

EFFECT OF CALCIUM ON EXCITATORY NEUROMUSCULAR TRANSMISSION IN THE CRAYFISH

BY H. BRACHO* AND R. K. ORKAND†

From the Department of Physiology, Centro de Investigacion y de Estudios Avanzados del I.P.N., Mexico 14, D.F., Mexico

(Received 28 May 1969)

SUMMARY

1. The effects of varying the external Ca concentration from 1.8 to 30 mM/l. ($\frac{1}{8}$ –2 times normal) have been studied at the *in vitro* crayfish excitatory neuromuscular junction. Electrophysiological techniques were used to record transmembrane junctional potentials from muscle fibres and extracellular junctional currents from the vicinity of nerve terminals.

2. The excitatory junctional potential amplitude was proportional to $[Ca]_0^n$, where n varied between 0.68 and 0.94 (mean 0.82) when $[Ca]_0$ was varied from 1.8 to 15 mM/l.

3. The increase in junctional potential amplitude on raising $[Ca]_0$ resulted primarily from an increase in the average number of quanta of excitatory transmitter released from the presynaptic nerve terminal by the nerve impulse.

4. The size of the quanta, synaptic delay, presynaptic potential and electrical properties of the muscle membrane were little affected by changes in calcium concentration in the range studied.

INTRODUCTION

Chemically mediated synaptic transmission of both excitation (Feng, 1936; Harvey & MacIntosh, 1940; Miledi & Slater, 1966) and inhibition (Otsuka, Iversen, Hall & Kravitz, 1966) depends on the presence of Ca in the extracellular fluid. The effect of changes in external Ca have been studied in detail only at the vertebrate neuromuscular junction. At this junction it has been found that Ca is necessary for the release of multi-molecular packets of acetylcholine (ACh) from the nerve terminal (Katz & Miledi, 1965*c*). In the absence of Ca the nerve impulse normally invades the presynaptic terminal but fails to release the neurotransmitter. Raising

* Fellow of the Instituto Nacional de la Investigacion Cientifica, Mexico.

† Grass Foundation Visiting Professor. Present address: Department of Zoology, UCLA, Los Angeles, California, U.S.A.

the Ca concentration increases the probability that a package of transmitter will be released. At low external Ca concentrations the e.p.p. amplitude is proportional to $[Ca]_o^n$ where n is about 4, suggesting that a number of Ca ions must combine with membrane receptors to lead to the release of the transmitter packets. At normal Ca the relation between e.p.p. amplitude and Ca is less steep (Dodge & Rahamimoff, 1967).

Excitatory neuromuscular transmission in crayfish is also quantal in nature, depending on the release of packets of excitatory transmitter from motor nerve endings (Dudel & Kuffler, 1961). An important difference is that this junction is not cholinergic; present evidence suggests that the natural chemical transmitter is closely related to, or is, glutamate (Takeuchi & Takeuchi, 1964). The present experiments utilize electrophysiological techniques to study the effects of varying Ca concentration on transmission at this junction.

METHODS

The experiments were done on the opener muscle of the claw of the Mexican crayfish (*Procambarus mexicanus*) which were purchased in the market place at Xochimilco. The muscle consists of a few layers of fibres 50–200 μ in diameter and 1–3 mm in length. It is innervated by one excitatory and one inhibitory axon. The preparation was dissected and set up in a chamber similar to that previously described by Dudel & Kuffler (1961). The excitatory axon runs in a fine branch of the nerve separately from the inhibitor and could be selectively stimulated through a suction electrode. For transmembrane recording conventional 3 M-KCl micro-electrodes of 5–15 M Ω were used. External recordings of synaptic potentials were obtained by searching the surface of the muscle fibres with low resistance, 1–5 M Ω micro-electrodes filled with 4 M-NaCl (Dudel & Kuffler, 1961). The recording system was conventional and consisted of a solid-state electrometer and Tektronix 565 oscilloscope. In a few experiments current was passed and recorded with a second intracellular micro-electrode which was part of a Wheatstone bridge (Martin & Pilar, 1963).

