Implication of frequenin in the facilitation of transmitter release in *Drosophila*

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1. We have investigated the possible role of frequenin in the modulation of synaptic facilitation at the larval *Drosophila* neuromuscular junctions. Excitatory junctional currents (EJCs) and presynaptic nerve terminal currents were recorded by external electrodes in normal larvae and in transgenic larvae carrying an extra insertion of the frequenin cDNA.

2. Motor nerve stimulation by twin pulses or trains of stimuli provoked EJC facilitation which was about three times higher in transgenic larvae compared to controls. Unconditioned EJCs revealed, however, similar quantal content and Ca^{2+} sensitivity in both *Drosophila* strains.

3. Differences between normal and transgenic *Drosophila* in the quantal content of the facilitated EJC do not depend on differences in the duration of the repolarization phase of the presynaptic action potential.

4. Perfusion of tetrodotoxin or of low-Na^+ solutions abolished the enhancement of the EJC facilitation observed in the transformants. These treatments only slightly affected the facilitation of normal junctions.

5. These results suggest that (i) internal Na^+ accumulation can enhance facilitation of transmitter release in *Drosophila* neuromuscular junctions overexpressing frequenin, and (ii) this effect possibly depends on a modulation of the activity of the Na^+–Ca^{2+} exchanger by frequenin.

It is generally admitted that the entry of calcium into presynaptic terminals with nerve impulses is the trigger for the phasic release of transmitter. This short-lived event is followed, in most synapses, by a much longer period of facilitation during which a second nerve impulse is much more effective than the first in raising the probability of release. Such use-dependent increase in synaptic efficacy is significant for optimizing the operation of synapses under a variety of physiological situations. This process has been termed short-term facilitation, augmentation, post-tetanic potentiation or long-term potentiation depending on its duration and the number of presynaptic action potentials required to elicit the effect (Silinsky, 1985; Wojtowicz & Atwood, 1986).

Short-term facilitation, usually referred to as facilitation, has been studied mostly in neuromuscular junctions, where it decays in less than 1 s (see Silinsky, 1985 for review). Its presence is important to ensure synaptic transmission by temporal summation of the endplate potentials in neuromuscular junctions with low safety margin of transmission (Grinnell & Herrera, 1980). Facilitation is believed to be due to a prolonged increase in intracellular calcium (called active calcium) remaining attached to specific molecules at the release sites (Katz & Miledi, 1968). Thus, calcium increments due to successive nerve impulses release more transmitter in a background of residual calcium because of a non-linear relationship between transmitter release and active calcium (Dodge & Rahamimoff, 1967; Katz & Miledi, 1968; Zucker & Lara-Estrella, 1983).

However, previous studies have shown that large variations in the ability to produce facilitation of release exist among different synapses. For instance in the crayfish, neuromuscular junctions showing high facilitation co-exist with junctions showing low facilitation (Bittner & Sewell, 1976). Also, in most cases the growth of facilitation during a train of impulses cannot be predicted by formalisms based on the residual calcium model (Zucker, 1974; Bittner & Sewell, 1976; Bittner & Schatz, 1981). Possibly, other factors, such as changes in the presynaptic action potential configuration or the action of regulatory mechanisms on

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calcium metabolism or on exocytosis may be involved in the facilitation of release, in addition to residual calcium.

The use of *Drosophila* mutants provides a powerful tool for identifying and manipulating the molecules that modulate the facilitation of transmitter release. For instance, the learning and memory deficient mutants dunce (*dnc*) and rutabaga (*rut*) exhibit a reduced synaptic facilitation possibly arising from a defective cyclic AMP cascade (Zhong & Wu, 1991). The bang-senseless (bss^{MW1}) mutant, which can suffer paralysis as a consequence of a mechanical shock, shows enhanced long-term facilitation which seems to be related to Na^+ accumulation in nerve endings (Jan & Jan, 1978; Ganetzky & Wu, 1982). Similarly, a previous study performed on *T(X;Y)V7* mutants of *Drosophila* (referred to as *V7*) (Mallart, Angaut-Petit, Bourret-Poulain & Ferrús, 1991) showed that neuromuscular junctions yielded abnormally high levels of transmitter output in a time- and frequency-dependent manner upon repetitive stimulation of motor nerves. This property prompted us to investigate in detail the nature of the molecular defect and its consequences for the facilitation of transmitter release.

Cloning and sequencing the DNA distal but close to the *V7* breakpoint on the X-chromosome lead to the identification of a 21 kDa Ca^{2+}-binding protein, called frequenin (Frq) (Pongs *et al.* 1993), which belongs to a newly recognized family of Ca^{2+}-binding proteins which act as guanylyl cyclase regulators (Yamagata, Goto, Kuo, Kondo & Miki, 1990; Dizhoor *et al.* 1991; Lambrecht & Koch, 1991). It was hypothesized that the enhanced facilitation of transmitter release observed in *V7* mutants could be related to the misexpression of Frq. This question was addressed in the present investigation by performing electrophysiological experiments on *Drosophila* transformants bearing an extra insertion of Frq cDNA under the control of the heat-shock promoter hsp70, and thus expected to overexpress Frq upon heat shock treatment. It was found that these transformants exhibit the same electrophysiological phenotype as *V7* suggesting that Frq is implicated in the modulation of synaptic facilitation.

METHODS

Third instar larvae dissection, nerve stimulation and neuromuscular junction recordings were performed as described previously (Mallart *et al.* 1991). Briefly, extracellular recordings were taken using macro-patch electrodes (5–10 μm diameter), filled with the bath solution and placed on selected synaptic boutons visualized at a magnification of ×500 using Nomarski optics. The area under the recording electrode included a portion of the postsynaptic membrane and 1–2 synaptic boutons which represent about one-fiftieth of the total number present in a single muscle fibre (authors' unpublished data). Electrode resistance was 0·5–1 MΩ in the medium and increased 3–6 times (seal factor) upon pressing the tip against the synaptic spots. Synaptic current amplitude was therefore obtained by multiplying the recorded signals by 1 + 1/seal factor (Cull-Candy, Miledi & Parker, 1980). Negativity at the electrode is indicated by downward deflections. Signals were recorded through a patch-clamp amplifier (List EPC-5: List-Electronic, Darmstadt, Germany, or Bio-logic RK 300: Bio-Logic, Claix, France) and analysed using the SCAN (J. Dempster, University of Strathclyde, Glasgow) or the pCLAMP (Axon Instruments Inc., Foster City, CA, USA) programs. The sampling rate was 20–100 μs. Quantal analysis of externally recorded synaptic currents was performed in low-calcium solutions by counting the number of individual quanta that compose the synaptic current (Katz & Miledi, 1965*b*; Johnson & Wernig, 1971). In most cases the counting was performed at 12 °C to improve the time resolution because of the increased temporal dispersion of quanta at low temperatures (Katz & Miledi, 1965*b*).

The standard bathing solution contained (mM): NaCl, 128; KCl, 2; $MgCl_2$, 4; Hepes, 5 (pH 7·2); sucrose, 36. The concentration of Ca^{2+} was varied to suit experimental requirements. In some experiments part of the Na^+ was isosmotically replaced by sucrose. The changes in the ionic composition of the medium were effected concomitantly in the bath and in the electrode. The temperature was maintained at the desired level by flowing the bathing solution through a spiral in contact with an electronically controlled Peltier device.

