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THE INTERACTION OF PRESYNAPTIC POLARIZATION WITH CALCIUM AND MAGNESIUM IN MODIFYING SPONTANEOUS TRANSMITTER RELEASE FROM MAMMALIAN MOTOR NERVE TERMINALS

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SUMMARY

1. The relationship between motor terminal polarization and miniature end-plate potential (m.e.p.p.) frequency was examined in the presence of various Ca, Mg and K concentrations ([Ca], [Mg] and [K]) and also at modified bathing osmolarity levels. The polarization changes were obtained with 'electrotonic' and 'focal' polarizing currents and with rapid changes in bathing [K].

2. M.e.p.p. frequency increased exponentially with electrotonic depolarizing currents, but failed to decrease similarly with hyperpolarizing currents. An increase in bathing [K] to 15 mM increased the sensitivity of the terminals to presynaptic hyperpolarization.

3. The slope, on semilogarithmic coordinates, of the function relating m.e.p.p. frequency to electrotonic polarizing currents (the release-current function) was unchanged when bathing [Ca] was raised from 2 to 8 mM. When [Ca] was reduced to 0·5 mM the slope of this function was reduced initially but eventually approached the same slope as in control [Ca]. A similar effect was also found in the presence of 15 mM-KCl.

4. The relationship between m.e.p.p. frequency and log [K], at various [Ca], resembled the relationships between m.e.p.p. frequency and presynaptic polarizing currents.

5. An increase in bathing [Mg] or osmolarity had a similar effect to a reduction of [Ca].

6. Tetrodotoxin (TTX) at a concentration of 10^{-6} g/ml. was found to reduce m.e.p.p. frequency, at various [K], by a constant fraction of about 30%.

7. In some of the junctions 'anodic break-down' was observed. An

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examination of this phenomenon with focal polarizing currents disclosed an unusual type of 'anodic break-down', with rapid 'on' and 'off' responses. This phenomenon may indicate that release depends on the influx of positively charged particles into the nerve terminals.

8. It is concluded that nerve terminal depolarization accelerates exponentially the activity of a membrane component bearing three Ca molecules, the rate of acceleration being independent of bathing [Ca].

INTRODUCTION

The kinetic model proposed by Hubbard, Jones & Landau (1968*b*) provided a quantitative explanation for the effects of Ca and Mg ions on transmitter release at a mammalian neuromuscular junction. It was assumed that these ions competed for three sites of an unknown membrane constituent, X, active in release, and that presynaptic depolarization accelerated transmitter release solely by increasing the activity coefficient (k_3) of one particular complex species of X, namely Ca_3X. This model was obtained by comparing the effects of Ca and Mg on miniature end-plate potential (m.e.p.p.) frequency and on end-plate potential (e.p.p.) quantal content. However, these two parameters represent rates of release differing by six to seven orders of magnitude (Hubbard *et al.* 1968*b*). Could the model also explain intermediate rates of release? What was the simplest function describing the relationship between the activity coefficient of Ca_3X (k_3) and presynaptic depolarization?

To answer these questions, the effects of changes in presynaptic polarization frequency on m.e.p.p. were studied at various bathing Ca, Mg and K concentrations ([Ca], [Mg] and [K] respectively). Presynaptic polarization was obtained by applying steady electrical currents in the manner described by Liley (1956), by Hubbard & Willis (1962, 1968) or by modifying [K]. Finally, the ability of the model of Hubbard *et al.* (1968*b*) to explain these results quantitatively was assessed.

METHODS

All experiments were performed *in vitro* upon the isolated hemidiaphragms of albino rats of the Wistar strain. The methods of mounting and recording and the bathing solutions used were described previously (Hubbard *et al.* 1968*a*). The [Ca] was varied between 0·5 and 8 mM, the [Mg] between 1 and 15 mM and the [K] between 2 and 70 mM.

Solutions with an increased osmolarity were obtained by the addition of suitable amounts of sucrose. The temperature in most experiments was 32–34° C, but in a few experiments was increased to 37–39° C. The pH of the bathing solution was 7·1–7·3. All solutions were bubbled with 95 % O_2 and 5 % CO_2 and contained tetrodotoxin (Sankyo, Japan), in a concentration of $0{\cdot}5 \times 10^{-7}$–10^{-6} g/ml. Preparations were allowed 2 hr for equilibration in the bathing solution before the start of an experiment.

The method of polarization of the motor nerve terminals was the one described by Liley (1956), with some modifications. The hemidiaphragm was divided by a cut parallel to the

fibres and immediately posterior to the point of entry of the anterior nerve branch. The anterior part of the hemidiaphragm was then mounted in a divided Perspex chamber with the cut edge as close to the Perspex partition as possible. The partition was 3 mm thick and beneath it, through a notch sealed with soft paraffin, the anterior branch of the nerve was led. Current was passed through non-polarizable electrodes between the two halves of the divided chamber, the lines of current flow converging upon and polarizing the nerve at the notch. The polarization of the nerve then spread electrotonically and different nerve endings were affected according to their distance from the notch and the length constant of the nerve. A diagram of the circuit and further details are to be found in Liley's original communication (1956). My method differed from that of Liley in two respects. First, I used the anterior rather than the posterior part of the hemidiaphragm and, second, I recorded at a distance of 1–0·5 mm from the notch instead of less than 0·2 mm in Liley's original method. This was done because the muscle fibres adjacent to the cut edge were found to be discoloured and very difficult to penetrate with the recording micro-electrode. On the other hand, in the anterior part of the hemidiaphragm, a small superficial twig usually branched off the main nerve trunk immediately after the point of entry. Nerve terminals were easily located along this branch and most recordings were done along it.