The normal bathing solution consisted of (mM): NaCl, 195; CaCl₂, 15; KCl, 5; MgCl₂, 3; Tris-maleate, 10; pH 7.1. Low Ca solutions were made by replacing CaCl₂ with equiosmotic amounts of sucrose; those of increased Ca were prepared by the addition of solid CaCl₂. Solutions were changed by overflowing the bath (2 ml.) with 10–15 ml. of new solution in 3–5 min. Measurements were begun 5–10 min after changing the bath. Experiments were run at room temperature, 20–23° C.

RESULTS

When the Ca concentration in the fluid bathing the muscle was reduced, the amplitude of the excitatory junctional potentials (e.j.p.s), recorded with an intracellular micro-electrode, decreased. Figure 1 shows a train of e.j.p.s produced by 5 impulses at 50 msec intervals in 15 mM-Ca, 3.7 mM-Ca, and after returning the preparation to 15 mM-Ca. The effects produced by changing the Ca concentration were complete after the preparation

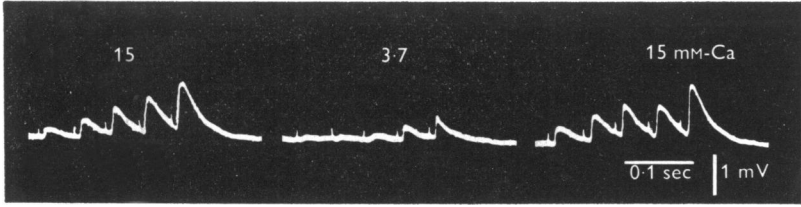


Fig. 1. Excitatory junctional potentials recorded with an intracellular micro-electrode from a single muscle fibre during stimulation of the excitatory axon five times at 50 msec intervals. Sequence of measurements from left to right in 15 mM-Ca, 3.7 mM-Ca and after returning the preparation to 15 mM-Ca.

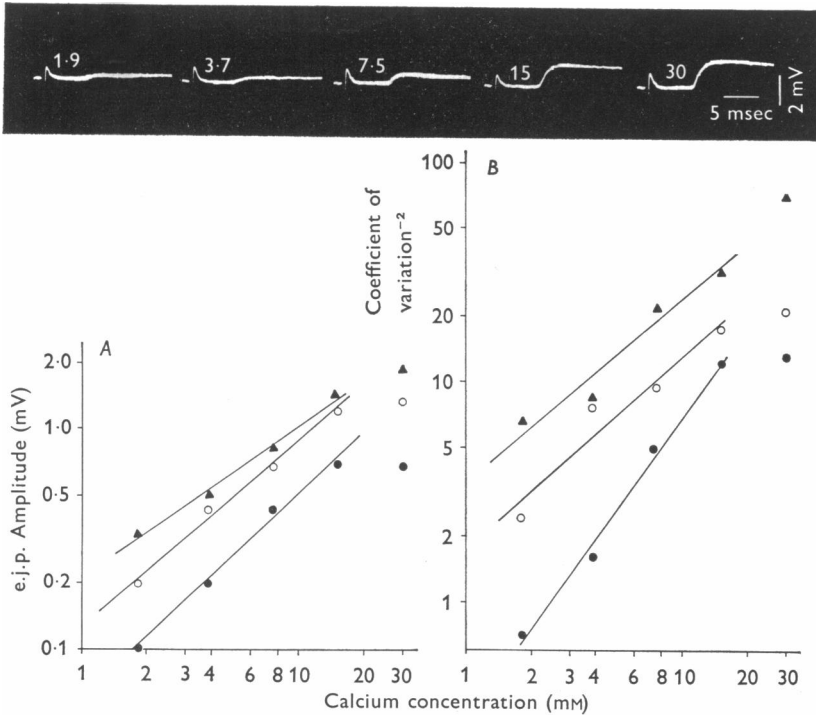


Fig. 2. Top: records of the rising phase of excitatory junctional potentials (e.j.p.) in the indicated Ca concentrations. *A*: e.j.p. amplitudes in varying Ca concentrations in three different experiments represented by different symbols. Each point is the average of fifty measurements; \pm s.d. contained within the points. *B*: calculated (coefficient of variation)⁻² for the same measurements as in *A*. Log-log plots in both *A* and *B*. Lines are calculated regression lines for points between 1.8 and 15 mM-Ca.

