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LANTHANUM AS A SURROGATE FOR CALCIUM IN TRANSMITTER RELEASE AT MOUSE MOTOR NERVE TERMINALS

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SUMMARY

1. The mechanism by which lanthanum (La^{3+}) causes an increased frequency of miniature end-plate potentials (m.e.p.p.s) was studied at the mouse neuromuscular junction.

2. At concentrations as low as 0·25 μM, La^{3+} caused a progressive rise in m.e.p.p. frequency, to a maximum of several hundred per second. 'Washing' with solution containing EDTA arrested the rise, but did not substantially reduce the raised m.e.p.p. frequency. At partially 'lanthanized' junctions high frequencies of m.e.p.p.s were maintained indefinitely, even in 0 Ca^{2+}/EDTA solutions.

3. The rate of development of high m.e.p.p. frequency was increased by repetitive nerve stimulation or by depolarization of the nerve terminal (high K^+ or focally applied current), and appeared to be proportional to the concentration of La^{3+} over the range of 0·25–5 μM.

4. At low concentrations of La^{3+} the rise of m.e.p.p. frequency depended upon the co-presence of a small amount of Ca^{2+} (> 10 μM) and was slowed and partially blocked by Cd^{2+}, or by Ca^{2+} at about 10 μM.

5. The quantal content of end-plate potentials was usually reduced in the presence of La^{3+}, but was increased over control values after removal of La^{3+} by 'washing' with solution containing EDTA, once a raised m.e.p.p. frequency had developed.

6. At partially lanthanized junctions the absolute increases in m.e.p.p. frequency produced by Ca^{2+} (in raised K^+), ethanol, or by nerve stimulation in the presence of Ba^{2+}, were greater than at control junctions, but in each case the increases in the logarithm of m.e.p.p. frequency were less than at control junctions.

7. It is concluded that La^{3+} causes transmitter release only after entry into the nerve terminal via voltage-sensitive channels, probably those that normally admit Ca^{2+}, that La^{3+} and Ca^{2+} may co-operate at internal sites to induce transmitter release, and that these ions both co-operate and compete at external sites that regulate their entry into the nerve terminal.

INTRODUCTION

It is well documented that at the neuromuscular junction Ca^{2+} enters the nerve terminal via voltage-gated channels and functions as the mediator linking transmitter release to nerve terminal depolarization (Katz, 1969; Silinsky, 1985). Among other divalent ions, it appears that Sr^{2+} and possibly Ba^{2+} may substitute, at least in part, for Ca^{2+} (Miledi, 1966; Silinsky, 1978). It was pointed out by Lettvin, Pickard, McCulloch & Pitts (1964) that Ca^{2+} and La^{3+} have roughly similar radii, but that with its higher valence La^{3+} might be bound much more strongly at some sites; it was verified that in terms of nerve blocking La^{3+} acts as if it were equivalent to an extraordinarily high Ca^{2+} concentration (Takata, Pickard, Lettvin & Moore, 1966). At the neuromuscular junction, Blioch, Glagoleva, Liberman & Nenashev (1968) found La^{3+} to be much more effective than divalent cations in increasing the frequency of miniature end-plate potentials (m.e.p.p.s), a result confirmed by Heuser & Miledi (1971) and DeBassio, Schnitzler & Parsons (1971), and suggested that La^{3+} might enter the terminal in the same way as Ca^{2+} and subsequently activate the release process. In agreement with this hypothesis Blioch *et al.* (1968) also reported a small increase of quantal contents of end-plate potentials (e.p.p.s) with La^{3+}, in nominally Ca^{2+}-free solution, but Miledi (1966, 1971), Heuser & Miledi (1971) and DeBassio *et al.* (1971) found only a profound depression of the e.p.p. by La^{3+}. It is notable, however, that La^{3+} appears to be essentially irreversible as an activator of release; the fall in m.e.p.p. frequency that occurs subsequent to a large rise can be attributed to vesicle depletion (Heuser & Miledi, 1971), and therefore one might expect, if Blioch *et al.* (1968) were correct, not an e.p.p. with La^{3+}, but rather a stepwise increase in m.e.p.p. frequency with each individual injection of La^{3+} by a nerve impulse.

In the present experiments we have tested the possibility that La^{3+} might indeed act to produce transmitter release only after entry into the nerve terminals via voltage-sensitive channels, and have found evidence that this may well be the case.

METHODS

The technique employed for the mounting, superperfusion and focal polarization of nerve terminals in the phrenic nerve diaphragm preparation have been described elsewhere (Cooke & Quastel, 1973*a*). In order to minimize the number of animals needed for a series of experiments, the whole diaphragm was excised from mice anaesthetized with ether, was mounted on Sylgard, and maintained in oxygenated buffer. Small strips were cut from this as needed and were used for each exposure to La^{3+}. Experiments were carried out at room temperature (25–29 °C).

Solutions

Because La^{3+} precipitates in bicarbonate/phosphate-buffered solutions, experiments were carried out in solutions bubbled with 100 % O_2, buffered with HEPES (3 mM), pH 7·3. In some experiments the chloride ion was largely replaced with nitrate, to increase the amplitude of m.e.p.p.s. Solutions were made isosmotic by the addition of the appropriate amount of sucrose. Except for experiments in which the nerve was stimulated, tetrodotoxin (0·5 μM) was added to solutions to prevent spontaneous generation of nerve action potentials, since it was previously observed that with very low [Ca^{2+}] spontaneous e.p.p.s and muscle twitching occurred immediately after switching to solution containing sufficient Ca^{2+} to permit neuromuscular transmission. For removal of La^{3+} the preparation was 'washed' with solution containing EDTA, either with or without an excess of Ca^{2+};

Silen & Martell (1971) quote the dissociation constant of La EDTA complex as $10^{-15.5}$, five orders of magnitude smaller than that for Ca EDTA ($10^{-10.5}$).

Estimation of m.e.p.p. frequency and quantal content of e.p.p.s

M.e.p.p.s were counted either from a Mingograf ink-jet record or using a PDP-12 computer; each computer count was monitored on the ink-jet recorder. For following the time course of changes in m.e.p.p. frequency (f_m) two protocols were generally used: (1) continuous recording at individual junctions; (2) 'multiple sampling': muscle fibres were penetrated randomly at a rate of 2–5 per minute, for up to 2 h, and the time of penetration and the f_m (counted from the ink trace) for each junction was recorded. The results were subsequently processed to obtain mean log f_m for non-overlapping sequential time 'bins'. To exclude junctions with unusually high f_m (perhaps due to damage to the nerve terminal by the micro-electrode) or unusually low f_m (see Results), individual values of log f_m were disregarded if more than two standard deviations from a running mean from three adjacent time bins. This procedure in effect eliminated about 7–10% of values, and made little difference to mean values.

Quantal content of e.p.p.s was estimated either by the method of failures (del Castillo & Katz, 1954) or as the ratio of e.p.p. height to the height of m.e.p.p.s.

RESULTS

Preliminary observations

Preliminary experiments were performed using solutions containing no added Ca^{2+}, so that it would be possible to vary the K^+ concentration without producing Ca^{2+}-dependent release. As previously reported by Heuser & Miledi (1971) for the frog neuromuscular junction, it was observed that application of La^{3+} caused a rise in spontaneous m.e.p.p. frequency. With continued application of La^{3+}, the frequency at various junctions reached a maximum usually in the range 100–1000 s^{-1}, and subsequently subsided slowly, taking at least an hour to revert to control values. However, the high frequency produced by La^{3+} was relatively well maintained if La^{3+} was used at a moderate concentration (<50 μM) and was apparently maintained indefinitely when La^{3+} was removed and the preparation was washed with solution containing EDTA (100 μM, either with or without extra Ca^{2+}) before or after m.e.p.p. frequency had attained its maximum. From one junction to another the behaviour was notably erratic with no consistent relationship between the concentration of La^{3+} and the rate of rise of m.e.p.p. frequency or the maximum frequency attained. In the result shown in Fig. 1 it is notable that m.e.p.p. frequency rose little if at all in the presence of 20 μM-La^{3+} until 1 min after the solution was switched from 5 to 15 mM-K^+, but at other junctions there sometimes appeared a lag of up to 10 min even if raised K^+ were present from the start. This Figure also illustrates a consistent finding, that the continuous rise of m.e.p.p. frequency could be interrupted by EDTA and re-established by re-application of La^{3+}, either in the presence or absence of Ca^{2+}. Simple withdrawal of La^{3+} usually did *not* interrupt the rise. Moreover, in the presence of raised K^+ a Ca^{2+}-sensitive component of m.e.p.p. frequency could often be detected (Fig. 1), provided the effect of La^{3+} had not reached its maximum.

