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AN ELECTROPHYSIOLOGICAL ANALYSIS OF THE EFFECT OF Ca IONS ON NEUROMUSCULAR TRANSMISSION IN THE MOUSE VAS DEFERENS

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1 A study has been made of the effects of changing the external calcium concentration $[Ca]_o$ and the external magnesium concentration $[Mg]_o$ on the synaptic potential due to noradrenaline release.

2 When $[Ca]_o$ was varied in the range 0.7 to 1.8 mM, the synaptic potential increased as about the second power of $[Ca]_o$.

3 Increasing $[Mg]_o$ depressed the synaptic potential; however, variation of $[Ca]_o$ in the presence of high $[Mg]_o$ did not significantly change the power relationship between the synaptic potential and $[Ca]_o$.

4 The facilitated increase in the synaptic potential during short trains of impulses at different frequencies was quantitatively predicted on the assumption that each impulse leaves residual Ca ions bound to release receptors in the nerve terminal.

Introduction

The dependence of the evoked release of acetylcholine at the cholinergic neuromuscular junction on calcium ions has been determined from an analysis of the effects of different external calcium concentrations $[Ca]_o$ on the amplitude of the endplate potential (Jenkinson, 1957). Evoked acetylcholine release increases as the fourth power of $[Ca]_o$ and this action is depressed by magnesium in such a way as to suggest that the release of transmitter is mediated by the formation of a Ca-receptor complex (CaX) (Dodge & Rahamimoff, 1967; Bennett, Florin & Hall, 1975). A natural consequence of this idea is that facilitation at this junction is due to a conditioning impulse leaving behind residual CaX at the nerve terminal, so that the amount of CaX present following a subsequent test impulse is increased, leading to an enhanced transmitter release (Katz & Miledi, 1968; Younkin, 1974).

The dependence of the evoked release of noradrenaline (NA) at the adrenergic neuromuscular junction on calcium ions has been studied by determining the NA overflow from either the spleen or vas deferens during short trains of nerve impulses in different $[Ca]_o$ (Kirpekar & Misu, 1967; Stjärne, 1973); evoked NA release was shown to be dependent on about the second power of $[Ca]_o$ in the range from 0.8 mM to 2.5 mM. In the present work we have examined the dependence of NA release on $[Ca]_o$ and $[Mg]_o$ by using the amplitude of the excitatory junction potential (e.j.p.) evoked in smooth muscle cells as a measure of NA release (Bennett, 1973a; Bennett & Middleton, 1975a); the dependence of the e.j.p. on $[Ca]_o$ and $[Mg]_o$ suggests that NA release is also mediated by a Ca-receptor complex (CaX) and that facilitation at the adrenergic neuromuscular junction is due to the accumulation of residual CaX following successive impulses.

Methods

The mouse isolated vas deferens was used in all experiments. The animals were killed with a cervical fracture, and both vasa deferentia dissected free and pinned out immediately in a Perspex organ bath of about 10 ml capacity. This was perfused with a modified Krebs solution of the following ionic composition (mM): Na 151, K 4.7, Ca 1.8, Mg 1.2, Cl 142, H_2PO_4 1.3, SO_4 1.2, HCO_3 16.3, glucose 7.8, and gassed continuously with 95% O_2 and 5% CO_2. Solutions were maintained at 34° to 36°C and flowed continuously through the bath at about 10 ml/minute. The intramural sympathetic nerves were stimulated with two platinum ring electrodes placed around the vas deferens and about 1 mm apart. Intracellular potentials were recorded from

the smooth muscle cells with glass microelectrodes filled with 2 M-KCl and having resistances of 30 to 70 MΩ. The signals were led through a high impedance unity gain amplifier, displayed on an oscilloscope and photographed on moving film.

Changes in the external calcium concentration $[Ca]_0$ or external magnesium concentration $[Mg]_0$ were made by changing the level of $CaCl_2$ or $MgCl_2$ present in the reservoir of Krebs supplying the organ bath; no compensation was made for tonicity changes by altering the NaCl. The combinations of $[Ca]_0$ and $[Mg]_0$ and the number of nerves stimulated were chosen such that the e.j.p. was in general less than 10 mV, even during trains of high frequency stimulation, in order to avoid serious errors due to non-linear summation (Martin, 1955; Williams & Bowen, 1974). The derivation of the mass action equation describing the competitive actions of Ca and Mg at some point or points in the release process, together with the general method for determining the dissociation constants in the equation, are given by Jenkinson (1957) and by Dodge & Rahamimoff (1967). The derivation of the method of analysis for the residual Ca*X* following conditioning impulses is given by Linder (1973), Younkin (1974) and Zucker (1974).

