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ACTIVATION OF TRANSMITTER RELEASE BY STRONTIUM AND CALCIUM IONS AT THE NEUROMUSCULAR JUNCTION

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SUMMARY

1. The interaction between Ca and Sr ions on quantal transmitter release at the frog neuromuscular junction was studied, using conventional electrophysiological techniques.

2. While Ca ions always activate transmitter release, the activating action of Sr ions depends on the Ca ion concentration in the medium; at low [Ca], strontium ions enhance the release, but at higher [Ca] they inhibit it. It is postulated that there is a [Ca] at which Sr ions do not affect transmitter liberation.

3. When Sr activates release, its effect and the effect of Ca add in a more than linear fashion.

4. Magnesium ions inhibit the release induced by Sr.

5. The results can be explained by assuming that Ca and Sr act on the same site, at some stage of the process of quantal transmitter release. The affinity of both ions towards the sites is approximately the same, but the effectiveness of Sr is much smaller.

INTRODUCTION

Calcium ions play an important role in the coupling between nerve terminal depolarization and acetylcholine release at the neuromuscular junction. In the absence of Ca ions the nerve action potential invades the terminals, but fails to induce liberation of transmitter (Katz & Miledi, 1965). It has been found recently (Miledi, 1966; Dodge, Miledi & Rahamimoff, 1969) that Sr ions are able to replace Ca ions in this respect (see also Mines, 1911). When Sr ions are substituted for Ca, the release of transmitter has similar properties to the normal release under calcium; it is liberated spontaneously and after nerve stimulation; the release is quantal in nature; when the mean number of quanta liberated per nerve impulse

(quantal content, m) is low, typical fluctuations in the end-plate potential (e.p.p.) amplitude appear, which are predicted accurately by the Poisson theorem (for review on normal properties of release, see Katz, 1969).

Though the activation of the release process by Ca and Sr seems to be qualitatively quite similar, there is an important quantitative difference in the action of these two ions. Under normal Ca concentration of 1·8 mM, the mean quantal content is several hundred (Katz, 1962). With similar strontium concentrations, the mean quantal content is only a few quanta.

There are several questions that arise regarding the action of Ca and Sr. First, are Sr and Ca enabling transmitter to be liberated by the same release mechanism? If so, what is the basis of the difference in potency? Is the shape of the relation between divalent ion concentration and release similar for both Ca and Sr? What is the effect of competitive inhibitors to Ca, such as Mg (see Jenkinson, 1957), on the release by Sr? Is it possible to employ the difference between Ca and Sr for further understanding of the release process? It is the purpose of this work to examine several of these questions.

METHODS

The experiments were performed on the sartorius nerve-muscle preparation of the frog *Rana ridibunda*, at room temperature of 19–24° C.

During the dissection and the mounting of the preparation, it was immersed in Ringer solution (standard composition: 116·0 mM-NaCl, 2·0 mM-KCl and 1·8 mM-$CaCl_2$). Thereafter, the divalent ion composition of the medium was varied by isotonic substitution for Na. It is unlikely that the variation in $[Na]_o$ has a significant effect on the transmitter release properties of the nerve endings under the present circumstances (e.g. see Kelly, 1965; Rahamimoff & Colomo, 1967). Two restrictions were imposed on the degree of variation of the divalent ion concentration. First, in most of the experiments, the total divalent ion concentration was never lowered below an arbitrary level of 1 mM in order to avoid large changes in the excitability of nerve (e.g. see Frankenhaeuser, 1957). Secondly, total divalent ion concentration was always adjusted in such a way as to keep the end-plate potential subthreshold for muscle excitation.

Special precautions were taken to avoid large artifacts when using Sr, due to Ca contamination. After the initial wash of the preparation and the bath with Ca-free medium, there is still a considerable amount of Ca in the muscle, most of it presumably in the sarcoplasmic reticulum (e.g. see Ebashi & Endo, 1968). This Ca leaks out during the course of the experiment. The amount of Ca liberated in the presence of Sr was estimated by Miss E. Eilander.

Frog sartorii were dissected and mounted on a platinum frame. They were incubated for 16 hr in a normal Ringer solution containing radioactive ^{45}Ca, at 4° C. Continuous bubbling of air through the incubation chamber was employed for mixing. After this period, the chamber and the muscle were transferred to room temperature and allowed to equilibrate. Thereafter, the muscle with the frame were transferred to successive test-tubes at 5–10 min intervals. The bubbling of air continued throughout the experiment. The washout of Ca was thus determined for 3–3½ hr. At the end of the experiment the muscle was dissolved in concentrated nitric acid (2 M-HNO_3). The radioactivity was determined by a liquid scintillation counter

(Tricarb-Packard) in the washout fluid and in the muscle. Most of the ^{45}Ca was released during the first hour. This presumably reflects mainly the diffusion from the extracellular space. Thereafter the contribution of the muscle to the bath [Ca] was relatively small. If one assumes that the specific activity of the released Ca is similar to that in the incubation mixture, the amount of Ca released to the bath is approximately 5.10^{-8} moles for a 30 min period. After the first hour, this amount will increase the [Ca] in a 5 ml. bath by approximately 10 μM.

For electrophysiological measurements only one of the surfaces of the muscle is fully exposed to the external medium, while in the washout tubes, both surfaces were in direct contact with the outside. Furthermore, during the washout continuous bubbling of air produced the necessary stirring, while during the electrical recording, the external medium was unstirred. To minimize the effects of the Ca efflux, frequent intermediate washings of the preparation were made. Initially, Ca was washed out by flowing several hundred ml. of a medium with low [Ca], during 90–120 min, through the preparation chamber of 5 ml. capacity. Subsequently, recordings were never taken for periods longer than 20 min without an intermediate exchange of the bathing medium. Even with these precautions, there is probably still a contamination with Ca of approximately 20–80 μM. However, such contamination does not have a noticeable influence on quantal release.