Subsequently, it was found that if the tissue were maintained in solution containing at least 50 μM-Ca^{2+}, chosen as sufficiently low to exclude virtually all Ca^{2+}-dependent K^+-evoked transmitter release (Cooke, Okamoto & Quastel, 1973), the behaviour at different end-plates became much less erratic, and the characteristic rise in m.e.p.p. frequency could be produced at concentrations of La^{3+} as low as 0·1 μM. However,

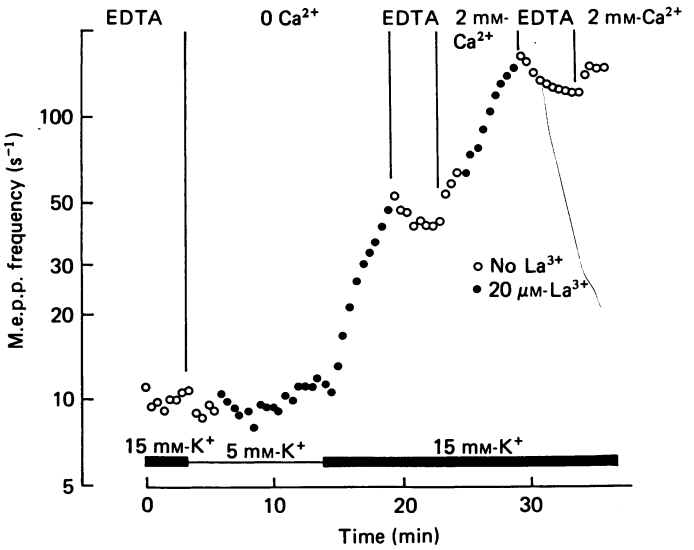

Fig. 1. Rise in m.e.p.p. frequency (f_m) produced by 20 μM-La^{3+} in 0 Ca^{2+} solution. Note failure of f_m to rise with exposure to La^{3+} (filled symbols) until 1 min after the switch from 5 mM to 15 mM-K^+. The progressive rise in f_m was halted by washing out the La^{3+} with EDTA, and re-established by re-application of La^{3+}, now in the presence of 2 mM-Ca^{2+}. Note the persistence of a relatively small Ca^{2+}-dependent component of f_m in 15 mM-K^+, superimposed on the high f_m produced by La^{3+}. The base-line f_m at this junction was relatively high (10 s^{-1} rather than about 1 s^{-1} or less) because of previous exposure of the preparation to La^{3+} and raised K^+.

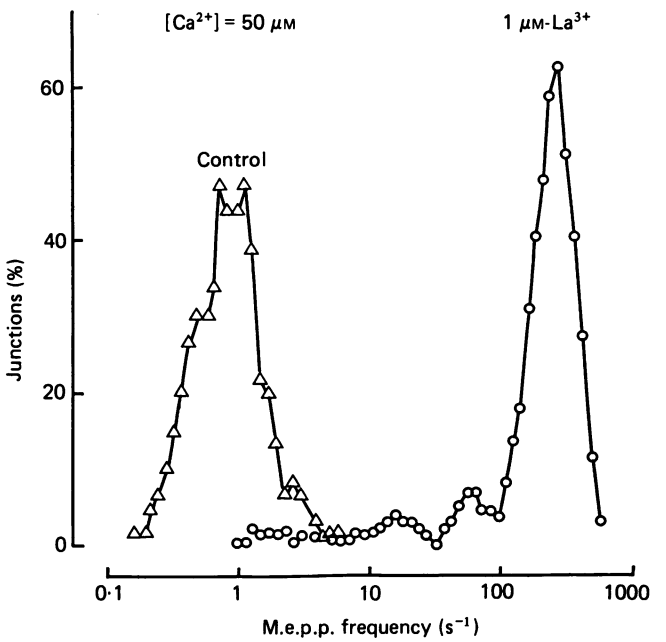

Fig. 2. Running-bin histogram of distribution of all m.e.p.p. frequencies found in a random sample of junctions in a diaphragm before (triangles) and after (circles) exposure to La^{3+} (1 μM for 2 h. [Ca^{2+}] was 50 μM). A substantial number of junctions appear to fail to respond to La^{3+} (m.e.p.p. frequency less than 100 s^{-1}). The bin size was 0·301 $\log_{10}$ units (i.e. 1 $\log_2$ unit), with individual steps of one-fifth this size.

there remained considerable variation in the response to La^{3+}, both between different preparations and between different junctions in the same preparation. Fig. 2 shows the variation from one junction to another in one diaphragm, obtained by 'multiple sampling' before and after 30 min exposure to 1 μM-La^{3+} in 15 mM-K^+. A substantial number of junctions do not have the high m.e.p.p. frequency typical of 'lanthanized'

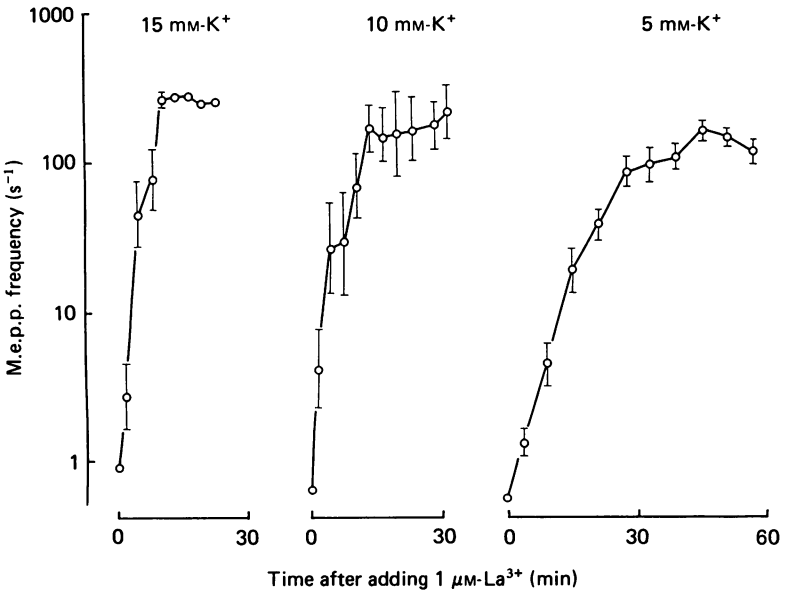

Fig. 3. Rise in m.e.p.p. frequency (f_m) produced by 1 μM-La^{3+}, in the presence of 50 μM-Ca^{2+}, in three different diaphragms equilibrated in 5, 10 or 15 mM-K^+ respectively. Each point represents the mean log f_m from five to twenty junctions, with S.E. of mean. The rate of rise of f_m produced by exposure to La^{3+} was greater in raised K^+ solutions, although the maximum f_m attained was similar in all cases.

junctions. There may be two reasons for the apparent failure to respond. (1) Junctions which are deep within the tissue may not have been sufficiently exposed to La^{3+}. Even with exposure times of the order of 1 h, we had the impression that junctions deep in the tissue consistently had lower m.e.p.p. frequencies than superficial junctions, suggesting that La^{3+} may not easily penetrate the tissue, perhaps because it is bound to extracellular sites and/or precipitated by bicarbonate produced by metabolism. (2) Some of the junctions with relatively low m.e.p.p. frequency might in fact have responded very quickly to the La^{3+}, giving a high m.e.p.p. frequency which subsequently declined (Heuser & Miledi, 1971).

