Journal of Physiology (1992), **455**, *pp.* 383–405
With 10 figures
Printed in Great Britain

MULTIPLICATIVE AND ADDITIVE Ca^{2+}-DEPENDENT COMPONENTS OF FACILITATION AT MOUSE ENDPLATES

BY A. I. BAIN AND D. M. J. QUASTEL*

From the Department of Pharmacology and Therapeutics, Faculty of Medicine, The University of British Columbia, 2176 Health Sciences Mall, Vancouver, BC, Canada V6T 1W5

(*Received 19 September 1991*)

SUMMARY

1. Facilitation of endplate potentials (EPPs) and frequency of miniature endplate potentials (MEPPs) were studied, in the presence of low Ca^{2+}/raised Mg^{2+}, in isolated mouse hemidiaphragm, using pseudo-random sequences of nerve stimulation and automated (computer) counting of MEPPs and quantal components of EPPs.

2. The facilitation in quantal content of EPPs (m) produced by one or more antecedent stimuli was accompanied by facilitation of MEPP frequency (f_m) that was similar in magnitude and substantially less than expected if facilitation reflects persistent (residual) intraterminal Ca^{2+}. The time course of 'phasic' quantal release, associated with the EPP, was little if at all altered with facilitation.

3. The magnitude and time course of facilitation was consistent with two distinct presynaptic processes, each manifest both in m and f_m, (i) an effect to multiply transmitter release, and (ii) residual Ca^{2+} which adds to Ca^{2+} brought in by nerve impulses. These have distinct time courses.

4. After loading nerve terminals with bis (*O*-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid (BAPTA), facilitation of m and f_m became very small.

5. At sufficiently low Ca^{2+}/raised Mg^{2+} facilitation of m and f_m became very small although latency histograms showed clear EPPs. However, the multiplicative component of facilitation became maximal at Ca^{2+}/Mg^{2+} concentrations giving an average m value less than 0·1, corresponding to about 5% of normal Ca^{2+} entry per pulse. At lower Ca^{2+}, facilitation was restored when EPPs were made larger using 4-aminopyridine.

6. With EPPs elicited by brief 'direct' nerve terminal depolarizations, facilitation was graded with pulse intensity (and m) and could be much less than with EPPs with similar m evoked by nerve stimuli at lower Ca^{2+} and/or higher Mg^{2+}.

7. It was concluded that fast facilitation is primarily multiplicative and reflects activity within the nerve terminal of a Ca^{2+}-sensitive process distinct from that generating Ca^{2+}-dependent release.

* To whom correspondence should be addressed.

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INTRODUCTION

There is now general agreement that the intense increase in quantal transmitter release that immediately follows a presynaptic nerve impulse, and is seen postsynaptically as an endplate potential (EPP) (del Castillo & Katz, 1956) depends upon entry of Ca^{2+} into the nerve terminal *via* selective channels that open in response to membrane depolarization (Douglas, 1968; Katz, 1969; Silinsky, 1985; Augustine, Charlton & Smith, 1987). Doubt persists, however, with regard to the mechanism(s) by which the quantal content of EPPs is increased by antecedent nerve impulses, namely, 'facilitation', 'augmentation', and 'tetanic potentiation', which have been distinguished from one another on the basis of rate of growth and decay (Zengel & Magleby, 1982), and are seen at a variety of synapses. In experiments by Katz & Miledi (1968) it was found that after single pulses, facilitation (lasting of the order of 100 ms) did not occur unless Ca^{2+} was present in the external medium at the time of the antecedent pulse, and they suggested that facilitation might be due to a residuum of Ca^{2+}, within the nerve terminal, adding to the Ca^{2+} caused to enter by the test pulse. It is consistent with this hypothesis that facilitation has been found to be little if at all modified by changes in extracellular Ca^{2+} (e.g. Linder, 1973, Zengel & Magleby, 1980).

Although support for a role of intracellular Ca^{2+} has recently been provided by the observation that facilitation (but not potentiation) is blocked by loading nerve terminals with bis (*O*-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid (BAPTA), a Ca^{2+} chelator (Kijima & Tanabe, 1988; Tanabe & Kijima, 1989), and that facilitation is absent when nerve terminals have been depleted of Ca^{2+} (Dudel, 1989), quantitative support for the hypothesis has been lacking. For example, facilitation at the crayfish neuromuscular junction does not grow with measured intracellular Ca^{2+} as predicted by the residual Ca^{2+} hypothesis (Delaney, Zucker & Tank, 1989). An old observation (Hubbard, 1963) also is at odds with the residual Ca^{2+} hypothesis, namely, the failure to find after one impulse or a short train of pulses a raised frequency of miniature endplate potentials (MEPPs) (i.e. continuation of release) of a magnitude corresponding to the (hypothetical) intraterminal Ca^{2+} producing facilitation of EPPs (see also Zengel & Magleby, 1981; Bain & Quastel, 1992, and below). The 'multiplicative' interactions of facilitation with augmentation and tetanic potentiation at the frog neuromuscular junction (Zengel & Magleby, 1982), also cannot fit a residual Ca^{2+} model for more than one of these processes.

These quantitative difficulties become more cogent in view of evidence that when Sr^{2+} is substituted for Ca^{2+} co-facilitation of EPPs and MEPP frequency (f_m) becomes fully consistent with a residual ion model, i.e. a process in which facilitation occurs only because accumulated Sr^{2+} within the nerve terminal *adds* to the amount of ion brought in by each impulse (Bain & Quastel, 1992), release rate being always proportional to the fourth (or fifth) power of internal Sr^{2+} concentration. The accumulated (residual) Sr^{2+} responsible for facilitation of EPPs is fully manifest in f_m.

In the present experiments, we have re-examined the behaviour of f_m and quantal content of EPPs (m) during facilitation, at the mouse motor nerve terminal, using stimulus protocols closely related to that of Hubbard (1963), with emphasis on the

relation between f_m and m during growth and decay of facilitation, and the Ca^{2+} dependence of the process. The results indicate that in addition to residual Ca^{2+}, facilitation includes a multiplicative component, not seen with Sr^{2+}, that probably depends upon entry of Ca^{2+} into the nerve terminal.

METHODS

All experiments were performed upon hemidiaphragms from adult mice anaesthetized with ether and killed by exsanguination, mounted and superperfused as previously described (Cooke & Quastel, 1973), at 25–28 °C. Standard solution contained (mM): 150 Na^+, 5 K^+, 24 HCO_3^-, 125 Cl^-, 1 $H_2PO_4^-$, 11 glucose, bubbled with 95% O_2–5% CO_2, with added Ca^{2+} and Mg^{2+} as required. In most experiments we used either 4 or 8 mM-Mg^{2+}; the latter tended to raise muscle fibre threshold and so permit relatively large EPPs without muscle twitching. In many experiments, in order to obtain enough MEPPs to provide well-defined estimates of MEPP frequency in limited time frames (see below), MEPP frequencies (and quantal content of EPPs) was increased by addition of dimethylsulphoxide (DMSO) (McLarnon, Saint & Quastel, 1987). Intracellular electrical recording, using microelectrodes filled with 3 M-KCl, was conventional.

Analysis of records was 'off-line', from PCM-records on videotape, by a microcomputer programmed to count MEPPs and quantal components of EPPs as previously described (Bain & Quastel, 1992). Briefly, the computer forms a template by averaging well-separated MEPPs in a record, and then searches the record for waveforms of corresponding shape. Serial subtractions of the template at appropriate times enables identification of the time of occurrence of each MEPP and every quantal component of the EPP. The accuracy of this program was checked by superimposing artificial MEPPs, with amplitude variation in the normal range, on typical recorded noise, of varied amplitude.

In all but a few experiments the phrenic nerve was stimulated with a suction electrode. For direct depolarization of nerve terminals we used large-tipped ($\sim 20\ \mu$m) 4 M-NaCl–agar electrodes, as previously described (Cooke & Quastel, 1973; Guan, Quastel & Saint, 1988), with tetrodotoxin (0·4 μM) added to the bathing solution to prevent generation of nerve terminal action potentials; tetraethylammonium (0·2 mM) was also added, to increase the effectiveness of depolarizing pulses (Saint, Quastel & Guan, 1987).

Computerized stimulation

A major objective in these experiments was to characterize the development and decay of facilitation produced by short trains of varied numbers of stimuli, in the same way as in the experiments of Hubbard (1963). From preliminary experiments it was evident that large numbers of iterations would be needed to provide sufficient numbers of quanta and MEPPs for meaningful data. For example, to obtain a total of only 100 MEPPs (for an estimate of frequency with S.E.M. = 10%), in a 100 ms 'bin', in a 2000 ms stimulation cycle, would require the recording of at least 2000 MEPPs, requiring more than 30 min of recording if MEPP frequency (f_m) was about 1 s^{-1}, and a stimulus protocol such as that of Hubbard (1963) would require this to be repeated for each particular train length. It was therefore necessary to devise a protocol, which could be continued indefinitely, in which trains of varying duration were represented and 'test' pulses were given at varying intervals after these trains. These requirements were met by a pseudo-random sequence generated by a computer which chose at random between intervals of 11 ms (more often) and intervals of 22, 44, 88, 176 etc. ms. A section of such a sequence is shown in Fig. 1. With such a sequence every pulse constitutes a 'test pulse' following a 'conditioning' pulse at a known interval, and each 'conditioning pulse' has, on average, the same conditioning by antecedent stimuli. Meanwhile, every pulse is a conditioning pulse and most pulses are members of a train of varied length. Moreover, corresponding to every stimulus one has a corresponding period, without a stimulus, in which to sample f_m.