Drosophila strains

Mutant strain. The mutant *T(X;Y)V7, ywf.* $B^s y+/C(1)M3$, named *V7*, was supplied by Dr A. Ferrús (Instituto Cajal, Madrid, Spain).

Transformants. The generation of the Frq transformants has been described previously (Pongs *et al.* 1993). Briefly, the protein coding region of Frq cDNA was inserted between the heat-shock promoter of the *Drosophila* hsp70 gene and its termination-processing sequences using the vector pWH1 according to standard procedures. The Frq–pWH1 construct was introduced into the germ line of white mutant (w^-) by P-element-mediated germ line transformation. Several independent w^+ transformed fly lines were established carrying pTFrq insertions on the 2nd or 3rd autosome. All lines are homozygous viable and produced TFrq mRNA upon raising transgenic larvae or flies at 30 °C. The strain TFrq2, used in the present experiments, has the insertion on the third chromosome. In practice, to activate the hsp70 promoter of transgenic flies, embryos and 1st instar larvae were allowed to develop at room temperature and then incubated at 30 °C during the 2nd and 3rd larval stages until the day of the experiment. TFrq2 larvae exhibited about 10 times the Frq protein content compared to controls (Pongs *et al.* 1993).

Controls. As controls, larvae from the w^- strain and from w^+ transformed stocks carrying an insertion lacking the Frq cDNA, exposed or unexposed to heat shock, were used initially. However, since preliminary electrophysiological tests failed to reveal any difference between these and normal Canton S (CS), the latter were routinely used as controls. Non-heat-shocked TFrq2 larvae were not used because of the basal level of activity of the hsp70 promoter at low temperature.

RESULTS

Quantal analysis of transmitter release

Amplitude distributions of spontaneous and stimulus-evoked quanta obtained at 12 °C from the same synaptic spot with 0·15 mM $[Ca^{2+}]_o$ are shown in Fig. 1*A* and *B*. The quantal nature of the steps in the excitatory junctional current (EJC) is shown by the agreement of amplitude distributions between single (or first) evoked releases and miniature excitatory junctional currents (MEJCs). The

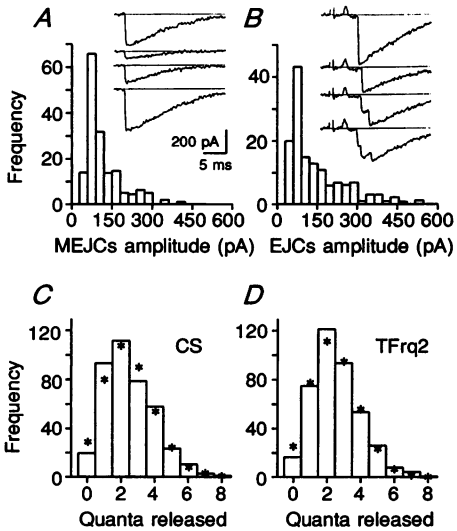


Figure 1. Quantal analysis of the EJC in CS and TFrq2 neuromuscular junctions
Amplitude histogram and samples (inset) of MEJCs (*A*) and stimulus-evoked EJCs (*B*) in a CS neuro-muscular junction. The presynaptic action potential is seen in the traces as a positive deflection preceding the EJC. In *A* and *B*, $[Ca^{2+}]_o = 0{\cdot}15$ mM, the temperature was 12 °C. Distribution of the quantal content of the EJC in CS (*C*) and TFrq2 (*D*). The theoretical distribution of m predicted by binomial statistics is indicated by asterisks. In *C* and *D*, $[Ca^{2+}]_o = 0{\cdot}20$ mM; the temperature was 10 °C.

average amplitude of the elementary synaptic current was 130 pA in this series of experiments, which is lower than the average value of 210 pA found in measurements performed at 20 °C in $[Ca^{2+}]_o$ of 2 mM (McLarnon & Quastel, 1987) because of reduced glutamate channel conductance at low temperature (Anderson, Cull-Candy & Miledi, 1978) and in low-Ca^{2+} solutions (Dudel, 1981). Taking 2 MΩ as the average input resistance of *Drosophila* muscle fibres (Jan & Jan, 1976) a quantum of 200 pA will depolarize the membrane by 0·4 mV, which is close to the value of 0·5 mV obtained by intracellular recording (Jan & Jan, 1976). Most quantal events had fast rise times in the range 200–500 μs. Quanta of low amplitude and rise times longer than 500 μs were also observed but not included in the analysis because they were thought to originate under the rim of the electrode and, therefore, to suffer from amplitude attenuation. Note the large fluctuations in quantal size and the skewed distribution of amplitudes of both spontaneous and evoked quanta, as already described for glutamate MEJCs in external (Bekkers, Richerson & Stevens, 1990) or whole-cell (Edwards, Konnerth & Sakmann, 1990) recordings from central neurones of vertebrates.

In solutions with $[Ca^{2+}]_o$ lower than 0·3 mM (and 4·0 mM Mg^{2+}) failures of release occur in reverse relationship with the external calcium. By carefully adjusting the Ca^{2+}:Mg^{2+} ratio one can make the number of failures of release represent only a few per cent of the total number of trials. Under these conditions, the fluctuation of the quantal content of the EJC follows binomial statistics according to which the quantal content m of the EJC is given by $m = np$, where n is the number of sites with releasable quanta and p the probability of release (Johnson & Wernig, 1971).

Quantal analysis of the EJC in TFrq2 and control larvae performed in $[Ca^{2+}]_o$ of 0·2 mM and $[Mg^{2+}]_o$ of 4 mM at 1 Hz nerve stimulation is shown in Fig. 1*C* and *D*. The observed frequency of failures and of responses containing 1, 2, 3, 4, etc. quanta agrees with the expected number of trials nx (indicated by asterisks) which release x quanta, as predicted by the binomial relation:

$$n_x = N\frac{n!}{(n-x)!\,x!}p^x(1-p)^{n-x}, \quad (1)$$

where N is the total number of trials in the series. The mean quantal content m was obtained by dividing the total number of quanta by N. The statistical release parameters p and n can be estimated by the relations $p = 1-(\text{var}\,m/m)$ (Johnson & Wernig, 1971) and $n = m/p$.

In this and in several other experiments, for a given $[Ca^{2+}]_o$, no significant differences were observed in mean quantal content or in the release statistics of individual EJCs between CS, *V7* mutants and transgenic larvae for frequencies of stimulation ≤ 1 Hz (Table 1).

Calcium dependence of transmitter release

The calcium dependence of transmitter release was examined by counting and averaging, at each calcium concentration, the number of quanta released by series of 150 stimuli at 0·3 Hz. $[Ca^{2+}]_o$ was varied in the range 0·08–0·2 mM and $[Mg^{2+}]_o$ kept constant at 4·0 mM. Higher values of the Ca^{2+}:Mg^{2+} ratio were not used because at high rates of release individual quanta could not be resolved in the recordings. In Fig. 2 the calcium dependence of transmitter

Table 1. Quantal content of the EJC (m) and paired-pulse facilitation (F) in wild-type (CS), transformants (TFrq2) and mutants (*V7*), measured in $[Ca^{2+}]_o$ of 0·12 mM

	m	F (Δt = 20 ms)
CS	0·86 ± 0·12 (9)	0·74 ± 0·12 (9)
TFrq2	0·84 ± 0·19 (9)	2·92 ± 0·28 (12)
V7	0·70 ± 0·11 (7)	2·10 ± 0·30 (11)

The mean quantal content has been estimated by counting the quantal events that compose the EJC as described in the text. Facilitation has been obtained using eqn (2). Results are given as means ± S.E.M., number of neuromuscular junctions is shown in parentheses.