Voltage dependence of increase of m.e.p.p. frequency by La^{3+}

Fig. 3 shows the response to 1 μM-La^{3+} in 5, 10 and 15 mM-K^+ solutions in the presence of 50 μM-Ca^{2+}; the data were obtained using multiple sampling in three different diaphragms. It is evident that the rate of development of high m.e.p.p. frequency was increased with raised K^+ concentration, while the maximum frequency attained was little if at all influenced.

In contrast to the rise produced by La^{3+} in solution containing 50 μM-Ca^{2+} and

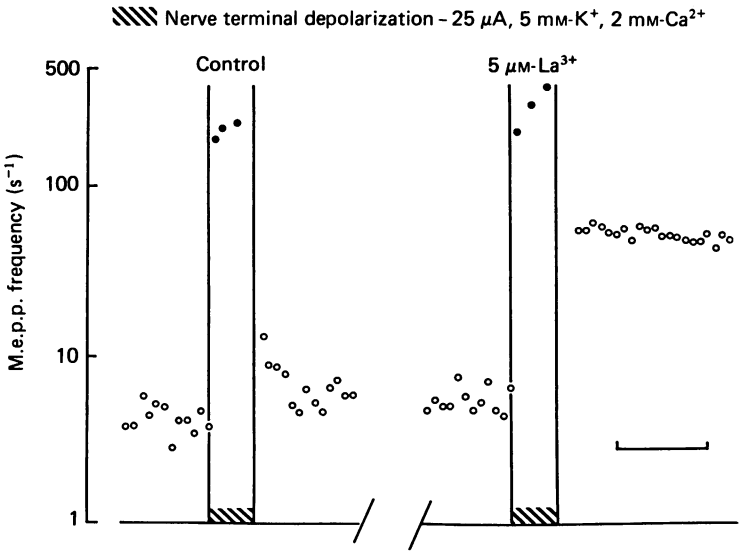

Fig. 4. Induction of 'lanthanization' by focal depolarization of the nerve terminal hatching). In the presence of 5 μM-La^{3+}, but not in the control, focal depolarization caused a maintained elevation of m.e.p.p. frequency. Solutions contained 5 mM-K^+, 2 mM-Ca^{2+}.

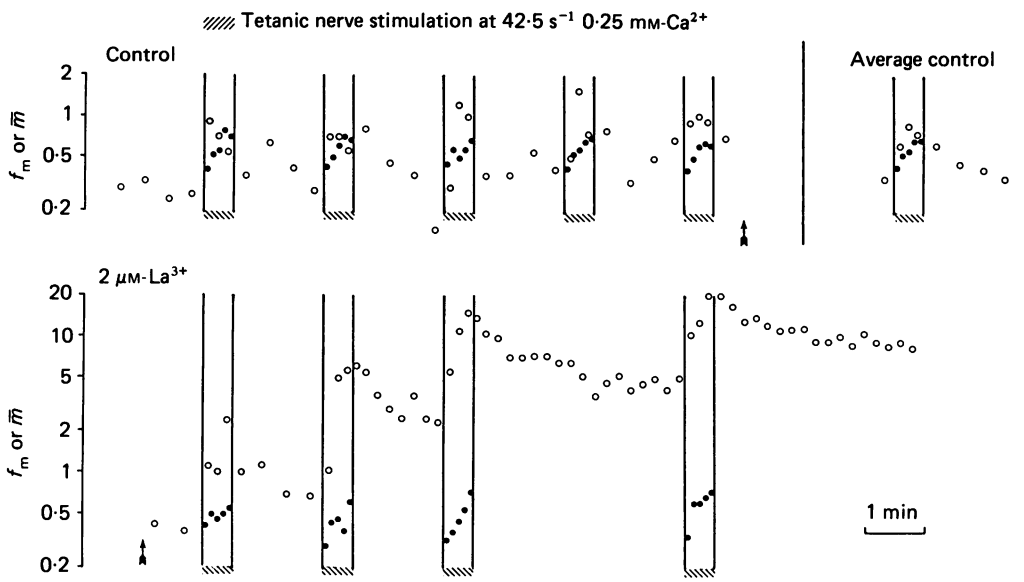

Fig. 5. Induction of 'lanthanization' by tetanic nerve stimulation (42·5 s^{-1}, 30 s). All solutions contained 0·25 mM-Ca^{2+}/5 mM-K^+. In the control (upper panel) the high m.e.p.p. frequency (f_m; ○) induced by each tetanus declined to normal between bouts of stimulation; 'average control' represents the mean of data from the five control tetani shown. 2 μM-La^{3+} was added at the arrow (end of upper panel, beginning of lower panel), and in the presence of La^{3+} each tetanus caused a maintained step rise in m.e.p.p. frequency (○) which was superimposed upon the transient post-tetanic potentiation that apparently continued unchanged. The quantal content of e.p.p.s ($\bar{m}$; ●), and the rise in $\bar{m}$ during each tetanus were apparently unaffected by the lanthanization.

5 mM-K^+, in 2 mM-Ca^{2+}/5 mM-K^+ there was no appreciable increase in m.e.p.p. frequency for at least 1 h, at least at concentrations of up to 10 μM-La^{3+}. However, when nerve terminals were depolarized by focally applied current in the presence of 5 μM-La^{3+}, there was a stepwise, irreversible increase (Fig. 4), again indicating that 'lanthanization' may reflect the entry of La^{3+} through voltage-sensitive channels. Surface potential effects (Frankenhaeuser & Hodgkin, 1957) might account for these channels being open more at 50 μM than at 2 mM-Ca^{2+}, in 5 mM-K^+.

TABLE 1. Modification by La^{3+} of m.e.p.p frequency and quantal content of e.p.p.s

	M.e.p.p. frequency			Quantal content		
Junction	Control	La^{3+}	Re-control	Control	La^{3+}	Re-control
			(A) No previous La^{3+}			
1	0·5	45	35	0·43	0·91	2·1
2	0·4	0·3	2·4	0·08	0·05	0·12
3	0·15	1·5	6·2	0·08	0·03	0·38
4	0·6	8·3	77	0·68	0·11	1·2
			(B) Previously partially lanthanized			
2	2·4	9·2	8·8	0·12	0·06	0·18
2	8·8	36	43	0·18	0·13	0·40
5	25	147	219	0·69	0·14	0·71
6	34	31	38	0·36	0·17	0·45

In each case the control and re-control solutions were the same (5 mM-K^+/low Ca^{2+}/raised Mg^{2+}) and exposure to La^{2+} (5 μM) was for several minutes. Nerve stimulation was continued at 5 or 10 Hz throughout. In the columns headed La^{3+} the values given are for immediately before 'washing' the preparation with control solution with added Ca EDTA (100 μM), and hence about 2 min before the 're-control'. Units of m.e.p.p. frequency are s^{-1}.

Repetitive stimulation of the nerve also produced an irreversible rise in m.e.p.p. frequency (f_m) in solutions containing La^{3+}. Fig. 5 shows the changes in f_m produced by repeated 30 s bouts of nerve stimulation at 42·5 s^{-1}, in 0·25 mM-Ca^{2+}. In the presence of 2 μM-La^{3+}, in contrast to the controls, each tetanus produced a maintained increase in f_m. It is notable that the facilitation of quantal content and of f_m during and after each tetanus appeared to persist, apparently unchanged, as the junction became 'lanthanized'. In this particular case the quantal content of the e.p.p.s ($\bar{m}$) was apparently little affected by the La^{3+}. However, when the preparation was subsequently 'washed' with solution containing 100 μM-added Ca EDTA and returned to control solution (0·25 mM-Ca^{2+}, no La^{3+}) '$\bar{m}$' was five times the control value. Tetanic stimulation continued to multiply '$\bar{m}$' and f_m to much the same extent as before and during exposure to La^{3+}. In Table 1 are listed other examples of increase in '$\bar{m}$' by lanthanization.