was perfused with the new solution for 3–5 min. The slow time course of the effect possibly reflects the time needed for Ca to diffuse to and from the vicinity of the nerve terminals (Katz & Miledi, 1967).

In order to study quantitatively the effect of Ca on the amplitude of the e.j.p.s fibres were selected in which the amplitude of a single e.j.p. in normal Ca could readily be measured. Figure 2 illustrates the results from three cells in which the effects of changes in Ca from 1.8 to 30 mM were examined. In Fig. 2*A* the amplitude of the intracellular e.j.p. (on a log scale) is plotted against the external [Ca] (on a log scale). Between 1.8 mM-Ca and 15 mM-Ca the amplitude of the e.j.p. increases as the Ca is raised. The average slope of the calculated regression lines for the points from 1.8 to 15 mM-Ca in these three experiments was 0.82 (range 0.68–0.94). Increasing Ca from 15 to 30 mM did not always lead to an increase in e.j.p. amplitude. At the frog neuromuscular junction an increase in Ca increases the amplitude of the e.j.p. primarily by increasing the number of quanta of acetylcholine released from the nerve. As the number of quanta released increases, the coefficient of variation (*CV*) of the amplitude distribution decreases (del Castillo & Katz, 1954). The quantal content, *m*, is equal to $1/ CV^2$. In the crayfish the identity of the excitatory transmitter is unknown. However, it is known that the substance is released in the form of discrete quanta (Dudel & Kuffler, 1961; cf. Atwood & Parnas, 1968). In Fig. 2*B* the calculated value of $1/ CV^2$ is plotted on a log scale against the $[Ca]_0$ on a log scale and it can be seen that this value increases in a manner similar to that for the amplitude of the e.j.p. (Fig. 2*A*). The average slope of the three calculated regression lines in Fig. 2*B* was 1.03 (range 0.83–1.37). This result suggests that an increase in Ca acts to increase the amplitude of the e.j.p. by increasing the number of quanta released. However, a measurement of quantum content based on coefficient of variation cannot be expected to be accurate in crayfish muscle fibres. These fibres have a multiterminal innervation; packets of transmitter affecting the membrane at some distance from the recording electrode will produce a smaller recorded depolarization. Thus, the coefficient of variation will reflect variation due not only to numbers of quanta but also to their spatial distribution along the muscle fibre. In order to circumvent this difficulty and also to gain information about the quantal size, synaptic delay and presynaptic potential change it was necessary to record the excitatory junctional currents from single synaptic spots with extracellular micro-electrodes.

Extracellular records from junctional spots

With an extracellular micro-electrode in the vicinity of a junctional region, potentials resulting from currents in both the presynaptic nerve terminal and the post-synaptic muscle fibre can be recorded (Katz & Miledi, 1965*a*; Dudel & Kuffler, 1961). Depending on the position of the electrode (Dudel, 1965) the presynaptic potential can be positive, negative, or diphasic whereas the post-synaptic potential appears as a negative potential. The procedure was to search along the surface of the muscle fibre in normal Ca while stimulating the excitatory axon at 2–5/sec and to locate a spot where both the presynaptic and post-synaptic currents could be recorded. The bath was then exchanged for fresh normal solution

