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FACTORS AFFECTING THE RATE OF INCORPORATION OF A FALSE TRANSMITTER INTO MAMMALIAN MOTOR NERVE TERMINALS

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SUMMARY

1. The incorporation of acetylmonoethylcholine (AMECh) into the transmitter store at the mammalian neuromuscular junction has been studied using electrophysiological techniques.

2. Incubation of rat muscle in the presence of 0·1 mM-monoethylcholine (MECh) and 40 mM-K^+ for 60–90 min produced a maximal reduction in the time constant of decay of synaptic currents and potentials, indicating that acetylcholine (ACh) had been replaced by AMECh in the released quanta.

3. Under resting conditions, muscles incubated for up to 24 hr in the presence of MECh showed no incorporation of AMECh into the released transmitter. In contrast, muscles pre-loaded with AMECh and then incubated in choline-containing medium showed a substantial reversion to ACh in the released transmitter within 4 hr.

4. It is suggested that this difference results from the rate of synthesis or packaging of transmitter being considerably slower with MECh than with choline, so that stimulation in the presence of MECh causes an over-all depletion of transmitter stores that does not occur with choline as the precursor. Measurements of m.e.p.c. amplitude following K^+-evoked release in the presence of MECh or choline confirmed this interpretation.

5. In order to test whether newly formed AMECh is incorporated into a single homogeneous pool of transmitter from which the released quanta are derived, the rate of incorporation of AMECh into the released transmitter was measured as a function of the number of quanta released when transmitter output was increased by various methods.

6. The number of quanta released before 63 % conversion of the released transmitter to AMECh was brought about varied from $1{\cdot}3 \times 10^5$ (nerve stimulation at 3 Hz) to about 4×10^5 (release by high-K^+ solution). With nerve stimulation in Mg^{2+}-blocked muscles the value was $2{\cdot}5 \times 10^5$.

7. Incorporation of AMECh into the quanta released by nerve stimulation appeared to take place more rapidly than its incorporation into spontaneously released quanta.

8. These results are discussed in terms of the compartmentation of transmitter stores in the nerve terminals.

INTRODUCTION

The present study was initiated in order to obtain further information about acetylcholine (ACh) metabolism at the neuromuscular junction. In a recent paper we have demonstrated that stimulation of motor nerves of isolated neuromuscular preparations in the presence of the choline analogue monoethylcholine (MECh) leads to a reduction in the amplitude and duration of miniature end-plate currents (m.e.p.c.s), the latter being evident as a reduced time constant of decay (τ) of the m.e.p.c.s (Colquhoun, Large & Rang, 1977). After an appropriate amount of nerve stimulation in the presence of MECh, $\tau_{m.e.p.c.}$ was depressed to 0·58 of the control value, which was in good agreement with the value of 0·56 found for the ratio of mean channel lifetimes of acetylmonoethylcholine (AMECh) and ACh, determined by noise analysis. It was concluded that when transmitter turnover was increased in the presence of MECh, this compound was taken up and acetylated by the presynaptic nerve terminals and was released spontaneously and in response to nerve stimulation. This previous work suggested an approach to study transmitter synthesis at the neuromuscular junction in which the change in the time course of synaptic currents or potentials could be used to determine the relative amounts of ACh and AMECh released spontaneously and by nerve stimulation at various stages of the incorporation of AMECh into the transmitter pool.

The first objective was to see if AMECh could be incorporated into the transmitter store when resting muscles were incubated in MECh and also whether muscles, preloaded with AMECh, would revert to synthesizing ACh on exposure to solutions containing choline. Such experiments might reveal differences in the ability of cholinergic nerves to take up and acetylate MECh in comparison to choline. Collier, Barker & Mittag (1976) found that stimulation of the cat superior cervical ganglion in the presence of either [^{3}H]choline or [^{3}H]MECh produced similar amounts of the acetylated derivatives, suggesting that MECh is as good a substrate as choline for choline acetyltransferase, even though *in vitro* studies have shown that the K_m value of MECh as a substrate of choline acetyltransferase is 7–8 times that of choline (Barker & Mittag, 1975).

The second objective was to study the effect of increasing transmitter turnover by nerve stimulation or by raising the K^+ concentration of the bathing solution on the incorporation of AMECh into the transmitter pool. From this type of experiment it was hoped to be able to calculate the normal size of the transmitter store at the neuromuscular junction. Using hemicholinium (HC-3), a compound which inhibits choline uptake, Elmqvist & Quastel (1965) found that the equivalent of 271,000 full quanta were released before the transmitter stores were exhausted. If newly synthesized AMECh is uniformly distributed throughout the releasable transmitter pool, measurement of the rate of replacement of ACh by AMECh should enable the size of the transmitter store to be calculated, and a similar value of store size should be obtained irrespective of the method employed to evoke transmitter release.

