

increases the sodium current associated with subsequent depolarization by about 50%.

3. These effects are described by stating that depolarization gradually inactivates the system which enables sodium ions to cross the membrane.

4. In the steady state, inactivation appears to be almost complete if the membrane potential is reduced by 30 mV. and is almost absent if it is increased by 30 mV. Between these limits the amount of inactivation is determined by a smooth symmetrical curve and is about 40% complete in a resting fibre at the beginning of an experiment.

5. At 6° C. the time constant of the inactivation process is about 10 msec. with $V=0$, about 1.5 msec. with $V=-30$ mV. and about 5 msec. at $V=+30$ mV.

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THE EFFECT OF CALCIUM IONS ON THE MOTOR END-PLATE POTENTIALS

By J. DEL CASTILLO AND L. STARK

From the Department of Physiology, University College, London

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Early studies of Locke (1894) revealed that neuromuscular transmission is greatly affected by the ionic composition of the surrounding medium, one of the important factors being the amount of ionized calcium present. Later work has shown that calcium affects the amplitude of the end-plate potential (Eccles, Katz & Kuffler, 1942; Katz, 1942; Coppée, 1943, 1946; Kuffler, 1944). It has been suggested by Feng (1936a, b), Feng & Shen (1937) and Cowan (1940a, b) that the amount of acetylcholine liberated at the motor nerve endings depends on the concentration of calcium ions. This hypothesis, which received support from the work of Harvey & MacIntosh (1940) on the cat's perfused cervical sympathetic ganglion, would also explain the action of calcium on the end-plate potential.

In this paper experiments are described which were performed with the purpose of establishing quantitative relationships between calcium concentration and the size of the end-plate potential, and obtaining additional information on the nature of this effect.

METHOD

End-plate potentials

The sciatic-sartorius preparation of the frog *Rana temporaria* was employed. The muscle was placed vertically in a bath so arranged that solutions could be changed readily and the fluid level adjusted accurately. One electrode was placed on the uppermost pelvic end, the other dipped into the fluid so that the surface of the bathing solution acted as the moving electrode. The nerve was lifted out of the bath and kept in fixed position on the stimulating electrodes; shocks of supra-maximal strength were applied.

The electrical apparatus consisted of platinum stimulating and recording electrodes, a two-stage condensed-coupled push-pull preamplifier, a cathode-ray oscilloscope with attached camera, and an electronic square-wave stimulator. A shielded transformer in the stimulating circuit reduced stimulus escape.

The procedure was to soak the muscle in the bathing solution for a period of about 30 min. to allow for equilibration. Then the fluid level was adjusted in 0.5 mm. steps until the locus of the

maximum end-plate potential was established; this generally occurred at about 1 cm. from the pelvic end. Once this point had been determined it could be easily found again throughout each experiment. There was a small variation in the height of the end-plate potentials taken successively, at the same place, and in plotting the changes of amplitude the largest one of each group was taken. All the experiments were performed at room temperature, about 21° C.

Depolarization experiments

A similar method to that described by Fatt (1950) was employed. The sartorius muscle was placed in a bath arranged so that the fluid could be run out at an approximately constant rate. Non-polarizable silver-silver chloride agar electrodes were used; contact was made by a wick soaked in Ringer's fluid. One electrode was placed on the pelvic end; the other dipped into the fluid, the surface of which acted as a moving electrode. The electrical recording apparatus consisted of a cathode follower, a direct-coupled amplifier and a pen recorder. The procedure was to record the potential difference between the electrode on the pelvic end of the muscle and the falling surface of the bathing solution. Thus the potential along the length of the muscle was recorded continuously. Without acetylcholine in the bathing fluid the muscle surface potential was relatively uniform; muscles which showed potential differences greater than 1 mV. were discarded. Special care was necessary to avoid even small injuries to the muscle fibres during dissection; the pelvic end proved to be particularly liable to damage. In the early experiments the muscles were soaked in Ringer's fluid overnight, at 4° C., to eliminate injury potentials; eventually, however, a careful dissection and immediate use of the muscles was adopted. After soaking the preparation with a solution which contained acetylcholine a depolarization of the muscle surface was observed corresponding to the spatial distribution of nerve endings.