At constant stimulus parameters, the amplitude of the e.j.p. at the stimulating electrode does not vary between smooth muscle cells as the synaptic potential in each cell occurs as a result of transmitter acting on cells throughout the smooth muscle electrical syncytium (Bennett, 1972); furthermore there is little difference between preparations in the amplitude of the e.j.p. recorded in cells at the stimulating electrode if the same stimulus parameters are used (Bennett & Middleton, 1975a). In the present experiments the number of nerves stimulated was kept approximately constant by stimulating with single impulses which were always of 0.5 ms duration and of 60 V strength, thus allowing the results from different cells to be pooled for any given set of ionic conditions. As the slowest component of facilitation declines exponentially with a time constant of 6 s (Bennett, 1973a), a minimum interval of 20 s was left between successive impulses when examining the effects of different $[Ca]_0$ on NA release.

Results

The effect of changes in $[Ca]_0$ on the release of noradrenaline by single impulses

The $[Ca]_0$ was changed in the range from 0.7 mM to 1.8 mM, and the amplitude of the e.j.p. due to the release of NA determined at each $[Ca]_0$. The amplitude of the e.j.p. increased with about the second power (2.3 ± 2.2 (3)) of $[Ca]_0$ over this concentration range in the presence of a constant $[Mg]_0$ (Figure 1). However, no changes in the amplitude of the m.e.j.ps were observed.

As the $[Ca]_0$ is increased from 0.7 mM to 1.8 mM there is a 10 mV hyperpolarization of the vas deferens smooth muscle cells (Kuriyama, 1964; Bennett, 1967) which will itself contribute a small increase to the e.j.p. amplitude. No correction has been made for the effect of this hyperpolarization.

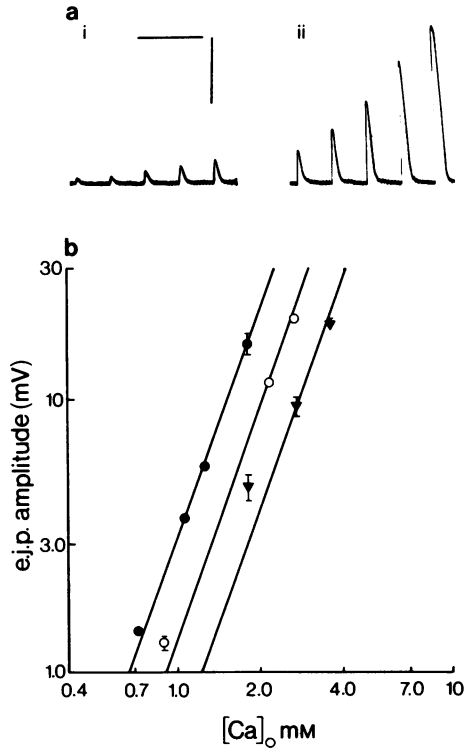

Figure 1 Dependence of the excitatory junction potential (e.j.p.) amplitude on external calcium concentrations $[Ca]_0$. (a) The effect of changing $[Ca]_0$ on the amplitude of the e.j.p. during short trains of impulses at low frequency (3 Hz); in (i) $[Ca]_0 = 0.7$ mM and in (ii) $[Ca]_0 = 1.8$ mM with $[Mg]_0 = 1.2$ mM constant; vertical calibration 20 mV and horizontal 0.6 s. (b) The effect of changing $[Ca]_0$ at three different $[Mg]_0$ is shown on log/log coordinates: (●), $[Mg]_0 = 1.2$ mM; (○), $[Mg]_0 = 6.2$ mM; (▲), $[Mg]_0 = 11.3$ mM; each point was determined from at least five e.j.ps in five cells. Vertical lines indicate ± s.e. mean for each point, where it is larger than the diameter of the point; the lines are drawn by eye and have a slope of 2.3. Stimulus parameters constant at 0.5 ms and 60 V.