A major problem in using this method involved the frequent occurrence of progressive m.e.p.p. frequency changes. When depolarizing current was switched on, the effect upon m.e.p.p. frequency was clearly established when the switching artifact (lasting about 10 msec) subsided. The early peak increase and subsequent depression of m.e.p.p. frequency described by Katz & Miledi (1967*a*, *b*) were not seen, probably because of the higher temperature range used in the present study. In many units, however, the frequency then continued to increase slowly over many seconds, at a nearly constant rate, and when the current was switched off, returned to a proportionally higher resting level. Sometimes when hyperpolarizing currents were passed, trends in the opposite direction appeared. This phenomenon was probably not due to K^+ fluxes in or out of the muscle fibres, because corresponding changes in m.e.p.p. amplitude were never observed, nor did m.e.p.p. frequency return quickly to control level in the manner reported by Hubbard & Willis (1968) for focal polarization. A possible explanation may be the accumulation or depletion of synaptic vesicles within the nerve terminals, as can be observed in electron-micrographs of nerve endings subjected to prolonged presynaptic polarizing currents (E. M. Landau & S. Kwan-bunbumpen, unpublished). As the magnitude of these progressive frequency changes varied greatly from junction to junction, it was decided to eliminate them from the results. To this end, the following procedure was adopted:

The resting frequency was determined at the beginning of each series of current applications and at frequent intervals during the series, usually after every application of current. About 20–30 m.e.p.p.s were recorded at each determination and the resting frequencies compared with the initial resting frequency. Records taken during the passing of a current which was followed by a significantly altered resting frequency (t tests, $P < 5\%$) were rejected. As an alternative, m.e.p.p.s were filmed continuously during an application of current, and the progressive frequency changes subsequently plotted by placing the m.e.p.p. frequencies, measured from successive oscilloscope sweeps, in the centre of the time intervals over which they were measured. A straight line was then drawn through the points and extrapolated to the instant the current was switched on, thus eliminating the effect of the slow trends in m.e.p.p. frequency. While an experiment was in progress, m.e.p.p. frequency was continuously monitored by a counting system similar to that described by Andersen & Curtis (1964) and care was taken not to proceed until resting frequency returned to the initial control level.

Another problem was presented by the occasional occurrence of 'giant' end-plate potentials, whose frequency was shown by Liley (1957) not to vary with presynaptic polarization. In order to exclude these, transients with a rising time in excess of 3 msec were not

counted. This was a simple criterion which did not necessitate extensive measurement of m.e.p.p. amplitudes. It had the additional virtue of excluding slow monophasic artifacts from the study.

In some experiments designed to examine 'anodic breakdown', the focal method of polarization reported by Hubbard & Willis (1962, 1968) was used. The full description of the method may be found in their original communications. In other experiments the exhibition of increased [K] was used to depolarize the nerve endings. Here the preparations were incubated for 2 hr in the appropriate control solution. M.e.p.p. frequency was recorded in ten junctions in the control solution, which was then removed by suction and the test solution containing an increased [K] allowed to flow rapidly (5–7 ml./min) into the bath, so that changes in the muscle membrane resting potential were complete within 2–3 min after change over. The m.e.p.p. frequency in eight to sixteen junctions was then recorded at 3–8 min after change over. This period of recording was chosen to avoid a second prolonged effect of [K] which is probably distinct from its depolarizing effects (Gage & Quastel, 1965). Each experiment was repeated twice, and the results averaged and computed as described elsewhere (Hubbard *et al.* 1968*a*).

RESULTS

The basic release–current function

The relationship between m.e.p.p. frequency and electrotonically applied currents is shown in Fig. 1*A*. At 32–34° C (filled circles) m.e.p.p. frequency increased exponentially with depolarization (cathodic currents), in confirmation of Liley's results (1956). The effect of hyperpolarizing (anodic currents), however, differed quite markedly from that found by Liley in the presence of 5 mM-K. Instead of decreasing exponentially with hyperpolarizing currents, m.e.p.p. frequency tended to stabilize at a level slightly below the control value. This phenomenon cannot be explained by the different temperature range used, as a very similar curve was obtained from the same junction at 37–38° C (Fig. 1*A*, open circles). It might be questioned whether the nerve terminals were actually hyperpolarized by the anodic currents. To test this, a unit was examined in normal [K], and found to be insensitive to hyperpolarizing currents (Fig. 1*B*, filled circles). The bathing [K] was then increased to 15 mM, and 30 min later the effect of currents upon m.e.p.p. frequency was again examined (Fig. 1*B*, open circles). Hyperpolarizing currents now produced a large fall in m.e.p.p. frequency, indicating that when the nerve terminal was depolarized by increased [K], hyperpolarization did reduce m.e.p.p. frequency in a nearly exponential manner. The difference between these and Liley's results cannot be attributed to progressive changes in m.e.p.p. frequency as these were checked for and eliminated (see Methods), nor can it be attributed to the low frequencies encountered, for at least 30 and usually 50 m.e.p.p.s were recorded in the hyperpolarizing range. Furthermore, the form of the curves shown in Fig. 1 was found repeatedly. In normal [K] it was found in all of fifteen junctions at 32–34° C and in seven units examined at 37–39° C. In high [K] the hyperpolarizing effect was

greatly enhanced in twelve junctions at 32–34° C and in five units at 37–39° C. In many units in high [K], the anodic depression of m.e.p.p. frequency eventually approached a limiting value (Figs. 2*A*, 4*A*), indicating that increased [K] served to depolarize the nerve terminals rather than to modify the basic release–current function. Similarly, the discrepancy between the present results and Liley's may be explained if the

