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A NOTE ON THE INTERACTION OF SPONTANEOUS AND EVOKED RELEASE AT THE FROG NEUROMUSCULAR JUNCTION

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SUMMARY

1. The interaction between spontaneous miniature end-plate potentials and evoked end-plate potentials was investigated at the frog neuromuscular junction using focal extracellular recording techniques.

2. End-plate potentials evoked immediately after a spontaneous miniature potential were facilitated by up to 20%. The percentage facilitation was negatively correlated with the average quantal content of the end-plate potential.

INTRODUCTION

Neurotransmitter is liberated from motor nerve endings in preformed packets, or quanta. At rest these quanta are released spontaneously, giving rise to miniature end-plate potentials (m.e.p.p.s, Fatt & Katz, 1952). The arrival of an action potential at the nerve terminal brings about a large, transient increase in the probability of quantal release; this nearly synchronous liberation of quanta produces the familiar end-plate potential (e.p.p.). Following the e.p.p. there is a prolonged tail of heightened release probability, manifested by an increase in the number of quanta released by a subsequent action potential (facilitation, Eccles, Katz & Kuffler, 1941; del Castillo & Katz, 1954*b*; Mallart & Martin, 1967), and by an increase in the frequency of m.e.p.p.s (del Castillo & Katz, 1954*b*; Miledi & Thies, 1971; Barrett & Stevens, 1972*b*; Rahamimoff & Yaari, 1973). Under certain experimental conditions (for example, high extracellular Ca, Rotshenker & Rahamimoff, 1970) an increase in the frequency of m.e.p.p.s also follows the occurrence of a single spontaneous m.e.p.p. In this study

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we investigated whether the evoked e.p.p. is also altered following a m.e.p.p. We employed extracellular focal electrodes (del Castillo & Katz, 1956; Katz & Miledi, 1965) to restrict the recording area to a few release sites along the long nerve terminal of the frog neuromuscular junction.

METHODS

Cutaneous pectoris nerve-muscle preparations of the frog *Rana pipiens* were dissected in Ringer solution (NaCl, 115·6 mM; KCl, 2 mM; $CaCl_2$, 1·8 mM) buffered to pH 7·2–7·5 with the Na salts of bicarbonate or phosphate. Isolated preparations were usually treated with a solution of 0·1 % (w/v) collagenase (Sigma, Type 1) in low Ca Ringer for 10–15 min at room temperature to loosen the connective tissue surrounding the end-plate region (Hall & Kelly, 1971; McMahan, Spitzer & Peper, 1972; Betz & Sakmann, 1973). During experiments the Ca concentration of the bathing solution was reduced to 0·25 mM, and 2 mM-Mg was added to keep the e.p.p. subthreshold. The motor nerve was stimulated with brief suprathreshold pulses via a suction electrode. E.p.p.s and m.e.p.p.s were recorded extracellularly from focal junctional regions of superficial fibres using micropipettes filled with 0·25–0·5 M-Ca and 1–2 M-NaCl (resistances 2–15 MΩ). All experiments were performed at room temperature (20–26° C).

When a focus with stable e.p.p.s and a sufficient rate (see below) of high-amplitude ($\geqslant$ 0·25 mV at peak) m.e.p.p.s was located, the stimulating circuit was switched to a mode in which the occurrence of a m.e.p.p. (detected by a capacitor-coupled discriminator) led to stimulation of the motor nerve (see diagram in Fig. 1*A*). The threshold of the m.e.p.p. detector was adjusted to trigger off only the larger extracellular m.e.p.p.s to minimize the likelihood of triggering off random noise fluctuations. The interval between detection of the m.e.p.p. and motor nerve stimulation was set to be alternately short ($\leqslant$ 1 msec) for test trials or long (35–100 msec) for control trials (Fig. 1), on the assumption that any effect of m.e.p.p.s on e.p.p.s would be greatest immediately after the m.e.p.p. At the m.e.p.p. rates seen here (0·06–3·3/sec, Table 1), very few trials showed m.e.p.p.s intervening between the triggering m.e.p.p. and the control e.p.p. Following each successful detection, the discriminator was inactivated for 8 sec to impose a minimal delay (dead time) between successive nerve stimulations. Since both test and control stimuli were time-locked to m.e.p.p.s, the interval between the stimuli fluctuated randomly (with a minimum of 8 sec), so that neither test nor control e.p.p.s were *selectively* depressed by the long-lasting effects of preceding stimulation (Takeuchi, 1958; Thies, 1965; Betz, 1970).