Increasing the $[Ca]_o$ in the presence of a fixed high concentration of $[Mg]_o$ produced an increase in the amplitude of the e.j.p. which again followed approximately a second power (Figure 1), although the curve was shifted to the right of that at the lower $[Mg]_o$. This result is to be expected if Mg competitively blocks the action of Ca in increasing NA release at some point in the release process, as it does the release of acetylcholine.

Dissociation constants governing noradrenaline release

If Mg competitively inhibits the action of Ca in increasing the amount of NA released by nerve impulses, then it is possible to derive the dissociation constants (K_1 and K_2) in the expression describing this competitive inhibition (see Dodge & Rahamimoff, 1967; Bennett *et al.*, 1975):

$$\text{e.j.p.} = L\left(\frac{[Ca]_o}{1+\frac{[Ca]_o}{K_1}+\frac{[Mg]_o}{K_2}}\right)^{2.3} \quad (1)$$

where L is a constant. The constant K_2 was determined from the data in Figure 1 by considering two sets of different $[Ca]_o/[Mg]_o$ solutions for which the e.j.p. amplitude was maintained constant. $K_2 = 10.75 \pm 2.9$ mM (3).

Rearrangement of equation 1 to the form

$$(\text{e.j.p.})^{\frac{1}{2.3}} = \frac{L^{\frac{1}{2.3}}[Ca]_o}{\left\{1+\frac{[Ca]_o}{K_1}+\frac{[Mg]_o}{K_2}\right\}} \quad (2)$$

allows a double-reciprocal plot of $(\text{e.j.p.})^{-1/2.3}$ against $[Ca]_o^{-1}$ to give a straight line (Figure 2). Each of these straight lines, for the three different $[Mg]_o$ given in Figure 1, should according to equation 2 intercept the ordinate scale of the double-reciprocal plot at the same point, which they do (Figure 2). Furthermore each line intercepts the abscissa scale at

$$\frac{1}{[Ca]_o}$$

where

$$K_1 = \frac{[Ca]_o}{1+\frac{[Mg]_o}{K_2}},$$

allowing three independent estimates of $K_1 = 8.61 \pm 0.31$ (3) mM. These values of K_1 and K_2 for the release of NA are greater than the equivalent values for the release of acetylcholine at the neuromuscular junction (Dodge & Rahamimoff, 1967; Bennett *et al.*, 1975), indicating that the former is less sensitive to changes in $[Ca]_o$ and $[Mg]_o$ than is the latter.

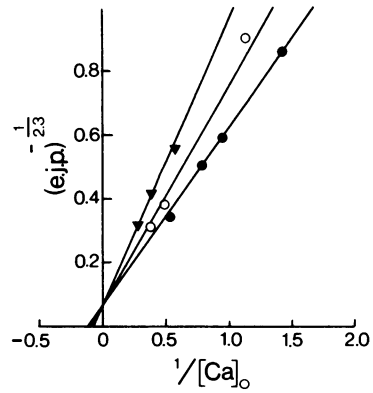

Figure 2 Double reciprocal plot for the relationship between excitatory junction potential (e.j.p.) and $1/[Ca]_o$. Linear coordinates. (●), $[Mg]_o = 1.2$ mM; (○), $[Mg]_o = 6.2$ mM; (▲), $[Mg]_o = 11.3$ mM. The data shown in Figure 1 have been replotted on these coordinates and lines of best fit drawn by eye.

The effect of changes in $[Mg]_o$ on the release of noradrenaline by single impulses.

In order to examine the suggestion further that $[Mg]_o$ competitively inhibits the action of Ca in releasing NA, $[Mg]_o$ was varied over a wide range while maintaining $[Ca]_o$ constant and the changes in e.j.p. amplitude compared with the predictions of equation (1). Increasing the $[Mg]_o$ decreased the e.j.p. amplitude over the entire range from 1.1 to 9.8 mM (Figure 3a); when the e.j.p. amplitude was plotted against.

$$\left(1+\frac{[Ca]_o}{8.6}+\frac{[Mg]_o}{10.8}\right)$$

on double logarithmic coordinates, the experimental values fell on a line of slope 2.3 as anticipated according to equation (1).