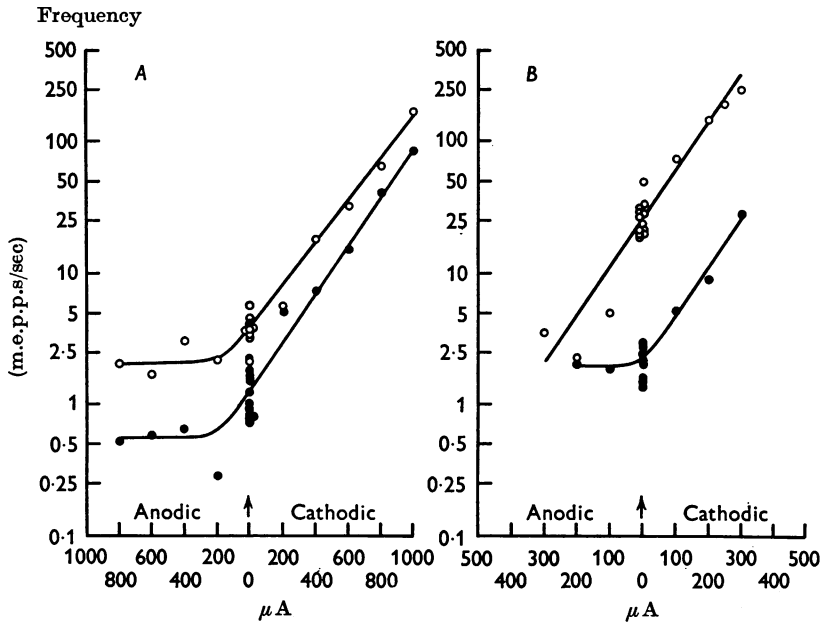

Fig. 1. The basic release-current function. Ordinates: m.e.p.p. frequency (note logarithmic scale). Abscissae: current strength in μA. To the right of the arrow cathodic currents, and to the left anodic currents.

A. The effect of temperature upon the release–current function. Filled circles: 32–34° C. Open circles: 37–39° C. Data obtained from a single junction.

B. The effect of increased [K] on the release–current function. Filled circles: control in the presence of 5 mM-KCl. Open circles: 30 min after change over to 15 mM-KCl. Data obtained from a single junction.

units studied by him were slightly depolarized, even when bathed in normal [K].

It is perhaps significant that in the unit illustrating an exponential decrease in m.e.p.p. frequency with presynaptic hyperpolarization (Liley, 1956, Fig. 4*A*), the control frequency is considerably higher than in the unit showing a deviation from this relationship (Liley, 1956, Fig. 4*B*). Liley's records, obtained within 200 μm of the cut edge of the muscle, may have been affected by K diffusing away from the damaged muscle fibres, thus depolarizing the junctions being studied.

The effect of Ca on the release–current function

The effect of Ca on the relationship between m.e.p.p. frequency and nerve terminal polarization is shown in Fig. 2*A*. In this experiment a junction was examined in normal [Ca] viz. 2 mM (open circles). [Ca] was then reduced to 0·5 mM and the polarization series repeated after 20 (filled circles) and 30 (filled diamonds) min. [Ca] was then returned to control level and the control series repeated after further 20 (open diamonds) and

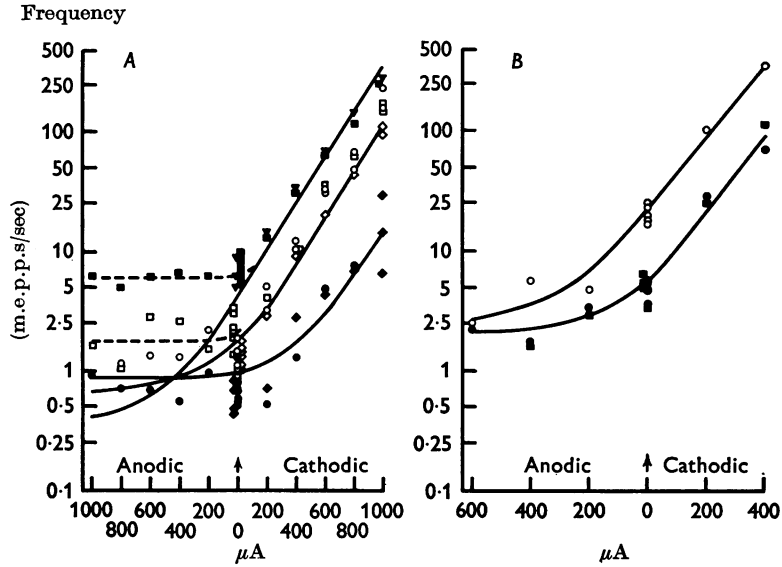

Fig. 2. The effect of Ca on the release–current function. Ordinates and abscissae as in Fig. 1.

A. The effect of Ca in normal bathing [K]. Control in 2 mM-$CaCl_2$: open circles. After 20 min in 0·5 mM-$CaCl_2$: filled circles. After 30 min in 0·5 mM-$CaCl_2$: filled diamonds. Twenty minutes after return to control: open diamonds. Thirty minutes back in control: open squares. Twenty minutes after change-over to 8 mM-$CaCl_2$: filled squares. Thirty minutes in 8 mM-$CaCl_2$: filled triangles. The continuous lines represent fitted curves. For details see Discussion. Data from a single junction.

B. The effect of Ca in the presence of 15 mM-KCl. Control series: open circles. Twenty minutes after change-over to 0·5 mM-$CaCl_2$: filled circles. Thirty minutes in 0·5 mM-$CaCl_2$: filled squares. Data from a single junction.

30 (open squares) min. Finally [Ca] was increased to 8 mM, and the series repeated 20 (filled squares) and 30 (filled triangles) min later. The increase in [Ca] (Figs. 3, 4*A*, filled triangles and squares) shifted the release–current function upwards. No significant change in the slope (i.e. exponential constant) of the function was found with depolarizing currents. In eight similar experiments the ratio between the slopes at 8 and 2 mM-Ca was $1{\cdot}04 \pm 0{\cdot}14$ (mean ± 1 S.E. of eight experiments). When [Ca] was reduced

(Fig. 2*A*, filled diamonds and circles) the release–current function appeared to have a smaller initial gradient which increased to approach that of the control function. A similar phenomenon was found in three more experiments. However, the scatter of the points prevented the exclusion of an alternative interpretation, namely, that reducing [Ca] produced an over-all reduction in the slope of the release–current function.

To elucidate this point further, three experiments were performed in preparations equilibrated for 2 hr in 15 mM-K, the results of one being shown in Fig. 2*B*. In such solutions the endings were only little depolarized (control frequency multiplied by about ten, see Figs. 6 and 7), but a second slow phase in K activity had further increased the frequency by 2·5–5 times. This phase was probably independent of the depolarizing effect of K (Gage & Quastel, 1965) but made the determination of m.e.p.p. frequency much easier. When [Ca] was reduced from 2 mM (open circles, Fig. 2*B*) to 0·5 mM (filled circles, squares, Fig. 2*B*), in the presence of 15 mM-K, the functions stayed parallel in the depolarizing range, but tended to approach each other in the hyperpolarizing range. This experiment thus confirmed both the tendency of the release–current function in low [Ca] to approach the same slope as in normal [Ca], and also its having a smaller initial gradient than in normal [Ca]. The same effect was found in two other similar experiments.

