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THE CALCIUM DEPENDENCE OF SPONTANEOUS AND EVOKED QUANTAL RELEASE AT THE FROG NEUROMUSCULAR JUNCTION

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SUMMARY

1. The quantal output from stimulated nerve terminals in the frog sciatic nerve–sartorius muscle preparation in low-Ca^{2+} Ringer solution was measured by the coefficient of variation and the failures methods. Adding sucrose to the Ringer to increase the tonicity or adding ethanol increased miniature end-plate potential (m.e.p.p.) frequency and also the end-plate potential (e.p.p.) amplitude. Earlier reports suggested that increases in tonicity did not increase evoked quantal release.

2. Concanavalin A has been reported to block the increase in m.e.p.p. frequency caused by increasing the tonicity of the Ringer (Gorio & Mauro, 1979). This effect was confirmed. The lectin-treated preparations also failed to show an increase in evoked quantal release when the tonicity was increased.

3. A model in which both spontaneous and evoked quantal releases depend on some power of the intracellular $[Ca^{2+}]$ is presented. The model predicts that rises in m.e.p.p. frequency will be accompanied by increased quantal output from stimulated nerve terminals. The maximum slope of the relationship between log (evoked quantal output) and log ($[Ca^{2+}]_{out}$) will be less than the true power. A theoretical analysis shows that, as the true power approaches infinity, the maximum slope will be slightly above 4. The value for the slope usually found experimentally at the frog neuromuscular junction is also about 4.

4. The model does not fit the experimental data. The observed increases in evoked quantal release are higher than those predicted for the observed increases in spontaneous release. There are several possible explanations for the discrepancy. Treatments that increase m.e.p.p. frequency may also increase Ca^{2+} influx into the stimulated terminal. However, we prefer the explanation that there is a fraction of spontaneous release that is independent of the $[Ca^{2+}]$ in the terminal; if this is true the model might account for the data.

5. The model can account for a variety of puzzling experimental observations, including: (*a*) the effect of hypertonic solutions and of diamine in decreasing the slope in the relation between log (evoked quantal output) and log ($[Ca^{2+}]_{out}$); (*b*) the slope of near 1 observed at the crustacean neuromuscular junction; (*c*) the decrease in the slope produced by treatment with botulinum toxin.

INTRODUCTION

At the neuromuscular junction quanta are released both spontaneously and following motor nerve stimulation (Katz, 1969). The quantitative relationship between the rate of spontaneous release and the quantal output following nerve stimulation is at present unknown. A number of treatments have been described that elevate both spontaneous and evoked release, some of which are listed in Table 1. Most of these treatments are progressive; both spontaneous and evoked release monotonically increase in time. The failure to achieve a steady level makes quantitative comparisons difficult. Treatment with the venom of the Australian tiger snake decreases both spontaneous and evoked release (Datyner & Gage, 1973), as does treatment with indomethacin and other prostaglandin synthase inhibitors (Madden & Van der Kloot, 1982).

TABLE 1. Treatments that increase both m.e.p.p. frequency and evoked quantal output

Treatment	Reference
Stretch	Hutter & Trautwein (1960)
Reduced $[Na^+]_{out}$	Elmquist & Feldman (1965)
Ouabain	Elmquist & Feldman (1965)
Anoxia	Hubbard & Løyning (1966)
Noradrenaline	Jenkinson, Stamenovic & Whitaker (1968)
Uncouplers	Glagoleva, Liberman & Kashayev (1970)
Antimycin A	Katz & Edwards (1973)
CN^-	Katz & Edwards (1973)
Caffeine	Onodera (1973)
Li^+	Crawford (1975)
X-537a	Kita & Van der Kloot (1974)
A23187	Stratham & Duncan (1976)
Diamine	Werman, Carlen, Kushnir & Kosower (1971)

There are other treatments, such as exposure to La^{3+}, which increase spontaneous release but depress evoked release. These agents probably act by blocking stimulated Ca^{2+} influx into the nerve terminal, thereby depressing evoked quantal release, and also by entering the terminal where they promote spontaneous release by mimicking the effects of elevated Ca^{2+} or by causing the release of Ca^{2+} from intracellular stores (Heuser & Miledi, 1971).

One possibility is that treatments that elevate both miniature end-plate potential (m.e.p.p.) frequency and end-plate potential (e.p.p.) amplitude act by elevating resting intracellular $[Ca^{2+}]$. To test this hypothesis we decided to study the effects of an increased tonicity of the Ringer solution, produced by adding sucrose, on spontaneous and evoked release. It has long been known that increases in the tonicity of the bathing solution substantially increase m.e.p.p. frequency (Fatt & Katz, 1952). However, moderate changes in the tonicity of the bathing solution have been reported to be without effect on evoked release (Furshpan, 1956), while large increases in tonicity depress evoked release (Thesleff, 1959; Hubbard, Jones & Landau, 1968; Kita & Van der Kloot, 1977). Recently Kita, Narita & Van der Kloot (1982) showed that increases in tonicity, produced by adding sucrose, NaCl or glycine to the Ringer solution, potentiated the increase in m.e.p.p. frequency elicited by tetanic stimulation

of the motor nerve in low Ca^{2+}–EGTA solutions. Decreases in the tonicity of the bathing solution, produced by lowering the [NaCl], decreased the m.e.p.p. frequency and the quantal output in response to tetanic stimulation (Kita *et al.* 1982). These results suggested the possibility of parallel changes in the rates of spontaneous and evoked quantal release produced by changes in tonicity, at least at low quantal outputs. The experimental advantage of using tonicity changes, in preference to most of the treatments shown in Table 1, is that the effects are readily reversible and, at least in the frog, the changes in m.e.p.p. frequency produced by a change in tonicity are relatively stable. Many of the treatments listed in Table 1 produce monotonically rising m.e.p.p. frequencies.

Our interest in the relationship between spontaneous and evoked release was stimulated by theoretical work on the co-operative action of Ca^{2+} in transmitter release. The theory predicts that at low quantal outputs a rise in m.e.p.p. frequency will be accompanied by a measurable increase in evoked output, so long as the amount of Ca^{2+} entering the nerve terminal as a result of the nerve action potential remains constant. These considerations prompted us to return to the study of the effects of changes in tonicity on spontaneous and evoked release, using experimental situations in which evoked release was kept low by decreasing the $[Ca^{2+}]_{out}$.

