Br. J. Pharmac. (1978), **63**, 485–493

ENHANCEMENT BY AN ANTAGONIST OF TRANSMITTER RELEASE FROM FROG MOTOR NERVE TERMINALS

E.M. SILINSKY

Laboratory of Presynaptic Events, Department of Pharmacology,
Northwestern University Medical School, 303 E. Chicago Avenue, Chicago, Illinois 60611, U.S.A.

1 The effect of Ba^{2+} on the synchronous release of acetylcholine from frog motor nerve terminals was studied by conventional electrophysiological techniques.

2 When Ca^{2+} and Ba^{2+} were the only divalent cations in the bathing fluid, Ba^{2+} caused a presynaptic reduction in the amplitude of the endplate potential (e.p.p.). This effect was surmountable by increasing the Ca^{2+} concentration.

3 The affinity constant (K_A) for Ba^{2+}, calculated on the assumption that Ba^{2+} is a competitive inhibitor of the agonist, Ca^{2+}, was 1.1 ± 0.4 mM^{-1} (mean $\pm$ s.e.mean, $n = 8$).

4 When e.p.ps were depressed by the addition of 1 mM Mg^{2+}, addition of Ba^{2+} (1 to 3 mM) caused either a further presynaptic depression of moderate magnitude or had no additional effect.

5 When e.p.p.s were depressed with $[Mg^{2+}] \geq 2$ mM, addition of $Ba^{2+} \geq 0.9$ mM enhanced the e.p.p. amplitude by a presynaptic mechanism.

6 The interaction of the divalent cation antagonists Mg^{2+} and Ba^{2+} with the agonist, Ca^{2+} is discussed. It is demonstrated that a model which considers the nonequilibrium, kinetic properties of binding can be used to describe interactions between divalent cations at the external surface of the motor nerve ending.

Introduction

Recent evidence suggests that Ba^{2+}, although unable to support the synchronous, phasic release of acetylcholine (ACh), is able to elicit the asynchronous, delayed secretion of enormous numbers of ACh quanta in response to repetitive nerve impulses (McLachlan, 1977; Silinsky, 1977a; 1978). Because antagonists of Ca^{2+} entry into nerve membranes (e.g. Mg^{2+}, Co^{2+}) competitively inhibit Ba^{2+}-mediated release with similar affinity to their corresponding antagonism of Ca^{2+}-mediated release, it has been suggested that Ba^{2+} is a direct agonist for the Ca^{2+} conductance pathway (Silinsky, 1978). In contrast to these results, experiments carried out on mammalian ganglia (McLachlan, 1977) demonstrated that Ba^{2+} does not antagonize the synchronous release of ACh in solutions containing Ca^{2+} and Mg^{2+}. It is difficult to envisage how an ion that exerts a pronounced effect on the Ca^{2+} conductance pathway yet does not support synchronous release, also fails to act as an antagonist of synchronous release. This paper offers an explanation for this apparent discrepancy.

The results suggest that Ba^{2+} antagonizes the synchronous, Ca^{2+}-dependent release of ACh when Mg^{2+} is absent from the bathing fluid. In the presence of Mg^{2+}, the effects of Ba^{2+} are complex but can be predicted by a model which considers nonequilibrium behaviour at the external surface of the motor nerve ending.

Methods

The isolated nerve-cutaneous pectoris preparation of the frog (*Rana pipiens*) was bathed in normal Ringer solution of the following composition (mM): NaCl 115, KCl 2, $NaHCO_3$, 2 and $CaCl_2$ 1.8. All experimental solutions contained approximately the same univalent ion concentration as normal Ringer with varying concentrations of divalent cations. In the majority of experiments, the Ringer solution contained tubocurarine chloride (1–3 μg/ml) to reduce the amplitude of the endplate potential (e.p.p.) below threshold. In such experiments the following procedure was employed. During the curarization process, the preparations were bathed in 1 mM Ca^{2+}, 0.25 mM Ba^{2+} Ringer. Ba^{2+} has been shown to produce a large depolarization of the muscle membrane (Werman & Grundfest, 1961). As the postsynaptic

effects appear to be near maximum at [Ba^{2+}] of 0.25 mM (Silinsky, 1978 and unpublished), it was possible to isolate the presynaptic effects of Ba^{2+} on Ca^{2+}-mediated release by the use of 1 mM Ca^{2+}, 0.25 mM Ba^{2+} Ringer as the control solution. In some experiments (e.g. Figure 1) Ba^{2+} solutions contained 1 mM ethylene-glycol-bis-(β-aminoethyl ether)-N,N'-tetra-acetic acid (EGTA) to reduce the extracellular [Ca^{2+}] to $< 10^{-8}$ M (Miledi & Thies, 1971). In a few experiments neostigmine methyl sulphate was employed to increase the size of miniature endplate potentials (m.e.p.ps), see e.g. Figures 1 and 5.

Preparations were transilluminated by a fibre optic system similar to that described by Dreyer & Peper (1974). Such illumination, in conjunction with a Wild M5 stereomicroscope used at 100× magnification enabled nerve terminals to be located visually (cf. Dreyer & Peper, 1974).