The effect of Mg on the release–current function

An experiment demonstrating the effect of Mg on the release–current function is shown in Fig. 3. In the presence of 15 mM-Mg, the initial gradient of the release–current function was reduced (Fig. 3, filled symbols), but the function subsequently exhibited a distinct effect of strong depolarizing currents on m.e.p.p. frequency. This junction was relatively sensitive to the effect of currents, as a tenfold increase in m.e.p.p. frequency (in the control solution) was produced by a depolarizing current of only 279 μA as compared, for instance, with 500 μA in the experiment of Fig. 2*A*. A similar effect was observed in two other identical experiments where the corresponding current values were 60 and 367 μA respectively. In a fourth identical experiment, the unit examined was less sensitive to polarizing currents, a depolarization of 460 μA being needed for a tenfold increase in m.e.p.p. frequency. When [Mg] was increased to 15 mM-Mg, both depolarizing and hyperpolarizing currents up to 800 μA had no effect on m.e.p.p. frequency, thus confirming the tendency of the release–current function to have a reduced initial gradient in high [Mg]. Similar effects were reported also by Liley (1956, Fig. 4*B*), and by del Castillo & Katz (1954, Fig. 5).

The effect of Mg on the release–current function was also examined in

the presence of 15 mM-K. An experiment, representative of three similar experiments, is shown in Fig. 4*A*. When [Mg] was increased from 1 to 8 mM, the effect of presynaptic polarization on m.e.p.p. frequency was greatly reduced (Fig. 4*A*, filled circles). However, it was clear that this was not brought about by a simple reduction of the 'exponential constant' of the release–current function. Rather, the gradient of this function was

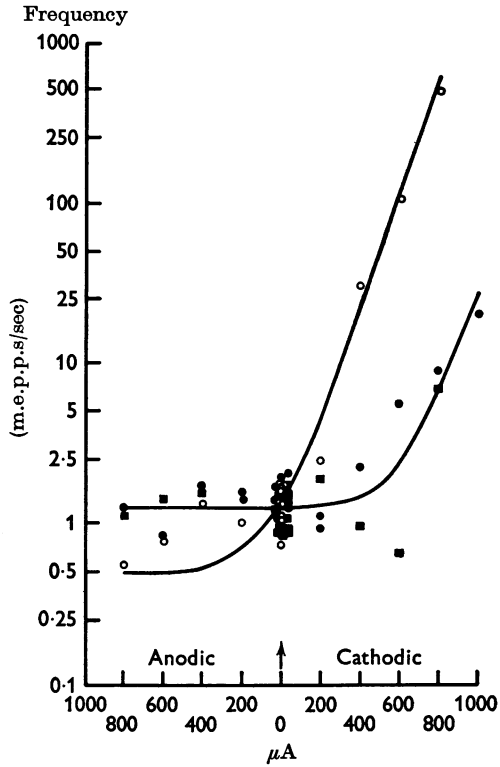

Fig. 3. The effect of Mg on the release–current function. Ordinate and abscissa as in Fig. 1. Control series: open circles. Series after 10 min in 15 mM-$MgCl_2$: filled squares. Series after 20 min in 15 mM-$MgCl_2$: filled circles. The lines are theoretically fitted curves. For details see Discussion. Data from a single junction.

reduced more at lower than at higher rates of release. Moreover, it could be predicted that the function in high [Mg] (Fig. 4*A*, filled circles), would eventually become parallel to the one in normal [Mg] (Fig. 4*A*, open circles). This prediction was substantiated in a further similar experiment, shown in Fig. 4*B*, where the upper region of the release–current function in 8 mM-Mg (Fig. 4*B*, filled circles), appeared to be parallel to the function in normal [Mg] (Fig. 4*B*, open circles). The results of four experiments in 15 mM-KCl, where bathing [Mg] was increased from 1 to 5 mM, were similar to those of the experiment shown in Fig. 4*B*. The control release–current

function and the one obtained in 5 mM-Mg appeared to be parallel to each other in the depolarizing range, but tended to approach each other in response to hyperpolarizing currents.

These results provided a strong indication that the effects of increased [Mg] on the relationship between spontaneous release and presynaptic polarization resembled closely those of reduced [Ca] (Fig. 2). This con-

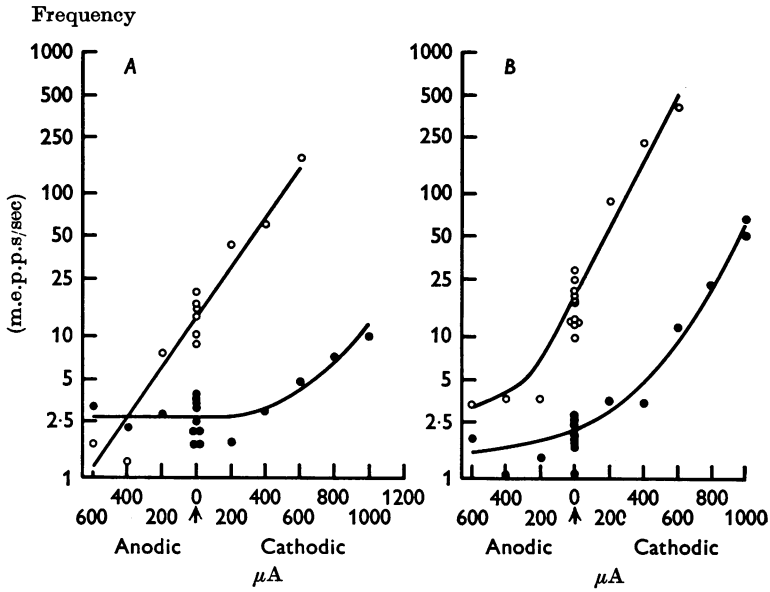

Fig. 4. The effect of Mg on the release–current function in the presence of 15 mM-K. Ordinates and abscissae as in Fig. 1. *A* and *B* show the results of two identical experiments, each obtained from a different junction. Dotted circles: control series in 1 mM-Mg. Filled circles: series obtained 10 min after the exhibition of 8 mM-Mg.