E.p.p.s were elicited at a rate of 0·1–0·25/sec in the presence of strontium and 0·2–0·5/sec in the presence of calcium. These relatively low rates of nerve stimulation were found necessary in order to avoid progressive accumulation of small after-effects (N. Moran & R. Rahamimoff, unpublished observations).

End-plate responses were recorded intracellularly with conventional 3·0 M-KCl-filled micropipettes. In the range of divalent ions concentrations used here, the e.p.p. amplitude fluctuated in accordance with the quantal release hypothesis (del Castillo & Katz, 1954*b*). Therefore a large number of e.p.p.s was used to estimate the quantal content by the three available methods (see del Castillo & Katz, 1954*b*; Martin, 1966). When the quantal content was estimated by the coefficient of variation method, a correction for non-stationarity was frequently used (Colomo & Rahamimoff, 1968). E.p.p. amplitude was usually averaged automatically by a computer for averaging transients (C.A.T. 1000, TMC).

RESULTS

Non-linear addition of the effects of Sr and Ca

The first question examined was whether Ca and Sr induce transmitter liberation by the same mechanism. If Ca and Sr ions are activating the nerve-evoked release of transmitter by two separate non-interacting processes, then one would predict that the combined effect of Sr and Ca is, at most, the algebraic sum of the individual effects. The combined post-synaptic effect might be of course less than the algebraic sum of the individual effects, due to effects of divalent ions on the post-synaptic sensitivity (del Castillo & Engbaek, 1954; Nastuk & Liu, 1966), and due to non-linear summation (del Castillo & Katz, 1954*b*; Martin, 1955). If, on the other hand, the two ions are operating on the same mechanism, then in a certain range of concentrations, the presence of one of the ions might facilitate the release by the other, and vice versa. Fig. 1 shows that the

combined action of Ca and Sr is not equal to, or less than, the sum of their separate actions, but is larger; in the presence of 0·3 mM-Ca and no Sr the averaged e.p.p. amplitude (R_{Ca}) is 0·117 mV; in the presence of 2·0 mM-Sr and no Ca the amplitude (R_{Sr}) is 0·119 mV; when the two divalent ions are present simultaneously, each at the same concentration as before, the e.p.p. amplitude ($R_{Ca,\,Sr}$) is 0·355 mV. This experiment illustrates that in a certain range of Ca and Sr concentration, their effects add in a more-than-linear way. The ratio $u = (R_{Ca,\,Sr})/(R_{Ca} + R_{Sr})$ is in this case 1·50. The ratio u was estimated in seven additional experiments and found to be in the range 1·20–3·33 with a mean value of 1·66.

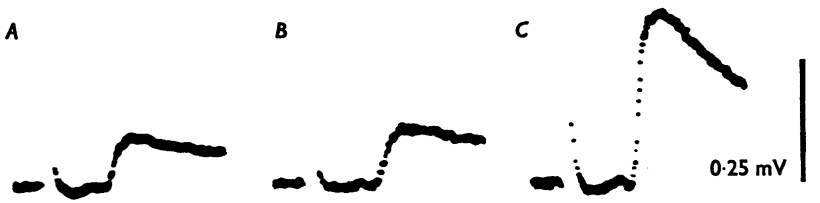

Fig. 1. Non-linear summation of the effects of Ca and Sr ions on transmitter release at the frog neuromuscular junction. *A*, Average response of 0·117 mV to 270 nerve stimuli. Mean quantal content of 0·61. Bathing medium contains 0·3 mM-Ca. *B*, average response of 0·119 mV to 135 nerve stimuli. Mean quantal content of 0·72. Bathing medium contains 2·0 mM-Sr. *C*, average response of 0·355 mV to 135 nerve stimuli. Mean quantal content 2·08. Bathing medium contains 0·3 mM-Ca *and* 2·0 mM-Sr.

Note that when the two activating divalent ions were present simultaneously, transmitter release is 56 % greater than the sum of the individual releases ($u = 2{\cdot}08/[0{\cdot}61 + 0{\cdot}72] = 1{\cdot}56$). 1 mM-Mg was present throughout the experiment. Voltage calibration of 0·25 mV applies to all three records. Averaging step 125 μsec. Stimulation rate of 0·5/sec in *A* and 0·25/sec in *B* and *C*.

The more-than-linear addition of the effects of Ca and Sr can be due either to an increase in the size of the quantum or to an increase in the quantal content. Analysis of the quantal contents of the above-mentioned experiments shows that the latter factor is predominant. For example in the experiment where the value $u = 3{\cdot}33$ was obtained, the quantal contents were $0{\cdot}92 \pm 0{\cdot}06$ (S.E. of mean) for Ca alone (m_{Ca}); $1{\cdot}39 \pm 0{\cdot}03$ (S.E. of mean) for Sr alone (m_{Sr}) and $7{\cdot}7 \pm 0{\cdot}09$ (S.E. of mean) for the combined action of Ca and Sr ($m_{Ca,\,Sr}$). The ratio $m_{Ca,\,Sr}/(m_{Ca} + m_{Sr}) = 3{\cdot}33$ shows that the origin of this *more-than-linear addition* is presynaptic and comes from an increase in the number of quanta released by the nerve impulse. These results also suggest that Ca and Sr act on some common mechanism in the release process. One can describe formally the quantitative dependence of transmitter liberation on [Ca] as a reaction between Ca

ions and a specific site X on the nerve terminal (del Castillo & Katz, 1954*a*; Jenkinson, 1957; Dodge & Rahamimoff, 1967):

$$\mathrm{Ca} + \mathrm{X} \underset{}{\overset{k_1}{\rightleftharpoons}} \mathrm{CaX}, \tag{1}$$

where K_1 is the dissociation constant. For release to occur at least four CaX (or Ca ions) have to co-operate. Mg ions compete with Ca for X, giving

$$\mathrm{Mg} + \mathrm{X} \underset{}{\overset{k_2}{\rightleftharpoons}} \mathrm{MgX}, \tag{2}$$

where K_2 is the dissociation constant for MgX.