Biochemical experiments on the superior cervical ganglion (Collier, 1969) and the rat diaphragm (Potter, 1970) have suggested, however, that labelled choline does not become uniformly distributed among the total releasable ACh stores; instead there appears to be a smaller pool of readily releasable transmitter which becomes labelled

more rapidly than the rest. If newly synthesized AMECh were also incorporated into this rapidly exchanging pool, we would expect estimates of store size obtained by following AMECh incorporation to be smaller than the estimate obtained by Elmqvist & Quastel (1965).

The results of our experiments show that the apparent size of the transmitter pool into which newly synthesized AMECh is incorporated varies quite markedly according to the procedure used to evoke transmitter release. In curarized muscle AMECh is incorporated rapidly during nerve stimulation and the estimated size of the rapidly exchangeable pool is about 130,000 quanta. In Mg^{2+}-blocked muscle during nerve stimulation, AMECh is incorporated much less readily and the size of the exchangeable pool is about 250,000 quanta, similar to that estimated by Elmqvist & Quastel (1965). With release evoked by increased K^+ the exchange is even slower, and the estimated size of the exchangeable pool is even greater.

METHODS

All experiments were carried out on the rat phrenic nerve-diaphragm preparation. Experimental details have been described previously (Colquhoun *et al.* 1977). Briefly, the preparations were pinned on to a bed of Sylgard resin and bathed with Krebs solution flowing at a rate of about 2 ml./min in a total bath volume of 2 ml. Normal Krebs solution contained (mM): NaCl 119, KCl 4·7, $CaCl_2$ 2·5, $MgSO_4$ 1·2, KH_2PO_4 1·2, $NaHCO_3$ 25, glucose 11, and was bubbled with 5% CO_2–95% O_2. In those experiments where either K^+ or Mg^{2+} concentration was increased, $MgCl_2$ or KCl was added without any reduction in the NaCl concentration. When muscle contraction was blocked by tubocurarine, a concentration of 1–2 μM-(+)-tubocurarine chloride was used. Experiments were carried out at 20 °C.

Electrophysiological recordings. In most experiments a two-electrode voltage clamp technique was used for recording m.e.p.c.s or end-plate currents (e.p.c.s). In all cases the intracellular micro-electrodes were filled with 3 M-KCl and usually had resistances of 5–10 MΩ. In some experiments, where prolonged impalement of muscle cells was required, 10–15 MΩ electrodes were used.

For recording m.e.p.c.s or end-plate potentials (e.p.p.s) in curarized or Mg^{2+}-blocked muscles, a conventional bath electrode (Ag/AgCl embedded in Agar) was used as a reference electrode, but for recording e.p.c.s in unblocked muscles a voltage follower with a differential input was employed. In this case the reference electrode was a micropipette, of similar characteristics to the voltage electrode, placed just over the cell being studied. This was done to eliminate interference from the gross extracellular field potential originating from the summed action potentials of the neighbouring fibres. All signals were stored on an FM tape recorder (Racal Store 4) and subsequently measurements were made by capturing the synaptic potentials or currents by means of a transient recorder (Datalab DL 901). The τ values were estimated by matching the signal, displayed on one beam of a twin-beam oscilloscope, with an exponential curve of variable time constant displayed on the second beam. About 10 m.e.p.c.s were normally recorded from each of 6–8 fibres in succession, and the mean m.e.p.c amplitude and time constant were determined.

E.p.c.s and m.e.p.c.s were recorded at a clamped membrane potential of -80 or -100 mV. The latter membrane potential was used in order to improve the signal to noise ratio in many experiments and should not affect the results of the experiments as the τ values of ACh and AMECh m.e.p.c.s are similarly sensitive to membrane potential (Colquhoun *et al.* 1977).

RESULTS

Time course of normal and false m.e.p.c.s

In a previous paper it was shown that replacement of ACh by AMECh brought about a characteristic shortening of the time constant of decay of m.e.p.c.s ($\tau_{m.e.p.c.}$)

together with a reduction in their amplitude. The maximum reduction in τ that could be achieved by nerve stimulation in the presence of MECh was from 1·84 msec to 1·06 msec, the ratio being 0·58 (Colquhoun *et al.* 1977). In the present studies we have checked to see whether the same degree of shortening occurs when transmitter release is evoked by raised K^+ concentration in the presence of 0·1 mM-MECh, and also whether the false m.e.p.c.s can be restored to normal by evoking transmitter release in the presence of 0·1 mM-choline.

