J. Physiol. (1968), **199**, *pp.* 11–35
With 16 *text-figures*
Printed in Great Britain

A STUDY OF THE MECHANISM OF QUANTAL TRANSMITTER RELEASE AT A CHEMICAL SYNAPSE

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(*Received* 1 *January* 1968)

SUMMARY

1. The nerve-muscle preparation of the cutaneous pectoris of the frog has been used to study quantal transmitter release.

2. When the osmotic pressure of the external solution is raised 1·5–2 fold, the frequency of miniature end-plate potentials (m.e.p.p.s) rises by 1·5–2 orders of magnitude. This effect is independent of the presence of Ca^{2+} ions and of the nature of the substances by which the osmotic pressure has been increased.

3. In Ca^{2+} free hypertonic solution the nerve impulse still invades the nerve terminals but does not alter the frequency of the m.e.p.p.s.

4. The arrival of the impulse in the terminals causes an immediate increase in the rate of quantal release, provided divalent cations are present whose passage through the axon membrane is facilitated by excitation (Ca^{2+}, Sr^{2+}, Ba^{2+}).

5. Divalent cations which penetrate only slightly (Mg^{2+}, Be^{2+}) lower the frequency of m.e.p.p.s and suppress the end-plate potential (e.p.p.) evoked by an impulse, in the presence of Ca^{2+} ions. Be^{2+} is a more effective inhibitor than Mg^{2+}.

6. In Ca^{2+} free solutions, adding Mg^{2+} causes an increase in the frequency of m.e.p.p.s evoked by depolarization of the nerve endings or by treatment with ethanol.

7. The trivalent cation La^{3+} is more effective than divalent cations are in increasing the frequency of m.e.p.p.s. The tetravalent cation Th^{4+} also raises the m.e.p.p. frequency.

8. The observations summarized in paragraphs 2–7 indicate that the frequency of m.e.p.p.s at a constant temperature depends only on the concentration of uni-, di- and trivalent cations inside the nerve ending. It

is suggested that the internal cation concentration influences the adhesion between synaptic vesicles and the membrane of the nerve ending.

9. For a model experiment, artificial phospholipid membranes have been used to study the effect of uni-, di-, tri- and tetravalent cations on the adhesion process. At pH 7–7·4, the time required for adhesion to take place decreases with increasing cation concentration in the bath. Ca^{2+} ions are 100–1000 times more effective than K^+ ions; La^{3+} and Th^{4+} ions are still more effective. The 'adhesion time' decreases when the pH is lowered; it increases greatly with lowering of temperature.

10. The hypothesis is put forward that the mutual adhesion of artificial vesicles made of phospholipid membranes, and the adhesion between synaptic vesicles and the membrane of the nerve ending arise by a common mechanism. In both cases, the important factor is the influence of cations on the electric double layer at the membrane surface.

INTRODUCTION

There is strong evidence for the view that at chemical synapses transmitter substances are released from the synaptic vesicles which are accumulated within the nerve endings (Katz, 1962). The synaptic vesicles, as well as the cell itself, are surrounded with lipoprotein membranes (de Robertis, de Lores Arnaiz & de Iraldi, 1962; Whittaker, Michaelson & Kirkland, 1964), and there is probably a common physico-chemical mechanism which underlies the release of transmitter substances at different synapses.

The nature of the interaction between synaptic vesicles and axon membrane is not known. The following hypothesis has been presented (Liberman, 1966): the membranes of synaptic vesicles are attracted to the membrane of the nerve ending by Van der Waals forces which become important when the distance between the two surfaces is less than 1000 Å. These forces of attraction are opposed by electrostatic repulsion arising from fixed negative charges present on the membrane surface. The physical theory of interaction between charged surfaces (see e.g. Kruyt, 1952) has been applied previously to the problem of interaction and adhesion of whole cells, by Curtis (1962).

We suppose that in a resting nerve ending, Van der Waals forces exceed the electrostatic forces from time to time in the course of thermal movements. This results in adhesion between vesicles and axon membrane, and leads to the quantal discharge of transmitter into the synaptic cleft, and so to the occurrence of a miniature end-plate potential. The electrostatic repulsion of the fixed negative charges depends on the concentration of cations within the nerve ending. An increase of the cation concentration

will result in screening of the surface charges; hence the probability of adhesion, and the frequency of m.e.p.p.s, should be increased.

In the present paper, experimental tests of this hypothesis are described.

METHODS

The end-plate potential and miniature e.p.p.s were recorded from m. cutaneous pectoris of the frog (*Rana temporaria*). This muscle has only a few layers of fibres. It is transparent, and with 10–20 fold magnification one can see the position of the nerve terminals. By inserting the micro-electrode at the end of the finest visible nerve twigs, one correctly locates the end-plate region of the muscle fibre. The muscle is innervated by the n. pectoralis proprius which is a small branch of the brachial nerve. The latter was cut near the spine (Fig. 1) and dissected down to the muscle. The nerve-muscle preparation was mounted in the chamber (Fig. 2) with the muscle gently stretched.

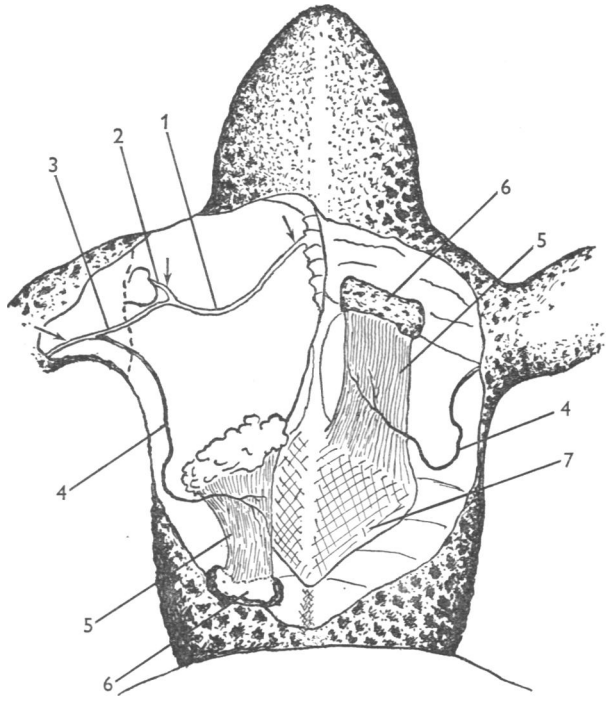

Fig. 1. Nerve-muscle preparation of m. cutaneus pectoris. (1) n. brachialis; (2) n. radialis; (3) n. ulnaris; (4) n. pectoralis proprius; (5) m. cutaneus pectoris; (6) strip of skin to which the upper tendon of m. cutaneus pectoris is attached; (7) ensiform cartilage—the attachment of the lower tendon of the muscle. The arrows show the sides where nerve was sectioned.

The solution was flowing continuously through the chamber, entering through tube (1) and the excess being sucked away through a cannula (2) with the help of a water pump. Stimulating pulses of 0·2 msec duration were applied to the nerve through Ag/AgCl electrodes.

Glass micropipettes, filled with 3M-KCl, of 0·1–0·5 μ tip size and 10–30 MΩ resistance were used for intracellular recording. For extracellular recording of m.e.p.p.s, e.p.p.s and nerve spikes, 0·5 M-NaCl-filled micropipettes of about 1 μ tip size (Katz & Miledi, 1965) or glass capillaries filled with Wood alloy and platinized tips, were used. The membrane potential was recorded with a cathode follower and d.c. millivoltmeter (UIPP-2). An a.c. amplifier (bandwidth 2–10,000 c/s) and oscilloscope were used to record nerve spikes, e.p.p.s and m.e.p.p.s. The frequency of m.e.p.p.s was monitored with a counter (PST-100).

