

Influence of Polyvalent Cations on the Activation of Muscle End Plate Receptors

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ABSTRACT The influence of polyvalent cations on the activation of end plate receptors has been studied in vitro on the sartorius muscle of the frog. In the absence of extracellular calcium, the sensitivity of the receptors to depolarizing quaternary ammonium salts was markedly reduced. Maximum receptor activation occurred in those fibers equilibrated in 1.8 mM calcium Ringer solution, with the response being reduced as the calcium concentration was raised or lowered. Magnesium was less efficient than calcium in regulating the sensitivity of the end plate receptors, the maximum receptor response occurring in those fibers equilibrated in 8 mM magnesium Ringer solution. In the presence of lanthanum the end plate response to carbamylcholine or acetylcholine was enhanced. Lanthanum increased the conductance change produced by carbamylcholine both in polarized and in potassium-depolarized fibers. The application of 10^{-2} mM lanthanum to the end plate increased MEPP's amplitude, rise time, and half-fall time by 19, 54, and 45 %, respectively. The results suggest that polyvalent cations influence postjunctional membrane receptor processes in addition to their well-documented prejunctional action.

INTRODUCTION

Magnesium and calcium ions, in high concentration, have been shown to decrease the response of the end plate receptors at the neuromuscular junction (del Castillo and Engbaek, 1954; Takeuchi, 1963; Mambrini and Benoit, 1964; Nastuk and Liu, 1966). Nastuk (1967) has suggested that these divalent cations competitively inhibit the cholinergic receptor.

In previous work, the influence of raising the extracellular calcium or magnesium concentration on the sensitivity of end plate receptors has been determined. Few studies have concerned receptor sensitivity in divalent ion-free media, primarily because of the changes in membrane excitability which occur under these conditions (Jenden and Reger, 1963; Koketsu,

1969). In the present study, the influence of calcium and magnesium on the sensitivity of end plate receptors has been reinvestigated. In addition, the influence of lanthanum ions on the response of the cholinergic receptor to carbamylcholine and acetylcholine has been studied.

MATERIALS AND METHODS

General Methods

All experiments were performed in vitro on the sartorius muscle of the frog (*Rana pipiens*) at room temperature (16–22°C). The muscles were dissected and mounted in a phosphate-buffered solution as described previously (Parsons, 1969 *a, b*). When the muscle was securely positioned, a Tris-buffered (tris (hydroxymethyl) amino-methane) Ringer solution was introduced into the muscle chamber and the preparation was allowed to equilibrate for at least 30 min. All experimental Ringer solutions used in this study were buffered with 1.0 mM Tris to avoid the precipitation of calcium. The pH of these solutions was maintained between 7.2 and 7.4. The composition of the test solutions is given in Table I except when indicated. In order to facilitate the removal of calcium, the calcium-free Ringer solutions contained 1 mM ethylene bis (oxyethylenetrilo) tetraacetic acid (EGTA).

Many of the experiments were done in solution C. In this medium, the resting potentials of individual muscle fibers ranged between 0 and –20 mv. Although the muscle fibers become electrically inexcitable when bathed in solution C, drug-induced changes in effective transmembrane resistance (EMR) at the end plate region can be measured (Parsons and Nastuk, 1969). It was found that prior to CARB application the EMR recorded from fibers equilibrated in solution C (0.154 ± 0.006 M Ω , mean \pm SE, in 87 fibers) was not significantly different (*t* test) from that obtained from fibers equilibrated in solution D (0.146 ± 0.009 M Ω , mean \pm SE, in 30 fibers). Consequently, depolarized muscle fibers were used in this study to avoid difficulties associated with changes in excitability which occur with calcium deprivation.

In other experiments, done in solution B, tetrodotoxin (1×10^{-7} g/ml) was often included in the bathing solution to eliminate muscle action potentials which would

TABLE I
COMPOSITION OF RINGER SOLUTIONS

Solution	NaCl	KCl	CaCl ₂	Na ₂ HPO ₄	NaH ₂ PO ₄	K propionate	Ca propionate	Tris	EGTA
	mM	mM	mM	mM	mM	mM	mM	mM	mM
A	111	2.5	1.8	2.55	0.45				
B	120	2.5	1.8					1	
C*						122.5	1.8	1	
D†						122.5		1	1

* In some instances the calcium concentration of solution C was altered as indicated in the text.