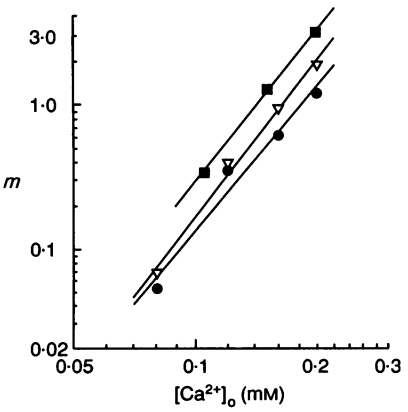


Figure 2. The relation between $[Ca^{2+}]_o$ and quantal content (m) of the EJC in CS (▽) *V7* (●) and TFrq2 (■), plotted in double logarithmic co-ordinates
Each series of points is from the same synaptic spot. Each point represents the average value of m obtained by counting the quantal events to 100–150 stimuli for each $[Ca^{2+}]_o$. The slope of the regression is 3·59 for CS, 3·31 for *V7* and 3·45 for TFrq2; the temperature was 12 °C.

release is shown by plotting the logarithm of the quantal content against the logarithm of $[Ca^{2+}]_o$. In three fibres of different CS larvae the slope of the double logarithmic plot was 4·06, 3·42 and 3·59, which is similar to that found by Jan & Jan (1976) in *Drosophila* or by Dodge & Rahamimoff (1967) in frog neuromuscular junctions. Comparable values of the slope were found in two *V7* fibres (4·26 and 3·31), and in one fibre of TFrq2 (3·45), which indicates that CS, mutant and transgenic neuromuscular junctions do not differ by their calcium sensitivity of transmitter release.

Facilitation

When one or more conditioning shocks are applied to the nerve supplying a presynaptic terminal, the number of quanta of transmitter released by a subsequent test shock may be increased. This phenomenon is called facilitation (F) and can be defined as the change in the quantal content of any EJC in the series (m) relative to the quantal content of the first EJC (m_0) (Mallart & Martin, 1967):

$$F = (m - m_0)/m_0. \quad (2)$$

Paired-pulse stimulation

Transgenic ($n = 5$) and CS ($n = 5$) neuromuscular junctions were subjected to a paired-pulse stimulation protocol at 12 °C and a $[Ca^{2+}]_o$ of 0·12 mM. The relationship between facilitation and interval was examined by varying the interval between the two stimuli and averaging the number of quanta obtained in fifty trials at each synaptic spot at any one interval. As shown in Fig. 3, the values of F were consistently higher at all time intervals in transgenic relative to control neuromuscular junctions. It was found by regression analysis that, in both cases, the decay of facilitation could be fitted by two exponentials with fast and slow time constants τ_1 and τ_2 respectively. In transgenics the values were $\tau_1 = 67$ ms and $\tau_2 = 500$ ms (Fig. 3, inset). In controls the corresponding values were 68 and 400 ms. The difference in τ_2 between both stocks is probably due to inaccuracies in the estimation of F at low levels of release. Nevertheless, in both cases, the values of τ_1 and τ_2 observed here are in the range, allowing for the effect of temperature on τ (Balnave & Gage, 1974), of the time constants of decay of the first and second components of facilitation reported in the literature for other animal species (Mallart & Martin, 1967; Bittner & Sewell, 1976; Charlton & Bittner, 1978; Silinsky, 1985). By contrast, the initial facilitation F_0, obtained by extrapolation to the zero intercept of data of Fig. 3, shows a marked difference between TFrq2 and control neuromuscular junctions with values of $F_0 = 4·80$ in the former and $F_0 = 2·11$ in the latter.

In another series of twin-pulse experiments, facilitation with a 20 ms time interval between conditioning and test stimuli was measured at room temperature. At least 100 paired sequences of conditioning–test EJCs were averaged in controls, in *V7* mutants and in TFrq2 transgenics. The

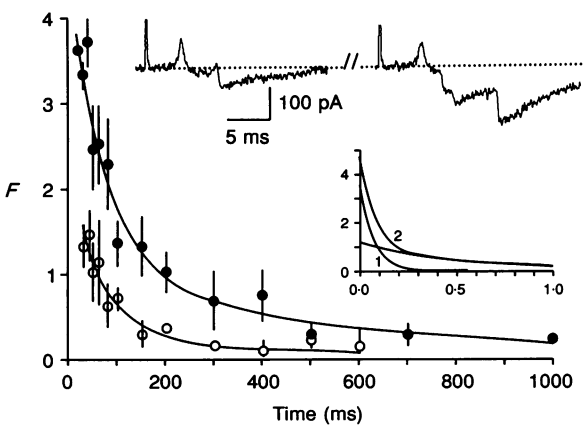


Figure 3. Time course of facilitation of transmitter release to paired-pulse stimulation
The relative quantal contents of the test EJC (m) and of the unconditioned EJC (m_0) (shown as an inset) have been used to calculate the facilitation (F) using eqn (2). F is plotted as a function of the interpulse interval in TFrq2 (●) and in CS (○). Each symbol represents the averaged value (± S.E.M) of F obtained from 200 to 250 paired stimuli delivered to 5 neuromuscular junctions; error bars have been omitted when shorter than the symbol. The curves have been drawn by exponential regression using a best fit program (Asystant, Asyst Software Technologies Inc.) to experimental data. The plot shown as an inset represents the two time constants (1, 2) of decay of F and the initial facilitation F_0 at the ordinate intercept obtained from the TFrq2 data. $[Ca^{2+}] = 0·12$ mM; the temperature was 12 °C.

average values of F shown in Table 1 indicate that facilitation in TFrq2 and *V7* was about 4 and 3 times larger than in normals, respectively.

Facilitation during trains of stimuli

The mean quantal content (± S.E.M.) of the EJC was measured in $[Ca^{2+}]_o$ of 0·10 mM during trains of fifty stimuli delivered at 1 or 5 Hz in heat-shocked TFrq2 transformants and in controls. Traces showing large bursts of quanta (which probably represent a different mechanism of transmitter release, see below) were excluded from the analysis. For 1 Hz stimulation, the average quantal content of the focal EJC in both stocks was comprised between 0·10 and 0·30 and remained practically constant throughout the duration of the train of stimuli. Under these conditions, the percentage of failures of release was close to 80 % in both experimental series. By contrast, for 5 Hz stimulation, the quantal content in TFrq2 junctions averaged $0·79 \pm 0·28$ during the first ten stimuli and reached a mean value of $2·79 \pm 0·69$ for the last ten stimuli of the series. The corresponding figures for normal neuromuscular junctions were $m = 0·14 \pm 0·04$ at the beginning and $0·18 \pm 0·06$ at the end of the 5 Hz train (Fig. 4*A*). It appears that, in agreement with the results of paired-pulse experiments, the motor endings of TFrq2 larvae show an unusually large degree of facilitation of transmitter release during the train. One would expect, accordingly, a decrease in the number of failures of release (del Castillo & Katz, 1954) in TFrq2, but not in CS endings, in the course of 5 Hz stimulation. The collected data from eight experiments in TFrq2 showed a mean (± S.E.M.) decrease in failures from 78 ± 5·6 % at 1 Hz stimulation (taken as the unfacilitated response) to 24 ± 9 % by the end of a series of fifty stimuli at 5 Hz; the corresponding values for CS neuromuscular junctions were 85 and 86 % respectively.