The residual CaX remaining after a single impulse

The present study has shown that the amplitude of the e.j.p. is dependent on about the second power of the external Ca concentration; furthermore, the action of Ca in releasing NA is antagonized by Mg in much the same way as is the release of acetylcholine at the endplate. The above observations suggest that facilitated release should be proportional to about the second power of

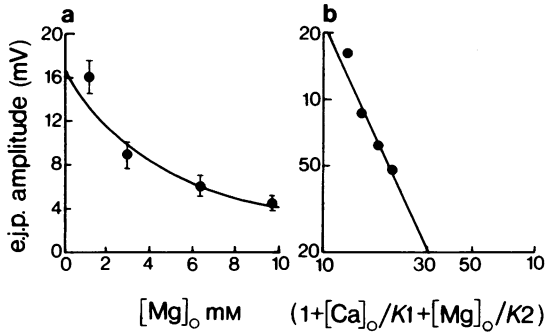

Figure 3 Dependence of excitatory junction potential (e.j.p.) amplitude on external magnesium concentration $[Mg]_o$. (a) The effect of changes in $[Mg]_o$ at a constant $[Ca]_o$ of 1.8 mM on the amplitude of the e.j.p. are shown on linear coordinates; each point was determined from at least seven e.j.ps in six cells and vertical lines indicate ± s.e. mean for each point; the line is drawn according to equation (1), with the appropriate values inserted. (b) Comparison between the predicted and observed results for the dependence of the e.j.p. amplitude on $[Mg]_o$; the data given in (a) have been replotted in (b) on log/log coordinates of e.j.p. amplitude against $(1 + [Ca]_o/K_1 + [Mg]_o/K_2)$; the line has been drawn by eye and has a slope of $2.3.K_1 = 8.6$ mM, $K_2 = 10.8$ mM.

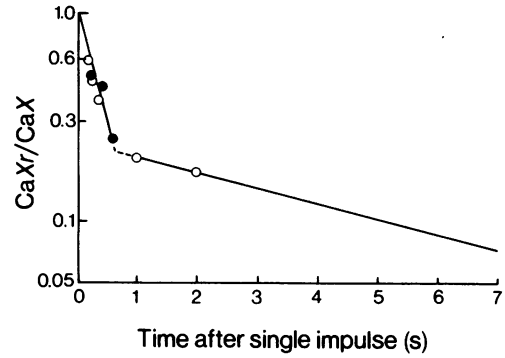

Figure 4 The decay of Ca*Xr* following a single impulse. (○) Value of Ca*Xr* determined from the decay of facilitation measured by a single test impulse at different times after a conditioning impulse; (●) value of Ca*Xr* determined from an anlysis (see text) of the facilitation which occurs during a train of four impulses at 5 Hz (see Figure 5). Straight line between 1.0 and 7.0 s, the value of Ca*Xr* determined from an analysis (see text) of the facilitation remaining after a train of four impulses at 5 Hz (see Figure 5). The decay of Ca*Xr* during the period indicated by the broken line was not determined. The s.e. mean for each (○) is less than 7% of the mean (number of observations ⩾ 17); the s.e. mean for (●) is the same as that in Figure 5.

Ca*X*. If this is so then the residual Ca*X* (Ca*Xr*) can be determined for various times after a single impulse, and used to predict the facilitated release of NA which arises as a consequence of this Ca*Xr* during trains of impulses. The residual Ca*X* at the time of a test impulse (Ca*Xr*) when expressed as a fraction of the Ca*X* formed at the time of a conditioning impulse (Ca*X*) (Linder, 1973; Younkin, 1974; Zucker, 1974) is in the present case

$$\frac{CaXr}{CaX} = (fac.)^{\frac{1}{2}} - 1 \qquad (3)$$

where *fac.* is the ratio of the amplitudes of the test e.j.p. to the conditioning e.j.p. Figure 4 shows that at adrenergic synapses the Ca*Xr* remaining at times greater than 100 ms after an impulse disappears along two exponentials, the first with a time constant of 0.4 s and the second with a time constant of 6 s (Bennett, 1973a); this may be contrasted with the Ca*X* remaining at times greater than 100 ms after an impulse at the endplate, which also decays along two exponentials (Barrett & Stevens, 1972; Younkin, 1974), one with a time constant of about 0.3 s (Mallart & Martin, 1967; Magleby, 1973; Younkin, 1974) and a much smaller component with a time constant of several seconds (Magleby, 1973).