For analysis, responses were averaged in two ways. (1) According to the interval (11, 22, 44, 88 etc. ms) from its preceding pulse. Corresponding f_m's were obtained by binning in periods 6–16, 16–30, 30–60 ms, etc., 'final' f_m being obtained from the latter half of the longest interval. The bins always excluded a 1 ms period immediately before a stimulus (where the computer might miss

MEPPs because of the ensuing stimulus artifact). Since the bins contained more sample periods from before the time of a next stimulus, the 'centre' time for each of these bins was close to the time of the corresponding stimulus. However, a small correction was applied to the average f_m (total number of MEPPs divided by total time in which MEPPs could be counted) based on the

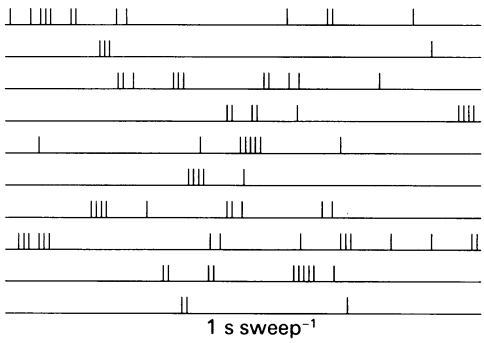

Fig. 1. Section of a computer-generated pseudo-random stimulation sequence with intervals of 11, 22, 44...704 ms. With 5 times as many 11 ms intervals as each of the others, the net effect is short trains of varied duration. Every stimulus is at the same time a conditioning pulse and a test pulse, at varying intervals from a single pulse or a short train. Pulses up to 44 ms from a preceding pulse may also be considered members of a train.

regression in time of f_m from bin to bin. (2) As trains. For this analysis all stimuli separated from a previous stimulus by 11 or 22 ms were considered as members of a train, and also stimuli 44 ms from a previous stimulus that was less than 44 ms from its antecedent. Stimuli 176 ms or more from a previous stimulus were taken as the first in a train. Thus, for stimuli with a maximum interval of 704 ms and 5 times as many 11 ms intervals as for the others (equally represented) the overall frequency of stimulation was 7·6 Hz, the 'trains' were at an average frequency of 64·8 Hz, and the interval before a train was, on average, 410·7 ms; the numbers of trains with n stimuli were 63% of the number with $n-1$ stimuli. In experiments where there were 40 times as many 11 ms intervals as for the others the overall frequency was 40·1 Hz and the mean frequency in the train was 82 Hz; the numbers of trains with n stimuli were 93% of the numbers with $n-1$ stimuli.

In a few experiments, nerve stimuli were applied with random choice between intervals of 11 ms and 2 s, so as to produce 90 Hz trains of randomly varying length.

In all cases quanta in the period 0–0·5 ms after a stimulus were ignored, since 'blanking' the stimulus artifact led to residua of MEPPs being sometimes counted as occurring just after the stimulus artifact. The quantal content was estimated as the total number of quanta recorded in the period 0·5–6 ms following a stimulus, 'corrected' by subtracting the number of quanta expected in 5 ms, on the basis of the f_m at the corresponding period without a stimulus. This definition was chosen in view of varying EPP latency from one junction to another and so that in the total absence of 'phasic' release the 'EPP' would have a quantal content equal to f_m in a 0·5 ms period; the calculated increment in the nth root of release rate, presumptively proportional to intracellular Ca^{2+} (Bain & Quastel, 1992 and see Results), would be 0.

For averaging data from separate stimulation series values of quantal content (m) and f_m were normalized relative to 'final' values (see above) and averaged without weighting when values were sufficiently well defined (as for Figs 5 and 6). Derived values, x and c_r (the multiplicative and additive components of facilitation; see Results), were obtained separately from m and f_m for each point in time in each series and then averaged. Where values from different series were not all well defined (because of relatively low total numbers of MEPPs or quantal components of EPPs) as for some of the data included in Fig. 9 and Table 2, normalized values were averaged with weighting, inversely with the variance (which was obtained from the total number of MEPPs and quantal components of the EPP).

To obtain average time constants of decay of facilitated m and f_m we used non-linear least squares to find the best-fitting time constant for *groups* of data obtained with the same Ca^{2+}, Mg^{2+}, and stimulation parameters. This was done using a bootstrap method (Efron, 1982) with a large number of iterations; in each iteration the weighting of the different series within each group was randomly varied. This gave means and confidence limits for the time constant for each group.

Selection of junctions

Although an effort was made to avoid bias in selecting junctions from which to obtain data series, this was not always possible. In general, in any given solution m and f_m varied greatly from one junction to another (mean S.D. of $\log_e m$ or f_m about 1), with m and f_m very significantly correlated. In solutions giving low m many junctions had values of m and/or f_m so low that it would have taken hours of recording to gather numbers of quanta sufficient to define facilitation with reasonable accuracy. As a result, the data presented for 0·05 and 0·1 mM-Ca^{2+} in 4 mM-Mg^{2+} pertain to the population of junctions where m and f_m were rather higher than average. It should be noted that in the range of m and f_m that were used neither was correlated with the magnitude of facilitation, for any one solution and stimulation protocol.

RESULTS

Facilitation of m and MEPP frequency in trains

Figure 2 illustrates a typical example of the growth of quantal content of EPPs (m) and of MEPP frequency (f_m) produced by brief tetanic trains at 90 Hz, in the presence of 0·8 mM-Ca^{2+} and 8 mM-Mg^{2+}. The 3-fold growth of m is perhaps a little more than usual (2- to 2·5-fold) and the growth of f_m perhaps a little less than usual (3- to 4-fold) but broad features are the same as in a number of experiments with a variety of combinations of lowered Ca^{2+} and raised Mg^{2+} producing (initial) m in the range of about 0·1 to 2 or 3, namely, the facilitation becomes near maximum within about ten pulses and facilitation of f_m is of the same order as facilitation of m. Within this range of m, beyond which it is difficult to quantify facilitation, there was no obvious correlation of the magnitude of facilitation with m.

In similar experiments using Sr^{2+} as a replacement for Ca^{2+} (Bain & Quastel, 1992) it was found that the co-modulation of m and f_m during facilitation conformed closely to the prediction of the residual ion model in which transmitter release rate at any moment is proportional to the nth power of ion concentration at release sites and the latter has two components, (a) transient, due to the Sr^{2+} that enters with each pulse and (b) 'residual', including 'resting' Sr^{2+} (and Ca^{2+}) responsible for 'spontaneous' MEPP frequency. That is,

$$r(t) = k(c(t)+c_r)^n, \tag{1}$$

and

$$r(t)^{1/n} = k^{1/n}c(t)+k^{1/n}c_r = k^{1/n}c(t)+f_m{}^{1/n}, \tag{1a}$$

where $c(t)$ is the added transient at time t, $r(t)$ is release rate, c_r is the sum of accumulated and resting ions, and k is a proportionality constant. The quantal content, m, is the integral of $r(t)$ over a period of about 2 ms and the MEPP frequency just before the impulse (f_m) is $kc_r{}^n$. On this basis, if $c(t)$ and k were constant, the increase in c_r (residual ion) that gives rise to an increase in m must also cause a certain rise in f_m. To fit the data obtained in the presence of Sr^{2+}, the Hill coefficient for co-operativity, n, had a value of 4, a value also to be found from other data and which also applies for Ca^{2+} (Guan *et al.* 1988).

The upper points in Fig. 2 represent the f_m expected if, in the presence of Ca^{2+}, facilitation of m is solely secondary to increase in c_r (residual Ca^{2+}), in the same way as was found for Sr^{2+} (and proposed by Katz & Miledi, 1968). Evidently, observed f_m is less than predicted by an order of magnitude. In other experiments of this type the

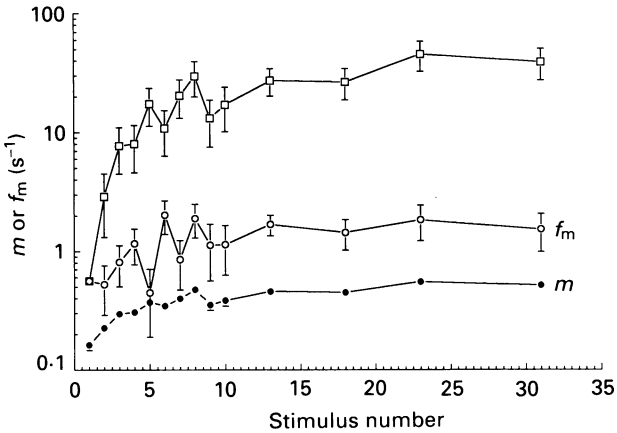

Fig. 2. Example of facilitation of quantal content (m, ●) and MEPP frequency (f_m, ○) during regular trains of 35 nerve impulses at 90 Hz, 2 s apart, at a junction in 0·8 mM-Ca^{2+}/8 mM-Mg^{2+}. Semilogarithmic plot. Values plotted for f_m are values from *before* the indicated stimulus. Values for after the tenth pulse have been averaged in groups of five up to stimulus number 25, then in a group of ten, to reduce standard errors. The uppermost plot (□) shows values of f_m predicted if facilitation of m reflects only residual Ca^{2+} (see text), from control f_m (at pulse 1) and the increase in m.

same discrepancy was always observed, although not always to the same extent, with Ca^{2+} as the mediator of release (cf. Hubbard, 1963; Zengel & Magleby, 1981). The one exception to this rule was on occasions when f_m rose to high levels, for no apparent reason, during repeated stimulation (see below).