MATERIALS AND METHODS

The experiments were performed on the sciatic nerve–sartorius muscle preparation from the frog, *Rana pipiens*, in January and February, at room temperatures (20–22 °C). The m.e.p.p.s and e.p.p.s were recorded in the usual way with a KCl-filled glass micro-electrode (d.c. resistance 3–8 MΩ) inserted in the muscle fibre at an end-plate. M.e.p.p.s were recorded on a Brush inkwriter. E.p.p.s were elicited by stimulating the nerve once every 3 s with a supramaximal stimulus. The e.p.p.s were captured on a Gould digital oscilloscope and stored on floppy disks. Later the amplitudes of the e.p.p.s were measured using a PDP 11/23 computer.

In most cases evoked quantal outputs were determined both by the method of failures (m_0) and by the coefficient of variation (m_2). The methods for calculating the output and the standard errors of the estimates are given by Martin (1955).

The Ringer solution usually contained (in mM): 120, NaCl; 2·0, KCl; 0·2, $CaCl_2$; 2·3, $MgCl_2$; 4·0, TES (*N*-tris(hydroxymethyl)methyl-2-aminoethanesulphonic acid) at pH 7·2; and 1 μg/ml neostigmine methylsulphate. The tonicity was raised by the additon of sucrose. The solution in the recording chamber was exchanged by the use of a regulated, two-channel pump, which simultaneously added and removed equal volumes of solution.

Concanavalin A pre-treatment followed the description by Gorio & Mauro (1979). The concanavalin A (Sigma) was dissolved in 1 M-NaCl at 3·5 mg/ml; 0·5 ml of this stock solution was added directly to the recording chamber containing the nerve–muscle preparation in about 5 ml of Ringer. Acccording to Gorio & Mauro (1979) the concanavalin A is not effective if simply dissolved in the Ringer, but this point was not re-examined.

RESULTS

Spontaneous and evoked release rates

Both the m.e.p.p. frequency and the quantal output (m) are usually increased to a new steady level by an elevation in the tonicity of the bathing solution. When the m.e.p.p. frequency rises, so does m. Fig. 1 shows a few of the experimental results which illustrate this point. Table 2 summarizes all of the results. The data show that usually both the m.e.p.p. frequency and m are monotonic functions of the tonicity

of the medium. Occassionally an increase in tonicity did not raise the m.e.p.p. frequency; when this occurred m also was not increased (Table 2, example C).

The quantal output was estimated by two techniques: from the fraction of failures (m_0) and from the coefficient of variation (m_2). The results from the two estimates were similar.

M.e.p.p. frequency can also be raised by adding ethanol to the Ringer, and it is known that the ethanol also increases evoked release (Okada, 1967; Quastel, Hackett & Cooke, 1971). Three examples of this effect are included in Table 2 (K, L, M).

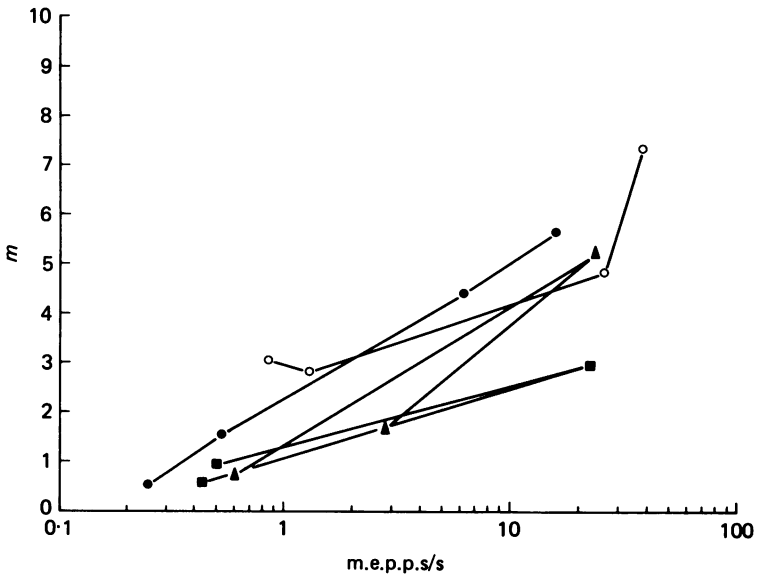


Fig. 1. The relation between the spontaneous quantal release rate and the evoked quantal output, m_2, from four of our experiments. The m.e.p.p. frequency was increased by adding sucrose to the Ringer. The effects of the change in tonicity are largely reversible.

The effects of concanavalin A

Gorio & Mauro (1979) reported that pre-treatment (see Methods) with concanavalin A eliminates the increase in m.e.p.p. frequency caused by elevated tonicity. The data in Table 3 confirm their report on the action of concanavalin A and also show that the treatment eliminates the effect of increased tonicity increasing evoked quantal output. This is further evidence that the two changes – the increase in m.e.p.p. frequency and the rise in m – go hand in hand.

Theory

Jenkinson (1957) and Dodge & Rahamimoff (1967) found that there is a steep relation between $[Ca^{2+}]$ and evoked quantal release. When plotted on logarithmic co-ordinates there was a linear relation over the lower range of $[Ca^{2+}]_{out}$ tested and an asymptote as the concentration was raised. The slope of the line in the linear region was about 4. This led Dodge & Rahamimoff (1967) to propose a model in which a number of Ca^{2+} ions (possibly four) interact with a saturable binding site on the outer surface of the nerve membrane.