The following equation, which is based on this co-operative assumption, describes the dependence of release on [Ca]:

$$m = K\left(\frac{[\mathrm{Ca}]/K_1}{1 + [\mathrm{Ca}]/K_1 + [\mathrm{Mg}]/K_2}\right)^4, \tag{3}$$

where K is a constant.

Since it seems that Sr ions are acting on the same sites as Ca, one may assume that

$$\mathrm{Sr} + \mathrm{X} \underset{}{\overset{k_3}{\rightleftharpoons}} \mathrm{SrX}, \tag{4}$$

where K_3 is the dissociation constant for SrX.

The inhibitory effect of Sr ions on transmitter liberation

If we continue with the formal description of the release properties, we may ask the following question. Why are Sr ions less effective than Ca ions? The difference in potency is obvious in Fig. 1. To obtain approximately the same response, 2·0 mM-Sr are needed, but only 0·3 mM-Ca. Such a situation may be either due to lower affinity of Sr to the site X, (i.e. a high dissociation constant of Sr) or due to lower effectiveness of the SrX complex. Of course both possibilities may co-exist. If the difference in potency between Sr and Ca is only due to lower affinity of Sr to X, addition of Sr, when the release is already substantially activated by Ca, will lead to a small increase in the quantal release. On the other hand, if the difference is due to lower effectiveness of SrX, then addition of Sr will reduce the fraction of sites occupied by Ca and will reduce the quantal output. Fig. 2 shows that indeed the effectiveness of SrX is lower; addition of Sr decreases the e.p.p. amplitude and the quantal content. In the presence of 1·8 mM-Ca, $\bar{R}$ is 4·44 mV and m, calculated by the coefficient of variation method, is 307. Addition of 2·0 mM-Sr reduces the $\bar{R}$ to 2·64 mV and the quantal content to 156. This effect is concentration dependent. When the concentration of Sr was progressively increased, the e.p.p. amplitude decreased accordingly, as illustrated in Fig. 3.

The relation between [Sr] and transmitter release

The analysis of the dependence of transmitter release at the frog neuromuscular junction on the extracellular Ca concentration, revealed that for a liberation of a quantum of transmitter a co-operative action of at least 4 CaX (or four Ca ions) is necessary. The value of four was obtained by estimating the initial slope of the log Ca–log release relation. At low [Ca]

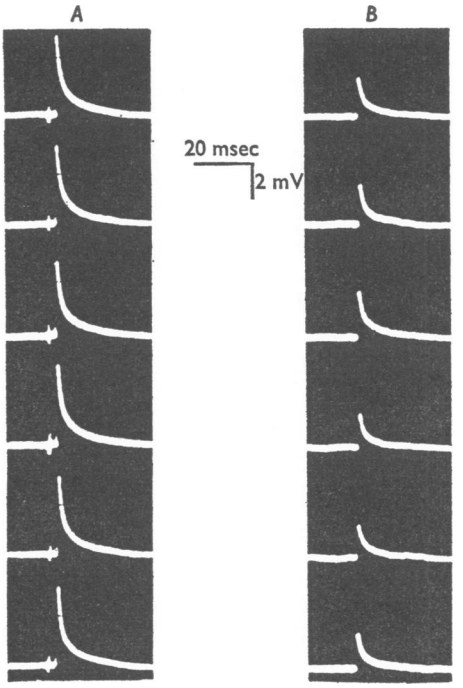

Fig. 2. Inhibition of transmitter release by Sr ions. The neuromuscular preparation was bathed in medium containing 1·8 mM-Ca and (+)-tubocurarine, 10^{-6} g/ml., throughout the experiment. *A*, Control with no Sr ions present. Average amplitude of e.p.p. = 4·44 mV. Quantal content calculated by the coefficient of variation method = 307 (number of responses (N) = 190). *B*, Addition of 2·0 mM-Sr reduced the average amplitude of e.p.p. to 2·64 mV and the quantal content to 156 (N = 190). Time calibration of 20 msec and voltage calibration of 2·0 mV apply to both sets of records.

the denominator of eqn. (3) can be considered approximately constant with small changes in [Ca] and therefore the slope of the log–log plot yields an estimate of the power of the relation (Dodge & Rahamimoff, 1967). It was found impossible to perform the same analysis for Sr; the average release of transmitter in the 10^{-4} M range of [Sr] is so low, that one needs many

thousands of responses to obtain reliable measures of the quantal content for every single Sr concentration. Since the optimal rate of stimulation cannot exceed 0·2–0·3/sec, in order to avoid accumulation of after effects (N. Moran & R. Rahamimoff, unpublished), it would have been necessary to spend several hours on each point, which is not feasible in practice. Therefore an alternative, though less satisfactory, procedure was employed. It