Thus, in the presence of external Ca^{2+}, the residual ion model does not account simultaneously for rise in f_m and for rise in m; either f_m fails to rise as much as it should, or m rises for reasons other than residual Ca^{2+}. An obvious alternative explanation for the rise in m is an increase in Ca^{2+} entry per pulse ($c(t)$) during the train. However, results from experiments with Ba^{2+} and Sr^{2+} where entry is signalled by 'tails' of raised f_m provide no support for any such increase (Quastel & Saint 1988, Bain & Quastel, 1992). Figure 3 illustrates that in agreement with Datyner & Gage (1980) the time course of 'phasic' release was little if at all changed during facilitation (cf. Barrett & Stevens, 1972). In a detailed analysis from twenty-six pseudo-random stimulation series (see below) the absolute latencies during a 'train' increased slightly but significantly from the first to third pulses and was steady thereafter (average change $0{\cdot}036 \pm 0{\cdot}004$ ms); the dispersion of latencies (within the period of occurrence of 95% of quanta between 0·5 and 6 ms after a stimulus), which was defined $\pm 1{\cdot}0$%, did not change significantly.

An example of the rise of m and f_m during 'trains' within a pseudo-random stimulation sequence (see Methods) is shown in Fig. 4*A*. Here, the increase in f_m is

rather more than the increase in m (in terms of multiplication) but, again, f_m remains much less than predicted by the residual Ca^{2+} model.

Time course of decay of facilitation

The data in Fig. 4*B* represent the same results as in Fig. 4*A*, but with values of m and f_m obtained by averaging on the basis of the intervals from a preceding

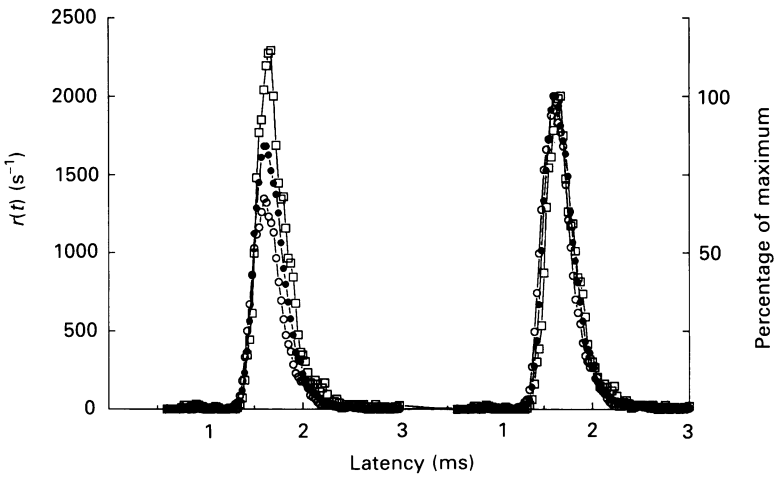

Fig. 3. Latency histograms of quanta, expressed as quantal release rate, $r(t)$, for the first impulse in a 'train' produced by pseudo-random stimulation (○, 5590 stimuli, $m = 0{\cdot}50$), impulses 2, 3, 4, and 5 combined (●, 7054 stimuli, $m = 0{\cdot}64$) and impulses from number 6 onward (□, 1244 stimuli, $m = 0{\cdot}83$). In the graphs on the right, points have been expressed as a percentage of maximum. Apart from a slight increase in latency (always found), there is no effect on the time course of 'phasic' release. 0·5 mM-Ca^{2+}, 8 mM-Mg^{2+}; 0·025 ms bins, double 2 point smoothing.

stimulus (see Methods), and here expressed relative to values of f_m close to the end of the longest interval (704 ms) and of m for stimuli at 704 ms from a previous stimulus. MEPP frequency has a relatively large early component that decays rapidly (before 11 ms) and the subsequent component is larger in magnitude than the facilitation of m. However, it is always much less than f_m predicted from m by the residual Ca^{2+} model (△). Semilogarithmic plots *versus* time of f_m minus final f_m and of m minus final m (Fig. 4*C*) show a good fit to an exponential decay with a time constant of 95 ms. This suggests that facilitation might fit a formula in which each pulse increments m and f_m linearly by factors that decay exponentially in time, with a time constant that in this case is 95 ms. However, construction, on this basis, of predicted f_m and m during 'trains' fails to account for the observed growth of f_m and m during 'trains' (Fig. 4*D*). Thus, the data does not fit a linearly additive 'model', without which the kind of analysis shown in Fig. 4*C* (cf. Mallart & Martin, 1967) has no theoretical basis.

Figure 5 shows averages of data for the facilitation of m and f_m, relative to final values (at more than 600 ms) after 'trains' of one stimulus, two and three stimuli (combined data), four, five and six stimuli, and more than six stimuli. Each point

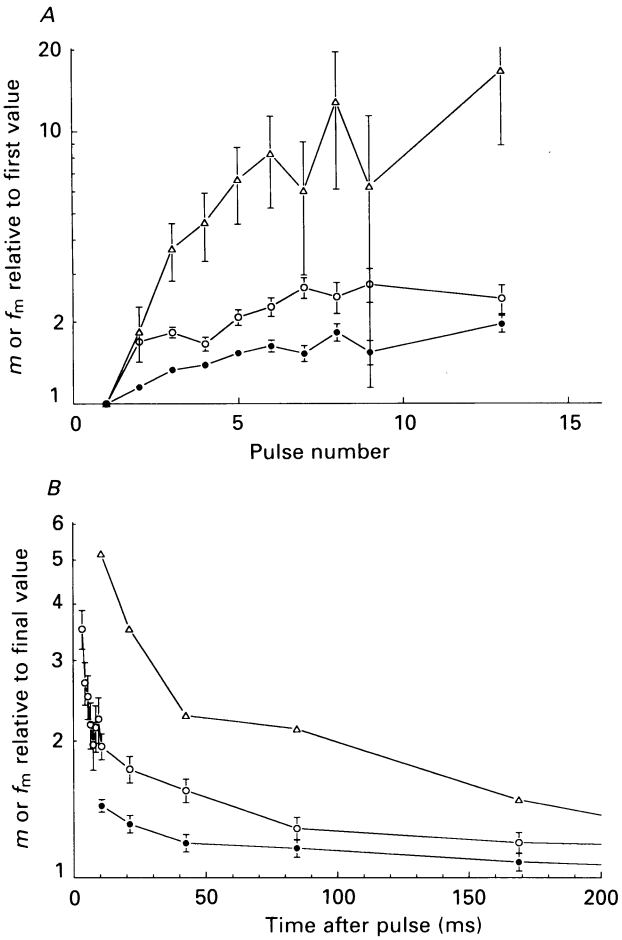

Fig. 4*A*,*B*. For legend see facing page.

represents the unweighted average (±S.E.M.) from twenty-six series, at twenty-three junctions where pseudo-random stimulation sequences were given at a mean overall frequency of 7·6 s^{-1} and interpulse interval in the train was, on average, 15·4 ms. At most of these junctions DMSO was added to the bathing medium, in order to raise f_m to a level where relatively small changes could be detected (see Methods). The lines without points represent averages of predicted increases of f_m, calculated on the basis of residual-ion model, in the same way as for Fig. 4*B*, but for $n = 5$ as well as $n = 4$. With $n = 12$ it was possible to obtain predicted values of f_m indistinguishable from those observed.

Components of facilitation

In terms of eqn (1) any change in release that is not explained simply by change in c_r must be explained in terms of alteration of $c(t)$ or of k, the multiplying factor. Thus, the failure of f_m to rise during facilitation as much as predicted by the rise in c_r calculated from the increase in m, assuming constant $c(t)$ and k (Figs 2, 4 and 5),