TABLE 2. The relation between m.e.p.p. frequency and evoked quantal output

Expt.	Solution	M.e.p.p. frequency ± S.E.M.	m_2 ± S.E.M.	m_0 ± S.E.M.
A.	Ringer	0·6 ± 0·08	2·1 ± 0·14	2·1 ± 0·25
	+100 mM-sucrose	19·5 ± 1·16	6·3 ± 0·25	—
	Ringer	0·9 ± 0·09	2·7 ± 0·67	2·5 ± 0·39
B.	Ringer	0·3 ± 0·05	0·5 ± 0·07	0·5 ± 0·08
	+25 mM-sucrose	0·5 ± 0·07	1·6 ± 0·12	1·4 ± 0·18
	+50 mM-sucrose	7·7 ± 0·62	3·3 ± 0·18	2·5 ± 0·34
	+75 mM-sucrose	6·2 ± 0·56	4·4 ± 0·21	3·0 ± 0·44
	+100 mM-sucrose	15·9 ± 1·60	5·6 ± 0·24	4·6 ± 0·99
C.	+50 mM-sucrose	3·5 ± 0·37	2·7 ± 0·16	2·8 ± 0·40
	+25 mM-sucrose	4·9 ± 0·45	2·9 ± 0·17	2·4 ± 0·32
	Ringer	1·6 ± 0·25	3·6 ± 0·19	2·1 ± 0·27
D.	Ringer	0·5 ± 0·07	0·9 ± 0·10	0·9 ± 0·13
	+50 mM-sucrose	22·6 ± 1·60	2·9 ± 0·17	2·9 ± 0·32
	Ringer	0·43 ± 0·06	0·57 ± 0·08	0·57 ± 0·08
E.	Ringer	0·6 ± 0·08	0·7 ± 0·09	0·6 ± 0·10
	+50 mM-sucrose	24·0 ± 3·34	5·2 ± 0·23	3·9 ± 0·70
	Ringer	2·8 ± 0·03	1·6 ± 0·13	1·6 ± 0·20
F.	Ringer	0·9 ± 0·12	3·0 ± 0·17	2·7 ± 0·34
	+100 mM-sucrose	38·9 ± 2·63	7·3 ± 0·26	—
	+50 mM-sucrose	1·3 ± 0·14	2·8 ± 0·17	2·5 ± 0·34
	+75 mM-sucrose	26·1 ± 2·16	3·8 ± 0·70	—
G.	Ringer	0·4 ± 0·06	0·8 ± 0·09	0·7 ± 0·10
	+25 mM-sucrose	0·7 ± 0·08	2·0 ± 0·14	1·8 ± 0·22
H.	Ringer	0·04 ± 0·02	0·2 ± 0·05	0·2 ± 0·05
	+50 mM-sucrose	1·3 ± 0·11	2·6 ± 0·16	2·1 ± 0·28
I.	+30 mM-sucrose	6·3 ± 0·71	2·3 ± 0·11	2·3 ± 0·22
	Ringer	2·0 ± 0·20	1·6 ± 0·09	1·6 ± 0·15
J.	+50 mM-sucrose	13·9 ± 1·32	6·3 ± 0·26	4·6 ± 0·98
	+25 Ringer	1·0 ± 0·12	1·6 ± 0·09	1·4 ± 0·13
K.	Ringer	0·9 ± 0·13	3·4 ± 0·18	3·3 ± 0·49
	+2% ethanol	7·8 ± 0·62	6·7 ± 0·26	4·6 ± 1·00
	Ringer	0·9 ± 0·12	3·0 ± 0·17	2·7 ± 0·34
L.	Ringer	0·43 ± 0·06	0·6 ± 0·08	0·6 ± 0·09
	+2% ethanol	5·3 ± 0·16	3·5 ± 0·19	3·0 ± 0·44
M.	1% ethanol	1·4 ± 0·15	1·6 ± 0·13	2·6 ± 0·36
	Ringer	0·5 ± 0·09	1·6 ± 0·13	1·5 ± 0·19
	2% ethanol	10·3 ± 0·65	1·8 ± 0·2	2·8 ± 0·56

m_0 is the evoked quantal output estimated by the method of failures.
m_2 is the evoked quantal output estimated by the coefficient of variation method.
In each experiment, m_0 and m_2 were measured from the responses to 100 stimuli.

A number of observations have suggested modifications to the basic kinetic scheme. It is now believed that the site of Ca^{2+} action is within the nerve terminal, since intracellular Ca^{2+} injection promotes transmitter release (Miledi, 1973). This raises the question of the relation between the $[Ca^{2+}]$ in the resting terminal and the Ca^{2+} added following the nerve action potential. We therefore decided to reconstruct

TABLE 3. The relation between m.e.p.p. frequency and evoked quantal output in preparations pretreated with concanavalin A

Expt.	Solution	M.e.p.p. frequency ±S.E.M.	m_2 ±S.E.M.	m_0 ±S.E.M.
A.	Ringer	0·06±0·02	0·33±0·06	0·39±0·07
	+50 mM-sucrose	0·05±0·02	0·17±0·04	0·18±0·05
B.	Ringer	0·08±0·03	1·1±0·11	1·0±0·13
	+50 mM-sucrose	0·09±0·03	1·4±0·12	1·0±0·14
	Ringer	0·08±0·03	1·9±0·13	1·9±0·23
C.	Ringer	0·15±0·03	1·7±0·13	1·9±0·24
	+50 mM-sucrose	0·25±0·06	0·52±0·07	0·67±0·09
	Ringer	0·11±0·03	0·8±0·09	1·01±0·13

m_0 and m_2 are the evoked quantal outputs calculated by the method of failures and the coefficient of variation method respectively.

In each experiment, m_0 and m_2 were measured from the responses to 100 stimuli.

the model of Dodge & Rahamimoff (1967), adding a term for the $[Ca^{2+}]$ in the resting terminal.

Suppose that spontaneous quantal release is also a function of the $[Ca^{2+}]$ in the resting nerve terminal; there is clear evidence that raising $[Ca^{2+}]$ by the use of ionophores markedly increases the m.e.p.p. frequency (Kita & Van der Kloot, 1976; Stratham & Duncan, 1976; Ito & Miledi, 1977). This suggests that the relation between the spontaneous quantal release rate, m.e.p.p. frequency, and the fraction of receptors with a bound Ca^{2+}, r, is

$$\text{m.e.p.p.f.} = k \cdot r^n, \tag{1}$$

where k is the theoretical maximum release rate of the terminal and n is the effective order of the release reaction. If Ca^{2+} binding to the receptor is first order (Crawford, 1974), then

$$r = [Ca^{2+}]_{in}/(K_m + [Ca^{2+}]_{in}), \tag{2}$$

where K_m is the equilibrium constant for the binding of Ca^{2+} to receptor. Defining b as

$$b = [Ca^{2+}]_{in}/K_m \tag{3}$$

and combining eqn. (1), (2) and (3) gives

$$\text{m.e.p.p.f.} = k/(1+1/b)^n. \tag{4}$$

For evoked release we defined m_s as the rate of additional release occurring shortly after stimulation (Crawford, 1973, 1974). It is given by

$$m_s = k/[1+1/(a+b)]^n - k/(1+1/b)^n, \tag{5}$$

where a is the increase in $[Ca^{2+}]_{in}$, as a function of K_m, produced by influx following the action potential. For the purposes of this discussion we assume that the Ca^{2+} entry is directly proportional to the $[Ca^{2+}]_{out}$ (Hodgkin & Keynes, 1957). The peak output from frog motor nerve terminals, k, has been measured by Katz & Miledi (1979). In normal Ringer at 20 °C the peak output is about 900 quanta released in less than a millisecond, giving an output of at least 900000 per second. This value can be

approximately doubled by increasing the $[Ca^{2+}]$ in the Ringer. When the action potential is prolonged by the use of K^+ channel blockers the ouput rises to about 46000 over a period of about 40 ms, giving an m_s of about $1{\cdot}1 \times 10^6$. Obviously k is greater than 10^6, but the actual value is unknown.