By analysing the growth of the facilitated release of NA which occurs during a high frequency train of impulses, and the decay of this facilitation at the end of the train (Figure 5), it is possible to estimate the Ca*Xr* remaining after different intervals following a single impulse on the basis that each impulse in the train develops the same Ca*X* and that this decays with the same time constants as for an isolated impulse (Linder, 1973; Younkin, 1974). The residual Ca*Xr* remaining from the first impulse at the time of the nth impulse during the train is

$$(CaXr)_n - (CaXr)_{n-1}$$

where $(CaXr)_n$ equals the residual present at the time of the nth impulse and $(CaXr)_{n-1}$ equals the residual present at the time of the $(n-1)$th impulse. Application of this analysis to the facilitation which occurs during four impulses at 5 Hz (Figure 5) gave values for the time course of decay of Ca*Xr* during the first 0.6 s following a single impulse similar to those previously obtained using only single conditioning-test impulses (Figure 4).

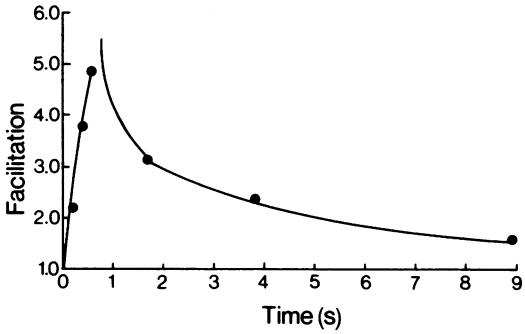

Figure 5 The facilitation of the excitatory junction potential (e.j.p.) during four impulses at 5 Hz. The decline of facilitation following the end of the train is shown for three times. The lines give the theoretical predictions for both the growth of facilitation during the train and the decline of facilitation at the end of the train, according to the theory outlined in the text. The s.e. mean is less than 10% of the mean for each point (number of observations $\geqslant 10$). $[Mg]_0 = 6.3$ mM, $[Ca]_0 = 1.8$ mM.

The decay of facilitation following the train of impulses in Figure 5 was determined at three different intervals, and these results used to determine the decay of Ca*Xr* after the train. As shown in Figure 6, Ca*Xr* decayed during the period from 1 to 9 s following the train along a single exponential of time constant 6 seconds. The initial Ca*Xr* component introduced by each of the four impulses which decays with a time constant of 0.4 s has completely decayed by 1 s after the end of the train, and only the cumulative effects of the second long-time course Ca*Xr* component introduced by each of the four impulses remains. It is therefore possible to use the decay of Ca*Xr* following the train of impulses (Figure 6) to determine the magnitude and time course of the slow component of Ca*Xr* introduced by each impulse. Thus for times greater than about 1 s after the train in Figure 5 (Linder, 1973; Younkin, 1974),

$$\mathrm{Ca}Xr = ce^{-(t-1.0)/\tau}(1 + e^{-\Delta t/\tau} + e^{-2\Delta t/\tau} + e^{-3\Delta t/\tau}) \qquad (4)$$

where c is the amount of Ca*Xr* remaining 1 s after a single impulse,
τ is the time constant of exponential decay,
t is the time after the end of the train,
Δt is the interval between impulses during the train.

For the train in Figure 5 and from Figure 6 $\Delta t = 0.2$ s, $\tau = 0.6$ s, and $c = 0.20$ so that

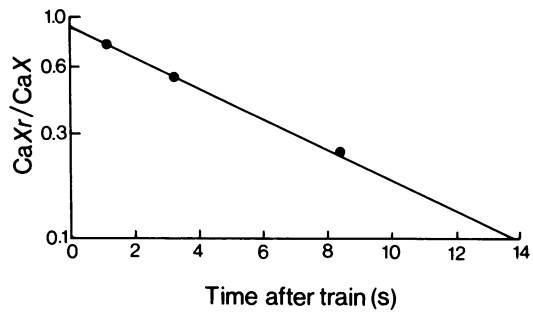

Figure 6 The decay of Ca*Xr* following a train of four impulses at 5 Hz. The straight line has a time constant of 6.0 seconds. The decay has been determined from an analysis of the data in Figure 5 (see text).