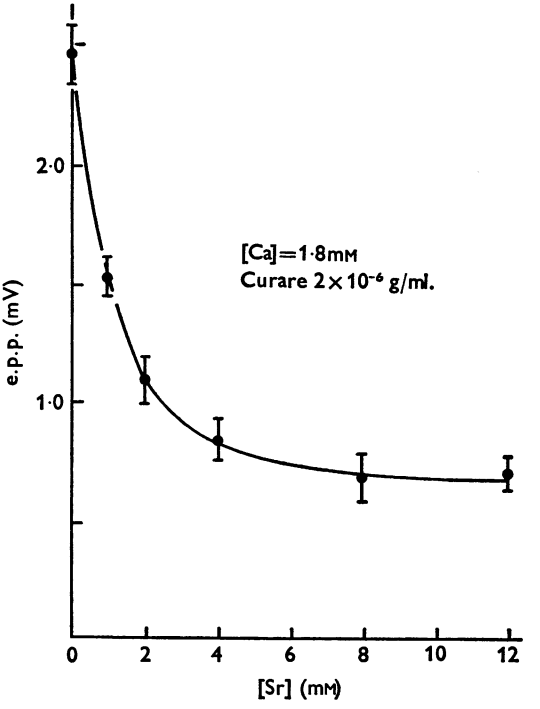

Fig. 3. Inhibition of e.p.p. amplitude by Sr ions. 1·8 mM-Ca and (+)-tubocurarine, $2 \cdot 10^{-6}$ g/ml., present throughout the experiment. Each point is an average response to sixty stimuli. Stimulation rate 0·25/sec. Vertical bars denote ± 1·0 S.E. of mean.

was assumed that since Ca and Sr are acting presumably on the same sites similar kinetics apply to both ions. Hence, the relations found for Ca (Dodge & Rahamimoff, 1967) were tested for Sr. An additional constant β was introduced to denote the relative effectiveness of SrX, so, if $\beta = 0{\cdot}5$, the effectiveness of SrX is one-half of that of Ca. Therefore the relation between quantal content and [Sr] is assumed to be

$$m = K\left(\frac{\beta[\mathrm{Sr}]/K_3}{1+[\mathrm{Sr}]/K_3}\right)^4. \qquad (5)$$

When both Ca and Sr are present, the relation between the divalent ion concentration and the release will be given by

$$m = K\left(\frac{[\mathrm{Ca}]/K_1 + \beta[\mathrm{Sr}]/K_3}{1 + [\mathrm{Ca}]/K_1 + [\mathrm{Sr}]/K_3}\right)^4, \tag{6}$$

(see also Discussion).

Estimation of K_3. Taking the fourth root of both sides of eqn. (5), one obtains a form similar to Michaelis and Menten formulation. The dissociation constant K_3 for Sr was then estimated using the double reciprocal Lineweaver & Burk plot (1934):

$$\frac{1}{\sqrt[4]{m}} = \frac{K_3}{K^{\frac{1}{4}}\beta[\mathrm{Sr}]} + \frac{1}{K^{\frac{1}{4}}\beta}. \tag{7}$$

If eqn. (5) describes the relation between [Sr] and m, then a plot of $1/\sqrt[4]{m}$ against 1/[Sr], should produce a straight line. The crossing point of this line with the abscissa gives $-1/K_3$.

Fig. 4 shows averaged e.p.p.s at different Sr concentration (no Ca ions were added to the medium). From the ratio *e.p.p. amplitude/miniature e.p.p. amplitude*, the quantal contents were calculated. Fig. 5*A* illustrates the relation of m against [Sr] on linear co-ordinates and Fig. 5*B* gives the Lineweaver & Burk (1934) plot. The resulting straight line is an indication that eqn. (5) can describe the relation between m and [Sr]. The value of K_3 was determined at six end-plates and the values were found to be in the range of 1·0–2·2 mM with mean of $1{\cdot}55 \pm 0{\cdot}38$ S.D. It is of interest to note that the mean value of K_3 for Sr, determined in this study, is not significantly different from the value of K_1 for Ca of $1{\cdot}1 \pm 0{\cdot}53$ S.D., determined by the same technique (Dodge & Rahamimoff, 1967). This suggests that under the present experimental conditions, there is not a large affinity difference between Ca and Sr toward the postulated sites X.

It is impossible in this set of experiments to distinguish between K and β in eqn. (5). However, the product $K^{\frac{1}{4}}\beta$ can be estimated from eqn. (7) and Fig. 5*B*. Having the value of $K^{\frac{1}{4}}\beta$, one can plot eqn. (5) over a wide range of Sr concentrations. The continuous line in Fig. 5*A* represents the equation

$$m = \left(\frac{2{\cdot}4[\mathrm{Sr}]/K_3}{1 + [\mathrm{Sr}]/K_3}\right)^4,$$

on linear co-ordinates. There is a reasonable fit between the experimental results and the theoretical line.

Estimation of β. Figs. 3 and 6 show that the effect of Sr on transmitter release depends on the extracellular Ca concentration. At low [Ca], addition of Sr increases the quantal output (Fig. 6) while at high [Ca] the same

concentration of Sr diminishes it. These findings were interpreted as a lower effectiveness of SrX compared to CaX in the process of transmitter liberation. The effectiveness term β cannot be determined directly from eqn. (5), but it can be estimated employing the first derivative of the