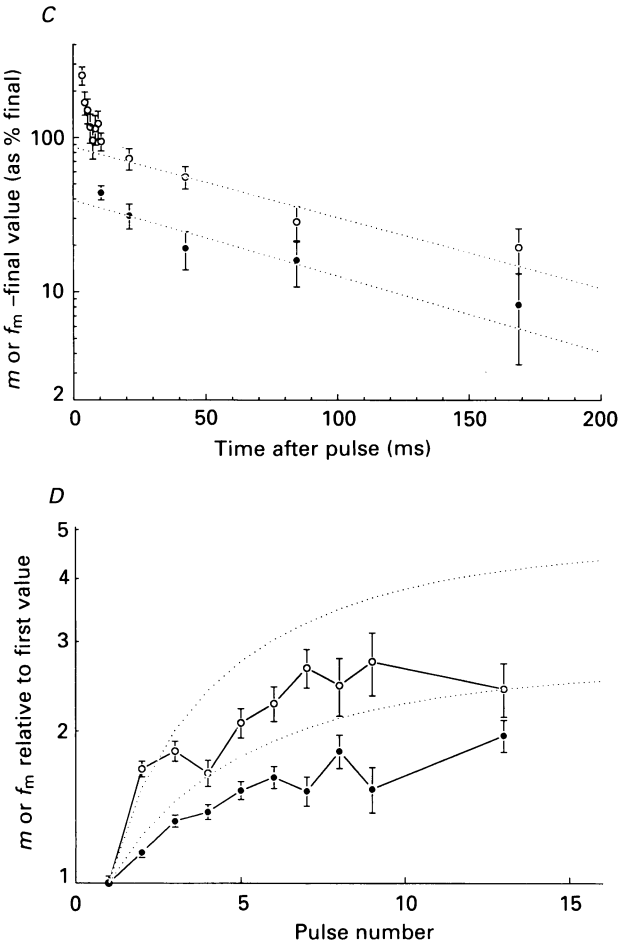

Fig. 4. Facilitation in a pseudo-random stimulation sequence. Same experiment as Fig. 3. *A* shows the growth of m (●) and f_m (○) in 'trains', each expressed relative to values for the first stimulus ($m = 0{\cdot}51 \pm 0{\cdot}01$, $f_m = 1{\cdot}63 \pm 0{\cdot}05$ s^{-1}). The growth of f_m is more than the growth of m, but much less than predicted by the residual Ca^{2+} model (△). The accuracy of points in the rising phase tends to decline with position in train because the number of samples declines with train length (see Methods). *B*, shows a plot of m, f_m, and predicted f_m, relative to unfacilitated 'final' values ($m = 0{\cdot}50 \pm 0{\cdot}02$, $f_m = 1{\cdot}62 \pm 0{\cdot}07$), at various intervals following each of the total of 20514 impulses. Symbols as in *A*. In *C* the differences of f_m and m from 'final' values are plotted semilogarithmically *versus* time; points from 22 ms onward fit fairly well to an exponential decay with a time constant of 95 ms. However, in *D*, the rise of m and f_m predicted by parameters obtained from the plot in *C* (lower and upper dotted lines respectively) fail to match observed values of m (●) and f_m (○) in trains. Predictions assume $\tau = 95$ ms.

indicates that m rises more than expected from the rise in c_r (manifest in f_m) because either k or $c(t)$ rises with antecedent pulses. As already pointed out, there is evidence that Ba^{2+} or Sr^{2+} entry per pulse is not facilitated (Quastel & Saint, 1988; Bain & Quastel 1992) and in experiments at the squid giant synapse (where facilitation appears in the same way as at the vertebrate neuromuscular junction) Ca^{2+} entry has

been reported not to be increased during facilitation (Charlton, Smith & Zucker, 1982). Nevertheless, we leave open the possibility that $c(t)$ might be increased with facilitation. Then, for any pairs of values for f_m and m, from eqn (1) and (1a)

$$k^{1/4}c(t) = r(t)^{1/4} - k^{1/4}c_r = r(t)^{1/4} - f_m{}^{1/4}.$$

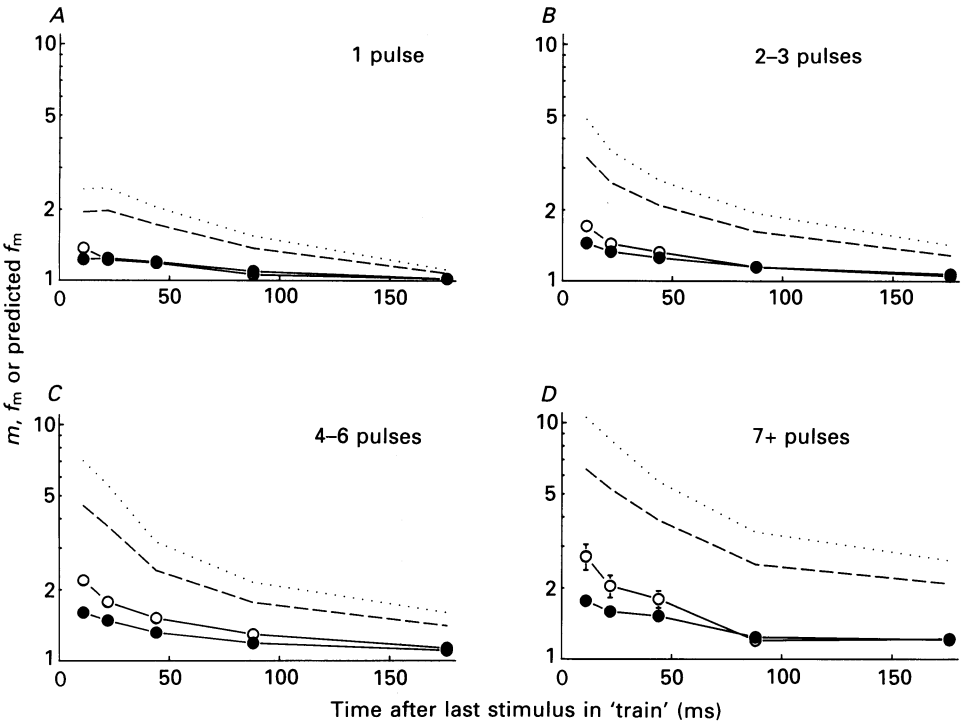

Fig. 5. Decay of facilitation of f_m and m after various numbers of conditioning pulses. The plots are for after a 'train' of : A, 1 pulse; B, 2 and 3 pulses (combined) (average 2·41); C, 4–6 pulses combined (average 4·74); and D, more than 7 pulses (average 9·1). Stimulation was with overall frequency of 7·6 Hz (see Methods) and the plotted points (m, ●; f_m, ○) are averages from twenty-six data series, from twenty-three junctions, in 8 mM-Mg^{2+} and (usually) 0·5 mM-Ca^{2+}. Twenty-three of the twenty-six series solutions contained added DMSO (2 or 4%). Dotted and dashed lines show 'predicted' f_m calculated by the residual Ca^{2+} model (see text) for $n = 4$ or 5, respectively.

This equation will also pertain to values of $r(t)$ and $c(t)$ at the peak of phasic release, and, from the latency histograms of release (e.g. Fig. 3) peak $r(t)$ can be closely approximated by quantal content, m, expressed as a rate in a period of 0·5 ms, giving a value R. This equation therefore provides a basis for analysing facilitation in terms of changes in $k^{1/4}c(t)$ and c_r (residual Ca^{2+}). The ratio of peak increments in the fourth root of release rate ($R^{1/4} - f_m{}^{1/4}$) gives a number, $x^{1/4}$, equal to the ratio of test to control $k^{1/4}c(t)$, at peak $r(t)$. If only k is altered, the ratio of test to control k is x, while if k is constant, the ratio of test to control peak $c(t)$ is $x^{1/4}$. For constant k, the ratio of test to control c_r is given simply by the ratio of test to control $f_m{}^{1/4}$, but if k is altered it affects f_m as well as m. If $c(t)$ is unchanged, the ratio of test to control c_r is the fourth root of the ratio of test to control f_m, divided by x.

Figure 6 shows, together with mean values of m and f_m, relative to control, mean values of x and increment in c_r, the latter being calculated (independently for each series) on the assumption of constant $c(t)$. The data is from the same twenty-six series as in Fig. 5. In other experiments with the same stimulus protocol facilitation of m and of f_m in the absence of DMSO were similar in magnitude and time course to that found with DMSO (see below, Table 2), but numbers of MEPPs and quanta recorded were insufficient for determination of changes in x and c_r at each junction. If it is $c(t)$ that alters with facilitation the increments in the fourth root of f_m (plotted) show the increments in c_r. Because these series were obtained at varied levels of external Ca^{2+}, and presumably various values of $c(t)$ and increment of c_r per stimulus, calculated values of c_r were normalized, before averaging, by dividing by calculated peak values of $c(t)$ ('c_p'), to give increment in c_r/c_p. Figure 6*A* shows average data for the rise of facilitation in 'trains'; rise in x accounts for most of the total rise in m while rise in c_r plays a large part in the rise of f_m. The increment in c_r produced by the first stimulus (at the time of the second stimulus), corresponds to about 1% of c_p; with Sr^{2+} the corresponding value was about 4% of c_p (Bain & Quastel, 1992).

The corresponding values for the decay of facilitation are shown in Fig. 6*B*, where the increments in x and c_r (after all pulses) are plotted semilogarithmically *versus* time. The increment in x declines exponentially with a time constant of about 84 ms while the increment in c_r seems to fit a double exponential.

In these calculations, the accuracy of determining c_r was intrinsically rather limited, since inaccuracy in values of x, arising from the stochastic distribution of numbers of quanta and MEPPs, causes a rather large inaccuracy of determination of c_r. However, each data series gave a fairly well-defined value of x at 11 ms, and gave well-defined values of f_m in time bins following a stimulus. If one simply assumes that x rises (because of increase in k) immediately after a stimulus and always falls with a time constant of 84 ms, one can obtain well-defined values for c_r. Figure 6*C* shows the average decay of f_m after a pulse (plotted semilogarithmically as the difference from final), before and after correcting for the increase in x by facilitation, and Fig. 6*D* shows corresponding values of c_r; the scale on the left shows values as a fraction of control c_r and the scale on the right values as a fraction of c_p.

In constructing the averages in Fig. 6, several sets of data were omitted: (a) data series obtained in very low Ca^{2+}, where facilitation was entirely or nearly absent (see below); (b) data series with insufficient numbers of quanta and/or MEPPs to define m and f_m to within $\pm 10\%$; and (c) three data series from junctions where f_m was unusually high and x did not alter. The reason for omitting the last group is shown in Table 1, which lists some of the data from a single junction. It may also serve as a numerical example to clarify the meaning of the parameters plotted in Fig. 6.