$\mathrm{Ca}X = 0.20e^{-(t-1.0)/0.6}$ describes the decay of Ca*X* for periods greater than 1 s after a single impulse, as is shown in Figure 4. The slow component of decay of Ca*Xr* determined by this analysis of the decay of facilitation following a train was the same as the decay of Ca*Xr* at these times when determined by single conditioning test impulses (Figure 4).

The residual CaX remaining during and after trains of impulses

In order to test the residual Ca*X* theory for the facilitated release of NA at synapses further, the growth of facilitation during and following trains at different frequencies was predicted, using the amount of Ca*Xr* remaining at different times after a single impulse (Figure 4), and these predictions compared with experimental observations. The rise in facilitation during stimulation at frequencies between 0.5 and 7.7 Hz with three to six impulses was very well predicted (Figure 7) using the results of Figure 4, as was the decay of facilitation following these trains of impulses (Figure 7).

Discussion

The role of Ca ions in the release of noradrenaline by single nerve impulses

The e.j.p. in the mouse vas deferens is likely to be due to the release of NA as pretreatment with 6-hydroxydopamine abolishes the synaptic potential (Furness, Campbell, Gillard, Malmfors, Cobb & Burnstock, 1970) and it is reduced by over 80% with phenoxybenzamine (10 μg/ml) and bretylium

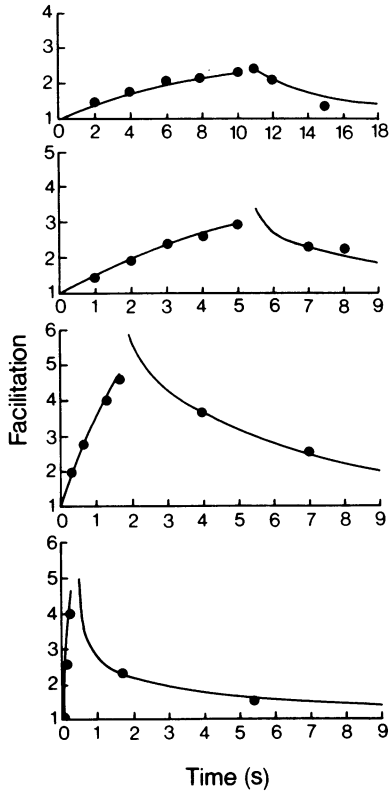

Figure 7 The growth of facilitation during trains at different frequencies, containing different numbers of impulses, together with the subsequent decay of this facilitation. From the top down the trains consist of 6,6,6, and 3 impulses at 0.5, 1.0, 3.0, and 7.7 Hz respectively. In each case these trains are followed by a test impulse triggered at various intervals to measure the rate of decay of facilitation following the train. The lines give the theoretical predictions for both the growth of facilitation during the the trains as well as the decline of facilitation following the end of the trains, according to the theory outlined in the text and the data in Figure 4. The s.e. mean is less than 10% of the mean for each point (number of observations = 5). $[Mg]_0$ = 6.3 mM, $[Ca]_0$ = 1.8 mM.

(10 μg/ml) but is unaffected by atropine (10 μg/ml) or hyoscine (10 μg/ml) (Bennett & Middleton, 1975a). It is likely that the amplitude of the e.j.p. is a measure of the amount of NA released per impulse as there is good correlation between the time course of changes in NA output from sympathetic nerves during long trains of impulses (Dearnaley & Geffen, 1966; Kopin, Breese, Kraus & Weise, 1968; Stjärne & Wennmalm, 1970) and changes in the e.j.p. amplitude (Bennett, 1973a); also, as shown in the present work, the e.j.p. amplitude is dependent on about the second power of $[Ca]_0$ and this dependence is the same as that determined when NA overflow is used as a measure of transmitter release (Kirpekar & Misu, 1967; Stjärne, 1973).