Fig. 2 shows some of the properties of the model. In Fig. 2*A* we have plotted on double logarithmic co-ordinates the output predicted by the model following stimulation for $k = 10^6$/s, m.e.p.p.f. = 1/s and $n = 5$ (the use of this relatively high value for n will be justifed shortly). Eqn. (4) was then used to calculate a value of b consistent with the assumptions. Then eqn. (5) was used to calculate a value for a that would give an m_s of 1000/s with these values of n, k, and of b. This value of a was assumed to be the level following nerve stimulation at a $[Ca^{2+}]_{out}$ of 0·2. The value of a was assumed to be a linear function of the $[Ca^{2+}]_{out}$, and then the complete curve was calculated. There are three major regions in the curve. At high levels of $[Ca^{2+}]_{out}$ the output approaches the maximum, k; the finite number of receptors is approaching saturation. When $[Ca^{2+}]_{out}$ is low then a is small compared with b; consequently increases in $[Ca^{2+}]_{out}$ produce only modest changes in m_s. At the mid point of the curve the slope, S, becomes larger. The value of S can be obtained by differentiating eqn. (5) with respect to a, which gives

$$S = \frac{na}{(a+b)(a+b+1)\left[1-\left(1+\frac{a}{ab+b^2+b}\right)^{-n}\right]}. \tag{6}$$

This equation shows the relation between the true power of the relation between m_s and $[Ca^{2+}]_{out}$, i.e. n, and the apparent power, which is obtained experimentally by measuring S. Since S is a function of both a and b, it can be far less than n. This is illustrated first in Fig. 2*A*, in which we show curves for $n = 5$, 20 and 100. Obviously there would be substantial difficulty in distinguishing experimentally between the three curves. The point is made a second time in Fig. 3, which shows the relation between the true power, n, and the maximum computed value of S, the apparent power, for two values of k (Barton, 1977). For low values of n (1 or 2) the apparent power is almost identical. But as n rises the apparent power reaches an asymptote, and for all true powers above 5 there is little increase in the apparent power. This maximum value of S is usually stated to be about 4 (Dodge & Rahamimoff, 1967). The importance of Fig. 3 is that it shows that if $S_{max} = 4$ then the true power may be anywhere from 5 upwards, and cannot be determined by measuring S_{max}.

The significance of the rather cumbersome eqn. (6) can be better appreciated by considering the limits as n tends to ∞. Then

$$S_{max} \approx \ln\left[\frac{k}{\text{m.e.p.p.f.}}\right]\Big/4. \tag{7}$$

For $k = 10^6$/s and m.e.p.p.f. = 1/s, $S_{max} = 3{\cdot}45$. If $k = 10^7$/s and m.e.p.p.f. = 1/s, $S_{max} = 4{\cdot}02$ (see Fig. 3). The rate of evoked quantal output at S_{max} is given by

$$m_s(S_{max}) \approx (\text{m.e.p.p.f.} \,.\, k)^{0{\cdot}5}. \tag{8}$$

From eqn. (7) and (8) we can estimate the maximum empirical slope, S_{max}, for the relation between $\log([Ca^{2+}]_{out})$ and $\log(m_s)$ when n tends to ∞ from the ratio of the