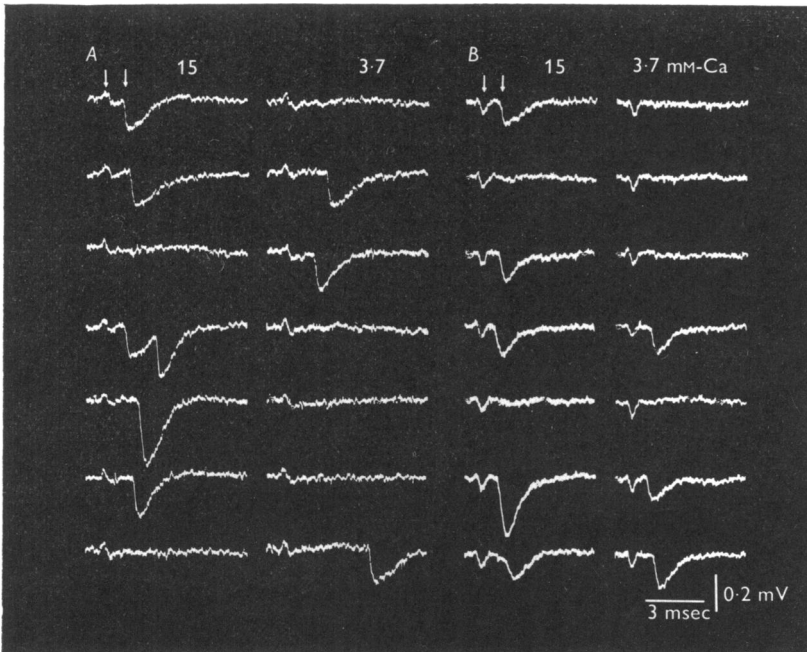


Fig. 3. Extracellular record of synaptic potentials in 15 mM-Ca and 3.7 mM-Ca from two experiments, *A* and *B*. Presynaptic nerve potential (first arrow) is positive in *A* and negative in *B*. Note variation in delay of post-synaptic potential (second arrow).

to check that the recording remained stable. Responses, 200–300 in number, were recorded and then the bathing solution was exchanged for one with a low Ca concentration of 3.7 mM; 400–600 responses were recorded before changing back to normal Ca and recording an additional 200–300 responses. Records were only measured if the before and after controls were

TABLE 1. Quantal content and extracellular synaptic currents

Fibre	Solution (mM-Ca)	Stimulus frequency (sec ⁻¹)	N	m	\bar{V} (μ V)	\bar{V}_1 (μ V)	Presynaptic potential	
							(μ V)	Shape
1	15	3	584	1.30	97.3	74.5	56 } 55 }	Positive
	3.7	3	579	0.42	33.6	80.5		
2	15	5	600	0.61	44.0	72.2	67 } 77 }	Diphasic
	3.7	5	600	0.18	11.5	64.0		
3	15	5	600	2.26	93.2	41.0	55 } 64 }	Negative
	3.7	5	600	0.47	26.0	52.0		
4	15	2	450	1.11	50.4	44.6	103 } 125 }	Negative
	3.7	2	450	0.66	33.0	50.0		
5	15	2	450	1.26	58.5	47.2	43 } 72 }	Negative
	3.7	2	450	0.18	12.0	66.6		

N is the number of trials. Quantal content, m , calculated from the number of failures = $\log_e N/N_0$, where N_0 is the number of failures. \bar{V} is the average size of post-synaptic potential obtained by summing all negative deflexions, following a single stimulus, and dividing by N . \bar{V}_1 is size of a single quantum obtained by dividing \bar{V} by m . Amplitude of diphasic presynaptic potential measured peak to peak.