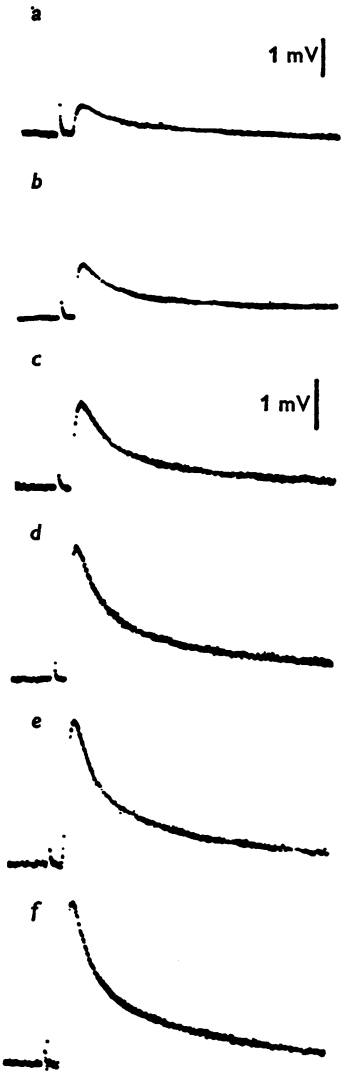

Fig. 4. The dependence of e.p.p. amplitude on Sr ion concentration. Each record is an average of sixty-six responses. Strontium concentrations are: a = 2·0 mM; b = 3·0 mM; c = 4·0 mM; d = 6·0 mM; e = 8·0 mM; f = 10·0 mM. Stimulation rate 0·16/sec. Averaging step 62·5 μsec. Upper calibration applied to a and b; lower calibration applied to c–f.

quantal content in eqn. (6) in respect of [Sr]. Solving $dm/dSr = 0$, one obtains

$$\beta = \frac{Ca'/K_1}{1+Ca'/K_1} = \frac{Ca'}{K_1+Ca'}. \tag{8}$$

This solution means that there is a Ca concentration-Ca', where addition of Sr ions does not produce any change in the quantal output. Fig. 7 illustrates three theoretical relations of m as function of [Sr], at three

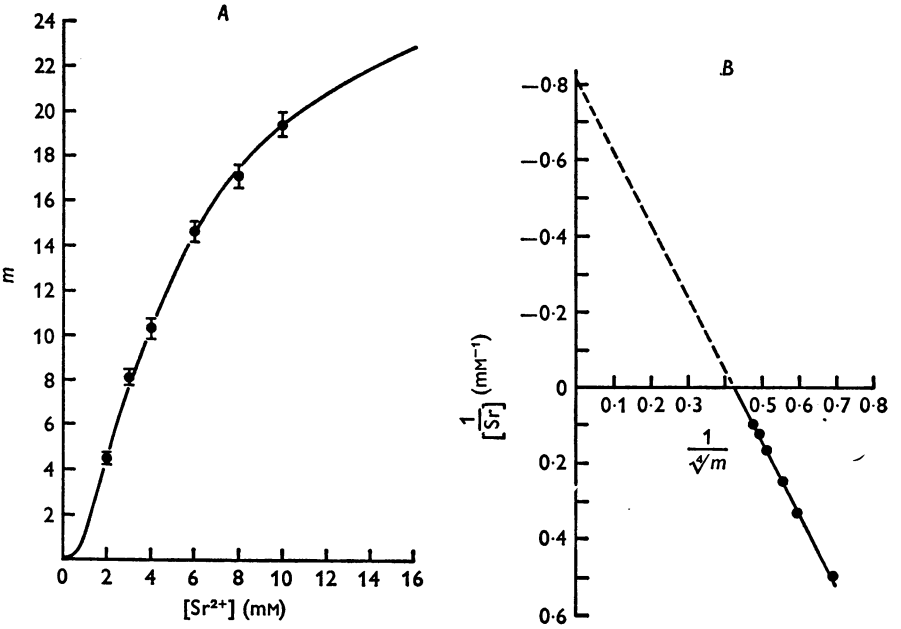

Fig. 5. The dependence of the quantal content on strontium ion concentration. The points were derived from the experimental results shown in Fig. 4. *A*, Linear co-ordinates. The points are the experimental results and the line is the theoretical relation

$$m = \left(\frac{1{\cdot}97\,[Sr]}{1+[Sr]/1{\cdot}22}\right)^4.$$

B. The same as *A* on double reciprocal relation (Lineweaver & Burk's plot (1934). From the intercept with the x-axis the value of $K_3 = 1{\cdot}22$ was estimated. From the slope and the intercept, the other constants of the equation were found.

different [Ca]([Ca] > Ca'; [Ca] = Ca'; [Ca] < Ca'). The upper curve corresponds experimentally to a situation similar to Fig. 3 and the lower curve to Fig. 6. In several experiments Ca' was estimated by interpolation and found to be 0·5–0·7 mM, giving values of β between 0·3 and 0·5. There-

fore the maximal release in Sr is $(0{\cdot}3)^4$ to $(0{\cdot}5)^4$ of the maximal release in Ca. In other words, at same molar concentrations the expected release in Sr is only 0·81–6·25 % of the corresponding release in Ca. Such ratios were indeed found in practice.

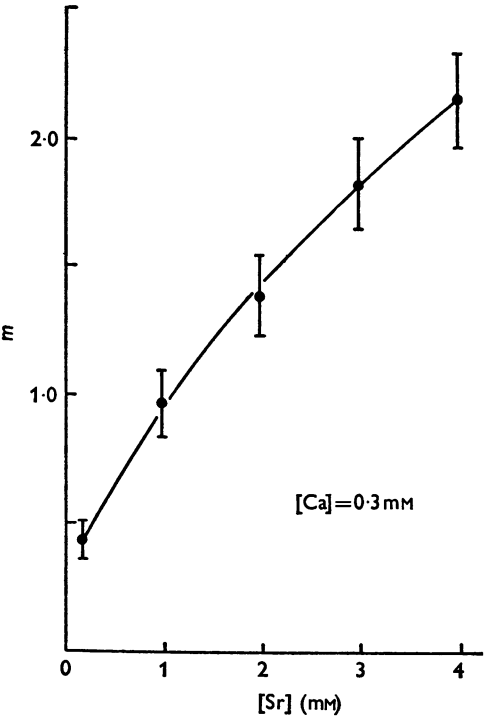

Fig. 6. The relation between [Sr] and transmitter release in the presence of Ca = 0·3 mM. Quantal content was estimated by the 'failure and success' method

$$m = \ln\left(\frac{\text{Number of stimuli}}{\text{Number of failures}}\right).$$

Vertical bars denote ± 1·0 S.E. of mean.