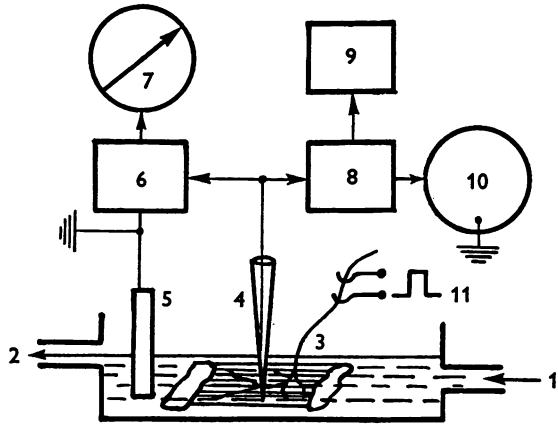

Fig. 2. Arrangement for recording membrane potential, end-plate potential and miniature potentials. (1) Inflow; (2) outflow; (3) nerve-muscle preparations; (4) recording micro-electrode; (5) reference electrode; (6) cathode follower; (7) millivoltmeter; (8) a.c. amplifier; (9) counter; (10) oscillograph; (11) stimulating electrodes.

In most experiments m.e.p.p. frequency was measured in the same fibre before and during application, and again after washing out, of test solutions. Some experiments were made by recording from many fibres and calculating the mean value for each solution.

Solutions. The Ringer and other solutions were prepared from isotonic stock solutions of the different salts (230 m-osmoles/l.). The composition of the solutions is shown in Table 1. The pH was maintained within 7–7·3. Solutions containing La^{3+} and Th^{4+} ions were prepared without $NaHCO_3$. All experiments were made at room temperature (22–24° C). In the solutions with La^{3+}, Th^{4+} and Be^{2+}, pH was 6·5–6 in some experiments.

Adhesion of phospholipid membranes. This was investigated by visual observation with the device shown in Fig. 3*a*. Phospholipids were extracted from cerebral white or grey matter by the method of Mueller, Rudin, Tien & Wescott (1963). After drying and dissolving in heptane (9–10 mg/ml.) the phospholipid solution was placed with a glass pipette over the ends of chlorvinyl tubes (1). Films of phospholipid were formed in this way, separating the aqueous solution in the bath (7) from that within the tubes (3 and 4). By applying pressure from syringes (2), the films were made to protrude from the ends of the tubes, and were brought into contact by a micrometer movement (5). The area of contact between the films was about 0·7 mm^2. From the moment of 'contact' (Fig. 3*b*, (1)) to the moment of 'adhesion' (Fig. 3*b*, (2)) a time interval elapsed which was measured with an accuracy of 0·2 sec, the process being observed with 5–20 fold magnification. Adhesion was indicated by a sudden change in the shape of the contact area; in some instances the transverse membrane broke, and a continuous phospholipid membrane tube was formed, connecting the aqueous solutions of the two chlorvinyl tubes but keeping them separated from the outside bath (Fig. 3*b*, (3)).

TABLE 1. Composition of solutions

Isotonic solutions. Concentration (mM)

Solutions	Cations			Other cations	Anions		Other anions
	Na^+	K^+	Ca^{2+}		Cl^-	HCO_3^-	
Ringer (R)	113	1·8	1	—	115	2·4	—
R − Ca + Mg	110–113	1·8	0–0·5	Mg^{2+} (0–5)	113–120	2·4	—
R − Ca + Mg + EDTA	110–113	1·8	—	Mg^{2+} (0–5)	110–120	2·4	EDTA (1–2)
R − Ca + exc. KCl + EDTA + Mg	95–100	15	—	Mg^{2+} (0–5)	110–113	2·4	EDTA (1)
R − Ca + $K_2C_2O_4$	95–100	15	—	Mg^{2+} (0–5)	100–110	2·4	$C_2O_4^{2-}$ (7·5)
R − Ca + Sr	113	1·8	—	Sr^{2+} (2)	116	2·4	—
R − Ca + Ba	110–113	1·8	—	Ba^{2+} (2)	116–117	2·4	—
R − Ca + Be	113	1·8	—	Be^{2+} (0·1–2)	112	2·4	CH_3COO^- (0·2–4)
R + Be	113	1·8	1	Be^{2+} (0·05–1)	115	2·4	CH_3COO^- (0·1–2)
R − Ca + La	110–113	1·8	—	La^{3+} (0·05–2)	115–118	—	—
R − Ca + La + exc. KCl	105	10	—	La^{3+} (1)	118	—	—

Hypertonic solutions. Concentration (mM)

	Cations				Anions		Neutral molecules	Osmotic pressure related to Ringer
	Na^+	K^+	Ca^{2+}	Mg^{2+}	Cl^-	HCO_3^-		
R + exc. NaCl	170–225	1·8	1	—	172–227	2·4	—	1·5–2
R + $MgCl_2$	113	1·8	1	38–76	190–270	2·4	—	1·3–1·7
R + sucrose	113	1·8	1	—	115	2·4	Sucrose 70–300	1·4–4·3
R − Ca + sucrose	113	1·8	—	—	113	2·4	Sucrose 115–210	1·6–2·25
Solutions with alcohol								
R − Ca + C_2H_5OH + Mg	110–113	1·8	—	0—10	115–130	2·4	Ethanol 430	—

In the table the total amount of ions is shown in all cases. The concentration of free ions especially La^{3+} and Be^{2+} ions, as well as Mg^{2+} ions in the solutions with EDTA and $K_2C_2O_4$, is lower.

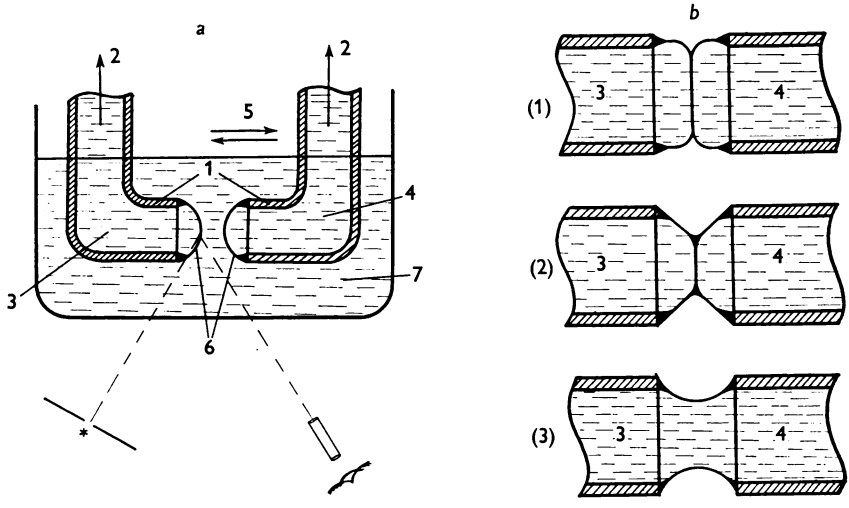

Fig. 3. Arrangement for study of the adhesion of phospholipid membranes. *a:* (1) Chlorvinyl tubes; (2) to the syringe; (3, 4, 7) aqueous solution; (5) direction of movement of tubes; (6) phospholipid membranes. *b:* illustrating the adhesion of the phospholipid membranes. (1) Membranes brought in contact, but not fused; (2) the membranes have fused and formed a 'unit membrane', separating solutions 3 and 4 (3*a*); (3) the fusion of the bimolecular membranes has led to the formation of a tube. Solutions 3 and 4 have joined.

RESULTS

Effects of increased osmotic pressure. According to the hypothesis mentioned above, the frequency of m.e.p.p.s should rise with an increase of cation concentration inside the nerve ending. Hence, the frequency should increase when the osmotic pressure is raised. This effect has been observed by several authors (Fatt & Katz, 1952; Furshpan, 1956; Boyd & Martin, 1956; Liley, 1956*a*; Babakov, Glagoleva & Liberman, 1963; Liberman & Blioch, 1968). It was necessary to check whether this increase in the frequency of m.e.p.p.s could be explained by the possible occurrence of a substantial depolarization of the nerve ending. Because of the small size of the nerve endings, it is difficult to measure their membrane potential. We examined, therefore, the action potential, and its ability to invade the terminals, in hyperosmolar solution, using the extracellular recording technique of Katz & Miledi (1965). The preparation was immersed in a solution whose Ca^{2+} content was partly or completely replaced by Mg^{2+} ions (e.g. 0 mM-Ca^{2+} and 1 mM-Mg^{2+}). Transmission was blocked, and the e.p.p. completely eliminated (Fig. 4*a*).