clusion is of particular importance as it excludes the possibility that the present results are produced by the effects of Ca^{2+} and Mg^{2+} on membrane resistance and 'delayed rectification' (Lüttgau, 1954; Weidmann, 1955; Frankenhaeuser, 1957; Frankenhaeuser & Hodgkin, 1957; Shanes, 1958; Shanes, Freygang, Grundfest & Amatniek, 1959). It is known that Ca^{2+} and Mg^{2+} have similar effects on these membrane properties (Frankenhaeuser & Hodgkin, 1957; Frankenhaeuser & Meves, 1958; Shanes, 1958), whereas in the present study the reduction of [Ca] had a similar effect to an increase in [Mg].

Effect of Ca on K-induced depolarization of the motor nerve terminals

The results obtained with electrotonic polarization of terminals could be confirmed in experiments in which the motor terminals were depolar-

ized by increased bathing [K]. M.e.p.p. frequencies were recorded 3–8 min after changes in [K] were affected, in order to avoid a second slow phase of K action which is probably distinct from its depolarizing effect (Gage & Quastel, 1965). The experiments shown in Fig. 5 were performed at a bathing osmolarity of 400 m-osmole/l. (range 391–411 m-osmole/l) to avoid osmotic effects due to the increased KCl concentrations. The effects of increasing [K] were similar in 2 and in 4 mM-Ca (Fig. 5, open and filled

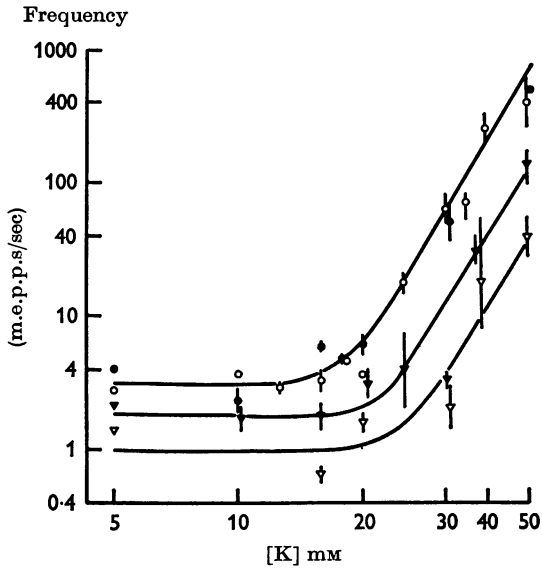

Fig. 5. The relationship between m.e.p.p. frequency and bathing [K]. Ordinate: m.e.p.p. frequency. Abscissa: [K] in mM. Note logarithmic scale of both axes. The period of recording was 3–8 min after changing bathing [K] from 5 mM to that indicated ('early' K-kinetics). Bathing osmolarity was 400 m-osmole/l. and the tetrodotoxin concentration 10 g^{-6}/ml. Each point represents frequencies recorded at eight to sixteen junctions at each of two experiments. The bars indicate ± 1 S.E. Where no bars are shown the S.E. was smaller than the symbol used. The experiments were performed in 2 mM-Ca: open circles; 4 mM-Ca: filled circles; 0·5 mM-Ca: filled triangles and 0·1 mM-Ca: open triangles. The symbols at 5 mM-KCl are the geometric means of all control readings at any given [Ca].

circles). When [Ca] was reduced to 0·5 mM (filled triangles) and 0·1 mM (open triangles) the function relating log. m.e.p.p. frequency to log [K] first showed a progressively smaller initial gradient and then tended to increase with a slope similar to that obtained in 2 mM-Ca. This series of experiments thus confirms the results obtained with electrotonic depolarization of motor nerve terminals. It can be calculated from the slope of the relationship between log m.e.p.p. frequency and log [K] using the Nernst formula, that a tenfold increase in release will be produced by

12·5 mV of depolarization. This is a steeper relationship than found by Hubbard, Jones & Landau (1967) using a similar method, and may be due to a quicker equilibration of the bathing solutions because of the higher flow rates used in the present study. The value of 12·5 mV may thus be an overestimate, but is of the same order of magnitude as the slope of the synaptic input–output transfer characteristic in the squid giant synapse (Katz & Miledi, 1967*c*). Liley (1956) performed a similar experiment using increased [Mg] instead of reduced [Ca] to block the effect of depolarization. His results (Liley, 1956, Fig. 6) suggest a simple linear relationship between log frequency and log [K] in the presence of high [Mg], but do not rule out a non-linear relationship of the type found in low [Ca] in the present series of experiments.

The effect of increased bathing osmolarity on the relationship between release and depolarization

It was of interest to note that even in 2 mM-Ca (Fig. 5, open circles) m.e.p.p. frequency remained insensitive to [K] lower than 20 mM. This contrasted with the results of Liley (1956) and also of Hubbard *et al.* (1967) who found log m.e.p.p. frequency to increase linearly with log [K] when [K] exceeded 10 mM. A possible explanation of this difference could be the effect of the increased osmolarity in the present experiments. To test this, the effect of K on m.e.p.p. frequency was examined in the normal osmolarity range (325–375 m-osmole/l., Fig. 6*A*, filled circles) and also at 600 m-osmole/l. (range 589–606, Fig. 6*A*, filled triangles). In the normal osmolarity range the osmotic pressure of the test solutions varied with the added KCl. However, only in one of these (30 mM-KCl) was there a change in osmolarity exceeding 10 %, which at this level of depolarization would probably have no additional effect (Hubbard *et al.* 1968*c*).