The effect of Mg ions on Sr-induced release

The above-mentioned experiments suggest that Ca and Sr exert their action by acting on the same postulated sites on the presynaptic nerve terminal. It is expected, therefore, that competitive inhibitors of Ca should inhibit also the Sr-induced release. Such an inhibitor is Mg (Jenkinson, 1957); and indeed addition of Mg decreases the mean amplitude of the e.p.p. and the mean quantal content (Fig. 8*A–F*).

The peculiar way of the interaction between Ca and Sr permits a further

prediction. The form of eqns. (6) and (8) for the case where Mg ions are also present, should be respectively

$$m = K\left(\frac{[\mathrm{Ca}]/K_1+\beta[\mathrm{Sr}]/K_3}{1+[\mathrm{Ca}]/K_1+[\mathrm{Mg}]/K_2+[\mathrm{Sr}]/K_3}\right)^4, \tag{9}$$

and

$$\beta = \frac{Ca'/K_1}{1+Ca'/K_1+[\mathrm{Mg}]/K_2}, \tag{10}$$

or rearranging eqn. (10) obtains

$$Ca' = \frac{\beta K_1(1+[\mathrm{Mg}]/K_2)}{1-\beta}. \tag{11}$$

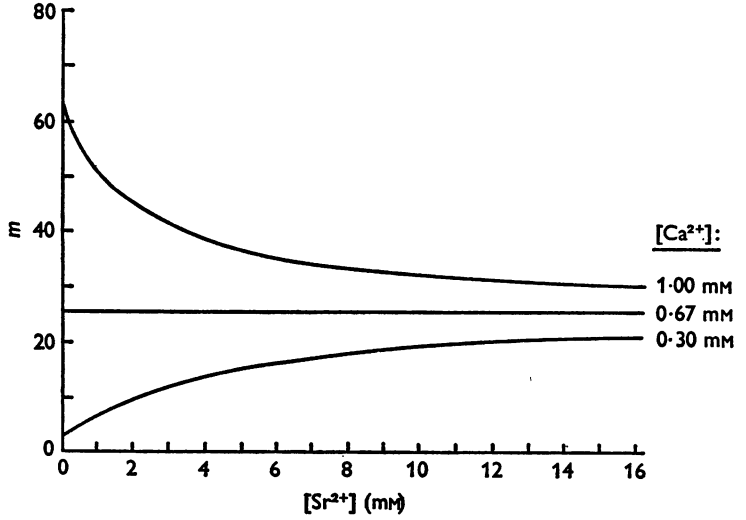

Fig. 7. Theoretical relation between [Sr] and quantal content at three different extracellular [Ca] as shown in Figure. The relations are according to the equation

$$m = K\left(\frac{\beta[\mathrm{Sr}]/K_3+[\mathrm{Ca}]/K_1}{1+[\mathrm{Ca}]/K_1+[\mathrm{Sr}]/K_3}\right)^4,$$

where $K = 1000$; $K_3 = 1{\cdot}5$; $K_1 = 1{\cdot}0$; $\beta = 0{\cdot}4$. Compare Fig. 3 with Fig. 6 and see text.

From eqn. (11) it follows that Ca' depends on the magnesium concentration in the medium. The higher the [Mg], the higher will be Ca'. This predicted property of the system suggested the following experiment: the averaged e.p.p. amplitude and quantal content were estimated at 1·0 mM-Ca (Fig. 9*A*). Addition of Sr (2·0 mM) as expected reduced $\bar{R}$ and m (Fig. 9*B*). Now the composition of the medium was changed to contain 1·0 mM-Ca and 4·0 mM-Mg (Fig. 9*C*). Since the competitive inhibitor Mg is present, mean R and m were smaller than in Fig. 9*A*. The addition of