Solutions

Frog Ringer's fluid was made up with the following ionic composition:

Na ⁺	115 mm.
K ⁺	2.1 mm.
Ca ⁺⁺	1.8 mm.
Cl ⁻	120.7 mm.

Calcium concentration was changed without altering the concentration of the other cations. Thus tonicity was altered within the range of 99–106 % of the Ringer's solution, unless otherwise noted.

D-Tubocurarine chloride (Barroughs Wellcome & Co.) was used throughout in a concentration of 3×10^{-6} . With this concentration, complete neuromuscular block was obtained and the end-plate potential could easily be recorded.

Neostigmine bromide (Prostigmine Roche) was used in a concentration of 1×10^{-6} in the depolarization experiments, and both it and acetylcholine chloride (Roche) were made up freshly each day.

RESULTS

End-plate potentials

In initial experiments calcium concentration was varied over a wide range, and the accompanying changes in the end-plate potential amplitude are shown in Fig. 1. It was possible to obtain end-plate potentials in calcium concentration ranging from 0.2 to 110 mm.; the maximum size occurred at about 18 mm. For calcium concentrations less than 7.2 mm. osmotic effects are likely to be negligible, but the increased tonicity may well account for the decline in

end-plate potentials seen at concentrations greater than 20 mm. From 0.45 to 7.2 mm. the amplitude of the end-plate potential appeared to be approximately directly proportional to the calcium concentration. Later experiments were devoted to the study of the effect of changes in calcium concentration within this range.

In Fig. 2A the results of one such experiment are shown. The full circles, falling along the sloping line, show the decay of the end-plate potential in the course of several hours, the muscle having been soaked in Ringer's solution.

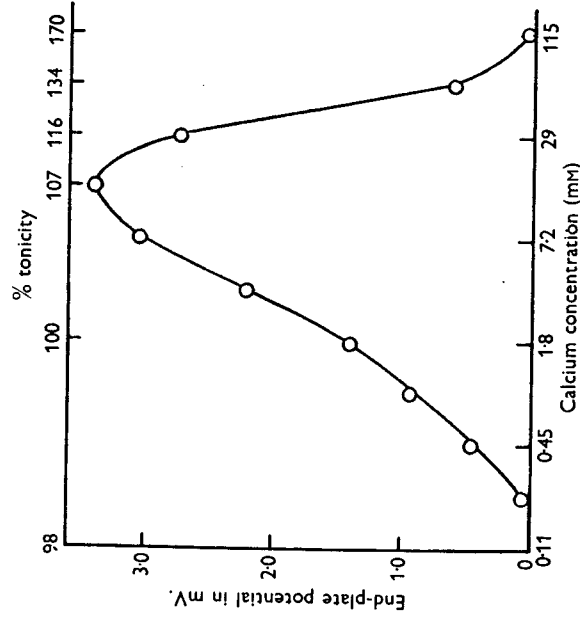


Fig. 1. Ordinates: amplitude of the end-plate potentials in mV. Abscissae: calcium concentration expressed in mm. on logarithmic scale. Note that increased calcium concentration involves increased tonicity of the solution as a percentage of the tonicity of Ringer's solution containing 1.8 mm. calcium, as shown in the upper border of the figure.

The hollow circles show the effect on the amplitude of the end-plate potential of changing the calcium concentration. The steady decline of end-plate potential was found in all the experiments; it was taken into account by comparing the amplitude of the end-plate potential in modified calcium solutions with the mean of their amplitudes in Ringer's fluid before and afterwards.

In Fig. 3A the results of sixteen experiments of this type are plotted. It is seen that the size of the end-plate potential is directly proportional to the calcium concentration over the range studied.

Plotted in Fig. 2B are the half-decay times of the end-plate potentials. Although the size of these potentials varied from 0.2 to 3 mV., there was no significant change in half-decay times either correlated with changes in calcium concentration or with the time from the beginning of the experiment.