Successsive values of the quantal content shown in Fig. 4*A* have been used to calculate the facilitation during a train of fifty stimuli. Practically no facilitation was found in TFrq2 endings for 1 Hz stimulation (Fig. 4*B*). With 5 Hz stimulation, taking 2·79 as the value of m at the end of the series, and 0·26 (the average quantal content at 1 Hz stimulation) as m_0, F was 9·7 by the end of the series of fifty stimuli at 5 Hz (Fig. 4*B*). It appears that the facilitation in TFrq2 endings grows steeply in an apparently linear fashion, reminiscent of the rate of rise of F observed in highly facilitating crayfish motor terminals (Bittner & Sewell, 1976). In contrast, controls stimulated at 5 Hz showed a small degree of facilitation which reached a plateau of $F = 1$ in about 2 s (Fig. 4*C*). With 20 Hz stimulation, facilitation in controls showed a steep initial rate of rise similar to that of transformants at 5 Hz but it reached a plateau of $F = 5$ in approximately 0·8 s. The growth of facilitation during trains of stimuli can be predicted at least qualitatively from data of paired-pulse experiments such as those of Fig. 3 which show, at all time intervals, higher values of F in transgenic larvae relative to controls. However, we failed to fit the present data using one of the classical models of facilitation, such as the 'linear summation' (Mallart & Martin, 1967) or the 'residual calcium' (or power) (Zucker & Lara-Estrella, 1983) models.

Large facilitated responses

Nerve stimulation of TFrq2 motor nerves for 10–15 s at 5 Hz or closely spaced paired stimuli delivered at 1 Hz for a few seconds occasionally gave rise to large bursts of quanta which appear superimposed to 'normal' EJCs in the

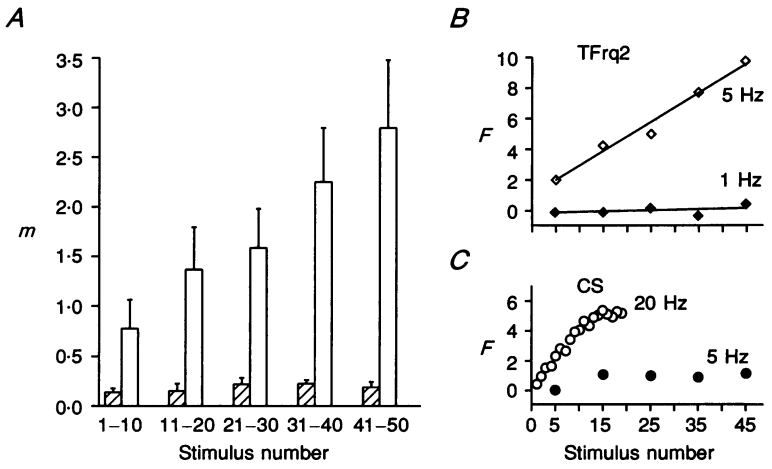


Figure 4. Facilitation of transmitter release by trains of stimuli
A, the quantal content (m) of the EJC is plotted as a function of the stimulus number in a train of 50 stimuli at 5 Hz. Releases elicited by groups of 10 successive stimuli were counted and the releases from corresponding groups from different synaptic boutons were pooled together and averaged. Each column represents the average number of quanta (± S.E.M) released by each group of 10 consecutive stimuli in TFrq2 ($n = 8$, open columns) and in CS ($n = 8$, hatched columns). *B*, the facilitation (F) of transmitter release in TFrq2 during a train of 50 stimuli was calculated for each group of 10 stimuli using eqn (2) taking the values of m shown in the graph in *A* and the average value of m obtained at 1 Hz stimulation as m_0. Symbols represent: ◇, 5 Hz stimulation; ◆, 1 Hz stimulation. *C*, facilitation in CS by trains of stimuli at 5 (●) and at 20 Hz (○). Note the large difference in facilitation at 5 Hz between TFrq2 and CS. $[Ca^{2+}]_o = 0·10$ mM; the temperature was 20 °C.

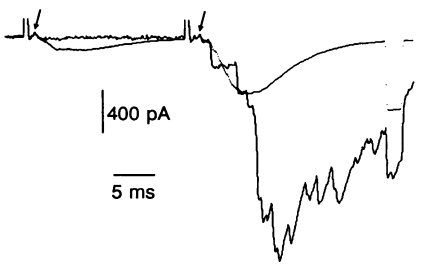


Figure 5. Paired-pulse facilitation and large facilitated responses in a TFrq2 neuromuscular junction
The smooth trace is the average of 90 conditioning–test EJC sequences upon which is superimposed a single trace showing a failure of release to the conditioning stimulus and a typical large facilitated response to the test stimulus. In this experiment a series of 100 pairs of stimuli delivered at 1 Hz gave four large facilitated responses to the test stimulus and were not included in the average. The average quantal contents were 0·4 and 1·4 for the conditioning and the test EJCs respectively but the large facilitated response consisted of at least 16 quanta. The arrows indicate the presynaptic action potential. The rightmost accident in the traces is a calibration pulse.

oscilloscope traces (Fig. 5). These events called 'large facilitated responses' (LFR), have been previously observed in *V7* for the same stimulation parameters as for TFrq2, and in normal neuromuscular junctions but only after 30–45 s nerve stimulation at 10 Hz or higher (Mallart *et al.* 1991).

In a $[Ca^{2+}]_o$ of 0·12 mM the quantal content of the LFRs of TFrq2 neuromuscular junctions varied between 10 and 20 while the average quantal content of the unconditioned EJC was 0·84 (Table 1). After the occurrence of a few bursts of quanta of this kind, transmitter release was depressed for several seconds. The mechanism of generation of the LFR is unclear. It does not seem to be triggered by re-excitation of the presynaptic membrane since we failed to observe any causal relationship between an eventual repetitive firing of nerve terminals and the triggering of LFRs. On the other hand, the presence of Ca^{2+} in the external medium is required, since nerve stimulation at 10 Hz in a Ca^{2+}-free medium containing 16 mM Mg^{2+} and 1 mM EGTA failed to evoke a LFR in TFrq2 endings although it promoted a clear increase in the frequency of MEJCs. The LFR may represent an extreme degree of synaptic facilitation caused by a sudden increase in the availability of Ca^{2+} inside nerve endings, perhaps through a calcium-induced calcium release mechanism.