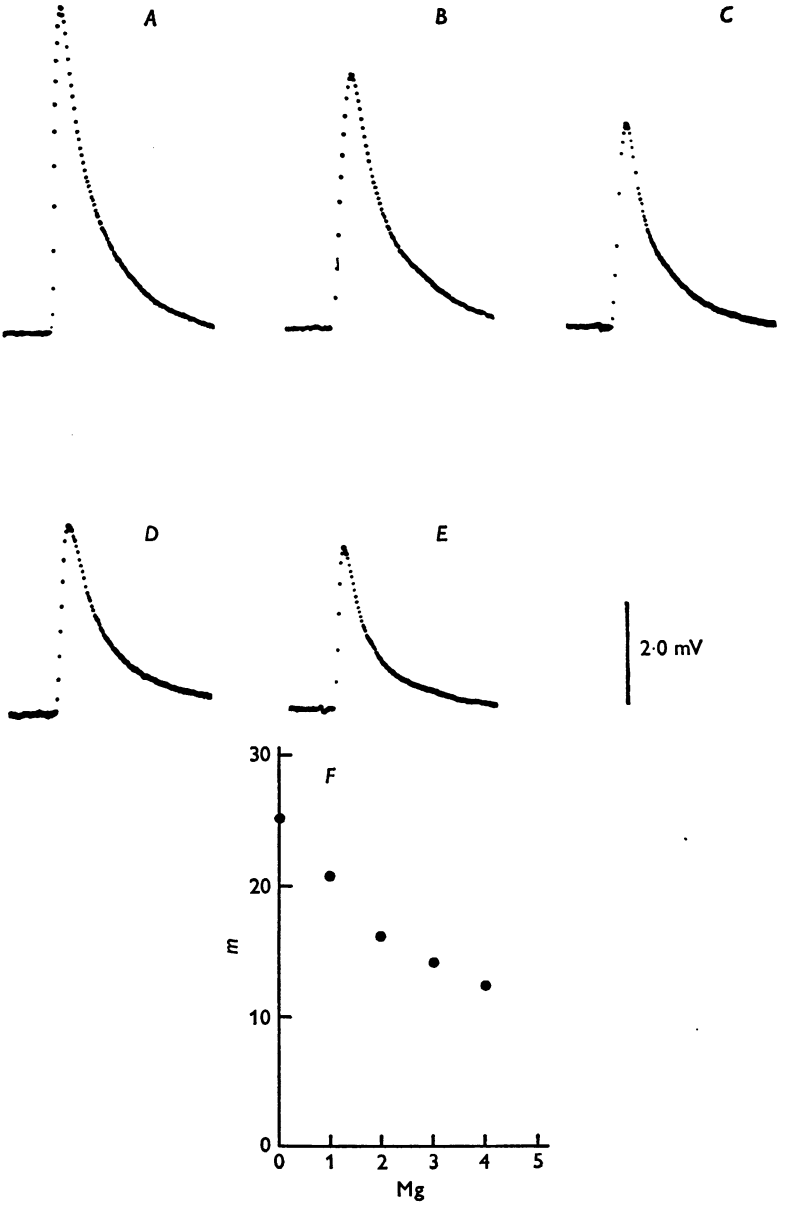

Fig. 8. Inhibitory effect of Mg ions on transmitter release. 4·0 mM-Sr present throughout the experiment. Average responses to 120 stimuli; *A*, Mg = 0·0 mM; *B*, Mg = 1·0 mM; *C*, Mg = 2·0 mM; *D*, Mg = 3·0 mM; *E*, Mg = 4·0 mM. Averaging step 250 μsec. Voltage calibration = 2·0 mV. Stimulation rate 0·22/sec. *F*, Quantal content as function of [Mg]. The points were derived from the experimental results in *A–E*.

2·0 mM-Sr under these circumstances *increased* $\bar{R}$ and m instead of reducing them (Fig. 9*D*). Therefore, one can convert the inhibitory effect of Sr to a facilitatory effect by introducing Mg ions into the system.

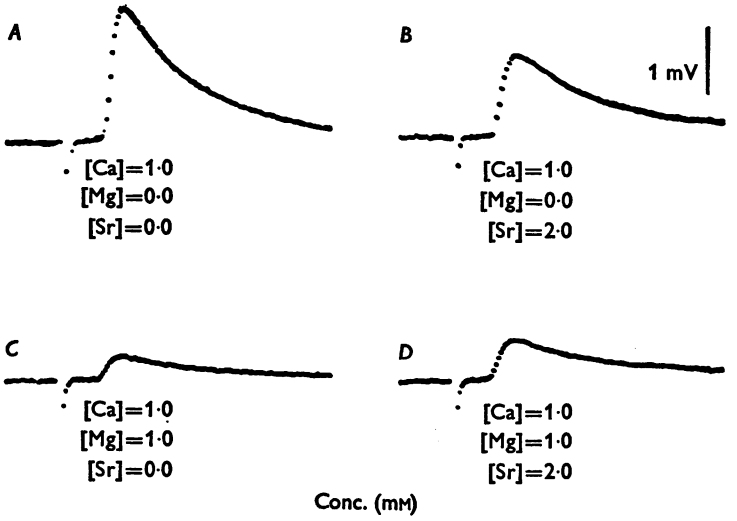

Fig. 9. Triple interaction among Ca, Sr and Mg ions. Concentration shown in Figure. Each figure is the average of fifty-five responses. Averaging step 125 μsec. Stimulation rate 0·1/sec. Quantal contents were estimated from N responses, which included the fifty-five shown in the average. $A, m = 150{\cdot}6$ ($N = 119$); $B, m = 107{\cdot}1$ ($N = 113$); $C, m = 25{\cdot}9$ ($N = 90$); D, $m = 33{\cdot}3$ ($N = 107$) (see text). $0{\cdot}7 \times 10^{-6}$ g/ml (+) −tubocurarine present throughout the experiment.

DISCUSSION

Among the various divalent ions examined until now, Sr is the only one that is able to replace Ca consistently in its action on transmitter liberation (Miledi, 1966; Dodge *et al.* 1969; Katz & Miledi, 1969*a*, *b*). The first point that needs to be discussed is whether the effect of Sr is a genuine one on the release, or whether Sr displaces some Ca ions from stores in the muscle. The results of Miss Eilander (see Methods) show that this possibility can be ruled out. The amount of Ca that diffuses out of the muscle is too small to create any appreciable effects on transmitter liberation. Furthermore, the competition between Ca and Sr, and the triple interaction of Ca, Sr and Mg cannot be explained by diffusion of Ca from the muscle to the extracellular space. Therefore, it can be concluded that Sr ions have a direct effect on quantal transmitter release.

The mode of action of Sr is quite similar to that of Ca. Both ions act presumably on the same presynaptic sites. The main difference in their influence is quantitative: Sr is less potent than Ca. This difference in

potency can formally be due to a lower affinity of Sr for the postulated sites X (high K_3), due to a lower effectiveness of the SrX complexes or due to a higher co-operative number (n). The latter possibility cannot be ruled out. However, the measured slopes of the log [Sr]–log release relations are between 1·0 and 3·0 for a range of concentration where Ca ions also produce similar slopes. Therefore, it was assumed that the same n applies to Sr and Ca. The minimal n that fitted the experimental data for Ca was 4 (Dodge & Rahamimoff, 1967), hence the same number was used for Sr.