† In other experiments Mg propionate was added to solution D as indicated in the text.

normally interfere with accurate measurements of drug-induced end plate depolarizations (Parsons, 1969 *a, b*).

The recording techniques employed to measure the resting membrane potential, miniature end plate potentials, and the EMR of single muscle fibers have been described previously (Parsons, 1969 *a, b*). The micropipettes used in this study for intracellular recording were filled with either 3 M potassium propionate (10–20 M Ω resistance) or 3 M KCl (8–12 M Ω resistance).

Drugs and Drug Application

In the present study carbamylcholine chloride (CARB) (K & K Laboratory, Plainview, N.Y.), an analogue of acetylcholine (ACh) which is resistant to hydrolysis by acetylcholinesterase, was most often used to activate the end plate receptors. The CARB was microperfused through a glass pipette (50–100 μ m in diameter) placed at the junctional region of individual fibers (Manthey, 1966; Parsons, 1969 *a, b*). In order to avoid the desensitization of receptors on adjacent muscle fibers, the muscle preparation was washed with CARB-free solution for at least 15 min prior to attempting another perfusion. Furthermore, only one CARB perfusion was done at any particular area on the muscle in which the end plate regions of individual fibers could be localized. The junctional regions of the individual muscle fibers were located visually under a magnification of 300 times using a Bausch and Lomb dynoptic microscope with a Leitz long working distance objective, by following nerve filaments to their termination. With this optical system (1.2 cm working distance) the microelectrode impalement and microperfusion could be performed under the same magnification. In those fibers equilibrated in solution B, MEPP's were recorded intracellularly at such junctions (Fatt and Katz, 1952).

The lanthanum chloride (LaCl₃·6H₂O) was microperfused onto the end plate region of individual fibers either alone or together with CARB.

Measurement of Receptor Activation

In most experiments the EMR of single muscle fibers was measured at the end plate region prior to, during, and immediately following microperfusion of CARB. In this case the decrease of the EMR produced by CARB was used as a measure of receptor activation. In other experiments, the resting potential in the region of the neuromuscular junction was monitored before, during, and after CARB microperfusion. In this instance the depolarization produced was used to estimate the extent of receptor activation. In a few experiments, changes in MEPP amplitude and time course were used to measure receptor sensitivity to ACh.

RESULTS

Response of the End Plate Receptors in Calcium-Free Ringer

In the first series of experiments, the end plate sensitivity to CARB was determined in potassium-depolarized muscles which were equilibrated in either the presence or the absence of calcium (solutions C or D, respectively). The sensitivity of the receptors was estimated by measuring the decrease of

the EMR produced by CARB.¹ Removal of calcium from the extracellular fluid reduced the sensitivity of the end plate receptors to CARB (Fig. 1). However, the maximum decrease of the EMR produced by 21.6 mM CARB was similar in those muscles equilibrated in either the calcium-free Ringer solution (solution D) or the Ringer solution containing 1.8 mM calcium (solution C).

Records which illustrate the decrease of the EMR produced by the application of 0.27 mM CARB to the end plate region of potassium-depolarized fibers are shown in Fig. 2. Example A is taken from a fiber equilibrated in

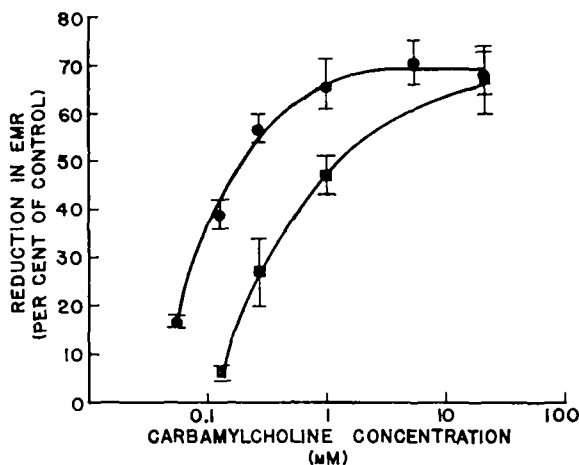


FIGURE 1. The effect of calcium deprivation on the EMR change produced at the end plate by CARB in potassium-depolarized fibers. Each point represents the mean response from at least five fibers. Reduction in EMR by CARB in muscles equilibrated in solution C (solid circles). Reduction in EMR by CARB in muscles equilibrated in solution D (solid squares). The vertical bars indicate standard error of the mean.