not significantly different. Figure 3 shows representative responses recorded from two junctional spots in normal, 15 mM-Ca, and at 3.7 mM-Ca. The most obvious change in response on lowering the Ca is the increase in the proportion of responses in which the nerve impulse fails to produce a detectable post-synaptic response. This increase in the incidence of failures indicates that the probability of release of a packet of the excitatory transmitter was reduced at low Ca. The quantum content of the e.j.p. can be calculated from the proportion of failures using the relation, $m = \ln N/N_0$, where m is the quantum content, N the number of nerve impulses and N_0 the number of failures (del Castillo & Katz, 1954). The results of this calculation for five experiments are shown in Table 1. For the five cells in Table 1 the reduction of Ca to 25% of normal reduced the quantum content to 0.31 ± 0.18 s.d. of normal. This value can be compared to an average decrease of the intracellularly recorded e.j.p. to 0.33 ± 0.04 in 3.7 mM-Ca as compared to 15 mM-Ca. The similarity of these two ratios is consistent with the hypothesis that the decrease in amplitude of the e.j.p. can be accounted for by the decrease in number of quanta of excitatory transmitter released.

Other factors to be considered are the possibilities that changes in Ca affect the size of the individual quanta, the sensitivity of the post-junctional membrane to the transmitter and the magnitude of the current through the muscle membrane. At the neuromuscular junction of the frog there is evidence to suggest that ACh slightly increases the Ca permeability of the post-synaptic cell (Takeuchi, 1963). If this were the case in the crayfish one might expect a decrease in inward current during the transmitter action when the external Ca was reduced. These factors, however, if present, play only a minor role in the action of Ca. It thus appears from Table 1 that the amplitude of the post-junctional current produced by a single packet of transmitter, \bar{V}_1 , was not reduced when the Ca was lowered.

One explanation for the increase in failures at low Ca could be that the nerve action potential fails to depolarize the nerve terminal or that such depolarization is reduced in low Ca. Table 1 and Fig. 3 indicate that this did not occur. In those recordings when the presynaptic potential was either diphasic or primarily negative, fibres 2-5, the amplitude of the presynaptic potential was consistently increased a significant amount when the Ca was reduced. When the potential was positive, fibre 1, the electrode was presumably closest to the excitatory nerve terminal (Dudel, 1965; Katz & Miledi, 1965*a*) and no effect of Ca on the amplitude of the potential was recorded.

In Fig. 4 the effect of Ca on the synaptic delay has been assessed. The time was measured from the peak of the nerve terminal potential to the

beginning of all the measurable quanta (Katz & Miledi, 1965*b*). The histograms are strikingly similar in 15 mM-Ca and 3.7 mM-Ca. In both cases the minimum latency was 0.6 msec with the most common value 1.0 msec and there was a long 'tail' to the distribution lasting 5–6 msec. Similar results were obtained from four other junctions. The probability of including a spontaneous miniature e.j.p. in these measurements was low; at these junctions the spontaneous frequency was about 1/min in the absence of damage to the terminal by the micro-electrode. In order to determine

