsky, 1978) is to be attributed to temporary

accumulation of Ba^{2+} within the nerve terminal (Quastel & Saint, 1988), with intracellular Ba^{2+} acting as an agonist on the transmitter release process. Guan et al (1988) showed that the rise in *m* that occurs concurrently with an increase in f_m conforms with the equation for a residual ion model (see below). This result and analysis was the first demonstration of a good fit of both *m* and f_m data to a residual ion model for enhancement of neurotransmitter release.

Tanabe and Kijima (1989) showed that at frog motor endplates an enhancement process identified by time course as 'augmentation' was uninfluenced by BAPTA-AM, a membrane permeant agent which is converted intracellularly to a high affinity chelator of Ca^{2+}. However, this result does not contraindicate a direct involvement of intracellular Ca^{2+} in augmentation (Zengel & Magleby, 1982a), since an additional intracellular buffer should only affect the time course, not the concentration-time integral, of the buffered intracellular cation.

5. Potentiation

First described electrophysiologically by Feng (1941), potentiation has been alternatively ascribed to an accumulation of intracellular Ca^{2+} and/or intracellular Na^{+}. Miledi and Thies (1971) and Hurlbut et al (1971) showed that potentiation can occur even if extracellular Ca^{2+} is strenuously removed. Misler et al (1987) showed that potentiation of both *m* and f_m grew in the absence of added

Ca^{2+}, but grew even further after the cessation of stimulation and simultaneous readmission of Ca^{2+}. Erulkar et al (1982) used various techniques to increase Na^{+} concentration in the nerve terminal and showed that Na^{+} is involved in potentiation. However, they concluded that the mechanism involves displacement of intracellular Ca^{2+} from Ca^{2+} stores in the nerve terminal. Misler et al (1987) were led to a conclusion that although presynaptic Na^{+} increase was likely involved in potentiation, the increased transmitter release (post-tetanically) was due to Ca^{2+} entering from the extracellular solution via the Na^{+}-Ca^{2+} exchanger.

A role of Na^{+} was reaffirmed by demonstration of the effects of Na/K ATPase (the sodium pump) inhibition by Na^{+} removal or ouabain addition (Nussinovitch and Rahamimoff, 1988). Thus, manipulations which increase the concentration of Na^{+} in the nerve terminal prolong the time course of decay of potentiation, even to the point of creating a new set point for the effectiveness of the release process. However, 90% of potentiation is sensitive to the presence of extracellular Ca^{2+}, in their experiments.

The importance of intracellular Ca^{2+} accumulation in the mechanism of potentiation was further indicated by work of Delaney et al (1989) in crayfish by imaging using Ca^{2+} sensitive dye. Intracellular Ca^{2+} rose in direct proportion to potentiation. That potentiation might have a major component that is not Ca^{2+} dependent was shown by Tanabe and

Kijima (1989) who showed its persistence under conditions of intracellular Ca^{2+} greatly reduced by intracellular BAPTA. However, although the intracellular free Ca^{2+} activity is buffered low in BAPTA, the time integral of exposure of intratterminal processes to Ca^{2+} may be identical or even higher than without BAPTA treatment, as discussed later.

As stated earlier for 'facilitation', models which include intracellular Ca^{2+} as the ultimate cause of potentiation are incompatible with a parallel multiplication of both m and f_m, when a Hill coefficient of 4 is assumed for the effect of Ca^{2+}.

6. Present data

In the present work (see Bain & Quastel, 1992a,b), emphasis was placed upon quantification of the relative magnitude of each enhancement phenomenon (excluding 'augmentation' in Ca^{2+} solution, for which a method of analysis had not yet been developed) upon phasic and non-phasic release. This approach was adhered to in recent analyses of release evoked in the presence of Ba^{2+} alone and Ba^{2+} and Ca^{2+} together (Quastel et al, 1989; Guan et al, 1988), based upon the assumptions that: (a) both types of release arise from a common pool of presynaptic vesicles and a single system for quantal release of neurotransmitter (Guan et al, 1988) and, (b) that a model of quantal neurotransmitter release mechanisms should account equally well for phasic and for non-phasic release. Accordingly, it is suggested that the magnitude of both types of release

will be enhanced by any enhancement phenomenon either multiplicatively or additively, according to the model formalized in:

$$R = k(C_i + C_r)^n \qquad (2)$$

On this basis, enhancement could correspond to either an increase in the multiplier k or an increase in the residual ion term c_r, respectively; a simultaneous enhancement of both types of release that is not analyzable as purely multiplicative or additive may be consistent with a mixture of effects on k and C_r which overlap in time, or with a non-constant divalent cation influx per stimulus, C_i. The Hill coefficient *n*, often assumed to be nearly 4, may appear to be less than 4 where it is determined by varying the effective number of Ca^{2+} channels, such as varied polarization of the terminal (Quastel et al, 1992).

Present data (Bain & Quastel, 1988; Quastel et al, 1989; Bain & Quastel, 1992b) show that potentiation can develop in the apparent absence of Ca^{2+} entry while facilitation cannot, confirming earlier data (potentiation - Misler & Hurlbut, 1983; facilitation - Dudel, 1990). More importantly it is demonstrated that, in mouse phrenic-diaphragm, most of both facilitation and potentiation is accounted for by a multiplicative effect, secondary to entry of Ca^{2+}, for the former, and of Na^{+}, for the latter.

II. Methods

A. Mouse hemidiaphragm

The mouse neuromuscular junction differs from the frog in its anatomy. In the adult mouse, a single nerve terminal innervates a single muscle fibre, and the nerve terminals usually form a single focussed synapse with the muscle fibre. In the frog, however, multiple convergent innervation is common, and nerve terminal synapses are elongated. These features make the mouse neuromuscular junction preferable for time course studies.

The isolated, superfused mouse hemidiaphragm preparation is similar to the same preparation from rat as described by Bulbring (1946). In the present experiments, diaphragms were removed from (ether) anaesthetized adult male white CD-1 mice with about 0.5 cm of each phrenic nerve left intact, rinsed immediately in cold bathing solution, trimmed of intercostal muscles and pleura, and cut in half from the sternum to the central tendon. Hemidiaphragms were pinned to silicone rubber (Sylgard) disks and mounted in a movable stage. The preparation was superfused via a glass tube with a tip about 0.5 mm diameter which was adjusted by a manipulator to provide a fast flow over the immediate area around the electrode.

B. Solutions and chemicals

The superfusion solution usually contained, in mM, 5 K^+, 24 HCO_3^-, 150 Na^+, and 1 $H_2PO_4^-$ and 11 D-glucose. K^+ concentration was increased to 10 mM for most experiments in which direct polarization was used. Mg^{2+} concentration was

varied between 1 mM and 12 mM; an attempt was made to maintain the total concentration of Mg^{2+} plus divalent agonist greater than 2 mM since low extracellular divalent cation concentration may cause depolarization due to surface charge effects. All solutions were bubbled continuously with 95% O_2 / 5% CO_2, maintaining a pH of 7.4. Ba^{2+}, Sr^{2+}, and Ca^{2+}, in the form of their chloride salts, were used in the range of 0 to 2 mM. All chemicals were of reagent grade purchased from local suppliers. Chemicals unavailable locally were purchased from Sigma Chemical and Calbiochem.

C. Stimulus delivery

Excitation-secretion coupling at the neuromuscular junction begins with the invasion of the nerve terminal by a depolarizing event. Physiologically, depolarization of the nerve terminal results from an action potential travelling orthodromically in the nerve. Experimentally, in addition to stimulation of the phrenic nerve, a motor nerve terminal in the diaphragm can be depolarized or hyperpolarized with a current clamp electrode placed focally (direct polarization).

Nerve stimulation was achieved by inserting the nerve with gentle suction into a glass tube one end of which is tapered to slightly larger diameter than the nerve, into the other end of which is inserted a chlorided silver wire. Current clamp pulses of 0.1 to 0.3 ms duration and 2 to 3 times threshold were used. Frequency of stimulation was kept below about 70 Hz for prolonged tetani and below 100 Hz

for any train, as higher frequencies of stimulation than these were often associated with nerve conduction failure. Nerve conduction failure was obvious at $m > 1$, but at low m it could be detected by its characteristic intermittency (runs of failures of quantal output or intermittently low m), or by an otherwise unexplained fall in potentiated f_m. An excess of failures over those predicted from the number of stimuli producing one quantum and the number producing two quanta, according to a Poisson distribution, was also used as an indication of nerve conduction failure.

The direct polarization technique (Cooke and Quastel, 1973a) utilises a large tipped (approximately 30-50 μm) glass electrode filled with 3 M NaCl-agar, connected by chlorided silver wire to a current clamp feedback circuit. The primary advantage of this technique for direct stimulus delivery to the nerve terminal is the uniformity of polarization, due to the large size of the electrode and the distance from which the current is delivered from the electrode to the membrane.

One disadvantage of the direct polarization technique is the sensitivity of the effectiveness of the stimulus to any drift in the positioning of the electrode; this was routinely overcome by bracketing in time the test protocols with control protocols. Care was taken to avoid any movement artifact in the voltage record or visual evidence of a localized muscle fibre movement (Cooke and Quastel, 1973a) after each stimulus. Consistent results required

that the polarizing electrode have a tip diameter of about 50 μm, a smooth aperature rim at right angles to the shaft, and be optimally placed such that the currents used to stimulate were minimized. These were made by breaking conventional intracellular electrodes with fine forceps, under a binocular microscope.

The briefness of the direct pulse possible was limited. Since the apparent time constant of the nerve terminal is in the order of 1 ms (Quastel & Saint, 1984), both the rise time and the decay time of nerve terminal depolarizations elicited by square current pulses were significant for pulses of duration less than 2 ms. In some experiments, in an attempt to overcome the capacitance of the nerve terminal and elicit depolarizations much more brief than the membrane time constant, a large brief depolarizing current pulse was followed after a delay by a hyperpolarizing current pulse. Using current pulses of only 50 to 100 μs duration and sizing the hyperpolarizing pulse to a magnitude sufficient to cancel the residuum of the partly decayed depolarization, the effective duration of the depolarization was close to the interpulse delay. Thus, for very brief current pulses, the magnitude of the effective depolarization and the magnitude of the hyperpolarizing pulse required to repolarize to resting membrane potential for a desired effective pulse duration, are approximated by:

$$V_d = I_d R_m [1-\exp(\delta t/\tau_m)], \text{ and}$$

$$I_h = I_d [1-\exp(-T/\tau_m)]$$

where:

V_d is the effective magnitude of depolarization (mV)

T is the interpulse delay, and is approximately the effective duration of the depolarization (ms)

δt is the duration of the current pulses, being much briefer than τ_m.

I_d and I_h are the current pulse magnitudes (μA)

τ_m and R_m are the membrane time constant and resistance (presumed constant at all membrane potentials in the presence of TTX, 4AP and TEA), respectively.

The presynaptic nerve impulse lasts for about 1 ms (Katz & Miledi, 1965) and differs from a direct depolarization of similar magnitude in that the repolarization subsequent to the former is subject only to the rate of Na^+ channel inactivation and K^+ channel activation (Hodgkin and Huxley, 1952), not to the time constant of the nerve terminal membrane as it is for 'direct' pulses. For a direct polarization in the presence of Na^+ and K^+ channel blockers (0.4 μM TTX and 0.2 to 0.4 mM TEA, 0.5 to 1 mM 4AP were used; Saint et al, 1987) the presynaptic membrane time constant is about 1 ms.

D. Stimulation protocol

Simultaneous measurement of both phasic and non-phasic release in the same experiment has historically been avoided because experiments which are designed to give sufficient output of e.p.p.s for statistical reliability do not usually

result in enough m.e.p.p. output, and *vice versa*. For the study of the magnitude and time course of facilitation in Sr^{2+} and in Ca^{2+}, stimulation sequences were generated by a computer program, delivered through a serial port, amplified, and used as trigger pulses for a conventional stimulator. These sequences were random, either in terms of train length (primarily for work with Sr^{2+}) or choice of interval between stimuli from a specified selection of possible intervals ranging from 11 ms to 2.8 s (primarily for characterizing facilitation in Ca^{2+}). An example of the random length train stimulation sequence is given in Fig. 1.

With conventional trains of stimuli, there is difficulty in obtaining data on the effect of various number of stimuli in the train on the enhancement of *m* and of f_m. Of the measured parameters, facilitated *m* is usually obtainable with the least number of repetitions, since a train of any number of stimuli gives data on *m* for each pulse number in trains of fewer stimuli, except that for pulses early in the train where *m* is unfacilitated, more repetitions are necessary to count enough quanta for statistical reliability. For f_m growth and decay, the difficulty in obtaining data for any particular train length is that sufficient repetitions of trains of that length are necessary for statistical reliability of quantal counts in each time bin after the last stimulus of the train; often the long time taken to carry out these repetitions is such

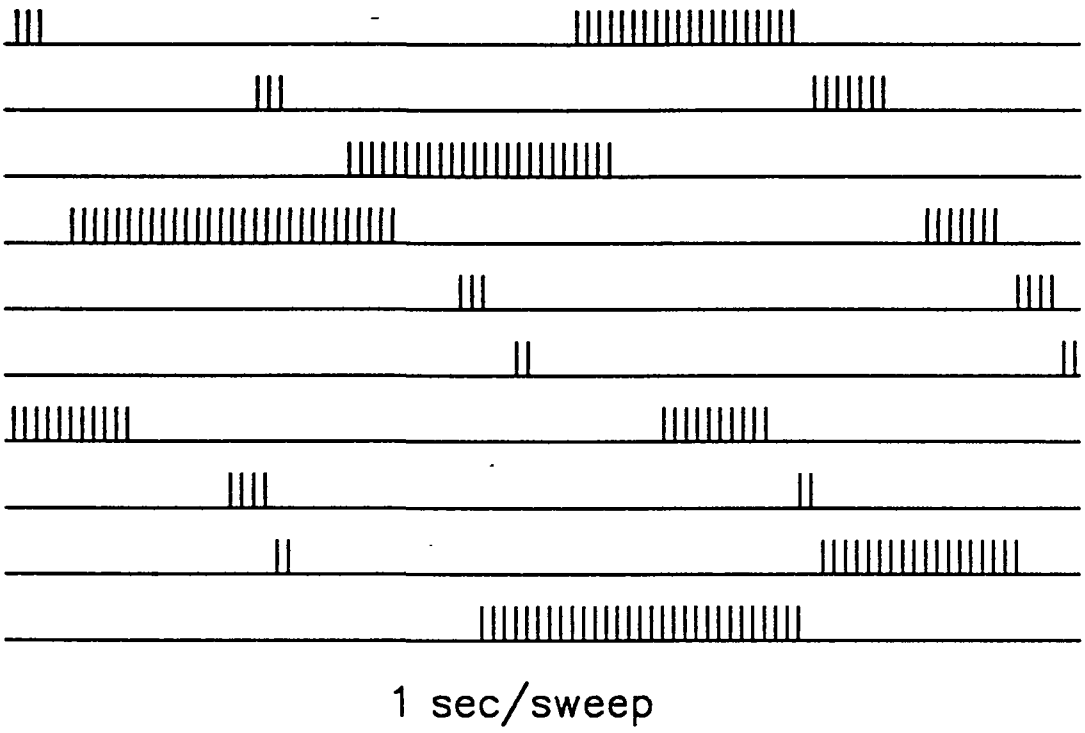


Fig. 1 Random train stimulation.

An example of the timing of pulses in the random train stimulation protocol used primarily for the work with Sr^{2+}. The short intervals used were invariably 11 ms, corresponding to a frequency in the train of about 91 Hz, while the long intervals most often used were 2000 ms (500 ms shown here). The average length of the trains was increased by increasing the probability of selecting a short interval at any one time.

that long term drift in *m* and f_m makes subsequent trials of other train lengths unreliable. The advantages of random length trains are twofold: first, the first algorithm provides for a lower frequency of occurrence of longer trains, which optimises the time required to gather data because fewer long trains are needed for statistical accuracy of the results since both phasic and non-phasic release build to high levels in the longer trains; second, the randomly varied train length means that for every stimulus in the train, there have been recorded responses to other trains which have terminated after fewer pulses, resulting in the ability to accurately determine the f_m at the time of each pulse in a train. Thus, these random trains provide data on both *m* and f_m during the entire growth of release during a train, as well as on the decay of f_m after trains of each length. This stimulation protocol was especially suitable for studying the effects of Sr^{2+}, since the random variable, train length, covers a range of time which encompasses the time constant of the major component of stimulation-induced enhancement in Sr^{2+}.

Random interval stimulation, or pseudo-random stimulation, allowed measurement of the *m* and the f_m at 11, 22, 44, 88, 176, 352, 704 and up to 1408 ms after a preceding stimulus. The highest stimulation frequency during the random series was 91 Hz. The time course of decay of the facilitation resulting from that stimulus and all its antecedent stimuli was then measured, at these

various intervals. A measurement of f_m at any particular interval was carried out in an interval twice or more as long; for example, the determination of f_m at 44 ms after a pulse would be done during 88 ms and longer intervals. A measurement of *m* at a particular interval resulted in another stimulation that formed part of the overall sequence. In the determination of the time course of facilitation after any pulse, any residual effects of enhancement due to the particular sequence prior the that pulse could be disregarded; since the sequence of intervals was random, such residual effects were, on overall average, the same for any stimulus. The primary advantage of this was that a measurement of *m* and f_m facilitation was made for many intervals with each interval being measured more than once every second. Variations on the computer program allowed increased weighting of the probability of occurrence of a short interval, resulting in an increase in the average amount of facilitation present at any one stimulus without compromising the consistency, on overall average, of the magnitude of that facilitation.

Random interval stimulation includes some of the advantages of random train stimulation, but the random nature of the series is addressed to study of a shorter time constant phenomenon, the major component of facilitation in the presence of Ca^{2+}. Thus, the stimulation series does not include trains at a constant interval, unless these appear in the random series of varied intervals. Since on average

a "train" containing stimuli at two different intervals, randomly occurring in the "train", can be described as a train of some intermediate interval, it was possible to obtain *m* and f_m growth and decay over the time of these "trains", in addition to data for the various intervals from 11 ms and longer.

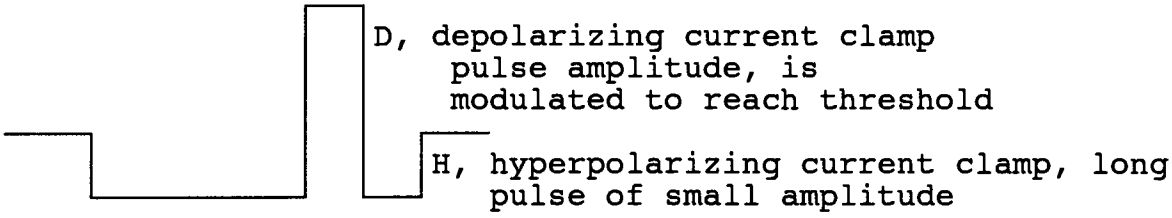
Similar to the random train protocol, random interval stimulation has the major advantage of providing *m* and f_m data which is affected very little by spontaneous drifts of quantal output which are normally experienced during recordings exceeding a few minutes. This is in contrast to the difficulty experienced in attempting to study an enhancement phenomenon where a two-fold effect is observed, but *m* or f_m is low and extended recordings are necessary, sometimes up to 30 minutes for each train length or interval, during which the drift of quantal output (either *m* or f_m or both) may be much more than two-fold.

E. Time constant of the nerve terminal

Two protocols of direct polarization of the nerve terminal (Cooke & Quastel, 1973) were used to estimate the effective time constant of the nerve terminal, as shown in Fig. 2. For one protocol, nerve terminal action potentials were evoked by current pulses of 0.1 ms duration during long hyperpolarizing pulses. As the delay between the onset of a hyperpolarizing pulse, H, and the depolarizing pulse, D, was decreased, the depolarization required to reach threshold

A

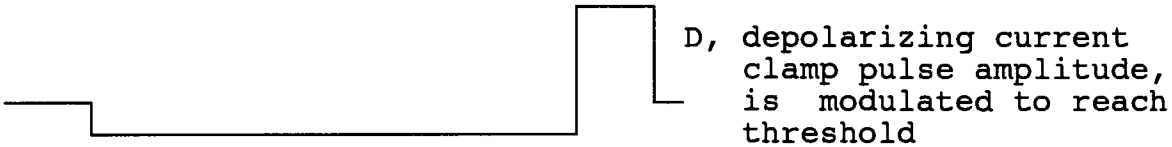
duration of test D pulse is held constant, 0.1 ms



delay (of D pulse, from beginning of H pulse) is decreased from rheobasic in small steps, with a determination of threshold D at each delay.

B

duration of test D pulse is changed in small steps, with a determination of threshold D at each duration of the D pulse



(long, preceding H is optional)

Fig. 2 Nerve terminal τ determination

Panel A shows a stimulation protocol which depends on the time course of the voltage response to a hyperpolarizing pulse prior to a test depolarizing pulse, whereas in panel B the test pulse itself is varied in duration and compared to a rheobasic depolarizing pulse.

for firing the presynaptic nerve terminal decreased to a minimum, D'. The slope of a plot of ln[(D'-D)/H] vs. delay (see Fig. 2) was an estimate of the time constant. An alternate method for estimating τ_m was to vary the magnitude of the depolarizing pulse to find threshold at many different durations, similar to the protocol used by Quastel & Saint (1986). In this case, D' was the minimal depolarization required to reach threshold at long pulse durations (rheobase). In most cases, the depolarizing pulse was overlapped by a preceding long, small amplitude hyperpolarizing pulse to remove g_{Na} (voltage-time dependent sodium conductance) inactivation. A plot of ln[1 - (rheobasic current/I_d) vs. d, where I_d is the threshold current at duration, d, gave a slope of $1/\tau$. The nerve terminal time constants were estimated by best fit (by eye) slopes.

F. Data recording

Recordings of m.e.p.p.s and e.p.p.s were made by placing conventional 3M KCl-filled microelectrodes intracellularly in muscle cells near nerve endplates. The signal from the electrode was converted into a voltage (1:1 Picometric preamplifier) and added to an inverted signal similarly recorded from the bath nearby. This data stream was then amplified 100 fold and recorded digitally on VHS videotape through a Medical Systems PCM-1 analog to digital converter. For computer analysis, the analog data stream,

either direct from the 100x amplifier 'on line' (during the actual experiment) or from playback through the PCM-1, was further amplified 10 to 20 fold and filtered through a Type 3A9 Tektronix amplifier and converted with 12 bit precision into digital form by a Tecmar Labmaster A/D converter at 40 kHz.

G. Computer programs

The computer analyses used in these experiments, developed in 'C' language and assembler by Dr. D. M. J. Quastel, were of three types. The first type of program was used for the experiments on putative Ca^{2+} channel time course using the tails of high f_m which follow trains of stimulation in Ba^{2+}. The second program was used for online monitoring of both *m* and f_m, with f_m counting accuracy remaining very high at f_ms of up to about 200/s, depending on noise level and quantal size. The third program, or suite of programs, allowed offline determination of the height and time of occurrence of every quantal component of a record, accurate in timing to about 0.05 ms.

1. Ba^{2+} tails program

In this program, the number of m.e.p.p.s was counted in the period 0.2 to 2.6 seconds following the last stimulus in a train. The data from this period were taken at a 40kHz sampling rate into the computer memory, then analysed in the following 10 sec or so. From the recorded data m.e.p.p.s were selected for their suitability for inclusion in an average for use as a template. Moving forward in time

through the data array, the program identifies deflections as m.e.p.p.s according to rise time and height criteria. Upon lining up the rising phase of each m.e.p.p. thus found, the m.e.p.p. template was subtracted from the record, prior to continuing the search for other m.e.p.p.s. The counting was validated by inspection of outputs of 0.2 s sections of data with markers indicating where the program had identified and counted m.e.p.p.s.

Accuracy of counts within time bins in the tail was assessed by visual inspection of the screen display. Measurements of Ba^{2+} tails which included false counts or under counts were excluded from overall averages if the counting in more than one 0.2 s time bin of the tail was miscounted. In addition, in the case where an individual bin had a count spoiled by noise in the record, the count in that bin was omitted from the tail measurement and replaced by a mean of the adjoining sections.

2. On line monitoring

The second type of program allowed on line counting of m.e.p.p.s and estimation of *m* from e.p.p. height. This program gives a monitor display of the data stream in 0.4 second lots, similar to the Ba^{2+} tail program, using subtraction of a quantal template (m.e.p.p. average updated every 0.4 s). Validity of the output of this program was established by monitoring the video display. As a further validity check, the ratio of the variance/mean of the f_ms in the 0.4 s sections was displayed every 30 s as an indicator

of non-Poisson counts, which may arise from 'giant' or 'monster' m.e.p.p.s or from erroneous counts associated with a voltage artifact in the data stream. The program also gave a running estimate of the average m.e.p.p. height, useful in establishing the rate of solution changeover when drugs were used which reduced or enhanced the postsynaptic effect of acetylcholine. This program was used during almost all experiments as a monitor and was sufficiently accurate in its m and f_m determinations to allow on line studies of potentiation and other long term processes. This on line estimation of m and f_m was accurate to about 95% as long as the signal/noise was 4 or better, the m was less than about 3 and the f_m was less than about 150/s.

3. Quantal deconvolution - off line

The third, and most extensive, type of computer acquisition and analysis of data on the time course of coupling mechanisms relied heavily upon programs which deconvoluted the data stream by the neurotransmitter quantum (m.e.p.p.). Validation of these programs was carried out both manually and automatically.

a) data logging

To begin with, the digitised data stream (Tecmar labmaster 40kHz analog digital converter) began with on or off line logging onto the computer disk. The data logging program was selective for suprathreshold events, anything in the data stream greater than a threshold set manually at about one-third of the mean m.e.p.p. height. To aid in

selection of threshold, the program caused an analog signal to be produced, displayed on an oscilloscope, each time an event was logged.

b) templates

From the data recorded on the hard disk of a computer (approximately 0.5 megabyte for each file, representing a minimum of about 6 seconds of continuous data in each file), two templates were constructed. First, templates were made of an average quantum. For this, a program sought individual m.e.p.p.s in the record (one file at a time, searching more than one file if necessary to obtain a smooth average quantum) which met criteria for height, rise time, superimposed noise level, baseline stability and isolation in time from other m.e.p.p.s. These quanta were lined up according to the starting point of their rising phase, and averaged. Care was taken to only make use of a particular template for later analysis of the data from which it was averaged, unless it was clear that there was no change in quantal (m.e.p.p.) size from one group of data to another and the data were from the same muscle cell penetration.

To obtain an average stimulus artifact, a program was used which displayed all stimuli and allowed manual selection of failures to be included in the artifact average.

Various difficulties were experienced in obtaining a stimulus artifact template which could be subtracted from the majority of the stimuli in the data stream leaving an

acceptably small residual. For example, although only failures were intended to be included in the stimulus artifact average, it was sometimes difficult to distinguish failures from single quantum e.p.p.s when the quantal size was small relative to the artifact transient. This difficulty, especially evident for the large current clamp pulses used in direct stimulation, was overcome by making two passes through the data to identify failures, the first pass averaging all stimuli and the second pass, after subtraction of the average from the first pass; those which showed the deepest negativity at the estimated time of the e.p.p. were then marked as failures to be included in the stimulus artifact template. In practice, the voltage record of pulses of a duration short enough to be clearly separated from the e.p.p. could be improved even further by neutralizing the capacitance at the tip of the recording electrode.

These methods for making templates of quanta and stimulus artifacts were modified widely to allow for m.e.p.p. averaging during high frequency stimulation and very high or very low f_m, and stimulus artifact averaging during high quantal content output when the stimulus artifact may rarely be seen without a response.

c) quantal deconvolution

The deconvolution program began by locating the stimuli in the data and subtracting the average stimulus artifact for which there was null response. Then, moving through the

data in the forward direction, quanta were located in the record by a threshold crossing algorithm. In preliminary screening of the putative quanta for voltage or movement artifacts, events which rose and fell much too quickly or too slowly were not further analysed. The time of initiation of each quantum was then established to the nearest 0.05 ms or so (about 2 points of digitisation at 40 kHz) by one of two methods. One method used was to correlate the data from about 0.1 ms just before the quantum to about the mid rising phase of the quantum with a hockey stick shape, the time of quanta initiation being taken as the time at the angle for the closest possible correlation. The other method used depended on extrapolation of the baseline forward toward the quantum and of the rising phase of the quantum backward, the point of intersection taken as the time of initiation. These methods are distinct in that the former fixes the angle between the "handle" and "blade" of the hockey stick shape, according to a preliminary estimate of m.e.p.p. rise time, then places the "hockey stick" at a position for which consequent variances of data to template are minimal, to then determine the latency of a particular quantum. The latter method, involving extrapolation, is less sensitive to noise which could occur at an inopportune time for the hockey stick method, for example noise occurring right at the point of rise of the quantum, but is more prone to error in counting as quanta

certain artifacts in the record whose rising phase is quite unlike that of a quantum.

After a putative quantum was located in time, the quantum template was lined up and subtracted, as exemplified in Fig. 3. If the residuum after subtraction had greater overall variance during the period of the quantum than before subtraction, the template was added back and the event was excluded. Multiple quantum e.p.p.s were not treated differently than m.e.p.p.s, in that the point of initiation of the event was found prior to each successive template subtraction and variance determination. This procedure was carried out progressively through the record, giving an output consisting of the time of occurrence (relative to the time of the preceding stimulus, which was also logged, to the nearest 0.025 ms) and the height of every quantum.

d) binning and statistical analysis

The standard output files from the quantal deconvolution program were analysed in a variety of ways. In general, the deconvoluted events were binned by latency from the stimulus into 0.1 ms bins and displayed as a latency histogram (eg. Fig. 4). Latency windows were imposed within which the number of 'phasic' events was counted for estimates of quantal content. For non-phasic release, average m.e.p.p. frequency was estimated from counts of quanta within time bins that were progressively

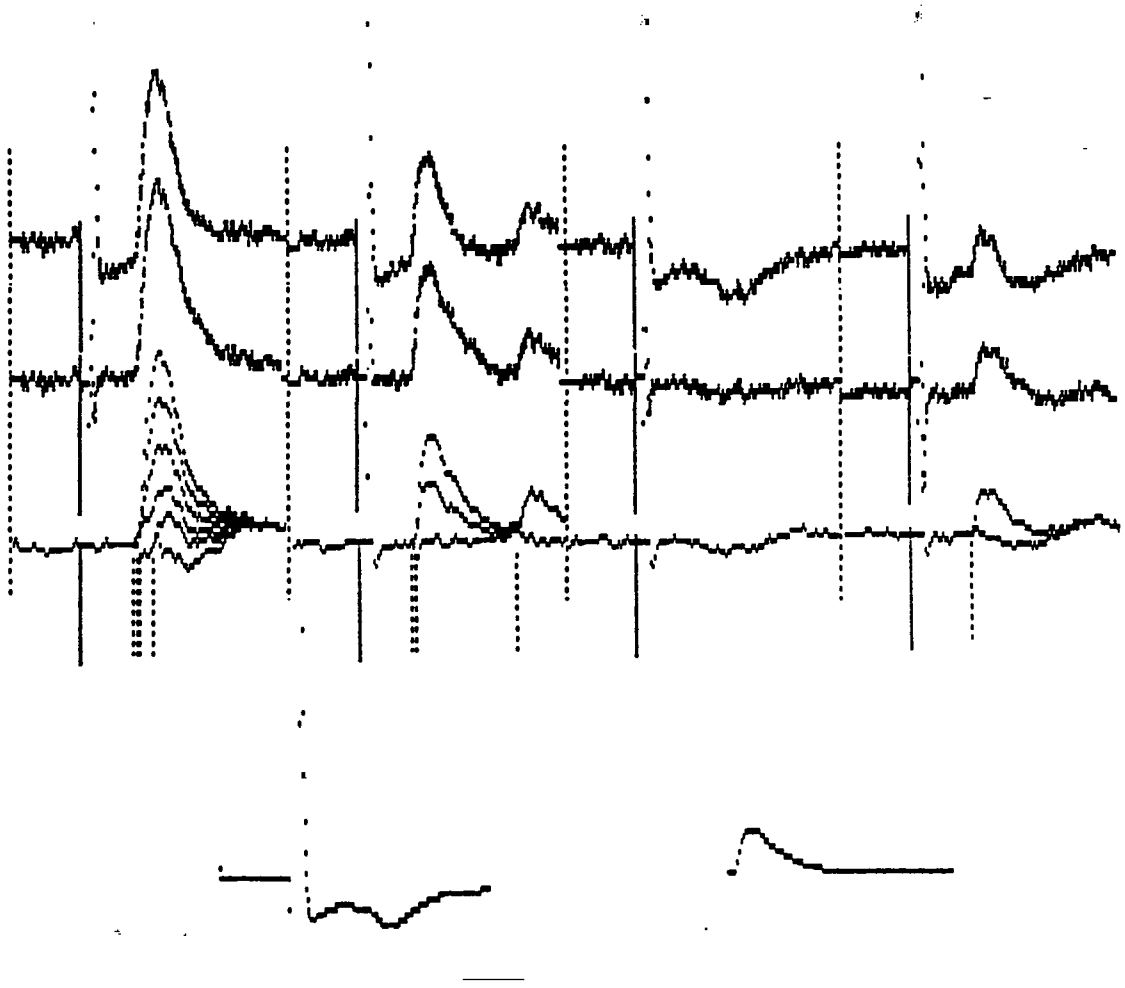


Fig. 3 Automated quantal deconvolution.