The results, shown in Fig. 6, indicated that the linear relationship between log m.e.p.p. frequency and log [K] was shifted along the log [K] axis in a parallel fashion with increased osmolarity. Thus, this linear relationship started at 10 mM in normal osmolarity (Fig. 6*A*, filled circles), at 20 mM-K in 400 m-osmole/l. (Fig. 6*A*, open circles), and at 30 mM in 600 m-osmole/l. (Fig. 6*A*, filled triangles). A similar effect could also be demonstrated by using the electrotonic method of depolarization. In a typical experiment (Fig. 6*B*), m.e.p.p. frequency in 600 m-osmole/l. started increasing only when a considerable depolarization was applied. Similar results were obtained in three more junctions, whereas in another the m.e.p.p. frequency increased even with small depolarizing currents, indicating a certain variability in the osmotic effect.

Two explanations can be suggested for this osmotic effect. First, an increased bathing osmolarity may increase intracellular [K], as found in

frog muscle by Adrian (1956), thus hyperpolarizing the terminals. In addition, the increased osmolarity may release membrane-bound Ca and reduce the concentration of Ca_3X, as suggested by Hubbard *et al.* (1968*c*).

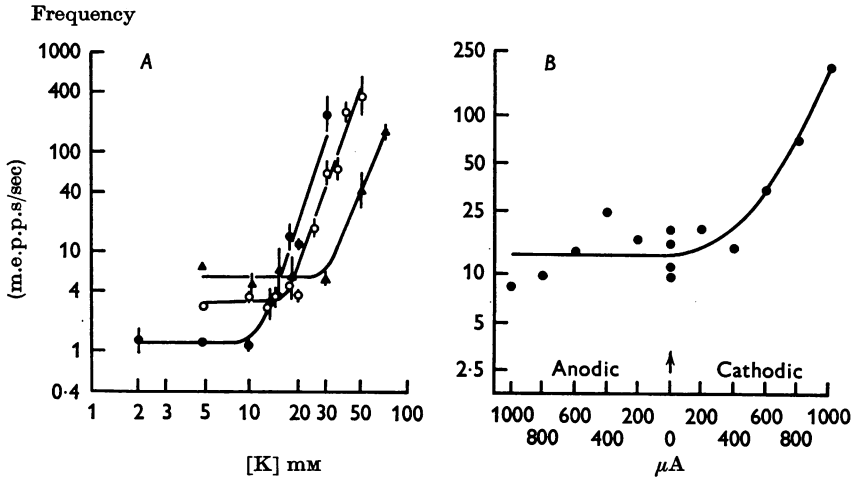

Fig. 6. The effect of increased bathing osmolarity on the release–current function. Ordinates: m.e.p.p.'s/sec. Note logarithmic scale.

A. The effect of increased bathing osmolarity on the 'early' K kinetics. Abscissa: [K]. Note logarithmic scale. The significance of the points and of the vertical bars is the same as in Fig. 5. The bathing osmolarity was 325–375 m-osmole/l.: filled circles; 391–411 m-osmole/l.: open circles; 589–606 m-osmole/l.: filled triangles. The data for the 391–411 range is the same as the data obtained in the presence of 2 mM-Ca in Fig. 5.

B. The relationship between m.e.p.p. frequency and polarizing currents. Ordinates and abscissae as in Fig. 1. Bathing osmolarity 608 m-osmole/l. Data from a single junction.

The effect of tetrodotoxins (TTX) on m.e.p.p. frequency

A chance observation prompted an investigation into the effects of TTX on the relationship between m.e.p.p. frequency and bathing [K]. The experiments were done in the same way as those in Figs. 5 and 6*A*. The results of Fig. 7*A* were obtained without osmotic correction, whereas those of Fig. 7*B* were done in solutions corrected to 400 m-osmole/l. In both situations the addition of TTX, 10^{-6} g/ml., to the bathing solution reduced m.e.p.p. frequency (Fig. 7*A*, *B*, filled circles), apparently by a constant factor. On the average m.e.p.p. frequency in TTX was depressed by $29{\cdot}8 \pm 5{\cdot}7\,\%$ (mean ± 1 S.E., $n = 15$). These results differ from those of Katz & Miledi (1967*a*) and Elmqvist & Feldman (1965). However, it cannot be excluded that the present results are due to some contaminant in the commercial preparation of TTX (Sankyo). Alternatively TTX may

reduce slightly the influx of Ca into the terminals (Watanabe, Tasaki, Singer & Lerman, 1967). Kusano, Livengood & Werman (1967) suggested a similar explanation for the block in synaptic transmission they found in relatively high TTX concentrations. As TTX reduced m.e.p.p. frequency by a constant factor over a wide range of m.e.p.p. frequencies, and as it

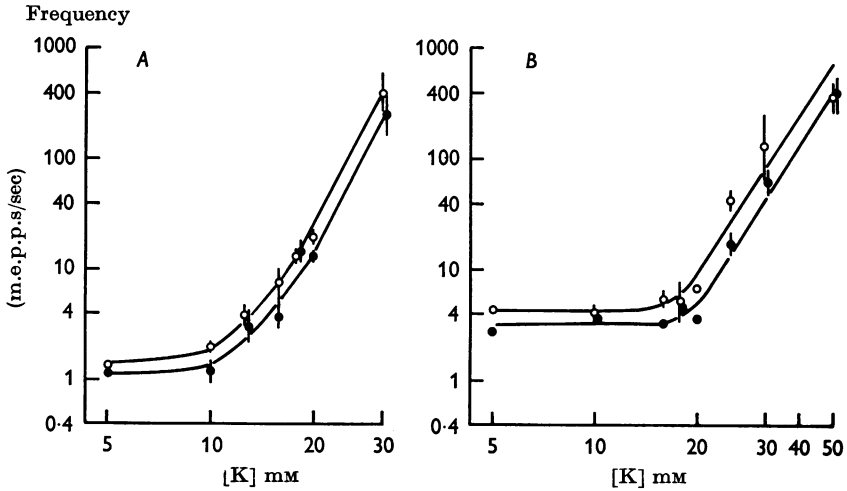

Fig. 7. The effect of TTX on the relationship between m.e.p.p. frequency and bathing [K]. Ordinates: m.e.p.p. frequency at 3–8 min after change-over ('early' K-kinetics). Abscissae: bathing [K] in mM. Note logarithmic scale. The significance of the points and of the vertical bars as in Fig. 5. Open circles: no TTX. Filled circles: TTX, 10^{-6} g/ml. In *A*, control osmolarity was 325 m-osmole/l. In *B* it was 400 m-osmole/l. Some of the data obtained with TTX (filled circles) is also shown in Fig. 5 (open circles).

was kept constant in any one experiment in the present study, this effect should not introduce any serious error into the results of the present investigation.