Isolated stimulation pulses produced by Grass S88 and SIU-15 units were delivered to the nerve through a suction electrode. Electrical potential changes were recorded intracellularly at endplate regions with glass microelectrodes filled with 3 M KCl, the reference electrode being a silver–silver chloride pellet (W.P. Instruments). Electrodes were filled by the fibreglass method of Tasaki, Tsukuhara, Ito, Wayner & Yu (1968) and had resistances ranging from 10 to 30 MΩ. Signals from the microelectrode were fed into a conventional preamplifier (W.P. Instruments-M701). The output of the preamplifier was delivered in parallel into an oscilloscope (Tektronix 5103 N), a pen recorder (Brush-Gould model 220), and a computer of average transients (Fabritek).

The majority of records in the text show the averaged e.p.p. produced in response to 128 stimuli presented at a frequency of 0.1 to 0.39 hertz. Such records were obtained by displaying the output of the computer on an oscilloscope and photographing this output with a Polaroid camera. As the average e.p.p. amplitude in all experiments was ≤ 5 mV, no correction was employed for non-linear summation (Martin, 1955; 1976) Miniature end-plate potential frequencies were determined from pen recorder traces and m.e.p.p. amplitudes from oscilloscope photographs. Experiments were carried out at room temperature. All results are from cells in which the resting membrane potential remained constant (± 2 mV) for the duration of the experimental period.

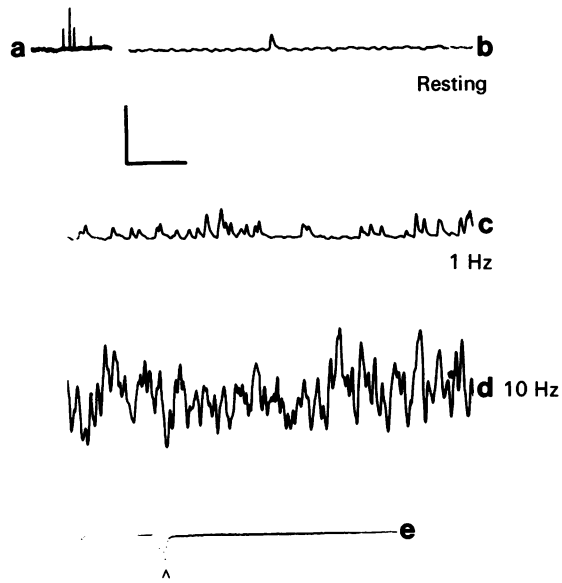

Figure 1 Evoked acetylcholine (ACh) release from preparation bathed in 3 mM Ba^{2+}-1 mM EGTA Ringer for 6 h before beginning the experiment. (a) and (b) show resting miniature end-plate potential (m.e.p.p.) frequency at 2 different recorder speeds (a) horizontal cal. 2 s, (b) to (d), horizontal cal. 125 ms, vertical cal. (a) to (c) 2.5 mV. (c) Continuous 1 Hz stimulation, steady-state m.e.p.p. frequency of ~63/second. The record was taken 30 s after stimulation started. (d) 10 Hz stimulation, m.e.p.p. frequency was elevated to an unmeasurable level within 1 s after stimulation. The irregular discharge in (d) is superimposed upon a steady membrane depolarization of 20 mV (which can be used to obtain an indirect measure of the m.e.p.p. frequency, see Katz & Miledi, 1972). Calculated m.e.p.p. frequency in (d) ~3000/second. Neostigmine methylsulphate (1 μg/ml) was included in the Ringer solution. (e) Computer averaged response to 32 stimuli made during trace (c). Arrow indicates end of stimulus artifact. Note absence of synchronous release (i.e. endplate potentials—e.p.ps) Vertical cal.: 10 mV, horizontal cal.: 4 ms.

Results

General observations on acetylcholine release in Ba^{2+} solutions

Figure 1 illustrates the behaviour of a preparation bathed in 3 mM Ba^{2+}-EGTA Ringer for 6 h before the start of the experiment. With respect to the *asynchronous* release process, continuous nerve stimulation at a frequency of 1 Hz within 30 s caused the control m.e.p.p. frequency of 1.5/s (Figure 1a, b) to increase to a steady-state level of 63/s (Figure 1c). Stimulation at 10 Hz caused an immediate (within 1 s) rise in the m.e.p.p. frequency to a level which could not be measured directly (Figure 1d). An estimate of this frequency by an indirect method (Heuser & Miledi, 1971; Katz & Miledi, 1972; Silinsky, 1978) suggests it to be approximately 3000/second. M.e.p.p.