Presynaptic action potentials and facilitation

The hypothesis that facilitation of transmitter release is due to use-dependent increases in amplitude or duration of presynaptic action potentials has been examined, and rejected, in the neuromuscular junction of the crayfish (Zucker, 1974) and in the giant synapse of the squid (Charlton & Bittner, 1978). More recently, experiments on presynaptic terminals of the neurohypophysis have shown frequency-dependent action potential broadening in relation to enhancement of $[Ca^{2+}]_i$ signals and facilitation of transmitter release (Bourque, 1990; Jackson, Konnerth & Augustine, 1991). Moreover, abnormal action potential repolarization and membrane re-excitation have been shown in the cervical connective of adult *V7* mutants (Tanouye, Ferrús & Fujita, 1981), which might be expressed also in nerve terminals and be the cause of enhanced release of transmitter. Therefore we re-examined the question and looked for activity-dependent changes in presynaptic action potentials which could explain the enhancement of facilitation present in *V7* and TFrq2. Presynaptic membrane currents were recorded in neuromuscular junctions where postsynaptic responses were abolished by bath perfusion of 10 mM sodium L-glutamate to desensitize the receptors or by using low Ca^{2+}:Mg^{+} ratio solutions to suppress transmitter release. According to Katz & Miledi (1965*a*), the current traversing the nerve fibre membrane near a closed end, which is the case for presynaptic terminals, is proportional to the first derivative of the action potential. This current is, therefore, recorded by external electrodes as a diphasic signal. Thus, the decay time of the action potential at the endings can be estimated from the duration of the negative deflection of the diphasic wave form. The conditioned and unconditioned presynaptic action potentials were compared in TFrq2 and controls using twin-pulse protocols of 10–20 ms interval or trains of stimuli at 5–10 Hz which usually give maximal facilitation. As shown in Fig. 6, conditioning and test action potential derivatives superpose exactly to each other, in control as well as in TFrq2. We can thus rule out any lengthening in action potential duration as an explanation of synaptic facilitation in TFrq2 and in controls.

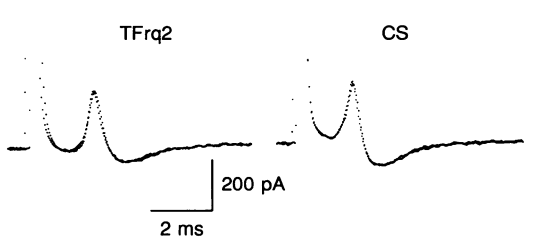


Figure 6. Externally recorded presynaptic action potentials in nerve terminals of transformant and CS larvae subjected to twin-pulse stimulation protocol
In each case the traces of conditioning and test action potentials have been superimposed. Glutamate (10 mM) was present in the bath to desensitize postsynaptic receptors. This dose of glutamate is unlikely to affect the shape of the presynaptic action potential since a similar configuration has been observed by recording and averaging the sweeps with failures of release in low-Ca^{2+}:high-Mg^{2+} solutions (Mallart *et al.* 1991). Each trace is the average of 150 sweeps. $[Ca^{2+}]_o$ = 0·12 mM; the temperature was 20 °C.

Sodium influx and facilitation

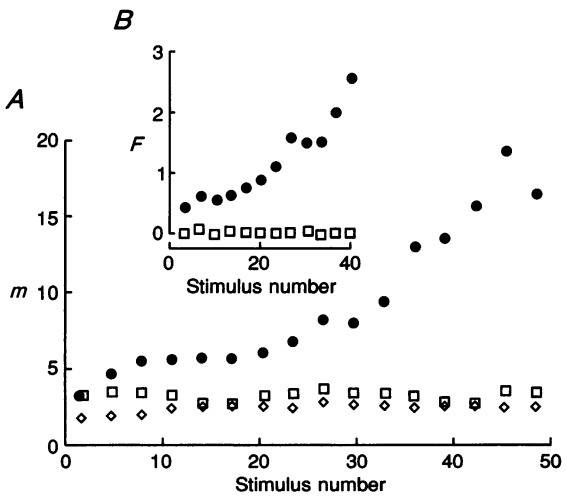
It is now well documented that elevations in $[Na^+]_i$ in nerve endings may promote an increase in both the spontaneous and the stimulus-evoked transmitter release by way of variations in Na^+–Ca^{2+} exchange (Birks & Cohen, 1968; Baker & Crawford, 1975; Atwood, Charlton & Thompson, 1983; Nussinovitch & Rahamimoff, 1988). Interestingly, bath application of the Na^+ ionophore monensin gives rise to large bursts of quanta in crustacean neuromuscular junctions (Atwood *et al.* 1983) which are strikingly similar to the LFRs observed in the present series of experiments or in *V7* neuromuscular junctions (Mallart *et al.* 1991). We therefore investigated whether such a Na^+-dependent mechanism may be the cause of the larger than normal facilitation and of the stimulation frequency-dependent upsurge of LFRs observed in TFrq2 and *V7*.

Facilitation during trains of stimuli at 5 Hz and by twin-pulse stimulation at a 20 ms interval was measured in TFrq2 neuromuscular junctions bathed in standard saline (0·12 mM Ca^{2+}) before and after the addition of tetrodotoxin to prevent Na^+ influx through voltage-sensitive channels. The stimulating suction electrode was positioned at 50–100 μm from the nerve endings to evoke release by electrotonic spread of current. EJC recordings from the same synaptic bouton were used to compare data obtained in control and tetrodotoxin solutions. In the experiment illustrated in Fig. 7*A*, performed in normal saline (circles), a fifty stimuli train at 5 Hz promotes a considerable growth of the quantal content of the EJC and the appearence of LFRs in the last third of the train. In this plot, unlike that of Fig. 4*A*, the quantal releases of the LFRs have been included. The facilitation of the EJC during this train has been calculated using eqn (2), after discarding the traces showing a LFR (Fig. 7*B*). This plot is comparable to that of Fig. 4*B*. Bath application of 2 μM tetrodotoxin abolished the growth of the quantal content of the EJC and the facilitation observed in TFrq2 neuromuscular junctions stimulated at 5 Hz (Fig. 7*A* and *B*, squares). The LFRs which usually occur in TFrq2 neuromuscular junctions after several stimuli at 5 Hz were also abolished by tetrodotoxin. CS nerve endings, which show practically no facilitation of release for this stimulation frequency (Fig. 4*A*), were unaffected by tetrodotoxin (Fig. 7*A*, diamonds).

The action of tetrodotoxin on twin-pulse facilitation was examined in two TFrq2 and four CS larvae. The amount of facilitation at a single synaptic spot was measured first in control solution and then during perfusion of 2 μM tetrodotoxin. Figure 8*A* shows that tetrodotoxin perfusion reduces considerably the amount of facilitation of TFrq2 endings to paired stimuli. In this example, *F* was 2·8 in control solution and 1·0 in the presence of tetrodotoxin. By contrast, the facilitation of transmitter release at control neuromuscular junctions was practically the same in control solution and in the presence of tetrodotoxin (Fig. 8*B*), in agreement with previous observations of Katz & Miledi (1967) in frog neuromuscular junctions.