An analysis of anticholinesterase action was made by Eccles & MacFarlane (1949) using the frog-sartorius preparation. Although changes in the size of the end-plate potentials were obtained, their most striking finding was the great increase in half-decay time with increasing anticholinesterase activity. The absence of any change in the half-decay times of the end-plate potentials

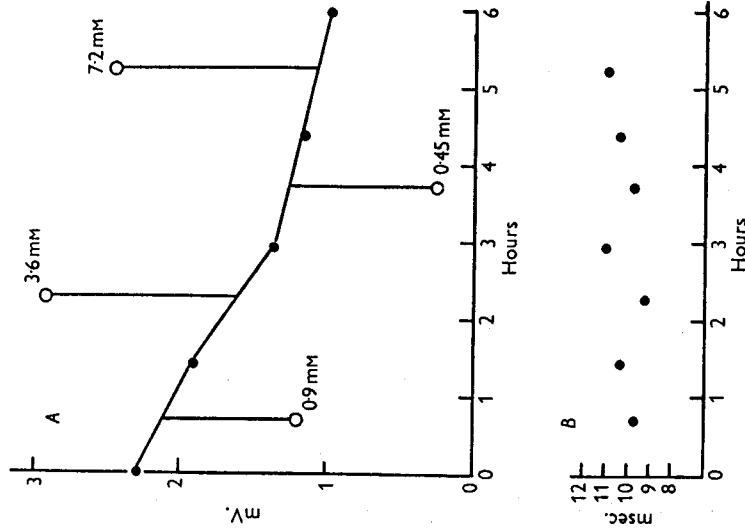


Fig. 2. *A.* Ordinates: amplitude of the end-plate potentials in mV; full circles, in Ringer's fluid; hollow circles, in fluid with modified calcium concentration as indicated. Abscissae: time in hours from the beginning of the experiment. *B.* Ordinates: half-decay times of the end-plate potentials in msec. Each point corresponds to the end-plate potential whose amplitude is represented in *A* on the same abscissa.

in our experiments, while their height varied about twenty-fold, indicates that calcium had no anticholinesterase effect.

The constancy of the half-decay times also provides some evidence that the recording was taken consistently from the region of maximum density of end-plates, since Eccles, Katz & Kuffler (1941) have shown that even small deviations from this point cause the half-decay time of the end-plate potentials to increase considerably.

The time interval between the stimulus and the peak of the end-plate potential includes excitation time, conduction of the nerve impulse into the

nerve endings and depolarization of the end-plate by liberated acetylcholine. The fact that this period remained constant suggests that the conduction of the nerve impulses into the fine nerve endings was not affected by changes in calcium concentration.

As D-tubocurarine was present in all these experiments the question had to be considered whether the effect of calcium concentration on the magnitude of the end-plate potential might be due to a direct anticholinergic action of the

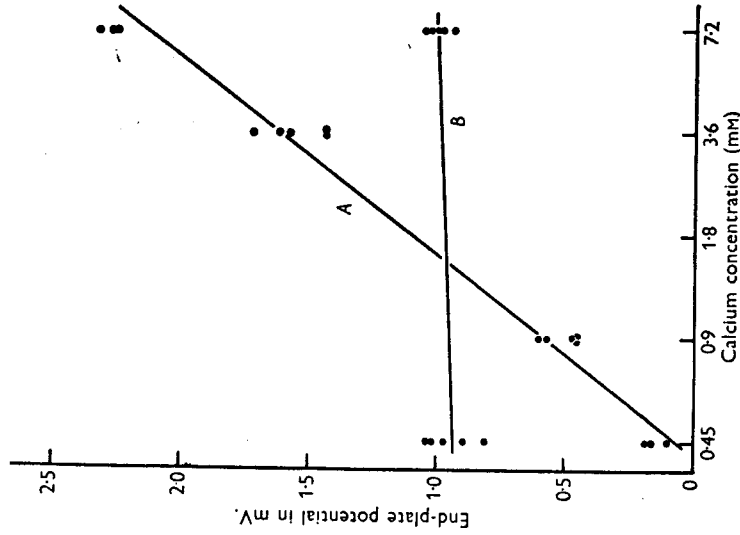


Fig. 3. *A.* Ordinates: amplitude of the end-plate potential in relative units with their amplitude in Ringer's solution (1.8 mM. calcium) taken as unity. Abscissae: calcium concentration in mM. plotted logarithmically. *B.* Ordinates: amplitude of the depolarization in relative units. Its amplitude in Ringer's solution is taken as unity. Abscissae: same as for *A.*

calcium ions. In order to investigate this matter a non-curarized muscle was soaked in a solution containing acetylcholine (0.2×10^{-6}) and prostigmine (2.5×10^{-6}) until block of neuromuscular transmission was obtained (cf. Fillenz & Hanafin, 1947). At this stage nerve stimulation elicited an end-plate potential similar in size to the curare end-plate potential. If the calcium concentration was then varied, as described above, the size of this 'acetylcholine-prostigmine end-plate potential' was affected in the same manner as that of the 'curarine end-plate potential'. It is clear, therefore, that the

effect of calcium can be obtained in the absence of curarine, and can hardly be explained by any specific antagonism between calcium ions and D-tubocurarine.