M.e.p.p.s and e.p.p.s were recorded at 7½ inches/sec on one channel of a FM tape recorder, and the pulse from the m.e.p.p. detector on another channel. Yet another channel was marked with a late pulse, occurring several hundred msec following detection of the m.e.p.p. By playing the tape backwards and using this late pulse to trigger an oscilloscope and an averaging computer, we could see the full time course of the (averaged) triggering m.e.p.p. on single and multiple trials. Such backwards records are shown photographically reversed in Figs. 1*B* and 2, so that time runs in the conventional left-to-right direction.

Test trials were summed separately from control trials. Thus, one summed record showed the test e.p.p. superimposed on the triggering m.e.p.p., while the other summed record showed the triggering m.e.p.p. followed 35–100 msec later by the control e.p.p. (Fig. 2). Paired records were summed over identical regions of tape to control for drift. The tail of the m.e.p.p. in the control record was used to extrapolate the tail of the m.e.p.p. in the test record (dashed line in test records of Fig. 2),

and the peak amplitudes and areas (measured with a polar planimeter) of the test and control e.p.p.s were then compared.

Data included in this study met the following criteria: (1) at least fifty test-control pairs (100 triggering m.e.p.p.s) in an hour, (2) reasonably stable e.p.p. amplitudes, and (3) no more than 12% of the trials triggered off random noise fluctuations instead of m.e.p.p.s. In sixteen of the twenty-three series in Table 1, noise triggered

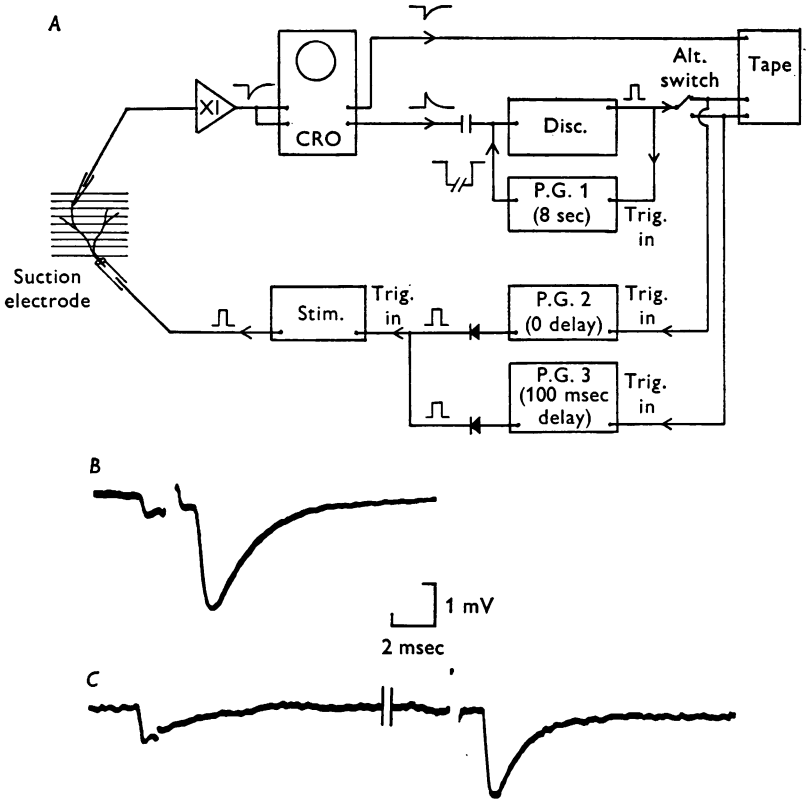

Fig. 1. *A*, schematic diagram of circuitry used to generate test and control trials following m.e.p.p. detection. X1, unity-gain preamplifier; CRO, cathode-ray oscilloscope; Disc., capacity-coupled m.e.p.p. discriminator; P.G., pulse generator. Test (0 delay between m.e.p.p. detection and nerve stimulation) and control (100 msec delay) trials alternated (Alt. switch) at a minimum interval of 8 sec; this interval was determined by inactivation of Disc. by P.G.1. The delay on P.G. 3 was actually 35, 40 or 100 msec in different experiments (Table 1).