solution C. The reduction of the amplitude of the membrane hyperpolarizations produced by the applied current pulses shows that CARB caused a rapid diminution of the input resistance of this fiber. Also notice that in example A the EMR returned rapidly toward control levels with the CARB perfusion sustained. This recovery we interpret from previous work to be due to desensitization of the receptors (Manthey, 1966; Parsons, 1969 *b*). Record B was obtained from a muscle fiber equilibrated in solution D. Note

¹ In Figs. 1, 3, 4, and 5 and Table II the reduction (decrease) in EMR was calculated as follows:

$$\left[\frac{\text{Control (preperfused) EMR} - \text{minimum EMR in the presence of CARB}}{\text{Control EMR}} \right] \times 100$$

For example in Fig. 2 A, the control EMR was 0.125 M Ω and the minimum EMR in the presence of CARB was 0.040 M Ω . Therefore, the reduction in EMR (per cent of control) in this fiber was 68%.

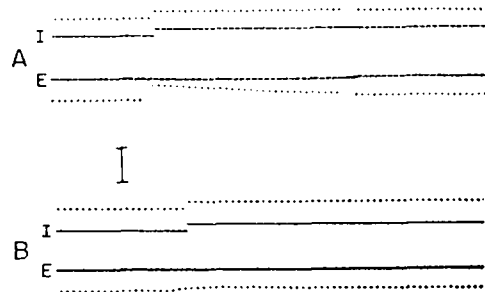


FIGURE 2. The effect of 0.27 mM CARB on the EMR measured at the end plate of potassium-depolarized fibers. In A and B the E trace represents membrane potential and the I trace indicates membrane current. The dots below the E trace denote the hyperpolarizations produced by constant amplitude, 400 msec current pulses delivered at 2 sec intervals. The magnitude of the current pulse, which was recorded as a potential change developed across a known resistance, is presented as dots above the I trace. The upward deflection of the I trace follows the onset of CARB application by 4–6 sec. Example A was obtained from a muscle fiber equilibrated in solution C whereas B represents the response recorded from a fiber equilibrated in solution D. The calibration represents 20 mv for E and 2×10^{-7} A for I. The break in A is a 60 sec period during which the recording was stopped.

that the decrease of the input resistance produced by CARB was considerably less.

In similar experiments performed using potassium-depolarized muscle preparations, we found that the removal of the extracellular calcium also reduced receptor sensitivity to 0.54 mM tetramethylammonium (TMA) and 0.5 mM succinylcholine. However, the maximum decrease of the EMR produced by 21.6 mM TMA or 21.6 mM succinylcholine was the same in muscle preparations equilibrated either in solution C or D.

Influence of Calcium and Magnesium on the Sensitivity of the End Plate Receptors

The maximum decrease of the EMR produced by application of 0.27 mM CARB was determined for potassium-depolarized muscle preparations equilibrated in various concentrations of extracellular calcium (Fig. 3). These experiments were done in solution D and solution C (with the calcium concentration varied in the range of 0.1 to 10 mM). The maximum EMR change (mean of at least eight fibers) produced by 0.27 mM CARB occurred in muscles equilibrated in the 1.8 mM calcium solution, with the response being reduced as the calcium concentration was raised or lowered.

Similar experiments were performed in muscles equilibrated in solution D containing magnesium propionate up to 12 mM. The results are summarized in Fig. 4, in which the change in the EMR (mean of at least five fibers) is