Relation between rate of rise of m.e.p.p. frequency and [La^{3+}]

The rise in m.e.p.p. frequency produced by 0·25, 0·5, 1, 2 and 5 μM-La^{3+}, in the presence of 15 mM-K^+ and 50 μM-Ca^{2+}, was followed at single junctions. The frequency eventually attained was much the same in all cases, but the rate of development depended on the concentration of La^{3+}. An arbitrary index of the rate of lanthanization, the inverse of the time taken for frequency to traverse the range

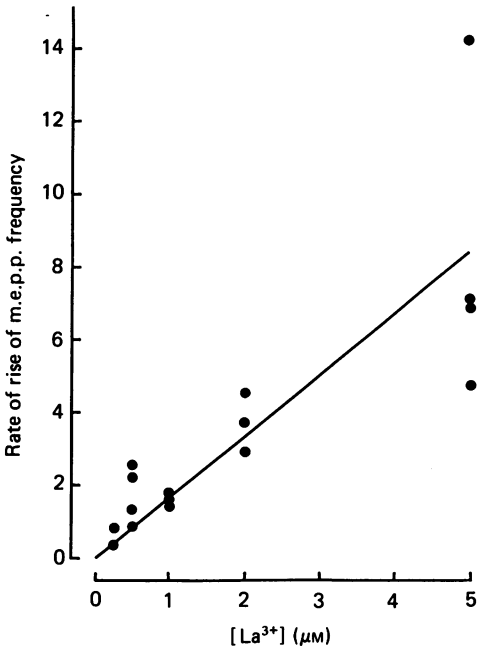

Fig. 6. Dependence of lanthanization on $[La^{3+}]$. Each point represents data from one junction in one diaphragm, in 15 mM-K^+ and 50 μM-Ca^{2+}. The 'rate of rise' of m.e.p.p. frequency (f_m) is in each case the inverse of the time taken for f_m to progress through the range 10–50 s^{-1}. Rate of rise of f_m appears to be linearly related to La^{3+} concentration.

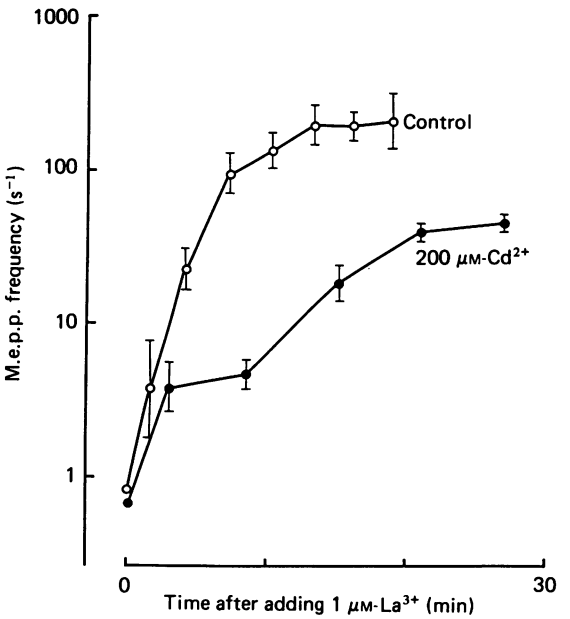

Fig. 7. Inhibition by 200 μM-Cd^{2+} (filled symbols) of the rise of mean m.e.p.p. frequency produced by 1 μM-La^{3+} in the presence of 15 mM-K^+/50 μM-Ca^{2+}. Compared to a control diaphragm (open symbols), 200 μM-Cd^{2+} slows the rate of rise and reduces the maximum attained. Error bars are ±S.E. of mean.

10–50 s^{-1}, is shown plotted *vs.* [La^{3+}] in Fig. 6. The results suggest that La^{3+} accumulates within the nerve terminal at a rate proportional to the external concentration.

Attempted blockade of La^{3+}-induced rise in m.e.p.p. frequency

If La^{3+} enters nerve terminals via the voltage-sensitive channels that normally admit Ca^{2+}, the response to La^{3+} would be expected to be blocked by agents that block Ca^{2+}-dependent transmitter release at depolarized nerve terminals. In control experiments we found that 200 μM-Cd^{2+} (cf. Shapovalov, 1962; Kostyuk & Krishtal,

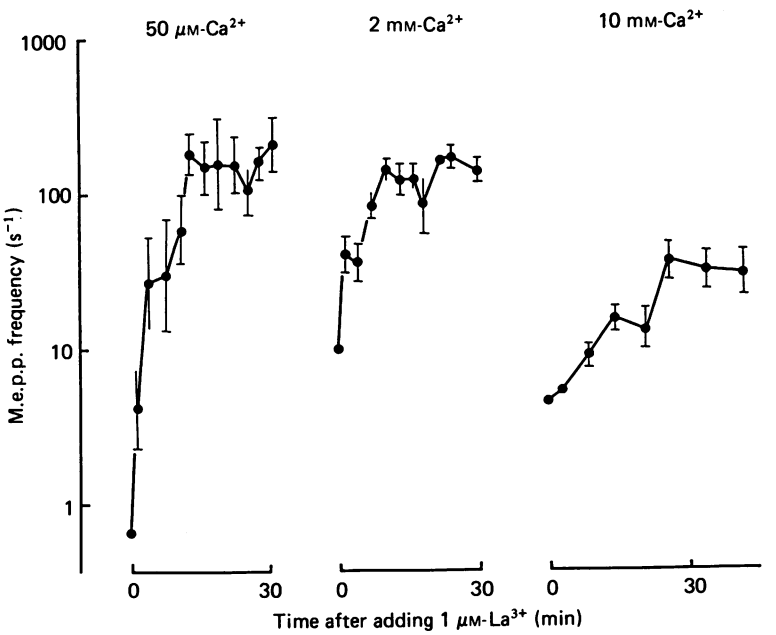

Fig. 8. Rise in m.e.p.p. frequency produced by exposure to 1 μM-La^{3+} in three diaphragms equilibrated in 50 μM, 2 mM and 10 mM-Ca^{2+} respectively, all in 10 mM-K^+. At 10 mM, Ca^{2+} reduces the rate of development of high m.e.p.p. frequency and the maximum attained. Error bars are $\pm$S.E. of mean.

1977) obliterated the response to Ca^{2+} or Ba^{2+} in 15 mM-K^+. As shown in Fig. 7, the rise of m.e.p.p. frequency produced by 1 μM-La^{3+} in 15 mM-K^+ was indeed slowed by this concentration of Cd^{2+}. However, the maximum frequency attained was also reduced which was not the case when the concentration of La^{3+} or K^+ was varied.