When the tonicity of the solution was increased by adding NaCl or

sucrose, the m.e.p.p. frequency rose steeply, while the nerve impulse continued to invade the terminal without eliciting an e.p.p. (Fig. 4*b*).

The experiment illustrated in Fig. 4 shows that the effect of increased osmotic pressure occurs even in the absence of external Ca^{2+} ions. This is

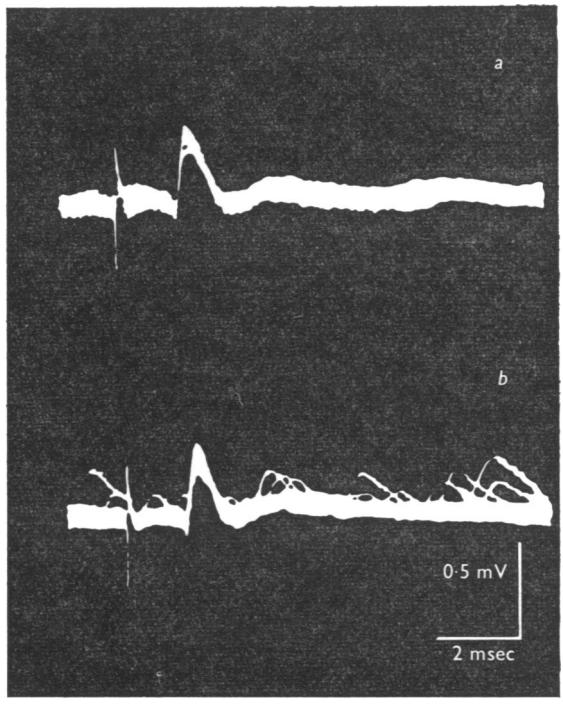

Fig. 4. Presynaptic spike and miniature end-plate potentials in Ca^{2+} free solution. The end-plate potential is absent. Extracellular recording. *a:* Ca^{2+} free Ringer solution, containing 1 mM-Mg^{2+} ions. *b:* hyperosmolar Ca^{2+} free Ringer solution, containing 1 mM-Mg^{2+} ions and 115 mM sucrose. In this and subsequent figures a positive-going potential change at the micro-electrode is shown as a downward deflexion.

to be expected from our present hypothesis, in spite of the fact that partial or complete removal of Ca^{2+} reduces the m.e.p.p. frequency (Hubbard, 1961; Elmqvist & Feldman, 1965; Liley, 1956*b*). For complete withdrawal of external Ca^{2+} ions, the preparation was kept in Ca^{2+} free solution for 24 hr, or in a solution containing 1–2 mM EDTA (ethylenediaminetetra-acetate Na) for 4 hr. The mean frequency of m.e.p.p.s under these conditions was much lower than in normal Ringer (see Table 2). Nor did nerve stimulation produce a noticeable change in m.e.p.p. frequency in the Ca^{2+} free medium. The effect of increased tonicity, by adding sucrose, NaCl or $MgCl_2$, however, was the same and led to an immediate rise in the

discharge. Figure 5 shows the relative increase in m.e.p.p. frequency obtained under various conditions by adding to the osmolarity of Ca^{2+} free solutions.

TABLE 2. Decrease of m.e.p.p. frequency in muscles kept more than 5 hr in Ca^{2+} free solutions

	Number of fibres	Frequency of m.e.p.p.s (sec^{-1}) Extreme values	Mean ± S.E. of value mean
Ringer	80	0·4–9	1·1 ± 0·25
Ca^{2+} free Ringer + Mg^{2+} (1–2 mM)	53	0·08–1	0·5 ± 0·05
Ca^{2+} free Ringer + Mg^{2+} (2 mM) + EDTA (1–2 mM)	32	0·01–0·2	0·07 ± 0·01

The frequency of m.e.p.p.s was recorded in several fibres of the preparations, both before and after keeping in Ca^{2+} free solution.

The effect of the depolarization of the active nerve ending on the release of ACh-quanta. It was shown by Katz & Miledi (1965) that in a Ca^{2+} free solution the action potential invades the nerve endings, but fails to evoke an e.p.p. This was confirmed in our experiments (Fig. 6*a*).

If the quantal release of transmitter from nerve endings depends on a balance between Van der Waals forces and electrostatic repulsion, then there is no reason why depolarization *by itself* should influence the rate of release, for the membrane depolarization of the order of 100 mV is associated with a change of the electric field only within the membrane and has practically no influence on the electric field in the solution near the membrane, due to fixed negative charges.

Now, apart from being accompanied by membrane depolarization, the arrival of the presynaptic impulse causes an influx of Na^+ ions into the axon and an efflux of K^+ ions. Both are univalent cations, and their effects on the electrostatic forces between axon and vesicular membranes should be identical. It is known, however (Hodgkin & Keynes, 1957; Bianchi & Shanes, 1959; Liberman & Tsofina, 1962), that depolarization of various excitable membranes leads to an influx of Ca^{2+} as well as Na^+ ions. Although the external concentration of Ca^{2+} is only about 1/100 of that of Na^+, nevertheless influx of Ca^{2+} ions would considerably reduce the electrostatic repulsion between the membranes because of the much greater effectiveness of divalent ions.

If this argument is correct, one would expect the e.p.p. to be restored, not only by Ca^{2+}, but by all divalent cations whose passage through the membrane is facilitated by depolarization. There is evidence, in muscle, that the membrane permeability to Sr^{2+} as well as to Ca^{2+} increases with

depolarization (Tsofina & Liberman, 1962; Edwards, Lorkovic & Weber, 1966). Also, it has been shown that Sr^{2+} and Ca^{2+} can restore the generation of action potentials in nerve and muscle fibres (Fatt & Ginsborg, 1958; Liberman, Tsofina & Vaintsvaig, 1961; Liberman & Voronin, 1963). It is reasonable to suggest, therefore, that the membrane permeability to these