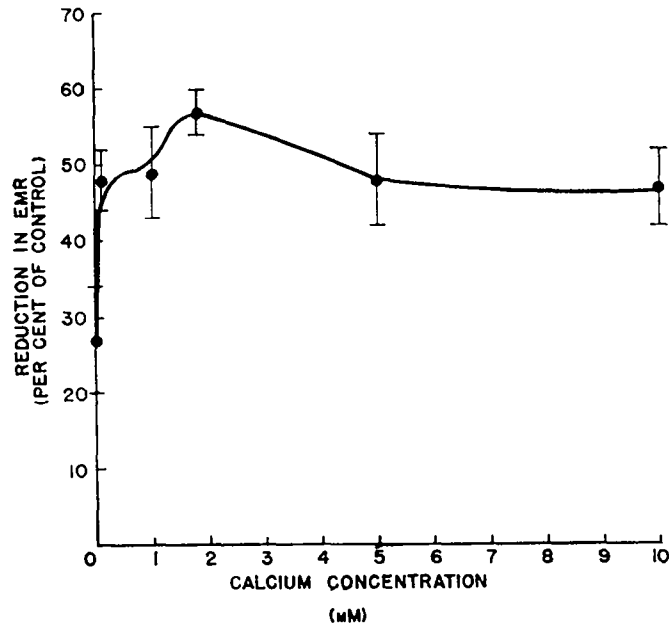


FIGURE 3. The effect of calcium on the EMR change produced at the end plate by 0.27 mM CARB in potassium-depolarized fibers. Each point represents the mean response from at least eight fibers. Vertical bars indicate standard error of the mean.

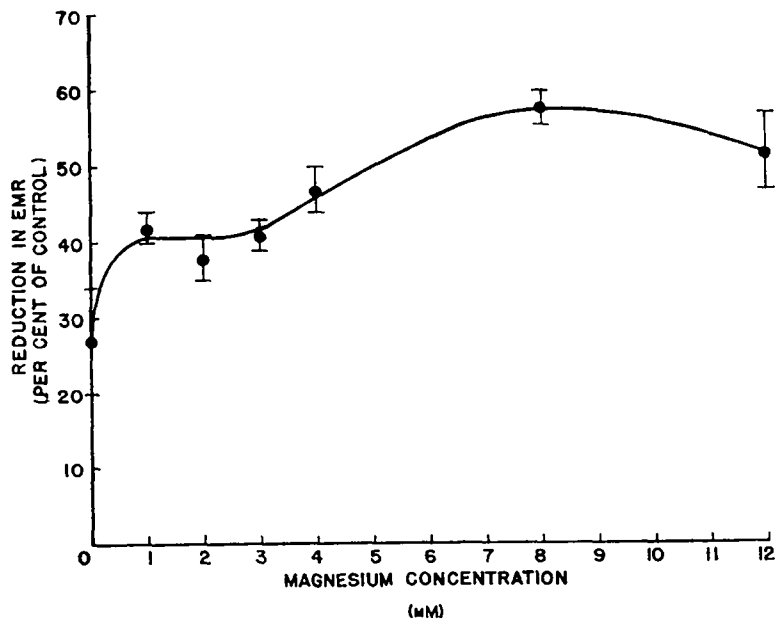


FIGURE 4. The effect of magnesium on the EMR change produced at the end plate by 0.27 mM CARB in potassium-depolarized fibers. Each point represents the mean response from at least five fibers. The vertical bars indicate the standard error of the mean.

plotted as a function of the extracellular magnesium concentration. The maximum change in EMR produced by 0.27 mM CARB occurred in muscles equilibrated in 8 mM magnesium; the mean response being depressed as the concentration of magnesium was raised or lowered.

Influence of Lanthanum on Receptor Activation in Depolarized Fibers

Lanthanum ions influence excitable membranes in a manner similar to very high calcium levels (Takata et al., 1966; Blaustein and Goldman, 1968). Because of the similarity between the influence of calcium and lanthanum on electrical excitability, a study of the influence of lanthanum ions on the end plate response to CARB and ACh has been undertaken. The initial experiments were performed on depolarized muscles equilibrated in solution C (calcium = 1.8 mM). The lanthanum ions (10^{-2} mM) and the CARB (at the concentrations indicated in Fig. 5) were combined in the microperfusate. The mean decrease of the EMR produced by CARB plus 10^{-2} mM lanthanum ions was greater than that produced by CARB alone even at high CARB concentrations (Fig. 5).

The influence of varying the lanthanum ion concentration (muscles equilibrated in solution C) in the range of 10^{-4} to 1 mM on the EMR change produced by 0.135 mM CARB is summarized in Table II. Lanthanum (up to 1 mM) increased the CARB-activated end plate response (Table II). However, in the absence of CARB, microperfusion of lanthanum (1 mM) did not produce any immediate change in the EMR of individual muscle fibers.