Silinsky (1977) has reported that Ca^{2+}, as an *antagonist* of Ba^{2+} entry into the terminal, has an apparent dissociation constant, K_i, of 0·12 $\pm$ 0·02 mM. Fig. 8 shows the effect of varying Ca^{2+} on the response to La^{3+} in 10 mM-K^+. Ca^{2+} at 2 mM did *not* apparently cause any decrease in the rate of rise of m.e.p.p. frequency caused by the La^{3+}; the picture is of course complicated by the 'base line' frequency, increased from < 1 s^{-1} in 50 μM-Ca^{2+} to $\simeq 10$ s^{-1} in 2 mM-Ca^{2+}. However, at 10 mM-Ca^{2+}, where m.e.p.p. frequency in raised K^+ is less than in 2 mM-Ca^{2+} (Cooke & Quastel, 1973*c*), the rate of increase induced by La^{3+} was lower than in low Ca^{2+}; in the same way

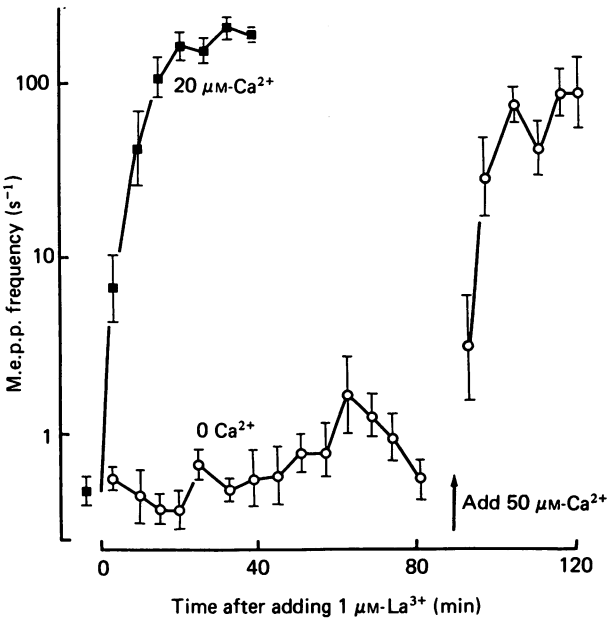

Fig. 9. Ca^{2+} dependence of the rise in m.e.p.p. frequency induced by 1 μM-La^{3+}. Open symbols represent multiple sampling data from a diaphragm equilibrated in 10 mM-K^+ and exposed to 1 μM-La^{3+} in the absence of Ca^{2+}; frequencies did not rise substantially until 50 μM-Ca^{2+} was added to the solution. Compare this to the data (filled symbols) from an experiment where 20 μM-Ca^{2+} was present throughout, in which frequencies rose rapidly immediately upon addition of La^{3+}. Error bars are $\pm$S.E. of mean.

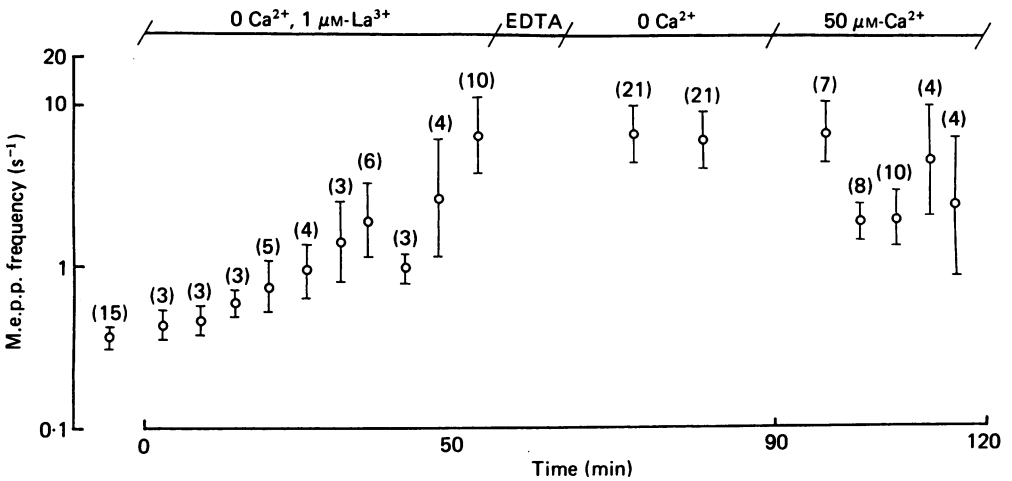

Fig. 10. Failure of 50 μM-Ca^{2+} to raise m.e.p.p. frequency (f_m) after exposure to La^{3+} in absence of Ca^{2+}, and removal of external La^{3+}. In 10 mM-K^+/0 Ca^{2+} mean f_m in a diaphragm was much less increased by 1 μM-La^{3+} in a 50 min period than normally seen in the presence of 20 or 50 μM-Ca^{2+} (cf. Fig. 9). After removal of La^{3+} by 'washing' with EDTA, f_m did not rise upon addition of 50 μM-Ca^{2+}. Numbers of junctions sampled in the time period are given in parentheses.

as seen with Cd^{2+}, the maximum m.e.p.p. frequencies were lower than in 2 mM- or 50 μM-Ca^{2+}. Subsequently, in this diaphragm, mean m.e.p.p. frequency gradually increased to its usual maximum with La^{3+}, when Ca^{2+} was reduced to 50 μM (La^{3+} still present).

Ca^{2+} dependence of La^{3+} effect

It has already been remarked that in Ca^{2+}-free solutions the response of m.e.p.p. frequency to La^{3+} was erratic and usually required much higher concentrations of La^{3+} than when 50 μM-Ca^{2+} was present, indicating that the rate of lanthanization

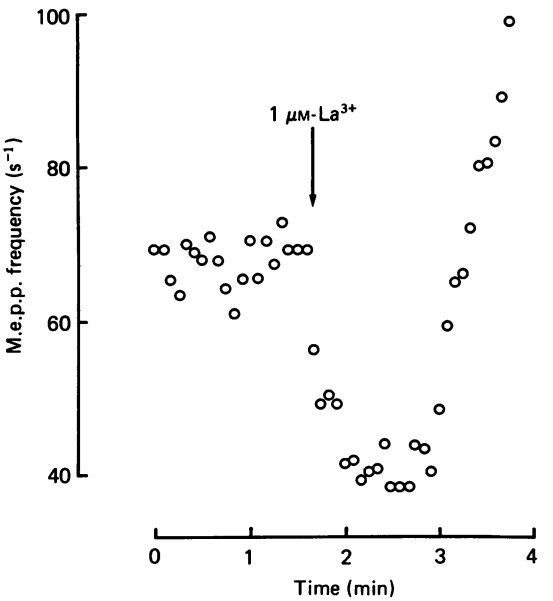

Fig. 11. Partial block by La^{3+} of Ca^{2+}-dependent transmitter release. Upon application of 1 μM-La^{3+} (in 20 mM-K^+, 1 mM-Ca^{2+}) m.e.p.p. frequency dropped quickly from about 70 s^{-1} to about 40 s^{-1}, then rose rapidly.

might be sensitive to low concentrations of Ca^{2+}. Fig. 9 illustrates that this is indeed the case. At this junction there was no significant rise in frequency during 80 min exposure to 1 μM-La^{3+} in 10 mM-K^+, nominally Ca^{2+}-free solution, but with the addition of 50 μM-Ca^{2+} m.e.p.p. frequency rose promptly. Lanthanization at low [La^{3+}] also could be found at 20 μM-Ca^{2+}, but at lower concentrations the responses to La^{3+} were erratic; in nominally Ca^{2+}-free solutions, lanthanization usually required at least 20 μM-La^{3+}.

One interpretation of the above result is that in the absence of Ca^{2+}, La^{3+} might enter the nerve terminal but fail to cause a high m.e.p.p. frequency. A test of this is shown in Fig. 10. The diaphragm was exposed to 1 μM-La^{3+} for an hour in 10 mM-K^+/Ca^{2+}-free solution (with a relatively small rise in m.e.p.p. frequency, compare Fig. 3) and then washed briefly with La^{3+}-free solution containing 100 μM-EDTA. Following this, exposure to 50 μM-Ca^{2+} caused no rise in m.e.p.p. frequency. It may therefore be concluded that Ca^{2+}, at concentrations lower than

those permitting transmitter release by raised K^+, accelerates La^{3+} entry into the nerve terminal.

Inhibition by La^{3+} of Ca^{2+}-mediated release

The action of La^{3+} to *block* transmitter release, presumably by interference with Ca^{2+} entry into the terminal (Miledi, 1971), could be seen both with raised K^+ and with e.p.p.s. In the example in Fig. 11 m.e.p.p. frequency in 20 mM-K^+/1 mM-Ca^{2+} was about 70 s^{-1}; the addition of 1 μM-La^{3+} caused an immediate *fall* to around 40 s^{-1}, followed by a rapid rise, similar to the La^{3+} effect in 20 mM-K^+/50 μM-Ca^{2+}. The quantal contents of e.p.p.s recorded before, during and after exposure to 5 μM-La^{3+} are listed in Table 1. In the presence of La^{3+} the e.p.p. was usually depressed, with immediate recovery on 'washing' with solution containing 100 μM-Ca EDTA, indicating that the inhibitory effect of La^{3+} can largely be reversed by removal of La^{3+} using EDTA. In each case the quantal content in La^{3+} should be compared with the re-control, recorded a minute or so later; the depression by 5 μM-La^{3+} varied from about 55% to over 90%.