B, sample test extracellular e.p.p. Calibrations for *B* and *C*: 1 mV, 2 msec.

C, sample control extracellular e.p.p. Rising phase of control e.p.p. retouched for clarity. The break on the decaying phase of the m.e.p.p. is an artifact produced by discharge of the m.e.p.p. discriminator. Note the discontinuity in trace between the m.e.p.p. and the control e.p.p., the interruption representing 85 msec.

fewer than 5% of the trials. False triggering was most accurately detected by playing the taped records of control trials backwards (see above). False triggering estimates based on test trials were less reliable because the stimulus artifact and e.p.p. were superimposed on the m.e.p.p., but these estimates usually agreed within a few percent with estimates based on control records.

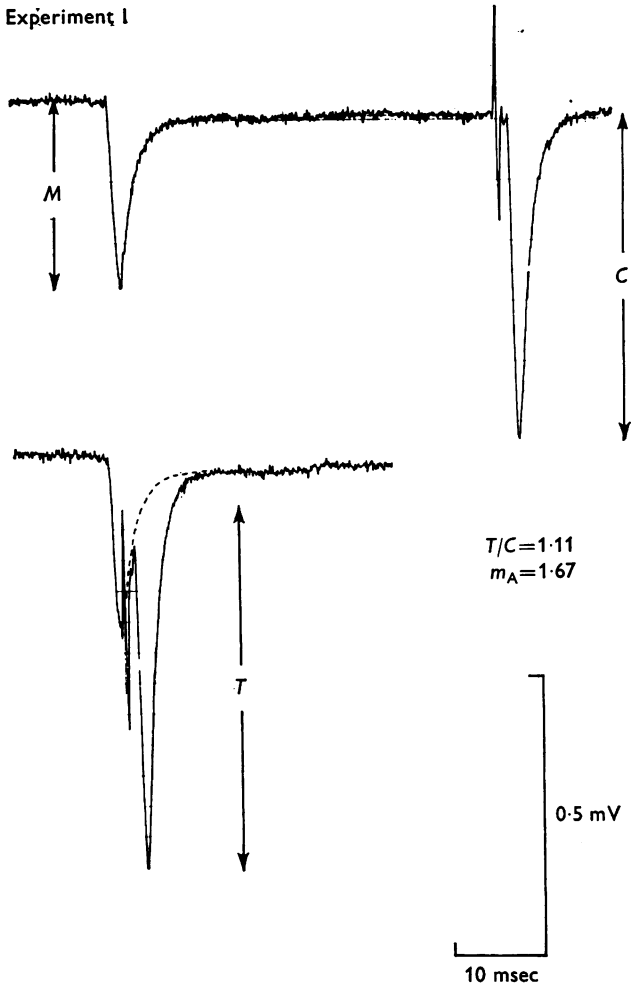

Fig. 2. For legend see facing page.

The average quantal content of the control e.p.p. was estimated as the ratio m_A, of the peak amplitudes of the averaged control e.p.p. and the averaged triggering m.e.p.p. (corrected for false triggering). Since the triggering mechanism selected large m.e.p.p.s, m_A probably under-estimated the true average quantal content slightly. Response failures detected in twelve series allowed an independent estimate of quantal content, m_F, the natural logarithm of the ratio of total trials to response failures (del Castillo & Katz, 1954*a*). In most cases m_F agreed quite well with m_A,

suggesting that in these twelve series the triggering m.e.p.p.s were not significantly larger than the average m.e.p.p. amplitude at the focus. Failures were not detected in the other eleven series, since quantal contents were high.