The effect of external Na^+ on the unusually large facilitation of release in TFrq2 endings was further investigated in five neuromuscular junctions by measuring, at the same spot, facilitation in standard solution and after changing to one with reduced $[Na^+]_o$. With $[Na^+]_o$ of 32 mM, the facilitation of transmitter release in TFrq2 endings was, on average, decreased by 50 % relative to that measured in control solution (Fig. 8*C*). The reduction of external sodium was practically without effect in normal endings, which

Figure 7. Effect of bath-applied tetrodotoxin on the facilitation of transmitter release
A, variation in *m* during the stimulation by trains at 5 Hz: TFrq2 neuromuscular junctions in standard saline solution (●) and in the presence of 2 μM tetrodotoxin (□); the behaviour of CS in tetrodotoxin is indicated by ◇. Data from CS in normal solution have been omitted in this graph for clarity but they are shown in Fig. 4*A*. The quantal content of the EJC was averaged by the 'moving bin' method: each bin consisted of 5 consecutive EJCs in the train and the bin was moved each time by 3 elements. Ten LFRs occurred in this TFrq2 synaptic spot in the control solution starting at the 34th stimulus of the train. The quanta released by the LFRs have been included in the corresponding points. In the tetrodotoxin experiments the stimulating suction electrode was positioned 50–100 μm from the recording site, which was the same in control and tetrodotoxin solutions. Stimulus duration, identical for conditioning and test pulses, was increased from 80 to 200–1000 μs in the presence of tetrodotoxin to evoke release by electrotonic spread of current and was adjusted to obtain similar unfacilitated EJCs in control and in tetrodotoxin solutions. *B*, facilitation (*F*) calculated by applying eqn (2) to the successive values of *m* shown in (*A*) where the average quantal content of the first bin was taken as m_0. Traces showing LFRs have been discarded. $[Ca^{2+}]_o = 0{\cdot}12$ mM; the temperature was 20 °C.



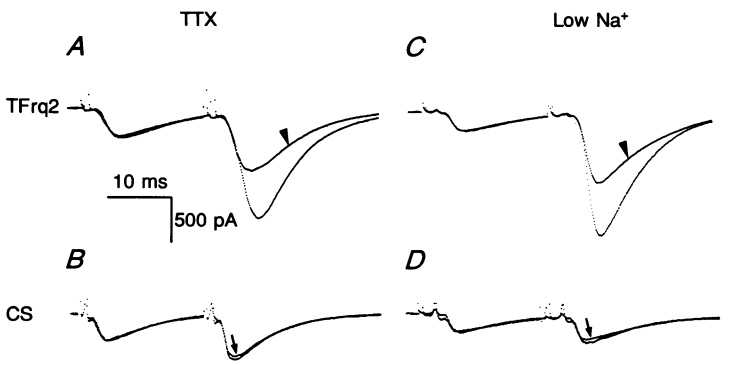


Figure 8. Effect of tetrodotoxin and low $[Na^+]_o$ on paired-pulse facilitation
A, the facilitation of the EJC in standard solution is decreased in TFrq2 endings during perfusion of 2 μM tetrodotoxin (arrowhead). *B*, small effect of tetrodotoxin perfusion on the facilitation in controls (arrow). *C*, the large facilitation of TFrq2 is decreased after changing to a 32 mM Na^+ medium (arrowhead). *D*, the facilitation of the EJC in normal neuromuscular junctions is little affected by low-Na^+ solutions (arrow). Each trace is the average of 150 sweeps. The traces have been scaled to give similar response amplitude to the conditioning stimulus in control and test solutions. The stimulating and recording conditions for the tetrodotoxin experiments were the same as in Fig. 7. $[Ca^{2+}]_o = 0{\cdot}12$ mM; the temperature was 20 °C.

showed about the same degree of facilitation in high- and low-Na^+ solutions (Fig. 8*D*), in agreement with the observations of Jan & Jan (1978). The results obtained with tetrodotoxin or low-Na^+ solutions in TFrq2 nerve endings indicate that the abnormally large facilitation of release elicited there by paired pulses or trains of stimuli is linked in some way to the influx of Na^+ through voltage-sensitive channels. In other words, the TFrq2 phenotype could be suppressed by preventing or reducing the nerve impulse-dependent influx of Na^+.

DISCUSSION

Our results indicate that transgenic *Drosophila* carrying an insertion of Frq cDNA present enhanced facilitation of neurotransmitter release at larval neuromuscular junctions. This enhancement of facilitation is similar to that observed in neuromuscular junctions of *V7* mutants (Mallart *et al.* 1991). This mutation is caused by a chromosomal translocation whose breakpoint on the X chromosome lies close to the 3′-end of the Frq transcription unit (Pongs *et al.* 1993). The *V7* mutation increases the expression of Frq RNA in the nervous system and heat-shocked TFrq2 larvae exhibit 10 times more Frq protein as shown by *in situ* hybridization and Western blots, respectively (Pongs *et al.* 1993). It is, therefore, likely that the *V7* and TFrq2 phenotype is due to overexpression of Frq.

Among the possible mechanisms by which Frq overexpression may affect facilitation of transmitter release, there is the possible action of Frq on the modulation of the outward currents which repolarize the action potential. Thus, Ca^{2+} influx facilitation by use-dependent broadening of the presynaptic spike might lead to synaptic facilitation (Bourque, 1990; Jackson *et al.* 1991). However, this mechanism can be excluded since we failed to find any presynaptic action potential broadening nor membrane re-excitation by twin pulses or trains of stimuli for intervals or frequencies which are expected to produce conspicuous facilitation. Since similar action potential configuration has been observed whether glutamate or a low Ca^{2+}:Mg^+ ratio were used to abolish synaptic transmission (Mallart *et al.* 1991), one can rule out the possibility that the absence of activity-dependent changes in action potential duration shown in Fig. 6 is a side-effect of glutamate.

Possible involvement of impulse-dependent Na^+ accumulation inside nerve terminals in the Frq-dependent enhancement of facilitation of transmitter release is strongly suggested by the tetrodotoxin and low-Na^+ experiments. It has long been recognized that increases in internal Na^+ promote enhanced transmitter release by nerve terminals (Birks & Cohen, 1968; Baker & Crawford, 1975; Atwood *et al.* 1983; Misler & Hurlbut, 1983; Nussinovitch & Rahamimoff, 1988). At least two possible mechanisms may be involved in the sodium effect on transmitter release: (i) reduction of the Ca^{2+} extrusion by the Na^+–Ca^{2+} exchanger leading to calcium retention; and (ii) release of calcium from internal stores.

The Na^+–Ca^{2+} exchanger is one of the major $[Ca^{2+}]_i$-regulating molecules present in all the excitable as well as non-excitable cells. It has been found with high density (Luther, Yip, Bloch, Ambesi, Lindenmayer & Blaustein, 1992) and activity (Tessari & Rahamimoff, 1991) in presynaptic plasma membranes. The Na^+–Ca^{2+} exchanger is primarily involved in the extrusion of Ca^{2+} to preserve calcium homeostasis during nerve activity. It can also work in the reverse direction, depending on the sodium gradient across the membrane, to produce net Ca^{2+} influx, and give rise to post-tetanic potentiation of transmitter release (Mulkey & Zucker, 1992). However, under physiological conditions, with moderate sodium accumulation with nerve impulses, Ca^{2+} retention is more likely to be caused by a reduction of the efflux as a consequence of the reduced sodium gradient, rather than by the reversal of the exchanger.