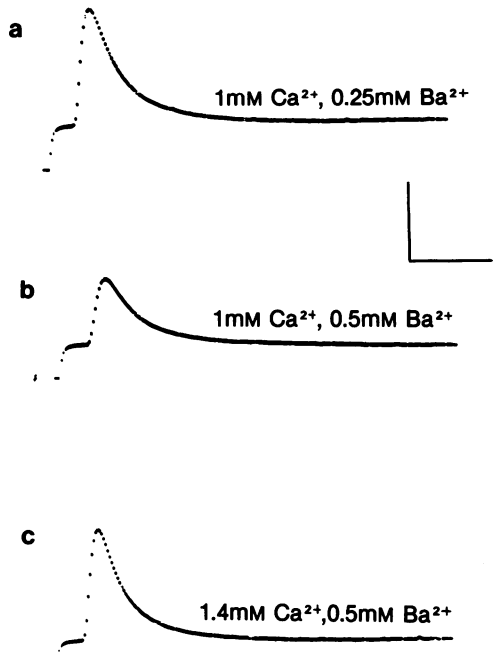

Figure 2 Antagonism by Ba^{2+} of the synchronous release of acetylcholine (ACh). (a) Averaged response in 1 mM Ca^{2+}, 0.25 mM Ba^{2+} Ringer solution. (b) Increasing the $[Ba^{2+}]$ to 0.5 mM depresses the endplate potential (e.p.p.) by approximately 50%. (c) Increasing the $[Ca^{2+}]$ to 1.4 mM (in the presence of 0.5 mM Ba^{2+}) allows the antagonism to be surmounted. Calibration: 2 mV, 4 ms.

discharges such as shown in Figure 1 can be antagonized competitively by Co^{2+} and Mg^{2+}, suggesting that asynchronous release is generated by Ba^{2+} movement through the conventional Ca^{2+} conductance pathway (Silinsky, 1978).

With respect to the *synchronized* release of many quanta from dispersed sites along the nerve terminal (i.e. the e.p.p.), it is apparent from Figure 1e (which is the averaged response to 1 Hz stimulation) that Ba^{2+} *cannot* support synchronous ACh release under conditions where this ion acts as a powerful mediator of asynchronous release.

Ba^{2+} as an antagonist of Ca^{2+}-mediated release, synchronous release

Figure 2 shows that elevation of the Ba^{2+} concentration from 0.25 mM (a) to 0.5 mM (b) in the presence of 1 mM Ca^{2+} caused a depression of the e.p.p. amplitude in curarized preparations. This effect was not due to changes in the sensitivity of the subsynaptic membrane (as identical increases in the $[Ba^{2+}]$ in the absence of curare failed to alter the amplitude of the m.e.p.ps) nor to a depression of the amplitude of the nerve terminal action potential (see Figure 1 in Silinsky, 1977a). The depression by Ba^{2+} could be completely surmounted by increasing the Ca^{2+} concentration to 1.4 mM (Figure 2c) suggesting that a competitive relationship *may* exist between the two ions. On the *assumption* that Ba^{2+} is a competitive antagonist of Ca^{2+}-mediated release (see Stephenson & Barlow, 1970, for precautions) it is possible to calculate the affinity constant (K_A) for Ba^{2+} as an antagonist as follows:

$$K_A = \frac{Ca_2 - Ca_1}{Ca_1Ba_2 - Ca_2Ba_1} \quad (1)$$

(see e.g. Jenkinson, 1957)

where Ca_1 and Ba_1 are the concentrations of Ca^{2+} and Ba^{2+} that produce an e.p.p. of a certain fixed, arbitrary amplitude; Ca_2 and Ba_2 are the (higher) concentrations of each ion that produce a matching e.p.p. Table 1 presents the K_A values for Ba^{2+} from eight experiments, with the mean $K_A = 1.1 \pm 0.4$ (mean $\pm$ s.e.mean).

Depression of the e.p.p. by Ba^{2+} *in the absence of curare* was observed under certain experimental conditions. In such studies, the preparation was pretreated with 1.8 mM Ba^{2+} Ringer and then bathed in drug-free, 1 mM Ca^{2+} Ringer (see Silinsky, 1978). Under these conditions, it is possible to observe e.p.ps uncontaminated by action potentials in many fibres (Silinsky, 1978). In such cells, Ba^{2+} caused a reduction in the e.p.p. without altering the mean m.e.p.p. amplitude, providing further evidence that the effect of Ba^{2+} in these experiments is exerted on the presynaptic membrane.

The effects of Ba^{2+} on the synchronous release of acetylcholine in solutions containing Ca^{2+} and Mg^{2+}

From these results it appears that Ba^{2+} is an antagonist of the synchronous release of ACh in the absence of other divalent cation antagonists. One possible explanation for the reported lack of effect of Ba^{2+} on ACh release (McLachlan, 1977) is that Mg^{2+}, which is generally included in the bathing solution of mammalian ganglia, may interact with Ba^{2+} in a complex fashion other than the additive antagonism that might be expected from equilibrium considerations.

Figure 3 illustrates an experiment in which the release was depressed from the control level (a) by the addition of 1 mM Mg^{2+} (b). Figure 3c shows that upon the addition of 2 mM Ba^{2+} a further depression of moderate magnitude was observed. Depression of evoked release by $[Ba^{2+}] \geq 2$ mM in the presence of 1 mM Mg^{2+} was observed in 2 other experiments. In

Table 1 Calculation of affinity constants (K_As) for Ba^{2+} as an antagonist of synchronous, Ca^{2+}-mediated acetylcholine release