An example of deconvolution by a suite of "C" and assembly language programs written by Dr. D. Quastel. Upper trace: raw data (digitised), the stimulus artifact adjacent to the vertical marker. Middle trace: after subtraction of the artifact. Lower trace: sequential lining up and subtraction of a average quanta, resulting in a nearly flat residuum. The time of each deconvoluted quantum is marked and recording in a file with its height. Bottom: the average stimulus artifact and the average m.e.p.p.

longer as they were further from the stimulus, minimizing the number of trials necessary to be averaged to achieve statistical significance (greater than 100 quanta per bin, for an accuracy of $\pm 100^{1/2}$ quanta per bin).

To observe the relative growth of f_m and m during a train, m.e.p.p.s in bins straddling each stimulus and e.p.p.s were averaged among trains according to their position in the train. For random train stimulation sequences, the stimuli were 11 ms apart, the m was determined from the quanta located in the first 6 ms, and the f_m was determined for bins after 6 ms, with separate sets of bins for each stimulus in a train. For random interval stimulation, sequences of stimuli were considered trains as long as there were only intervals of the shortest two types in the sequence.

e) Poisson tests

Throughout the analyses, it was found that only rarely did release not follow a Poisson distribution, and it was generally assumed that if this were not the case the most likely reason was error, of one kind or another. Although at high quantal contents variance of numbers of quanta per e.p.p. appears less than the mean, i.e. release appears to be governed by binomial statistics (del Castillo & Katz, 1954), it has been shown that upon correction for the post-synaptic non-linear effect of neurotransmitter on voltage, e.p.p. amplitude distribution appears to be Poisson, even at

3	3	2.090	■■
4	2	1.889	■■
5	1	1.588	■
6	2	1.889	■■
7	6	2.486	■■
8	6	2.486	■■■
9	6	2.486	■■
10	0	0.000	■
11	1	1.588	■■
12	2	1.889	■■■■■■
13	18	3.272	■■■■■■■■■■■■■■■
14	50	4.224	■■■■■■■■■■■■■■■■■■■■■■■■■■■
15	92	4.919	■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■
16	89	4.879	■■
17	72	4.627	■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■
18	54	4.306	■■■■■■■■■■■■■■■■■■■■■■■■■■■■
19	29	3.686	■■■■■■■■■■■■■■■■■■■
20	15	3.126	■■■■■■■■■■■■
21	22	3.440	■■■■■■■■
22	6	2.486	■■■■■■
23	6	2.486	■■■■
24	5	2.375	■■
25	1	1.588	■■
26	4	2.246	■■
27	4	2.246	■■
28	3	2.090	■■
29	5	2.375	■■
30	5	2.375	■■
31	3	2.090	■■
32	4	2.246	■■
33	1	1.588	■■
34	5	2.375	■■
35	2	1.889	■■
36	4	2.246	■■
37	4	2.246	■■
38	1	1.588	■
39	2	1.889	■
40	2	1.889	■
41	1	1.588	■
42	1	1.588	■
43	1	1.588	■
44	2	1.889	■
45	2	1.889	■
46	2	1.889	■■
47	7	2.584	■■
48	6	2.486	■■
49	3	2.090	■■
50	2	1.889	■■
51	5	2.375	■■
52	5	2.375	■■
53	0	0.000	■■
54	5	2.375	■■
55	5	2.375	■■
56	2	1.889	■
57	0	0.000	■
58	1	1.588	■
59	1	1.588	■
60	2	1.889	■

Fig. 4 Typical latency histogram.

Phasic release (e.p.p.) was calculated to include all quanta in the region of spanning the peak release phase, less the number of quanta expected from non-phasic release. In this typical output, the values are (from left) latency in tenths of ms, number of quanta in the 0.1 ms time bin and the fourth root of quantal frequency in the bin. Experiment 89D05A, 0.3 mM Ca^{2+} 4 mM Mg^{2+}, m=0.357.

high *m* (Martin, 1955). Furthermore, it has been shown (Vere-Jones, 1966; Hubbard et al, 1969) that even if the final step in release is binomial in nature, the entire release process would appear to follow a Poisson distribution if the preceding step involved a Poisson process.

From the output of the deconvolution program, the number of phasic responses of each quantal content, ie. 0, 1, 2, etc., was counted. From the observed mean quantal content, the predicted number of each was calculated. In this way, it became obvious whenever there arose a non-Poisson distribution of quantal contents, either due to biological problems, such as an intermittently failing nerve terminal action potential, or a problem in the deconvolution program, as occurred for a variety of reasons, especially during early development of the algorithms. As a quick check of Poisson nature, the variance to mean ratio of quantal contents was routinely checked by the computer program, a ratio differing more than 10% from unity being sufficient reason to reanalyse the data.

In some analyses, especially at higher quantal contents, there was considerable difficulty determining the quantal size. Scaling the quantal template would obviously skew the distribution from a Poisson: too small a template skewing toward higher quantal contents, too large skewing toward lower quantal contents. Sometimes, the best approach

was to perform the deconvolution repeatedly, each time scaling the quantal template to improve the fit of the observed distribution to a Poisson.

Goodness of fit to a Poisson was often determined by the lack of regression of quantal content calculated from the frequencies of occurrence of each pair of adjacent numbers of quanta in the Poisson distribution. For a Poisson, the frequency of occurrence (F_q) of an e.p.p. with a particular number of quanta (q) is given as:

$$F_q = (q\ e^{-m})/q!$$

Thus, one can obtain several estimates of *m* according to:

$$m(q,q-1) = qF_q/F_{q-1}$$

For failures (q=0): $m(0) = -\ln(F(0))$, giving a further estimate of *m*.

If these estimates of *m* regress upward with number of quanta, an overcount of quanta is indicated (possibly due to an undersized quantal template); the obverse is also true.

H. Data analysis

1. Estimation of m

Quantal content of the e.p.p. (*m*) is an arbitrary measure of phasic release that usually includes some contribution from at least three processes which are non-phasic in nature. These include:

1 spontaneous release of m.e.p.p.s, which are present in the absence of stimulation and may occur at the latency at which phasic release is expected,

2 a component of facilitation (as defined above) from previous stimuli, and

3 a fast component of non-phasic release elicited by the stimulus in question, classified as non-phasic solely on the basis of the disparity of its time course with that of phasic release, about 6 ms decay vs. about 0.5 ms decay time constants.

In the present experiments, *m* was estimated from the latency histogram produced from quantal deconvolution of the raw data, mentioned earlier. The estimate of *m* was a sum of all quanta between latency limits which were chosen according to the following criteria:

1 the early limit was chosen early enough to include the beginning of the e.p.p. (that is, the obvious sharp rise in probability of quantal release that begins within one or two 0.1 ms bins), but late enough to minimize the risk of including artifactual quantal counts which were sometimes associated with the settling of the stimulus artifact;

2 the late limit was chosen to include the major component of the decay of phasic release, estimated as the latency at which release rates do not trend downward in more than about five 0.1 ms bins.

In practice, the limits for estimating *m* from the latency histogram were set at 0.3 ms and 6 ms for experiments in which the phrenic nerve was stimulated. For e.p.p.s evoked

by a focally placed stimulating electrode, the lower limit was set according to the duration of the stimulus artifact, striking a balance between the risk of inaccuracy due to artifactual counts of quanta arising from quanta-like residua of stimulus artifacts after subtraction of the average stimulus artifact and the risk of undercounting the earliest of the quanta actually released very close to the stimulus artifact. The upper limit was adjusted, for some data, when a late or spread out e.p.p. occurred, or when an estimate of *m* without its late phase (seen as high non-phasic release rate between the e.p.p. and about 10 ms, see DISCUSSION) was required.

In the estimation of *m* under conditions of high f_m preceding the stimulus, from the number of quanta summed for the e.p.p. was subtracted the number of quanta during the e.p.p. time window (0.3 - 6 ms) that could be attributed to f_m in the absence of a stimulus. This correction made a significant difference to calculations only at low *m* or high f_m.

2. Estimation of f_m

Frequency of occurrence of m.e.p.p.s, called f_m in general referring to any non-phasic release rate, was estimated in various time bins around a stimulus, excluding the region where phasic release occurs. Quanta occurring in time bins within about 0.1 ms of the beginning or end of the stimulus artifact were also excluded from f_m determination.

In order to determine the decay time course of *m* and f_m facilitation, the random interval stimulation protocol was used. In this analyis, f_m corresponding to each stimulus interval was determined in a window which spanned from mid way to the next shorter interval to midway to the next longer interval. In the binning program, a correction was calculated to take into account that the decay of f_m was curvilinear within the span of the f_m windows; this correction was generally very small. It should be emphasized that in the present data, wherever a particular *m* and f_m are compared, the f_m was determined from a section of the record for which the previous history corresponds to that preceding the stimuli leading to the *m*.

3. Data averaging

In experiments where data for *m* or f_m from many cells were to be averaged, an assumption was made that these data were log normally distributed among junctions and a log transform was done on the data from each cell prior to averaging.

Outlying data in terms of *m* or f_m were not included in averages and were those neuromuscular junctions at which the f_m spontaneously rose to high frequencies, either gradually during the recording or in brief bursts lasting 1 to 30 s. These phenomena both correlated with a declining viability of the cell since both become very common when the preparation is made hypoxic.

For experiments in which nerve stimulation was used, the exclusion criterion was simply failure of the nerve terminal action potential, seen as a lack of any phasic release, as distinct from release failure in accord with a Poisson distribution of *m*. Nerve action potential failure often occurred in conjunction with spontaneous high f_m. For direct stimulation experiments, junctions were excluded, in practice after preliminary recording, if the f_m was more than about 10 fold greater than the average.

Weighting of data, while giving the advantage of avoiding bias toward junctions at which very little data was acquired, was a problem in that it created a bias toward those junctions in which the resting f_m was higher. When means were weighted, it was according to the inverse of the variance of each mean.

4. Derivations

The model which has been used for analysis of the present data is based on the Ca^{2+}-release model (above). Those events which are generally accepted to occur intracellularly in the presynaptic nerve terminal prior to quantal release lead to the following model. First, intracellular Ca^{2+} binds a site which is able to selectively bind certain divalent metallic cations, the divalent agonists (as well as some trivalents, eg. Curtis et al, 1986), with a cooperativity of 4. Thus, $C_4B = B_t\ C^4\ /\ (K_C{}^4 + C^4)$, where C_4B represents an

intracellular receptor for polyvalent cation agonist with four such cations bound, B_t is the maximum number of binding sites, C is the concentration of intracellular cation agonist and K_C is the apparent affinity.

Release is expressed as $R = k' C_4B$, where k' is the 'intrinsic activity' of the C_4B complex and a proportionality constant, and $R = k'' C^4/(K_C{}^4+C^4)$, where $k''=k'B_t$. Assuming that $C^4 << K_C{}^4$, since the quantal release rates in the present experiments are about 1/100th those at normal extracellular Ca^{2+} concentration, this can be simplified to $R = k C^4$ where $k = k''/K^4$ (Quastel et al, 1992).

Thus, inherent in the k term is a dissociation constant (K_C), a factor associated with the number of release sites (B_t), and the average readiness of the release sites for Ca^{2+} induced release (k'). If one or more factors of the k term modulated during repetitive stimulation, the effect would be multiplicative on all release evoked by the divalent agonist.

If the amount of divalent agonist at the receptor site changes, as with an accumulation from one stimulus to the next, or from the time of phasic release to the time of non-phasic release, it is the intracellular concentration of the divalent agonist at the receptor sites averaged for the actively exocytotic portion of the nerve terminal that becomes the term 'C'. Thus, the equations that have been

derived for phasic and non-phasic release and facilitation by an accumulation of divalent agonist are as follows.

Spontaneous release, present in the absence of stimulation, assumed to be due to resting intracellular Ca^{2+} concentration, is expressed as $f_o = k\ C_o^4$. Release at any moment following a stimulus, including phasic release, is also taken as a release rate, $R(t) = k\ (C(t)+C_o)^4$, where $C(t)$ is the intracellular concentration of divalent agonist at the release sites due to the stimulation-evoked influx, which rapidly changes during phasic release. Defining C_t as an average of $C(t)$ over a short time (less than 1 ms) corresponding to, but phase shifted by excitation-secretion coupling latency from, the peak of phasic release, one can write $R_o = k\ (C_t + C_o)^4$ for the phasic release rate produced by an isolated stimulus. If there is any residuum of C_t after a stimulus, the miniature frequency is given at any moment by $f_m = k\ C_r^4$, where C_r is sum of C_o and the residuum of C_t, that is, total residual divalent agonist at the intracellular receptor at that time. A stimulus delivered in the presence of a residuum gives $R = k\ (C_t+C_r)^4$. From these equations, a value in arbitrary units can be derived for entry of divalent agonist, C, and for k:

$$f_m^{1/4} = k^{1/4}\ C_r, \text{ and}$$

$$R^{1/4} = k^{1/4}\ (C_t+C_r), \text{ therefore}$$

$$R^{1/4} - f_m^{1/4} = k^{1/4}\ C_t \qquad (3)$$

whether C_r includes a residuum from previous C_t, or C_r is simply C_o in which case f_m is f_o and R is R_o. This

difference is termed the phasic delta fourth root, when C_r is taken just before the pulse.

Another measure of entry and k can be derived from f_m elevated by the presence of a residuum from previous pulses. If the elevated $f_m = k\ C_r^4$ and $f_o = k\ C_o^4$, then

$$f_m^{1/4} - f_o^{1/4} = k^{1/4}\ (C_r - C_o). \qquad (4)$$

This difference is termed the non-phasic delta fourth root.

The usefulness of $(C_r - C_o)$ as a indication of the magnitude of C_t depends on the assumption that this difference is the fraction of C_t remaining at the time of observation, and should modulate with C_t with changes in extracellular divalent agonist concentration, nerve terminal stimulus, or other influence on entry.

5. Enhancement calculation

Where enhancement processes which are multiplicative overlap in time those which are additive, assumptions must be made with regard to the sequence in which events occur leading to neurotransmitter release, and their corollaries in the model. The approach made is to first isolate the effects of pure multiplicative enhancement and pure additive enhancement according to the model.

First, if it is supposed that facilitation is purely additive, that is, due to an addition of a residuum of C_t from a previous stimulus to the C_t of the next stimulus, leading to a larger *m*, and an addition of the same residuum to the resting C_o for a higher f_m, then it is sufficient that C_r changes and k remains constant. Alternatively, if

facilitation is multiplicative, either $C_r = C_o$, there is no significant residuum at the time of observation, or C_r is unchanging in the time frame between stimuli. In this case, k modulates with a time constant of the same order of magnitude as the interval between stimuli. Multiplicative facilitation is calculated as m/m_o and f_m/f_o. For a measure of additive facilitation, the amount of C_r is calculated, relative to C_t.

In order to separate the additive and multiplicative components, it is first assumed that any changes in the multiplier in the model, k, correspond physiologically with actions on release at a point in the release process after those processes which determine the absolute magnitude of C_t or C_r. This assumption will hold, unless the apparently multiplicative component of facilitation in fact reflects an increase in C_t, an unlikely possibility discussed later. The following example, typical of data from nerve stimuli given in trains in the presence of about 0.3 mM Ca^{2+} and 2 mM Mg^{2+} illustrates the method of separation of the components (subscript f denotes facilitated):

1 Let: $m = 0.5$ $m_f = 1$ $f_m = 1$ $f_{mf} = 3$

2a phasic delta fourth root $= (m \cdot 1000)^{1/4} - f_m^{1/4}$

$= 500^{1/4} - 1 = 3.73$

2b facilitated " " " " $= (m_f \cdot 1000)^{1/4} - f_{mf}^{1/4}$

$= 1000^{1/4} - 3^{1/4} = 4.31$

3 Multiplicative component, X, is the 4th power of the ratio of 2b to 2a, and, since the C_t cancels on the

assumption that entry does not change,

$$= (k_f^{1/4} / k^{1/4})^4$$

$$= 4.31/3.73 \quad = 1.78$$

4 Now, $f_{mf}^{1/4} = k_f^{1/4} C_{rf}$, and $f_m^{1/4} = k^{1/4} C_r$, so

$$f_{mf}/f_m = X^4 (C_{rf}/C_r)^4,$$

$$C_{rf}/C_r = (f_{mf}/f_m/X)^{1/4}$$

$$C_{rf}/C_r = (3/1.78)^{1/4} = 1.13$$

Thus, for this example, where *m* is 0.5 and it doubles and f_m is 1 and it triples, the multiplier k increases by 78% and residual Ca^{2+} increases by 13%.

It should be noted that if the integration time ascribed to C_t is taken as 0.5 ms instead of 1 ms, the difference is very small. Table 2 shows X and the increment in C_r relative to control for assumed e.p.p. integration times of 0.1, 1, and 10 ms. It is clear that the longer the time over which the e.p.p. is integrated, the multiplicative component, X, is less and the additive component, C_r, is more.

6. Assumptions

In this analysis of the present studies, a number of simplifying assumptions have been made. The assumptions which are essential to the models proposed are (a) that quantal release of neurotransmitter occurs by mechanisms which are common for both evoked and spontaneous release, and (b) that the quanta involved in both types of release are from a single pool. Two lines of evidence support these

Table 2: Effect of period of integration of phasic release on derived values of facilitation and residual calcium.

			Integration time of e.p.p. (ms)		
			0.1	1	10
	m	f_m	phasic delta fourth roots		
control	0.5	1	7.41	3.73	1.66
facilitated	1	3	8.68	4.31	1.85
X			1.89	1.78	1.53
increase in C_r			0.12	0.14	0.18

The time over which phasic release is integrated in order to arrive at an average phasic release rate has an effect on the interpretation of the data and fitting to a particular model in terms of the relative facilitation of *m* and f_m. The above data are mock data which are typical of facilitation data from trains of about 5 pulses at 90 Hz in low Ca^{2+} raised Mg^{2+} solution.

assumptions: first, the equivalence of e.p.p. quanta and m.e.p.p. quanta, as evidenced by comparisons of evoked and spontaneous end plate potentials (del Castillo & Katz, 1954; Elmqvist & Quastel, 1965a,b) and second, the additivity of the effect of Ba^{2+} or Sr^{2+}, remaining in the nerve terminal from previous stimulation events, with the effect of the influx of that ion with a subsequent stimulus. That this additive relation obeys four or five power kinetics has been demonstrated (Quastel & Saint, 1988) and is part of this thesis (Bain & Quastel, 1992).

7. Definitions

As previously explained, the meaning of certain words will be closely defined, to avoid unnecessary coining of new terms. "Enhancement" will refer to any stimulation induced increase in neurotransmitter release. "Facilitation" and "potentiation" will be used as they are usually used in the literature, that is enhancement processes which are differentiated solely on the basis of their gross time course of growth or decay, less than one second for the former and minutes for the latter.

III. Results

A. Time course of stimulus-secretion coupling

1. Does the presynaptic Ca channel inactivate?

Prior to commencing experiments which are directed to the nature of events known to occur late in the process of stimulus-secretion coupling, experiments were conducted to establish some of the characteristics of the nerve terminal Ca^{2+} channel, an early component of the coupling mechanism. In particular, the question asked in this section was: do Ca^{2+} channels inactivate? If so, the process underlying facilitation would be more difficult to resolve, since Ca^{2+} channel opening would be likely to change upon each stimulus given in succession.

a) Studies with Ba^{2+}

In order to study the activation and inactivation characteristics of the Ca^{2+} channel at the mammalian neuromuscular junction, the Ba^{2+} entry paradigm (Quastel and Saint, 1988) was employed. In solution in which Ba^{2+} was substituted for Ca^{2+} (TTX present), a train of depolarizing current pulses delivered to a single nerve terminal elicited a buildup of f_m during the train and a 'tail' of m.e.p.p.s, which lasted for several seconds following the train. Assuming the model of Quastel and Saint (1988) whereby the difference of fourth roots of f_m in the tail and before the train (non-phasic delta fourth root for Ba^{2+}, see METHODS) is a linear function of Ba^{2+} entry, variations in the duration of direct depolarizing pulses should yield information about

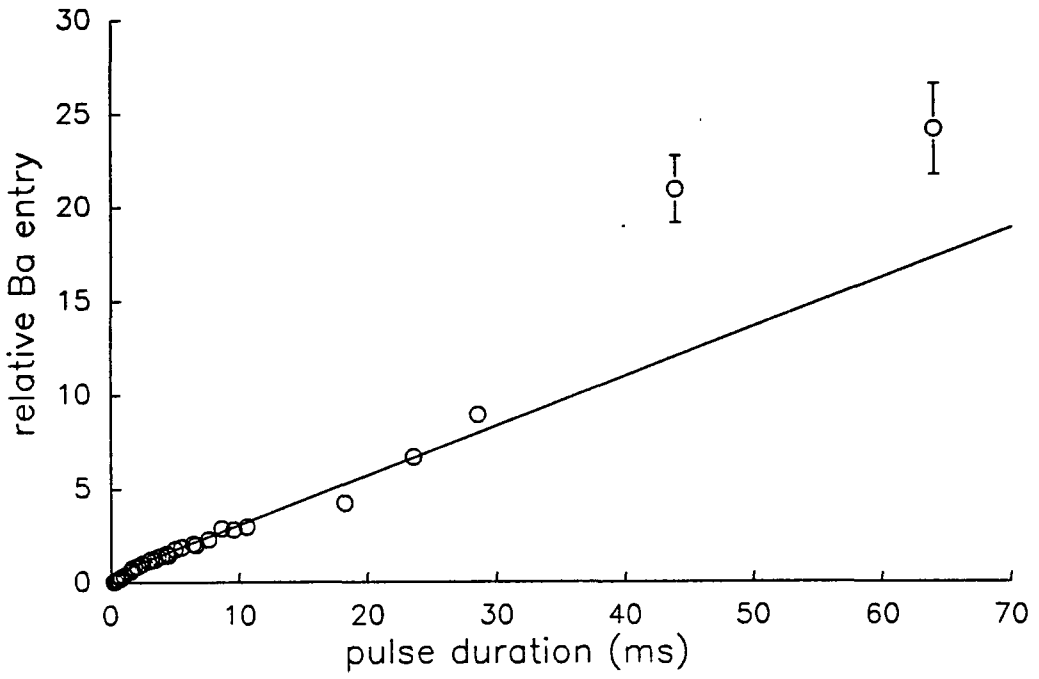


Fig. 5 Effect of pulse duration on "Ba entry".
Direct depolarizing pulses (TTX present) were given in short trains. Pulse duration and number of pulses per train were varied approximately inversely, in order to prevent excessive f_m. Data points are means from 11 cells, weighted by the number of repeats for each cell, ± s.e.m. Trains of pulses of each duration were bracketed one minute before and after by trains of 2.4 ms pulses. Values plotted at each duration are for 100•('Ba entry') relative to the average of that of the bracketing trains of 2.4 ms pulses, where Ba entry is defined as $(f_{tail}^{1/4} - f_o^{1/4})$ (see equation 4). Superfusion solution was (mM) 10 KCl, 120 NaCl, 2 $MgCl_2$ and bicarbonate buffer.

the time course of the effective nerve terminal depolarization and the time dependency of overall Ca^{2+} channel opening at the neuromuscular junction. Typical experimental results with pulse durations varied between 0.15 ms and 100 ms are shown in Fig. 5. At first appearance, the apparent Ba^{2+} entry per pulse is linearly related to the duration of the direct depolarization pulse, at least for durations less than about 30 ms; a best fit line is drawn (by eye) to the data for durations less than 30 ms. That is, every millisecond increment in duration of the presynaptic depolarization appeared to increase the apparent Ba^{2+} entry a constant amount.

The slope of plots such as Fig. 5 expressed per μA•ms of pulse duration is a measure of the average effectiveness of the direct depolarization pulse, in arbitrary units of rate of Ba^{2+} entry per charge passed from the current electrode. This effectiveness is very steeply dependent on the distance of the polarizing electrode from the endplate, but for any given polarizing electrode position, gradient for Ba^{2+} (or other divalent cation) across the nerve terminal membrane (dictated largely by extracellular Ba^{2+} concentration), and the number of channels available for activation, this effectiveness is a measure of a combination of variables which may be involved in the coupling of the stimulus to channel opening. These variables include the efficiency of conversion of the clamped current pulse into a voltage transient across the membrane, dependent on the

resistance and capacitance of the membrane and voltage or time dependent changes in these, and the voltage sensitivity of the presynaptic Ca^{2+} channel gate, and any voltage or time dependent changes.

In perfusates containing from 0.5 to 2 mM Ba^{2+} (10 K^{+}, TTX present), this type of plot was almost linear over a wide range of pulse widths between 5 ms and 100 ms. That is, the apparent effectiveness of the depolarization was constant, regardless of whether it was delivered as a small number of long pulses or a larger number of shorter pulses. This was not true, however, for pulse widths of 5 ms or less; Fig. 6 is an average from 11 junctions. At most junctions studied, the slope was very low for durations up to about 1 ms, after which the slope increased to a maximum at about 2 ms, decreased to nearly zero, subsiding thereafter to an intermediate slope which was quite constant over the longer pulse widths used. Fig. 7 shows the differential of the average plot.

In the following two sections, the experiments were designed to dissect from this overall coupling effectiveness those components whose role is in the ultimate delivery of a depolarizing voltage transient across the Ca^{2+} channels. With these components removed, or accounted for, the remaining components may be predominated by the putative voltage and time dependent Ca^{2+} channel at the motor nerve terminal.

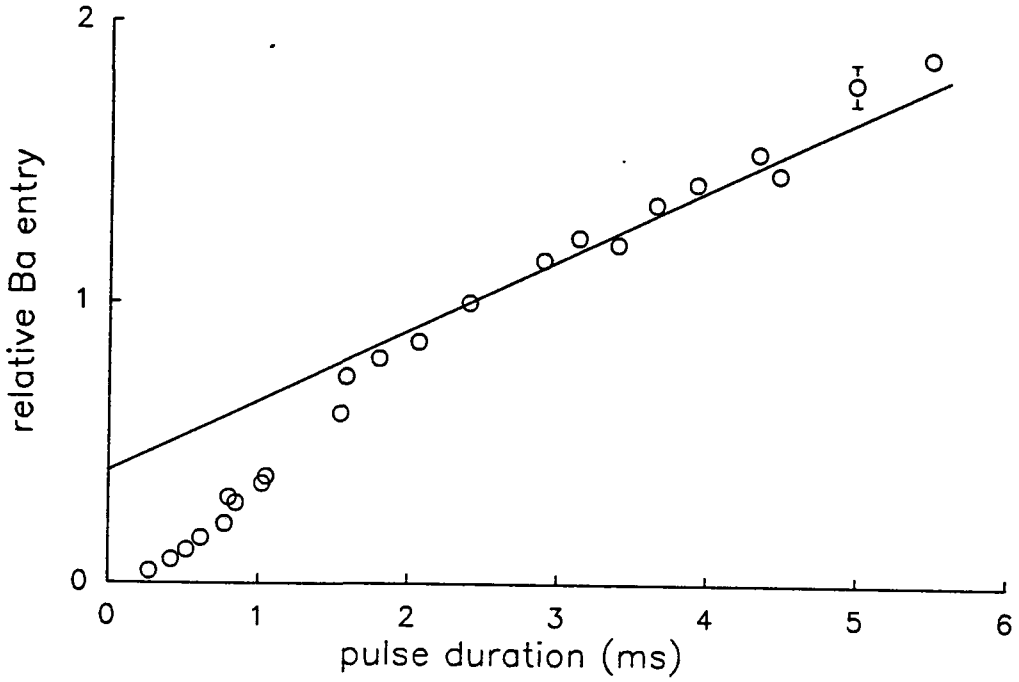


Fig. 6 Non-linear relation of Ba entry and pulse duration for brief pulses.

Detail of data shown in figure 5, showing the changes in apparent effectiveness of pulse prolongation in eliciting Ba entry.

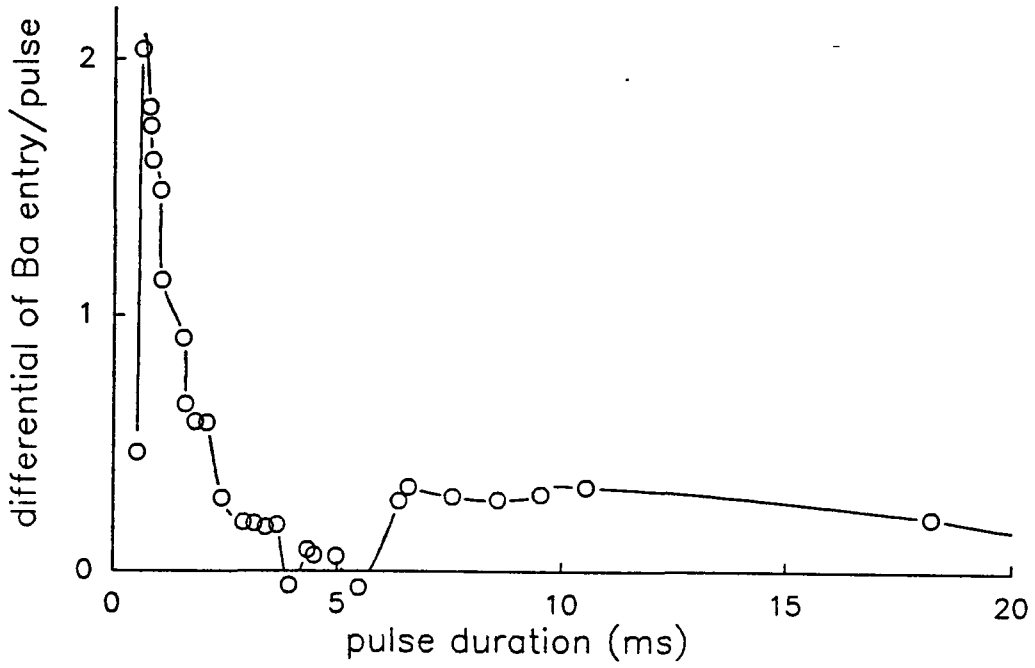


Fig. 7 Differential effectiveness of increases in pulse duration.