In contrast to the usual behaviour of junctions stimulated with the usual pseudo-random sequence (at 7·6 Hz overall in the presence of 2% DMSO), at which unfacilitated f_m (f_{m0}) remained steady after a small rise in the first minute or so of stimulation, at this junction unfacilitated f_m gradually climbed from 8 to 49 s^{-1} over the stimulation period of 32 min. With the rise in f_m there was also a rise in m, but facilitation declined, especially the facilitation in m. Calculations show values of unfacilitated $k^{1/4}c_p$ ($k^{1/4}c_{p0}$, values of $R^{1/4}-f_m{}^{1/4}$ for first pulses in 'trains') nearly constant, i.e. the increase of m accords with a progressive rise in 'resting' c_r.

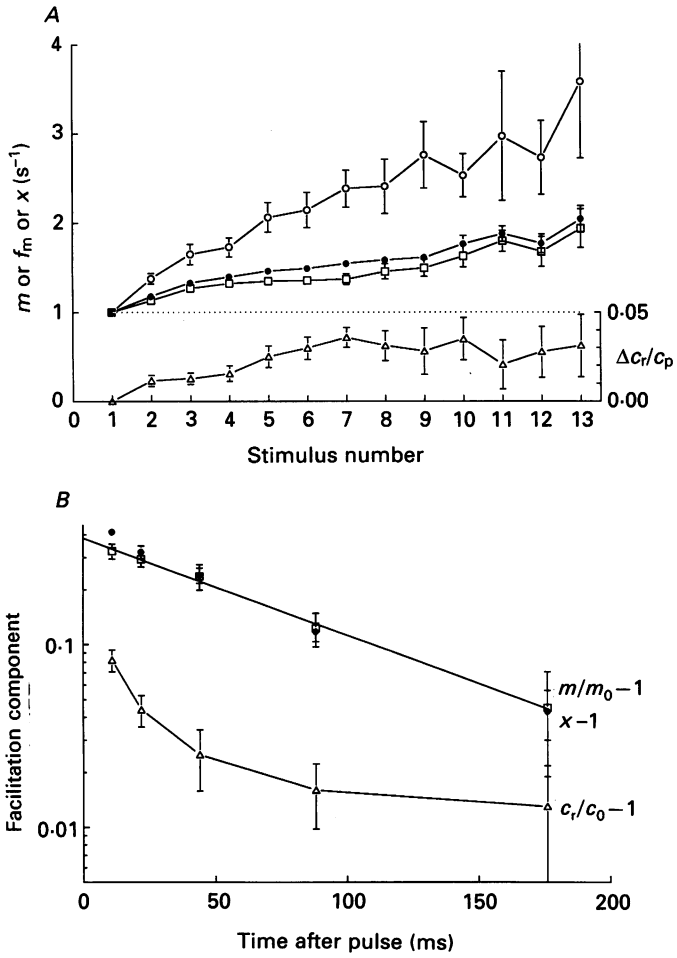

Fig. 6*A*,*B*. For legend see facing page.

Considering facilitation at 11 ms from preceding pulses, values of $k^{1/4}c_p$ were consistently higher than $k^{1/4}c_{p0}$; their ratio is $x^{1/4}$. If one assumes that c_p is unchanged with facilitation, the tabulated values of x are the facilitation of m and f_m attributable to increase of k and values of c_{r1}/c_{r0} are the ratios of facilitated to unfacilitated c_r. The tabulated values of y_m and y_{fm} are the factors by which m and f_m would have increased solely from the increase in c_r. It is intrinsic to the mathematics that the overall facilitations of m and f_m are the products xy_m and xy_{fm} respectively, i.e. the two components are 'multiplicative'. If it is $c(t)$ that alters with facilitation while k is unchanged, c_{r1}/c_{r0} is given by the 1/4 power of the tabulated value of f_{m1}/f_{m0} and x is the factor by which c_p^4 is increased.

Clearly, in this series, the decline in facilitation of m, with spontaneously rising f_m, relates to a progressive decline in the increase of x produced by each stimulus, with the increment of c_r produced by each pulse being roughly constant if x changes because of change in k. Thus, the facilitation of m began as mainly due to increase of k and eventually declined, to where it was due only to residual Ca^{2+}. In view of data (see below) showing dependence of rise in x upon entry of Ca^{2+} it seems likely

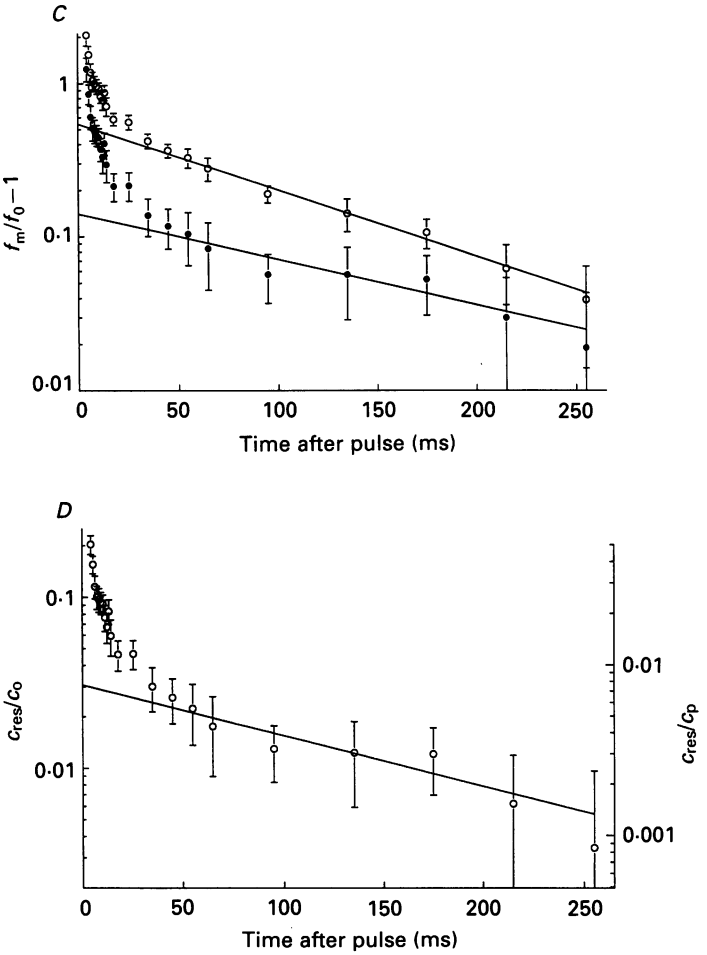

Fig. 6. *A*, growth of m (●) and f_m (○) during 'trains' for same data series as in Fig. 5. relative to initial values, m for the first stimulus in a 'train' and for f_m the value before the first stimulus. These averaged 0·66 and 4·4 s^{-1} respectively. Each plotted point for f_m pertains to the same time, following the same number of antecedent stimuli, as the corresponding point for m; values of x (□) and c_r (△, scale on right) were calculated (see text) independently for each data series and then averaged; the latter are expressed relative to c_p, apparent Ca^{2+} influx per pulse (see text). Calculated values of x account for most of the rise in m. *B* shows plots *versus* time for the decay of facilitation after all pulses. The plots are semilogarithmic, of increments in m, increments in x, and increment of c_r (relative to final c_r, c_o, plotted at 10 times actual values); points are averages (±S.E.M.) of values derived independently from the same twenty-six data series as in *A*. The increment of x (which accounts for nearly all of the increase in m) falls with a time constant of 84 ms (upper line). The line through the points for (c_r/c_o-1) merely joins the points. *C*, plots of the decay of the increment of f_m relative to final f_m (f_0) before (○) and after (●) 'correcting' f_m by dividing by x, assuming that x follows an exponential decay with an 84 ms time constant (see text). The lines are drawn with time constants of 100 and 150 ms. *D*, calculated residual Ca^{2+} ($c_{res} = c_r - c_o$, where c_o is final c_r), relative to c_o (scale on left) or relative to c_p (scale on right). The line represents an exponential fall with a time constant of 150 ms.

that the gradual increase in f_m was produced by a rise in intracellular Ca^{2+} that was sufficient to saturate the system by which Ca^{2+} causes rise in x.

Notwithstanding the sequence of events shown in Table 1, which also occurred at a few other junctions, in most data series where there was non-stationarity in absolute levels of m and f_m there were no significant changes in the magnitudes of fast

TABLE 1. Modification of facilitation with spontaneous rise in f_m

Minutes	f_{m0}	m_0	$k^{1/4}c_{p0}$	f_{m1}/f_{m0}	m_1/m_0	x	c_{r1}/c_{r0}	y_m	y_{fm}
0–8	8·1	1·04	5·07	1·85	1·47	1·36	1·08	1·08	1·36
8–15	13·4	1·25	5·16	1·76	1·34	1·21	1·10	1·11	1·46
15–21	20·9	1·41	5·15	1·58	1·38	1·30	1·05	1·06	1·21
21–25	30·7	1·59	5·15	1·60	1·29	1·17	1·08	1·11	1·38
25–32	49·3	1·92	5·22	1·58	1·18	1·01	1·12	1·17	1·57

Results for 0·5 mM-Ca^{2+}, 8 mM-Mg^{2+} 2% DMSO pseudo-random stimuli (11, 22, ... 704 ms). f_{m0} and m_0 are averages of values for 176, 352 and 704 ms. f_{m1} and m_1 are values at 11 ms after a preceding stimulus. x is facilitation of m produced by change in kc_p^4. y_m and y_{fm} are facilitations of m and f_m respectively that would be produced by increase in c_r if x were held constant.

facilitation, either of m or f_m. Thus, parameters of facilitation estimated from pooled data from any one series (as used in Figs 5 and 6) were essentially the same as parameters estimated from parts of each data series where m and f_m varied little.