	Control response		*Matching response*		
Experiment	$[Ba^{2+}]$ *mM*	$[Ca^{2+}]$ *mM*	$[Ba^{2+}]$ *mM*	$[Ca^{2+}]$ *mM*	K_A (mM^{-1})
1	1.0	2	2.75	2.0	0.59
2	1.0	1	1.50	1.25	1.00
3	0.6	2	1.00	2.25	0.38
4	0.6	2	2.00	3.50	0.76
5	0.25	1	0.50	1.30	1.43
6	1.00	1.5	1.50	2.00	2.00
7	1.5	1.25	2.00	2.25	0.80
8	1.5	1	2.00	1.8	2.00
					(1.1 ± 0.4) mean ± s.e.mean

All preparations were depolarized in 1 mM Ca^{2+}, 0.25 mM Ba^{2+} Ringer before experimentation. The K_A was calculated using equation 1 in the text. For assumptions of this method, see Stephenson & Barlow (1970).

one experiment, when $[Ba^{2+}]$ added was only 1 mM (1.25 mM total), no change in e.p.p. amplitude was observed.

In contrast to these results, when the control e.p.p. (Figure 4a) was depressed with 2 mM Mg^{2+} (Figure 4b), addition of 2 mM Ba caused a presynaptic *enhancement* of the e.p.p. amplitude (Figure 4c). Increases in synchronous release were also observed in five other experiments where the $[Ba^{2+}]$ added ranged from 2.00 to 3.35 mM. It should be noted that

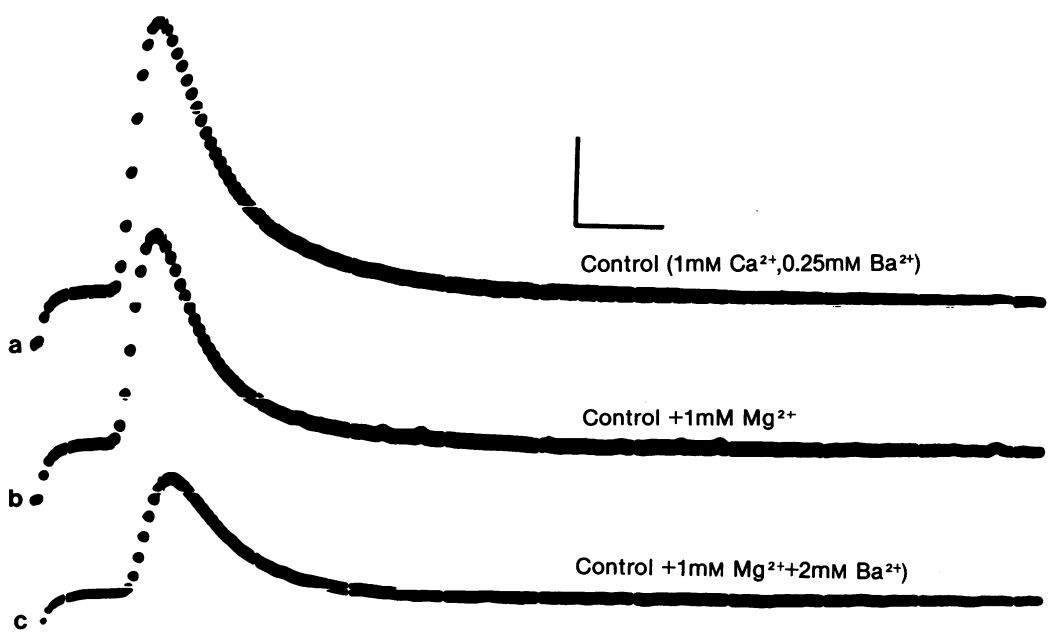

Figure 3 Antagonism by Ba^{2+} of Ca^{2+}-mediated acetylcholine (ACh) release in 1 mM Mg^{2+} solutions. Preparation was curarized. (a) 1 mM Ca^{2+}, 0.25 mM Ba^{2+} Ringer-control response. (b) 1 mM Ca^{2+}, 0.25 mM Ba^{2+}, 1 mM Mg^{2+}, note moderate depression by Mg^{2+}. (c) 1 mM Ca^{2+}, 2.25 mM Ba^{2+}, 1 mM Mg^{2+}, note further depression of e.p.p. by Ba^{2+} and increase in latency. It should be noted that changes in e.p.p. amplitude reflect presynaptic events. Calibration 1 mV, 1 ms.