Differential derived from data in figure 5 (s.e.m. omitted), showing the changes in apparent effectiveness of pulse prolongation in eliciting Ba entry.

b) K^+ channel blockers

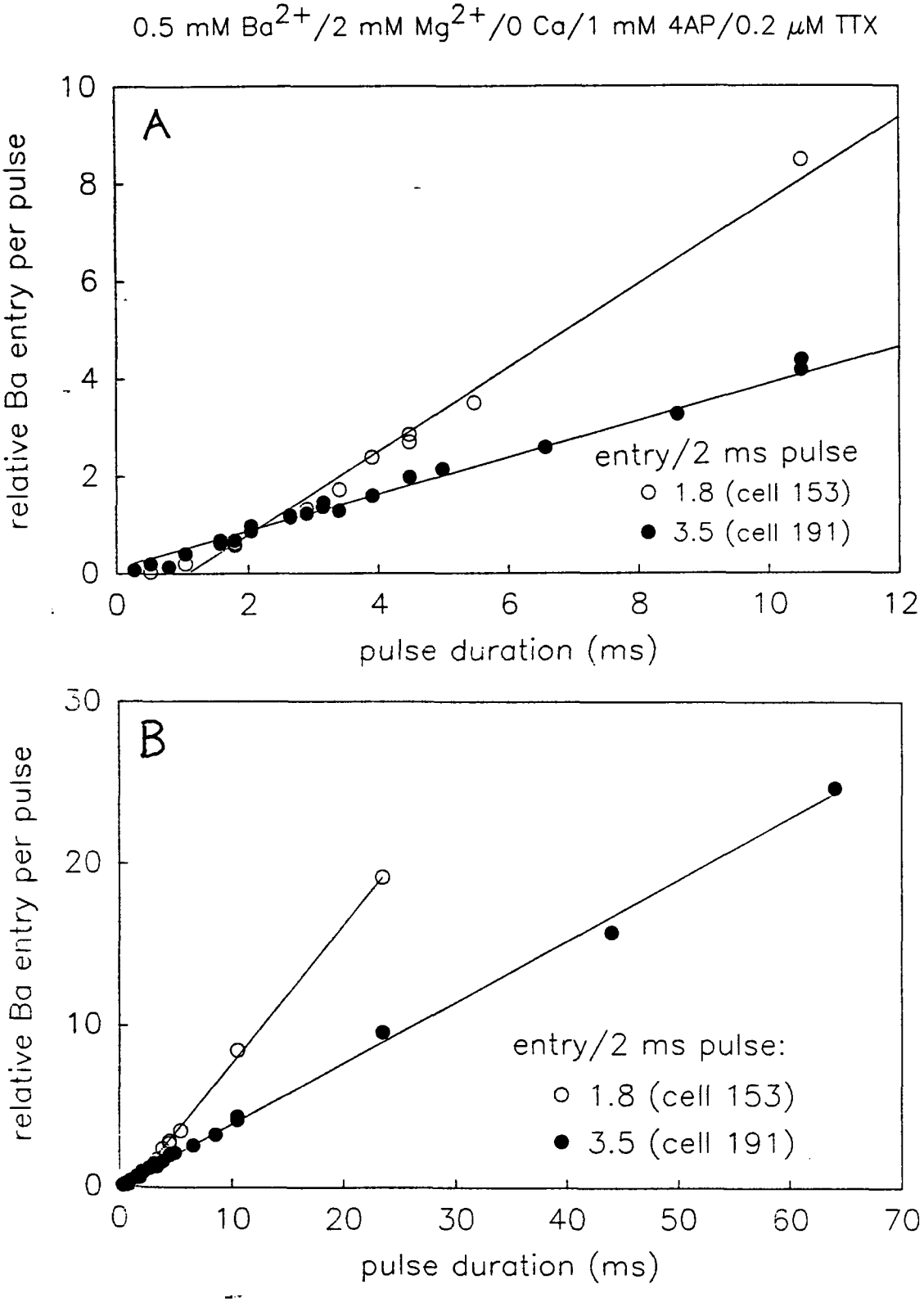
For a given clamped current stimulus, the magnitude of the voltage transient across the membrane is made greater by decreasing membrane conductance (Cooke and Quastel, 1973a). At the time of stimulation, the presence of tetraethylammonium (TEA) ions at the junction increases the effectiveness of the current pulse (Saint et al, 1987), presumably by a reduction in the membrane leak current and thus an increase in the voltage transient. Such a change in membrane resistance with TEA could not be large, since there was no significant difference in the membrane time constant (membrane resistance multiplied by membrane capacitance) when measured in the presence or the absence of 1 mM TEA using a rheobasic comparison method (see below). Only a very small change in membrane resistance and thus the voltage excursion elicited by an applied current pulse is necessary, however, to make a large difference to divalent cation entry, inferred from the observation that the fourth root release rate, proportional to Ca channel opening, appears to be very steeply graded with membrane potential.

The preparation was very sensitive to the presence of 4AP. With the addition of 0.2 to 1 mM 4AP, Ba^{2+} entry (delta fourth root release rate) was usually an order of magnitude greater than in the absence of 4AP. The effect of 4AP was immediate in onset but very slowly and usually incompletely reversible, in accord with previous observations (Saint et al, 1987).

Fig. 8A Linearity of relationship between pulse duration and Ba entry in the presence of 4AP.

Fig. 8B Linearity of response to prolonged pulses.

The relation between direct depolarization duration and non-phasic delta fourth root is used as an index of Ba^{2+} entry. Results from two different cells are shown in each panel, the top panel showing the shorter pulse durations, the bottom panel showing the longer durations. For both cells (separate experiments), the solution contained 0.5 Ba^{2+}, 2 Mg^{2+}, with 1 mM 4AP (and TTX) present. The Panel A shows linearity around the 1 to 4 ms region, in contrast to what was observed in the absence of 4AP; panel B shows that the same slope persists, for each cell, even to very long durations. The lines in the two panels are drawn with the same parameters (slope and intercept) for each cell, respectively. The difference in slope between the two cells shown was characteristic of the variation seen.


0.5 mM Ba^{2+}/2 mM Mg^{2+}/0 Ca/1 mM 4AP/0.2 μM TTX
A
relative Ba entry per pulse
pulse duration (ms)
entry/2 ms pulse
1.8 (cell 153)
3.5 (cell 191)
B
relative Ba entry per pulse
pulse duration (ms)
entry/2 ms pulse:
1.8 (cell 153)
3.5 (cell 191)

When either or both of the potassium channel blockers 4AP (1 mM) or TEA (0.5 mM) was present in the superfusate during the experiments with varied duration direct pulses (Ba^{2+}-containing solution, TTX present), the deviation from linearity of Ba^{2+} entry per pulse with increasing pulse duration was abolished, as shown in the example in Fig. 8 for 4AP.

c) Membrane time constant

A non-linearity which persisted in all bathing solutions (shown in Table 3) consisted of a decrease in pulse effectiveness for durations less than about 1 ms, consistent with a membrane time constant in the order of 1 ms. Values for membrane time constant, determined in a variety of conditions, using differing protocols (see METHODS), are given in Table 3. These data are consistent with those of Quastel & Saint (1986).

Direct pulses of durations less than the nerve terminal time constant synthesized from a combination of a depolarizing followed by a hyperpolarizing pulse, (see METHODS) appeared relatively equi-effective to longer depolarizing pulses in evoking release in Ba^{2+}, indicating that Ca^{2+} channel activation kinetics are much faster than the membrane time constant.

2. Timing of phasic release

a) Minimum latency - direct pulses

In Ca^{2+}, the average latency of peak phasic release

Table 3: Time constant of the nerve terminal measured under various conditions.

K^+	TEA	Ca^{2+}	Mg^{2+}	method	τ
10	.5	1	1	delay	0.7
10	.5	1	1	delay	0.7
10	.5	1	1	delay	1.2
5		8	1	duration	0.5
5		8	1	duration	0.7, 0.2
5	0.5	1	1	duration	0.5
5		1	1	duration	1.0, 0.5
5		1	1	duration	0.5
5		1	1	duration	1.3, 0.6
5	1.0	1	1	duration	1.4, 0.5

At some junctions, the transform did not yield a clear single time constant, rather an indication of two linear sections of the plot, wherefrom two slope estimates and two estimates of τ were made.

after the beginning of a direct depolarizing pulse focally applied to the nerve terminal (TTX present) was about 0.4 ms, consistent among junctions. Precision for minimum latency with direct pulses was limited by occasional interference with the residuum of the stimulus artifact after subtraction. Even with the best data in which the artifact was minimal and apparently consistent among stimuli, there was enough residuum after subtraction of the stimulus artifact to cluster the latency measurement of the first quanta released. Due to this limitation, further precision in determining minimum latency was not sought.

b) Phasic release time course

Phasic release consequent to a nerve action potential usually occurs in a period much less than 1 ms, 90% within about 0.2 ms. According to a fourth power intracellular Ca^{2+} model for release (see equation 2), the latency histogram of the fourth root of quantal release might be expected to fit first order growth and decay kinetics. In the example shown in Fig. 9, the onset and decay time constants were estimated by interpolating to find the latency of the peak of release, then iterating according to fitting equation derived from the sum of two exponentials:

$$y = A\ (e^{-at} - e^{-bt}),$$

where y is the fourth root of the instantaneous release rate, a and b are the inverses of the onset and decay time

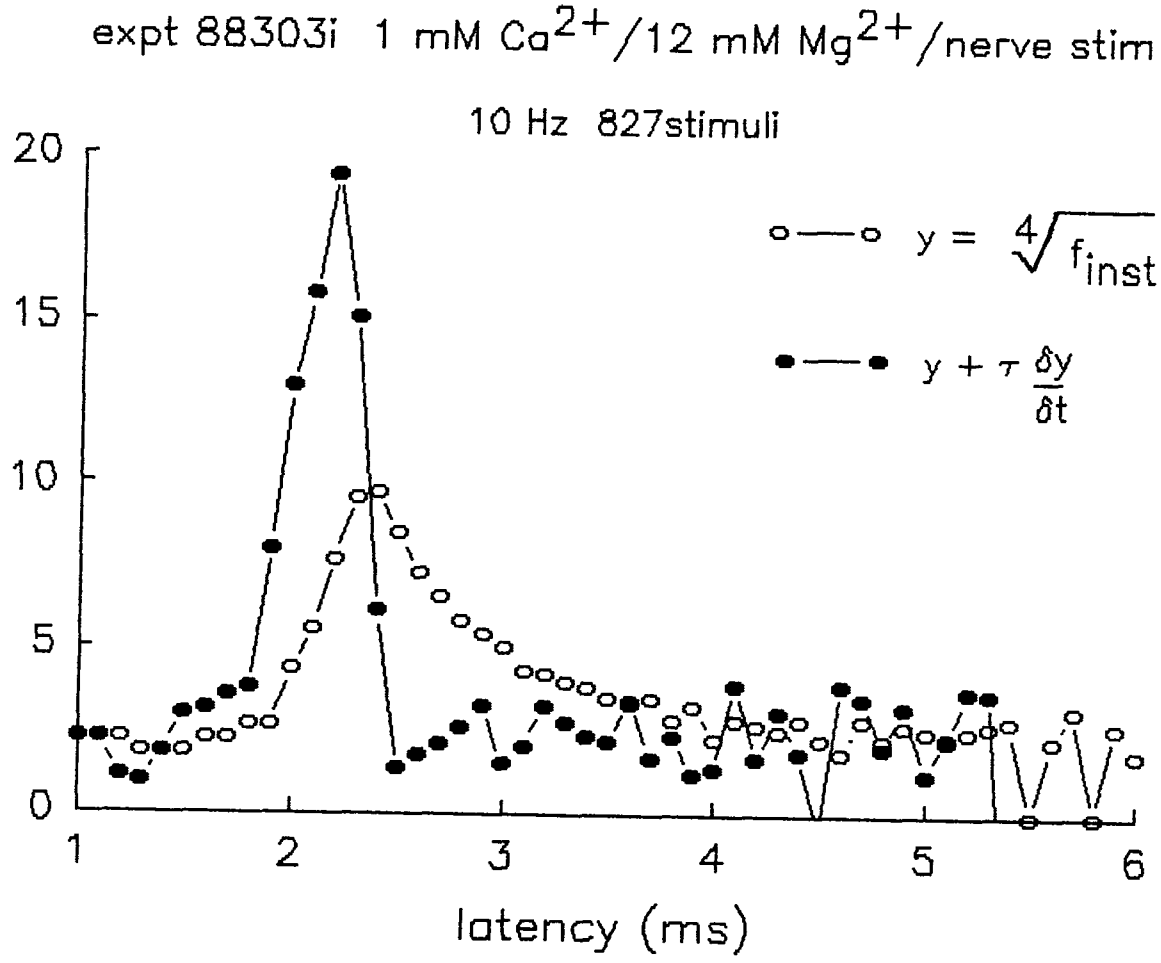


Fig. 9 Latency distribution of an e.p.p. and its derivative.

This example was recorded in 1 mM Ca^{2+} and 12 mM Mg^{2+} at 10 Hz, 827 nerve stimuli. Iterative best fitting of τ_r and τ_d (rise and decay time constants) was done by eye, according to a formula (see text). The ordinate is explained in the fiqure legend, in arbitrary units.

constants, respectively. The differential of y with respect to time is

$$dy/dt = A\ (-ae^{-at} + be^{-bt}).$$

At the time of the peak, P, dy/dt = 0, so

$$b/a = e^{-aP} / e^{-bP}, \text{ and}$$

$$P = \ln(b/a) / (b-a)$$

The differential curve (e.g. fig. 9, closed circles), should approach an impulse function, without any 'undershoot', when the appropriate time constants are chosen. Upon iteration, best fit time constants were found to be about 0.2 ms (=1/a) and 0.5 ms (=1/b), respectively.

The time course of the process can be determined directly from a histogram of either the latency of every quantum released, or the latency of the first quantum to be released after each stimulus, the first latencies method (Barrett & Stevens, 1972a). Fig. 10 shows that for recordings in conditions of low quantal content, ie. less than about 2, the difference between the latency histograms from these two methods is very slight.

c) Sources of variance in timing of phasic release

Autocorrelations of quantal release and of multiple quanta e.p.p.s show very close coupling in time of the release process with the nerve terminal action potential, with more variability among the apparent times of arrival of nerve action potentials at the nerve terminal, as shown in Fig. 11.

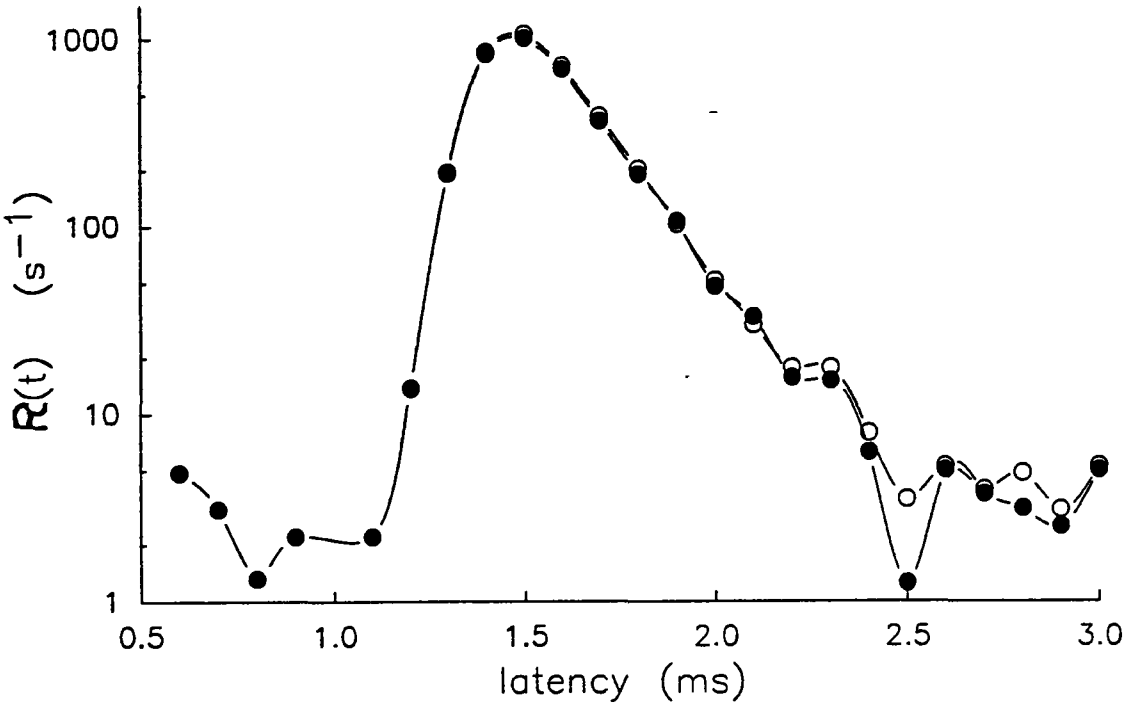


Fig. 10 Latency distributions of all quanta and of first quanta.

An example of the latency distribution of phasic release, expressed as instantaneous release rates in each 0.1 ms bin, with nerve stimulation. The upper curve is the latency histogram of all quanta; the slightly lower curve is the histogram of the latency of the first quantum after each stimulus. Ordinate: instantaneous release rate, measured in 0.1 ms bins after each stimulus. Abscissa: latency, or time in ms after the stimulus.

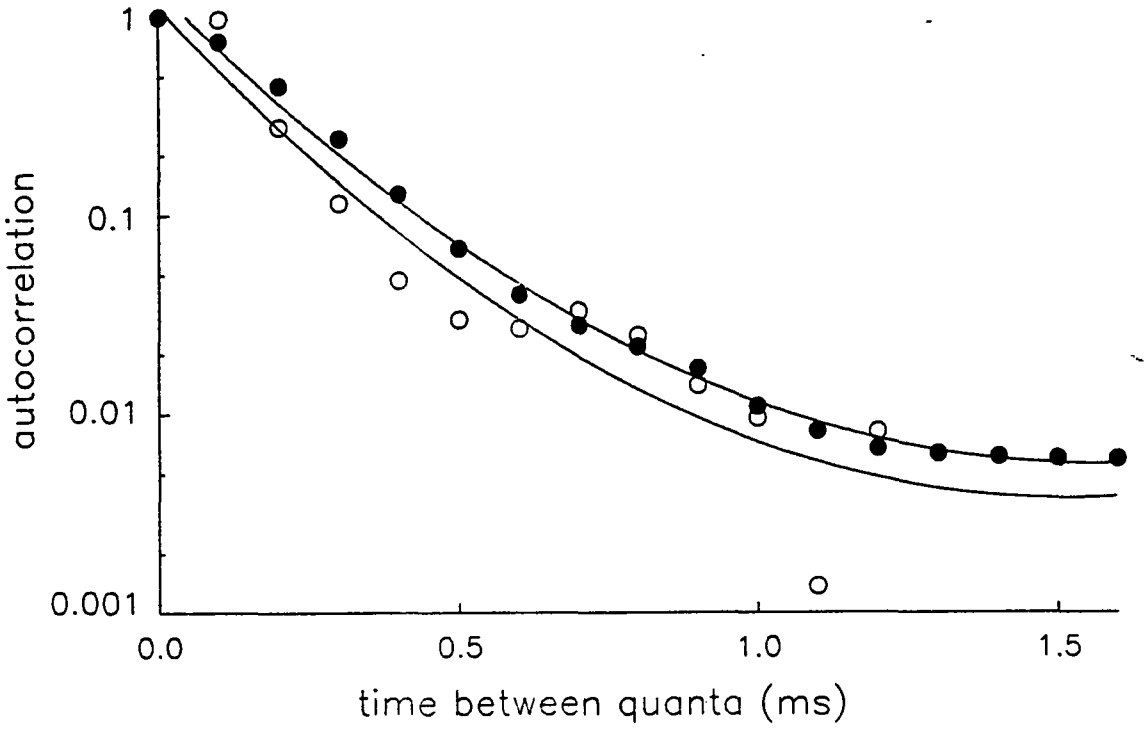


Fig. 11 Autocorrelation of quantal latencies within and among e.p.p.s.

Comparison of the autocorrelation of quantal latencies within multiple quantum e.p.p.s (open circles) with the autocorrelation of all quantal responses (closed circles). The data are from the same junction as in Fig. 10.

Such autocorrelations were used to determine the viability of the nerve terminal action potential. Those which were viable had an autocorrelation among all responses which at 50% of the responses was no more than 0.1 to 0.2 ms wider that the autocorrelation within multiple quanta responses. Non-viable nerve terminal action potentials resulted in autocorrelations among responses that were spread out over 1 ms or more, while the autocorrelation within e.p.p.s remained tightly distributed. The drawback to the routine use of the autocorrelation test for nerve terminal action potential viability is that it is not useful for experiments in which the quantal content is much less than one, since there are not enough multiple quantum e.p.p.s from which to construct the autocorrelation within the e.p.p.

Another source of variance in nerve evoked release arose in prolonged tetanic stimulation. Tetani were accompanied by an increase of 0.1 to 0.4 ms in latency and spreading of the phasic release latency histogram, depending on the stimulation frequency, as exemplified in Fig. 12. In terms of autocorrelation, both the among and within correlations were broadened, indicating that the variability in timing had increased in both the nerve terminal action potential and the coupling of the action potential with release, respectively. The latter variability in timing may indicate a broadening of the presynaptic action potential.

Another source of e.p.p. timing variability is one which is endogenous to the nerve terminal itself, the effect

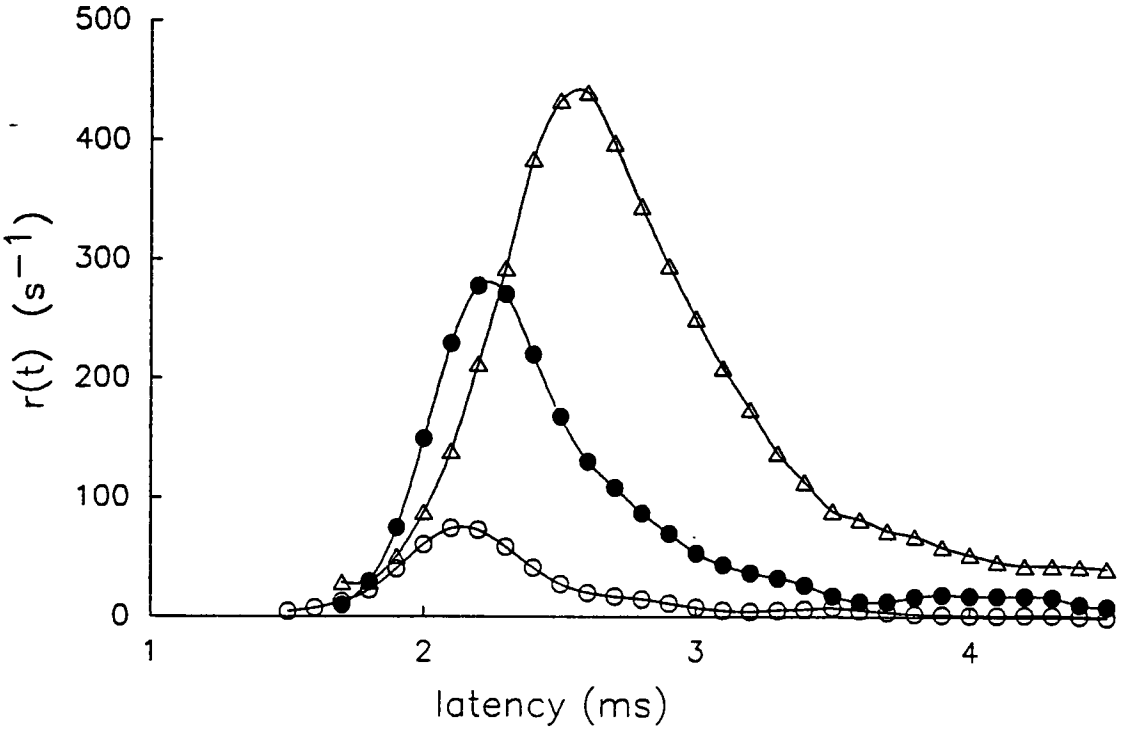


Fig. 12 Effect of prolonged tetanus on quantal latencies. Prolonged tetanus in low Ca^{2+} raised Mg^{2+} results in increase in synaptic delay and spreading of the time course of phasic release. This example is Expt. 88328a; the perfusate included 0.4 mM Ca^{2+}, 2 mM Mg^{2+} and 66 μM bekanamycin (to maintain low *m*). Stimulation was 55 Hz; progressively higher curves correspond to first, second and third 30 s samples of a 90 s tetanus. Ordinate and abscissa as in Fig. 10.

or correlate of spontaneous 'bursts' of m.e.p.p. frequency. These bursts are characterised by increases in f_m by one or two orders of magnitude which persist for seconds. During a burst, the latency of phasic release increases slightly, recovering to normal as the burst subsides.

Bursts can also be elicited, somewhat repeatably, by stimulating a terminal with a hyperpolarizing current (Cooke and Quastel, 1973a). In solution containing 2 mM Ca^{2+}, 1 mM Mg^{2+}, 10 mM K^+, and no TTX, it was possible to deliver long hyperpolarizing pulses of 5 to 15 ms in duration (< 1 Hz) at a magnitude between 5 and 15 μA such that about half of the pulses elicited a burst. Under these conditions in which a burst was sometimes present within the first milliseconds of a long hyperpolarization, a brief (about 1 ms) depolarization of magnitude just at threshold for firing a nerve terminal action potential was superimposed on the hyperpolarization at about 3 ms delay. Whenever a burst was present during the hyperpolarizing pulse, the latency of the e.p.p. response decreased, indicating increased excitability of the nerve terminal occurred concomitantly with an elicited burst.

Finally, alterations in the latency, or variability thereof, of phasic release can be induced by various pharmacological agents. In preliminary experiments, the K^+ channel blocker 4AP (0.2–1 mM) caused a prolongation (and enlargement) of the phasic component of the latency histogram.

B. Stimulation-induced enhancement of release

21. Residual ion: Sr

a) Stimulation in Sr^{2+}

Short trains of nerve stimuli at 90 Hz in the presence of Sr^{2+} (no Ca^{2+}) elicited phasic and non-phasic release which grew with each additional pulse during the train, the non-phasic release subsiding within seconds after the train to pre-train levels. In the series of experiments reported here, the number of pulses in the train was randomized to between 1 and 35, with progressively fewer trains as train length increased, according to the weighted randomization algorithm (see METHODS). Each train was followed by 2 seconds without stimuli to permit measurement of the declining non-phasic release rate, f_m. Fig. 13 shows an example of the growth of *m* and f_m during a train and the f_m tail after.

As with the results of Quastel and Saint (1988) with Ba^{2+}, it was observed early in the experiments with Sr^{2+} that for short trains, the increase in the f_m after the train depended upon the number of pulses in the train. As a first approximation, the data were analysed in a similar manner to those of Quastel and Saint (1988), according to a fourth power residual ion model for enhancement of non-phasic release. For short trains, it was clear that the non-phasic delta fourth root (see Equation 4, METHODS) increased linearly with number of pulses, consistent with a

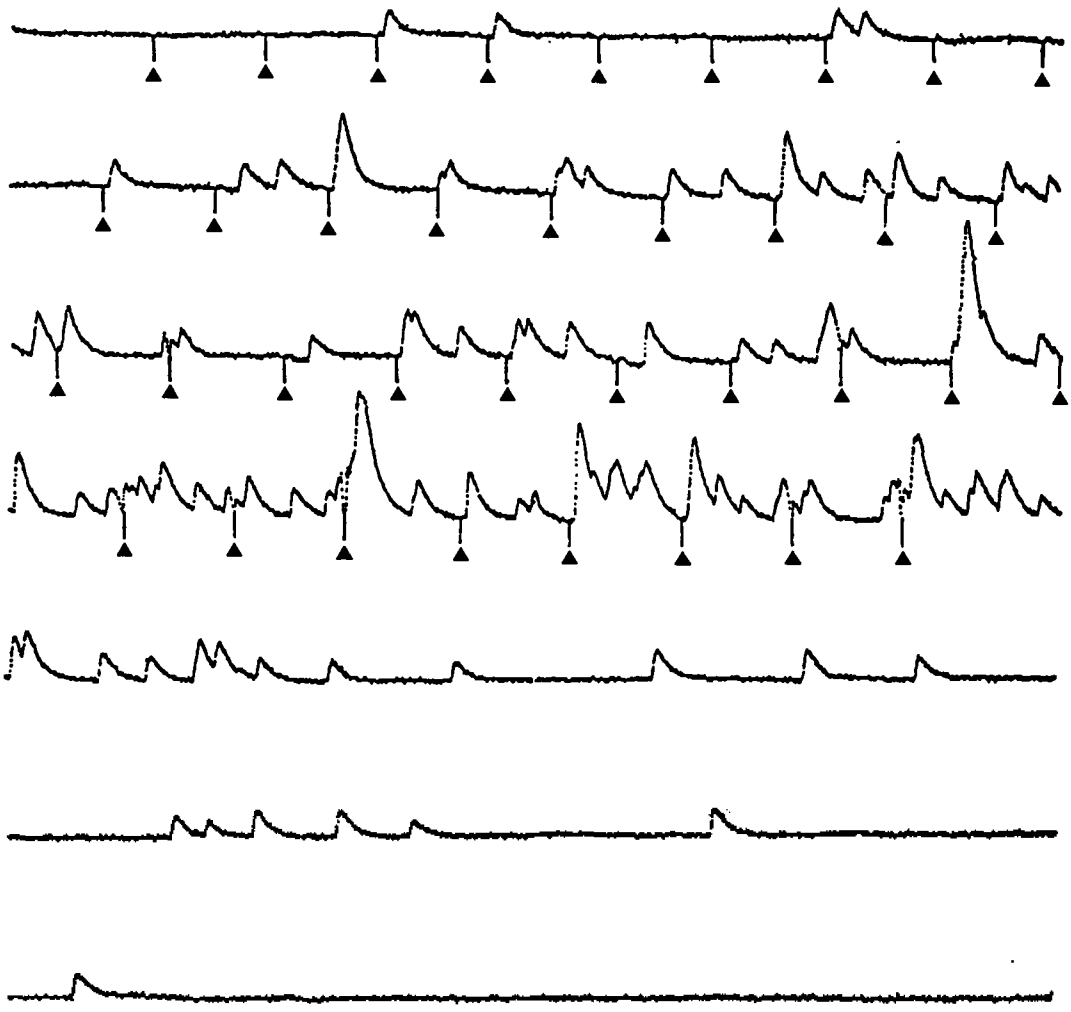


Fig. 13 Buildup and decay of *m* and f_m during and after a train of stimuli in Sr^{2+}.

An example of the *m* and f_m response to one train of nerve impulses at 11 ms intervals in Sr^{2+} containing solution. Experiments were carried out with random trains in the presence of 0.5 to 1 mM Sr^{2+} with 2 to 8 mM Mg^{2+}, this example is 1.3 mM Sr^{2+} with 8 mM Mg^{2+}. Stimuli as marked.

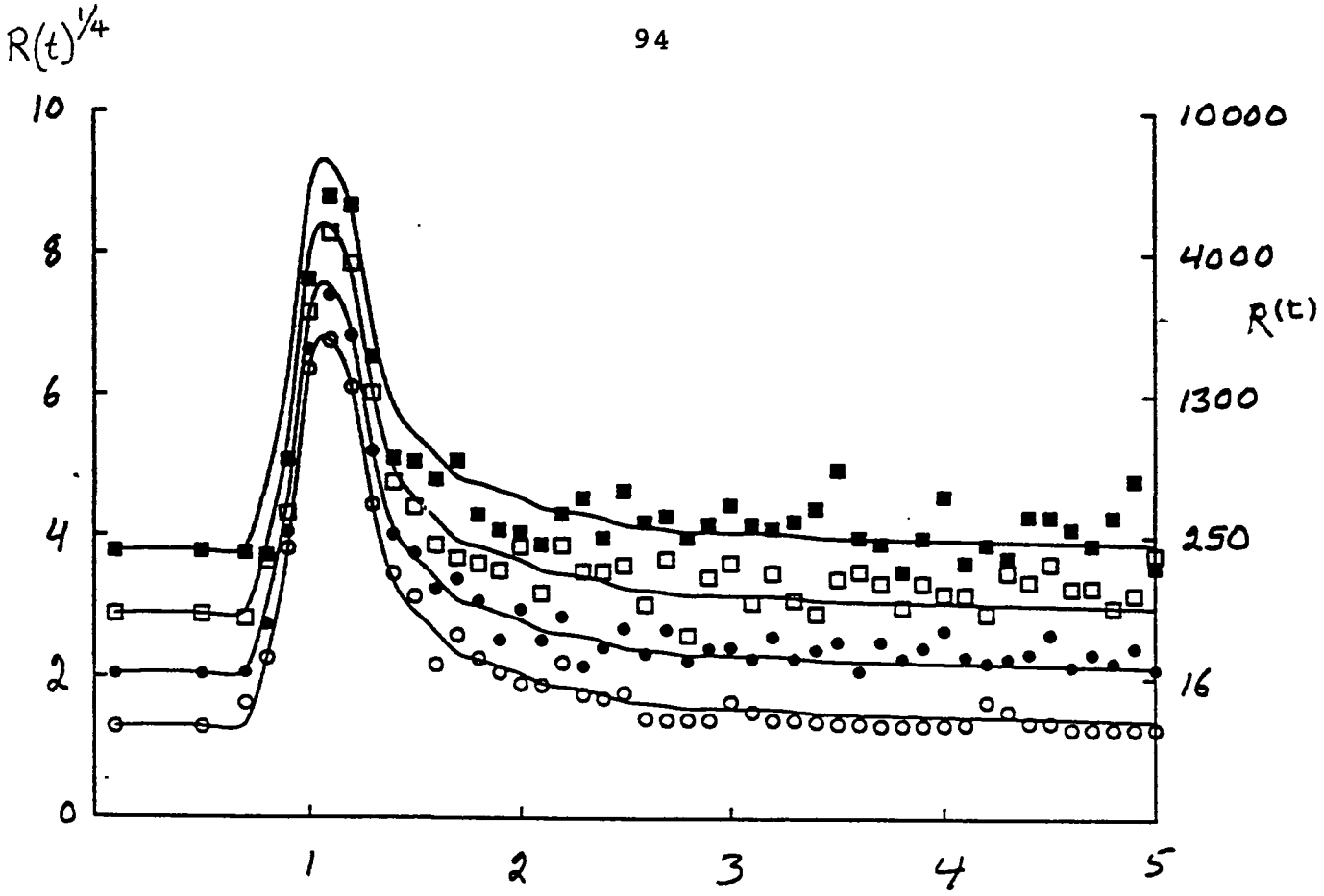


Fig. 14 Co-modulation of phasic and non-phasic release in Sr^{2+}.