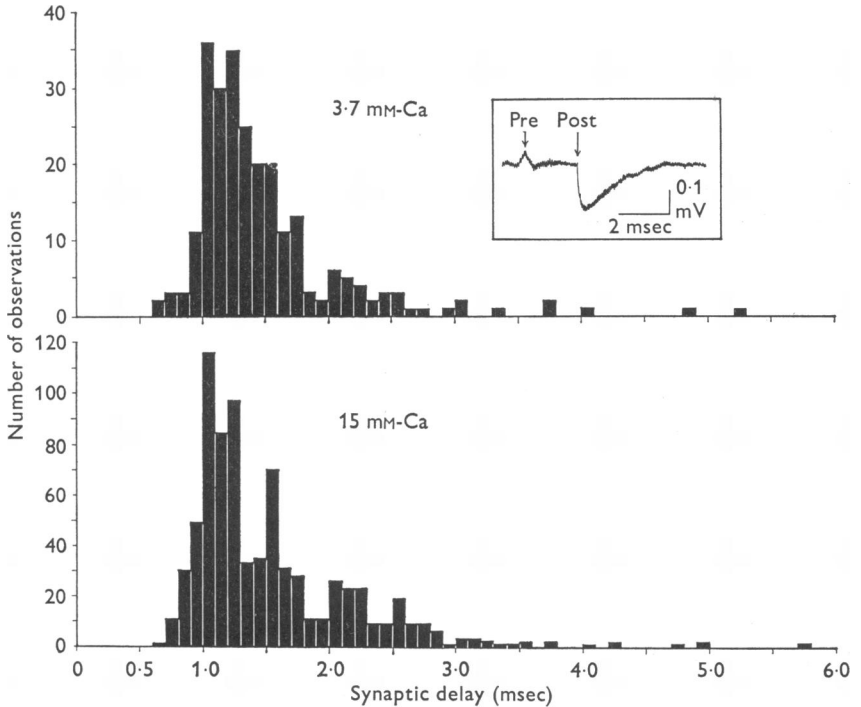


Fig. 4. Histogram of synaptic delays from a single junctional spot in 3.7 mM-Ca (top) and 15 mM-Ca (bottom). Data from fibre 1, Table 1.

that most of the units were resolved and therefore that the distributions are accurate, the number of units actually measured was compared to that predicted to occur on the basis of the number of failures observed (Katz & Miledi, 1965*b*). In 15 mM-Ca there were 158 failures out of 584 tests and the response should have contained 754 discrete units; 746 were actually measured. In 3.7 mM-Ca there were 380 failures in 579 responses and whereas 240 units were predicted 232 were measured. These results suggests that a significant number of units was not lost due to the simultaneous occurrence of quanta.

Effect of calcium on electrical properties

The amplitude of the intracellularly recorded e.j.p. depends not only on the synaptic current but also on the input resistance and the resting potential of the muscle fibre. In order to determine the input resistance in different Ca concentrations, two micro-electrodes were inserted in a single fibre, one to pass current across the membrane and the second to record the membrane potential and the change in potential produced by the applied current. Current-voltage curves were measured in 15 mM-Ca, 1.8 mM-Ca and again in 15 mM-Ca in the same fibre. In four fibres the input resistance (0.18–0.20 M Ω) was reduced by an average of 11 % (range 5–15 %) in the solution with reduced Ca. Thus, a small part of the reduction of intracellular e.j.p. amplitude can be attributed to an effect of Ca on the membrane resistance of the cell. Changes in resting membrane potential of more than 1–3 mV were not observed under these conditions.

DISCUSSION

These experiments show that Ca is a necessary cofactor for excitatory neuromuscular transmission in the crayfish as it is at other synapses. A comparison between the effect of Ca in these experiments and those at the frog neuromuscular junction is difficult because the crayfish normally has a greater blood Ca concentration. Thus, at normal Ca concentrations, 15 mM in crayfish, 1.8 mM in frog, the relation between e.j.p. or e.p.p. amplitude is proportional to $[Ca]_0^n$, where n is about 1 (Dodge & Rahamimoff, 1967). The difference is that when the Ca concentration is reduced to 15–25 % of normal the value of n approaches 4 in frog, whereas it remains about 1 in crayfish. The finding that as the Ca was raised to 30 mM the e.j.p. usually either increased little or even decreased in amplitude, suggests either that the Ca receptors on the nerve terminal were saturated or possibly that conduction of the nerve depolarization into the fine nerve terminals was impaired. Calcium increases the threshold for nerve excitation and the branch points of the axon would be the point of lowest safety factor for conduction.

Although a small part of the decrease in e.j.p. amplitude on lowering Ca can be attributed to a decrease in the effective resistance of the cell, it is clear that the most important effect of decreasing Ca is a reduction in the probability of a packet of excitatory transmitter being released by a depolarization of the presynaptic nerve terminal (cf. Katz & Miledi, 1965*c*).