After assuming that $n = 4$, it was possible to check whether the difference in potency is due to the affinity constant or the effectiveness. The results presented in Figs. 2, 3 and 5 show that the dissociation constants for Ca and Sr are similar, and that the main difference is in the effectiveness of the complexes: SrX is β times less effective than CaX. Fig. 10 shows the effect of various β on a theoretical relation between divalent ion concentration and the average number of quanta liberated per nerve impulse, assuming equal affinity constants. For example, when β is 0·4, the appropriate quantal content is only 2·56 % of the value at $\beta = 1{\cdot}0$. The suggested general equation describing the relation between the concentration of a divalent ion combining with X (MeX) and release is therefore

$$m = K\left(\frac{\beta_{Me}[Me]/K_{Me}}{1+[Me]/K_{Me}}\right)^n, \qquad (12)$$

where β_{Me} is the relative potency of the MeX complex compared to CaX, and K_{Me} is the dissociation coefficient of the $Me + X \rightleftarrows MeX$ reaction. As can be seen from Fig. 10, divalent ions with small β (less than 0·15) will produce negligible release. These ions will compete with Ca (or with Sr) on the postulated sites X, and serve therefore as competitive inhibitors. The effect of an inhibitor, present at a constant concentration throughout an experiment, is to change the apparent values of the dissociation constants. Therefore the [Na] term was omitted from the various equations (see Colomo & Rahamimoff, 1968). The equation relating the concentrations of the two activating divalent ions (Ca and Sr in this case) and release (eqn. 6), is based on the assumptions that Ca and Sr are acting on the same sites and that the two ions can co-operate. The fraction of sites occupied by Ca is given by: $CaX = ([Ca]/K_1)/(1+[Ca]/K_1+[Sr]/K_3)$ and by Sr: $SrX = ([Sr]/K_3)/(1+[Ca]/K_1+[Sr]/K_3)$. If the relative effectiveness is β, then the total fraction of active sites is $(MeX)_{active} = CaX + \beta SrX$. Quantal release is then: $m = K(MeX)^n_{active}$; the explicit form of this relation is given by eqn. (6).

The physical basis of the reduced effectiveness of SrX depends on the interpretation of what the critical releasing sites X are. There is strong

evidence that the entry of calcium ions through the presynaptic nerve membrane caused by depolarization is the important factor in the coupling between depolarization and transmitter release (Katz & Miledi, 1967). Recently the evidence was strengthened by experiments performed by Katz & Miledi (1969*b*) on the tetrodotoxin- and tetraethylammonium-treated stellate ganglion of the squid. In this preparation presumably an

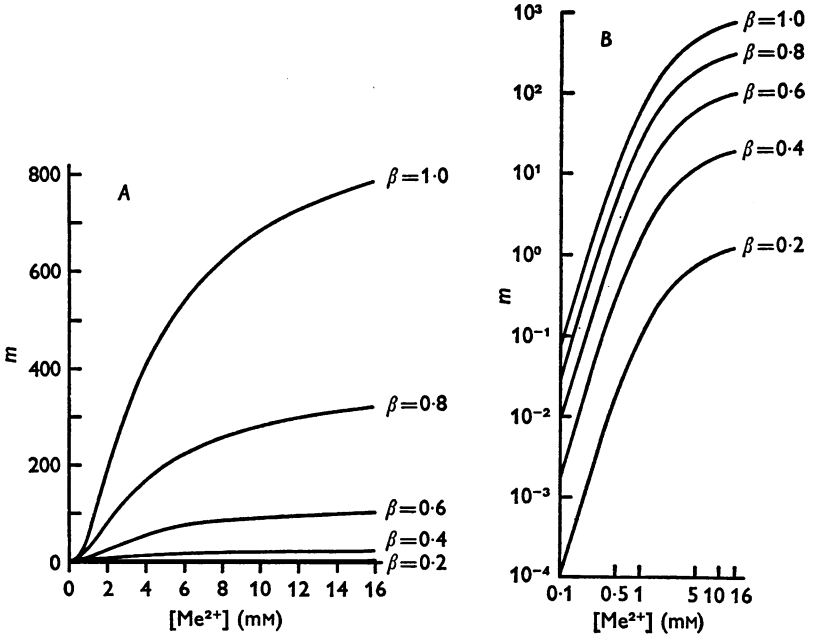

Fig. 10. Influence of β on the theoretical relation between m and activating divalent ion concentration. *A*, Linear co-ordinates; *B*, double logarithmic co-ordinates. The relations follow the equation

$$m = K\left(\frac{\beta_{Me}[Me]/K_{Me}}{1+[Me]/K_{Me}}\right)^4,$$

where $K = 1000$, $K_{Mc} = 1{\cdot}0$ mM and β_{Me} has the successive values of 1·0, 0·8, 0·6, 0·4 and 0·2 (see text).

entry of Ca ions occurs, associated with transmitter release. This Ca hypothesis can be easily applied to our case. Assuming that the sites X are the channels through which Ca and Sr enter the nerve terminal, the similar affinity of both ions could be interpreted as equal probabilities of occupying the channel. If the important parameter is the concentration of the activating divalent ion on the inner side of the nerve membrane or the concentration *in* the nerve terminal, then it might be expected that the heavier Sr ions will move slowly, will create a lower concentration in the

relevant location and therefore will be less effective on a molar basis. It would be expected also that the reverse movement of Sr to the outer surface of the nerve membrane will be slower and therefore the residual increased probability of release after the e.p.p. will be prolonged (N. Moran & R. Rahamimoff, unpublished).

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