On a fourth root basis, indicating the amount of Sr^{2+} present in the nerve terminal, repetitive stimulation resulted in additive, residual ion facilitation. Latency histograms show transmitter release as rates determined in each 0.1 ms bin after a stimulus. The curves, progressing upward, correspond to the average histogram for stimulus numbers 1-3, 4-10, 11-17 and 18-35 of a train. The lines are a best fit to the average of the two lowest curves, vertically displaced to superimpose each curve for comparison purposes.

fourth power residual ion (Sr^{2+}) model, such that $f_m = k(N \cdot C_i + C_o)^4$, where N is number of pulses, C_i is Sr^{2+} influx per pulse, and C_o is Sr^{2+} activity in the nerve terminal in the absence of stimulation. For longer trains (trains lasting longer than about 60 ms, more than about 5 stimuli at 90 Hz, for example), the non-phasic delta fourth root, or apparent Sr^{2+} entry, appeared to increase less than predicted by the model. It was obvious that the rapid decay of f_m in Sr^{2+} during the delivery of the train compared to that in Ba^{2+} ($\tau \approx 3$ s) would necessitate correction of the actual number of pulses given in a train to an 'effective' number of pulses. The 'effective' number of stimuli in a train would be the number of pulses that would raise f_m an equivalent amount if they were delivered at one time at the beginning of the train, before any decay of the purported residual Sr^{2+} giving rise to the non-phasic release could occur. In order to make this correction, the time constant of the putative residual Sr^{2+} was required and was estimated based on a first order decay of non-phasic delta fourth root determined for a series of tails of raised f_m. Using the corrected number of pulses, N' for each train, it was clear that for trains of any length, the putative Sr^{2+} entry (non-phasic delta fourth root) increased linearly with the 'effective' number of pulses given, consistent with a residual ion model (Bain and Quastel, 1992).

The apparent buildup of intracellular residual Sr^{2+} during trains also enhances the phasic component of release

in strict accord with a residual ion model. Figure 14 shows that the peak amplitude of release histograms for successive stimuli in trains grows with position in the train by the same amount as the non-phasic component for the same stimuli grows, on a fourth root basis.

b) Estimation of *n* and τ

In order to avoid building a bias toward a fourth power release model into the analysis of the Sr^{2+} data, a method was used which assessed the most likely power, *n*, at the same time as the time constant of decay of apparent intracellular Sr^{2+} activity. A similar, although less extensive, analysis was previously carried out for Ba^{2+} (Quastel & Saint, 1988). In the present analysis, it was observed that the decay curve of f_m after the train could be linearized with a log transform, whatever the root ($1/n$) taken prior to the transform. Likewise, the curve describing the growth of f_m could also be linearized with any root. A least squares best fit was carried out by computer (program by Dr. D. M. J. Quastel) for each line generated for each of several values of *n* greater than 1. Each plot (Figure 15 is an example) gave an estimate of τ as a slope and a goodness of fit as a correlation coefficient. It was found that for various sets of data, either multiple stimulation series from the same junction or from different junctions, the *n* of the particular plot which gave the highest correlation coefficient was not consistent. That is, for best fit linearization of the apparently first order

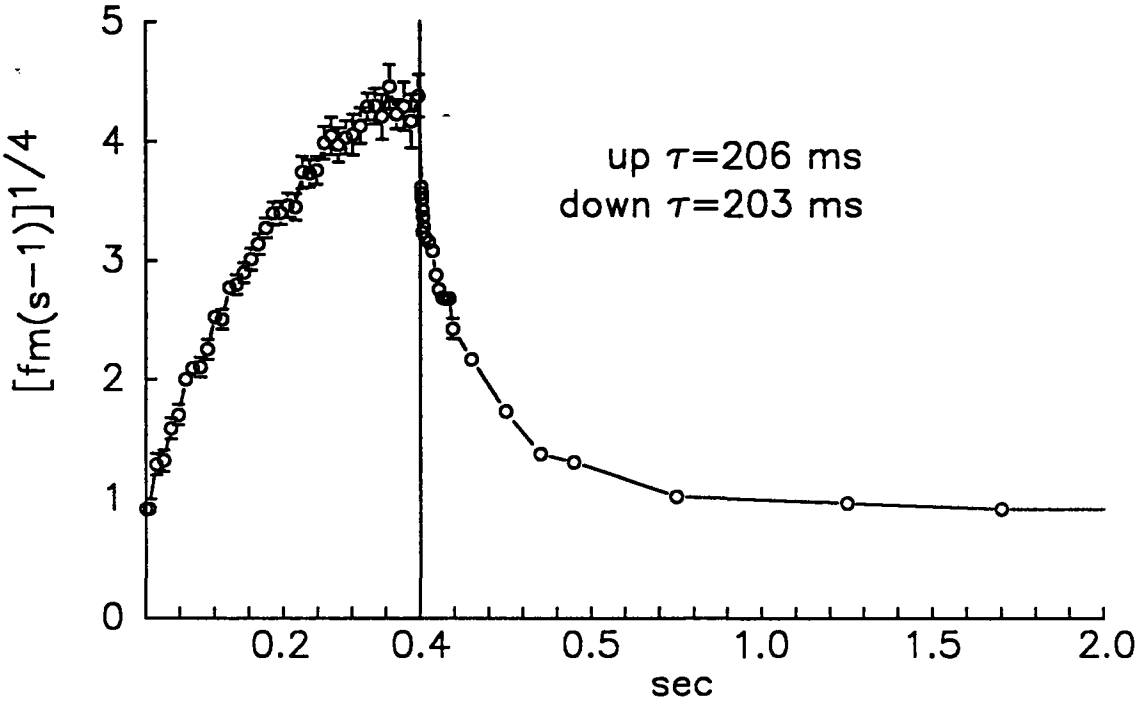


Fig. 15 Fourth root transform of f_m buildup and decay in Sr^{2+}.

Non-phasic release buildup during random length trains and the decay of f_m after the train. Note the consistency of the first order time constant for the growth and decay of the non-phasic release rate when the chosen transform is with $n = 4$. The solution was 1.3 Sr^{2+}, 8 Mg^{2+} (Expt. 891212e).

process of f_m growth and decay in Sr^{2+}, one particular *n* did not appear to have a clear advantage. Thus, the best fit line for each *n* gave an estimate of the τ for that proposed *n*, but the *n* and τ combination which was consistent with any particular model was not apparent from considering the growth and the decay of f_m separately.

A definitive measurement of *n* and τ was achieved upon making the assumption that the same first order decay process occurs both during the buildup of f_m during the train (putative buildup of intracellular Sr^{2+}) and as the f_m tail subsides (putative loss of intracellular Sr^{2+}) after a train. It is also assumed that the same *n* pertains during buildup and decay. Thus, the best fit τ was calculated for *n* between 1 and 8, for both the rising and the decay phases of f_m. For the rising phase, the larger the chosen *n*, the shorter the best fit τ, whereas for the falling phase, the higher the *n*, the longer the τ. In this way, it was observed that there could be only one combination of *n* and τ that would provide a good fit for both the rising and falling phase, and thus satisfy the above assumptions. Fig. 15 exemplifies the consistency of growth and decay τ in Sr^{2+} when the chosen *n* is 4. Fig. 16 is an example of one such analysis in which *n* and τ were determined as the coordinates at the intersection of plots of the rising and falling phase best fits for *n* and τ. According to this analysis, the means of *n* and τ ($\pm$ s.e.m.) were 4.23 $\pm$ 0.22 and 248 $\pm$ 10, respectively, n = 22 junctions. Recordings which were

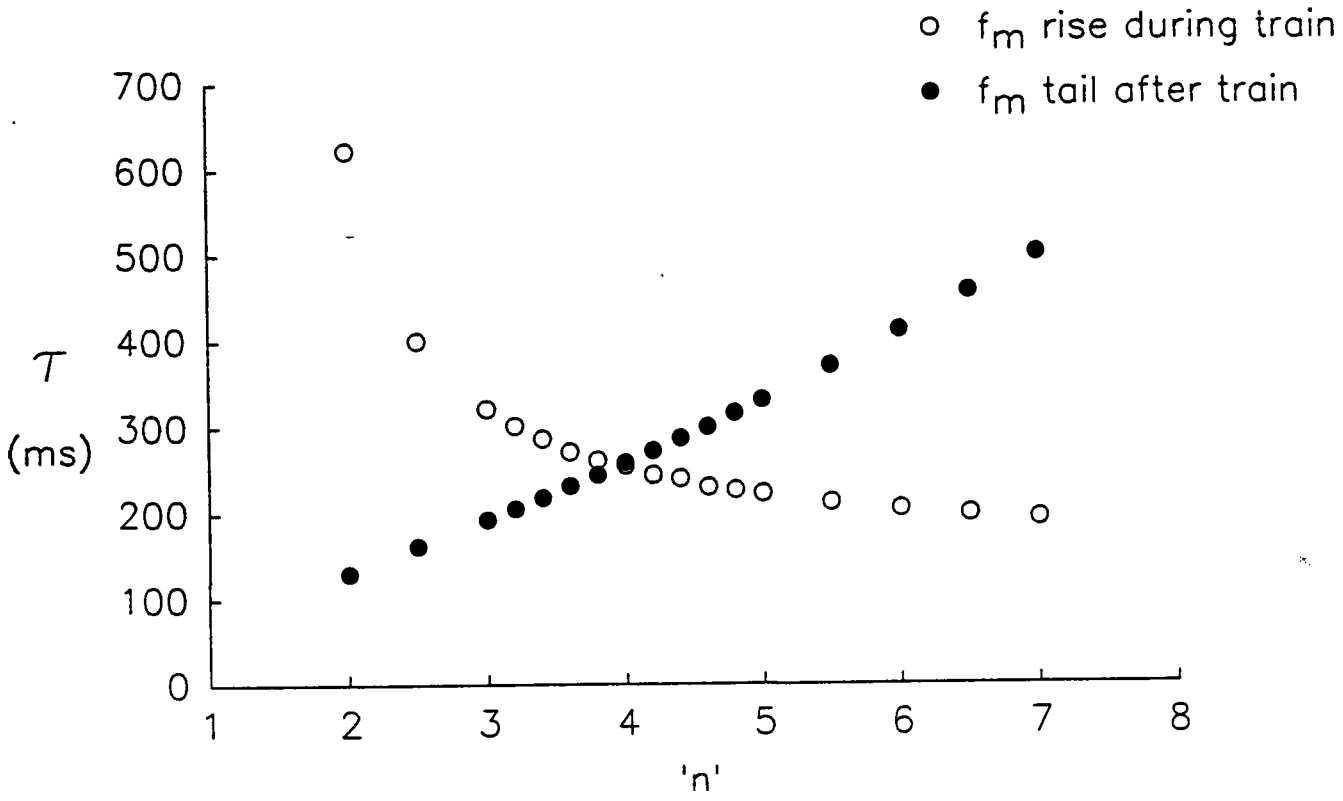


Fig. 16 Estimation of τ and *n* for Sr^{2+} by best fit.

An example of the best fit routine for *n* and τ on the growth of non-phasic release during a train in 1 Sr^{2+}, 8 Mg^{2+} (open circles) and after the train (filled circles). The intersection was always well defined for junctions which had a moderate to low resting f_m, with *n* about 4 and τ about 220 ms. (Expt. 90104-2505).

excluded from this analysis included those in which the f_m was persistently high, defined in practice as an f_m over 10/s persisting in the absence of stimulation.

Thus, while the observation of linearity of delta fourth root with the 'effective' number of pulses clearly supports a residual ion model for the short term enhancement seen in Sr^{2+} containing solutions, the above analysis also supports the residual ion model for Sr^{2+}, but without requiring assumption of a particular *n*. Instead, the only assumption was that the process(es) with the predominant influence on the growth and on the decay of f_m had a time constant in common.

c) Estimation of Sr^{2+} entry

For each series of data, usually consisting of 100 to 300 random trains or more for cells or conditions of low quantal content, two parameters were estimated: entry per pulse, and the time constant of decay, τ. There are three variables involved in the calculation: Sr^{2+} entry, which is equivalent, according to the model, to the non-phasic delta *n*th root at the time of the e.p.p., Sr^{2+} time constant of elimination, and the *n* for Sr^{2+} action in the nerve terminal. Of these variables, an assumption is made that *n* is known to be 4 (according to the independent method of determination mentioned above). If the time constant was assumed to be 200 ms (which was approximately the mean of the time constants as determined above), entry could be calculated from the f_m in the tail. Non-phasic delta fourth

root was extrapolated back to the time of the pulse according to a first order decay, *n* of 4, with time constant τ of 200 ms. The initial value of Sr^{2+} or amount of Sr^{2+} entry, according to the model, was determined by least squares fitting of non-phasic delta fourth roots from the tail to:

$$\ln S(t) = \ln S_o - t/200$$

where S is the non-phasic delta fourth root at any time t and S_o is S extrapolated to the time of peak Sr^{2+} entry, time 0. In some analyses, entry and τ were estimated by iteration of the above equation with varied τ to find the entry and τ combination for which the correlation coefficient was highest for the decay curve. However, in practice it was found that the error variance, which increased greatly toward the end of the tail where f_ms were relatively low, appeared to introduce an unacceptably high variability into the entry and τ best fits to make them much greater or less than the mean values among series, despite very little improvement of the correlation coefficient over that determined with the assumption of τ = 200 ms.

d) Comparison of Sr^{2+} with Ca^{2+} and Ba^{2+}

Assuming a residual ion model, for any one nerve impulse or direct stimulus, divalent cation agonist enters the nerve terminal in a small bolus through channels which are opened in a phasic (although not simultaneous, Quastel et al, 1992) manner, resulting in phasic release of neurotransmitter followed by diffusion of the divalent

agonist throughout the nerve terminal cytoplasm. The 'residual ion' concentration at release sites resulting from diffusion then causes non-phasic release of neurotransmitter until the active ion concentration is reduced through a process which removes the ion from the cytoplasm.

With this assumption, the difference among the divalent agonists in the manner in which they support neurotransmitter release can be quantified in terms of a "dilution factor" (Bain and Quastel, 1992a). If the increment of non-phasic delta fourth root per pulse is taken as an indicator of intracellular bulk Sr^{2+} concentration and the increment in the phasic delta fourth root for the same pulse indicates the average peak Sr^{2+} at the release sites, then the ratio of the latter to the former could be considered a factor by which the effect of Sr^{2+} becomes "diluted" just after it enters the nerve terminal. This dilution factor does not depend on the potency (inherent in k) of the divalent agonist, since the factors involved in potency are inherent in both delta fourth roots, and thus cancel. On the other hand, for Ca^{2+}, Sr^{2+} and Ba^{2+}, phasic release (as indicated by phasic delta fourth root), dilution factor and potency correlate. The dilution factor observed for Sr^{2+} was 24.8 ± 1.0 (s.e.m.), less than that estimated for Ca^{2+} but more than for Ba^{2+}.

Although the apparent time course of phasic release is identical in Ba^{2+} and Ca^{2+} (Quastel et al, 1989) as well as in Sr^{2+} and Ca^{2+} (Fig. 17) the divalent agonists differ

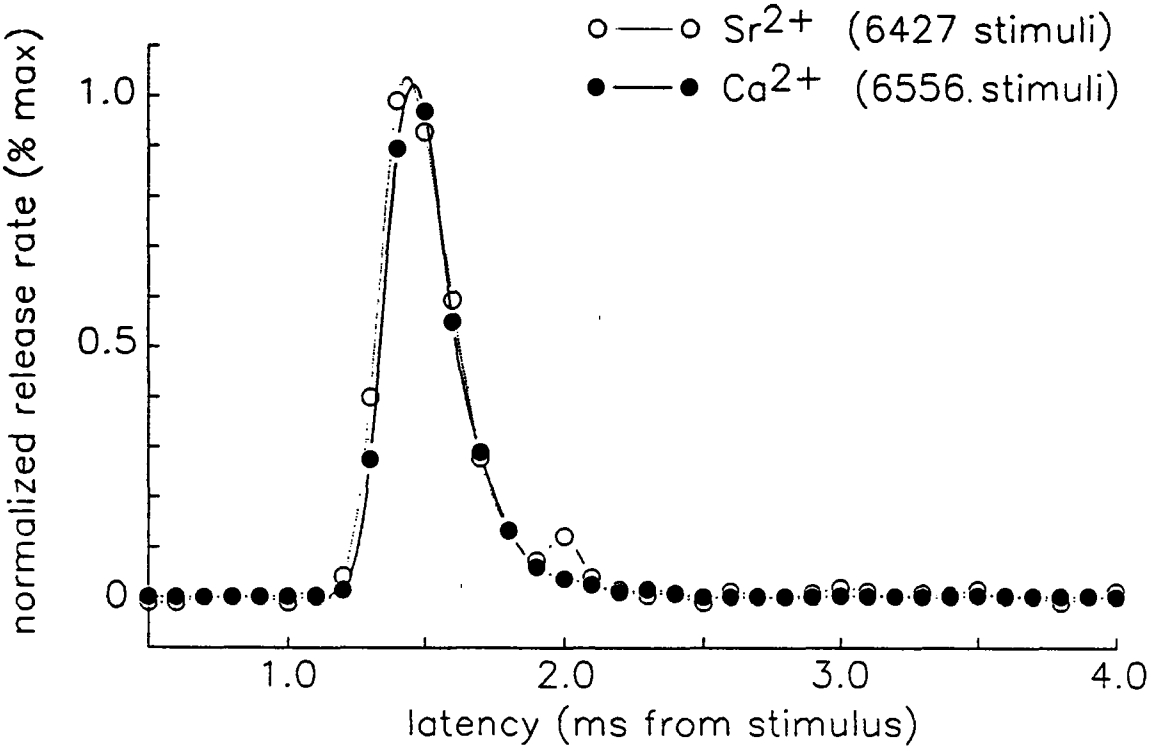


Fig. 17 Demonstration of the identity in time course of phasic release in Ca^{2+} as in Sr^{2+}.

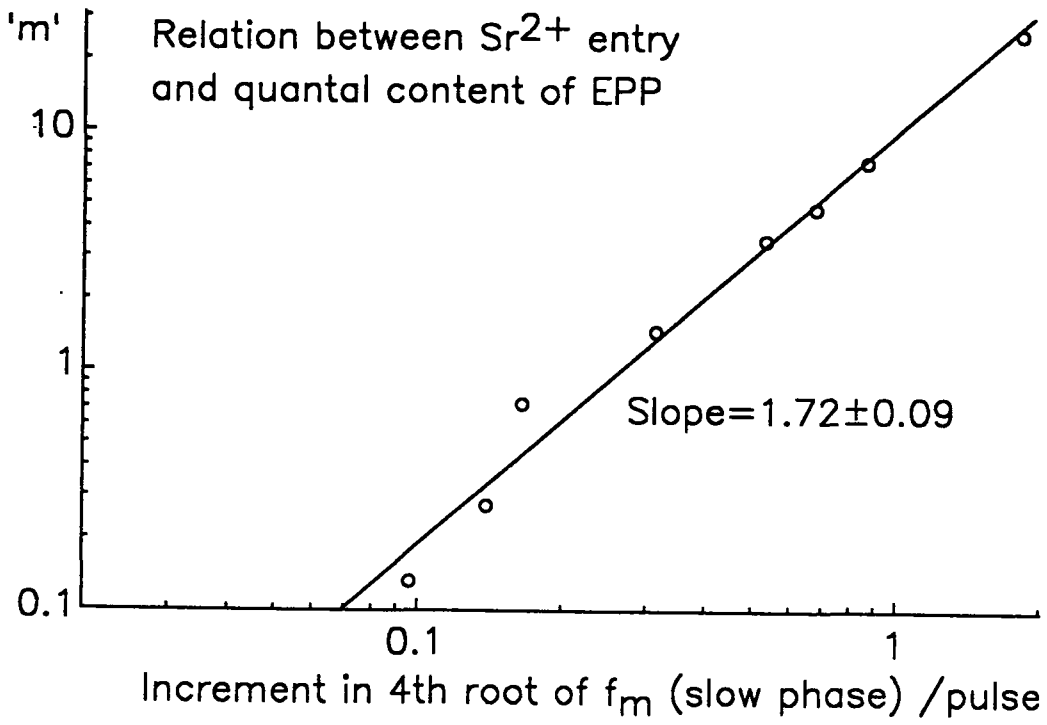


Fig. 18 Relation between Sr^{2+} entry and quantal content of the e.p.p.

Direct depolarizations (0.5ms duration) in 1 Sr^{2+}, 1 Mg^{2+}, 0.2 TEA, 0.5 4-AP, and 0.4 μM TTX. While the *n* appears to be 4 for non-phasic release, it is nearly 2 for phasic release.

widely in terms of apparent absolute potency and timing of their intra-terminal distribution and disposal, reflected in the peak phasic release rate for a given extracellular concentration and in some of the non-phasic time courses, respectively.

Using direct pulses with TTX and K^+ channel blockers TEA and 4AP present, the dilution factor, calculated as above, decreased as the depolarization was increased. This negative correlation stems from the same mechanism underlying the result that a log-log plot of *m* versus non-phasic delta fourth root per pulse (putative Ba^{2+} entry) gives a slope of two rather than four in Ba^{2+} (Quastel et al, 1992) and in Sr^{2+}, as shown in Fig. 18, when *m* was modulated by changing direct polarization intensity. This bias toward phasic release at smaller intensities of nerve terminal depolarizations can be explained in terms of a small number of channels per release site, the presence of stochastic heterogeneity among channels and release sites, and thus a decreased dominance of the intracellular cooperativity of 4, as proposed by Quastel et al (1992).

e) Effect of BAPTA/AM

In consideration of the indirect manner in which the majority of the data with Sr^{2+} supported a residual ion model for stimulation induced enhancement in the presence of Sr^{2+}, a more direct assay of residual Sr^{2+} was desirable. A method for direct removal of free Sr^{2+} ions from the cytoplasm by chelation would allow determination of the

contribution of these ions to stimulation induced enhancement of neurotransmitter release. BAPTA-AM is apparently able to diffuse into the cytoplasm, where its aminomethyl group is enzymatically cleaved, producing a chelator for Ca^{2+} which is trapped in the cell by its ionic nature (Tsien, 1981).

After incubating a diaphragm in 500 μM BAPTA/AM for 5-15 minutes followed by return to control superfusate, both phasic and non-phasic release were considerably and irreversibly reduced at every junction so treated. Upon examination of the 'tails' of raised f_m after trains in BAPTA treated cells in the presence of Sr^{2+}, it was found that the reduction of magnitude of non-phasic release was accompanied by a prolongation of the apparent time constant of intracellular Sr^{2+} (determined as the best fit slope of log transformed non-phasic delta fourth root against time). In various cells, all of which had a time constant for non-phasic delta fourth root in Sr^{2+} of about 200 ms prior to BAPTA, the time constant after BAPTA varied between 200 ms to 500 - 2000 ms. As shown in Fig. 19, after BAPTA/AM the prolongation of the time constant was correlated with the reduction in apparent Sr^{2+} accumulation per pulse, ie. non-phasic delta fourth root per pulse. At the same time, while the phasic and non-phasic delta fourth roots in various cells were correlated in the absence of BAPTA, they were similarly correlated after BAPTA loading, except that the

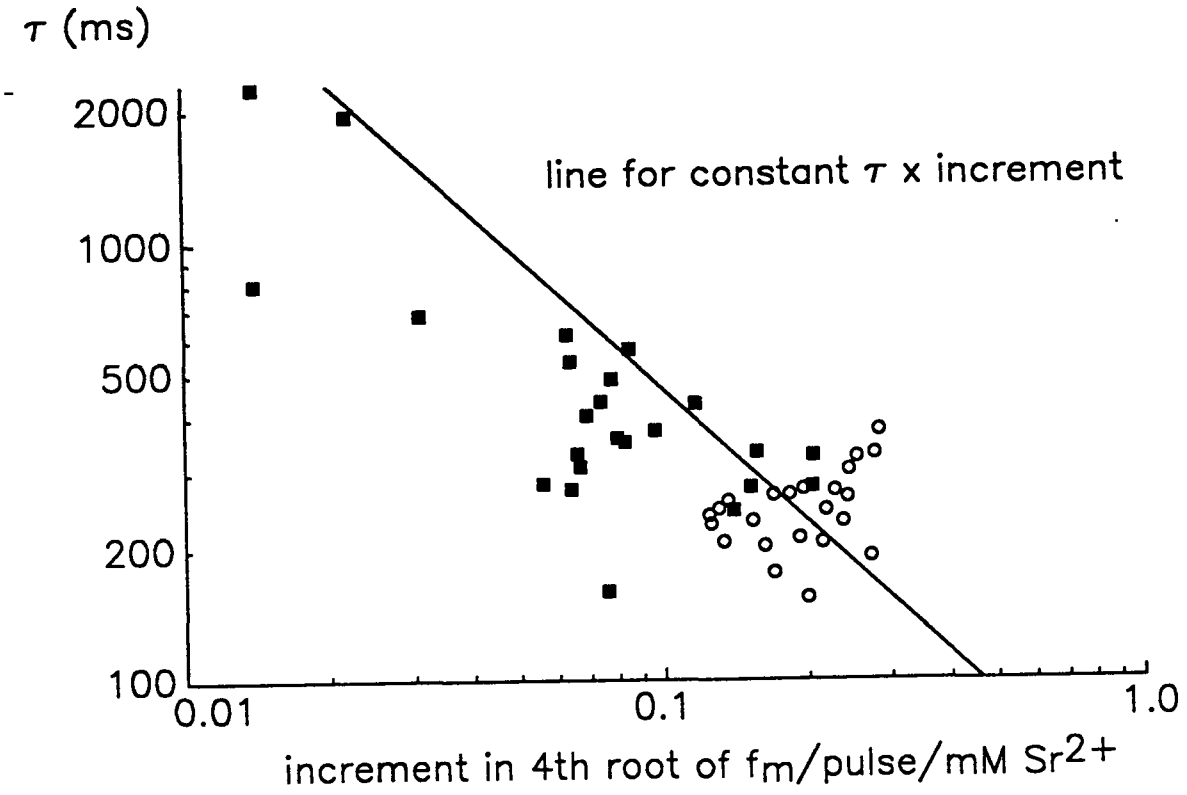


Fig. 19 Effect of BAPTA-AM on τ and apparent entry in Sr^{2+}.

Modification by BAPTA-AM exposure of the response to stimulation in Sr^{2+} included a prolongation of the f_m decay τ. The control τs (circles) were close to 220 ms, while after exposure ot BAPTA-AM (squares), τ was prolonged, to a degree correlated with the decrease in non-phasic delta fourth root (apparent Sr^{2+} entry).

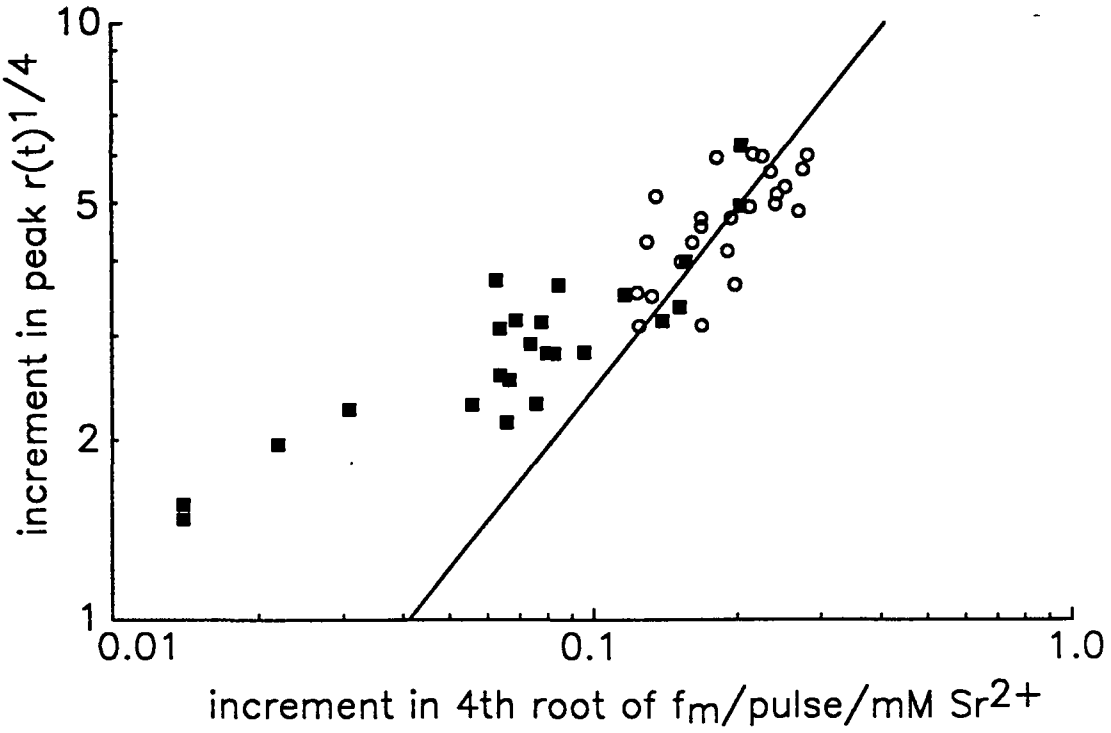


Fig. 20 Relative effect of BAPTA-AM on phasic and non-phasic release in Sr^{2+}.

BAPTA reduced the non-phasic delta fourth root more than it reduced the phasic delta fourth root. BAPTA-treated (squares); control (circles).

non-phasic delta fourth root was depressed more than was the phasic delta fourth root, as seen by an inflection (at an abscissa value of about 0.05) in the correlation of the two measurements in Fig. 20. This result gives further support for the residual ion hypothesis for Sr^{2+} by showing that the presence of an intracellular buffer for Sr^{2+} reduced the apparent magnitude of the tail of f_m, presumptively by removing a fraction of free intracellular Sr^{2+} ions from the cytoplasm. The slowing of overall rate of removal (prolonged time constant of non-phasic delta fourth root) would be expected on this basis, from a reduction of the ratio of free to bound Sr^{2+}.