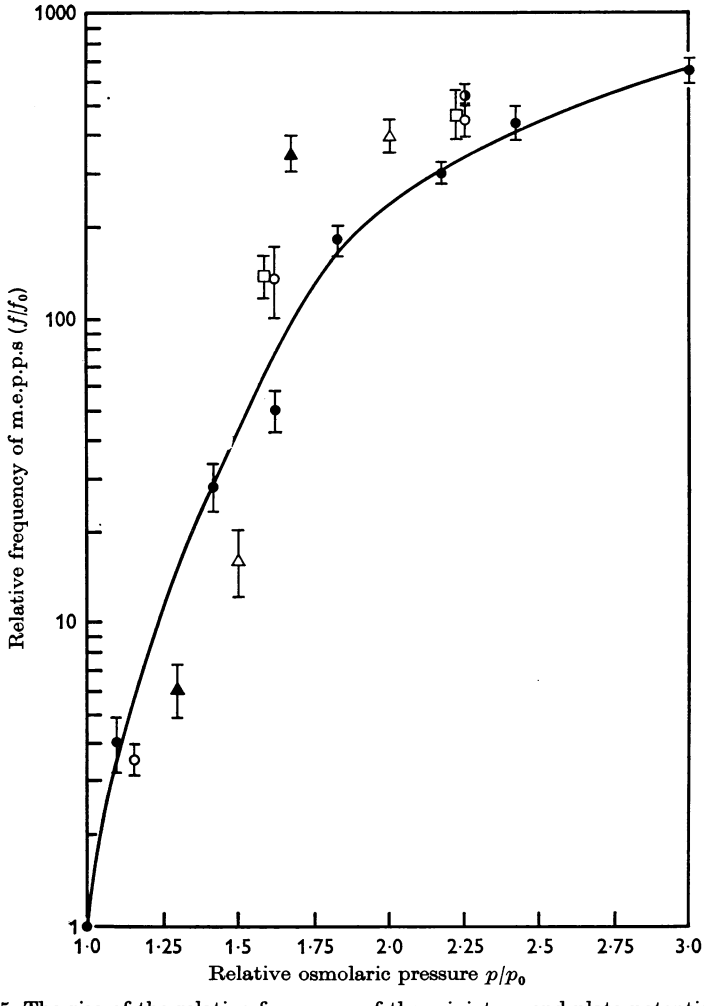

Fig. 5. The rise of the relative frequency of the miniature end-plate potentials as result of increasing the relative osmotic pressure of the bathing solution, by addition of sucrose, NaCl or $MgCl_2$. In Ringer solutions: ●, sucrose; ▲, $MgCl_2$; △, NaCl. In Ca^{2+} free Ringer solutions: ○, 1 mM-$MgCl^{2+}$ sucrose; □, same solution, the frequency of m.e.p.p.s being recorded immediately after nerve stimulation at 30 c/s for 2 min; ◑, same solution but preparation was pre-treated with Ca^{2+} free solution containing 1 mM EDTA. Osmotic pressure (P_0) and frequency of m.e.p.p.s (f_0) in iso-osmotic solutions was taken as unity.

ions in the nerve ending increases during depolarization. Figures 6, 7 and 8 show the restoration of e.p.p.s when Ca^{2+}, Sr^{2+} and Ba^{2+}, respectively, were added to the Ca^{2+} deficient solution.

Restoration of full transmission was also observed in some muscle fibres.

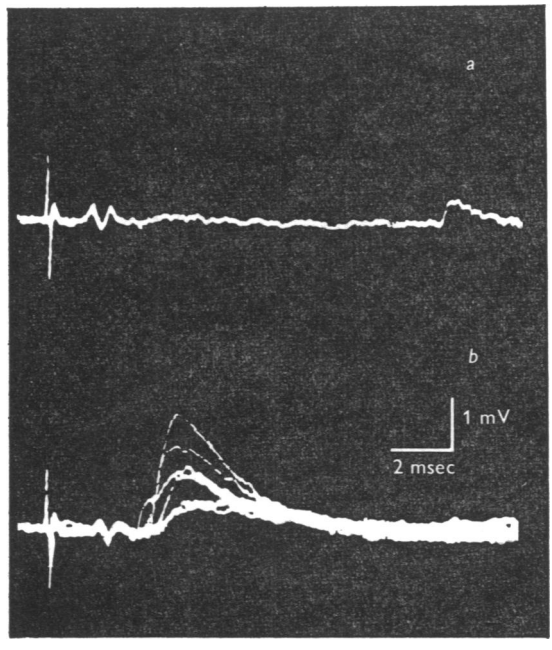

Fig. 6. Restoration of end-plate potential by addition of Ca^{2+} ions to a Ca^{2+} free solution. Extracellular recording. *a:* Ca^{2+} free Ringer solution, containing 1 mM-Mg^{2+} ions; *b:* after adding 1 mM-Ca^{2+}; vertical scale—1 mV, horizontal scale—2 msec.

Effects of Mg^{2+} and Be^{2+} ions. There is lack of information concerning the permeability of excitable membranes to Mg^{2+} ions. It is known, however, that this ion, in contrast to Ca^{2+}, Sr^{2+} and Ba^{2+}, fails to maintain action potentials. One may suggest, therefore, that the Mg^{2+} permeability rises, if at all, only slightly during excitation of the nerve endings. This would explain the absence of e.p.p.s in a medium in which Mg^{2+} has replaced all Ca^{2+}.

In the presence of Ca^{2+} ions, the addition of Mg^{2+} to the solution is known to suppress the quantal release of ACh (del Castillo & Engbaek, 1954; del Castillo & Katz, 1954; Hubbard, 1961). We have now studied the effect of adding Mg^{2+} to a Ca^{2+} free solution. It is seen in Fig. 9 and Table 3 that, under these conditions, excess K^+, or a period of nerve stimulation, causes the frequency of m.e.p.p.s to rise.

Furthermore, the effect of ethanol in raising m.e.p.p.s frequency is inhibited by Mg^{2+} when Ca^{2+} ions are present (Okada, 1967). When Mg^{2+} is added to a *Ca^{2+} free* solution containing 2% ethanol, however, the

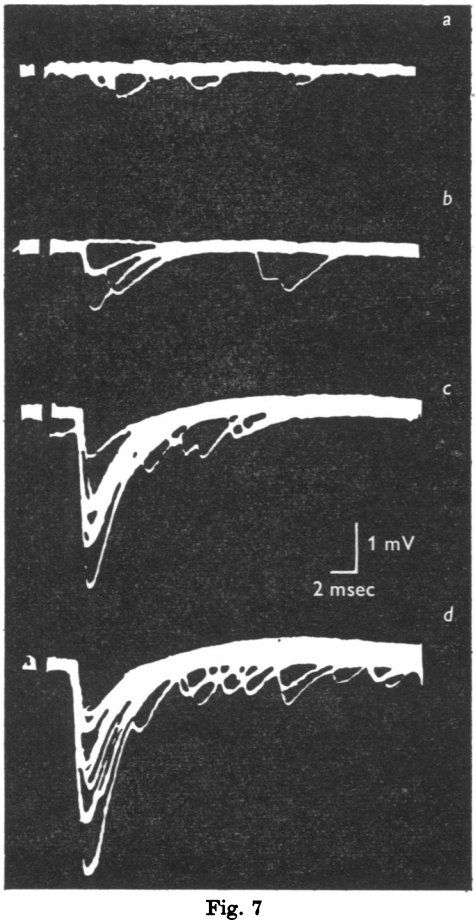

Fig. 7

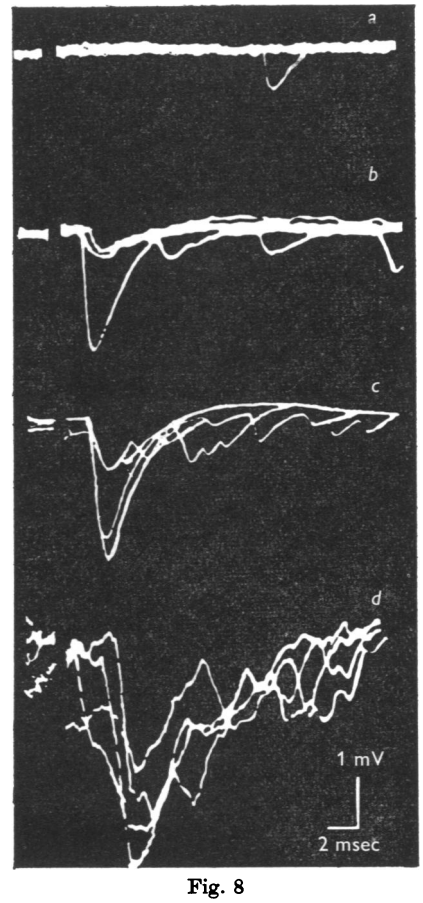

Fig. 8

Fig. 7. Restoration of end-plate potential by adding Sr^{2+} ions to Ca^{2+} free solution. Intracellular recording. *a:* 1 min; *b, c, d:* 5–10 min after replacing the Ca^{2+} free Ringer solution, containing 1 mM-Mg^{2+} ions, by the same solution with 2 mM-Sr^{2+} ions added. Frequency of stimulation: *b:* 1 c/s, *c:* 3 c/s, *d:* 10 c/s.

Fig. 8. Restoration of end-plate potential by adding Ba^{2+} ions to Ca^{2+} free Ringer solution. *a:* Ca^{2+} free solution, containing 1 mM-Mg^{2+} ions; *b, c, d:* Mg^{2+} was replaced by Ba^{2+} (4 mM); 5–7 min after change of solution. Frequency of stimulation: *b:* 1 c/s, *c:* 3 c/s, *d:* 10 c/s.

frequency of m.e.p.p.s increases (Fig. 10), though this increase is appreciably smaller than that obtained with corresponding concentrations of Ca^{2+} ions.