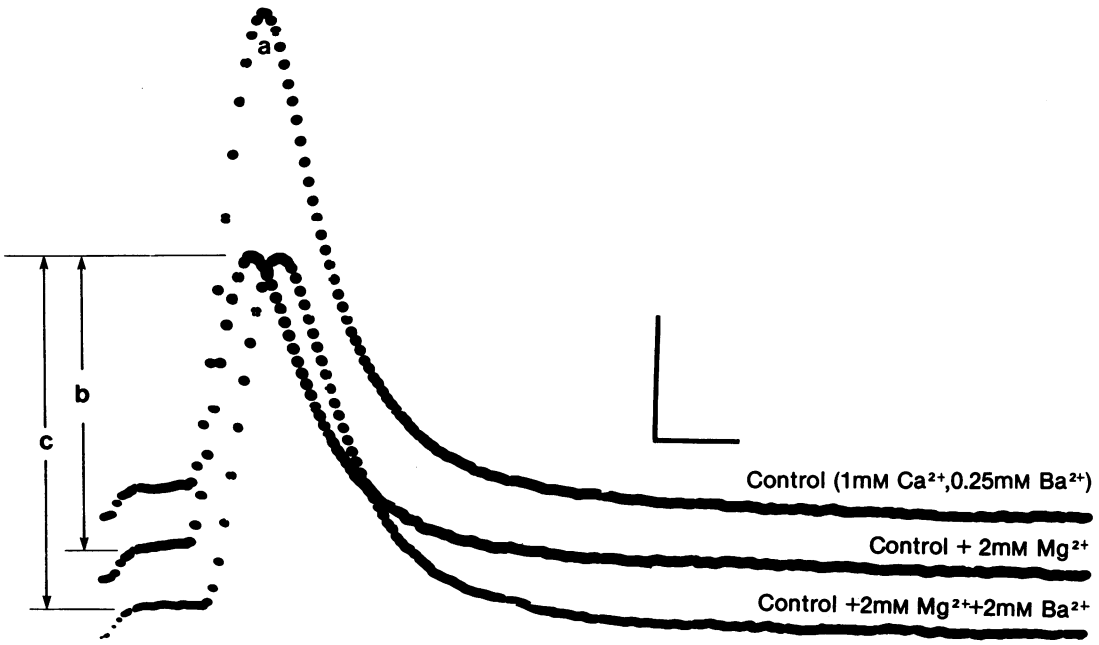

Figure 4 Enhancement by Ba^{2+} of Ca^{2+}-mediated acetylcholine (ACh) release in 2 mM Mg^{2+} solutions. Preparation was curarized. (a) 1 mM Ca^{2+}, 0.25 mM Ba^{2+} Ringer-control response. (b) 1 mM Ca^{2+}, 0.25 mM Ba^{2+}, 2 mM Mg^{2+}, note depression of control response. (c) 1 mM Ca^{2+}, 2.25 mM Ba^{2+}, 2 mM Mg^{2+}, note (i) enhancement of e.p.p. (by a presynaptic mechanism) and (ii) increased latency and time to peak of the e.p.p. Calibration: 0.5 mV, 1 ms.

for the sake of clarity, the changes of e.p.p. amplitudes have been described in a qualitative fashion in this section. All of the experiments will be reconsidered quantitatively in the Discussion (see Table 2).

From these results, it appears that between 1 mM and 2 mM Mg^{2+} (the range of the normal Mg^{2+} concentration in the bathing fluid of mammalian ganglia), there is a transition point at which the effect of Ba^{2+} on Ca^{2+} mediated-release is minimal. Although the mechanism of evoked ACh release at ganglionic synapses may differ somewhat from the motor nerve ending, a complex interaction between divalent cations could explain the absence of antagonism by Ba^{2+} on Mg^{2+}-bathed preganglionic nerve endings (McLachlan, 1977).

Regardless of whether Ba^{2+} acts to depress or increase ACh release in 1 to 2 mM Mg^{2+} solutions, there appears to be a change in latency of the e.p.p., resulting in a shift in the e.p.p. peak to the right (see record (c) of Figures 3 and 4). Although the cause of this behaviour is unknown, it is possible that this temporal shift in the e.p.p. is related to changes in nerve terminal action potential (unpublished observations). In the present experiments, where the data are used quantitatively to predict only the *direction* of the effect of Ba^{2+} on synchronous ACh release (i.e. whether release increases, decreases or remains unchanged, see Discussion) the change in latency can be ignored.

Figure 5 shows an experiment in which a low concentration of Ba^{2+} (0.9 mM) produced a forceful enhancement of synchronous ACh release. In this experiment, the e.p.p. produced in 0.9 mM Ca^{2+}, 9 mM Mg^{2+} Ringer (Figure 5a) was nearly doubled by the addition of 0.9 mM Ba^{2+} (Figure 5b) without a temporal shift of the e.p.p. peak. In this experiment, m.e.p.p. amplitudes were unaltered as a result of the addition of 0.9 mM Ba^{2+}, suggesting that the effect of Ba^{2+} was exerted on the presynaptic membrane; more precisely, on the process whereby depolarization of the nerve ending is coupled to the synchronous release of ACh quanta.

Discussion

The results suggest that, depending upon the concentration of Mg^{2+}, Ba^{2+} can either antagonize, enhance

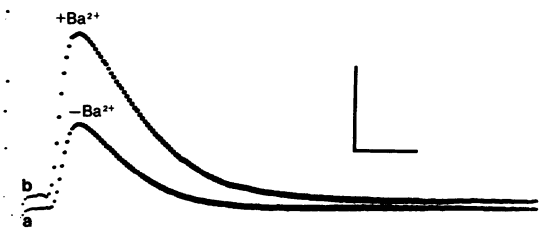

Figure 5 Enhancement of transmitter release in 0.9 mM Ca^{2+}, 9 mM Mg^{2+} (a) by Ba^{2+} (0.9 mM) (b). Neostigmine methylsulphate (1 μg/ml) was used in this experiment. In (b), mean m.e.p.p. amplitude was the same as in (a) suggesting a presynaptic enhancement of the e.p.p. Note absence of alterations in the e.p.p. configuration in trace (b) (cf. Figures 3 and 4). Calibration: 2 mV, 2.5 ms.