2. Facilitation in Ca

a) Time components

(1) Trains and postpulses

Facilitation was observed within and after short trains of stimuli at up to 100 Hz, in superfusates containing low Ca^{2+} raised Mg^{2+}. In the first experiments, an attempt was made to determine the time course of facilitation of phasic and non-phasic release using post-train test pulses at varied intervals. From these data it was obvious that *m* and f_m facilitation under these conditions was similar to that reported by Hubbard (1963), with *m* and f_m rising and falling nearly in parallel. The decay time constant of the prominent phase of facilitation was approximately 80 ms. Fig. 21 (lower two curves) shows that the maximum facilitation during a train appeared to be reached after 5

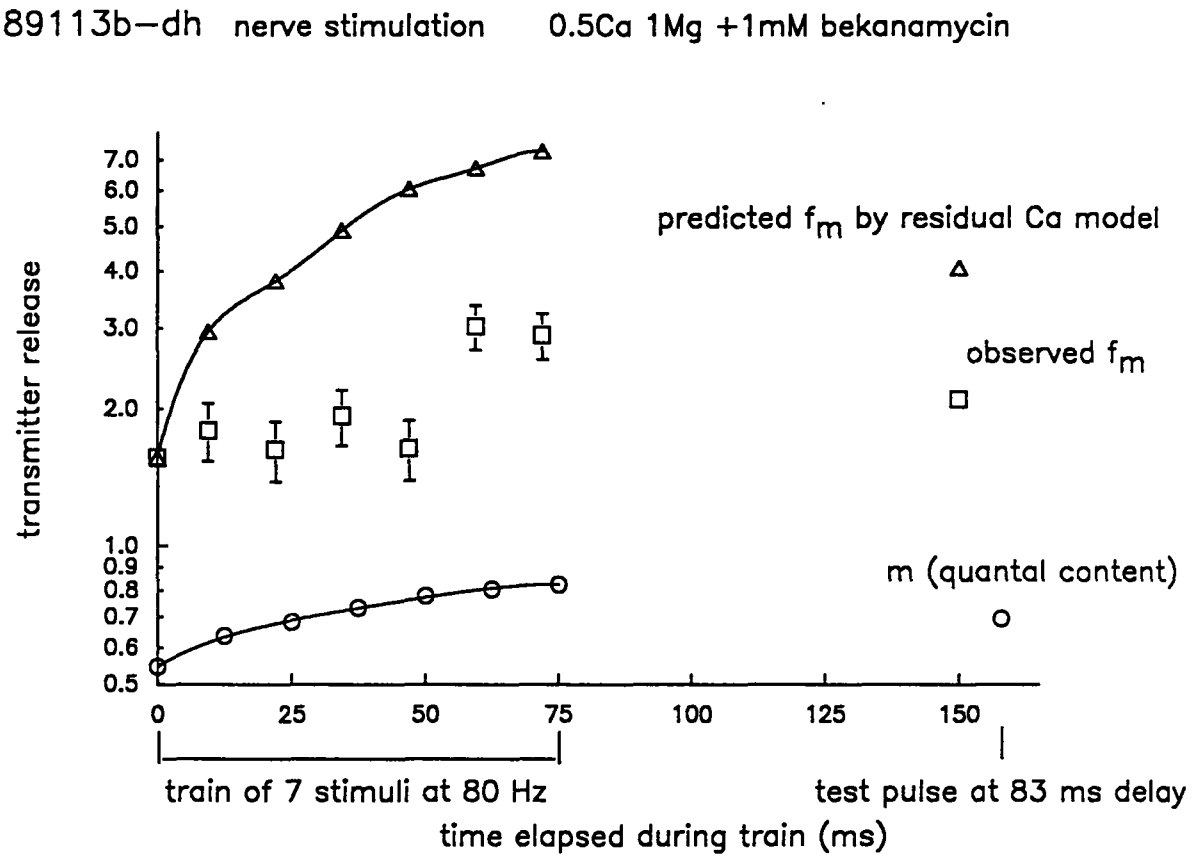


Fig. 21 Observed and predicted facilitation of *m* and f_m by short trains in Ca^{2+}.

An example of septuplets of nerve stimuli at 80 Hz with a post-pulse at delays which were varied from series to series (83 ms delay shown, expt. 89113b-dh). The solution contained 0.5 Ca^{2+} and 1 Mg^{2+}; 1 mM bekanamycin was added in place of higher Mg^{2+} to reduce quantal content to below the threshold for muscle action potential and twitching.

or 6 pulses (at 80 Hz stimulation rate), corresponding to a similar time constant for growth of the process as for its decay. However, this method was not practical for obtaining more accurate data on facilitation time course for two reasons:

1) at usual f_ms, there were not enough m.e.p.p.s in time bins brief enough to allow determination of the time course of f_m facilitation with reasonable accuracy (±10%, s.e.m); and
2) the time of data acquisition in order to acquire sufficient numbers either of phasic quanta, under low *m* conditions, or of non-phasic quanta, under the usual low f_m conditions for reasonable accuracy was so long that variation in the overall *m* or f_m as they drift in time would often far exceed the magnitude of the short term facilitatory effects, and the relative and absolute facilitation of *m* and f_m often changed with drifts to higher and lower *m* and f_m.

Despite these shortcomings, the simple train and post-pulse stimulation protocol was able to clearly demonstrate, in all cells in which facilitation was evident, that facilitation in Ca^{2+} could not be accounted for by a simple residual Ca^{2+} model, in contrast to the facilitation in Sr^{2+} shown earlier. The uppermost line in Fig. 21 shows the f_m that would be predicted by such a model, such that the putative residual Ca^{2+} would be $(m_f \cdot 1000)^{1/4} - (m \cdot 1000)^{1/4}$, and the facilitated f_m would be the fourth power of the sum of

residual Ca^{2+} and $f_o^{1/4}$. Even with large standard deviations in the low f_m measured between stimuli, it was clear in most junctions that the predicted f_m exceeded the observed by nearly an order of magnitude.

(2) Pseudo-random stimulation

Experiments conducted to determine the time course of facilitation in the presence of Ca^{2+} were made much easier and more accurate by the use of random interval, or 'pseudo-random', stimulation (see METHODS). A typical example of the phasic and non-phasic response to pseudo-random stimulation is shown in Fig. 22a and 22b. In this example, as above with the septuplets, the predicted f_m according to the residual Ca^{2+} hypothesis is shown (uppermost symbols). The growth and decay of *m* and f_m, shown on a log scale, are normalized relative to the *m* or f_m measured after the longest of the intervals, in this example 0.5 and 1.6/s, respectively.

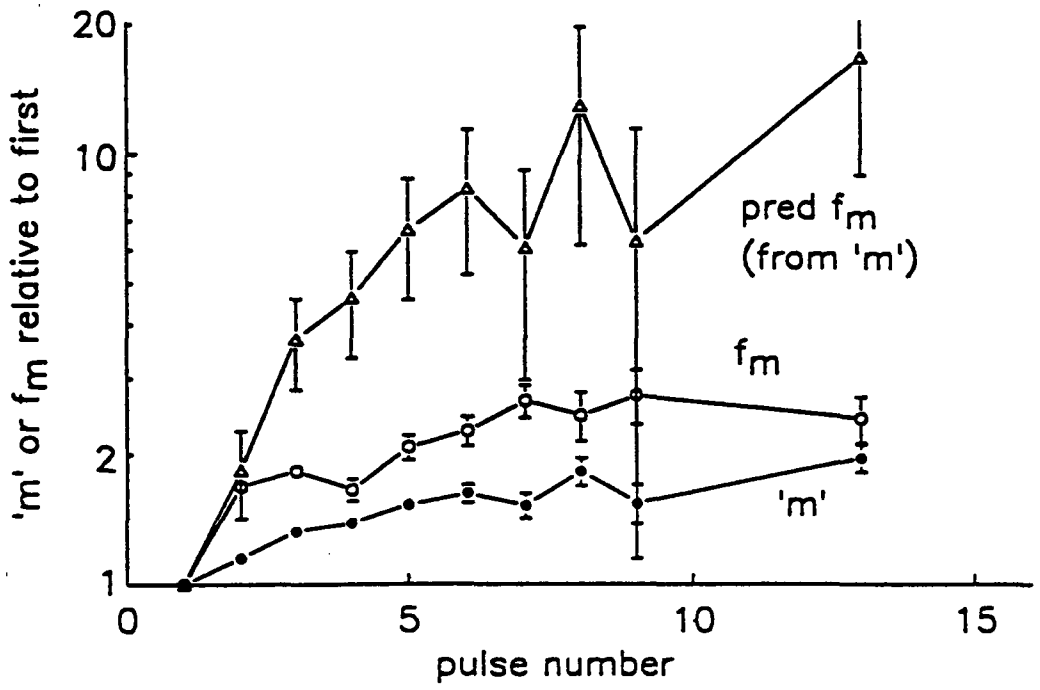
(3) Resolution of components

Since enhancement of release by stimulation in the presence of Ca^{2+} does not conform to the residual Ca^{2+} model, a variety of alternative models can be proposed (see DISCUSSION). In the present work, resolution of various components of facilitation was not based upon time course, but instead upon components of the model proposed for all release, as given in Equation 2, repeated here: $R = k(C_i + C_r)^n$.

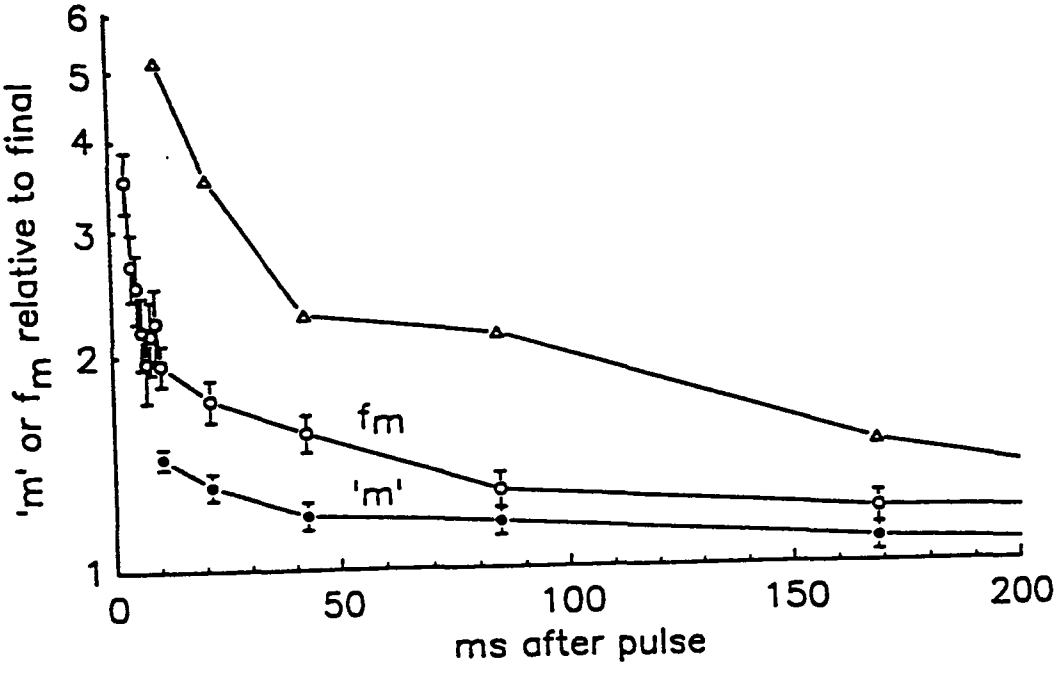
Fig. 22 Facilitation in Ca^{2+} using continuous stimulation with random intervals.

Using repetitive nerve stimulation with randomly selected intervals between 11 ms and 0.7 s, facilitation was observed as it developed during each "train" (see Methods) and as it subsided during the longer intervals (panels A and B, respectively). The pattern of facilitation under this continuous but random interval stimulation was virtually identical to that seen with individual trains (Fig. 21). The solution was 0.5 Ca^{2+}, 8 Mg^{2+}, (expt. 90317h).

A


20
10
2
1
0
5
10
15
'm' or f_m relative to first
pulse number
pred f_m
(from 'm')
f_m
'm'

B


6
5
4
3
2
1
0
50
100
150
200
'm' or f_m relative to final
ms after pulse
fm
'm'

From series of data such as the above, two components of enhancement of non-phasic release, whose time courses partially overlap, could be resolved. As previously discussed (see METHODS, Enhancement Calculations), the analysis involves finding the contributions of increases in k, a multiplier, and increases in C_r, residual Ca^{2+}, to the enhancement of phasic and non-phasic release in the period starting with about 10 ms and ending with about 500 ms following a stimulus. The assumption being made that the mechanisms which would contribute to k are distal to intracellular concentration of Ca^{2+} in the effector pathway, it was possible to begin resolution of components by establishing values (as a function of time since previous stimulation) for X, the multiplicative component of enhancement. Calculation of the multiplicative component X as the fourth power of the ratio of facilitated to unfacilitated phasic delta fourth root is unaffected by the possible simultaneous presence of a residual ion process, unlike the more conventional calculation of multiplicative facilitation as simply the ratio of facilitated to unfacilitated *m* (Hubbard, 1963). While multiplicative facilitation calculated according to the latter calculation is descriptive of the major component of *m* and f_m facilitation often seen in Ca^{2+}, the former, more explicit calculation is necessary to dissect out a multiplicative component where a significant residual ion component is present.

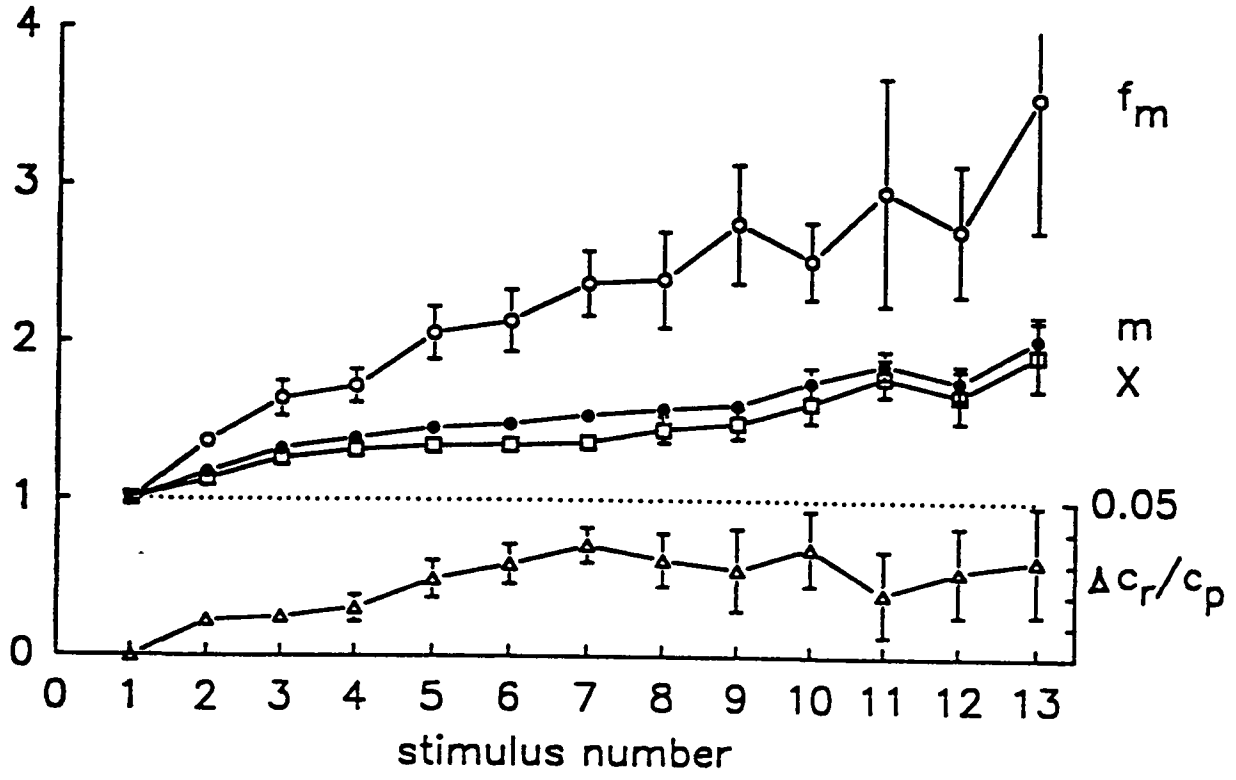


Fig. 23 Growth of facilitation in Ca^{2+}: multiplicative and 'residual Ca^{2+}' components.

Growth of *m*, f_m, residual Ca^{2+} and factor X for 26 series of data. In 23 series, 2-4% DMSO was present to increase f_m and *m* in order to improve the counting statistics. In most cases, the solution included 0.5 Mg^{2+} and 8 Mg, although this was altered on occasion to maintain *m* lower than threshold for postsynaptic activation.

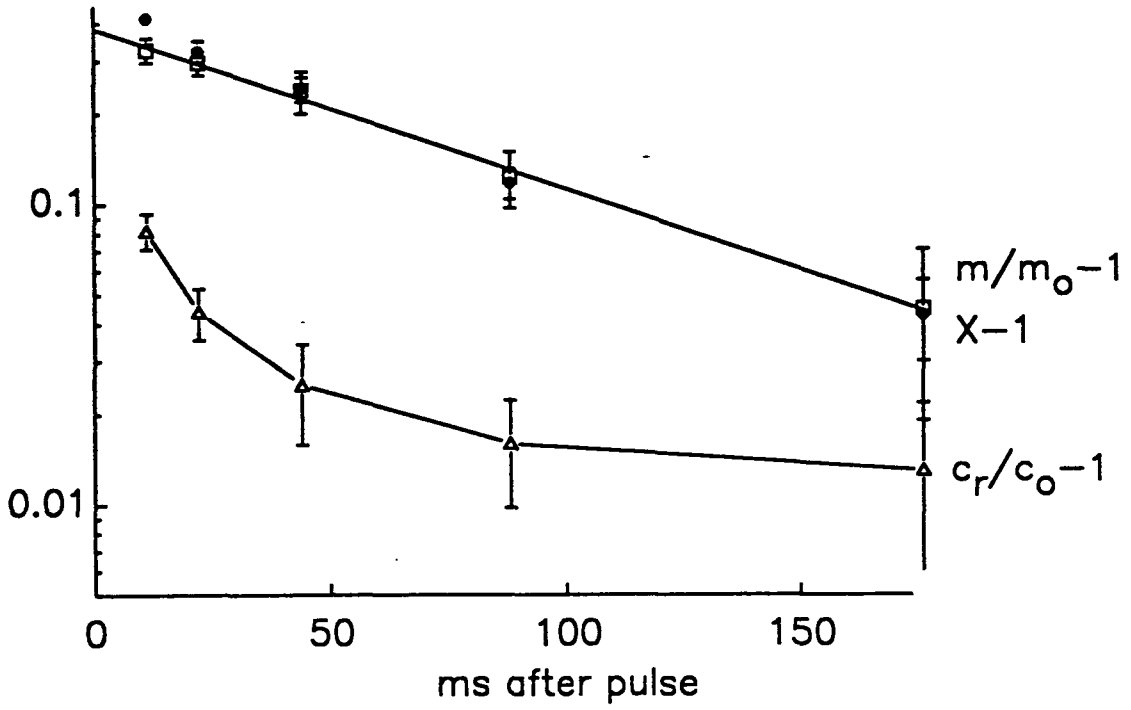


Fig. 24 Decay of facilitation in Ca^{2+}: multiplicative and 'residual Ca^{2+}' components.

Same series of experiments as in Fig. 23, showing the decay of components of facilitation. The points are calculated at each progressively longer (doubled) interval. Decay of the 'residual Ca^{2+}' component does not fit a single exponential.

Results from random interval stimulation in the presence of Ca^{2+} were analysed first by determining the value of X from the phasic delta fourth roots (as described above) for each time bin. Then, the residual Ca^{2+} component was determined from the enhancement of f_m, after correcting for the contribution of the multiplier X. In this manner, the components were resolved for all time bins. Assuming that the underlying processes responsible for both components followed first order kinetics, a best fit τ was determined for each: for the multiplicative component τ was about 80 ms and for the residual ion component (additive on a fourth power basis) τ was about 150 ms.

Fig. 23 and Fig. 24 show the growth and decay of *m*, f_m, putative residual Ca^{2+} and the multiplicative factor X for 26 series of data. In the plot of facilitation growth, putative accumulation of intracellular Ca^{2+} is shown as a rising ratio of c_r / c_p (ratio of total accumulated to peak intracellular at the time of phasic release). In the decay phase, the plot of the expression (c_r / c_o − 1) shows that the ratio of intracellular Ca^{2+} added as a result of stimulation to resting intracellular Ca^{2+}, does not fall with a single exponential. On the other hand, the ratio of the increment in *m* to the unfacilitated *m* does fall with a single exponential (for which τ = 84 ms), as does the multiplicative factor X (subtract 1, the X in the absence of multiplicative facilitation). It is noteworthy that the time course of the X factor almost superimposes with that of

m, confirming that under the conditions of this series the multiplicative form of facilitation predominates, seen as a parallel facilitation of *m* and f_m.

In order to study further the time course of decay of the additive, residual Ca^{2+}, component of facilitation, instead of considering only those f_m measurements which coincide in time with specific stimulation events, the non-phasic release after all stimuli was binned according to the latency from the final stimulus of each 'train', regardless of the antecedent stimulation pattern. Taking the fourth root of these binned values for f_m, then dividing by the calculated X factor corresponding to each particular time bin, a value was calculated for each time bin for residual Ca^{2+}. In Fig. 25, these values, less c_o, are plotted as ratios to c_o and to c_p, on a log scale. Apart from a very fast decay phase (see below), the plot was linear, with a time constant for decay of residual intracellular Ca^{2+} of about 150 ms.

Fig. 25 shows a fast component of release, evident during and immediately after the period of phasic release and persisting until about 10 or 15 ms after the stimulus, as an excess of f_m enhancement over that expected from multiplicative facilitation and the slow component of additive facilitation. This fast component was more prominent under conditions of greater Ca^{2+} entry - higher Ca^{2+} concentration in the superfusate. Its time course was such that it would not contribute significantly to

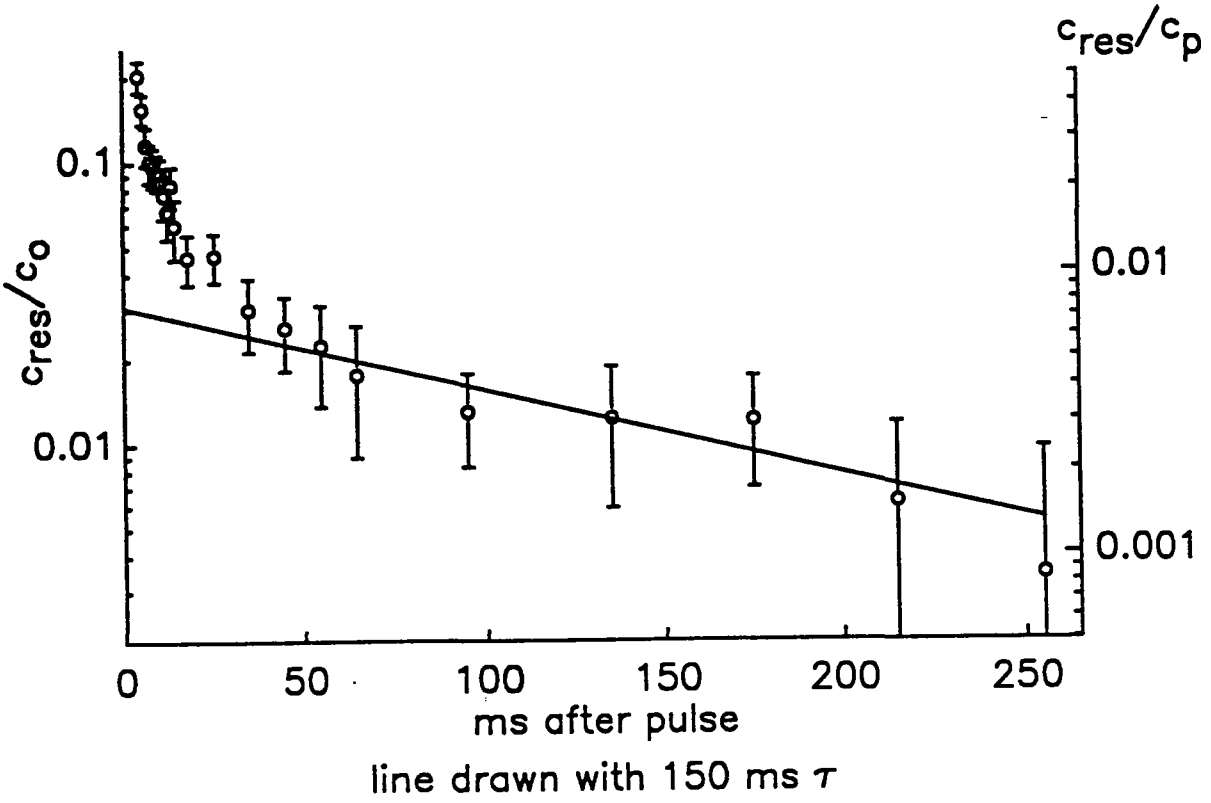


Fig. 25 Time course of decay of residual Ca^{2+}.

Decay of residual Ca^{2+} component of facilitation after all pulses of 26 random interval series. The expression c_{res} is equivalent to $(c_r - c_o)$. For greater resolution of the time course, the points are calculated from f_ms determined at smaller increment in interval than the doubling used for the increment in interval between stimuli. The line is drawn with τ = 150 ms.

facilitation measured at 11 ms or later after a conditioning pulse.

b) Ca dependency of facilitation

In order to find a possible relationship between extracellular Ca^{2+} and the magnitude, time course, or relative contribution of multiplicative and additive (residual Ca^{2+}) components of facilitation, the Ca^{2+} in the perfusate was varied from 0.05 mM Ca^{2+}, at which the quantal content was barely detectable, through 0.1, 0.2 and 4 mM Ca^{2+} (4 mM Mg^{2+} in all solutions). In Table 4, it is evident that facilitation of both *m* and f_m was nearly absent (multiplicative factor X could not be calculated) in solution containing only 0.05 mM Ca^{2+}. For concentrations of Ca^{2+} 0.1 mM and higher the facilitation is clearly evident (see also Fig. 26, same data) and appears to grow with increased Ca^{2+} concentration. However, the multiplicative component X appears to be present at the same magnitude for Ca^{2+} concentration 0.1 mM or greater. These data imply that:

1) multiplicative and additive components of facilitation of release in the presence of Ca^{2+} are dependent on the concentration of extracellular Ca^{2+};

2) with increasing extracellular Ca^{2+}, the additive component of facilitation is increased, seen as an increase in the departure of the curves for f_m and *m* from being parallel,

Table 4: Dependence of multiplicative facilitation on extracellular Ca^{2+}.

The solution contained 4 mM Mg^{2+}; stimulation was random interval, with an overall frequency of 40.1 Hz (see also Fig. 26). 'Total facilitation' in this Table refers to the ratio of facilitated to unfacilitated *m* or f_m. The τ is for the decay of *m* facilitation at the end of the train.

$[Ca^{2+}]$ (mM)	*m*	f_m (s^{-1})	total facilitation of *m*	total facilitation of f_m	X	τ (ms)
0.05	0.012	4.0	1.17±0.11	1.15±0.04	n.s.	48±20
0.1	0.031	3.4	1.42±0.07	1.60±0.09	1.45±0.17	62±7
0.2	0.031	3.4	1.52±0.06	1.77±0.11	1.40±0.10	49±14
0.3	0.320	2.1	1.67±0.04	2.34±0.13	1.47±0.07	53±4
0.4	0.460	2.0	1.66±0.06	2.33±0.18	1.44±0.06	48±4

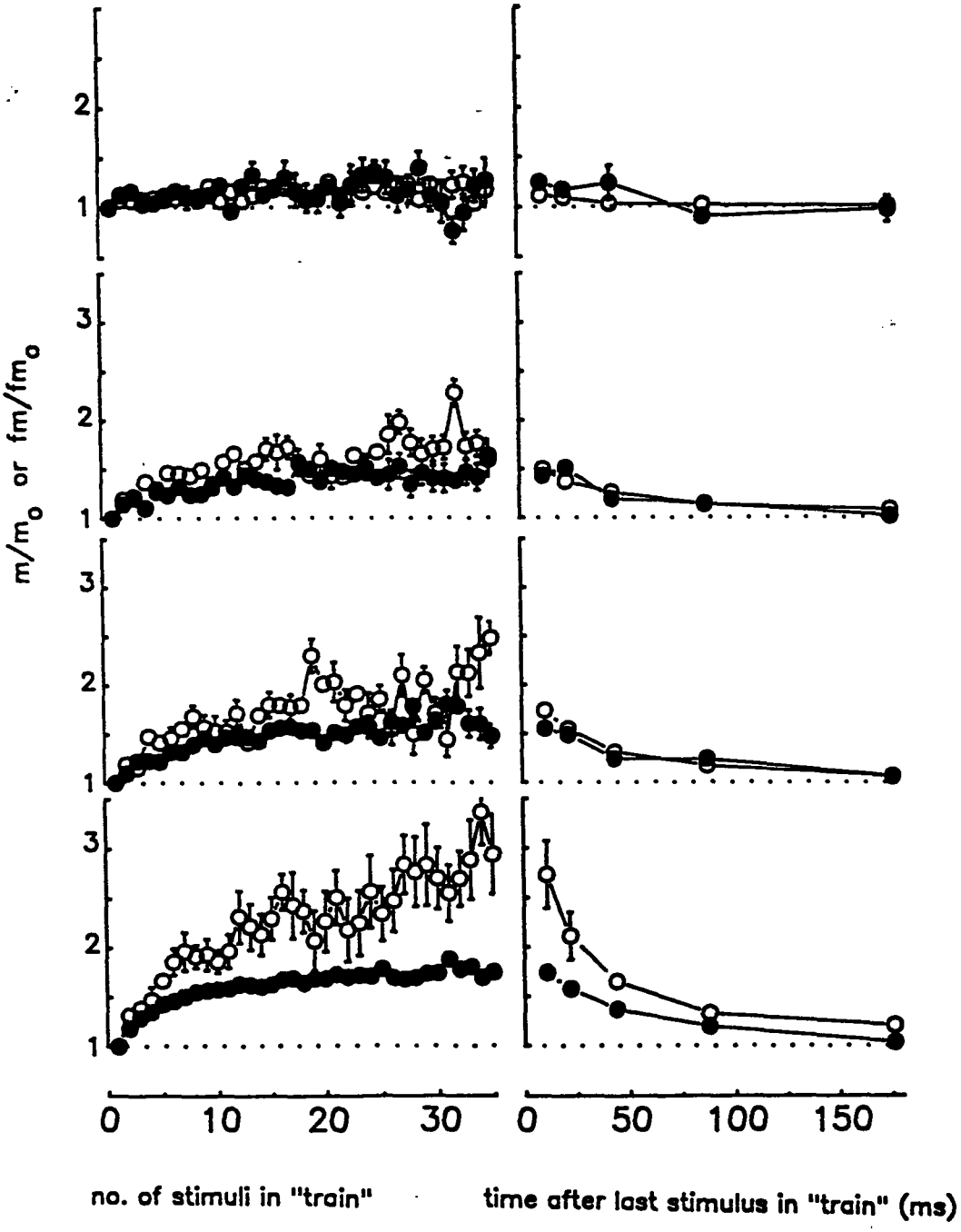
with no indication in the data obtained that this increase saturates in the range of Ca^{2+} concentrations in which e.p.p.s can be measured concomitantly with m.e.p.p.s without disruption of the recordings from suprathreshold postsynaptic activation; and

3) the multiplicative component of facilitation appears to be steeply graded with extracellular Ca^{2+}, nearly absent at extracellular Ca^{2+} concentration of 50 μM and maximal at 100 μM.

In order to determine whether multiplicative facilitation was dependent on the presence of resting intracellular Ca^{2+}, which might have become depleted during a prolonged exposure to bathing solution containing very low Ca^{2+}, or Ca^{2+} which entered during the nerve terminal action potential, 4AP (0.2 - 1.0 mM) was added to very low Ca^{2+} solution in which facilitation was absent or too small to measure. 4AP increases Ca^{2+} entry (by enlarging and/or prolonging the presynaptic a.p., Saint et al, 1987; review: Thesleff, 1980), while resting intracellular Ca^{2+} should remain virtually unchanged. As expected, 4AP greatly increased *m* with little or no effect on f_m. The effect of the 4AP on facilitation was to restore the multiplicative component to its normal magnitude seen in higher Ca^{2+} concentrations in the absence of 4AP. At some junctions, multiplicative facilitation not only reappeared after 4AP but was more than normal.