Interaction of transmitter release induced by La^{3+} and release by Ca^{2+}, Ba^{2+} and ethanol

At junctions where lanthanization had been allowed to proceed to completion, i.e. where m.e.p.p. frequency had reached its maximum before washing with EDTA, we were generally unable to find any discernible e.p.p. upon nerve stimulation; nor was there any response of m.e.p.p. frequency to Ca^{2+} (in raised K^+) or to focal depolarization of nerve terminals in the presence of Ca^{2+}. There was also no response to ethanol (Gage, 1965; Okada, 1967) which was previously observed to produce constant multiplication of transmitter release under a variety of conditions, including absence of Ca^{2+} (Quastel, Hackett & Cooke, 1971).

In contrast, terminals at which m.e.p.p. frequency had been raised moderately by La^{3+} remained responsive, and in terms of amount of transmitter release responses were actually enhanced. As listed in Table 1, quantal content of e.p.p.s was increased after each exposure to La^{3+} and subsequent 'wash' with Ca EDTA (which had no effect in controls). As shown in Table 2, the same was true for responses of m.e.p.p. frequency to Ca^{2+}, in raised K^+, and to ethanol. However, expressed in terms of multiplication of m.e.p.p. frequency (change in $\log f_m$), responses were reduced. It is notable that the attenuation of responses in terms of change in $\log f_m$ was most at those junctions where f_m was highest, i.e. those probably most lanthanized, and that the attenuation of change in $\log f_m$ is prominent at m.e.p.p. frequencies far less than can be attained with lanthanization.

A form of release in some respects different from that seen with e.p.p.s is the 'asynchronous' release manifest as a shower of m.e.p.p.s following nerve stimulation in the presence of Ba^{2+} (Silinsky, 1978). With this kind of release we found the same rule to apply as with Ca^{2+}-depolarization release, ethanol and e.p.p.s. For example, at one junction spontaneous m.e.p.p. frequency was $0{\cdot}4 \pm 0{\cdot}05$ s^{-1} in the presence of 2 mM-Ba^{2+} and 0·1 mM-Ca^{2+}, and in the period of 0·2–1·2 s after a fifty impulse tetanus there occurred twenty-five m.e.p.p.s. Following nerve stimulation in the presence of 5 μM-La^{3+} and 'wash' with EDTA, spontaneous m.e.p.p. frequency was raised to 19 s^{-1}; at 0·2–1·2 s after the fifty impulse tetanus, the number of m.e.p.p.s was 141.

In both cases and at an intermediate level of lanthanization, the logarithm of number of m.e.p.p.s in the 'after-discharge' was linearly related to the number of impulses in the tetanus, with a slope that became less with lanthanization.

TABLE 2. Modification by lanthanization of increases in m.e.p.p. frequency (f_m) induced by ethanol (EtOH) and by Ca^{2+}

	f_m			Δf_m		$\Delta \ln f_m$	
Junction	Control	EtOH	Ca^{2+}	EtOH	Ca^{2+}	EtOH	Ca^{2+}
			(A)Before La^{3+}				
1	0·5	3·6	9·1	3·1	8·6	1·95	2·87
4	0·7	3·4	13·7	2·7	17·9	1·61	3·31
3	0·8	2·6	5·8	1·8	5·0	1·19	1·98
6	1·1	4·4	15·7	3·3	14·6	1·41	2·67
2	1·1	6·8	13·7	5·7	12·6	1·82	2·52
5	1·2	4·7	4·8	3·5	3·6	1·37	1·38
			Mean	3·4	10·4	1·56	2·45
			S.E. of mean	±0·5	±2·3	±0·12	±0·28
			(B) After La^{3+}				
10	3·2	13·1	17·8	9·9	14·6	1·41	1·72
9	5·3	18·9	29·2	13·6	23·9	1·27	1·70
7	8·2	26·8	40·5	18·6	32·3	1·19	1·60
12	16·9	49·3	56·1	32·4	39·2	1·07	1·20
11	17·5	59·7	81·1	42·2	63·6	1·23	1·53
8	29·1	74·9	66·9	45·8	37·8	0·95	0·83
			Mean	27·1	35·2	1·18	1·43
			S.E. of mean	±6·1	±6·8	±0·06	±0·14

Control solution contained 60 μM-Ca^{2+}/15 mM-K^+ (HEPES buffer). Test Ca^{2+} was 2 mM. Test ethanol ('EtOH') was 0·4 M. La^{3+} was applied for 5 min at 0·25 μM, and the preparation 'washed' with control solution with added 100 μM-Ca EDTA for 4 min. Junctions in each group are listed in order of control f_m; the junctions are numbered in the order that recordings were made. Units of f_m are s^{-1}.

DISCUSSION

The present results concur with previous studies in showing two distinct effects of La^{3+}: (1) an action to inhibit transmitter release, manifest both on e.p.p.s and on m.e.p.p. frequency, when the latter is raised by high K^+ in the presence of Ca^{2+}, and (2) an action to raise m.e.p.p. frequency. Similar effects have previously been described with Y^{3+}, Pr^{3+} and Er^{3+} (Bowen, 1972; Alnaes & Rahamimoff, 1974; Metral, Bonneton, Hort-Legrand & Reynes, 1978), and with Mn^{2+}, Co^{2+}, Hg^{2+} and Pb^{2+} (Balnave & Gage, 1973; Weakly, 1973; Manalis & Cooper, 1975; Juang, 1976; Binah, Meiri & Rahamimoff, 1978; Washio, 1982; Manalis, Cooper & Pomeroy, 1984). Since the inhibitory action of La^{3+} disappears rapidly with removal of La^{3+}, there is no reason to believe that it represents anything but blockade of voltage-gated Ca^{2+} entry, from an extracellular site, in the same way as seen with Mg^{2+} and a variety of divalent cations (Jenkinson, 1957; Shapovalov, 1962; Dodge & Rahamimoff, 1967; Manalis *et al.* 1984).

The action of La^{3+} to raise m.e.p.p. frequency is essentially irreversible and high frequencies are maintained for hours if external La^{3+} is withdrawn, indicating that

the secondary fall seen with prolonged exposure and/or relatively high concentrations of La^{3+} (Heuser & Miledi, 1971; Washio & Miyamoto, 1983) may be due to an excess accumulation of La^{3+} inside the nerve terminal. The acceleration of 'lanthanization' by nerve terminal depolarization (focally-applied current or raised K^+) or by nerve action potentials strongly suggests that La^{3+} acts to stimulate release only after entering the terminal via voltage-sensitive channels, although it is conceivable that La^{3+} might become tightly bound to certain external membrane sites in a voltage-dependent manner and exert its action via 'receptors' extending across the presynaptic membrane.