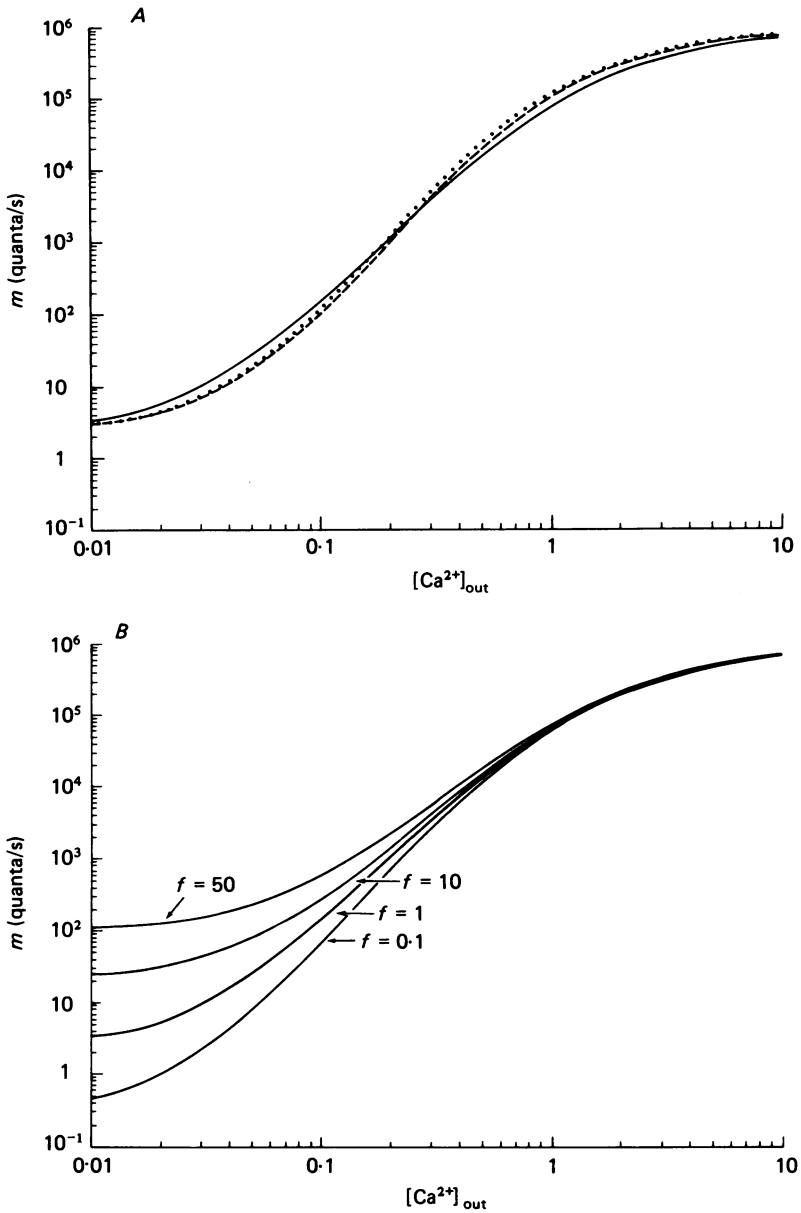
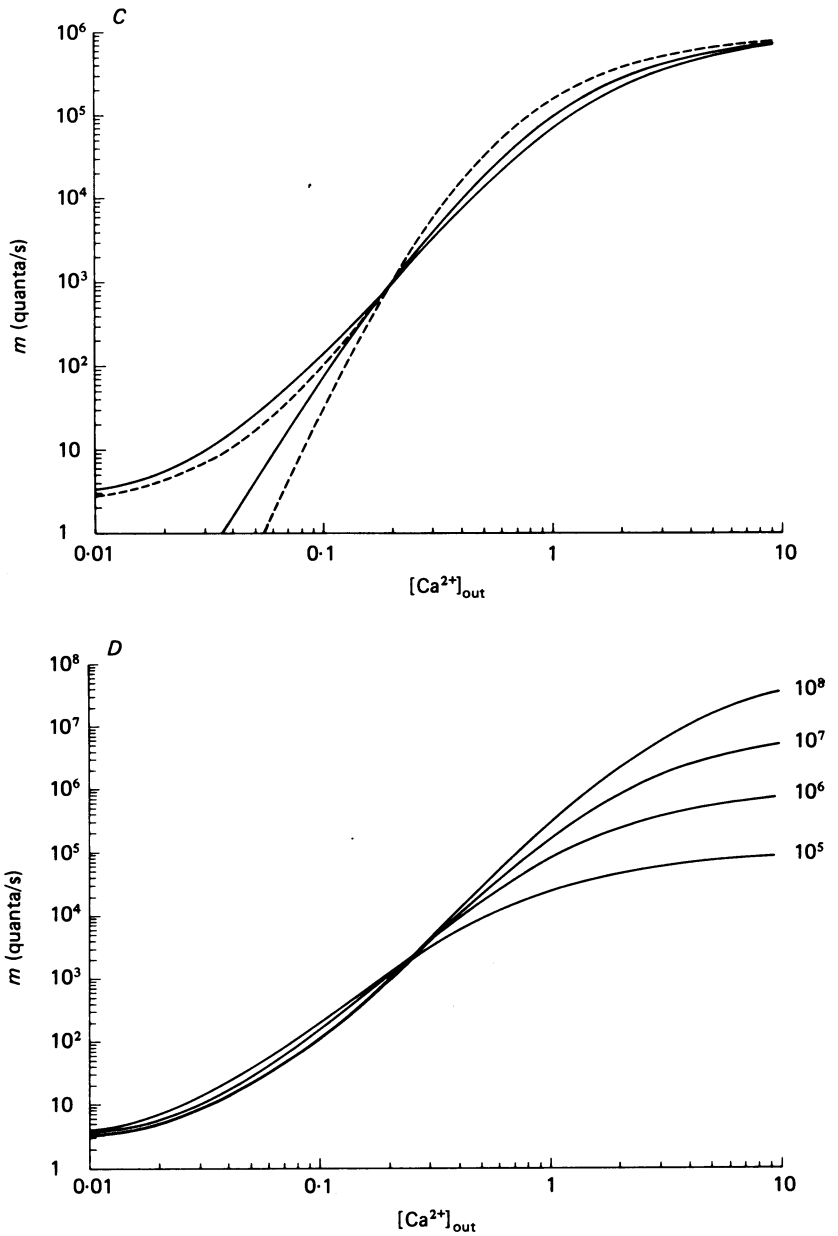


Fig. 2. Some of the predictions from the model developed in the text. The methods of calculation and the assumed values for the parameters are given in the text. *A*, the predicted relationship between $[Ca^{2+}]_{out}$ and the quantal output following stimulation. $k = 10^6/s$; $n = 5$ (continuous line), 20 (dashed line) or 100 (dotted line). Note the double logarithmic co-ordinates. *B*, the effect of changes in m.e.p.p. frequency on the relation between $[Ca^{2+}]_{out}$ and evoked quantal output. $k = 10^6/s$; $n = 5$. *C*, the relation between



$[Ca^{2+}]_{out}$ and evoked quantal release predicted by the model, either taking into account the spontaneous release rates (continuous lines: $n = 5$) or neglecting them (dashed lines: $n = 20$). The spontaneous release rates are important for determining the left-hand portion of the curve and also affect the maximum slopes. *D*, the effects of changes in k, the maximum quantal release rate from the stimulated terminal, on the relation between $[Ca^{2+}]_{out}$ and evoked quantal output. The choice of k affects both the maximum evoked output and the slope of the relationship.

evoked and the spontaneous release rates:

$$S_{max} = \ln[(m_s^2/\text{m.e.p.p.f.}^2]/4. \quad (9)$$

Two other features of the model are also shown in Fig. 2. Fig. 2*B* shows the results of calculations using $n = 5$, $k = 10^6$/s, assuming that a is a linear function of $[Ca^{2+}]_{out}$. The variable is b, which is adjusted to give resting m.e.p.p.f. values of 0·1, 1, 10 and 50/s. At very low $[Ca^{2+}]_{out}$ the output is dominated by b, which determines the resting output from the terminal and represents the base line level of internal Ca^{2+} already present when the nerve is stimulated. An elevated value for b reduces the maximum apparent slope, S_{max} (see eqn. (9)). However, these predicted changes in S_{max} are