The release of Ca^{2+} bound to intracellular pools by elevations in internal Na^+ has been proposed to explain the increase in miniature endplate potential frequency or hormone release promoted by Na^+ influx in Ca^{2+}-free medium (Baker & Crawford, 1975; Lowe, Richardson, Taylor & Donatch, 1976; Misler & Hurlbut, 1983). However, the time scale (in the order of minutes) of the reported

$[Ca^{2+}]_o$-independent effects of internal Na^+ accumulation is much longer than the millisecond time scale of the effect on the facilitation of transmitter release observed in the present experiments. This makes it very unlikely that the Ca^{2+} released from internal stores is involved in the Frq-dependent enhancement of facilitation. One might argue that phasic $[Na^+]_i$ elevations may displace Ca^{2+} from calcium-binding proteins, more specifically from frequenin, with a speed compatible with the time scale of the phenomenon observed here. This model, though attractive, cannot be retained since, as described by Pongs *et al.* (1993), the submicromolar Ca^{2+}-dependent regulatory effect of frequenin on guanylyl cyclase *in vitro*, is observed in the presence of 100–150 mM Na^+. We, therefore, favour the hypothesis that the enhancement of synaptic facilitation by frequenin is likely to depend on changes in the activity of systems that regulate the influx and/or the outflux of Ca^{2+} across the presynaptic membrane, such as the Na^+–Ca^{2+} exchanger.

We propose that the increment of facilitation found in TFrq2 (and perhaps also in normals) in excess of the amount believed to depend on residual calcium (Katz & Miledi, 1968), is Na^+–Ca^{2+} exchanger dependent. One might argue whether the exchanger is fast enough to produce noticeable changes in $[Ca^{2+}]_i$ that will affect short-term facilitation. It is not possible to measure the activity of the exchanger in *Drosophila* nerve endings, but data obtained in *Torpedo* electric organ synaptosomes indicate a Ca^{2+} flux of 11·33 $\pm$ 5·93 nmol (mg protein)$^{-1}$ s^{-1} (Tessari & Rahamimoff, 1991). This activity is equivalent to a current density of approximately 0·5 μA μF^{-1}, assuming that 1 mg protein is equal to 2000 cm^2 membrane surface area, the membrane capacitance is 1 μF cm^{-2} and the stoichiometry is 3 Na^+ per 1 Ca^{2+} (Kimura, Miyae & Noma, 1987; Reeves & Philipson, 1989). With this rate of efflux, it will take only a few tens of milliseconds to expel the calcium entering into nerve terminals with each nerve impulse, which makes plausible the explanation of the present observations in terms of a Frq-dependent regulation of the Na^+–Ca^{2+} exchanger.

If the existence of a Na^+-dependent modulation of the neuromuscular facilitation appears clearly demonstrated in the TFrq2 transformants because of the intensity of the effect, we failed to observe any conspicuous action of Na^+ on facilitation in controls. One cannot exclude, however, the possibility of some Na^+–Ca^{2+} exchanger-mediated frequenin action in the regulation of the normal synaptic facilitation. Thus, the considerable differences in facilitation described so far among synapses (Bittner & Sewell, 1976) might depend on the intensity of this regulatory mechanism.

We can only speculate about the mechanism by which the misexpression of Frq leads possibly to modulation of the Na^+–Ca^{2+} exchanger. Frq is a protein which belongs to a newly recognized family of Ca^{2+}-binding proteins which includes visinin and recoverin (Yamagata *et al.* 1990; Dizhoor *et al.* 1991; Lambrecht & Koch, 1991; Pongs *et al.* 1993). Frequenin shares with these proteins the property of being regulators of the photoreceptor guanylyl cyclase and, thus, it modulates the synthesis of cyclic GMP (Pongs *et al.* 1993) which, in turn may be involved in the modulation of several cellular functions. Indeed, it has been shown in vascular smooth muscle cells that cyclic GMP stimulates the Na^+–Ca^{2+} exchanger (Furukawa, Oshima, Tawada-Iwata & Sigekawa, 1991), which supports the view of a Frq- and Na^+–Ca^{2+} exchange-dependent modulation of synaptic facilitation. Possibly, the fine-tuned regulation of the exchanger is altered in the *V7* mutation and in TFrq2 transformants, perhaps because of the overexpression of the Frq gene product.

In this context, it is worth recalling that a phenotype similar to *V7* and TFrq2 has been described in the bss^{MW1} mutation (Jan & Jan, 1978; Ganetzky & Wu, 1982). As in *V7* and TFrq2, the abnormally large facilitation of transmitter release found in bss^{MW1} seems to be dependent on Na^+ accumulation in the nerve terminals (Jan & Jan, 1978). Apparently, the bss^{MW1} phenotype is due to the action of an unidentified mutant gene product (Ganetzky & Wu, 1982). It would be of interest to see whether a similarity exists between the proteins affected by these two mutations.

REFERENCES

Anderson, S. R., Cull-Candy, S. G. & Miledi, R. (1978). Glutamate current noise: post-synaptic channel kinetics investigated under voltage clamp. *Journal of Physiology* **282**, 219–242.

Atwood, H. L., Charlton, M. P. & Thompson, C. S. (1983). Neuromuscular transmission in crustaceans is enhanced by a sodium ionophore, monensin, and by prolonged stimulation. *Journal of Physiology* **335**, 179–195.

Baker, P. F. & Crawford, A. C. (1975). A note on the mechanism by which inhibitors of the sodium pump accelerate spontaneous release of transmitter. *Journal of Physiology* **247**, 209–226.

Balnave, R. J. & Gage, P. W. (1974). On facilitation of transmitter release at the toad neuromuscular junction. *Journal of Physiology* **239**, 657–675.

Bekkers, J. M., Richerson, G. B. & Stevens, C. F. (1990). Origin of variability in quantal size in cultured hippocampal neurons and hippocampal slices. *Proceedings of the National Academy of Sciences of the USA* **87**, 5359–5362.

Birks, R. I. & Cohen, M. W. (1968). The influence of internal sodium on the behaviour of motor nerve endings. *Proceedings of the Royal Society* B **170**, 401–421.

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

Bittner, G. D. & Sewell, V. L. (1976). Facilitation at crayfish neuromuscular junctions. *Journal of General Physiology* **109**, 287–308.

Bourque, C. W. (1990). Intraterminal recordings from the rat neurohypophysis *in vitro*. *Journal of Physiology* **421**, 247–262.

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

Cull-Candy, S. G., Miledi, R. & Parker, I. (1980). Single glutamate-activated channels recorded from locust muscle fibres with perfused patch-clamp electrodes. *Journal of Physiology* **321**, 195–210.

del Castillo, J. & Katz, B. (1954). Statistical factors involved in neuromuscular facilitation and depression. *Journal of Physiology* **124**, 574–585.

Dizhoor, A. M., Ray, S., Kumar, S., Niemi, G., Spencer, M., Brolley, D., Walsh, K. A., Philipov, P. P., Hurley, J. B. & Stryer, L. (1991). Recoverin: a calcium sensitive activator of retinal rod guanylate cyclase. *Science* **251**, 915–918.

DODGE, F. A. & RAHAMIMOFF, R. (1967). Co-operative action of calcium ions in transmitter release at the neuromuscular junction. *Journal of Physiology* **193**, 419–432.