Since La^{3+} is known to be an extremely potent inhibitor of active accumulation of Ca^{2+} by mitochondria (Mela, 1968, 1969) and might well displace Ca^{2+} from binding sites or interfere with non-mitochondrial disposition of Ca^{2+}, it would seem reasonable to suppose that La^{3+} acts to release transmitter by increasing the intracellular Ca^{2+} concentration (Rahamimoff, 1978). However, there are reasons for doubting that this is the case. The more cogent is that with full lanthanization m.e.p.p. frequency is usually much less than can be maintained indefinitely in high K^+ with Ca^{2+} present (e.g. Cooke *et al.* 1973). Unless release is limited because of yet another effect of La^{3+}, e.g. an interference with production or mobilization of quantal units, this indicates that the release system is far from saturated with Ca^{2+} at the (hypothetical) Ca^{2+} level achieved with lanthanization, i.e. the system should still respond to Ca^{2+} entry. In fact, there is a complete blockade of all depolarization/Ca^{2+}-dependent release including e.p.p.s, even after removal of external La^{3+} using EDTA. Secondly, if La^{3+} were acting by increasing the Ca^{2+} close to release sites it is difficult to understand why the increase in m.e.p.p. frequency at partially lanthanized junctions is not eventually reversed with raised K^+/0 Ca^{2+} and EDTA, with loss of Ca^{2+} from the nerve terminal via open Ca^{2+} channels. According to Reuter & Scholz (1977) Ca^{2+} channels in heart muscle (which may or may not be similar to those at nerve terminals; see Hagiwara & Byerly, 1981) carry current in accord with constant-field theory, i.e. rectification appears only because of the asymmetry of Ca^{2+} activities on either side of the membrane (Tsien, 1983), rather than an inability of channels to pass Ca^{2+} outward. Moreover, Rahamimoff, Lev-Tov & Meiri (1980) and Lev-Tov & Rahamimoff (1980) have reported results that strongly suggest that internal Ca^{2+} can indeed escape from nerve terminals via Ca^{2+} channels when external Ca^{2+} is very low. It therefore seems likely that La^{3+} itself replaces Ca^{2+} as an activator of transmitter release within the nerve terminal, as proposed by Blioch *et al.* (1968).

On the assumption that La^{3+} enters the terminals via voltage-gated channels and causes release by binding to sites inside the terminal, two further questions arise. (1) Are the channels through which La^{3+} enters those that normally admit Ca^{2+}? (2) Are the internal binding sites those that normally bind Ca^{2+}? Lanthanization is indeed slowed by Cd^{2+} (Fig. 7), and by high concentrations of Ca^{2+} itself. Moreover, the blockade by La^{3+} of Ca^{2+}-dependent release, exerted extracellularly, presumably indicates a competition between La^{3+} and Ca^{2+} at sites governing Ca^{2+} entry. One can tentatively conclude, therefore, that La^{3+} enters the terminal through channels that normally admit Ca^{2+}. If this is true, then the marked acceleration of lanthanization by a low concentration of Ca^{2+} takes on a particular significance, since it implies that either (1) opening of these channels itself normally requires the presence

of a low concentration of Ca^{2+} or (2) Ca^{2+} in some way 'co-operates' with La^{3+} for entry of the latter. With regard to the first possibility, it may be noted that Cooke & Quastel (1973*b*) found that a small ($\sim \mu$M) level of extracellular Ca^{2+} was required for the slow acceleration of m.e.p.p. frequency that takes place when nerve terminals are depolarized in very low Ca^{2+}, i.e. some Ca^{2+} may be necessary for the integrity of certain membrane responses to depolarization. With regard to the second hypothesis, the results of Cooke *et al.* (1973) suggested that Ca^{2+} entry into nerve terminals depends upon the square of extracellular [Ca^{2+}]. This may reflect the same ion dependence of selectivity as reported by Hess & Tsien (1984) for Ca^{2+} channels in cardiac muscle.

The question remains as to whether the internal sites at which La^{3+} acts are those which normally mediate Ca^{2+}-dependent release. At partially lanthanized terminals, depolarization (raised K^+)/Ca^{2+}-dependent release is enhanced in terms of absolute change in m.e.p.p. frequency (Table 2), and in the same way there is an increase in quantal content of e.p.p.s (in low Ca^{2+}/raised Mg^{2+}) that constitutes an increase of 'synchronous' release *rate* (number per unit time) that is more than the increase of m.e.p.p. frequency (f_m). For example, at junction 1 in Table 1 f_m was increased by partial lanthanization from 0·5 to 35 s^{-1}, a 70-fold change, while $\bar{m}$ was increased from 0·4 to 2·1, a 5-fold change. However, the control $\bar{m}$ corresponds to a frequency of about 400 s^{-1} in a 1 ms period (cf. Miledi & Thies, 1971) and the $\bar{m}$ of 2·1 to a release rate of about 2100 s^{-1}; the increase of 1700 s^{-1} is much more than the increase in f_m. These data exclude the possibility that La^{3+} acts on a transmitter pool distinct from that involved in normal release, and are most easily interpreted in terms of La^{3+} and Ca^{2+} in some way co-operating in the release of transmitter, i.e. La^{3+} substituting for Ca^{2+} at sites where Ca^{2+} ions normally co-operate (Dodge & Rahamimoff, 1967; Cooke *et al.* 1973; Charlton, Smith & Zucker, 1982). With La^{3+} as a partial agonist (implied by the rather low maximal release rate obtainable with La^{3+}, compared to that with Ca^{2+}; see Silinsky, 1985) this model also accounts for the complete blockade of e.p.p.s and other Ca^{2+}-dependent release that occurs with complete lanthanization.

The proposal by Blioch *et al.* (1968) that La^{3+} and Ca^{2+} act merely by neutralizing surface charges is fully consistent with co-operation between La^{3+} and Ca^{2+} if a number of positively charged ions are required for each vesicle–membrane adhesion, but is inconsistent with the very high efficacy of Ca^{2+} relative to other cations in evoking release. For example, even Ba^{2+}, which enters nerve terminals via Ca^{2+} channels as readily as Ca^{2+} (Brigant & Mallart, 1982), does not support an e.p.p. (Silinsky, 1978). Other models (discussed by Silinsky, 1985) suppose that for each quantum to be released there exist a number of sites at which Ca^{2+} (or surrogates) may bind, with release of each quantum either being absolutely contingent upon a fixed number of sites ('n') being occupied (Dodge & Rahamimoff, 1967) or made progressively more probable the more sites are in the liganded state (Cooke *et al.* 1973). With the former model the present data regarding potentiation of Ca^{2+}-dependent release with partial lanthanization can be accommodated if it is supposed that for quanta with 'n' sites occupied and therefore with a release probability greater than zero, this probability varies with the partition of sites between Ca^{2+} and La^{3+}. With the other model (continuously graded probability) it is only necessary to postulate that sites liganded to La^{3+} cause less multiplication of release probability (which is

more than zero even in the absence of activating cations) than do sites liganded to Ca^{2+}; we find that either model can be fitted to the data in Table 1 and Table 2 (with ethanol acting to increase the affinity of receptors for Ca^{2+} without altering La^{3+} binding). With either of these models it makes little or no difference whether La^{3+} is irreversible because of irreversible binding to the 'receptor' or simply because it is unable to escape from the nerve terminal. Moreover, a permanent or quasi-permanent alteration of receptors after transient La^{3+} binding would be indistinguishable from an alteration dependent upon the continued attachment of La^{3+}. In this connexion it may be recalled that after intense and prolonged focal depolarization of nerve terminals, in the absence of any foreign ions, the release system temporarily behaves in a manner that closely resembles that of partially lanthanized terminals in terms of the interaction of a high Ca^{2+}-insensitive m.e.p.p. frequency with Ca^{2+}/depolarization-dependent release (Cooke & Quastel, 1973*b*).

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REFERENCES

ALNAES, E. & RAHAMIMOFF, R. (1974). Dual action of praseodymium (Pr^{3+}) on transmitter release at the frog neuromuscular synapse. *Nature* **247**, 478–479.

BALNAVE, R. J. & GAGE, P. W. (1973). The inhibitory effect of manganese on transmitter release at the neuromuscular junction of the toad. *British Journal of Pharmacology* **47**, 339–352.

BINAH, O., MEIRI, U. & RAHAMIMOFF, H. (1978). The effects of mercuric chloride and mersalyl on mechanisms regulating intracellular calcium and transmsitter release. *European Journal of Pharmacology* **51**, 453–457.