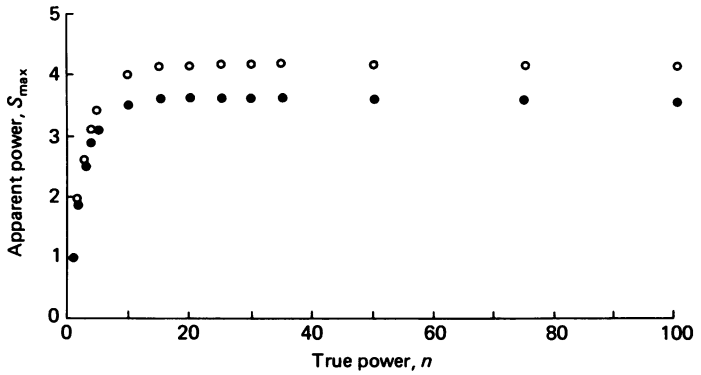


Fig. 3. The relationship between the true power of the relationship between $[Ca^{2+}]$ in the terminal and the quantal release rate, and the apparent power, measured from the maximum slope in plots like that shown in Fig. 2. The apparent and real powers are close only when the true power is low. The apparent power at the frog neuromuscular junction is usually about 4, so it is impossible to state what the true power may be except that it is greater than 4. ○, $k = 2{\cdot}5 \times 10^7$/s; ●, $k = 2{\cdot}5 \times 10^6$/s.

small and might be rather difficult to detect experimentally; a much more striking prediction is that increasing the m.e.p.p. frequency at low $[Ca^{2+}]_{out}$ should also increase m_s: this was the reason for the design of the present experiments.

Fig. 2*C* also shows that at high values of $[Ca^{2+}]_{out}$ the output is dominated by the value of a, which is the same for each of the examples, so the curves overlap. Fig. 2*C* shows the effect of neglecting b, the basal level of Ca^{2+} in the terminal; this completely changes the relationship at low $[Ca^{2+}]_{out}$ and also increases the apparent slope, S.

Fig. 2*D* shows the effects of changing k, the maximum number of quanta that can be released. As expected, a lower k leads to lower values for m_s at high $[Ca^{2+})_{out}$. It was less obvious that a decreased k would lead to some increase in m_s at low $[Ca^{2+}]_{out}$.

Applying the theory to the data

On the basis of the assumptions we have made, the m.e.p.p. frequency and the rate of evoked quantal release are described by eqns. (4) and (5). There are four unknowns: a, b, n and k. To see whether the theory can account for the data we first took the examples in which the preparation was transferred from Ringer solution to Ringer

+50 mM-sucrose. The mean values for m.e.p.p. frequency are 0·7/s and 10·4/s. Assuming that evoked release occurs over a 1 ms time period, m_s in Ringer is 1100 quanta/s and in Ringer + sucrose it is 3300 quanta/s. Then values for k and n were chosen, and the value of b in Ringer, b_r, was calculated from eqn. (4). The value of b in 50 mM-sucrose, b_s, was also calculated. If we assume that the nerve action potential is the same in Ringer and in Ringer + sucrose, the value of a should be the same in the two conditions. Knowing b_r, b_s, and assuming a reasonable value for k (see above) and any value for n, should give two simultaneous versions of eqn. (5) with unknown, a, appearing in each. The equations have a solution only if the changes in evoked output are accounted for by an elevation in base line $[Ca^{2+}]$. For the majority of the data sets no solution exists, which suggests that the elevation of base line $[Ca^{2+}]$ could not account for the changes in evoked release regardless of the power of the reaction, n.

The deviation of the data from the theory could be illustrated by undertaking the following step-by-step calculations.

(*a*) The mean data from Ringer and from Ringer + 50 mM-sucrose were used to calculate b_r, as described in the last paragraph.

(*b*) The value of a for the data from Ringer was calculated from eqn. (5).

(*c*) A series of values for b_s were chosen and used to calculate the m.e.p.p. frequency and m_s.

The results were plotted by showing the predicted relation between the ratio of the m_s values in hypertonic solution to Ringer, as a function of the ratio of the m.e.p.p. frequencies in the two conditions. Fig. 4*A* shows the results of these model calculations for a series of values of n and for two values of k. Fig. 4*B* shows some of the same curves plotted on a different scale, along with points taken from the data in Table 2. Obviously most of the ratios of evoked outputs far exceed the predictions of the theory: the rise in the evoked output is greater than predicted from the rise in the spontaneous release rate.

There are several alternative explanations. Possibly both the increase in tonicity and the addition of ethanol have a second effect: increasing the influx of Ca^{2+} into the stimulated nerve terminal. A weak point in this explanation is that then the concanavalin A pre-treatment must have blocked both the effect of increased tonicity on m.e.p.p. frequency and the effect of increased tonicity on the influx of Ca^{2+} into the stimulated terminal. Probably the most parsimonious explanation is that our application of the theory is slightly oversimplified. In the development of the theory we have assumed that the m.e.p.p. frequency is totally dependent on the $[Ca^{2+}]$ in the resting nerve terminal (eq. (4)). There is evidence that a fraction of the spontaneous release does not depend on internal $[Ca^{2+}]$ (Hubbard *et al.* 1968; Quastel *et al.* 1971). There is also evidence that Mg^{2+} has some effect on spontaneous release rates and that there is a significant $[Mg^{2+}]$ in the nerve terminals (see Van der Kloot, 1978, for references). Suppose, for example, that in the data used to calculate Fig. 4 there is a Ca^{2+}-independent release of 0·7/s, in both Ringer and in Ringer + sucrose. The calculations can then be repeated with new values for the m.e.p.p. frequencies; now the pair of simultaneous equations can be solved. The calculated values are $a = 0{\cdot}22$, $n = 7{\cdot}9$. By making similar assumptions we could calculate a and n for all of our examples, but considering the number of assumptions that must be made there