Mean m.e.p.p. rates were estimated by counting all distinguishable m.e.p.p.s occurring in a 1–2 sec interval during the latter half of each 8 sec dead time. These counts probably included some m.e.p.p.s too small to trigger the stimulator.

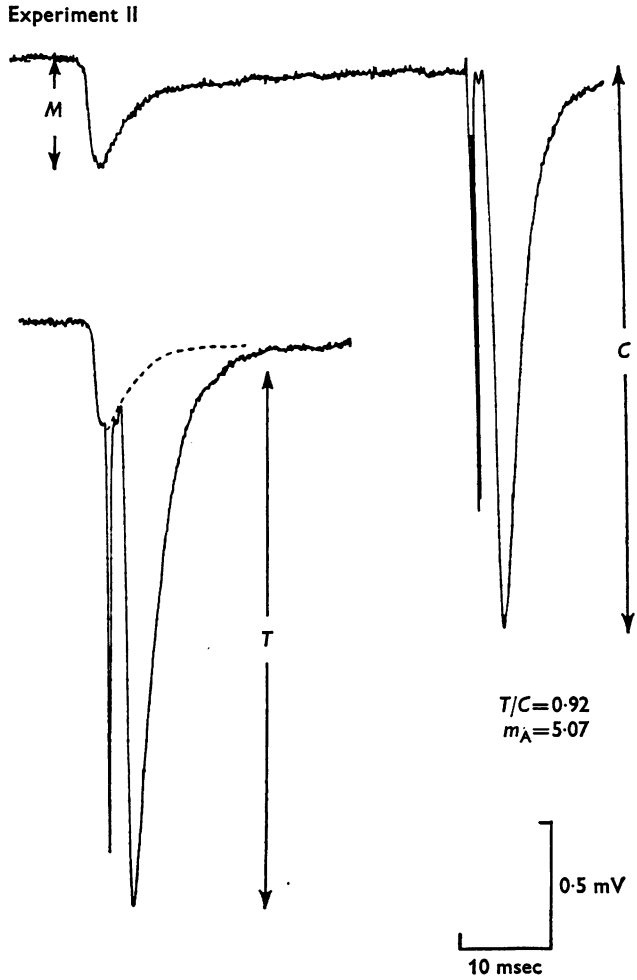

Fig. 2. Averaged test and control e.p.p.s from two extracellular foci, I from 110 paired trials at focus D1b (Table 1), II from seventy-five paired trials at focus F1a. *T*, *C* and *M* represent the averaged amplitudes of the test e.p.p., control e.p.p. and triggering m.e.p.p., respectively. The averaged triggering m.e.p.p. from control trials was used to estimate the contribution of the triggering m.e.p.p. to test records (dashed lines in test records). *T*/*C* is the ratio of test and control e.p.p. amplitudes; m_A is a quantal content estimate obtained from the ratio *C*/*M*. Calibrations: 0·5 mV, 10 msec.

RESULTS

E.p.p. amplitude ratios: Variation with average quantal content

Fig. 2 illustrates averaged records from two experiments in which test e.p.p.s (evoked less than 5 msec following a spontaneous m.e.p.p.) were compared with control e.p.p.s (evoked about 40 msec following the m.e.p.p.). In Experiment I (preparation D1b of Table 1) the averaged test e.p.p. had a peak amplitude about 11% larger than that of the averaged control e.p.p., while in Experiment II (preparation F1a of

TABLE 1. Comparison of e.p.p.s evoked at short (test) and long (control) intervals after m.e.p.p.s. Data are arranged in order of decreasing test/control e.p.p. amplitude ratios. Capital letters A–K designate different preparations; where present, numbers 1–4 indicate different extracellular foci in the same preparation. At the five foci where more than 150 paired trials were collected, data were analysed in two parts, labelled *a* and *b*. E.p.p. amplitudes, average quantal contents m_A and m_F, and m.e.p.p. rates were measured as described in Methods. E.p.p. areas were measured over comparable 7–20 msec periods with a polar planimeter. * Indicates focis howing significant m.e.p.p. bursting (see text). The approximate interval between the triggering m.e.p.p. and the control e.p.p. was 35 msec in H, K; 40 msec in A, D, F; 100 msec in B, C, E, G, I, J.