Interaction of tetanic potentiation and facilitation

In experiments using pseudo-random stimulation at a mean frequency of 40·1 Hz (40 times as many 11 ms intervals as intervals of 22, 44, ... 352 ms) fast facilitation was just as great, or greater, than in experiments using stimulation at a mean frequency of 7·6 Hz. Such stimulation invariably caused a progressive rise of m and f_m, by as much as 20-fold, with a rather variable time course (time constants between about 15 s and several minutes), consistent with tetanic potentiation. During this rise the magnitudes of fast facilitation did not alter significantly, in accord with previous observations that potentiation and facilitation are mutually multiplicative (Magleby, 1973; Zengel & Magleby, 1982). In agreement with the result of others (Miledi & Thies, 1971; Misler, Falke & Martin, 1987), m and f_m were multiplied nearly in parallel during tetanic potentiation, which in terms of eqn (1) is equivalent to a rise in k rather than c_r. The increase in f_m permitted evaluation of changes in k and c_r with facilitation without the addition of DMSO to bathing solutions, and for this reason relatively high-frequency pseudo-random stimulation was used to evaluate Ca^{2+} dependence of facilitation (see below). The magnitude and time course of decay of facilitation after 'trains' of various numbers of stimuli for the data obtained using 40·1 Hz overall stimulation frequency, in the presence of 0·3 or 0·4 mM-Ca^{2+} and 4 mM-Mg^{2+}, were similar to those shown in Fig. 5.

BAPTA AM

It was reported by Kijima & Tanabe (1988) that at frog neuromuscular junctions loading nerve terminals with BAPTA, a Ca^{2+} chelator, by exposure to BAPTA AM (acetoxymethyl ester form) (Tsien, 1981) blocks facilitation. Confirmation of this

result for the mouse junction is shown in Fig. 7 (compare Fig. 4*B*), at a junction in a preparation which had been exposed to BAPTA AM. In this preparation previous experiments in the presence of Sr^{2+} had shown the reduced magnitude and prolongation of time course of Sr^{2+} 'tails' characteristic of loading with BAPTA

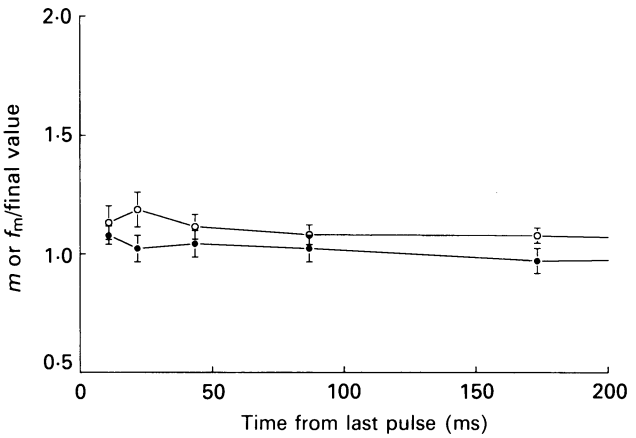

Fig. 7. Virtual lack of facilitation after loading with BAPTA. Plot same as in Fig. 4*B*. Values of 'final' m and f_m were $1{\cdot}92 \pm 0{\cdot}05$ and $11{\cdot}3 \pm 0{\cdot}2\ s^{-1}$. The bathing solution contained added 4% DMSO to accelerate f_m and raise m. 1 mM-Ca^{2+}, 8 mM-Mg^{2+}.

(Bain & Quastel, 1992). DMSO (4%) was added to the bathing solution to produce a relatively high f_m as well as quantal content.

Calcium dependence of facilitation

Although facilitation was apparently insensitive to external Ca^{2+} and Mg^{2+} over a wide range of m (to as high as could be obtained without eliciting suprathreshold EPPs), at sufficiently low external Ca^{2+}, facilitation became small or disappeared. In the experiment illustrated in Fig. 8, pseudo-random stimuli (at 7·6 Hz overall) were applied at the same junction in the presence of 8 mM-Mg^{2+} and 0·2, 0·5 or 1 mM-Ca^{2+}. At 0·2 mM-Ca^{2+} the quantal content of the EPP was very low (0·014), but clearly present (Fig. 8*A*). However, in contrast to what was seen in 0·5 or 1 mM-Ca^{2+} there was no obvious continued acceleration of quantal release after 2 ms from the stimulus, and there was little or no facilitation of f_m (Fig. 8*B*) or of quantal content (Fig. 8*C*). The facilitation of f_m, but not of m, was more for 1 mM-Ca^{2+} than for 0·5 mM-Ca^{2+}. To pursue this observation experiments were done using relatively high frequency pseudo-random stimulation (at 40·1 Hz overall), in 4 mM-Mg^{2+} and 0·05, 0·1, 0·2, 0·3 and 0·4 mM-Ca^{2+}. Results are shown in Fig. 9 and Table 2. Essentially, facilitation in 0·05 mM-Ca^{2+} was substantially less than at higher Ca^{2+}; during trains the growth of m and f_m was barely detectable at individual junctions and significant values for facilitation were obtained only by averaging values from the thirteen junctions studied. It may be noted that unfacilitated f_m was on average rather more than at higher Ca^{2+}, perhaps because we tended to select for study those junctions where an EPP could be detected (see Methods). At higher Ca^{2+} concentrations

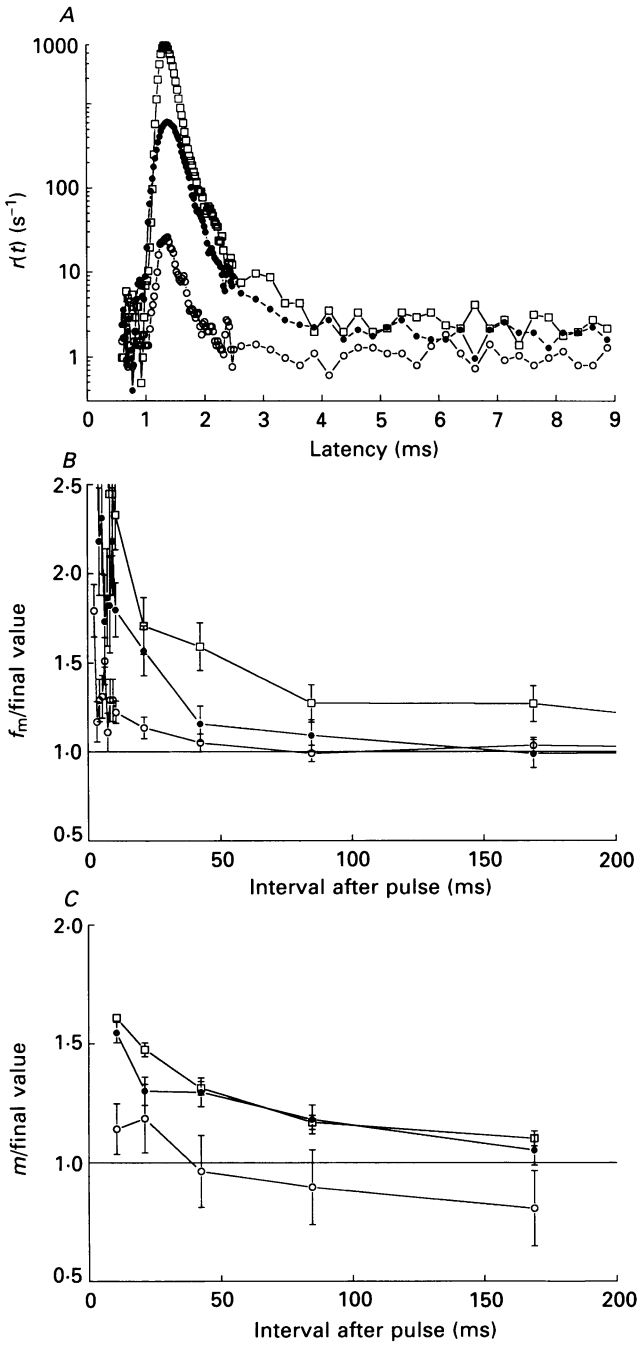

Fig. 8. Gradation of facilitation with Ca^{2+} entry and m. A, latency histograms of quantal release, expressed as release rate, $r(t)$, obtained at a single junction in 8 mM-Mg^{2+} and 1 mM-Ca^{2+} (□, 20396 stimuli), 0·5 mM-Ca^{2+} (● circles, 24926 stimuli) and 0·2 mM-Ca^{2+} (○, 65962 stimuli). The pseudo-random stimulation sequence gave a mean frequency of 13·6 Hz. Note the absence of a late phase of increased release (from 2·5 ms on) in 0·2 mM-Ca^{2+}; 'final' values of f_m (at past 264 ms from a preceding pulse) were 0·87, 0·91 and 0·76 s^{-1} in 1, 0·5 and 0·2 mM-Ca^{2+}, respectively. B, facilitation of f_m for same data as in A,

facilitation of both m and f_m grew with Ca^{2+}, but this was more marked for f_m than for m. Calculated values of maximum x were not significantly different from 0·1 to 0·4 mM-Ca^{2+} (Table 2) and the same was true for growth of x in trains (data not shown). That is, the increase of facilitation from 0·1 to 0·4 mM-Ca^{2+} is to be attributed primarily to increase in the increment in c_r (residual Ca^{2+}).