Fig. 26 Ca^{2+} dependence of facilitation.

Composite showing the growth and decay of *m* and f_m and the dependency of facilitation on extracellular Ca^{2+}. Ca^{2+} was increased from 0.05 mM (top) through 0.1, 0.2 and 0.4 mM Ca^{2+}. The values plotted are normalized relative to the *m* or f_m after a long delay.


m/m$_o$ or fm/fm$_o$
2
1
3
2
1
3
2
1
3
2
1
0
10
20
30
0
50
100
150
no. of stimuli in "train"
time after last stimulus in "train" (ms)

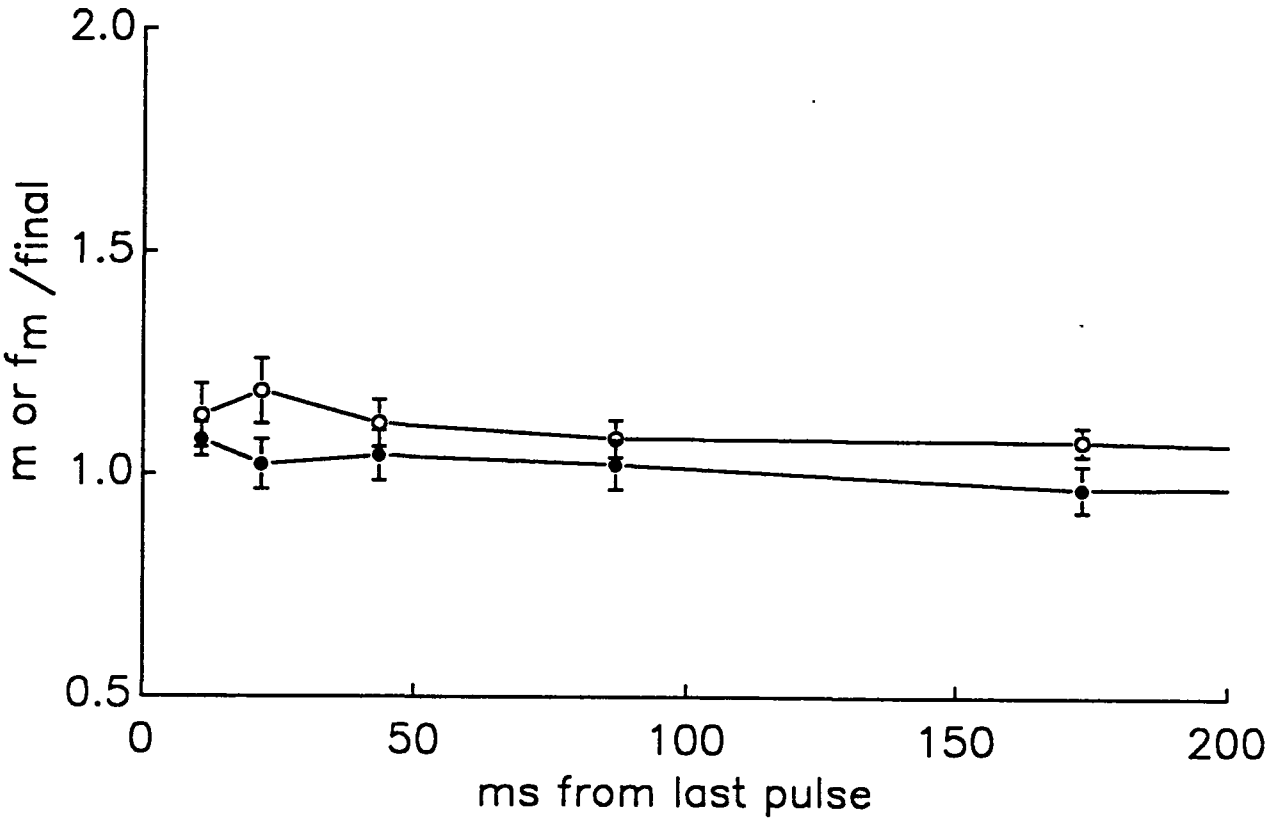


Fig. 27 Lack of facilitation in Ca^{2+} solution after loading with BAPTA.

Data for *m* (filled circles) and f_m (open circles) after a preceding pulse in random interval stimulation are plotted at the time at which they occur after the preceding stimulus, as a ratio to their unfacilitated values. Unfacilitated *m* and f_m were 1.92 and 11.3/s (with 4% DMSO present), respectively.

Finally, in agreement with the results of Kijima & Tanabe (1988) for the frog neuromuscular junction and Hochner et al (1991) for crayfish neuromuscular junction, facilitation was blocked by prior loading of the nerve terminal with BAPTA. In solution containing 1 mM Ca^{2+}, 8 mM Mg^{2+} and 4% dimethylsulfoxide to increase *m* and f_m, conditions under which both multiplicative and additive components of facilitation were normal, incubation for 5 to 15 minutes in 500 μM BAPTA-AM virtually abolished facilitation, as shown in Fig. 27.

c) Dependency on presynaptic stimulus

Fig. 28 shows that facilitation is dependent on the magnitude of a presynaptic 'direct' depolarization. In this figure is shown the facilitation of *m* and f_m after stimulation with various magnitude 'directs', in the presence of 2 mM Ca^{2+} and 1 mM Mg^{2+}, TTX, K^+-channel blocker TEA, and K^+ elevated to 10 mM. In this solution, the stimulus magnitude was chosen to elicit an *m* (final) of only 0.2 for the lowest current (10μA, 0.4 ms) and 3.53 for the highest current (20μA, 0.4 ms). For comparison, in similar solution, a normal presynaptic action potential would elicit an e.p.p. with a quantal content of over 100 (Elmqvist and Quastel, 1965b).

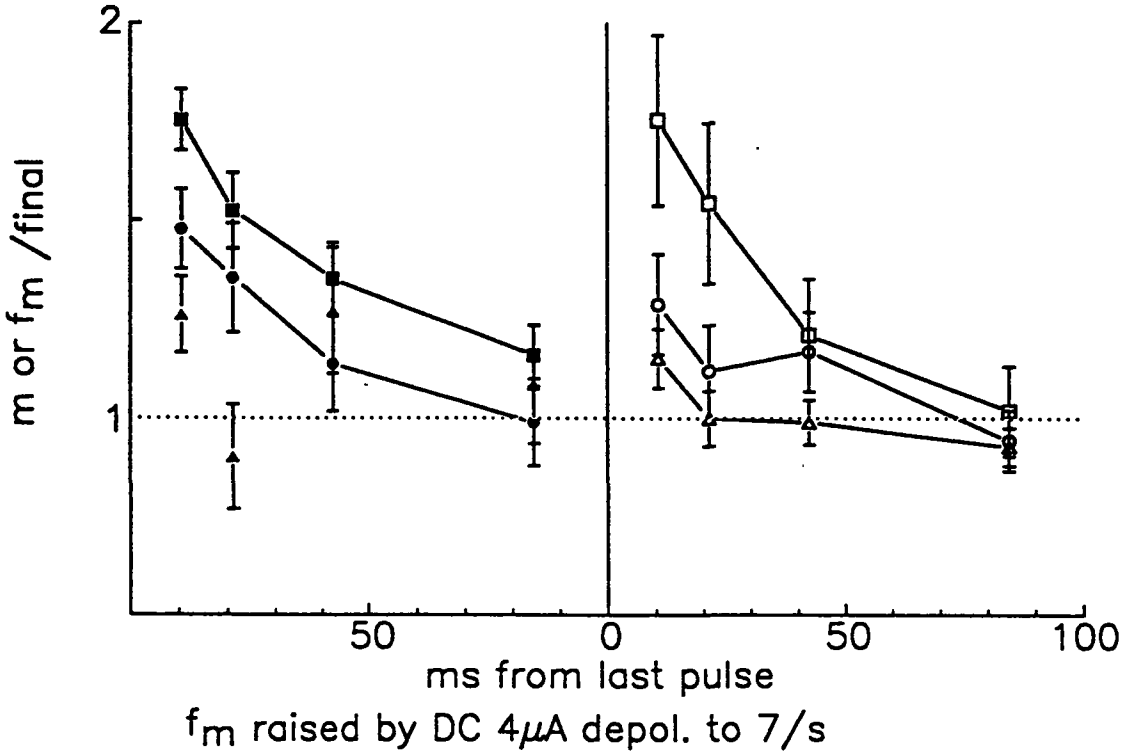


Fig. 28 Dependency of facilitation on depolarization amplitude.

Facilitation with direct pulse (TTX present) random interval stimulation with three different pulse intensities, normalized *m* the left three plots, normalized f_m on the right. The pulse intensities were 10 μA - triangles, 15 μA - circles, and 20 μA - squares; actual unfacilitated *m*s were 0.2, 0.66 and 3.53. The solution contained (mM) 10 K^+, 2 Ca^{2+}, 1 Mg^{2+}, 0.2 TEA, and 0.4 μM TTX.

While the quantal content for the lowest current used (10 μA, m = 0.2) was still much greater than that for the 0.1 mM Ca^{2+}/4 mM Mg^{2+} situation shown in Fig. 26 (with nerve stimulation) where the multiplicative facilitation was fully expressed, the facilitation here was absent. As stimulus intensity was increased, facilitation became clearly evident. In experiments where stimulus intensity was increased to give still higher quantal content, there was no indication of any increase in multiplicative facilitation greater than that shown in Fig. 28.

3. Deviations from the models

a) Decreasing stimulus

In principle, additive fourth power facilitation would be mimicked, even if only multiplicative facilitation existed, if the stimulus were to decrease progessively during the train (see DISCUSSION). In the case of stimulation via action potentials, this could occur either as a result of an increasing probability of action potential failure, or by a decrease in the effectiveness of each action potential to open Ca^{2+} channels. Usually, action potential failure was found to occur primarily at stimulation frequencies more than 100 Hz (avoided in the present experiments). An apparent decrease in the action potential effectiveness (as evidenced by a decrease in the phasic delta fourth root) was found to occur whenever f_m rose spontaneously, or after prolonged stimulation, to more than 200/s or so, either in Ca^{2+}, Sr^{2+} or Ba^{2+}. The latter

indicates a possible role of raised intracellular Ca^{2+} (or Sr^{2+} or Ba^{2+}) in suppressing the action potential.

Apparent decrease in effective stimulation was not observed within a short train of *direct* stimuli.

b) Increasing stimulus

In principle, it is possible for a graded change from stimulus to stimulus in the voltage excursion in the nerve terminal, or its effectiveness to open Ca^{2+} channels, to mimic multiplicative facilitation. If a nerve terminal were normally to facilitate in accord with the residual ion model (as seen in Ba^{2+} or Sr^{2+} containing solution) but the action potential grew or became more effective with each successive stimulus, the e.p.p. would grow more than predicted from the residual accumulated divalent agonist calculated from raised f_m. Conceivably, the growth in f_m due to accumulated divalent agonist could be matched by the growth in *m*, resulting mainly from the increased stimulus, appearing as a parallel or near parallel multiplication of release. However, if this were the case one would expect enhancement of the stimulus itself sometimes to be evident as a facilitation of *m*, with less facilitation of f_m than predicted by a parallel multiplication. This was never seen. Moreover, the results with Sr^{2+} (e.g. Fig. 15) cannot be reconciled to a model in which impulse effectiveness rised within a train.

c) Ultra fast facilitation

There was a component of increased probability of release which was not specifically investigated, but was evident from latency histograms of release in Ca^{2+}-containing solution. This component of increased non-phasic release appears to be of the residual Ca^{2+} type of enhancement, since the m.e.p.p. frequency at 11 ms after a previous stimulus often exceeded that predicted by the multiplicative effect of facilitation of the quantal content at a corresponding time (eg. Fig. 25). Furthermore, this early tail of non-phasic release was always more pronounced under conditions of greater Ca^{2+} entry, ie. higher extracellular Ca^{2+} or less Mg^{2+}. A detailed analysis of this component of enhancement could not be carried out using nerve terminal action potentials (focally evoked or by nerve trunk stimulation), since action potential generation and conduction is not reliable when the interval between nerve impulses is less than about 10 ms.

4. Potentiation

a) Ca^{2+} dependency

(1) Effect of bekanamycin

With tetanic stimulation of the nerve under conditions of low quantal content in the presence of Ca^{2+}, m and f_m continue to rise after the facilitation, as described above, is complete. This tetanic enhancement of release, called potentiation, is similar to the short term enhancement of release in Ca^{2+} (usually referred to as facilitation), in

that it appears to be nearly multiplicative with the addition of a component, usually developing after persistent stimulation (usually for more than 30 s), in which f_m growth exceeds *m* growth. Suspecting that potentiation might be a purely multiplicative process supplemented by an extra gradual buildup of f_m due to intracellular Ca^{2+} accumulation during the tetanus, and to rule out the possibility that accumulation of intracellular Mg^{2+} might play a part in enhancement of release by prolonged trains, an attempt was made to inhibit the influx of Ca^{2+} using bekanamycin, an aminoglycoside blocker of the neuromuscular junction Ca^{2+} channel (Uchiyama et al, 1981). A typical result is shown in Fig. 29; the plots of log f_m and log *m* against frequency of tetanic stimulation are linear and parallel. This linearity implies that a multiplicative unit of the underlying process of potentiation is added with each stimulus, such that with p amount of potentiation adding over r pulses in a tetanus, where r is large enough that the potentiation is fully developed, the amount of potentiation present (P) is:

$$P = p + pz + pz^2 + pz^3 + pz^4 + pz^r$$

$$= p[(1-z^r)/(1-z)]$$

where:

$z = e^{-1/f\tau}$

f is the frequency of stimulation (sec^{-1})

τ is the time constant of decay of potentiation

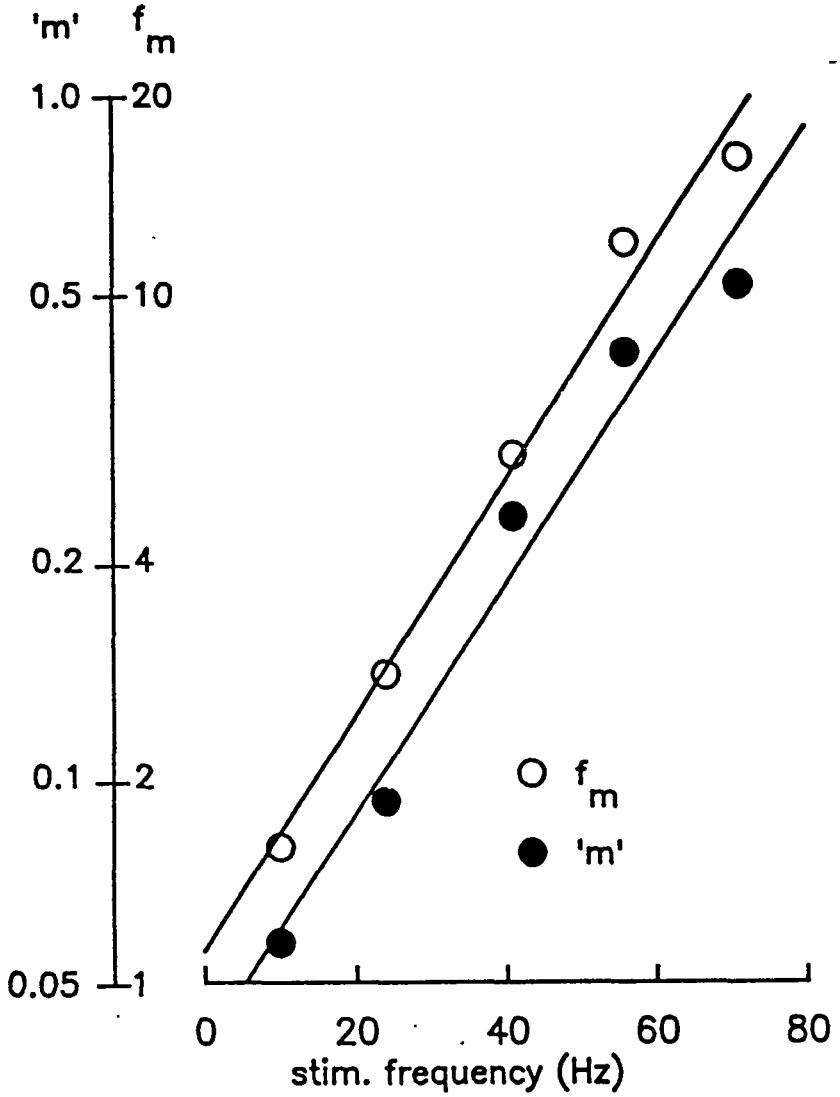


Fig. 29 Potentiation: a log-linear relation between release and tetanic stimulation frequency.

An example in a single junction of tetanic potentiation with nerve impulses under conditions of low quantal content, with 66 μM bekanamycin present, 0.2 Ca^{2+}, 2 Mg^{2+}.

This approximates to:

$$P = p \bullet f \bullet \tau$$

where $f \bullet \tau >> 1$

Thus, the amount of potentiation at its full development is directly proportional to the stimulation frequency, and if it is a multiplicative factor in release, then a logarithmic plot of release against stimulation frequency should be linear.

The parallel nature of the plots implies that the same multiplicative process affects both phasic and non-phasic release equally.

(2) Fourth root transform

In the absence of bekanamycin and especially when the tetanus was prolonged and at a high frequency (> 40 Hz), the f_m potentiation generally outstripped that of *m*, as seen in Fig. 30. On the assumption that this outstripping of *m* by f_m might reflect a component of f_m supported by intracellular Ca^{2+} (or Mg^{2+}), the phasic delta fourth root was plotted for each 2 s time bin. Fig. 30 shows that the multiplicative component of potentiation actually is completely developed rather early on in a tetanus, the continuing creep upwards of *m* and more dramatic rise in f_m with persistent stimulation being attributable to a gradual accumulation of intracellular divalent agonist. The apparent time constant τ for multiplicative potentiation at the junction shown was 9.1 s. That tetanic stimulation

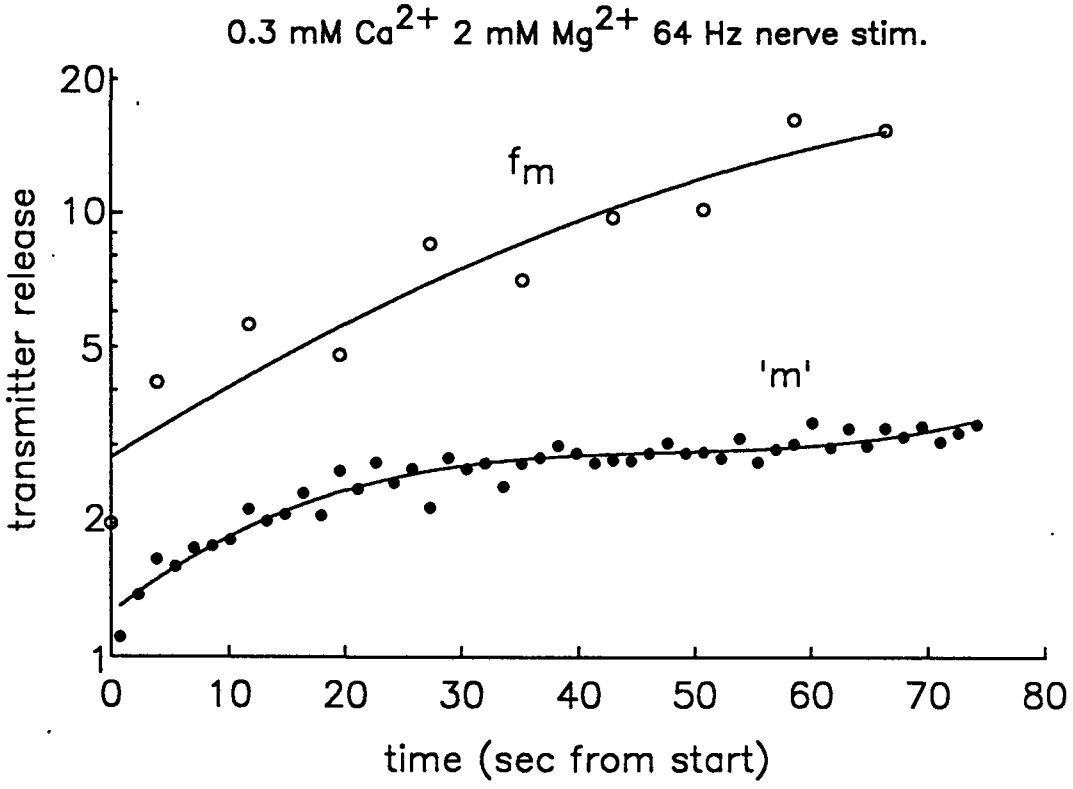


Fig. 30 Relative effects on *m* and f_m of prolonged tetanic nerve stimulation.

Stimulation was at 64 Hz under conditions of low quantal content, 0.3 Ca^{2+}, 2 Mg^{2+} (Expt. 88923e).

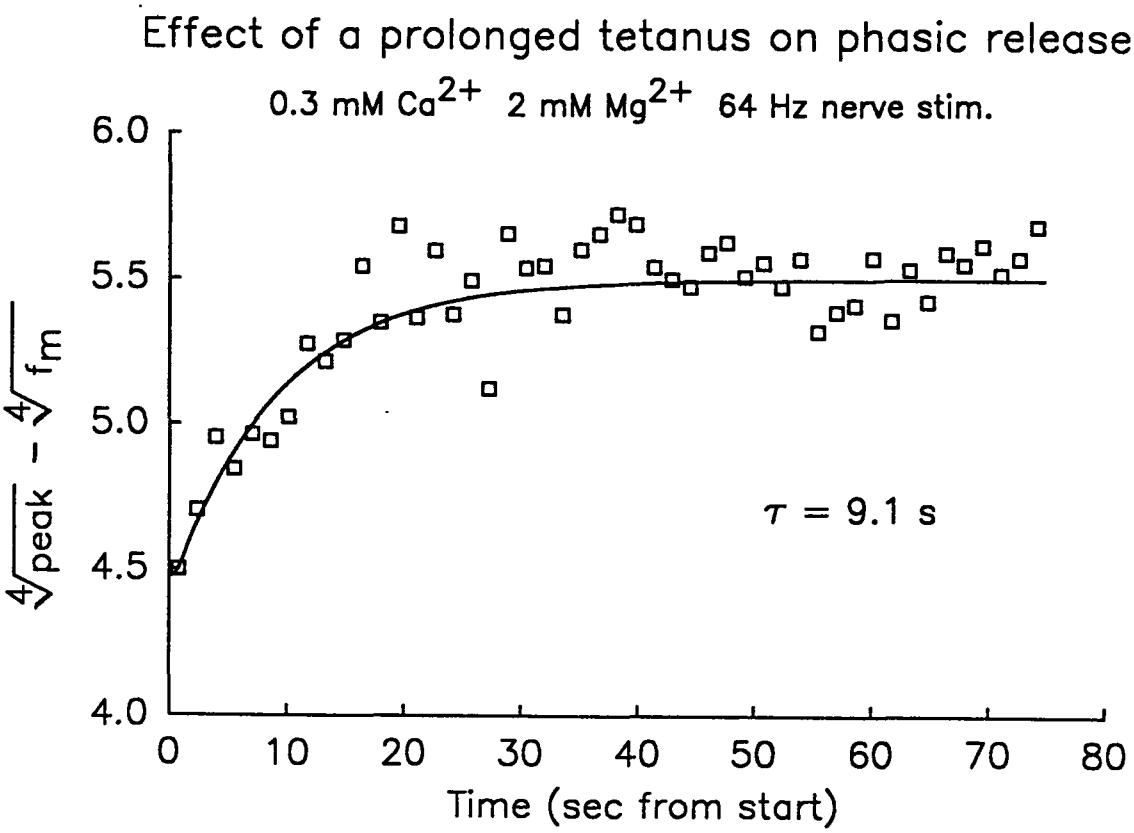


Fig. 31 Plot of phasic delta fourth root over duration of a tetanus.

Same data as in Fig. 30, plotted as the phasic delta fourth root transform. The best fit τ for the growth phase observed was 9.1 s.

might lead to increased intracellular Ca^{2+} has been suggested by a number of workers and there have been two major mechanisms proposed: (1) simple accumulation of Ca^{2+} (Katz and Miledi, 1968), and (2) increase of intracellular Ca^{2+} secondary to Na^{+} accumulation (eg. Misler et al, 1987; Nussinovitch and Rahamimoff, 1988). In the present experiments, the stimulation protocol was not designed to allow determination of the time constant of the apparent Ca^{2+} accumulation phase of potentiation, although in preliminary experiments with prolonged high frequency (50-100 Hz) stimulation, f_m appeared to continually creep upward, in excess of multiplicative potentiation, for minutes. This is consistent with the time constant of about 100 s determined by Nussinovitch and Rahamimoff (1988).

(3) Near absence of extracellular Ca^{2+}

The predominantly multiplicative nature of potentiation was best demonstrated by its apparent independence from Ca^{2+} entry. In order to eliminate any significant Ca^{2+} entry, the preparation was superfused with solution containing 2 mM Mg^{2+} and 50 μM bekanamycin to reduce Ca^{2+} influx, extracellular Ca^{2+} reduced to 10 μM (total) with 100 μM EGTA added to chelate extracellular Ca^{2+}, and 6% dimethylsulfoxide (DMSO) added to increase f_m to allow an accurate determination of release rate in 0.1 ms bins. In Fig. 32, the latency histogram of release shows a clear but tiny e.p.p. and frequency-dependent potentiation of both

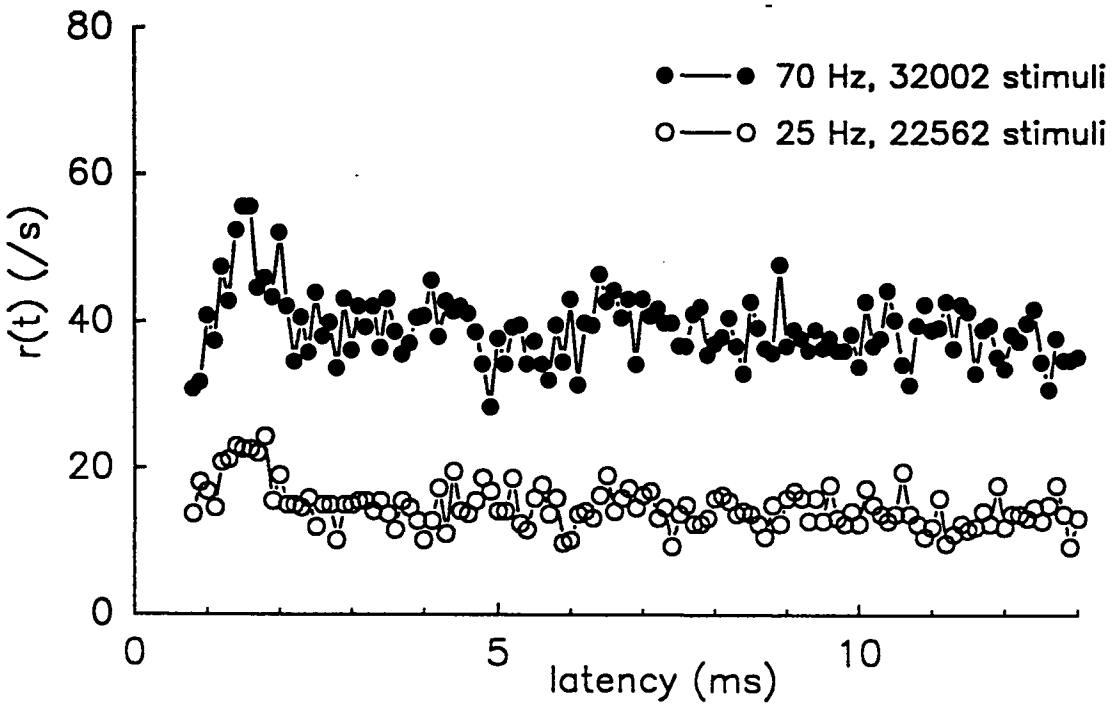


Fig. 32 Survival of potentiation in the absence of Ca^{2+} entry.

Prolonged tetanic stimulation in the presence of very low Ca^{2+} with sufficient Ca^{2+} chelator present to render the extracellular Ca^{2+} concentration effectively nil, also with Ca^{2+} channel blocker bekanamycin present to further decrease any possible entry of Ca^{2+} or any other cation. The solution was 2 mM Mg^{2+}, 50 μM bekanamycin, 10 μM Ca^{2+}, 100 μM EGTA, and 6% DMSO (Expt. 88509d).

phasic and non-phasic release. It is noteworthy that the potentiation was greater at 70 Hz than at 25 Hz and that f_m, which is in the expected range for 6% DMSO regardless of extracellular Ca^{2+} concentration, was potentiated about 9-fold at 70 Hz, within the normal range for potentiation at low extracellular Ca^{2+} concentration. The potentiation of phasic release is present despite a maximum phasic release rate less than double the non-phasic release rate, ie. an *m* of less than 1/10,000 normal physiological quantal content at the neuromuscular junction. It is striking that phasic release can occur, although it is very minute, under conditions in which it seems just as likely that the driving force for Ca^{2+} be in the outward direction as it would be in the inward direction (Rotshenker et al, 1976) since extracellular concentration of free Ca^{2+} ions was approximately 10^{-8}M (estimated from data in Fabiato and Fabiato, 1979).

b) Na^+ dependency

Using direct, current clamp pulses of various durations between 0.1 and 0.5 ms and of various amplitudes, potentiation was not attainable, despite the presence of clear e.p.p.s., in the presence of TTX to block the presynaptic voltage dependent Na^+ channels. Fig. 33 shows a typical example of the progress of *m* and f_m during a 20 s tetanus for which the 'direct' stimulus was just sufficient, in solution containing 1 mM Ca^{2+} and 1 mM Mg^{2+} (and TTX), to elicit an e.p.p. similar in magnitude to that commonly seen

with nerve stimulation in low Ca^{2+} raised Mg^{2+} containing solution (eg. 0.3 mM Ca^{2+} and 2–4 mM Mg^{2+}), that is, an *m* at the beginning of the tetanus of about 1. Upon commencing a tetanus with directs, *m* and f_m appeared only to facilitate in the first few pulses (not visible in the figure, since data from 100 stimuli (1 s) are binned for clarity), then the *m* declined to a minimum of about one-half the *m* observed with a single pulse. This lack of potentiation was not likely due to any kind of depletion, since the quantal content was less than 1 in most experiments.