BLIOCH, Z. L., GLAGOLEVA, I. M., LIBERMAN, E. A. & NENASHEV, E. A. (1968). A study of the mechanism of quantal transmitter release at a chemical synapse. *Journal of Physiology* **199**, 11–35.

BOWEN, J. M. (1972). Effects of rare earths and yttrium on striated muscle and the neuromuscular junction. *Canadian Journal of Physiology and Pharmacology* **50**, 603–611.

BRIGANT, J. L. & MALLART, A. (1982). Presynaptic currents in mouse nerve endings. *Journal of Physiology* **333**, 619–636.

CHARLTON, M. P., SMITH, S. J. & ZUCKER, R. S. (1982). Role of presynaptic calcium ions and channels in synaptic facilitation and depression at the squid giant synapse. *Journal of Physiology* **323**, 173–193.

COOKE, J. D., OKAMOTO, K. & QUASTEL, D. M. J. (1973). The role of calcium in depolarization–secretion coupling at the motor nerve terminal. *Journal of Physiology* **228**, 459–947.

COOKE, J. D. & QUASTEL, D. M. J. (1973*a*). Transmitter release by mammalian motor nerve terminals in response to focal polarization. *Journal of Physiology* **228**, 377–405.

COOKE, J. D. & QUASTEL, D. M. J. (1973*b*). Cumulative and persistent effects of nerve terminal depolarization on transmitter release. *Journal of Physiology* **228**, 407–434.

COOKE, J. D. & QUASTEL, D. M. J. (1973*c*). The specific effect of potassium on transmitter release by rat motor nerve terminals, and its inhibition by calcium. *Journal of Physiology* **228**, 435–458.

DEBASSIO, W. A., SCHNITZLER, R. M. & PARSONS, R. L. (1971). Influence of lanthanum on transmitter release at the neuromuscular junction. *Journal of Neurobiology* **2**, 263–278.

DEL CASTILLO, J. & KATZ, B. (1954). Quantal components of the end-plate potential. *Journal of Physiology* **124**, 560–573.

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

FRANKENHAEUSER, B. & HODGKIN, A. L. (1957). The action of calcium on the electrical properties of squid giant axons. *Journal of Physiology* **137**, 218–244.

GAGE, P. W. (1965). The effect of methyl, ethyl, and *n*-propyl alcohol on neuromuscular transmission in the rat. *Journal of Pharmacology and Experimental Therapeutics* **150**, 236–243.

HAGIWARA, S. & BYERLY, L. (1981). Calcium Channel. *Annual Review of Neuroscience* **4**, 69–125.

HESS, P. & TSIEN, R. W. (1984). Mechanism of ion permeation through calcium channels. *Nature* **309**, 453–456.

HEUSER, J. & MILEDI, R. (1971). Effect of lanthanum ions on function and structure of frog neuromuscular junctions. *Proceedings of the Royal Society* B **179**, 247–260.

JENKINSON, D. H. (1975). The nature of antagonism between calcium and magnesium ions at the neuromuscular junction. *Journal of Physiology* **196**, 75–86.

JUANG, M. S. (1976). An electrophysiological study of the action of methylmercuric chloride and mercuric chloride on the sciatic nerve–sartorius muscle preparation of the frog. *Toxicology and Applied Pharmacology* **37**, 339–348.

KATZ, B. (1969). The release of neural transmitter substances. In *The Sherrington Lecture No. 10*. Liverpool: University Press.

KOSTYUK, P. G. & KRISHTAL, O. A. (1977). Separation of sodium and calcium currents in the somatic membrane of mollusc neurones. *Journal of Physiology* **270**, 545–568.

LETTVIN, J. Y., PICKARD, W. F., MCCULLOCH, W. S. & PITTS, W. (1964). A theory of passive ion flux through axon membranes. *Nature* **202**, 1338–1339.

LEV-TOV, A. & RAHAMIMOFF, R. (1980). A study of tetanic and post-tetanic potentiation of miniature end-plate potentials at the frog neuromuscular junction. *Journal of Physiology* **309**, 247–273.

MANALIS, R. S. & COOPER, G. P. (1975). Evoked transmitter release increased by inorganic mercury at the frog neuromuscular junction. *Nature* **257**, 690–691.

MANALIS, R. S., COOPER, G. P. & POMEROY, S. L. (1984). Effects of lead on neuromuscular transmission in the frog. *Brain Research* **294**, 95–109.

MELA, L. (1968). Interaction of La^{3+} and local anesthetic drugs with mitochondrial Ca^{2+} and Mg^{2+} uptake. *Archives of Biochemistry and Biophysics* **123**, 286–293.

MELA, L. (1969). Inhibition and activation of calcium transport in mitochondria, effect of lanthanides and local anesthetic drugs. *Biochemistry* **8**, 2481–2496.

METRAL, S., BONNETON, C., HORT-LEGRAND, C. & REYNES, J. (1978). Dual action of erbium on transmitter release at the frog neuromuscular junction. *Nature* **271**, 773–775.

MILEDI, R. (1966). Strontium as a substitute for calcium in the process of transmitter release at the neuromuscular junction. *Nature* **212**, 1233–1234.

MILEDI, R. (1971). Lanthanum ions abolish the 'calcium response' of nerve terminals. *Nature* **229**, 410–411.

MILEDI, R. & THIES, R. (1971). Tetanic and post-tetanic rise in frequency of miniature end-plate potentials in low-calcium solutions. *Journal of Physiology* **212**, 245–257.

OKADA, K. (1967). Effects of alcohols and acetone on the neuromuscular junction of frog. *Japanese Journal of Physiology* **17**, 245–261.

QUASTEL, D. M. J., HACKETT, J. T. & COOKE, J. D. (1971). Calcium: is it required for transmitter secretion? *Science* **172**, 1034–1036.

RAHAMIMOFF, R. (1978). Intracellular and extracellular calcium ions in transmitter release at the neuromuscular synapse. *Annals of the New York Academy of Sciences* **307**, 583–598.

RAHAMIMOFF, R., LEV-TOV, A. & MEIRI, H. (1980). Primary and secondary regulation of quantal transmitter release; calcium and sodium. *Journal of Experimental Biology* **89**, 5–18.

REUTER, H. & SCHOLZ, H. (1977). A study of the ion selectivity and the kinetic properties of the calcium dependent slow inward current in mammalian cardiac muscle. *Journal of Physiology* **264**, 17–47.

SHAPOVALOV, A. I. (1962). A study of action of drugs on neuromuscular transmission with the aid multibarrelled intracellular microelectrodes. *Biochemical Pharmacology* **9**, 213–220.

SILEN, L. C. & MARTELL, A. E. (1971). Stability constants. *The Chemical Society*. Special publication **25**, suppl. 1.

SILINSKY, E. M. (1977). An estimate of the equilibrium dissociation constant for calcium as an antagonist of evoked acetylcholine release: implications for excitation–secretion coupling. *British Journal of Pharmacology* **61**, 691–693.

SILINSKY, E. M. (1978). On the role of barium in supporting the asynchronous release of acetylcholine quanta by motor nerve impulses. *Journal of Physiology* **274**, 157–171.

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

TAKATA, M., PICKARD, W. F., LETTVIN, J. Y. & MOORE, J. W. (1966). Ionic conductance changes in lobster axon membrane when lanthanum is substituted for calcium. *Journal of General Physiology* **50**, 461–471.

TSIEN, R. W. (1983). Calcium channels in excitable cell membranes. *Annual Review of Physiology* **45**, 341–358.

WASHIO, H. (1982). A dual effect of cobalt ions on the spontaneous release of transmitter at insect motor nerve terminals. *Journal of Experimental Biology* **98**, 53–361.

WASHIO, H. & MIYAMOTO, T. (1983). Effect of lanthanum ions on neuromuscular transmission in insects. *Journal of Experimental Biology* **107**, 405–414.

WEAKLY, J. N. (1973). The action of cobalt ions on neuromuscular transmission in the frog. *Journal of Physiology* **234**, 597–612.