With pseudo-random stimulation at 7·6 Hz overall (in 2 mM-Mg^{2+} and no DMSO) facilitation, of m or f_m, was the same in 0·3 as in 0·2 mM-Ca^{2+} (Table 2) despite a 3-fold increase in m. These data were omitted from the series shown in Figs 5 and 6 because values of f_m were too low to permit determination of changes in c_r, but it is evident that the average magnitude of facilitation was much the same as in Figs 5 and 6. Values for increase of f_m by facilitation were rather variable and ill-defined because of the low values of f_m in these series.

On average, the time constant for decay of facilitation of m was 50 ± 3 ms for all the series listed in Table 2. There was no significant correlation of the time constant with extracellular Ca^{2+} and the time constants were the same in the series at 7·6 and at 40·1 Hz. The average time constants for decay of f_m (not listed) were somewhat shorter, 35 ± 7 ms, perhaps because the data included some of the fast component of facilitation of f_m (Fig. 6*C* and *D*). Where it could be determined (in solutions giving a mean m more than 0·1) the time constant of decay of x was 69 ± 11 ms, which is not significantly different from that in Fig. 6*B*. The time course of development of facilitation, in trains, corresponded closely to the time course of decay, and was not correlated with external Ca^{2+}.

The above results indicated that facilitation depends upon the presence of a minimal level of extracellular Ca^{2+} but left open the possibility that facilitation (rather than 'phasic' release) might be generated by a 'direct' effect of membrane depolarization (cf. Parnas & Parnas, 1988), contingent upon extracellular or intracellular Ca^{2+}. In an attempt to test this, experiments were done using tetraethylammonium (TEA) and 4-aminopyridine (4-AP) to increase Ca^{2+} entry into the nerve terminal, presumably by prolonging and/or increasing the presynaptic action potential (e.g. Saint *et al.* 1987). However, it was found that TEA did not increase the EPP in the presence of 0·05 mM-Ca^{2+} and 4 mM-Mg^{2+}. The data for facilitation with added 4-AP (0·2 mM) are listed in Table 2; in 0·05 mM-Ca^{2+}–4 mM-Mg^{2+} facilitation was restored, and most of the facilitation was of the 'multiplicative' type (manifest in x). Facilitation was greater with 4-AP, in both 0·05 mM-Ca^{2+} and 0·1 mM-Ca^{2+}, than in any of the series without 4-AP; at 0·2 mM or higher Ca^{2+} the effect of 4-AP on facilitation could not be tested because EPPs became suprathreshold. In the series with 0·05 and 0·1 mM-Ca^{2+} the magnitude of facilitation was uncorrelated with initial m.

'Direct' EPPs

Several experiments were done using 'direct' depolarization of nerve terminals in the presence of tetrodotoxin to block action potential generation, on the grounds that if facilitation depends on Ca^{2+} entry it should be graded with the intensity of

plotted as in Figs 4*B* and 5. *C*, facilitation of m, plotted in the same way as *B*. Final values of m were 1·00, 0·21 and 0·014 respectively. Note near absence of facilitation in 0·2 mM-Ca^{2+} and, in contrast to facilitation of f_m, nearly equal facilitation of m with either 0·5 or 1 mM-Ca^{2+}.

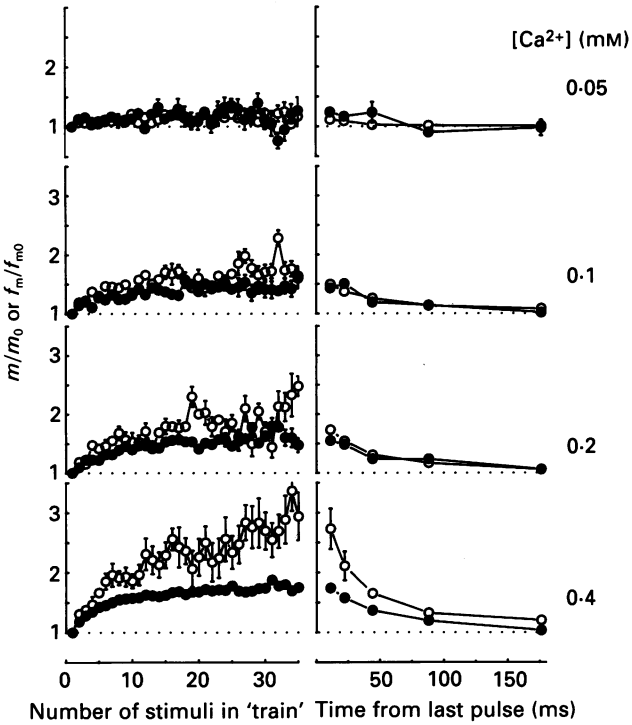

Fig. 9. Facilitation of m (●, lower lines) and of f_m (○) with growth in trains (left) and decay after all pulses (right) in 4 mM-Mg^{2+}, with pseudo-random stimulation at an overall frequency of 40·1 Hz. From top to bottom the graphs are for 0·05, 0·1, 0·2 and 0·4 mM-Ca^{2+}. All points are averages of normalized values. Other data from these series are given in Table 2.

TABLE 2. Ca^{2+} dependence of facilitation

Ca^{2+} (mM)	n	m	f_m (s^{-1})	Maximum facilitation m	Maximum facilitation f_m	X factor	τ (ms)
4 mM-Mg^{2+}, random stimulation at 40·1 Hz overall							
0·05	13	0·012	4·0	1·17±0·11	1·15±0·04	n.s.	48±20
0·1	11	0·031	3·4	1·42±0·07	1·60±0·09	1·45±0·17	62±7
0·2	11	0·105	2·8	1·52±0·06	1·77±0·11	1·40±0·10	49±14
0·3	16	0·320	2·1	1·67±0·04	2·34±0·13	1·47±0·07	53±4
0·4	13	0·460	2·0	1·66±0·06	2·33±0·18	1·44±0·06	48±4
0·2 mM-4-AP–4 mM-Mg^{2+}, random stimulation at 40·1 Hz overall							
0·05	7	0·050	0·88	1·70±0·08	2·45±0·39	1·69±0·33	41±14
0·1	6	0·34	1·8	2·09±0·20	3·32±0·37	1·90±0·25	84±8
2 mM-Mg^{2+}, random stimulation at 7·6 Hz overall							
0·2	15	0·17	0·48	1·68±0·14	1·25±0·29	1·66±0·19	42±13
0·3	19	0·50	0·53	1·55±0·08	2·12±0·41	1·46±0·10	51±10

Tabulated values of m and f_m are geometric means of unfacilitated values. 'Maximum facilitation' is mean for responses in trains for the tenth impulse and after, except for the experiments with 7·6 Hz overall, where it is for the seventh and subsequent impulses. Except for the value with 0·05 mM-Ca^{2+} and 4-AP, where the quoted τ pertains to f_m, the tabulated values of τ are for the decay of facilitation of m after all impulses.

depolarization and with m. The result shown in Fig. 10 is from an experiment where Mg^{2+} was raised to 2 mM and Ca^{2+} lowered to 0·3 mM because, rather unexpectedly, direct depolarizations that produced low quantal content EPPs (m = 0·1–0·5) in the presence of 2 mM-Ca^{2+} and 1 mM-Mg^{2+} were consistently not productive of significant

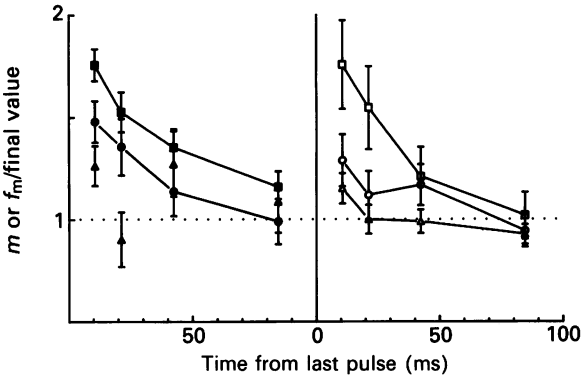

Fig. 10. Gradation of facilitation with intensity of direct depolarizing pulses of 0·4 ms duration, in the presence of 0·4 μM-tetrodotoxin and 0·2 mM-TEA, plotted as in Fig. 8*B* and *C*, with m in the left panel and f_m in the right panel. Values of final m were 3·53, 0·66, and 0·20 for pulses of 20 μA (squares), 15 μA (circles and 10 μA (triangles) respectively. Final f_m was the same, 7 s^{-1}, for all three sets of trials, having been raised about 1 s^{-1} by a maintained (DC) depolarizing current of 4 μA. Points for normalized m and f_m for 10 μA pulses, past 11 ms, were not significantly different from unity. The magnitudes of facilitation of m and f_m, at each pulse intensity, are not significantly different; facilitation was graded with pulse intensity but far from proportional to m.

facilitation. A gradation of facilitation with pulse intensity is evident, with facilitation of f_m (much better defined than for m) nearly absent at the lowest stimulation intensity. What is striking in this result is that facilitation nearly disappeared at an m of about 0·2, at which facilitation of both f_m and m were *always* in the normal range (Table 2) when evoked by nerve impulses. This finding does not necessarily indicate that facilitation is a function of nerve terminal depolarization *per se*, since other evidence indicates that combinations of numbers of channels opening and Ca^{2+} entry per channel that produce the same m will have less total Ca^{2+} entry, the higher the Ca^{2+} entry per channel (Quastel, Bain, Guan & Saint, 1989).