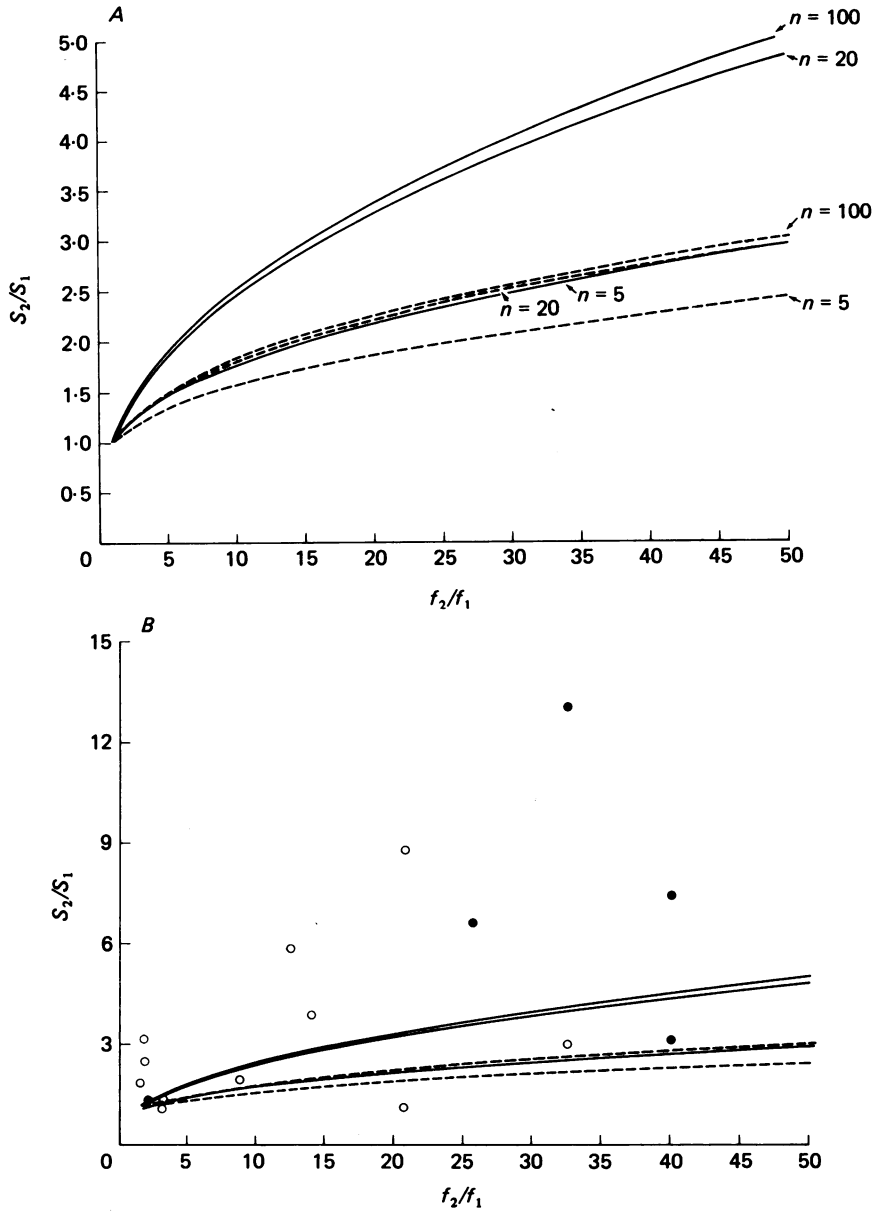


Fig. 4. *A*, calculated curves for the relationship between the ratio of the m.e.p.p. frequency at two different tonicities and the ratio of the evoked quantal outputs at the same tonicities. The method of calculation is described in the text. Continuous lines: $k = 10^6$/s. Dashed lines: $k = 10^8$/s. *B*, three of the curves from Fig. 4*A* replotted with a different scale on the ordinate. Continuous line: $n = 5$, $k = 10^6$/s. Dotted line: $n = 5$, $k = 10^6$/s. Dashed line: $n = 100$, $k = 10^8$/s. The points show data from Table 2. The open circles are for the results in Ringer and in Ringer + 50 mM-sucrose. As described in the text the parameters for the equations were estimated from the mean values from these experiments. The filled circles show data obtained with changes in tonicity other than addition of 50 mM-sucrose or by the addition of ethanol. Most of the experimental points fall far above any of the theoretical curves, showing that the changes in evoked output are greater than predicted by the model for the observed changes in spontaneous release.

appears to be nothing to be gained – the principal point is that the theory can describe all of the data, when it is modified to include a background, Ca^{2+}-independent spontaneous release rate.

DISCUSSION

The results show a clear relation between the frequency of spontaneous quantal release and the number of quanta released from the stimulated nerve terminal in experimental conditions in which quantal release (m) is low and in which the spontaneous release rate is increased by raising the tonicity of the Ringer or by adding ethanol. The effect of increased tonicity on m is strikingly obvious when m is low. Presumably earlier investigators failed to detect the effect because they measured evoked release in conditions in which m was relatively high (Thesleff, 1959; Hubbard *et al.* 1968; Kita & Van der Kloot, 1977).

The effect of tonicity in increasing the spontaneous release rate is blocked by pre-treatment with concanavalin A, which also blocks the effect of raised tonicity on increasing m. Our results therefore confirm the report by Gorio & Mauro (1979) on this effect of concanavalin A pre-treatment. At present we have no idea how this plant lectin achieves this effect, but the observation significantly advances our thinking on how changes in tonicity might change the m.e.p.p. frequency. One line of speculation on how tonicity changes spontaneous release has focused on alterations in intracellular ion concentrations produced by changing the volume of the nerve terminal (Van der Kloot & Kita, 1973). Since there is no reason to believe that the concanavalin A pre-treatment blocks the movement of water in and out of cells in response to osmotic pressure gradients, this line of speculation becomes less attractive. The idea that changes in tonicity act indirectly by membrane alterations that lead to shifts in $[Ca^{2+}]$ in the terminal (Shimoni, Alnaes & Rahamimoff, 1977) becomes more attractive.

The effects of tonicity and of ethanol in increasing evoked release can be accounted for by a model that assumes that these treatments elevate intra-terminal $[Ca^{2+}]$, and that the spontaneous release has both Ca^{2+}-dependent and Ca^{2+}-independent components. The model suggests that many of the treatments that increase both spontaneous and evoked release may act by elevating the $[Ca^{2+}]$ in the motor nerve terminal.

If the model is applicable, the true power of the relation between $[Ca^{2+}]$ and quantal release, which is the number of Ca^{2+} ions that interact co-operatively in promoting the release of a quantum of transmitter, cannot be determined by taking the maximum slope of the relation between log (e.p.p. amplitude) and log ($[Ca^{2+}]_{out}$), as is often done. If the maximum slope is 4, the true power may be several orders of magnitude higher. Many Ca^{2+} ions may act co-operatively in promoting quantal release, perhaps by changing the physical properties of a region on the inner face of the nerve terminal to favour vesicle fusion. The rather large body of literature on the apparent power of the co-operative reaction and treatments that apparently change the power should be re-examined in the light of our model (see Parnas & Segal, 1981; Nachsen & Drapeau, 1982).