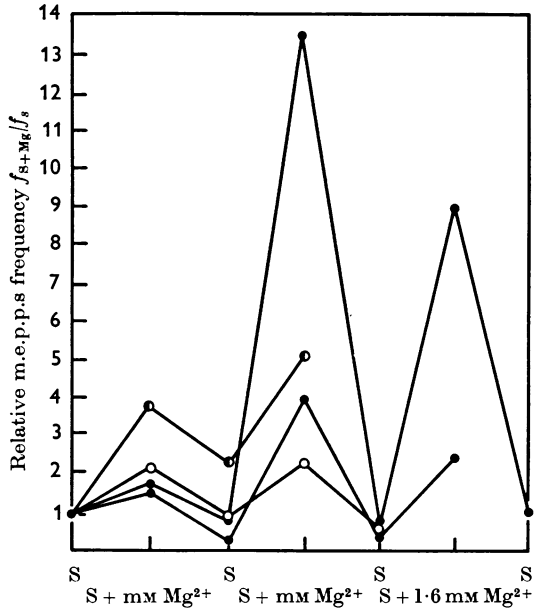

Fig. 9. Relative changes of m.e.p.p. frequency during successive changes of Ca^{2+} free Ringer solution (S) for the same solution, containing Mg^{2+} ions (S + Mg). All solutions contained 15 mM-K^+. M.e.p.p. frequency was recorded 15 min after each solution change. f_s-initial frequency of m.e.p.p. in Ca^{2+} free solution. ◑, preparation had initially been kept in Ca^{2+} free Ringer solution + 1 mM EDTA; ●, 1 mM EDTA is present in the test solutions; ○, 7·5 mM-$C_2O_4^{2-}$ is present to chelate Ca^{2+} ions.

TABLE 3. Increase of m.e.p.p. frequency shortly after nerve stimulation (30–40 c/s for 20 sec) in Ca^{2+} free solution, containing Mg^{2+} ions

No. of fibre	Concentration of Mg^{2+} ions in Ca free solution (mM)	Mean frequency of m.e.p.p.s. ($\bar{f}$) (sec^{-1})	
		Rest	After stimulation
1	0·1	0·18	0·16
2		0·26	0·6
3	0·5	0·35	0·7
4	1	0·9	1·5
5		0·37	0·8
6		1·1	1·4
7		4·6	55
8	5	8	50
9		9	42
10		8·2	20
11		7	40
12	10	14	27
13		13	27
14		14	31
15	20	5	13
16		20	30
17	38	4·7	8·2

$\frac{\text{S.E.}}{\bar{f}} \times 100\ \%$ is less than 10 % in all measurements.

These effects in Ca^{2+} free solutions can best be explained if the membrane permeability to Mg^{2+} ions increases relatively slightly.

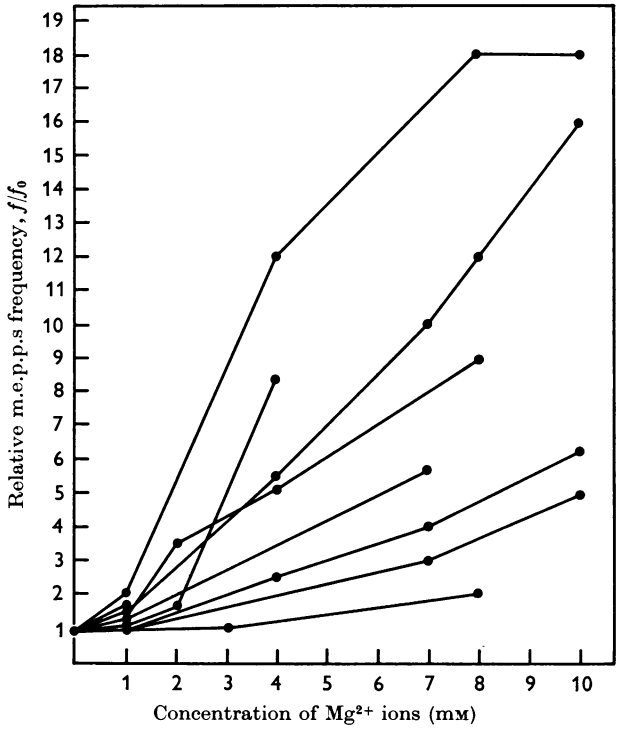

Fig. 10. Increase of frequency of m.e.p.p.s with increased concentration of Mg^{2+} ions in Ca^{2+} free solution, containing 2 % ethanol. f_0—frequency of m.e.p.p.s in the Mg^{2+} free solution.

Be^{2+} ions would be expected to have a similar action to that of Mg^{2+}. Indeed, Be^{2+} reduced and blocked e.p.p.s in even lower concentration than Mg^{2+}, and produced quantal fluctuations in successive e.p.p.s, characteristic of those obtained in Ca^{2+} deficient or Mg^{2+} blocked preparations (Fig. 11). The addition of Be^{2+} also resulted in a reduction of frequency of m.e.p.p.s in most fibres. In low concentration Be^{2+} ions act mainly presynaptically. With concentrations above 0·5 mM, however, the amplitude of m.e.p.p.s decreases markedly. At even higher concentrations m.e.p.p.s disappear. When Be^{2+} was replaced by Ca^{2+}, Sr^{2+} or Ba^{2+}, the e.p.p. was restored just as in the case of Mg^{2+} block (Figs. 12, 13). It is probable that an increase in Be^{2+} concentration causes a reduction in sensitivity to acetylcholine, but this would be insufficient to account for the depression in transmission and the marked fluctuations in the synaptic responses, which were observed with low Be^{2+} concentration in Ca^{2+} deficient solu-

tions (Ca^{2+} 0·5 mM, Be^{2+} 0·05 mM, pH 6·0), when the m.e.p.p.s amplitudes were almost unaffected. An increase of Ca^{2+} concentration to 2 mM in this case reverses the Be^{2+} block.

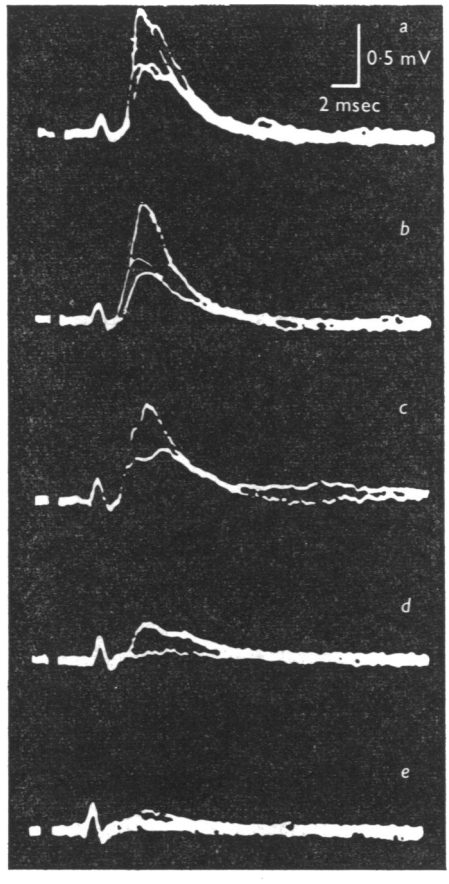

Fig. 11. Effect of Be^{2+} ions on the end-plate potential. Extra-cellular recording. *a*, *b*, *c*: 1, 10, 40 min, respectively, after adding 0·5 mM-Be^{2+} to the bath; *d*, *e*: 2 and 10 min, respectively, after adding 1 mM-Be^{2+}. The pH was 7·5. The concentration of free Be^{2+} ions, at this pH, is well below the total concentration stated here (about 0·05 mM and 0·1 mM-Be^{2+}).