or exert no effect at all on the synchronous Ca^{2+}-dependent release of ACh. These experiments appear to provide a plausible explanation for the absence of antagonism by Ba^{2+} in Ca^{2+}-Mg^{2+} solutions (McLachlan, 1977). Concerning the mechanism responsible for this behaviour, interactions such as those described here may occur when two competitive antagonists with markedly different dissociation rate constants are present simultaneously at a receptive surface (Stephenson & Ginsborg, 1969; Ginsborg & Stephenson, 1974). For example, when a rapidly dissociating antagonist (fast antagonist = F) is applied after a slowly dissociating antagonist (S) has been allowed to equilibrate with receptors, rather than further depressing the response to a transiently applied agonist (A), under many conditions F will increase the response produced by A. One interpretation of this interaction is that S may not enter into a competitive relationship with A if the antagonist dissociation time is long compared to the rate of agonist application (Stephenson & Ginsborg, 1969; Ginsborg & Stephenson, 1974). Addition of F (in the presence of S) now allows the agonist to compete with F for some of the receptor sites that F has wrested from S, thus increasing the fraction of binding sites occupied by the agonist. As the conventional model of the drug-receptor interaction (Stephenson & Barlow, 1970) has provided a valuable framework for interpreting the ion-receptor interaction at the motor nerve ending (see Silinsky, 1977b, Introduction), it would appear to be of interest to test the Stephenson-Ginsborg model quantitatively at the external binding site on the motor nerve terminal. To this end, the following assignments were employed:

Agonist = A = Ca^{2+}, $K_A = 8\,mM^{-1}$ (for justification of K_A see Silinsky, Mellow & Phillips, 1977; Silinsky, 1978).

Slowly dissociating antagonist = S = Mg^{2+}, $K_A = 0.27\,mM^{-1}$ (see Jenkinson, 1957; Dodge & Rahamimoff, 1967; Crawford, 1974).

Fast antagonist = F = Ba^{2+}, $K_A = 1.1\,mM^{-1}$ (see Table 1). It should be noted that all K_A values were calculated for the ion as an *antagonist* using null methods. The affinities thus represent a fairly reliable estimate of the ion's 'affection' for an external site, a site that presumably regulates the entry of divalent cation agonists into the nerve ending (Crawford, 1974; Silinsky, 1977b; 1978). The fraction of receptors occupied by X ('occupancy' of X = p_X) was calculated from equation 2

$$p_X = \frac{K_A[X]}{1 + K_A[X]} \qquad (2)$$

where X = A, S, or F. Next, using these *calculated* values, the *predicted* change in occupancy was read directly from Figure 7 of Ginsborg & Stephenson (1974) and this prediction then compared with the *observed* changes in e.p.p. amplitude. As Table 2, experiments B-D show, when the addition of F (in the presence of S) predicts a decrease in the occupancy of A ($[p_A(S, F)/p_A(S)] < 1$) (column 5) then the ratio of the e.p.p. amplitude in the presence of both S and F to the e.p.p. amplitude in the presence of S alone [e.p.p. (S, F)/e.p.p. (S)] was less than 1 (column 6). Where the model predicts an increase in the agonist occupancy as a consequence of adding S, experiments E-L illustrate that release was indeed enhanced (columns 5 and 6). It is intriguing that the transition between depression and enhancement, namely the level of occupancy at which $[p_A(S, F)/p_A(S)] = 1$, is accurately predicted by the model (see experiment A).

As a general rule then, it appears that with a low occupancy of S, ($[Mg^{2+}] = 1$ mM), F acted as an antagonist while at higher occupancies of S ($[Mg^{2+}] \geq 2$ mM), F enhanced release. More precisely, Ginsborg & Stephenson (1974) predicted that in order for F to enhance release, $p_S + p_F > 1$. Table 2, column 7 shows this was indeed the case.

Before such an appealing binding model can be used to explain the interaction of divalent cations at the external surface of the nerve terminal, it is necessary to include some additional assumptions in order to meet the criteria prescribed by Ginsborg & Stephenson (1974). For example, because the K_A (= association rate constant/dissociation rate constant) for Ba^{2+} is higher than for Mg^{2+}, it is necessary to assume that the effective association rate constant for Ba^{2+} is considerably greater than for Mg^{2+} (i.e., Mg^{2+} must have a lower effective dissociation rate constant than Ba^{2+}, otherwise Mg^{2+} would be an inappropriate choice for S). If one assumes that dehydration (or partial dehydration) is a necessary prerequisite for ion binding (see e.g. Diamond & Wright, 1969), then the rate constant for dehydration

Table 2 A comparison of the experimental results with the predictions of the Stephenson–Ginsborg model (Ginsborg & Stephenson, 1974, Figure 7)