Preparation	No. paired trials	Test/control e.p.p. ratios		Average quantal content		Mean m.e.p.p. rate, sec^{-1}
		Amplitude	Area	m_A	m_F	
A1	70	1·19	1·15	2·72	—	0·52
B	75	1·17	1·21	1·12	1·37	0·88
C1	70	1·14	1·17	0·95	1·82	1·69
D1b	110	1·11	1·05	1·67	1·48	0·24
D1a	100	1·10	1·06	2·71	2·81	0·15
C2a	75	1·10	1·22	1·98	2·01	0·61
D2	60	1·095	1·08	1·83	2·23	0·44*
C2b	75	1·07	1·07	1·38	0·82	0·34
E	50	1·06	1·195	1·31	2·12	0·145
A2	80	1·06	1·06	3·56	—	0·83
F1b	75	1·03	1·02	4·59	—	3·26*
G	115	1·03	1·05	2·49	—	1·03*
H	70	1·015	1·04	1·16	0·99	0·43*
I	70	1·01	0·96	2·59	2·64	0·096
F2b	100	1·00	0·955	2·36	—	0·53*
F3	90	0·985	1·01	2·34	—	2·54*
F4a	75	0·98	0·95	3·98	—	2·13*
J	100	0·97	1·00	4·21	—	0·505
D3	80	0·97	0·99	4·17	3·64	0·06
F2a	120	0·97	0·95	4·28	—	0·67
K	84	0·97	0·93	2·47	2·64	0·41
F4b	74	0·95	0·98	2·90	—	1·25*
F1a	75	0·92	0·955	5·07	—	2·23*
Mean ± S.D.	—	1·04 ± 0·07	1·05 ± 0·09	—	—	0·91 ± 0·87

Table 1) the averaged test e.p.p. amplitude was about 8% smaller than the control e.p.p. amplitude. Table 1 summarizes results from twenty-three experiments in eleven preparations. Test/control e.p.p. amplitude ratios ranged from 0·92 to 1·19, with a mean of 1·04. Test/control area ratios usually agreed within ±5% with the corresponding peak amplitude ratio ($r = +0{\cdot}83$), and exhibited a range of 0·93 to 1·22 (mean 1·05). Neither mean ratio is significantly different from 1.

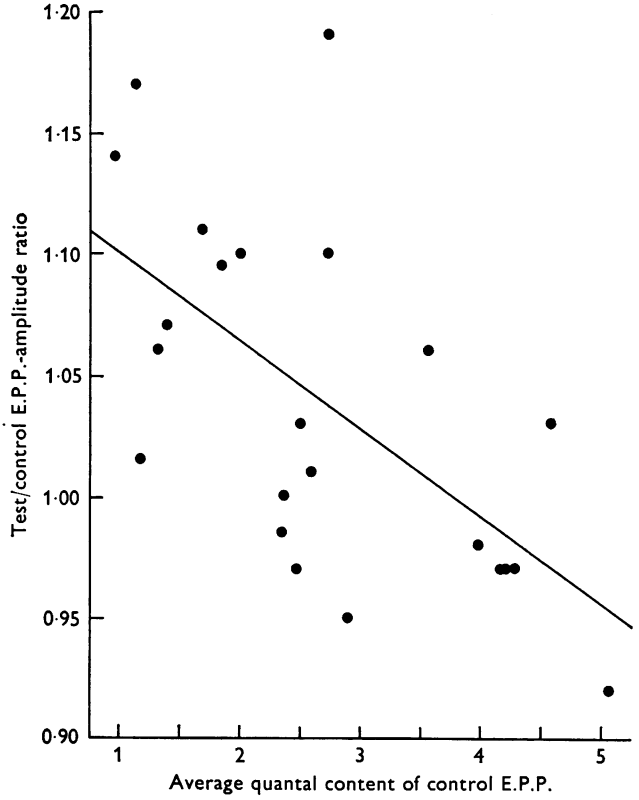

Fig. 3. Variation of test/control e.p.p. amplitude ratio with control average quantal content, m_A, for the 23 series of Table 1. Calculated regression line has slope −0·037, intercept 1·138; $r = -0{\cdot}62$, $P < 0{\cdot}05$. The regression line is included merely to emphasize the direction of the relationship between the amplitude ratio and quantal content; it is not meant to imply that this relationship is linear.