Depolarization experiments

Additional information was needed to decide whether the effect of varying calcium concentration might be due to a change of the sensitivity of the end-plates to a constant amount of acetylcholine or to a variation of the amount of acetylcholine released at the nerve terminals following an impulse. Some information on this point was obtained by studying the depolarizing action of applied acetylcholine on the end-plate region of the muscle. This depolarization is a more direct index of end-plate sensitivity than the muscle twitch or spike and is also more immediately comparable to the end-plate potentials.

TABLE 1. Effect of D-tubocurarine and neostigmine on the concentration of acetylcholine needed to produce a depolarization of about 3 mV.

D-Tubocurarine (3×10^{-6})	Neostigmine (1×10^{-4})	Acetylcholine ($\mu\text{g./ml.}$)
-	-	4 (1.5-5.0)
+	-	60 (30.0-70.0)
-	+	0.8 (0.4-1.2)
+	+	6 (1.5-12.0)

In Table 1 are shown the approximate concentrations of acetylcholine needed to produce a depolarization of about 3 mV. in this preparation under a variety of conditions. In this experiment two concentrations of acetylcholine differing by a factor of two or three were used to ensure that the observed depolarizations were below maximal, and that changes in acetylcholine sensitivity could readily be detected.

The amount of depolarization was found to vary with the time allowed after adding acetylcholine to the bathing solution. Immediately after application of acetylcholine a transient twitching of the muscle frequently occurred; the depolarization then rose to a maximum in 3-5 min., and then slowly began to decline.

By removing the acetylcholine from the bathing solution repolarization was generally completed in 15-20 min. Although the amount of depolarization varied with time as described above it was found that changing the calcium content of the fluid did not alter the time sequence of depolarization. The maximum depolarization was always measured for quantitative comparisons.

Fig. 4 shows a typical experiment on a preparation treated with D-tubocurarine and neostigmine. The two lines represent the amplitude of the depolarization elicited by two different concentrations of acetylcholine. The full circles correspond to the depolarization produced when the muscle has been equilibrated in ordinary Ringer's fluid. The amplitude of the depolari-

zation decreases progressively during the experiment, the decline being more marked with the higher concentration of acetylcholine. This gradual decay is similar to that already described for the end-plate potential.

The open circles represent the magnitude of the depolarization after soaking the muscle in solutions containing 7.2 and 0.45 mm. calcium. It can be seen that these changes in calcium concentration have no significant effect on the amount of depolarization.

The results of a series of ten experiments are plotted in Fig. 3B in the same manner as the earlier results on the end-plate potential.

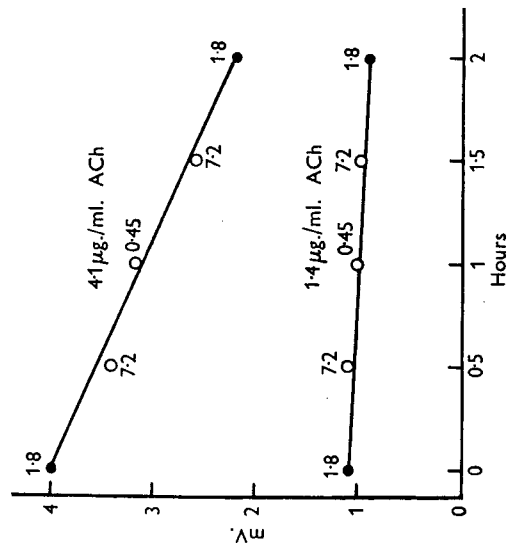


Fig. 4. Depolarization elicited by two different concentrations of acetylcholine. Ordinates: amplitude of the depolarization in mV.; full circles, in Ringer's fluid; hollow circles, in fluid with modified calcium concentrations as indicated. Abscissae: time in hours from the beginning of the experiments.