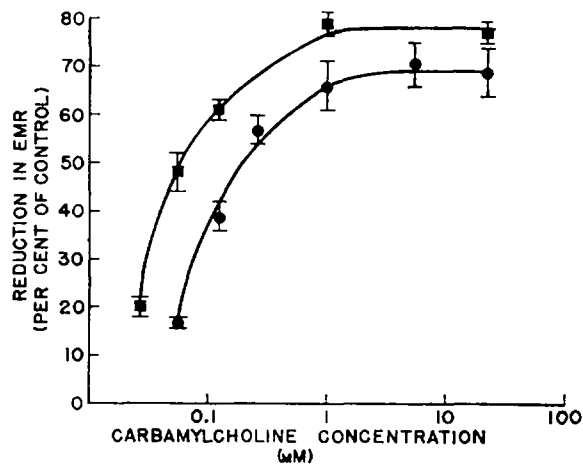


FIGURE 5. The influence of lanthanum on the EMR change produced at the end plate by CARB in potassium-depolarized fibers. Each point represents the mean response from at least six fibers. Reduction in EMR by CARB in muscles equilibrated in solution C (solid circles). Reduction in EMR by CARB + 10^{-2} mM La^{3+} in muscles equilibrated in solution C (solid squares). The vertical bars indicate the standard error of the mean.

TABLE II
INFLUENCE OF LANTHANUM IONS ON THE
END PLATE RESPONSE TO 0.135 mM CARB FOR
FIBERS EQUILIBRATED IN SOLUTION C

La ³⁺ concentration	EMR decrease	No. of fibers
<i>mM</i>	<i>% of control</i>	
—	39.4±3.0*	5
10 ⁻⁴	40.7±1.2	6
10 ⁻²	60.6±2.3	6
1	58.0±2.1	6

* Mean ± SE.

Influence of Lanthanum on CARB Depolarization in Polarized Fibers

Additional experiments were done to determine the influence of lanthanum ions on receptor activation in muscles equilibrated in solution B to which tetrodotoxin (10⁻⁷ g/ml) was added. The mean resting membrane potential was -94 mv in 29 fibers. In these experiments the depolarization produced by 0.011 mM CARB alone was compared to that produced by 0.011 mM CARB microperfused simultaneously with lanthanum in the range of 10⁻⁴ to 10⁻¹ mM. The results, summarized in Table III, demonstrate that lanthanum in the range of 10⁻³ to 10⁻¹ mM markedly increased the depolarization produced by 0.011 mM CARB. Examples of the depolarization produced by 0.011 mM CARB in the absence and presence of 10⁻² mM lanthanum ions are shown in Fig. 6.

During the sustained application of quaternary ammonium compounds to muscle fibers, the membrane is rapidly depolarized, but then more slowly repolarizes (Thesleff, 1955; Nastuk, 1967; Parsons, 1969 *b*). Under these conditions, the receptors are said to be "densitized." In those fibers microperfused with 0.011 mM CARB (in the absence of lanthanum) the

TABLE III
INFLUENCE OF LANTHANUM IONS ON
0.011 mM CARB DEPOLARIZATIONS

PJM* potential control	CARB perfused PJM- minimum potential	Change in PJM potential	La ³⁺ concentration	No. of fibers
<i>mv</i>	<i>mv</i>	<i>mv</i>	<i>mM</i>	
-93.1±1.1‡	-76.0±1.0	17.1±0.9	—	8
-94.8±1.5	-78.0±1.6	16.8±0.1	10 ⁻⁴	4
-93.3±1.3	-55.3±2.0	38.0±1.0	10 ⁻²	6
-93.6±1.6	-60.6±2.0	33.0±1.7	10 ⁻²	7
-98.5±1.3	-65.0±2.4	33.5±3.4	10 ⁻¹	4

* Postjunctional membrane.

‡ Mean ± SE.

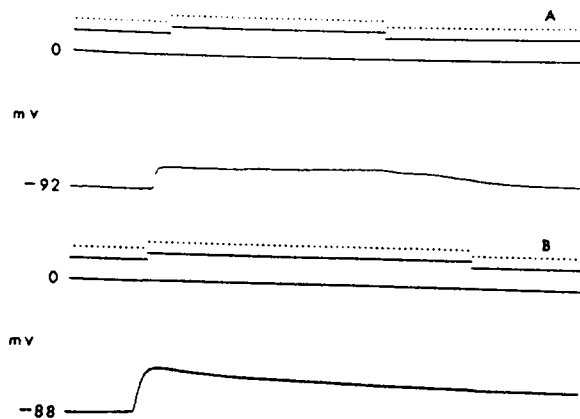


FIGURE 6. Examples of the depolarization produced during microperfusion of 0.011 mM CARB from two different fibers equilibrated in solution B: A, CARB applied alone; B, CARB simultaneously perfused with 10^{-2} mM La^{3+} . The upward deflection of the time base follows the onset of the CARB perfusion by approximately 4 sec. The downward deflection of the time base marks the termination of the perfusion.