DISCUSSION

The essential conclusion from the present experiments is that facilitation at the mouse motor nerve terminal, although dependent upon Ca^{2+}, does not occur primarily because the Ca^{2+} that enters with each presynaptic action potential is supplemented by Ca^{2+} that has accumulated within the nerve terminal. Instead, in contrast to what is observed when Sr^{2+} is substituted for Ca^{2+} (Bain & Quastel, 1992), facilitation is mainly 'multiplicative'. Moreover, the magnitude and time course of this multiplicative effect is apparently unaffected by concurrent augmentation and potentiation (of longer time course, see Zengel & Magleby, 1982), produced by the overall stimulation frequencies of 7·6 and 40 Hz used in these experiments.

That facilitation depends upon Ca^{2+} entry is suggested by three results: (a) blockade by (presumed) intracellular Ca^{2+} chelation after exposure to BAPTA AM;

(b) its virtual disappearance at sufficiently low extracellular Ca^{2+}/high Mg^{2+}, and restoration by 4-aminopyridine; and (c) its non-appearance with 'direct' depolarization at low m, and reappearance with relatively large depolarizations in low Ca^{2+}/raised Mg^{2+}, to an extent dependent upon the magnitude of depolarization. Nevertheless, we cannot rule out the possibility that multiplicative facilitation is due (in whole or in part) to a 'direct' effect of brief nerve terminal depolarization (presumably exerted via voltage-sensitive membrane-bound proteins) that is contingent upon intracellular Ca^{2+} and (perhaps) extracellular Ca^{2+}, especially in view of the apparent increase of facilitation by 4-AP (Table 2).

Essentially our basis for rejecting the model for facilitation proposed by Katz & Miledi (1968) is the failure of MEPP frequency (f_m) to rise as much as predicted by the 'residual-ion hypothesis', although with Sr^{2+} (Bain & Quastel, 1992) and with Ba^{2+} (Guan *et al.* 1988) the fit of data to the residual-ion model indicates that f_m responds to intracellular ion in the same way as 'phasic' release. To maintain the residual-ion hypothesis it would be necessary to postulate either a mechanism whereby intracellular Ca^{2+} suppresses the increase in f_m that it would otherwise cause, or a mechanism whereby with Ca^{2+} (but not Sr^{2+} or Ba^{2+}), 'phasic' release is made much more than it would otherwise be by a 'direct' effect of depolarization (Parnas & Parnas, 1988). However, the time course of phasic release is the same with Ca^{2+} as with Sr^{2+} (Bain & Quastel, 1992). Robitaille & Charlton (1990) have reported that at the frog neuromuscular junction the time course of facilitation (of m) was affected by a Ca^{2+} chelator in a manner incompatible with the residual Ca^{2+} hypothesis. Any attempt to explain facilitation on the basis of bound Ca^{2+} within the terminal runs into the difficulty that the magnitude and time course (for rise and fall) of facilitation is insensitive to external Ca^{2+}, above that necessary to give m above 0·1, corresponding to about 5% of normal Ca^{2+} entry. Nevertheless, facilitation produced by 2 or 3 antecedent pulses is more than that produced by 1 pulse. That is, the terminal 'remembers' how many pulses were given in the previous 100 ms or so, but does not 'remember' how much Ca^{2+} entered.

Mathematically, the 'multiplicative' component ('x') could be interpreted as an increase in Ca^{2+} entry per impulse rather than an increase in k. However, there is no indication that this occurs at the squid giant synapse (Charlton *et al.* 1982) or at the neuromuscular junction with Ba^{2+} (Quastel & Saint, 1988) or with Sr^{2+} (Bain & Quastel, 1992). Nevertheless, it could be argued that this occurs only with Ca^{2+}, and an increase of the magnitude required to account for facilitation at the mouse junction, of the order of 10%, could have escaped detection in experiments at the squid synapse. Another difficulty with the idea that facilitation may depend upon increase in per pulse Ca^{2+} entry is that it provides no reason for facilitation of f_m always being at least as great as facilitation of m and often nearly the same (Fig. 9, Table 2). This points directly to a process that multiplies 'phasic' and 'non-phasic' release to the same extent, in the same way as can be observed with tetanic potentiation (Misler *et al.* 1987; Quastel *et al.* 1989). On this basis, both facilitation and tetanic potentiation represent increases in the multiplying factor, k, in eqn (1). It is possible, of course, that per pulse Ca^{2+} entry falls slightly during a train. If so, our calculations will have consistently overestimated c_r and underestimated the increase in k.

These experiments also showed that it is possible to have phasic release, producing clear EPPs, together with little or no facilitation. With 'direct' EPPs produced by nerve terminal depolarization this occurred with quite large EPPs and the same was true at nerve terminals loaded with BAPTA. This does not necessarily indicate that the sites of action of Ca^{2+} to cause facilitation re far removed from the release sites, since the co-operativity of Ca^{2+} for release entails that if release sites are heterogeneous with regard to Ca^{2+} concentration (because Ca^{2+} channels open stochastically) most release is from sites which happen to have higher than average Ca^{2+} concentrations. Particularly in the case of 'direct' EPPs, where one has relatively few channels opened but relatively high Ca^{2+} entry per channel, a fairly high m could co-exist with a low total Ca^{2+} entry and low average Ca^{2+} per release site (Quastel *et al.* 1989). It would seem unlikely that increment in 'bulk' intracellular Ca^{2+} is normally the cause of multiplicative facilitation, since this apparently decays with a time constant of about 150 ms (Fig. 6*D*), while multiplicative facilitation (increase in 'x') decays with a time constant of about 80 ms. More likely, facilitation is triggered by the relatively prolonged transient of intracellular Ca^{2+} that appears as a fast phase of decay of f_m and 'c_r' (Fig. 6*C* and *D*), following 'phasic' release for up to about 10 ms. In this period Ca^{2+} (at release sites) is not much raised above 'resting' and it would therefore seem reasonable that facilitation can be absent when 'resting' Ca^{2+} is depleted (Dudel, 1989) or chelated by BAPTA (Kijima & Tanabe, 1988). Since facilitation is activated at very low Ca^{2+} entry and evidently saturates at not much higher Ca^{2+} entry it is likely that resting Ca^{2+} normally activates the system and maintains release at a level higher than would otherwise be the case.

It is very striking that when release is evoked in the presence of Ca^{2+} there is much less increase of f_m following impulses than occurs in the presence of Ba^{2+} (Quastel & Saint, 1988) or Sr^{2+} (Bain & Quastel, 1992); this relates to a briefer time course of residual ion (c_r) for Ca^{2+}, especially relative to Ba^{2+}, but also to a much reduced absolute magnitude, a least after 10 ms or so from a preceding impulse. Presumably this occurs because of proteins(s) that bind Ca^{2+} much more effectively than they bind the other ions (Kwan & Putney, 1990). The apparent time course of decay of c_r is also faster for Ca^{2+} (τ about 150 ms) than for Sr^{2+} (τ about 250 ms), although most Ca^{2+} is presumably bound, suggesting that the removal mechanism, if it is the same for all the ions, removes free Ca^{2+} at about 10 times the rate for free Sr^{2+} and 200 times the rate for Ba^{2+}.

With regard to the mechanism whereby transiently raised intracellular Ca^{2+} causes 'multiplicative' facilitation, we can only speculate. One attractive possibility is that it activates an enzyme that produces an intracellular modulator that acts multiplicatively, in much the same way as dimethylsulphoxide (McLarnon *et al.* 1987) or alcohols (Gage, 1965; Quastel, Hackett & Cooke, 1971). The same applies to tetanic potentiation where the mediating ion is probably Na^+ (Atwood, Swenarchuk & Gruenwald, 1975; Misler & Falke, 1987). It is tempting to link the two by postulating that submembrane Ca^{2+} does not act directly to activate the (hypothetical) enzyme, but instead gives rise to an increase in intracellular Na^+, *via* the Ca^{2+}–Na^+ exchanger (Barzilai, Spanier & Rahamimoff, 1984; Mullins, Requena & Whittenbury, 1985; Lux & Bruns, 1991). This is the reverse of the mechanism for

Na^+ involvement in tetanic potentiation suggested by Misler *et al.* (1987), but the concept of Na^+ acting by increasing intracellular Ca^{2+} in potentiation is untenable since this would appear as an increase in c_r rather than an increase in 'x', i.e. cause rises in f_m much larger than the rises in m, rather than the parallel or nearly parallel multiplication that is observed (Misler *et al.* 1987; Quastel *et al.* 1989). One should note that the multiplicative parameter k in eqn (1) implicitly includes all factors in transmitter release except release site intracellular Ca^{2+} *per se*, namely, availability of quanta for release (Hubbard, 1963), affinity of Ca^{2+} for its (probable) internal receptor, and effectiveness of the Ca^{2+}–receptor complex.

This work was supported by grants from the Muscular Dystrophy Association of Canada and the Medical Research Council.

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