In Fig. 3, therefore, the results of the two series of parallel experiments can be easily compared, and this shows that changes in the calcium content of the surrounding medium have no significant effect on the acetylcholine sensitivity of the end-plate, even though they have an obvious influence on the size of the end-plate potential.

DISCUSSION

According to current views, neuromuscular transmission can be divided into three stages: (i) arrival of the nerve impulses at the motor-nerve endings with subsequent liberation of acetylcholine, (ii) depolarization of the end-plate by the released acetylcholine, and (iii) initiation of a propagated action potential in the neighbouring region of the muscle fibre.

On this view, the amplitude of the end-plate potential may vary as a result either of a change in the amount of acetylcholine liberated at the nerve endings

or a change in the sensitivity of the end-plate to the depolarizing action of that substance. Under our experimental conditions changes in calcium concentration seemed to have no appreciable effect on the sensitivity of the end-plate to depolarization, whereas the amplitude of the end-plate potential varied considerably. It is concluded, therefore, that the amount of acetylcholine released by a single maximal motor volley is a direct function of the concentration of calcium ions.

This interpretation agrees with the suggestions made by Feng (1936*a, b*), Feng & Shen (1937) and Cowan (1940*a, b*). Their evidence was based on the following observations: increase of calcium concentration (*a*) favoured the development of neuromuscular failure which occurred when a nerve was stimulated at high frequency, (*b*) restored or partially restored the response evoked by a single maximal shock from partly curarized or fatigued preparations, and (*c*) augmented the contracture which follows the main response to tetanic stimulation of the nerve of an eserine-treated preparation. They interpreted these phenomena in terms of an increased liberation of acetylcholine by the motor-nerve endings when the calcium concentration is increased.

A different source of evidence concerning the action of the change of calcium concentration on transmitter liberation comes from Harvey & MacIntosh's paper (1940) on the cat's perfused cervical sympathetic ganglion. They found that low calcium concentration in the perfusion fluid caused block of synaptic transmission, while the ganglion cells still retained their sensitivity to acetylcholine. By direct assay they found that the acetylcholine content in the perfusate of stimulated ganglia was diminished. Furthermore, Brown & Vianna Dias (1947) have shown that during perfusion of frog muscle with NaH_2PO_4 , which presumably reduces the amount of ionized calcium, a neuromuscular block appears although the end-plates retain their sensitivity to acetylcholine.

Evidence has been obtained indicating that the relationship between the magnitude of the end-plate potential and the calcium content in the surrounding fluid, i.e. between calcium concentration and the amount of acetylcholine liberated, is due neither to an anticholinesterase effect of calcium nor to a specific antagonism between calcium ions and D-tubocurarine. A blocking effect on nervous conduction when low calcium concentrations are used cannot be entirely excluded, although the constancy of the latent period of the end-plate potential argues against this being a major factor. Fatt & Katz (1952), however, have recently shown that the end-plate potential, recorded with internal microelectrodes, diminishes stepwise with reduced calcium concentration, and this suggests that the effect might be due to successive blockage of individual nerve terminals.

SUMMARY

1. The relationship between calcium concentration and the amplitude of the end-plate potentials has been studied. Within a range of calcium concentration between 0.45 to 7.2 mm. there appears to be approximately direct proportionality.
2. Calcium has no effect on the time course of the end-plate potentials, suggesting that it has no anticholinesterase activity.
3. The effect of calcium is not due to a direct anticholinesterase action since it can be obtained in non-curarized muscles blocked with acetylcholine and prostigmine.
4. The sensitivity of the end-plate region to the depolarizing action of applied acetylcholine was studied in the presence of varied calcium concentrations. It was found that the amount of ionized calcium has no significant effect on the sensitivity of the end-plates.
5. The amplitude of the end-plate potential may vary as a result either of a change in the amount of acetylcholine liberated at the nerve endings or of a variation in the sensitivity of the end-plate to the depolarizing action of that substance. The experiments described in (1), (2) and (3) suggest, therefore, that the amount of acetylcholine released by a single maximal motor volley is a function of the concentration of calcium ions.

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