depolarization was maintained which indicated that no significant amount of receptor desensitization occurred (Fig. 6 A). In contrast, a marked repolarization following the initial depolarization was often observed when CARB (0.011 mM) and lanthanum ions (10^{-2} – 10^{-1} mM) were applied together. These results suggest that lanthanum ions in addition to increasing the extent of the CARB depolarization also accelerate receptor desensitization.

Influence of Lanthanum on the CARB Reversal Potential

The influence of lanthanum (10^{-2} mM) on the CARB reversal potential has been determined in fibers equilibrated in solution B made approximately 2.3 times hypertonic by the addition of 300 mM sucrose. The hypertonic solution was used because in this medium mechanical activity of the muscle fiber is greatly diminished (Hodgkin and Horowicz, 1957; Manthey, 1966). Previously, Parsons (1969 *b*) demonstrated that the CARB reversal potential is shifted to a more negative value in those fibers equilibrated in the hypertonic sodium-sucrose Ringer solution. In fibers equilibrated in the hypertonic Ringer solution, 10^{-2} mM lanthanum ions did not shift the CARB reversal potential. These results, which are summarized in Table IV, suggest that lanthanum does not selectively alter the sodium or potassium conductances.

Influence of Lanthanum on Miniature End Plate Potential Amplitude and Time Course

The influence of lanthanum ions on the amplitude and time course of MEPP's was also determined. In these experiments, 10^{-2} mM lanthanum ions

TABLE IV
INFLUENCE OF LANTHANUM IONS ON
THE CARB REVERSAL POTENTIAL IN HYPERTONIC
SODIUM-SUCROSE RINGER

PJM* potential control	CARB perfused PJM- minimum potential	CARB concentration	La ³⁺ concentration	No. of fibers
<i>mv</i>	<i>mv</i>	<i>mM</i>	<i>mM</i>	
-93.0±1.1‡	-23.3±1.4	5.4	—	12
-98.0±1.8	-22.5±1.2	5.4	10 ⁻²	4

* Postjunctional membrane.

‡ Mean ± SE.

were microperfused directly onto the junctional regions of individual muscle fibers equilibrated in solution B, so that MEPP's recorded prior to lanthanum application were compared to those obtained during lanthanum perfusion in each fiber. Physostigmine sulfate (10⁻⁶ g/ml) was included in the bathing solution to inhibit the acetylcholinesterase. In some of the experiments the tonicity of the solution was increased 10% by the addition of sucrose to increase the MEPP frequency. In the presence of 10⁻² mM lanthanum ions, the mean amplitude, the mean rise time, and mean half-fall time of the MEPP's increased, the mean change for eight fibers being 19, 54, and 45%, respectively. In order to demonstrate the change in MEPP configuration which occurred in the presence of lanthanum, the results obtained in one fiber are presented in Table V. In this fiber, 10⁻² mM lanthanum significantly increased the MEPP amplitude, rise time, and half-fall time; the changes being 20% (*t* test, *p* < 0.01), 68% (*p* < 0.001), and 65% (*p* < 0.001), respectively. The change in MEPP configuration were not due to an increase in membrane resistance. No significant change in EMR (recorded from the region of the neuromuscular junction in four fibers which were not pretreated with physostigmine) was noted immediately following the perfusion of 10⁻² mM lanthanum, the mean preperfused and perfused EMR values being 0.466 MΩ and 0.471 MΩ, respectively. In addition to the postjunctional action,

TABLE V
INFLUENCE OF LANTHANUM ON
MEPP CONFIGURATION AS RECORDED
FROM A SINGLE FIBER

La ³⁺ concentration	MEPP amplitude	MEPP rise time	MEPP half-decay time	No. of MEPP'S
<i>mM</i>	<i>mv</i>	<i>msec</i>	<i>msec</i>	
—	0.80±0.04*	1.27±0.05	3.37±0.14	32
10 ⁻²	0.96±0.04	2.13±0.10	5.55±0.25	32

* Values are means ± SE mean.

lanthanum (10^{-2} mM) markedly increased MEPP frequency. For example, in three other fibers (not treated with physostigmine) the MEPP frequency increased from $2.3 \pm 0.9/\text{sec}$ to $165.0 \pm 32.8/\text{sec}$ after 10 min of microperfusion with 10^{-2} mM lanthanum. Similar results have been reported by Blioch et al. (1968).