Effects of tri- and tetravalent cations on e.p.p. and frequency of m.e.p.p.s. It could be argued that the restoration of the e.p.p. in Ca^{2+} free Ringer by Sr^{2+} or Ba^{2+} might be due to a similarity in the chemical actions of these ions rather than to their influence on the surface charge of the membranes. To obtain further evidence, we have studied the influence of trivalent cations, because on the present hypothesis trivalent ions should

be even more effective than divalent ones in screening or reducing the membrane surface charges. The action of La^{3+} was therefore studied.

Addition of 1 mM-La^{3+} to the Ringer solution, in the presence or absence of Ca^{2+}, produced a significant rise in m.e.p.p.s frequency (Fig. 14). An appreciable increase in m.e.p.p.s frequency by La^{3+} was also observed when the nerve ending had been partially depolarized by isotonic substitution of 10–20 mM-KCl for NaCl (Table 4). Furthermore, La^{3+} gave rise to an increased rate of discharge of m.e.p.p.s following nerve impulses.

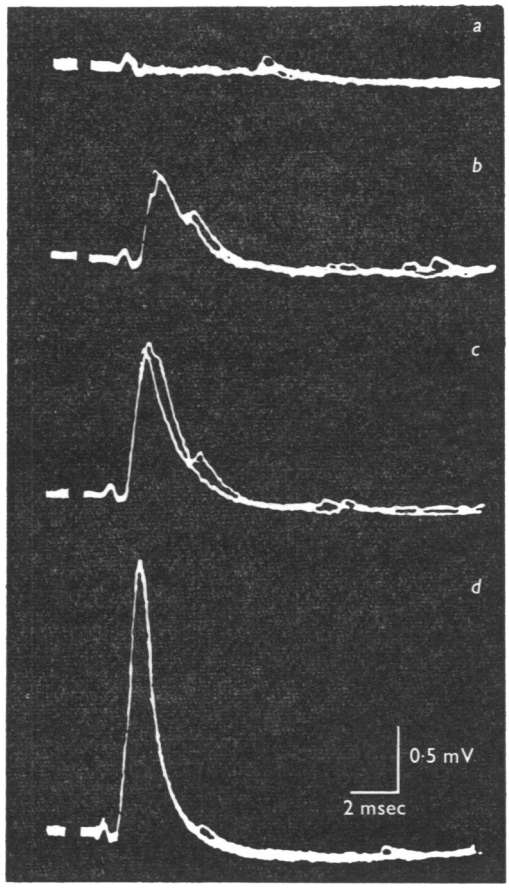

Fig. 12. Restoration of end-plate potential after replacing Be^{2+} by Ca^{2+} ions. Extracellular recording. *a:* Ringer solution + 1 mM-Be^{2+}; *b, c, d:* Ringer solution, containing 2 mM Ca^{2+} ions; after 3, 5, 10 min respectively.

When the preparation had been pre-treated with a solution containing 0·5 mM-Ca^{2+} and 2 mM-Mg^{2+}, resulting in about 60% failures of e.p.p.s, it was found that replacement of Ca^{2+} by 0·1 mM-La^{3+} caused the e.p.p. amplitude to increase and muscle contractions to occur in response to a

nerve stimulus. But within 5–7 min (at this particular concentration) while the frequency of m.e.p.p.s rose to its maximum value, neuromuscular transmission became blocked again, and e.p.p. responses diminished and became indistinguishable from m.e.p.p.s (Fig. 14).

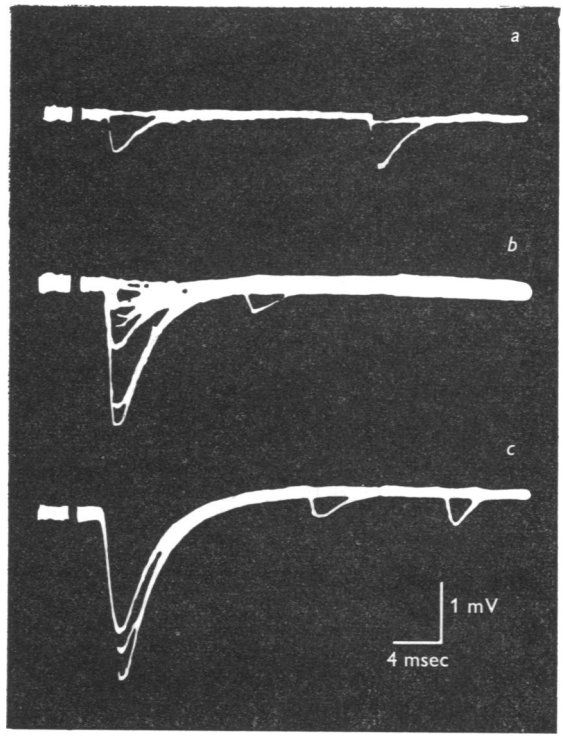

Fig. 13. Restoration of end-plate potential after replacement of Be^{2+} by Sr^{2+} ions (2 mM). Intracellular recording. *a:* 1 min, *b:* 3 min, *c:* 5 min after change of solution.

Addition of 0·1 mM-La^{3+} to preparations which had been kept in Ca^{2+} free solution produced recovery of e.p.p. responses within 5 min, and irregular contractions of individual muscle fibres were sometimes observed. Again, as the m.e.p.p.s frequency rose, the amplitude of the e.p.p. response declined, and eventually no e.p.p.s occurred, although the nerve spikes still invaded the terminals at that stage. Replacement of this solution by Ringer produced an immediate recovery of e.p.p. and neuromuscular transmission.

Professor B. Katz called our attention to recent papers by Miledi (1966) and Miledi & Thies (1967). The results presented above are substantially in accordance with their experiments, except for the distinct increase of m.e.p.p.s frequency in the presence of La^{3+} ions obtained in our experiments.

Tetravalent Th^{4+} ions were also able to increase the m.e.p.p. frequency when they were added to Ca^{2+} free solution. Th^{4+} was, however, less effective than La^{3+}; this difference might be explained because of a lower membrane permeability for this large cation and probably also because it is difficult to keep a sufficient quantity of Th^{4+} in solution at pH 7.

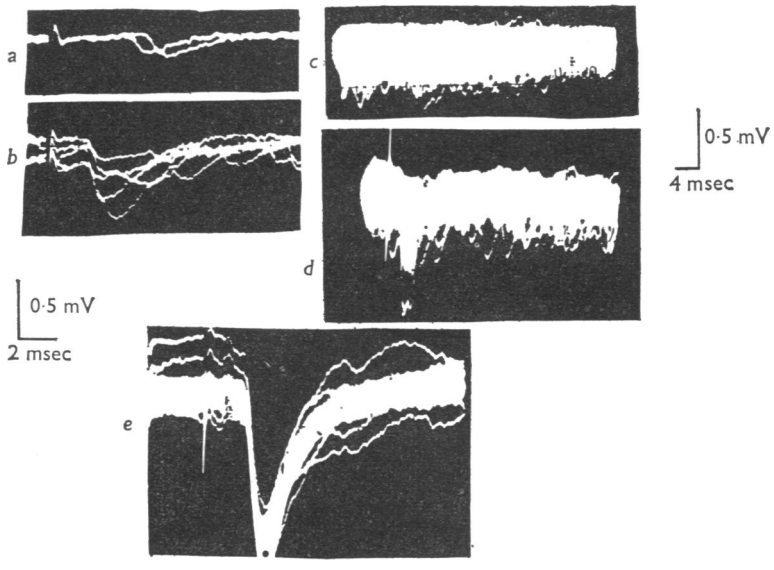

Fig. 14. Effect of La^{3+} ions on m.e.p.p.s and on end-plate potential. Intracellular recording. *a:* m.e.p.p.s in Ca^{2+} free solution with 2 mM-Mg^{2+} ions; the e.p.p. response is absent. *b:* the e.p.p. in the same solution with 0·05 mM-La^{3+}; m.e.p.p. frequency has increased. *c–d:* m.e.p.p.s and e.p.p., respectively, from other fibres in the same solution; more than 100 traces superimposed. *e:* restoration of e.p.p. after 2 min in Ringer solution; same fibre as in *c* and *d*. Vertical scale, 0·5 mV, horizontal scale, in *a*, *b*, *e:* 2 msec, in *c*, *d:* 4 msec.