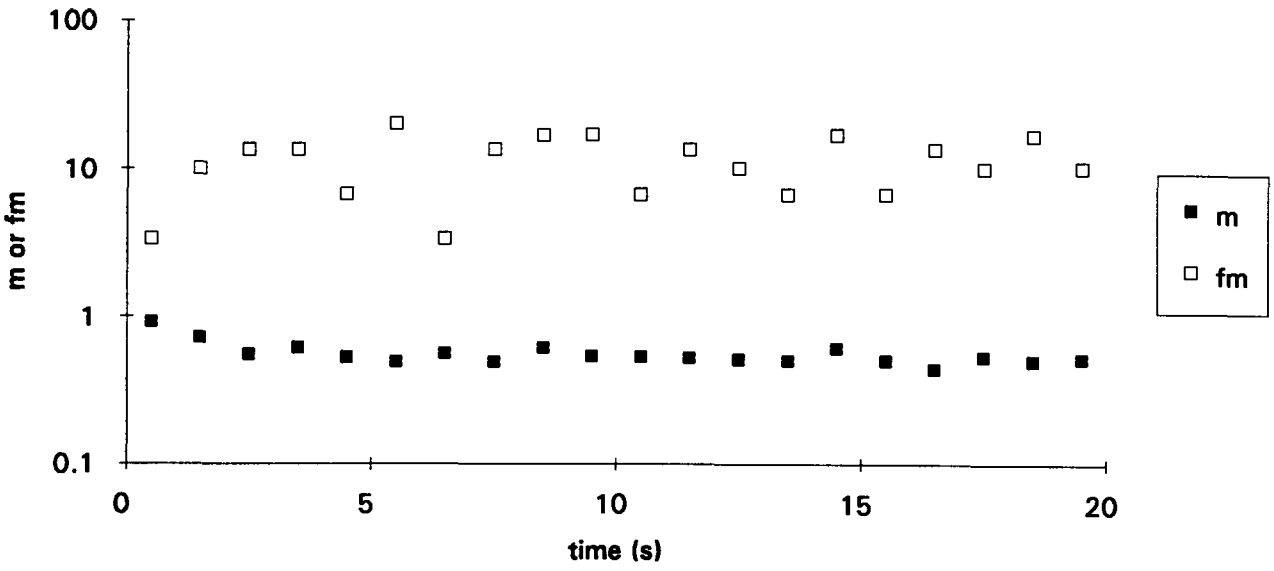


Fig. 33 Lack of potentiation with 'direct' pulses. Potentiation was not obtainable when tetani were carried out using direct pulses in the presence of TTX. In this typical example, the stimuli were current clamped pulses of 5 μA and 0.15 ms at 100 Hz; the solution contained 1 mM Ca^{2+}, 1 mM Mg^{2+}, 0.4 μM TTX and total K^{+} of 10 mM. The data were averaged in 1 s bins for clarity.

IV. Discussion

A. Phasic release time course

1. the presynaptic Ca^{2+} channel

According to the Ca^{2+} hypothesis for release, the time course for phasic release should be predominantly affected by the time course of the presynaptic voltage-dependent Ca^{2+} channel. Present evidence indicates that the type of Ca^{2+} channel present at the neuromuscular junction is one which either does not inactivate (tested with stimulus durations up to 100 ms in the presence of Ba^{2+}) or inactivates partially with a time course too fast to observe with the present technique.

The activation kinetics of the neuromuscular junction Ca^{2+} channel could not be studied by the present technique of indirect measurement using tails of raised f_m in presence of Ba^{2+} since no delay of time course of activation was noticeable upon stimulation with direct pulses of less than 1 ms.

Thus, according to classification of Ca^{2+} channels by their activation and inactivation kinetics and voltage and drug sensitivity (Nowycky et al, 1985), the Ca^{2+} channel at the neuromuscular junction appears not to be one of the L, T, or N types which are represented in a wide variety of other electrically active tissues, but has some of the pharmacological characteristics of the P channel (Uchitel et al, 1992). While voltage and drug sensitivity of the neuromuscular junction Ca^{2+} channel were observed in the

present study, they were studied only as they pertained to experiments with other objectives.

2. similarities for divalent agonists - temperature studies

Since Ca^{2+}, Sr^{2+} and Ba^{2+} exhibit the same values for the three time parameters of phasic release (minimum latency, rise time constant of probability function and decay time constant), then two possibilities are as follows:

1 the forward and reverse rate constants for binding and for effect are very fast, and the observed time course of release reflects primarily the time course of phasic divalent cation entry during channel opening and subsequently the diffusion limited onward rate constant at the receptor, or

2 binding and efficacy, and all the rate constants inherent therein contribute substantially to the time course of release, but are very similar for the three divalent agonists.

The above possibilities may be further investigated using temperature as a probe (between 5°C and 35°C). If the first one represents reality, then Q_{10}'s for phasic release time course should be near unity and the time course for phasic release should be invariant with temperature changes. It is noteworthy that although the Q_{10} for all three time parameters is greater than unity, especially for temperatures lower than 15°C, the three parameters appear to be equally prolonged by temperature reduction, consistent

with the first scenario with temperature affecting the exocytosis process at a step after those promoted by divalent agonist (Datyner & Gage, 1980). Datyner & Gage suggest that the inflection in the temperature dependency toward high Q_{10} seen as the temperature was dropped below 15°C may reflect a lipid phase change. These data are consistent with a model for phasic release whereby a bolus of divalent agonist appears and disappears at the intracellular active site with diffusion limited rates, triggering an exocytotic event for which the rate limiting step involves the reorganization of vesicular and plasma membrane lipids.

If the second possibility represents reality, then the above result of Datyner and Gage (1980) with Q_{10}s as large as 4 could be consistent with a rate limiting mechanism for phasic release involving protein, for which a high Q_{10} is not uncommon, but it would not explain the non-linearity of temperature dependence on the basis of a single rate determining step.

3. model-fitting in retrospect

Some features of the work of Barrett and Stevens (1972a,b) and of Datyner and Gage (1980) on phasic release time course can be explained in terms of a combined model for phasic release and facilitation. Given in more detail later, this combined model proposes that a transient of Ca^{2+} elicits phasic release, while facilitation can be due to residual Ca^{2+}, or a multiplier secondary to the Ca^{2+}

transient, or a combination, as suggested by Silinsky (1985) and others. The combined model presented here for neurotransmitter release simplifies the analysis of data in which there are apparent non-exponential or multi-exponential time courses of phasic release, as follows.

First, the time course of the e.p.p. decay as studied by Barrett and Stevens (1972b) did not give a linear relation between ln R(t) and time where R(t) is the frequency of quanta during each small increment of time (eg. 0.1 ms) after the peak of the e.p.p. Although it is not possible to attempt further quantitative analysis on their data as published, it is possible that a linear relation would have been achieved through a transformation according to the fourth power residual ion model, ie. a plot of ln $[R(t)^{1/4} - f_m{}^{1/4}]$. The fourth root difference should be a measure of the magnitude of the transient intraterminal Ca^{2+} at any time during the e.p.p. and is best suited to the model for a rate limiting factor in the release process which may decay exponentially.

Second, in frog (with bathing solution containing Ca^{2+}), in which much of the previous work has been done, the apparent time constant of phasic release decay, obtained from the slope of log release vs time, increases as facilitation develops. The same result would be obtained for the time constant of phasic release decay determined in the same way in Sr^{2+}, according to the present data. These results are not in accord with a linear release process

decaying exponentially and summing between successive stimulus pulses, since this would require a further factor to explain the change in apparent time constant. On the other hand, a linear process to which release is related by a power function would give a consistent time constant despite various amounts of residuum from previous pulses. In the data of Barrett and Stevens (1972b) it is not possible to determine which power transform would maximize the consistency of the time constant of e.p.p. decay among various stimulation rates.

Third, the apparent discrepancy between the work of Datyner and Gage (1980), in which the decay time course of phasic release did not change with successive stimuli, and the work of Barrett and Stevens (1972b), in which the phasic release appeared to be prolonged with successive stimuli, might actually reflect a time course of Ca^{2+} at the intracellular release sites which is not altered by repetitive stimulation and is consistent in both cases. The time constant would be obtained from a plot of the difference in fourth roots of the phasic and non-phasic release rates prior to measuring decay time course. Thus, if residual Ca^{2+} played a predominant role in the facilitation observed by Barrett and Stevens at 11°C in the frog, the increase in the amount of residual Ca^{2+} present after a pulse would be seen experimentally as an increase in interpulse f_m which greatly exceeds the increase in m as the train proceeds. The slope of the log of the difference

between initial and final (just before the next pulse) phasic release rates plotted against time (apparent inverse τ_m) would increase with successive stimuli, whereas the slope of the log of the difference between initial and final fourth roots (according to postulated time course of intracellular Ca^{2+} during phasic release) would remain constant. From Barrett and Stevens' published data it is not possible to determine whether the fourth root transform would eliminate the phenomenon of prolonged phasic release decay with successive stimuli in a train, but their report that test ERP (phasic release) is facilitated less than is the tail of release (f_m) indicates that this is so. Furthermore, that the facilitation observed by Barrett and Stevens resulted from stimulus intervals of 100 ms (10 Hz), whereas that observed by Datyner and Gage and reported herein was only significant at intervals of about 20 ms or shorter, also points to a predominant residual Ca^{2+} component in the data of Barrett and Stevens, since according to the present work Ca^{2+} has a longer decay time than the multiplicative component of facilitation.

Finally, the jump in the temperature dependency of release from Q_{10}s of about 1 over 15C to about 4 under 15C may not indicate a single process such as lipid phase change (as postulated by Datyner and Gage, 1980), but rather a change from one process to another process being the rate limiting step as the temperature changes. That the Q_{10} for one process is near 1 may simply indicate that that process

is limited by diffusion. At lower temperatures, a Q_{10} for release in elevated K^+ in the presence of Ca^{2+} of nearly 4 may not necessarily indicate a highly temperature sensitive protein involved in release, since a temperature change giving a 4-fold change in release may do so by changing the Ca^{2+} channels in such a way as to change intracellular Ca^{2+} by only 1.4-fold. It is apparent that in temperature studies a suitable model is needed on which to base transformation of the data prior to constructing a van't Hoff plot. If the model used is the combined model, concurrent data on *m* and f_m must be obtained in order to distinguish between temperature effects.

4. time course of BAPTA effects

In Sr^{2+}, after BAPTA loading, the time constant of non-phasic release was prolonged and the magnitude of non-phasic release was reduced to a greater extent than was phasic release. These data fit a model in which BAPTA at the intracellular release sites chelates a fraction of the Sr^{2+} as it enters through the channel, not allowing it to bind the nearby release site and promote release. This would explain the observed reduction in phasic release in Ca^{2+} and in Sr^{2+} after BAPTA loading. Subsequently, Sr^{2+} which was immediately chelated upon entry and Sr^{2+} which was chelated after binding the release site during phasic release is released from the BAPTA, allowing it to bind the release site and play a part in promoting non-phasic release. The greater the concentration of BAPTA in the nerve terminal,

the greater the probability that Sr^{2+} will remain either bound to BAPTA or to the release site. Thus, with Sr^{2+} able to oscillate between these two bound forms intracellularly, the time constant of the non-phasic delta fourth root is increased, as follows.

In the absence of BAPTA, the concentration of Sr^{2+} at the release site is

$$Sr(t) = Sr_{max}e^{-t/\tau}$$

With the addition of BAPTA, after the rapid equilibration of Sr^{2+} with BAPTA, this becomes

$$Sr(t) = Ae^{-t/\tau 1} + Be^{-t/\tau 2}$$

where A and B add to give total Sr^{2+} entry.

This is a pharmacokinetic consideration, analogous to the prolongation of elimination time constant for a drug which is highly protein bound relative to one which is not, all other factors being equal.

Assuming the above, it should be possible at low *m* to show that the inhibition by BAPTA of non-phasic delta fourth root disappears if one integrates over a time period much longer than the apparent time constant of Sr^{2+} removal.

B. Models of release enhancement

1. Residual ion model

The residual Ca^{2+} hypothesis is that Ca^{2+} is a common factor for evoked release and for facilitation. The residual Ca^{2+} model for facilitation (see INTRODUCTION) assumes the Ca^{2+} release model of Katz & Miledi (1965) and

further postulates that a fraction of the Ca^{2+} which enters phasically after a stimulus remains in the nerve terminal, adding to the Ca^{2+} which enters after a subsequent stimulus (Katz & Miledi, 1968), according to $R = k (C_t+C_r)^4$.

For experiments carried out in the presence of Ca^{2+}, the present data confirm that there is a component of facilitation which can be ascribed to an increase in residual Ca^{2+}, C_r, but that most facilitation (particularly of *m*) is due to a multiplicative process expressed as an increase in k. This concurs with the multiplicative character of facilitation as previously described by Hubbard (1963).

For Sr^{2+}, as previously shown for Ba^{2+} (Quastel and Saint, 1988), the present data are consistent with a pure residual ion model based on the residual Ca^{2+} model, according to a number of tests.

First, the apparent entry of Sr^{2+} (difference in fourth roots of non-phasic release rate just after a short high frequency train and of release rate in the absence of stimulation) appears to be linear with the number of pulses, as shown previously for Ba^{2+}. In Sr^{2+}, however, the time constant of apparent loss of Sr^{2+} from the active site is much less than that of Ba^{2+}, requiring a correction for loss during the stimulation-induced buildup.

Second, facilitations of the phasic component and of the non-phasic component of release in Sr^{2+}, i.e. of *m* and

f_m, are mutually predictive according to the residual ion model.

Third, for stimulation in the presence of Sr^{2+}, the results fit a residual ion model, with an n of 4 or 5. This fit is confirmed by the equality of development and decay time constants of f_m after transformation to the fourth or fifth root.

In the following section, various models which have been studied in attempts to accomodate the discrepancy of facilitation in Ca^{2+}-containing solutions from a simple, additive fourth power residual ion model, while maintaining the underlying assumption of that model, are discussed.

2. Variations of the residual ion model

a) cooperativity

In order for the present results to be interpreted solely in terms of a residual ion model, various modifications of the model could be entertained. One such model would be that in which the apparent cooperativity of Ca^{2+} (n) must be very high under low m conditions, but decreases with increased m to a minimum of 4 at high m (presumed high Ca^{2+} entry and residual Ca^{2+}. However, the data for Sr^{2+} (see above) and for Ba^{2+} (Quastel and Saint, 1988) show no evidence for n not always being the same.

b) inhibitor of f_m

Another possible complication of the residual Ca^{2+} hypothesis that can account for apparently multiplicative facilitation is the inclusion of an unknown inhibitor

specific for non-phasic release which is not fully expressed except under those conditions where the apparent multiplicative component is expressed. There is no evidence for the existence of such mechanism, which requires the invocation of separate release systems for phasic and non-phasic release, contrary to previous and present findings (eg. Guan et al, 1988; see also present RESULTS and DISCUSSION re Sr^{2+}).

c) Ca voltage hypothesis

The hypothesis put forward by Parnas' group (Parnas et al, 1986) is sufficient to account for the observation of multiplicative facilitation and potentiation in the presence of Ca^{2+} while being able to maintain that these processes arise as a result of the accumulation of intracellular Ca^{2+} from previous stimulation. Parnas' Ca-voltage hypothesis proposes voltage activated affinity for Ca^{2+} of an intracellular receptor (K_C, see Derivations, in METHODS). Thus, for conditions under which the amount of intracellular Ca^{2+} is sufficiently low that the Ca^{2+} receptor is far from saturation, this model can be restated in terms of Equation 2 as:

$$r(t) = k(V_m) \left(C_t + C_r + C_o \right)^4,$$

where the multiplier 'k' is modulated by the membrane potential V_m.

In this model, Ca would persist in the terminal enhancing both phasic and non-phasic release. However, the Ca entry resulting from the nerve terminal depolarization

would be necessarily rather small, to account for the relatively low f_m even during facilitation. For example, one would observe two-fold multiplicative facilitation (for both *m* and f_m) and account for it entirely by residual Ca, with the two- to three-order of magnitude greater instantaneous release rate corresponding to *m* than f_m explained by a large phasic increase in 'k', as follows:

eg. facilitation of $m = 2$.

$$m'/m = (C_r + C_t + C_o)^4 / (C_t + C_o)^4 = 2$$

$$f_m'/f_m = (C_r + C_o)^4 / C_o^4 = 2$$

For equal multiplication of *m* and f_m, $C_t = 0$ and the multiplier k, which cancels out from the equations for *m* and f_m facilitation as shown above, is then the only factor responsible for the phasic component of release, and must be a voltage-dependent variable whose magnitude is approximately equal to the ratio of phasic to non-phasic release rates and whose time course is mirrored in the time course of phasic release.

The Ca-voltage hypothesis thus accounts for the time course of phasic release simply by postulating that this reflects predominantly the time course of k, as a function of membrane potential. This is consistent with the observations that the time course of phasic release is independent of the particular active divalent cation which is present to support release (Ca, Sr or Ba; Datyner and Gage, 1980; Quastel et al, 1989) and their apparent

differences in time course of sequestration in or removal from the nerve terminal (Zengel and Magleby, 1981).

The problems with this model are threefold. First, it does not account for phasic release in Sr^{2+} and in Ca^{2+} without imposing large differences between the ions either for intracellular potency or for permeability through the open voltage dependent Ca^{2+} channel, neither of which is supported. For example, with $m = 1$ and $f_m = 1/s$, a voltage dependent k would have to increase phasically by about 1000 fold in the absence of a large Ca^{2+} transient, such absence being necessary to maintain the fit of the Ca-voltage model to multiplicative facilitation. The present evidence shows that Sr^{2+} is able to support phasic release in the same order of magnitude as Ca^{2+}, but without such a phasic, voltage-dependent increase in k (see Fig. 14). To be consistent with the present results, Sr^{2+} would need to be much more potent than Ca^{2+}, by a factor of about 1000. This is inconsistent with the work of Silinsky (review: 1985) in which combinations of Ca^{2+} and Sr^{2+} were used after pretreatment with La^{3+} to reduce the number of intracellular receptors for Ca^{2+} or Sr^{2+}; the data showed that the efficacy of Ca^{2+} and Sr^{2+} range from 9 to 20 and from 0.2 to 0.5, respectively (Silinsky, 1981). The Ca-voltage hypothesis is thus not consistent with the present Sr^{2+} results.

Second, while the consistent time course among the active divalent cations for phasic release has been used in

support of the Ca-voltage hypothesis, this consistency casts doubt on the validity of the hypothesis in view of the preceding paragraph. That is, if phasic release in the presence of Sr^{2+} is triggered only by a transient Sr^{2+} at the intracellular active sites secondary to voltage dependent cation channel opening, whereas phasic release in the presence of Ca^{2+} is triggered only by a phasic change in a multiplier (k), it is unlikely that such disparate mechanisms for phasic release would be associated with the same minimum latency and time course of rise and decay as shown in the present results (Fig. 13).

Finally, although the argument is still ongoing whether or not a depolarization in the absence of Ca^{2+} (or surrogate) entry can evoke phasic release using novel caged calcium compounds (Hochner et al, 1989; Mulkey & Zucker, 1991), the observation that agents which can be shown to block Ca^{2+} entry are able to block phasic release (eg. bekanamycin: Bourret & Mallart, 1989; Guan et al, 1988) suggests that if a voltage effect exists which is contingent on resting or slightly elevated intracellular Ca^{2+}, it should be manifest in a minimum e.p.p. which is not further reduced by increasing the concentration of any one of these entry blockers. In the present results, a minimum e.p.p. was evident after stringent measures were taken to prevent Ca^{2+} entry and intracellular Ca^{2+} was depleted, but the phasic effect of the stimulation was very small.

On the other hand, Neher (1988) demonstrated that for mast cells, resting intracellular Ca^{2+} concentration is sufficient for exocytotic release to occur, provided that GTP-gamma-S is present intracellularly, but in its absence transient increases in intracellular Ca^{2+} did not evoke release.

d) saturation of Ca^{2+} dependency

Katz and Miledi (1968), Younkin (1974), and many others have observed that facilitation is dependent on the presence of some extracellular Ca^{2+} ions. However, Charlton and Bittner (1978a), Dudel (1989) and others have shown that facilitation of *m* is largely independent of extracellular Ca^{2+} over a range of concentrations modulating *m* up to 12-fold. The present results are consistent with this observation for facilitation of *m*. Although the residual Ca^{2+} hypothesis can theoretically predict such behaviour of *m* facilitation (as described in the INTRODUCTION), it does not concur with the current evidence that although facilitation stays quite constant over changes in extracellular Ca^{2+} concentration which change *m* more than 10-fold, resting f_m does not modulate over a similar range as *m*. That the residual ion model was originally postulated based on *m* data without including concurrent f_m data explains the difficulties in trying to account for facilitation of both *m* and f_m.

3. Multiplicative model

A multiplicative model would require a mechanism which (1) would only be active in the presence of minimal Ca^{2+} entry (extracellular Ca of more than about 50μM in 4 mM Mg^{2+}) and (2) would be strictly multiplicative in nature. This multiplier would act by a mechanism which would increase release either by affecting the affinity of an intracellular Ca-binding active site or by lowering the energy barrier for fusion of the neurotransmitter vesicle with the presynaptic membrane. The latter was alluded to by Mallart and Martin (1967) in their speculation that the release mechanism is rendered hyperexcitable by a preceding stimulus.

In accord with the present evidence that the interaction of a putative facilitator mechanism with the rest of the release system is multiplicative, there is also the question of how the facilitator interacts with itself. That is, does additional multiplier add to or multiply with that present (from previous stimulation). For ethanol and DMSO, pharmacological multipliers of neurotransmitter release (Quastel et al, 1971; McLarnon et al, 1987), all release present is multiplied, even that which is the result of an unrelated multiplicative process, such as facilitation. It is not evident from the present results whether the hypothetical multiplier responsible for facilitation multiplies all release, or only the non-facilitated release rate. There are some regions of the

crayfish opener muscle where the facilitation builds multiplicatively and other regions where it builds additively (Robitaille and Tremblay, 1991).

If multiplicative facilitation does indeed build multiplicatively, it would be acting in a fashion consistent with a model whereby intracellular Ca^{2+}, raised for a short time after each stimulus, evokes the formation, or intracellular release, of a multiplicative modulator of release, which acts in a manner similar to ethanol or DMSO. Biochemically, there are a great many candidates, since intracellular Ca^{2+} activates many enzymes whose products are potentially multiplicative modulators of neurotransmitter release. Lipids, possibly one or more species involved in the phosphoinositide pathway, or of neurotransmitter vesicular origin during exocytosis and reuptake, are candidates. Small molecule metabolite products of Ca^{2+} activated enzymes are also candidates. A systematic assessment of the effect of many normal metabolites on phasic and non-phasic release, and on multiplicative facilitation, has not been done.

A model for facilitation involving enzyme or active carrier activity is suggested by temperature studies of facilitation time course (Balnave and Gage, 1970) which showed that the time course of decay of facilitation of high quantal content m was slowed with low temperature with a Q_{10} of about 4. It is noteworthy that this result does not support the residual Ca^{2+} hypothesis, since the simple

addition of a Ca^{2+} residuum from preceding stimulation should have no different temperature sensitivity than Ca^{2+}-influx-evoked release itself. On the other hand, it is important that the Q_{10} for growth of facilitation is shown to parallel that for its decay for a hypothesis involving a metabolite as multiplier to be valid.

4. Combined model - multiplicative and additive

In order to test the present hypothesis that distinct multiplicative and additive (residual ion) processes contribute to facilitation, two effects on release rate which have time course and magnitude in the same order must be accurately and appropriately separated. Such a separation was clearest when experimental conditions were varied to maximally express one or the other. With high *m* and nerve stimulation both components are expressed but the additive component is usually predominant, while at the lowest extracellular Ca^{2+} concentration at which multiplicative facilitation is still fully expressed (low m) the additive component was almost completely absent.

C. Mechanisms of multiplicative facilitation

1. presynaptic ion channels

a) action potential

Action potential prolongation is not likely to play a role in the induction of facilitation. In the present work, we have shown that although the presynaptic spike becomes progressively delayed upon the commencement of a high frequency train stimulation (70-90 Hz), there is no evidence

for spike broadening, which would presumably prolong Ca^{2+} influx and thus prolong the histogram of times of quantal release, since the release histogram is virtually the same at different periods during a train except for the latency change. Likewise, changes in the electrical excitability of the nerve terminal or in spike amplitude are unlikely to induce facilitation since facilitation can be observed using direct pulses given in the presence of TTX and K^+ channel blockers.

b) membrane potential

It has been shown that nerve stimulation can result in time dependent changes in presynaptic membrane potential which outlast the action potential (Gage and Hubbard, 1966). Either hyperpolarization or depolarization or a convolution of both in time after the action potential (or direct stimulus) have been demonstrated in several neuronal types, usually in somata. If a change in polarization of the presynaptic nerve terminal were in some way dependent on presynaptic Ca^{2+}, and if this change in polarization altered the phasic component of intracellular Ca^{2+} without significantly altering its apparent time course, this could be a mechanism of *m* facilitation, in conjunction with an additive residual Ca^{2+} component to raise f_m. Both of the following alternative explanations for parallel facilitation involve residual Ca^{2+} while at the same time attempt to explain the fact that observed f_m facilitation is much less than that predicted by the residual Ca^{2+} model, based on hte

observed extent of *m* facilitation. Thus, the possibility is raised that perhaps residual Ca^{2+} can account for f_m facilitation, and that *m* facilitation depends on a process which is in turn dependent on residual Ca^{2+}. However, it is unlikely that an enhancement of *m* and f_m resulting from such a mechanism would be as well stereotyped as observed multiplicative facilitation in the presence of Ca^{2+}.

(1) endogenous presynaptic hyperpolarization

Post stimulus hyperpolarization, called after hyperpolarization (AHP), is usually attributed in neuronal somata to an increase in K^+ conductance (gK_{Ca}) activated by Ca^{2+} whose concentration decays after the bolus entry consequent to the somatic action potential. That AHP occurs in nerve terminals and sums during a tetanus has been shown under certain conditions (Gage and Hubbard, 1966). It is possible that if this occurred, it may result in a larger action potential without any change in duration, the larger voltage swing being somehow responsible for facilitation. Although hyperpolarization can remove Na^+ channel inactivation, it is unlikely that under normal or low quantal content conditions a large enough fraction of the nerve terminal Na^+ channels would be inactivated such that a hyperpolarization-induced increase in action potential size would be evident. Presynaptic hyperpolarization has been shown to enhance neurotransmitter release (Parnas et al, 1986), although it is not apparent whether the enhancement is predominantly multiplicative, nor is it obvious that an

endogenous hyperpolarization would have the same effect. Nevertheless, presynaptic hyperpolarization, resulting from gK_{Ca}, could account for an increase in *m*, with f_m increasing almost in parallel due to residual Ca^{2+}, both of which are dependent on Ca^{2+} entry and its residuum after the Ca^{2+} channels close, but only the f_m enhancement due to an additive effect of residual Ca^{2+}.

(2) endogenous presynaptic depolarization

Presynaptic depolarization which is initiated with each stimulus and decays thereafter could be involved in multiplicative facilitation through an increase of action potential size, although a mechanism by which a persistent presynaptic depolarization could be dependent on Ca^{2+} entry and its residuum is not known. A small presynaptic depolarization will reduce the size of a depolarizing stimulus necessary to elicit a presynaptic action potential if the membrane potential is much more negative than the activation voltage of the Na^+ channels, but will increase the size of the stimulus required if the depolarization is sufficient to inactivate some of the voltage dependent Na^+ channels. Thus, a mechanism by which presynaptic depolarization increases the action potential size is not clear. Furthermore, a Ca^{2+}-dependent post-stimulus depolarization has not been shown.

Depolarization, as a mechanism possibly involved in multiplicative facilitation, is attractive because of its consistency with the possibility that depolarization-induced

raised f_m in Ca^{2+} containing solution is a result of frequent, local influxes of Ca^{2+} rather than global Ca^{2+} accumulation in the nerve terminal. If depolarization of the nerve terminal were sufficient to account for facilitation of the e.p.p. (perhaps by increasing the a.p. size or by priming the Ca^{2+} channels in some way), then it is possible that the same depolarization could account for a relatively small facilitation of f_m which in Ca^{2+}-containing solution is much smaller than predicted by an additive residual Ca^{2+} model.

c) Ca^{2+} entry per pulse

Nerve terminal Ca^{2+} current inconsistency from stimulus to stimulus could result from a variety of processes known to occur within various excitable cells, including stimulation induced changes in the action potential, changes in the transmembrane Ca^{2+} gradient and priming or inactivation of the Ca^{2+} channel. Any of these changes during repetitive stimulation would play a major role in determining the relative facilitation of phasic and non-phasic release, and would also largely determine the time course of the buildup of facilitation as the series of stimuli progressed.

If Ca^{2+} entry per pulse decreased with each stimulus, facilitation that would otherwise appear multiplicative could appear additive: as long as the entry of Ca^{2+} were not reduced sufficiently to block the development of multiplicative facilitation of both *m* and f_m, *m* would suffer

from the decrease in Ca^{2+} entry while f_m would not. Thus, the facilitation would appear similar to a residual Ca^{2+} hypothesis prediction assuming consistent Ca^{2+} entry. In the present work, this was occasionally seen to occur when the nerve terminal action potential failed late in high frequency trains.

For synapses at which facilitation is predominantly multiplicative and is maximal at about 2-fold, a progressive decrease in Ca^{2+} entry might progressively detract from quantal content with a time course similar to the development of facilitation, the concurrence of the two distinct processes resulting in an apparent reduction or abolition of facilitation. It has been pointed out (Wang and Quastel, 1991) that this might be the explanation of the apparent block of facilitation after the addition of Cd^{2+} (Dudel, 1990; Zengel et al, 1988), Zn^{2+} (Zengel et al, 1988), and Pb^{2+} (Wang and Quastel, 1991).

On the other hand, an increase in Ca^{2+} entry per pulse would tend to increase quantal content more than predicted by a residual ion model, possibly appearing as a multiplicative effect on *m* and f_m even if the facilitation were predominantly due to an underlying residual ion process (involving divalent cations such as Sr^{2+} or Ba^{2+}).

2. Potentiation and facilitation

a) Shared mechanisms?

Both potentiation and facilitation apparently have multiplicative and additive components (discussed later),

and are mutually multiplicative. Is it possible that all multiplicative components of neurotransmitter release enhancement, without regard to time course, arise from the same presynaptic process?

The presence of potentiation neither obviates nor occludes facilitation. This is consistent with the combined model, with potentiation sharing a distal step in its mechanism with facilitation, with the following provisos:

1) the process leading to potentiation must not involve a further rise in intracellular Ca^{2+} from that which occurs for facilitation, since present findings indicate that very low intracellular Ca^{2+} concentration is necessary to saturate Ca^{2+} dependent multiplicative facilitation;

2) the shared step, if saturable, must be far from saturation under the conditions of the present experiments; and

3) if the shared step is the final step, it apparently must be capable of being activated by two different proximate effectors.

If facilitation and potentiation, as described, represent phenomena which have discrete mechanisms, it is possible that they share the ultimate mechanistic step which leads to the multiplication of release. That the two phenomena do not share all steps in their mechanisms is evident from the differences observed experimentally.

Comparison of multiplicative potentiation and facilitation.

observation	potentiation	facilitation
blocked by tetrodotoxin	yes	no
blocked by botulinum toxin A *	yes	no
prolonged τ_{decay} in low K **	yes	no
occluded or obviated by the other	no	no
time course	20s to minutes	~80ms

* P. Sun & D. M. J. Quastel (unpublished observations)

** G. Polyakov & D. M. J. Quastel (unpublished observations)

b) Intracellular Na^+

(1) Cooperation with Ca^{2+}

While intracellular Na^+ has been proposed in the combined model to promote the intracellular appearance of a multiplicative factor, a direct role for Na^+ in release must be considered. A simple model including Na^+ is as follows:

$$r = k[C(t) + C_o + qN(t)^n + qN_o^n]^4$$

where r is release at any time t, C and N are intracellular Ca^{2+} and Na^+ concentrations at the release site (C_o and N_o in the absence of phasic influx of the ion), q is a constant which refers to the affinity of Na^+ as a fraction of that of Ca^{2+} for the putative binding site, k includes the affinity and intrinsic activity of the bound

receptor, and n is the number of Na^+ ions that must be bound to take the place of one Ca^{2+} ion bound.

This model does not account for the multiplicative component of facilitation, since Na^+ is merely acting as a substitute for Ca^{2+}. Unless the Na^+-dependent component was predominant and its n>>1, the overall apparent cooperativity would be close to 4, precluding prediction of parallel changes in the log of *m* and of f_m. By the same logic, Na^+ cannot produce multiplicative potentiation via an increase in intracelluar Ca^{2+}.

(2) Indirect multiplier, F-actin

A possible mechanism for indirect multiplicative action, presumably through 'mobilisation' (Hubbard, 1963), Na^+ may act intracellularly to increase k by activating the vesicular transport mechanism, increasing the availability of quanta docked for release at the 'active zones' (Heuser et al, 1979; Smith and Augustine, 1988). Na^+ has been shown to activate F-actin function (Bernstein and Bamburg, 1989) and botulinum toxin has been shown to inhibit F-actin function. Both Na^+ (Misler et al, 1987) and botulinum toxin type A (Molgo et al, 1987) appear to be multiplicative in their action on release; that is, both appear to affect k in Equation 2, albeit in opposite directions. That a ouabain-induced increase in intra-terminal Na^+ might be able to partially counteract the effect of botulinum A is suggested by Molgo et al (1987).