Taken as a group then, these amplitude and area ratios suggest that the occurrence of a m.e.p.p. has no significant effect on the magnitude of subsequent e.p.p.s. However, we noted that the twelve experiments with control average quantal contents (m_A) below 2·5 had a higher mean test/

control amplitude ratio (1·06 ± 0·02 S.E.) than the eleven experiments with control quantal contents exceeding 2·5 (1·01 ± 0·02 S.E.). In order to determine whether the test/control amplitude ratio varied with the level of release, this ratio was plotted against m_A. Fig. 3 indicates a significant negative relationship between m_A and the test/control amplitude ratio ($r = -0{\cdot}62$, $P < 0{\cdot}05$). The relationship between m_A and the test/control area ratio is similarly significant. Thus, at quantal contents below 2, the test e.p.p. tends to be facilitated after a m.e.p.p. released at the same location (Fig. 2, Expt. I), but at higher quantal contents the facilitation either diminishes or disappears. At the highest quantal contents studied here (4–5), the test e.p.p. may even be slightly depressed after the preceding m.e.p.p. (Fig. 2, Expt. II). Thus, some (but certainly not all) of the variability in the test/control e.p.p. ratios can be related to the observed variation in average quantal content.

In eight of the data series of Table 1, the interval between the triggering m.e.p.p. and the control e.p.p. was 100 msec, while in the remaining fifteen series this interval was 35–40 msec (see legend). For the eight 100 msec series the average test/control e.p.p. amplitude ratio was high (1·07), and this ratio was steeply related to and highly correlated with the quantal content of the control e.p.p. ($r = 0{\cdot}85$, $P < 0{\cdot}05$). The fifteen series with a m.e.p.p.-control e.p.p. interval of only 35–40 msec gave a lower average test/control amplitude ratio (1·02), which was much less steeply and significantly related to control quantal content ($r = -0{\cdot}44$). Differences in this direction would be expected if m.e.p.p.s were followed by a facilitation in the presynaptic terminal lasting more than 40 msec: a control e.p.p. evoked 35–40 msec after a m.e.p.p. might still be facilitated, and thus exhibit relatively little difference from the test e.p.p. (hence a low test/control amplitude ratio), while a control e.p.p. evoked 100 msec after a m.e.p.p. would be less facilitated, and thus allow a higher test/control amplitude ratio. However, the control quantal content of the 100 msec series averaged 2, while that of the 35–40 msec series averaged 3, a difference which could obviously contribute to the observed difference in average test/control amplitude ratio (Fig. 3).

Spontaneous m.e.p.p.s: bursting

Because trials were initiated by m.e.p.p.s, and because it was difficult to obtain stable recordings from a single focus for more than an hour, this study selected for rather high m.e.p.p. rates. Mean m.e.p.p. rates ranged from 0·06 to 3·3/sec, higher than those reported previously for extracellular foci (about 0·01–0·1/sec, del Castillo & Katz, 1956). M.e.p.p. rates were possibly elevated by the hypertonic NaCl-$CaCl_2$ solution in the recording pipette (Fatt & Katz, 1952; Furshpan, 1956; Blioch, Glagoleva,

Liberman & Nenashev, 1968), or by mechanical damage to the nerve terminals. Test/control e.p.p. amplitude ratios were not significantly correlated with m.e.p.p. rate ($r = 0{\cdot}24$, $P > 0{\cdot}05$), nor was m.e.p.p. rate significantly correlated with average quantal content, m_A ($r = +0{\cdot}39$, $P > 0{\cdot}05$).