TABLE 4. Effect of La^{3+} ions on m.e.p.p. frequency

Solution	Concentration of La^{3+} ions (mM)	Relative change of m.e.p.p. frequency	No. of fibres
Ringer + La^{3+}	0·1	50	1
Ca^{2+} and HCO_3^- free Ringer + La^{3+}	1	100–300	10
Ca^{2+} and HCO_3^- free Ringer + La^{3+} + KCl (10 mM)	1	500–800	8
* Ca^{2+} and HCO_3^- free Ringer + Mg^{2+} (1 mM) + La^{3+}	0·5	100–150	7
	0·05	20–50	5

* Preparations pre-treated with a solution containing 1 mM EDTA for 2–3 hr.

M.e.p.p. frequency was recorded in the same fibre before (f_0) and after (f_{La}) addition of La^{3+} ions. f_{La}/f_0 is shown; error in determining this value was less than 20 %.

The adhesion between phospholipid membranes in aqueous solutions at different ion concentrations. Technical difficulties preclude, at present, a direct experimental test of the effect of intracellular cation concentration on the adhesion between synaptic vesicles and the axon membrane. We used, therefore, artificial phospholipid membranes (Mueller *et al.* 1963; Babakov, Ermishkin & Liberman, 1966) for model experiments. The present results refer mainly to 'coloured' membranes (about a few hundred Å thick). Qualitatively, similar results were obtained with 'black' bimolecular phospholipid membranes.

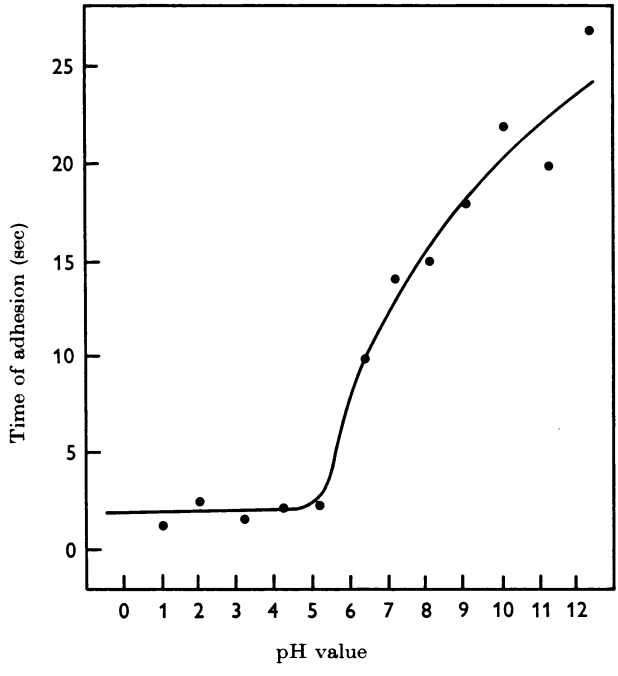

Fig. 15. The dependence of the adhesion time of phospholipid membranes on the pH value (100 mM-KCl, 20° C).

Figure 15 and Table 5 show the dependence of the 'adhesion time' on the pH value, and on the concentration of the various cations, in the aqueous solution surrounding the membranes.

Figure 15 shows that the adhesion time diminished when the pH was lowered, which results in a reduction of the negative charges on the membrane surface.

The influence of the cation concentration was studied at a constant pH of 7·4, corresponding to the pH value inside the cell (Kostyuk & Sorokina, 1961). In distilled water at this pH neither coloured nor black membranes

fused. By adding KCl and raising its concentration up to 100 mM, the adhesion time was reduced down to a few seconds. Doubling the KCl concentration caused an approximately 3–4-fold reduction in adhesion time. Divalent cations proved to be much more effective than univalent ions; by comparison, only about 1/1000 of the concentration was needed for a divalent ion to reach the same adhesion time (Table 5). It is interesting to note that Ca^{2+} ions appear to be more effective than Mg^{2+}. Addition of 1 mM-Ca^{2+} ions to a solution containing 100 mM-KCl reduced the adhesion time about sixfold; the addition of 1 mM-Mg^{2+} decreased it only about threefold.

TABLE 5. Average 'adhesion times' of phospholipid 'coloured' and 'black' membranes in solutions of various cations

Solutions	Concentration of cations (mM)	Time of adhesion (sec) Coloured (means ± S.E. of mean)	Time of adhesion (sec) Black (means ± S.E. of mean)
KCl	100 K^+	16 ± 2	32 ± 4
KCl	200 K^+	4·8 ± 0·5	7 ± 2
$CaCl_2$	0·1 Ca^{2+}	5·2 ± 0·6	—
$CaCl_2$	1 Ca^{2+}	2·7 ± 0·3	—
$MgCl_2$	0·1 Mg^{2+}	60	—
$MgCl_2$	1 Mg^{2+}	6·9 ± 0·7	—
$LaCl_3$*	0·01 La^{3+}	0·6 ± 0·3	—
$KCl + CaCl_2$	100 K^+ + 1 $CaCl_2$	2·6 ± 0·3	5 ± 1
$KCl + MgCl_2$	100 K^+ + 1 Mg^{2+}	4·9 ± 0·5	—
$KCl + LaCl_3$*	100 K^+ + 0·01 La^{3+}	0·7 ± 0·3	—

* Without buffer.

(Temp. = 20° C; pH = 7·4; 10 mM-K_2HPO_4 buffer, K^+ ion concentration shown includes the buffer.) The number of observations is 30 in each case.

The trivalent cation La^{3+} was more effective than the divalent ions, and the tetravalent Th^{4+} even more effective. Replacement of univalent by multivalent (2, 3 or 4) anions had practically no effect on the adhesion time.

The influence of temperature on the mean value of the adhesion time was also examined. The results of a typical experiment are shown in the graph of Fig. 16. For comparison, the dependence of the average frequency of m.e.p.p.s on the temperature is also shown in Fig. 16. If the occurrence of a m.e.p.p. is the result of the 'adhesion' of a synaptic vesicle with the membrane of the nerve ending, then the frequency of the m.e.p.p.s is inversely proportional to the average 'life time' $\bar{\tau}$ of the synaptic vesicle. The variation of $\bar{\tau}$ with temperature T in different muscles is, in general, similar to that of the adhesion time of the phospholipid membrane model, though the slopes of the curves differ. However, the anomalous effect

reported by Liley (1956*a*) in the range of 25–15° C had no parallel in the observations on phospholipid membranes (with 0·1 M-KCl at pH 7·4).

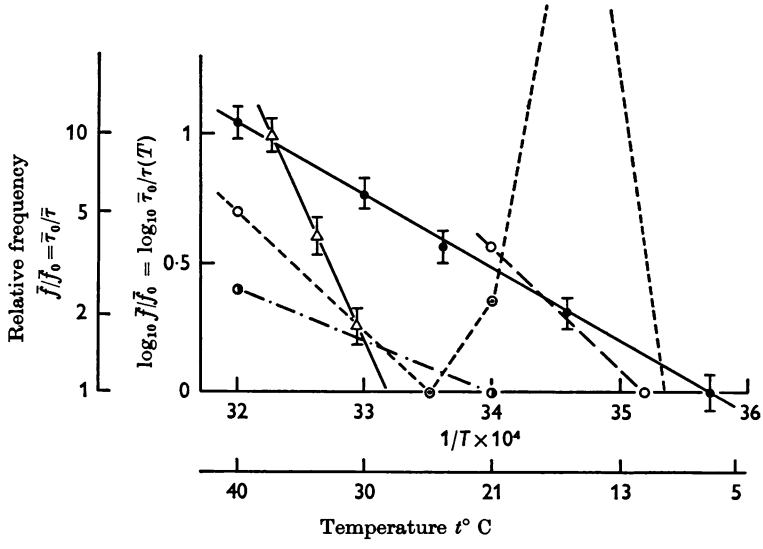

Fig. 16. Effect of temperature on frequency of m.e.p.p.s and on the adhesion time of artificial phospholipid membranes. $\bar{f}$—frequency of m.e.p.p.s at a given temperature; $\bar{f}_0$—frequency of m.e.p.p.s at the lowest temperature in a given experiment; $\bar{\tau}$, adhesion time of phospholipid membranes at a given temperature; $\bar{\tau}_0$, adhesion time at the lowest temperature; ——— ● — artificial phospholipid membranes (100 mM-KCl, pH 7·4); — — — ○ — m.e.p.p.s at the neuro-muscular junction of the frog (Fatt & Katz, 1952); — · — · — ◑ — m.e.p.p.s in cat muscle (Boyd & Martin, 1956); ——— △ — m.e.p.p.s in rat muscle (Hofmann, Parsons & Feigen, 1966); — — — ⊙ — m.e.p.p.s in rat muscle (Liley, 1956*a*).