If the internal calcium concentration at a synapse $[Ca]_I$ is raised then transmitter is released (Miledi, 1973), and as the $[Ca]_I$ following an impulse is momentarily raised by an amount linearly related to $[Ca]_0$ (Hodgkin & Keynes, 1957; Baker, Hodgkin & Ridgway, 1971), the second power relationship between $[Ca]_0$ and NA release is likely to be due to the formation of a Ca-receptor complex (CaX) within the nerve terminal and not on the external surface of the terminal membrane. At the cholinergic neuromuscular junction transmitter release is governed by binominal statistics (Bennett & Florin, 1974; Bennett *et al.*, 1975), in which the quantal release parameter n is dependent on the third power of $[Ca]_0$ whilst the probability parameter p is dependent on the first power of $[Ca]_0$ suggesting that n is proportional to the third power of a calcium-receptor complex but that p is only dependent on the first power of a calcium-receptor complex. As the structural basis of the binomial statistical parameters is not known, it is not clear if the same calcium-receptor complex is likely to control both n and p, or whether this calcium-receptor complex resides in the terminal membrane or wholly within the nerve terminal (Cooke, Okamoto & Quastrel, 1973). It is not possible to test if NA release at the adrenergic neuromuscular junction obeys binomial statistics because of the syncytial couplings between the smooth muscle cells (Bennett, 1972), so that it is not known if the second power relationship between NA release and $[Ca]_0$ arises as a consequence of the sensitivity of the parameters n and p on the formation of Ca-receptor complex nor whether this complex resides in the terminal membrane or inside the terminal or both.

The antagonism by Mg of the action of Ca in increasing NA release by an impulse observed in the present study, and in a previous study by Kirpekar & Misu (1967) using NA overflow as a measure of release, does not help to determine the site at which Ca-receptor complexes are formed in the nerve terminal. Mg antagonizes the entry of Ca into axons which accompanies the nerve impulse (Baker *et al.*, 1971) as well as itself entering the axon during the impulse (Baker & Crawford, 1972). Thus the effects of Mg observed in the present study could be due to this ion blocking Ca movement through the membrane or entering the terminal or both.

The role of Ca ions in the release of noradrenaline by trains of nerve impulses

The facilitation of NA release by successive impulses during and following a short train can be successfully predicted on the basis that each impulse leaves residual Ca*X* in the nerve terminal in an amount determined from a study of the potentiating effects of a conditioning impulse on a test-impulse, and that transmitter release is proportional to the second power of Ca*X*. At low frequencies (< 1 Hz) there is less than 10% difference between the predictions of this theory based on a second power relationship and a linear relationship (Bennett, 1973a), so that it is not possible to distinguish experimentally between the two. However, after a few impulses at high frequencies (> 2 Hz) there is a greater than 30% difference between the predictions for facilitation based on a second power relationship and a linear one, and it is easy to distinguish that the second power relationship (Figure 7) gives the better fit to the experimental observations.

During a short train of high frequency impulses, the amplitude of the e.j.p. increases with successive impulses at the beginning of the train and then decreases until a depressed steady amplitude is reached (Bennett, 1973a). In the present work the first few facilitated e.j.ps during high frequency trains were predicted on the basis of the accumulation of residual Ca*X* but this hypothesis does not readily provide an explanation for depression; a similar change in the amplitude of synaptic potentials during short high frequency trains is observed at the cholinergic neuromuscular junction (Mallart & Martin, 1968), where the depression is attributed to a growing depletion in the number of quanta of transmitter which are readily available for release. It might be argued that the depression observed at the adrenergic neuromuscular junction is due to a growing α-adrenoceptor mediated autoinhibition of NA release, in which the NA released by an impulse acts on the nerve terminal to decrease the entry of Ca during a subsequent impulse, this more than counteracting the effects of residual Ca*X* (Stjärne, 1973). Reserpine (Bennett & Middleton, 1975a) as well as the α-adrenoceptor blockers (Bennett, 1973b; Bennett & Middleton, 1975b), reverse the normal depression which develops during a short high frequency train to a facilitation, so that it is unnecessary for a drug to possess an α-adrenoceptor blocking capacity in order to relieve this depression. If both types of drug act at a common point in the release process to reverse depression to facilitation it is possible that they both act within the nerve terminal to prevent the depletion of quanta that are readily available for release by nerve impulses.

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