In several preparations m.e.p.p.s tended to occur in bursts (*, Table 1); a frequency histogram of m.e.p.p. counts per interval (see Methods) showed significant disagreement from that predicted for a random Poisson process. Such bursts could reflect mechanical damage to the nerve terminal: high m.e.p.p. rates and bursting were most prominent in preparation F (Table 1), in which collagenase pretreatment was omitted, perhaps because more pressure was required to push the pipette through the connective tissue ensheathing the junctional region. Such bursts could also be related to the high Ca concentration under the pipette tip (Rotshenker & Rahamimoff, 1970; Dennis, Harris & Kuffler, 1971).

DISCUSSION

This study demonstrates that an extracellular e.p.p. evoked immediately after the occurrence of a spontaneous extracellular m.e.p.p. is facilitated, and that the percentage facilitation is negatively correlated with the quantal content of the e.p.p. This facilitation of evoked release is similar to Rotshenker & Rahamimoff's (1970) finding that in high Ca solutions the probability of 'spontaneous' m.e.p.p. occurrence is increased following a 'conditioning' m.e.p.p. It is still uncertain whether this post-m.e.p.p. facilitation of e.p.p.s and m.e.p.p.s is related to events preceding or following the conditioning m.e.p.p.; *i.e.* does the occurrence of the m.e.p.p. merely signal a temporary state of facilitation within the nerve terminal, or does the occurrence of the m.e.p.p. itself produce this facilitated state? One argument against the latter idea is that the quanta released during the e.p.p. do not interact (Barrett & Stevens, 1972*a*).

The variation of post-m.e.p.p. facilitation with quantal content shown in Fig. 3 is reminiscent of reports by Mallart & Martin (1968) and Rahamimoff (1968) that the magnitude of two-pulse facilitation is also negatively correlated with the average quantal content of the e.p.p. In both these earlier studies, facilitation decreased more or less exponentially with increasing quantal content of the *conditioning* e.p.p. However, the conditions in the present experiments are somewhat different. The 'quantal content' of the conditioning event is always unity. The relation in Fig. 3 is between facilitation and quantal content of the *control* e.p.p. Because we used focal extracellular recording techniques, the evoked quantal contents reported here should be multiplied by 10–100 times to be

comparable to the intracellularly recorded quantal contents in these previous studies.

Numerous studies have demonstrated that Ca ion is important in many aspects of quantal release. Evoked release increases steeply with the external Ca concentration (Jenkinson, 1957; Dodge & Rahamimoff, 1967), two-pulse facilitation occurs only if Ca is available during the conditioning depolarization (Katz & Miledi, 1968), and m.e.p.p.s appear to facilitate subsequent m.e.p.p.s only in high Ca solutions (Rotshenker & Rahamimoff, 1970). Thus it is tempting to interpret the present results in terms of the Ca hypothesis of transmitter release (Katz & Miledi, 1970; see also Rahamimoff, 1968; Barrett & Stevens, 1972*b*) as follows: Suppose that the occurrence of a m.e.p.p. reflects a transient increase, Δ Ca, in the Ca concentration at local release sites. Suppose further that the quantal content of the e.p.p. is positively related to the amount of Ca at such sites. If an amount Ca_{AP} enters during the action potential, then this amount will give rise to the control e.p.p., and likewise an amount ($Ca_{AP} + \Delta$ Ca) will give rise to the test e.p.p. Thus, facilitation would be a function of the factor ($1 + \Delta$ Ca/Ca_{AP}). Such a simple scheme would partially explain the data of Fig. 3: as Ca_{AP} (and thus the quantal content of the e.p.p.) decreases, the ratio (Δ Ca/Ca_{AP}) increases, and the facilitation of e.p.p.s following m.e.p.p.s increases. Of course, many other schemes would also explain these limited data.

The post-m.e.p.p. facilitation of e.p.p.s reported here is so small ($\leqslant 20\%$) that it cannot be detected in intracellular recordings (Y. Yaari & R. Rahamimoff, unpublished observations), and with present techniques it is difficult to record a sufficient number of m.e.p.p.-triggered trials from single extracellular foci. Hopefully, improved experimental conditions allowing extensive data collection at foci with lower and more stable quantal contents will increase the magnitude and reduce the variability of this facilitation and allow accurate study of its time course and mechanism.

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