DUDEL, J. (1981). The effect of reduced calcium on quantal unit current and release at the crayfish neuromuscular junction. *Pflügers Archiv* **391**, 35–40.

EDWARDS, F. A., KONNERTH, A. & SAKMANN, B. (1990). Quantal analysis of inhibitory synaptic transmission in the dentate gyrus of rat hippocampal slices: a patch-clamp study. *Journal of Physiology* **430**, 213–249.

FURUKAWA, K.-Y., OHSHIMA, N., TAWADA-IWATA, Y. & SHIGEKAWA, M. (1991). Cyclic GMP stimulates Na^+/Ca^{2+} exchange in vascular smooth muscle cells in primary culture. *Journal of Biological Chemistry* **266**, 12337–12341.

GANETZKY, B. & WU, C. F. (1982). Indirect suppression involving behavioral mutants with altered nerve excitability in *Drosophila* melanogaster. *Genetics* **100**, 597–614.

GRINNELL, A. D. & HERRERA, A. A. (1980). Physiological regulation of synaptic effectiveness at frog neuromuscular junctions. *Journal of Physiology* **307**, 301–317.

JACKSON, M. B., KONNERTH, A. & AUGUSTINE, G. J. (1991). Action potential broadening and frequency-dependent facilitation of calcium signals in pituitary nerve terminals. *Proceedings of the National Academy of Sciences of the USA* **88**, 380–384.

JAN, L. Y. & JAN, Y. N. (1976). Properties of the larval neuromuscular junction in *Drosophila* melanogaster. *Journal of Physiology* **262**, 189–214.

JAN, Y. N. & JAN, L. Y. (1978). Genetic dissection of short-term and long-term facilitation at the *Drosophila* neuromuscular junction. *Proceedings of the National Academy of Sciences of the USA* **75**, 515–519.

JOHNSON, E. W. & WERNIG, A. (1971). The binomial nature of transmitter release at the crayfish neuromuscular junction. *Journal of Physiology* **218**, 757–767.

KATZ, B. & MILEDI, R. (1965*a*). Propagation of electric activity in motor nerve terminals. *Proceedings of the Royal Society* B **161**, 453–482.

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

KATZ, B. & MILEDI, R. (1967). Tetrodotoxin and neuromuscular transmission. *Proceedings of the Royal Society* B **167**, 8–22.

KATZ, B. & MILEDI, R. (1968). The role of calcium in neuromuscular facilitation. *Journal of Physiology* **195**, 481–492.

KIMURA, J., MIYAMAE, S. & NOMA, A. (1987). Identification of sodium–calcium exchange current in single ventricular cells of guinea-pig. *Journal of Physiology* **384**, 199–222.

LAMBRECHT, H. G. & KOCH, K. W. (1991). A 26 kd calcium binding protein from bovine rod outer segments as modulator of photoreceptor guanylate cyclase. *EMBO Journal* **10**, 793–798.

LOWE, D. A., RICHARDSON, B. P., TAYLOR, P. & DONATCH, P. (1976). Increasing intracellular sodium triggers calcium release from bound pools. *Nature* **260**, 337–338.

LUTHER, P. W., YIP, R. K., BLOCH, R. J., AMBESI, A., LINDENMAYER, G. E. & BLAUSTEIN, M. P. (1992). Presynaptic localization of sodium/calcium exchangers in neuromuscular preparations. *Journal of Neuroscience* **12**, 4898–4904.

MCLARNON, J. G. & QUASTEL, D. M. J. (1987). A voltage clamp study of the glutamate responsive neuromuscular junction in *Drosophila melanogaster*. *Canadian Journal of Physiology and Pharmacology* **66**, 321–327.

MALLART, A., ANGAUT-PETIT, D., BOURRET-POULAIN, C. & FERRÚS, A. (1991). Nerve terminal excitability and neuromuscular transmission in *T(X;Y)V7* and *Shaker* mutants of *Drosophila melanogaster*. *Journal of Neurogenetics* **7**, 75–84.

MALLART, A. & MARTIN, A. R. (1967). An analysis of facilitation of transmitter release at the neuromuscular junction of the frog. *Journal of Physiology* **193**, 679–694.

MISLER, S. & 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 Sciences of the USA* **80**, 315–319.

MULKEY, R. M. & ZUCKER, R. S. (1992). Posttetanic potentiation at the crayfish neuromuscular junction is dependent on both intracellular calcium and sodium ion accumulation. *Journal of Neuroscience* **12**, 4327–4336.

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

PONGS, O., LINDEMEIER, J., ZHU, X. R., THEIL, T., ENGELKAMP, D., KRAH-JENTGENS, I., LAMBRECHT, H.-G., KOCH, K. W., SCHWEMER, J., RIVOSECCHI, R., MALLART, A., GALCERAN, J., CANAL, I., BARBAS, J.A. & FERRÚS, A. (1993). Frequenin – a novel calcium binding protein that modulates synaptic efficacy in the *Drosophila* nervous system. *Neuron* **11**, 15–28.

REEVES, J. P. & PHILIPSON, K. D. (1989). Sodium–calcium exchange activity in plasma membrane vesicles. In *Sodium–Calcium Exchange*, ed. ALLEN, T. J. A., NOBLE, D. & REUTER, H., pp. 27–53. Oxford University Press, Oxford, New York, Tokyo.

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

TANOUYE, M. A., FERRUS, A. & FUJITA, S. C. (1981). Abnormal action potentials associated with the *Shaker* complex locus of *Drosophila*. *Proceedings of the National Academy of Sciences of the USA* **78**, 6548–6552.

TESSARI, M. & RAHAMIMOFF, H. (1991). Na^+–Ca^{2+} exchange activity in synaptic plasma membranes derived from the electric organ of *Torpedo ocellata*. *Biochimica et Biophysica Acta* **1066**, 208–218.

WOJTOWICZ, J. M. & ATWOOD, H. L. (1986). Long-term facilitation alters transmitter releasing properties at the crayfish neuromuscular junction. *Journal of Neurophysiology* **55**, 484–498.

YAMAGATA, K., GOTO, K., KUO, C.-H., KONDO, H. & MIKI, N. (1990). Visinin: a novel calcium binding protein expressed in retinal cone cells. *Neuron* **2**, 469–476.

ZHONG, Y. & WU, C. F. (1991). Altered synaptic plasticity in *Drosophila* memory mutants with a defective cyclic AMP cascade. *Science* **251**, 198–201.

ZUCKER, R. S. (1974). Characteristics of crayfish neuromuscular facilitation and their calcium dependence. *Journal of Physiology* **241**, 91–110.

ZUCKER, R. S. & LARA-ESTRELLA, L. O. (1983). Post-tetanic decay of evoked and spontaneous transmitter release and a residual calcium model of synaptic facilitation at crayfish neuromuscular junctions. *Journal of General Physiology* **81**, 355–372.

Acknowledgements

We thank Dr M. Gho for helpful comments on the manuscript. This research was supported, in part, by grants no. 88-096 and 91-090 from the Direction d'Etudes, Recherches et Techniques, D.G.A. We are grateful to the Association Française contre les Myopathies and to the European Science Foundation for support of this work. R.R. was recipient of an A.F.M. fellowship.

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Received 22 April 1993; accepted 5 July 1993.