Crawford (1974) reported that at low quantal outputs the slope of the relationship between log (e.p.p. amplitude) and log ($[Ca^{2+}]_{out}$) approaches 1. He suggested that this

occurs because there is a 'change in gears' in the relation between $[Ca^{2+}]$ and the release mechanism. The model predicts that at low $[Ca^{2+}]$ release will be dominated by the resting level of Ca^{2+} in the terminal. Andreu & Barrett (1980) made similar measurements using a longer delay between stimuli and Mn^{2+} or Co^{2+} as the Ca^{2+} antagonist. They report that the relationship stays fourth-power down to release at 2–4 quanta/1000 stimuli. Since they do not give the values for m_s and m.e.p.p. frequency for each of the concentrations they studied, we cannot test their data with our model.

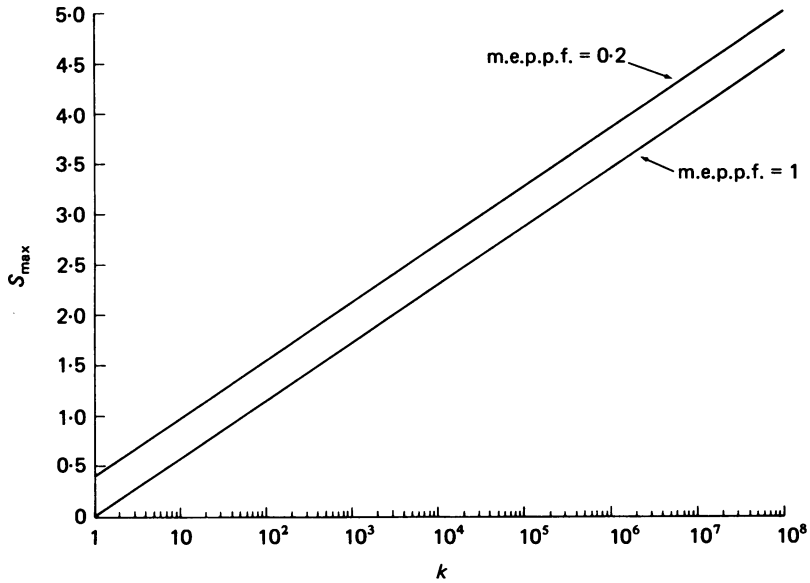


Fig. 5. The relationship between the maximum quantal output from the stimulated terminal, k, and the maximum slope, S_{max}, of the relationship between log ($[Ca^{2+}]_{out}$) and log (stimulated quantal output). For further discussion see the text.

Our model helps to account for some other unexplained data. Kita & Van der Kloot (1973) reported that in solutions containing 200 mM-sucrose the maximum slope of the relation between log (m.e.p.p. amplitude) and log ($[Ca^{2+}]_{out}$) was decreased to about 1·5 (the controls showed maximum slopes of between 3 and 4). They also found that the treatment increased (relative to controls) the e.p.p. amplitudes at the lower values of $[Ca^{2+}]_{out}$ tested. Both effects are predicted by the model (see Fig. 2*B*).

Diamine increases the spontaneous rate of transmitter release and also reduces the slope of the relation between log (m) and log ($[Ca^{2+}]_{out}$) at the frog neuromuscular junction from about 4 to 3 (Carlen, Kosower & Werman, 1976). According to our model, this effect is accounted for in the same way as that produced by an elevation in the osmotic pressure (see Fig. 2*B*).

At the crustacean neuromuscular junction the maximum slope in the relation between log (excitatory junctional potential amplitude) and log ($[Ca^{2+}]_{out}$) is often reported to be about 1 (see Atwood, 1976, for references). This has led to some speculation about a mechanism in which a single Ca^{2+} ion can promote a quantal release. However, it is also clear that k, the maximum quantal release rate, is low

in the crustacean compared with the frog or the mouse. Fig. 5 shows that relation between k and S_{max} (eqn. (7)). The model predicts that S_{max} will decline as k falls (see Fig. 2*D*). The low S_{max} found in the Crustacea is not necessarily produced by a different number of Ca^{2+} ions co-operating in promoting quantal release.

Botulinum toxin markedly reduces transmitter release from mammalian motor nerve terminals, and at the same time the slope of the plot of log (e.p.p. amplitude) against log ($[Ca^{2+}]_{out}$) is reduced from about 2·7 to 1·7 (Cull-Candy, Lundh & Thesleff, 1976). The decrease in slope is easily accounted for by a reduction in the maximum output, k, without assuming that there is any change in the number of Ca^{2+} ions co-operating in release (see Fig. 2*D*).

Finally, it is worth considering the functional advantages of a steep relation between $[Ca^{2+}]_{in}$ and quantal release. Only a modest Ca^{2+} influx per action potential is required to accelerate release greatly. This allows for relatively little quantal loss of transmitter from the resting terminal, and relatively low energy utilization for restoring ionic gradients after terminal depolarization. Conversely, relatively small changes in resting $[Ca^{2+}]_{in}$ can have marked effects on evoked release with relatively slight effect on spontaneous release.

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Note added in proof. Niles & Smith (1982) studied the effects of hypertonic solutions on spontaneous and evoked quantal release at the crayfish neuromuscular junction. They found that hypertonic solutions, ranging from 0·4 to 1·3 Osm, decreased evoked output, m, but increased the rate of spontaneous quantal release. Kita & Van der Kloot (1977) reported the same effect at the frog neuromuscular junction, using solutions with tonicities from 0·4 to 0·56 Osm. In the present paper, to avoid this effect, the highest tonicity used is 0·31 Osm. Barton (1977) presented evidence that at the frog neuromuscular junction increases in tonicity decrease the maximum output from the terminal, k. We suggest that at levels of tonicity above about 0·4 Osm the effect on the evoked release is dominated by the decrease in the maximum release rate. The rise in base line Ca^{2+} still increases spontaneous release, despite the decrease in the maximum release rate. The pertinent calculations for our model are in Fig. 2*D*.

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