An examination of 'anodic breakdown'

A phenomenon was occasionally encountered in the present investigation, which had not been found by Liley (1956): the occasional occurrence of greatly increased m.e.p.p. frequency in the presence of hyperpolarizing currents ('anodic breakdown', del Castillo & Katz, 1954; Katz & Miledi, 1965). This could be explained by the finding (cf. Fig. 1) that the nerve endings examined by Liley (1956) were probably slightly depolarized.

In four units out of fifteen at 32–34° C, and in two out of seven at 37–39° C, the application of strong electrotonic hyperpolarization resulted in a sudden dramatic increase in m.e.p.p. frequency. The onset, though abrupt, was not linked to the onset of the current and the frequency

stayed up for many seconds after the current was withdrawn. During the application of the current, the m.e.p.p.s sometimes occurred in short, high-frequency bursts. In general, these phenomena resembled the 'anodic breakdown' found by del Castillo & Katz (1954) and Katz & Miledi (1965) in frog neuromuscular junctions. To rule out the possibility of the 'break-down' occurring in the phrenic nerve near the aperture where the current was most intense, rather than at the nerve terminals, the focal method of polarization of Hubbard & Willis (1962, 1968) was employed. Experiments were performed both in normal [Ca] and in a [Ca] reduced to 10^{-5} M by the use of an ethylenediaminetetra-acetic acid buffer (Hubbard *et al.* 1968*a*). The currents applied with this method were smaller than with the electrotonic method, and usually did not exceed 100 μA. Due to the increased efficiency of the method, such currents were sufficient to produce intense depolarization of the terminals. It was noted, however, that local muscle contracture developed during the passage of focal polarizing currents (Lucas, 1908; Kuffler, 1946; Huxley & Taylor, 1958). The focal experiments had to be performed, therefore, in the presence of increased osmolarity (600 m-osmole/l., Hodgkin & Horowicz, 1957; Howarth, 1958), which was found upon microscopic inspection (enlargement by 40) to abolish the muscle contracture.

When hyperpolarizing currents were applied, 'anodic break-down' could be obtained readily, and was found in nine units with normal [Ca] and in five units with reduced [Ca]. An interesting variant of this phenomenon was also found, the increase in m.e.p.p. frequency occurring within 1–2 sec after the application of the anodic current and subsiding within 1–2 sec after the current was switched off. This was found in three units in normal [Ca] and in two units in reduced [Ca], m.e.p.p. frequency in all but one being too low for closer definition. However, in one experiment in normal [Ca], illustrated in Fig. 8, the effect was large enough to show that this type of anodal acceleration had a very rapid phase in both the 'on' and 'off' responses, which were clearly evident when the switching artifact (about 10 msec) subsided.

This rapid onset of the anodic acceleration of m.e.p.p. frequency could be explained by assuming that transmitter release was mediated by the influx of some positively charged particles across the presynaptic membrane (cf. Katz & Miledi 1967*b*, *c*; Kusano *et al.* 1967). A step increase in the potential difference (p.d.) across the membrane would be expected to cause an instantaneous increase in the influx of these particles and, hence, a rapid increase in transmitter release. A similar consideration also applies to the rapid 'off' effect shown in Fig. 8. The fact that this phenomenon was not found in all instances where a strong anodal current was applied presynaptically indicates that other factors must be taken into account.

Primarily one will have to consider the effects of the anodic current on the permeability of the presynaptic membrane. However, at present this effect remains obscure and cannot be controlled experimentally.

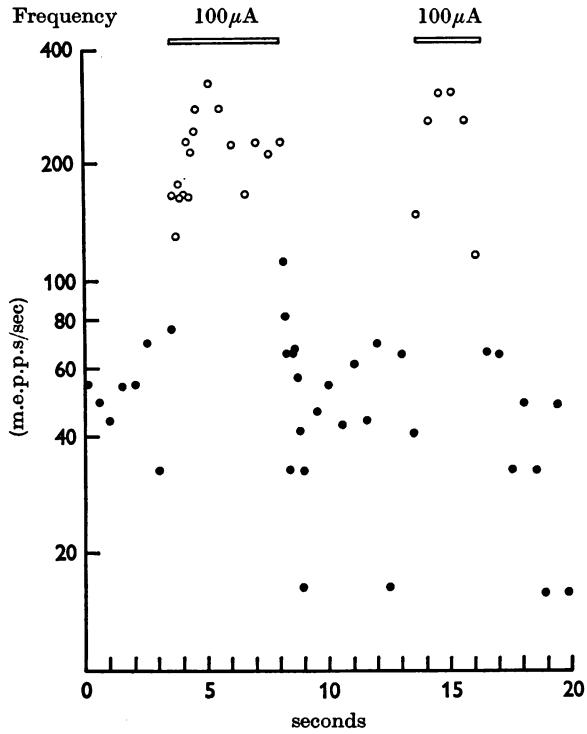

Fig. 8. The effect of a focally applied anodic current on m.e.p.p. frequency. Ordinate: m.e.p.p. frequency. Note logarithmic scale. Abscissa: time in sec. Resting frequency: filled circles. Frequency during the application of current (100 μA): open circles. Horizontal bars denote periods of application of current. Data from a single junction.