When an excitatory crayfish axon is repetitively stimulated the amplitude of the e.j.p. increases. Under these conditions Dudel (1965) observed a decrease in the amplitude of the externally recorded presynaptic current

at some distance from the terminal and an increase in the positive nerve current close to the terminal. In our experiments an increase in e.j.p. amplitude, produced by an increase in Ca, led to a reduction in the amplitude of the presynaptic current recorded away from the terminal, but no change was observed in the positive current presumably coming from the end of the presynaptic fibre (Katz & Miledi 1965*a*). In Dudel's experiments the relation between e.j.p. amplitude and positive nerve terminal current was almost linear. If such a relation held for changes in Ca, an increase in presynaptic current of some 400% on raising Ca from 3.7 to 15 mM would be expected; an increase was not observed.

The similarities in the synaptic latency histograms obtained at different Ca concentrations indicates that the time course of the increase in probability of excitatory transmitter release is independent of external Ca. This result is consistent with the suggestion of Katz & Miledi (1967) that the time course of the entrance of Ca or 'active Ca' into the nerve terminal is a brief event compared to the subsequent unknown events leading to the rise and fall of release probability.

We are grateful to D. Erlij for helpful discussions and criticism.

This work was supported in part by U.S.P.H.S. grant no. NB 08346.

REFERENCES

- ATWOOD, H. L. & PARNAS, I. (1968). Synaptic transmission in crustacean muscles with dual motor innervation. *Comp. Biochem. Physiol.* **27**, 381-404.
- DEL CASTILLO, J. & KATZ, B. (1954). Quantal components of the end-plate potential. *J. Physiol.* **124**, 560-573.
- DODGE, F. A. & RAHAMIMOFF, R. (1967). Co-operative action of calcium ions in transmitter release at the neuromuscular junction. *J. Physiol.* **193**, 419-432.
- DUDEL, J. (1965). Potential changes in the crayfish motor nerve terminal during repetitive stimulation. *Pflügers Arch. ges. Physiol.* **282**, 323-337.
- DUDEL, J. & KUFFLER, S. W. (1961). The quantal nature of transmission and spontaneous miniature potentials at the crayfish neuromuscular junction. *J. Physiol.* **155**, 514-529.
- FENG, T. P. (1936). Studies on the neuromuscular junction. II. The universal antagonism between calcium and curarizing agencies. *Chin. J. Physiol.* **10**, 513-528.
- HARVEY, A. M. & MACINTOSH, F. C. (1940). Calcium and synaptic transmission in a sympathetic ganglion. *J. Physiol.* **97**, 406-416.
- KATZ, B. & MILEDI, R. (1965*a*). Propagation of electric activity in motor-nerve terminals. *Proc. R. Soc. B* **161**, 453-482.
- KATZ, B. & MILEDI, R. (1965*b*). The measurement of synaptic delay, and the time course of acetylcholine release at the neuromuscular junction. *Proc. R. Soc. B* **161**, 483-495.
- KATZ, B. & MILEDI, R. (1965*c*). The effects of calcium on acetylcholine release from motor nerve terminals. *Proc. R. Soc. B* **161**, 496-503.
- KATZ, B. & MILEDI, R. (1967). The timing of calcium action during neuromuscular transmission. *J. Physiol.* **189**, 535-544.

- MARTIN, A. R. & PILAR, G. (1963). Dual mode of synaptic transmission in the avian ciliary ganglion. *J. Physiol.* **168**, 443-463.
- MILEDI, R. & SLATER, C. R. (1966). The action of calcium on neuronal synapses in the squid. *J. Physiol.* **184**, 473-498.
- OTSUKA, M., IVERSEN, L. L., HALL, Z. W. & KRAVITZ, E. A. (1966). Release of gamma-aminobutyric acid from inhibitory nerves of lobster. *Proc. natn. Acad. Sci. U.S.A.* **56**, 1110-1115.
- TAKEUCHI, A. & TAKEUCHI, N. (1964). The effect on crayfish muscle of iontophoretically applied glutamate. *J. Physiol.* **170**, 296-317.
- TAKEUCHI, N. (1963). Effects of calcium on the conductance change of the end-plate membrane during the action of transmitter. *J. Physiol.* **167**, 141-155.