DISCUSSION

In the present study, removal of divalent cations from the external fluid markedly reduced the response of the end plate receptors elicited by CARB. The addition of either calcium or magnesium to the extracellular fluid increased the receptor response, although calcium was found to be more efficient than magnesium. Maximum receptor activation occurred in the presence of 1.8 mM calcium or 8 mM magnesium (Figs. 3 and 4). Similarly, Nastuk and Liu (1966) demonstrated in polarized fibers that maximum receptor activation by CARB occurred in the presence of 1.8 mM calcium in the extracellular fluid. The decrease of receptor activation observed in the absence of divalent cations suggests that in addition to the well-documented prejunctional influence of calcium and magnesium (Simpson, 1968), these divalent cations may regulate the sensitivity of the end plate receptors.

An increase in the extracellular calcium above 2 mM or in the extracellular magnesium above 8 mM also decreased the response of the end plate to CARB. Comparable results have been reported by del Castillo and Engbaek (1954), Takeuchi (1963), and Nastuk (1967). Takeuchi (1963) postulated that calcium decreased the sodium conductance change and to a lesser extent the potassium conductance change produced by ACh at the end plate. Nastuk (1967) has suggested that these divalent cations may also competitively inhibit the end plate receptors. A raise in the extracellular calcium or magnesium has also been shown to depress the sodium (primarily) and potassium conductances associated with the action potential in excitable membranes, although calcium is much more effective than magnesium (Frankenhaeuser and Hodgkin, 1957; Blaustein and Goldman, 1968).

Lanthanum ions increased the response of the end plate receptors to CARB or ACh. For instance, the conductance change at the end plate produced by CARB was increased in the presence of lanthanum (Fig. 5). Accordingly, the depolarization produced by 0.011 mM CARB and the MEPP amplitude were increased in the presence of lanthanum although the CARB reversal potential was not altered. Furthermore, lanthanum increased the maximum end plate conductance change produced by high concentrations of CARB. The mechanism by which lanthanum increases the end plate conductance change produced by CARB is not clear. However, it is apparent that the effect of lanthanum on the end plate conductance system differs from that produced by lanthanum on the conductance system of excitable mem-

branes. For example, lanthanum decreases the sodium and potassium conductances in nerve associated with the action potential so that excitability is reduced (Takata et al., 1966; Blaustein and Goldman, 1968; Hafemann, 1969).

Although lanthanum increases the maximum end plate conductance change produced by CARB or ACh, lanthanum apparently slows receptor activation. This effect is apparent from the change in MEPP time course in the presence of lanthanum, in particular the slowing of the rising phase of the MEPP's.

From previous studies on nerve, it has been suggested that La^{3+} is 20 times more effective than calcium in reducing excitability (Takata et al., 1966). If we assume that lanthanum is also approximately 20 times more potent than calcium at the end plate, one would expect to observe depression of the end plate conductance change produced by CARB in the presence of 1 mM lanthanum. For instance, Nastuk and Liu (1966) have shown that in high concentration (10–20 mM) calcium reduced the end plate depolarization produced by CARB. Similarly, Takeuchi (1963) demonstrated that increasing the extracellular calcium from 1.8 to 18 mM decreased the end plate current produced by ACh. However, in the present study no depression of the end plate response produced by La^{3+} was observed although concentrations greater than 1 mM were not used (Table II).

Figs. 5 and 6 demonstrated that lanthanum accentuates the CARB-activated end plate conductance change. However, the results in Fig. 6 also show that lanthanum accelerates the rate at which end plate conductance turns off during prolonged CARB application; the latter effect is observed as end plate desensitization. Therefore, although calcium (in high concentrations) and lanthanum have dissimilar actions on end plate receptor activation, these cations exhibit similar effects on end plate desensitization.

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