	1	2	3	4	5	6	7
$p_A = 0.9$ *for all experiments*	[F] (mM)	p_F	[S] mM	p_S	$p_A(S, F)/p_A(S)$ *(predicted)*	*e.p.p. (S, F)/ e.p.ps (S)*	$p_S + p_F$
Control condition ($\approx p_S$)	0.25	0.21	1	0.21			
Experiment							
A	1.0	0.54	1	0.21	1	1	0.76
B	2.25	0.71	1	0.21	0.8	0.67	0.92
C	2.25	0.71	1	0.21	0.8	0.63	0.92
D	3.25	0.78	1	0.21	0.75	0.64	0.97
Control condition ($\approx p_S$)	0.25	0.21	2	0.35	—	—	—
Experiment							
E	2.25	0.71	2	0.35	1.1	1.8	1.06
F	2.25	0.71	2	0.35	1.1	1.4	1.06
G	2.75	0.75	2	0.35	1.1	1.2	1.10
H	3.00	0.76	2	0.35	1.1	1.2	1.11
I	3.25	0.78	2	0.35	1.1	1.2	1.13
J	3.60	0.80	2	0.35	1.1	1.4	1.5
Control condition ($= p_S$)	—	—	9	0.75	—	—	—
K	0.9	0.5	9	0.75	1.6	1.8	1.25
L	0.9	0.5	9	0.75	1.6	1.6	1.25

A = agonist = Ca^{2+}, occupancy $p_A = 0.9$, F = fast (rapidly dissociating) antagonist = Ba^{2+}. Column 2, occupancy of Ba^{2+} calculated from equation 2 (see text) using [F] from column 1. S = slowly dissociating antagonist = Mg^{2+}. Column 4, p_S = calculated occupancy of Mg^{2+} (equation 2), using [S] from column 3. Column 5, predicted ratio (Ginsborg & Stephenson, 1974) of agonist occupancy (p_A) in the presence of both S and F (p_A(S, F)) to the agonist occupancy in the presence of S alone (p_A(S)). Column 6, experimental results, ratio of e.p.p. amplitude in the presence S and F to e.p.p. amplitude in the presence of S alone. Column 7, total occupancy of both antagonists. Note that the Stephenson-Ginsborg model prediction ($p_S + p_F > 1$ for enhancement) is borne out by the experimental results. Letters refer to different experiments. The control antagonist occupancy in experiments A to D and E to J can be considered equivalent to p_S alone as the occupancy of F at 0.25 mM is negligible. Further details in text.

would appear as part of the 'effective' equilibrium constant—a low dehydration rate constant contributing to a low K_A. The observation that Mg^{2+} loses its inner hydration sphere three or four orders of magnitude more slowly than Ca^{2+} or Ba^{2+} (Diebler, Eigen, Ilgenfritz, Maab & Winkler, 1969) could then account for the low K_A and give Mg^{2+} a lower *effective* association rate constant than Ba^{2+}, apart from any interactions at the membrane binding site. At the binding site itself, a low *effective* dissociation rate constant for Mg^{2+} might be predicted if it is considered that divalent cation entry through open conductance channels is an important force in dissociating ions from the external binding site. Thus Mg^{2+}, which behaves as an impermeant under the conditions of these experiments, would not dissociate readily from the external site whilst Ba^{2+}, which can enter the nerve ending once the conductance pathway is opened, can, in effect, dissociate rapidly from this site.

The other condition of the Stephenson-Ginsborg model is more difficult to meet, namely that the agonist is transiently applied to the receptive site. At the motor nerve ending, the agonist Ca^{2+}, rather than acting directly to stimulate synchronous ACh release, is thought to bind externally (Silinsky, 1977b) in the absence of the physiological stimulus. Ca^{2+} can thus equilibrate with *both* F and S under resting conditions. Once provoked by an individual nerve action potential, Ca^{2+} can leave the external site, move briskly down its concentration gradient through the

nerve terminal membrane and elicit the release of ACh. Now, under these non-equilibrium conditions, as a Ca^{2+} ion departs from the external site during the early part of the conductance change, it leaves a vacant site which appears to require *reoccupation* by many Ca^{2+} ions to elicit ACh release (see e.g. Llinas, 1977). If one considers that the action potential is a vehicle for transiently applying and reapplying Ca^{2+}, by allowing numerous cycles of occupation-permeation to occur during the fleeting period of the conductance change, Ca^{2+} may behave, in effect, as transiently applied agonist.

An alternative, perhaps simpler way of viewing transient Ca^{2+} application is to assume that the site of interest is an *intra-terminal* releasing site. As Ca^{2+} (i) is the only ion of the three under study that can produce e.p.ps (i.e. that can traverse the nerve terminal rapidly and activate a releasing site during the brief conductance change produced by the action potential) and (ii), can be removed rapidly from the release site (see Baker, 1972), this ion is, in effect, transiently applied to the intraterminal release site. As so little is known about intraterminal release site(s), even in a qualitative sense, it would seem presumptuous to make assumptions about the affinities of various divalent cations for such a site (see Discussion in Silinsky, 1977b; 1978; Silinsky & Mellow, 1978). However, it may be argued that such a brief yet forceful application of Ca^{2+} to an intraterminal site, when considered in light of the numerous Ca^{2+} ions that enter each channel (Llinas, 1977) implies that the action potential transiently applies Ca^{2+} to the external site as well.