Much evidence (eg. Atwood and Wojtowicz, 1986; Misler et al, 1987; Nussinovitch and Rahamimoff, 1988) including present evidence suggests that the increase in phasic and non-phasic release by a factor of about 20 during 50 to 100 Hz nerve stimulation at the neuromuscular junction is due to Na^+ accumulation. The possibility exists that the neurofilaments which guide the neurotransmitter vesicles along their journey from the endoplasmic reticulum to the active zones for exocytotic release, such as demonstrated by freeze-fracture electron micrscopy (Heuser et al, 1979), may be the site at which many agents act. It is conceivable that enhancement of actin function would result in a multiplicative effect, a change in the k of Equation 2, by providing closer approximation of the vesicle to the active zone, reducing the energy requirement for fusion and increasing the probability of fusion. It is also conceivable that ethanol, DMSO and other agents which affect release multiplicatively also exert their actions through an effect on nerve terminal actin.

c) Presynaptic proteins

Whether the proximate step between nerve terminal excitation and multiplicative facilitation is a rise in intracellular Ca^{2+} or Na^+, or a change in membrane potential, there are a number of candidate protein targets, in addition to F-actin mentioned above, for which an alteration in function could result in a change in k.

Synapsin, for example, forms tetramers upon binding Ca^{2+} (Thomas et al, 1988), and other proteins isolated from nerve terminals appear to be fusogenic. A number of presynaptic proteins appear to be activated by Ca^{2+} in vitro with a cooperativity of 4 and with apparent dissociation constants (cell-free systems) in the region of Ca^{2+} concentrations anticipated during nerve terminal activation (Crompton et al, 1988; Plattner, 1989).

D. Mechanism of additive components

Only one hypothesis seems plausible to explain the additive components of neurotransmitter release enhancement, without respect to time course, the residual Ca^{2+} hypothesis. The distinctiveness of time course among various multiplicative and additive components of enhancement makes the separation of the components into multiplicative and additive very much easier, and is important evidence in favour of the residual Ca^{2+} hypothesis for the additive component. This evidence is summarized as follows:

1 Release rate or probability is proportional to the fourth power of the Ca^{2+} at the release site (see INTRODUCTION), and an additive component of enhancement is defined as one which contributes equally to phasic and non-phasic release fourth roots. Facilitation and potentiation, as previously defined by the short and long time courses, respectively, usually include such an additive component.

2 The additive components are of greater magnitude under conditions where Ca^{2+} entry might exceed the capacity of one or more of the processes responsible for its removal rate from the release site. For facilitation, this would arise from increases in extracellular Ca^{2+} (see Fig. 22) and from decrease in interval between pulses to intervals less than about 10 ms. Under the very brief condition of Ca^{2+} loading imposed by short trains, the additive component would not be expected to be very large, and would resolve itself with a very rapid decay, as observed in the present data. For potentiation, on the other hand, where the nerve terminal has a large metabolic demand placed on it due to prolonged tetanization, it is conceivable that Ca^{2+} loading, and thus an additive component, might result from a growing inability of an energy consuming Ca^{2+} removal process to keep up with Ca^{2+} entry.

In the present work, additive and multiplicative components of facilitation and of potentiation have been dissected and, with trains or brief tetani, the multiplicative component predominates. With respect to the additive component, whether or not intracellular Ca^{2+} concentration actually rises with a magnitude and time course consistent with that predicted from the residual Ca^{2+} hypothesis for the observed additive enhancement is not obvious. While 'direct' measurements using fluorescent Ca^{2+}

indicators have shown that intracellular Ca^{2+} does rise during repetitive stimulation and fall thereafter to a baseline, these measurements have not yet been made at a mammalian neuromuscular junction. In addition, a linear correlation between the magnitude of enhancement of release and the intracellular Ca^{2+} concentration (eg. Zucker et al, 1991) is difficult to reconcile to either an additive or a multiplicative model.

E. Ultra fast facilitation

The stimulation induced enhancement of release which occurs as an early component of non-phasic release may indicate a late component of the phasic release Ca^{2+} transient. However, any visible elevation of f_m after, but close to, the period of phasic release (ie. about 4 to 10 ms latency), if it were ascribed to residual Ca^{2+}, would necessitate the existence of a third component of intracellular Ca^{2+} decay. That this early component of non-phasic release cannot be simply the residuum of the e.p.p. transient Ca^{2+} falling with a single time constant can be shown by the following example.

One must first assume that the decay of intracellular Ca^{2+} follows first order kinetics, that is, that its decay is rate limited in any one component by processes other than diffusion, such as binding and unbinding reactions. If the transient Ca^{2+} responsible for phasic release decays with a single time constant, then given a f_o and m, one can find the τ required for a $r(6\ ms)$ which is 5% greater than f_o.

Thus, for m=1, f_o=1/s and a 5% elevation in release rate at 6 ms latency (for example) relative to the release rate measured later (say at 20-40 ms; decay of multiplicative facilitation has little effect),

$$c(t)=c_p \exp(-t/\tau)$$

$$c_p \approx (2000m)^{1/4} \approx 7$$

$$c(6\text{ ms}) = (1.05/s)^{1/4} - 1^{1/4} = 0.0123, \text{ and}$$

$$\tau = 6/(\ln 7 - \ln 0.0123) \approx 0.9\text{ms},$$

much longer τ than that observed in the latency histograms, about 0.1 to 0.3 ms. The τ prediction gets worse for greater elevations of f_m.

Thus, if this ultra fast facilitation is mediated by residual Ca^{2+}, another process for the decay of transient Ca^{2+} is suggested, intermediate in time course between the diffusion limited fall of transient Ca^{2+} at the active sites ($\tau \approx 0.2$ms), corresponding to the predominant termination of phasic release, and the much slower process of Ca^{2+} extrusion ($\tau \approx 200$ms). One possible scenario is that this ultra fast (τ of milliseconds) facilitatory process might represent the rate of Ca^{2+} unbinding from release sites which have been activated.

F. Spontaneous release

1. Role in the combined model

The present analytical method depends upon a model in which all non-phasic release is dependent upon divalent agonist at specific intracellular active sites for promoting release. Whether the source of this active divalent agonist

is the extracellular solution via permeation through membrane channels or the source is release from intracellular stores (such as mitochondria) makes no difference to the analysis, since any increase in active intracellular divalent cation is simply an increase in c_r in Equation 2, as applied to that ion.

2. Cause of spontaneous release

a) Ca^{2+}-dependent

Not all spontaneous quantal release, in the absence of antecedent or concurrent nerve terminal depolarization, is Ca^{2+}-dependent. However, high spontaneous release rate can be attributed to intracellular Ca^{2+} when removal of extracellular Ca^{2+} reduces the rate, and also when nerve stimulation in Ca^{2+}-free solution containing EGTA reduces the rate (by allowing Ca^{2+} out of the nerve terminal down its gradient) (Rotshenker et al, 1976).

b) Ca^{2+}-independent

However, in the present experiments there were some cells in which the spontaneous f_m was orders of magnitude greater than 1 and was insensitive to any measure to reduce intracellular Ca^{2+}. It is possible that this type of spontaneous release is supported by Mg^{2+}, since prolonged stimulation in solutions containing high Mg^{2+} concentration sometimes results in raised f_m with similar characteristics (Hubbard et al, 1968).

An alternative explanation for apparently Ca^{2+}-independent high spontaneous release rate is that the cell

has a large Na^+ leak current, resulting in a persistent potentiation, according to the model in which potentiation results from Na^+ accumulation (eg. Nussinovitch & Rahamimoff, 1988 results with ouabain) during tetanus and in some way multiplies release. The Na^+ explanation might explain why nerve terminals exibiting this behaviour often do not fire action potentials.

A final explanation for Ca^{2+}-independent release is simply that there exists endogenous mechanisms which produce a substance or an effect similar to that of ethanol, such that release probability is increased without the necessity of the presence of Ca^{2+} (Quastel et al, 1971). Such an effect may indicate a nerve terminal in which normal metabolism is compromised.

V. Conclusion - Fitting data to a model

Data from a system such as the neuromuscular junction whose mechanisms are unknown can only be analysed in a bootstrapping fashion. That is, a preliminary model is proposed and the experimental data is analysed according to that model. From this analysis, the model is refined to explain any lack of fit. It is generally considered valid for this process to be repeated either until the model can correctly predict the data or until the model is discarded in favour of simpler models which are equally predictive. For the neuromuscular junction, it is apparent from the literature and from the present data that the present

combined model has the greatest predictiveness for the time course of phasic release under many conditions of stimulation and contents of the superfusate, and for stimulation induced enhancement of phasic and non-phasic release.

References

Atwood, H. L., Swenarchuk, L. E. & Gruenwald, C. R. (1975) Long-term synaptic facilitation during sodium accumulation in nerve terminal. *Brain Research* **10**, 198-204

Atwood, H. L. & Wojtowicz, J. M. (1986) *International Review of Neurobiology* **28**, 275-362

Augustine, G. J. & Eckert, R. (1984). Divalent cations differentially support transmitter relase at the squid giant synapse. *Journal of Physiology* **346**, 257-271.

Augustine, G. J., Charlton, M. P., & Smith, S. J. (1987) Calcium action in synaptic transmitter release. *Annual Reviews of Neuroscience* **10**, 633-693

Bain, A. I. & Quastel, D. M. J. (1988). Time course of transmitter release and calcium channels at the mouse motor nerve terminal. Society for Neuroscience Abstracts, 1988.

Bain, A. I. & Quastel, D. M. J. (1992a) Quantal transmitter release mediated by strontium at the mouse motor nerve terminal. *Journal of Physiology* **450**, 63-87.

Bain, A. I. & Quastel, D. M. J. (1992b) Multiplicative and additive Ca^{2+}-dependent components of facilitation at mouse end-plates. *Journal of Physiology* **455**, 383-405.

Baldo, G. J., Cohen, I. S., & Van der Kloot, W. (1986). Estimating the time course of evoked quantal release at the frog neuromuscular junction using end-plate current latencies. *Journal of Physiology* **374**, 503-513.

Balnave, R. J. and Gage, P. W. (1970) Temperature sensitivity of the time course of facilitation of transmitter release. *Brain Research* **21**, 297-300

Barrett, E. E. & Stevens, C. F. (1972a). Quantal independence and uniformity of presynaptic release kinetics at the frog neuromuscular junction. *Journal of Physiology* **227**, 665-689.

Barrett, E. E. & Stevens, C. F. (1972b). The kinetics of transmitter release at the frog neuromuscular junction.*Journal of Physiology* **227**, 691-708.

Barzilai, A., Spanier, R. & Rahamimoff, H. (1984) Isolation, purification and reconstitution of the Na^{+} gradient dependent Ca^{2+} transporter from brain synaptic plasma

membranes. *Proceedings of the National Academy of Sciences, U. S. A.* **81**, 6521-6525.

Bernstein, B. W. and Bamburg, J. R. (1989) Cycling of actin assembly in synaptosomes and neurotransmitter release. *Neuron* **3**, 257-265.

Bittner, G. D. and Schatz, R. A. (1981) An examination of the residual calcium theory for facilitation of transmitter release. *Brain Research* **210**, 431-436.

Blaustein (1988) Trends in Neuroscience **11**, 438.

Blioch, Z. L., Glagoleva, I. M., Liberman, E. A. & Nenashev, V. A. (1968). A study of the mechanism of quantal transmitter release at a chemical synapse. *Journal of Physiology* **199**,11-36.

Bourret, C. & Mallart, A. (1989) Depression of calcium current at mouse motor nerve endings by polycationic antibiotics. *Brain Research* **478**, 403-406

Braun, M., Schmidt, R. F. & Zimmerman, M. (1966a). Facilitation at the frog neuromuscular junction during and after repetitive stimulation. *Pflugers Arch. ges. Physiol.* **287**, 41-55.

Braun, M. & Schmidt, R. F. (1966b). Potential changes recorded from the frog motor nerve terminal during its activation. *Pflugers Arch. ges. Physiol.* **287**, 56-80.

Bulbring, E. (1946). Observations on the isolated phrenic nerve diaphragm preparation of the rat. *British Journal of Pharmacology* **1**, 38-61.

Charlton, M. P. & Atwood, H. L. (1977) Modulation of transmitter release by intracellular sodium in the squid giant synapse. *Brain Research* **134**, 367-371.

Charlton, M. P. & Augustine, G. J. (1990). Classification of presynaptic calcium channels at the squid giant synapse: neither T-, L- nor N-type. *Brain Research* **525**, 133-139.

Charlton, M. P. and Bittner, G. D. (1978a). Transmitter release facilitation at squid synapses. *Journal of General Physiology* **72**, 4??-48?

Charlton, M. P. and Bittner, G. D. (1978b). Presynaptic potentials and facilitation of transmitter release in the squid giant synapse. *Journal of General Physiology* **72**, 487-511

Charlton, M. P., Smith, S. J. & Zucker, R. S. (1982) Role of presynaptic calcium ions and channels in presynaptic facilitation and depression at the squid giant synapse. *Journal of Physiology* **323**, 173-193

Cohen, I. S., Van der Kloot, W. (1986) Facilitation and delayed release at single frog neuromuscular junctions. *Journal of Neuroscience*. **6**, 2366-2370.

Cooke, J. D. & Quastel, D. M. J. (1973a). Transmitter release by mammalian motor nerve terminals in response to focal polarization. *Journal of Physiology* **228**, 377-405

Cooke, J. D. & Quastel, D. M. J. (1973b). Cumulative and persistent effects of nerve terminal depolarization on transmitter release. *Journal of Physiology* **228**, 407-434

Cooke, J. D., Okamoto, K. & Quastel, D. M. J. (1973). The role of calcium in depolarization-secretion coupling at the motor nerve terminal. *Journal of Physiology* **228**, 459-497

Crompton et al (1988) Diversity in the lipocortin/calpactin family. *Cell* **55**, 1-3

Curtis, M. J., Quastel, D. M. J. & Saint, D. A. (1986) Lanthanum as a surrogate for calcium in transmitter release at mouse motor nerve terminals. *Journal of Physiology* **373**, 243-260

Dale, H. H. & Feldberg, W. & Vogt, M. (1936). Release of acetylcholine at voluntary motor nerve endings. *Journal of Physiology* **86**, 353-380.

Datyner, N. B. & Gage, P. W. (1980). Phasic secretion of acetylcholine at a mammalian neuromuscular junction. *Journal of Physiology* **303**, 299-314.

Del Castillo, J. & Katz, B. (1954). Quantal components of the end-plate potential. *Journal of Physiology* **124**, 560-573.

Del Castillo, J. & Katz, B. (1956) Biophysical aspects of neuromuscular transmission. *Progress in Biophysics and Biophysical Chemistry* **6**, 121-170

Delaney. K. R., Zucker, R. S. and Tank, D. W. (1989). Calcium in motor nerve terminals associated with posttetanic potentiation. *The Journal of Neuroscience* **9**, 3558-3567.

Dodge, F. A. & Rahamimoff, R. (1967). Cooperative action of calcium ions in transmitter release at the

neuromuscular junction. *Journal of Physiology* **193**, 419-432

Douglas, W. W. (1968) Stimulus-secretion coupling: the concept and clues from chromaffin and other cells. *British Journal of Pharmacology* **34**, 451-474

Dudel, J. (1989) Calcium and depolarization dependence of twin-pulse facilitation of synaptic release at nerve terminals of crayfish and frog muscle. *Pflugers Archiv - European Journal of Physiology* **415**, 304-309.

Dudel, J. (1990) Inhibition of Ca^{2+} inflow at nerve terminals of frog muscle blocks facilitation while phasic transmitter release is still considerable. *Pflugers Archiv - European Journal of Physiology* **415**, 566-574.

Elmqvist, D. and Quastel, D. M. J. (1965a) Presynaptic action of hemicholinium at the neuromuscular junction. *Journal of Physiology* **177**, 463-482.

Elmqvist, D. and Quastel, D. M. J. (1965b) A quantitative study of end-plate potentials in isolated human muscle. *Journal of Physiology* **178**, 505-529.

Erulkar, S. B., Meiri, H., and Rahamimoff, R. (1982) The ionic basis of high-frequency synaptic activation. In *Physiology and Pharmacology of Epileptogenic Phenomena*, M. R. Klee, ed. Raven Press, New York, 1982, pp 197-205.

Fabiato, A. and Fabiato, F. (1979) Calculator programs for calculating the composition of the solutions containing multiple metals and ligands used for experiments in skinned muscle cells. *Journal of Physiology (Paris)* **75**, 463-505

Feng, T.P. (1940) Studies on the neuromuscular junction XVIII. The local potentials around N-M junctions induced by single and multiple volleys. *Chin. J. Physiol* **15**,367-404.

Feng, T.P. (1941) Studies on the neuromuscular junction XXVI. The changes of the endplate potential during and after prolonged stimulation. *Chin. J. Physiol* **16**,341-372.

Gage, P. W. & Hubbard, J. I. (1966) The origin of the post-tetanic hyperpolarization of mammalian motor nerve terminals. *Journal of Physiology* **184**, 335-352.

Guan, Y. -Y., Quastel, D. M. J., & Saint, D. A. (1988). Single Ca^{2+} entry and transmitter release systems at the neuromuscular synapse. *Synapse* **2**, 558-564

Heuser, J. E, Reese, T. S., Dennis, M. J., Jan, Y., Jan, L., & Evans, L. (1979). Synaptic vesicle exocytosis captured by quick freezing and correlated with quantal transmitter release. *Journal of Cell Biology* **81**, 275-300

Hille (1984) *Ion Channels of Exitable Membranes*, Sinauer and Assoc., Sunderland, Mass., USA.

Hochner, B., Parnas, H. and Parnas, I. (1991) Effects of intra-axonal injection of Ca^{2+} buffers on evoked release and on facilitation in the crayfish neuromuscular junction. *Neuroscience Letters* **125**, 215-218.

Hochner, B., Parnas, H., & Parnas, I. (1989). Membrane depolarization evokes neurotransmitter release in the absence of calcium entry, *Nature* **342**, 433-435.

Hubbard, J. I., Llinas, R. and Quastel, D. M. J. (1969) *Electrophysiological Analysis of Synaptic Transmission*, London: Edward Arnold, p.129.

Hubbard, J. I. (1963). Repetitive stimulation at the mammalian neuromuscular junction and the mobilisation of transmitter. *Journal of Physiology* **169**, 641-662

Hubbard, J. I. and Schmidt, R. F. (1963). An electrophysiological investigation of mammalian motor nerve terminals. *Journal of Physiology* **166**, 145-165.

Hubbard, J. I., Jones, S. F. & Landau, E. M. (1968a). On the mechanism by which calcium and magnesium affect the spontaneous release of transmitter from mammalian motor nerve terminals. *Journal of Physiology* **194**, 355-380.

Hubbard, J. I., Jones, S. F. & Landau, E. M. (1968b). On the mechanism by which calcium and magnesium affect the release of transmitter by nerve impulses. *Journal of Physiology* **196**, 75-86.

Katz, B. & Miledi, R. (1965a). The effect of calcium on acetylcholine release from motor nerve terminals. *Proc. R. Soc. Lond. B. Biol. Sci.* **161**, 496-503.

Katz, B. & Miledi, R. (1965b). The effect of temperature on the synaptic delay at the neuromuscular junction. *Journal of Physiology* **181**, 656-670.

Katz, B. & Miledi, R. (1965c). The measurement of synaptic delay, and the time course of acetylcholine release at the neuromuscular junction. *Proc. R. Soc. Lond. B. Biol. Sci.* **161**, 483-495.

Katz, B. & Miledi, R. (1967). The release of acetylcholine from nerve endings by graded electric pulses. *Proc. R. Soc. Lond. B. Biol. Sci.* **167**, 23-28.

Katz, B. & Miledi, R. (1968). The role of calcium in neuromuscular facilitation. *Journal of Physiology* **195**, 481-492.

Katz, B. (1969). *The Release of Neural Transmitter Substances*. Springfield: Charles C. Thomas

Kijima, H. & Tanabe, N. (1988). Calcium independent increase of transmitter release at frog end-plate by trinitrobenzene sulphonic acid. *Journal of Physiology* **403**, 135-149.

Kwan, C. Y. & Putney, J. W. (1990). Uptake and sequestration of divalent cations in resting and methacholine-stimulated mouse lacrimal acinar cells. Dissociation by Sr^{2+} and Ba^{2+} of agonist-stimulated divalent cation entry from the refilling of the agonist-sensitive intracellular pool. *Journal of Biological Chemistry* **265**, 678-684

Landau, E.M., Smolinsky, A., Lass, Y. (1973) Post-tetanic potentiation and facilitation do not share a common calcium-dependent mechanism. *Nature New Biology* **244**, 155-157

Liley and North (1953). An electrical investigation of effects of repetitive stimulation on mammalian neuromuscular junction. *Journal of Neurophysiology* **16**, 509-527.

Linder, T. M. (1973) Calcium and facilitation at two classes of crustacean neuromuscular synapses. *Journal of General Physiology* **61**, 56-73.

Lux, H. D. & Bruns, D. (1991) Photolytically induced rapid increase of intracellular Ca^{2+}. Studies on Ca channels function and synaptic transmission. Abstracts, Third IBRO world congress of Neuroscience, p 428

Magleby, K. L. & Stevens, C. F. (1972) A quantitative description of end-plate currents. *Journal of Physiology* **223**, 173-197

Magleby, K. L. (1973) The effect of tetanic and post-tetanic potentiation on facilitation of transmitter release at

the frog neuromuscular junction. *Journal of Physiology* **234**, 353-371.

Mallart A., Molgo J., Angaut-Petit D. & Thesleff S. (1989) Is the internal calcium regulation altered in type A botulinum toxin-poisoned motor endings?. *Brain Research* **479**, 167-171

Mallart, A. & Martin, A. R. (1967) An analysis of transmitter release at the neuromuscular junction of the frog. *Journal of Physiology* **193**, 679-694.

Mallart, A. & Martin, A. R. (1968). The relation between quantum content and facilitation at the neuromuscular junction of the frog. *Journal of Physiology* **196**, 593-604.

McLarnon, J. G., Saint, D. A. & Quastel, D. M. J. (1987) The actions of dimethylsulfoxide on neuromuscular transmission. *Molecular Pharmacology* **30**, 631-638

Martin, A. R. (1955) A further study of the statistical composition of the end-plate potential. *Journal of Physiology* **130**, 114-122.

Mellow AM (1979) Equivalence of Ca^{2+} and Sr^{2+} in transmitter release from K^{+}-depolarised nerve terminals. Nature **282**, 84-85.

Miledi, R. & Thies, R. (1971) Tetanic and post-tetanic rise in frequency of miniature end-plate potentials in low-calcium solutions. *Journal of Physiology* **212**, 245-257.

Miledi, R. (1966). Strontium as a substitute for calcium in the process of transmitter release at the neuromuscular junction. *Nature* **212**, 1233-1234

Misler, S. and Hurlbut, W. P. (1983) Post-tetanic potentiation of acetylcholine release at the frog neuromuscular junction develops after stimulation in Ca^{2+}-free solutions. *Proceedings of the National Academy of Science (USA)* **80**, 315-319.

Misler, S. & Falke, L. C. (1987) Dependence on multivalent cations of quantal release of transmitter induced by black widow spider venom. *American Journal of Physiology* **253**, C469-476.

Misler, S., Falke, L. & Martin, S. (1987). Cation dependence of posttetanic potentiation of neuromuscular transmission. *American Journal of Physiology* **252**, c55-62

Molgo, J., Angaut-Petit, D. & Thesleff, S. (1987) In botulinum type A-poisoned frog motor endings ouabain induces phasic transmitter release through Na+-Ca+ exchange. *Brain Research* **410**, 385-389.

Mulkey, R. M. & Zucker, R. S. (1991). Action potentials must admit calicum to evoke transmitter release. *Nature* **350**, 153-155.

Neher, E. (1988) The influence of intracellular calcium concentration on degranulation of dialysed mast cells from rat peritoneum. *Journal of Physiology* **395**, 193-214

Nowycky, M. C., Fox, A. P. and Tsien, R. W. (1985). Three types of neuronal calcium channel with different calcium agonist sensitivity. *Nature* **316**, 440-443.

Nussinovitch I. & Rahamimoff R. (1988) Ionic basis of tetanic and post-tetanic potentiation at a mammalian neuromuscular junction. *Journal of Physiology* **396**, 435-455.

Parnas H., Dudel, J., & Parnas, I. (1986). Neurotransmitter release and its facilitation in crayfish. VII. Another voltage dependent process beside Ca entry controls the time course of phasic release. *Pflugers Archiv - European Journal of Physiology* **406**, 121-130

Parnas I. & Parnas H. (1986) A new method for determining co-operativity in neurotransmitter release. *Journal of Theoretical Biology* **119**, 481-499.

Parnas I. & Parnas H. (1988). The `Ca-voltage' hypothesis for neurotransmitter release. *Biological Chemistry* **29**, 85-93.

Parnas, I., Parnas, H. and Dudel, J. (1986). Neurotransmitter release and its facilitation in crayfish. VIII. Modulation of release by hyperpolarizing pulses. *Pflugers Archiv - European Journal of Physiology* **406**, 131-137

Philipson, K.D. (1985) *Annual Review of Physiology* **47**, 561

Plattner, H. (1989) Regulation of membrane fusion during exocytosis. *International Review of Cytology* **119**, 197-286.

Quastel, D. M. J. (1974) Excitation-secretion coupling at the mammalian neuromuscular junction. In: *Synaptic Transmission and Neuronal Interaction*, Raven Press, New York.

Quastel, D. M. J. Guan, Y. -Y. & Saint, D. A. (1992) The relation between transmitter release and Ca^{2+} entry at the mouse motor nerve terminal: role of stochastic factors causing heterogeneity. *Neuroscience* **51**, 657-671.

Quastel, D. M. J., Hackett, J. T. & Cooke, J. D. (1971) Calcium: is it required for transmitter secretion? *Science* **172**, 1034-1036

Quastel, D. M. J. & Saint, D. A. (1986) Modification of motor nerve terminal excitability by alkanols and volatile anaesthetics. *British Journal of Pharmacology* **88**, 747-756

Quastel, D. M. J. & Saint, D. A. (1988). Transmitter release at mouse motor nerve terminals mediated by temporary accumulation of intracellular barium. *Journal of Physiology* **406**, 55-73

Quastel, D. M. J., Bain, A. I., Guan, Y.-Y. & Saint, D. A. (1989). Ionic cooperativity in transmitter release. In: *Neuromuscular Junction*. ed. Sellin, L. C., Libelius, R. & Thesleff, S. Elsevier.

Quastel, D. M. J., Hackett, J. T. & Cooke, J. D. (1971). Calcium: is it required for transmitter secretion? *Science* **172**, 1034-1036.

Robitaille, R., Adler, E. M. & Charlton, M. P. (1990) Strategic location of calcium channels at transmitter release sites of frog neuromuscular synapses. *Neuron* **5**, 773-779

Robitaille R. & Charlton, M. P. (1990) Frequency facilitation is not caused by residual ionized calcium at the frog neuromuscular junction. *Society for Neuroscience Abstracts*, **16**, 503

Robitaille, R. and Tremblay, J. P. (1991) Non-uniform responses to calcium along the frog neuromuscular junction effects on the probability of spontaneous and evoked transmitter release. *Neuroscience* **40**, 571-586

Rotshenker, S., Erulkar, S. D. & Rahamimoff, R. (1976). Reduction in frequency of miniature end-plate potentials by nerve stimulation in low calcium solutions. *Brain Research* **101**, 362-365

Saint, D. A., Quastel, D. M. J. & Guan, Y. -Y. (1987) Multiple potassium conductances at the mammalian motor nerve terminal. *Pflugers Archiv - European Journal of Physiology* **410**, 408-412.

Silinsky, E. M. (1978) On the role of barium in supporting the asynchronous release of acetylcholine quanta by motor motor nerve impulses. *Journal of Physiology* **274**, 157-171.

Silinsky, E. M. (1985) The biophysical pharmacology of calcium-dependent acetylcholine secretion. *Pharmacological Reviews* **37**, 81-132.

Silinsky, E. M. (1981) On the calcium receptor that mediates depolarization-secretion coupling at cholinergic motor nerve terminals. *British Journal of Pharmacology* **73**, 413-429

Tanabe, N. & Kijima, H. (1989) Both augmentation and potentiation occur independently of internal Ca^{2+} at the frog neuromuscular junction. *Neuroscience Letters* **99**, 147-152.

Thesleff, S. (1980) Aminopyridines and synaptic transmission. *Neuroscience* **5**, 1413

Thomas, L., Hartung, K., Langosch, D., Rehm, H., Bamberg, E. Franke, W. W., Betz, H. (1985) Identification of synaptophysin as a hexameric channel protein of the synaptic vesicle membrane. *Science* **242**, 1050-1053

Tsien, R. Y. (1981). A non-disruptive technique for loading calcium buffers and indicators into cells. *Nature* **290**, 527-528

Uchitel, O. D., Protti, D. A., Sanchez, V., Cherksey, B. D., Sugimori, M. and Llinas, R. (1992) P-type voltage-dependent calcium channel mediates presynaptic calcium influx and transmitter release in mammalian synapses. *Proceedings of the National Academy of Science USA* **89**, 3330-3333

Uchiyama, T., Molgo, J., Lemeignan, M. (1981) Bekanamycin blockade of transmitter release at the motor nerve terminal. *European Journal of Pharmacology* **72**, 271-280

Van der Kloot, W. (1988a). Estimating the timing of quantal releases during end-plate currents at the frog neuromuscular junction. *Journal of Physiology* **402**, 595-603.

Van der Kloot W. (1988b). The kinetics of quantal releases during end-plate currents at the frog neuromuscular junction. *Journal of Physiology* **402**, 605-626.

Vere-Jones, D. (1966) Simple stochastic models for the release of quanta of transmitter from a nerve terminal. Australian Journal of Statistics 8, 53-63

Wang, Y. X. and Quastel, D. M. J. (1991) Actions of lead on transmitter release at mouse motor nerve terminals. *Pflugers Archiv - European Journal of Physiology* **419**, 274-280.

Younkin, S. G. (1974). An analysis of the role of calcium in facilitation at the from neuromuscular junction. *Journal of Physiology* **237**, 1-14

Zengel, J. E. & Magleby, K. L. (1980). Differential effects of Ba^{2+}, Sr^{2+}, and Ca^{2+} on stimulation-induced changes in transmitter release at the frog neuromuscular junction. *Journal of General Physiology* **76**, 175-211

Zengel, J. E. & Magleby, K. L. (1981). Changes in miniature endplate potential frequency during repetitive nerve stimulation in the presence of Ca^{2+}, Ba^{2+}, and Sr^{2+} at the frog neuromuscular junction. *Journal of General Physiology* **77**, 503-529

Zengel, J. E. & Magleby, K. L. (1982a). Augmentation and facilitation of transmitter release: a quantitative description at the frog neuromuscular junction. *Journal of General Physiology* **80**, 583-611

Zengel, J. E. & Magleby, K. L. (1982b) A quantitative description of stimulation-induced changes in transmitter release at the frog neuromuscular junction. *Journal of General Physiology* **80**, 613-638

Zengel, J. E., Lee, D. T., Van Veelen, M. L. and Moser, D. R. (1988) Effects of divalent cations on stimulation-induced changes in transmitter release at the frog neuromuscular junction (abstract). *Biophysical Journal* **53**, 363.

Zucker, R. S., Delaney, K. R., Mulkey, R. & Tank, D. W. (1991) Presynaptic calcium in transmitter release and posttetanic potentiation. *Annals of the New York Academy of Sciences* **635**, 191-207

Zucker, R. S. & Haydon, P. G. (1988). Membrane potential has no direct role in evoking neurotransmitter release. *Nature* **335**, 360-362.

Zucker, R. S. & Fogelson, A. L. (1986). Relationship between transmitter release and presynaptic calcium influx when calcium enters through discrete channels. *Proceedings of the National Academy of Science USA* **83**, 3032-3036.

Zucker, R. S. (1987). The calcium hypothesis and modulation of transmitter release by hyperpolarizing pulses. *Biophysical Journal* **52**, 347-350.