DISCUSSION

The experiments described above give qualitative support to the hypothesis that adhesion occurs between synaptic vesicles and the membrane of the nerve ending because of screening or reduction of the surface charge of the membranes.

On the basis of our hypothesis concerning the role of Van der Waals and electrostatic forces, it should be possible to calculate the relative frequency of occurrence of adhesion between axon and vesicular membranes under various conditions. The theory predicts satisfactorily the relation between m.e.p.p. frequency and osmotic pressure of the outside solution.

The action of Ca^{2+} can be explained by assuming that external Ca^{2+} ions, after passing through the outer membrane, are bound by the negatively charged sites on the inner surface, which leads to a great reduction

of the surface charge. This is in line also with the recent work of Kimizuka, Nakahara, Uejo & Yamauchi (1967). However, it is necessary to suppose that the concentration of Ca^{2+} and Mg^{2+} ions in the region between outer membrane and synaptic vesicles is very small ($< 0{\cdot}1$ mM). For Ca^{2+} ions this is probably the case, a Ca^{2+} is actively accumulated by mitochondria and microsomes (Lieberman, Palmer & Collins, 1967). Concerning Mg^{2+} ions there are no data available. A. S. Spirin (personal communication) has called our attention to the need for a high concentration of Mg^{2+} ions (3–7 mM) for a normal functioning of ribosomes. The membrane ATPase also requires Mg^{2+} ions (Skou, 1965). There may, nevertheless, be a specific mechanism for lowering Mg^{2+} ion concentration in the region between axon membrane and synaptic vesicles.

The reduction in m.e.p.p. frequency and in e.p.p. amplitude by increased concentration of Mg^{2+} can be explained by competition for 'pores' or sites on the external membrane surface, between the poorly penetrating Mg^{2+} and the readily penetrating Ca^{2+} ions. Blockage of these 'pores' by external Mg^{2+} or Be^{2+} ions would prevent the influx of Ca^{2+} ions. The same type of competitive blockage might account for the diminution in m.e.p.p. frequency by increased external Na^{+} concentrations (Birks & Cohen, 1965). An excess of Ca^{2+} concentration beyond a certain level may act in the same manner (Elmqvist & Feldman, 1966; Gage & Quastel, 1966). It would be interesting to test these suggestions by examining the influence of various concentrations of Mg^{2+}, Be^{2+} and Ca^{2+} ions on the influx of Ca^{2+} in the giant axon of the squid.

According to our hypothesis, in a Ca^{2+} free medium Mg^{2+} ions would be expected to act like Ca^{2+} ions. This is borne out by the results (see Table 3, and Figs. 9 and 10).

It would be interesting to study the effect of direct intra-axonal injection of Ca^{2+} and of other di- and trivalent cations, but this is not feasible at the neuromuscular junction. Such an experiment, however, has been performed by Miledi & Slater (1966) on the presynaptic terminal in the giant synapse of the squid. In their experiments, no effect such as would be predicted by our hypothesis was observed. It is possible that the calcium injected into the interior of the axon may have been bound immediately by intracellular anions, and by microsomes and mitochondria (Lieberman *et al.*1967), and in this way failed to reach the region between inner surface of the axon membrane and synaptic vesicles. This situation differs from the influx of external Ca^{2+} ions directly into that region during depolarization of the axon membrane. On the present hypothesis one would expect the intracellular application of Ca^{2+} into the presynaptic ending to result in an increased rate of transmitter release, leading to a slow post-synaptic depolarization of the giant axon. This may be difficult to observe, and a

negative result is perhaps not decisive. We would not expect, on our hypothesis, to see restoration of the post-synaptic response to a nerve impulse if the preparation were kept in Ca^{2+} free solution, because intracellular injection of Ca^{2+} would not facilitate influx of Ca^{2+} during depolarization.

The proposed hypothesis allows one to explain, not only the effect of osmotic pressure, but also that of temperature on the m.e.p.p. frequency. The large effect of temperature was well known (Fatt & Katz, 1952; Boyd & Martin, 1956; Liley, 1956*a*), but has not previously been explained. The theory of interaction between charged particles in electrolyte solutions which has been developed by Overbeek, Derjagin, Landau and others (see Kruyt, 1952) shows that the frequency of adhesions increases, not only with the concentration of oppositely charged ions, but also with the temperature. This effect was observed in our model experiments with phospholipid membranes (Fig. 16). But with both, m.e.p.p.s and artificial membranes, the dependence on temperature was steeper than predicted by the theory. It is even more difficult to explain the decrease of m.e.p.p. frequency with rise of temperature in the range of 15–25° C, which was observed by Liley (1956*a*) in the rat diaphragm. Possibly this effect arose from an influence of temperature on biochemical reaction rates which alter the properties, and perhaps lead to an increase in the surface charge, of the membrane.

It may be recalled in this context that Mg^{2+} ions increase the activity of the membrane ATPase (see review by Skou, 1965). This would tend to increase the surface charge of the membrane and so reduce the frequency of m.e.p.p.s.

It is reasonable to suggest that the mechanism of transmitter liberation from synaptic vesicles is closely related to the process of secretion of other substances from intracellular membrane-bound particles. The results obtained by Douglas & Poisner (1962, 1964) on the secretion from the adrenal medulla and neurohypophysis and their dependence on Ca^{2+} and Mg^{2+} ions favour this suggestion.

For a direct test of our hypothesis further experiments using intracellular application to nerve endings of Ca^{2+} and other multivalent cations will be needed. As an indirect approach, we have used agents which uncouple oxidative phosphorylation. It is known that the mitochondria maintain a high concentration of Ca^{2+} ions and release them under the influence of various uncoupling agents. In this experiment the frequency of m.e.p.p.s increased strikingly, while the preparation was kept in Ca^{2+} free solution.

The present hypothesis suggests that a reduction of electrostatic repulsion between membrane surfaces may lead not only to a fusion between

synaptic vesicles and axon membrane but to a fusion between the vesicles themselves. However, mutual fusion between vesicles (e.g. in hypertonic media) would lead to a different distribution of m.e.p.p. amplitudes than is observed. To account for this situation one may suppose that the density of the negative charges on the inner side of the axon membrane is less than that on the synaptic vesicles. This difference in charge density can strongly increase the probability of the fusion. One of the possible reasons for reduced density of negative charge on the inside of the surface membrane is the crossing of Ca^{2+} ions from the outside.

Removal of Ca^{2+} ions from the external medium could lead to an increase of negative charge density on the surface membrane, leading in turn to a decrease of m.e.p.p. frequency. The observed slow decrease of the frequency in Ca^{2+} free solutions is in accord with the long time required for elimination of Ca^{2+} ions from the surface of phospholipid monolayers (Kimizuka *et al.* 1967). The change in the surface charge caused by Ca-inflow during excitation probably plays an essential part in the quantal release.

It is clear that the present hypothesis can account only for the first step in the mechanism of quantal release. It would be followed by chemical interactions between the specific membrane sites involved, whose nature would be much more difficult to elucidate.

The authors are deeply grateful to Professor B. Katz for useful discussion and advice, and for help in preparing this paper.

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