To summarize, it appears that a model which considers non-equilibrium kinetic features of *binding* as the rate-limiting step in the ion-receptor interaction is capable of explaining complex interactions between divalent cations at the *external* surface of the motor nerve terminal. These results by no means exclude other possible explanations. For example, very similar quantitative predictions are made by a model that considers *diffusion* in the presence of binding as the rate-limiting step (see e.g. Thron & Waud, 1968; Roberts & Stephenson, 1976). One of the generalizations of a diffusion-binding model, however, is that S has a very high affinity and is thus used in lower concentrations than F. This does not seem to be the case in the present experiments as Ba^{2+} has a higher affinity than Mg^{2+}. It appears that measurements of the rate constants for the onset and offset of antagonism are needed before any firm distinction between these and other models is possible.

This work was supported by grant NS 12764 from the U.S. Public Health Service.

References

BAKER, P.F. (1972). Transport and metabolism of calcium ions in nerve. *Progr. Biophys. molec. Biol.*, **24,** 177–223.

CRAWFORD, A.C. (1974). The dependence of evoked transmitter release on external calcium ions at very low mean quantal contents, *J. Physiol.*, **240,** 255–278.

DIAMOND, J.M. & WRIGHT, E.M. (1969). Biological membranes: the physical basis of ion and non-electrolyte selectivity. *Ann. Rev. Physiol.*, **31,** 581–646.

DIEBLER, H., EIGEN, M., ILGENFRITZ, G., MAAB, G. & WINKLER, W. (1969). Kinetics and mechanism of reactions of main group metal ions with biological carriers. *Pure appl. Chem.*, **20,** 94–115.

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

DREYER, F. & PEPER, K. (1974). A monolayer preparation of innervated skeletal muscle fibres of the M. cutaneous pectoralis of the frog. *Pflügers Arch. ges. Physiol.*, **348,** 257–262.

GINSBORG, B.L. & STEPHENSON, R.P. (1974). On the simultaneous action of two competitive antagonists. *Br. J. Pharmac.*, **51,** 287–300.

HEUSER, J. & MILEDI, R. (1971) Effect of lanthanum ions on function and structure of frog neuromuscular junctions. *Proc. Roy. Soc. B. (Lond.)*, **179,** 247–260.

JENKINSON, D.H. (1957). The nature of the antagonism between calcium and magnesium ions at the neuromuscular junction. *J. Physiol.*, **138,** 434–444.

KATZ, B. & MILEDI, R. (1972). The statistical nature of the acetylcholine potential and its molecular components. *J. Physiol.*, **224,** 665–699.

LLINAS, R.R. (1977). Calcium and transmitter release in squid synapse. In *Approaches to the Cell Biology of the Neuron.* ed. Cowan, W.M. & Ferrindelli, J.A. Bethesda, Md: Society for Neuroscience.

MARTIN, A.R. (1955). A further study of the statistical composition of the end-plate potential. *J. Physiol.*, **130,** 114–122.

MARTIN, A.R. (1976). The effect of membrane capacitance on non-linear summation of synaptic potentials. *J. theor. Biol.*, **59,** 179–187.

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

McLACHLAN, E.M. (1977). The effect of strontium and barium at synapses in sympathetic ganglia. *J. Physiol.*, **267,** 497–518.

ROBERTS, F. & STEPHENSON, R.P. (1976). The kinetics of competitive antagonists on guinea-pig ileum. *Br. J. Pharmac.* **58,** 57–70.

SILINSKY, E.M. (1977a). Can barium support the release of acetylcholine by nerve impulses? *Br. J. Pharmac.*, **59,** 215–217.

SILINSKY, E.M. (1977b). An estimate of the equilibrium dissociation constant for calcium as an antagonist of evoked acetylcholine release: implications for excita-

tion-secretion coupling. *Br. J. Pharmac.*, **61,** 691–693.

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

SILINSKY, E.M. & MELLOW, A.M. (1978). On the relation between strontium and other divalent cations in the process of transmitter release from cholinergic nerve endings. In *Strontium and Biological Systems*, ed. Skoryna, S.C. (in press).

SILINSKY, E.M., MELLOW, A.M. & PHILLIPS, T.E.R. (1978). Conventional calcium channel mediates asynchronous acetylcholine release by motor nerve impulses. *Nature, Lond.*, **270,** 528–530.

STEPHENSON, R.P. & BARLOW, R.B. (1970). Concepts of drug action, quantitative pharmacology and biological assay. In *A Companion to Medical Studies*. Ch. 3, pp. 1–19. ed. Passmore, R. & Robson, J.S., Oxford: Blackwell.

STEPHENSON, R.P. & GINSBORG, B.L. (1969). Potentiation by an antagonist. *Nature, Lond.*, **222,** 790–791.

TASAKI, K., TSUKUHARA, Y., ITO, S., WAYNER, M. J. & YU, W.Y. (1968). A simple, direct and rapid method for filling microelectrodes. *Physiol. Behav.*, **3,** 1009–1010.

THRON, C.D. & WAUD, D.R. (1968). The rate of action of atropine. *J. Pharmac. exp. Ther.*, **160,** 91–105.

WERMAN, R. & GRUNDFEST, H. (1961). Graded all-or-none electrogenesis in arthropod muscles. II The effects of alkali earth and onium ions on lobster muscle fibres. *J. gen. Physiol.*, **44,** 997–1027.

(Received November 11, 1977.
Revised February 1, 1978.)