DISCUSSION

An interesting prediction can be made from the model of Hubbard *et al.* (1968*b*). Namely, that when log. m.e.p.p. frequency is plotted against increasing membrane depolarization, the slope of the resulting function will become increasingly independent of changes in [Ca] and [Mg]. This derives from the finding (Hubbard *et al.* 1968*b*) that the only effect of presynaptic depolarization is to increase the activity coefficient (k_3) of a single membrane component (X) bearing three Ca molecules (Ca_3X). Thus for high rates of release, when Ca_3X is the predominant active complex of X, the following formula holds (Hubbard *et al.* 1968*b* eqn. 2)

$$\text{Quantal release} = k_3 . [Ca_3X]_m,$$

where $[Ca_3X]_m$ is the concentration of Ca_3X in the presynaptic membrane. If only k_3 varies with presynaptic depolarization, then the relationship between log. quantal release and depolarization will clearly be shifted in a parallel manner along the quantal release axis when [Ca] and [Mg] (and hence $[Ca_3X]_m$) are modified. This prediction contrasts with the generally accepted view (del Castillo & Katz, 1954; Liley, 1956; Katz, 1966) that changes in [Ca] and [Mg] modify the 'exponential constant' of the function relating release to presynaptic depolarization.

However, this view is based on rather limited evidence, and the present results (Figs. 2–4) seem to bear out the prediction based on the model of Hubbard *et al.* (1968*b*). It must be emphasized, however, that the present data are not conclusive, because the relationship between log. m.e.p.p. frequency and polarizing currents was non-linear over most of the range being studied. It is felt that further experiments, perhaps with large presynaptic depolarizing pulses (Katz & Miledi 1967*a*, *b*) may extend the range of release rates observed and help to test the model of Hubbard *et al.* (1968) in a more rigorous manner.

The findings in the squid giant synapse (Katz & Miledi, 1967*c*; Kusano, 1968) that variations in [Ca] and [Mg] modified the exponential constant of the input–output transfer characteristic of that synapse, cannot be evaluated in the present context, because of the lack of a full kinetic description of the effect of Ca^{2+} and Mg^{2+} on transmitter release from squid giant nerve endings.

The findings that m.e.p.p. frequency failed to fall exponentially with hyperpolarizing currents (Figs. 1–4), in contrast to the findings of Liley (1956), indicated that some of the previously reported work on mammalian neuromuscular junctions was done on slightly, but significantly, depolarized nerve endings. This could also explain the fact that the 'anodic break-down' found in the present investigation had previously been alleged not to exist in the mammal (Liley, 1956). This insensitivity of spontaneous transmitter release to presynaptic hyperpolarization could be explained conveniently with the help of the scheme proposed by Hubbard *et al.* (1968*b*). In this scheme, other complex forms of X besides Ca_3X (i.e. X, CaX, Ca_2X, Mg_3X), could release transmitter, but were insensitive to changes in the presynaptic membrane potential. When the activity coefficient of Ca_3X (k_3) was reduced, the other complexes of X would become dominant in release and produce the observed insensitivity of release to presynaptic hyperpolarization. To test whether this explanation could account quantitatively for the present findings, an attempt was made to fit theoretical curves to the data of Figs. 2*A* and 3, with the additional assumption that k_3 increased exponentially with depolarization.

The kinetic formulae and constants used were those of Hubbard *et al.*

1968*b* (cf. their eqn. (1)). The fitting procedure began by computing the expected m.e.p.p. frequency values at various [Ca] and [Mg] for a tenfold increase or decrease in k_3, for a 100-fold increase or decrease in k_3 and so on. A problem here was that the control m.e.p.p. frequency in individual units in normal [Ca] was slightly different from the average value reported by Hubbard *et al.* (1968*a*) and used in computing the various rate and dissociation constants (Hubbard *et al.* 1968*b*). To overcome this, the whole set of computed m.e.p.p. frequencies was normalized in each case to the actual control frequency in 2 mM-Ca. This would be formally equivalent to a similar modification in Xt, the total concentration of all X species. The curves in Fig. 2*A* were thus normalized to a control frequency 1·8/sec (instead of 2·3/sec) and those of Fig. 3 were normalized to a control frequency of 1·3/sec. In the next stage the current strength was determined at which the observed m.e.p.p. frequency in normal [Ca] corresponded to the one computed for a ten-fold increase in k_3. This was 500 μA in the case of Fig. 2*a* and 279 μA in Fig. 3. The estimates for a 100-fold increase in k_3 were then placed at twice this current strength, and so on. Similarly, the estimates for 0·1 k_3, etc., were placed at corresponding points in the negative (hyperpolarizing) direction. The same current scale was used for both depolarizing and hyperpolarizing currents and for all the curves in the same figure. As can be seen in Fig. 2*A*, the fit with the results in the depolarizing range was reasonably good. A large deviation, however, occurred in the hyperpolarizing range, which was larger the higher the [Ca]. This was so because the depression of m.e.p.p. frequency by hyperpolarization could be expected to increase in higher [Ca], as the effect of a reduction in k_3 would be more prominent the higher the concentration of Ca_3X. In contrast, the release–current function in high [Ca] was practically flat in the hyperpolarizing range (Fig. 2*A*), and this was found in all eight experiments in high [Ca]. Similarly, the agreement between the theoretical curve and the data of Fig. 3 was better in the depolarizing than in the hyperpolarizing range. The deviation in the hyperpolarizing range was observed, predictably, at the lower [Mg], where a larger concentration of Ca_3X was expected to occur. It must be concluded therefore that although the model of Hubbard *et al.* (1968*b*) provides an explanation for the effect of depolarizing currents on m.e.p.p. frequency at various [Ca] and [Mg], it does not explain the failure of hyperpolarizing currents to reduce m.e.p.p. frequency, unless it is assumed, further, that k_3 cannot be reduced below some constant value.

Within the framework of the model of Hubbard *et al.* (1968*b*), the present findings thus define in some detail the relationship between presynaptic polarization and the activity of Ca_3X, the Ca-membrane complex responsible for excitation release coupling. Further speculations as to

the processes underlying this relationship seem unprofitable at present. Although the present study cannot be taken to provide definite proof for the model of Hubbard *et al.* (1968*b*), it points to the necessity for considering the effects of Ca^{2+} on current-evoked release, when formulating a kinetic model for transmitter release in general.

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