TABLE 1. Exchange of transmitter evoked by raised potassium concentration

A, conversion from normal to false transmitter by evoking release in the presence of MECh

Experiment	MECh concn. (mM)	K^+ concn. (mM)	Time in raised K^+ (min)	Final $\tau_{m.e.p.c.}$ / Initial $\tau_{m.e.p.c.}$
1	0·2	40	120	0·59
2	0·1	40	75	0·56
3	0·1	25	60	0·59

B, conversion from false to normal transmitter. Muscles were soaked in 40 mM-K^+ and 0·1 mM-MECh for 90–100 min, allowed to recover in normal Krebs solution containing 0·1 mM-MECh. M.e.p.c.s were recorded, and the muscles were then transferred to high K^+ solution containing choline as shown

Experiment	Choline concn. (mM)	K^+ concn. (mM)	Time in raised K^+ (min)	Initial $\tau_{m.e.p.c.}$ / Final $\tau_{m.e.p.c.}$
4	0·1	40	90	0·58
5	0·1	40	90	0·57

Over-all mean 0·58 ± 0·01

Table 1 shows that m.e.p.c.s are shortened to the same extent (mean ratio 0·58) as was previously reported, when raised K^+ (to 25 or 40 mM) is used to evoke release. Since 25 mM-K^+ applied for 60 min had the same effect as 40 mM-K^+ applied for 120 min we conclude that the process of exchange had gone to completion in both cases. This is confirmed by the finding (Table 1) that when muscles were intially exposed to 40 mM-K^+ for 90 min in the presence of 0·1 mM-MECh and then, after recording the m.e.p.c.s in Krebs solution containing MECh, exposed to 40 mM-K^+ for 90 min in the presence of 0·1 mM-choline, the m.e.p.c. time course returned fully to normal as judged by the identical ratio of the τ values.

The miniature end-plate potential (m.e.p.p.) frequency is difficult to measure with certainty in K^+ concentrations exceeding 20 mM, but certainly exceeds 500 Hz in the presence of 40 mM-K^+. The number of quanta released during 90 min would therefore exceed 2·5 million. This is about ten times the estimated number of quanta releasable without replenishment by frog motor nerve terminals (Ceccarelli & Hurlbut, 1975) and mammalian motor nerve terminals (Elmqvist & Quastel, 1965) so it is not surprising to find that complete exchange of ACh for AMECh occurred within this period.

In subsequent calculations we have taken the ratio $\tau_{false}/\tau_{normal}$ for m.e.p.c.s to be 0·58. This ratio is too close to unity for synaptic currents generated by a mixture

of ACh and AMECh to be resolved into two exponential components. In calculating the relative contributions of ACh and AMECh at intermediate stages of exchange, the time constant of the best-fitting single exponential was determined, and the fraction of AMECh was assumed to be directly proportional to the observed shortening, i.e. fraction of

$$\text{AMECh} = \frac{1}{0{\cdot}42}\left(1 - \frac{\tau}{\tau_{\text{normal}}}\right).$$

Where prolonged impalements were necessary, e.p.p.s were recorded without a voltage clamp. Because of the passive cable properties of the membrane, the shortening of the e.p.p.s and m.e.p.p.s associated with the conversion from ACh to AMECh was smaller and more variable than that of the underlying synaptic currents. The decay of synaptic potentials is not strictly exponential, but was sufficiently close for the 'template' method to be used with good reproducibility. In a series of experiments where maximal transmitter exchange was brought about by nerve stimulation or raised K^+, the mean ratio $\tau_{\text{false}}/\tau_{\text{normal}}$ for e.p.p.s or m.e.p.p.s was $0{\cdot}74 \pm 0{\cdot}01$ (twenty cells).

Transmitter turnover in resting muscle

A number of experiments were carried out to see whether nerve terminals would release AMECh in the form of spontaneously occurring m.e.p.c.s if the muscles were exposed to MECh without nerve stimulation, and also to see whether the m.e.p.c.s recorded from muscles loaded with AMECh by exposure to 40 mM-K^+ solution in the presence of MECh would spontaneously revert to normal if the muscle was soaked in a choline-containing medium without a further period of K^+ stimulation. The experimental results are summarized in Table 2.

Soaking a normal muscle in Krebs solution containing 0·1 mM-MECh even for as long as 22 hr, did not produce any shortening of the m.e.p.c.s. In these experiments, muscles were placed in 2 l. Krebs solution containing MECh overnight (continuously bubbled with O_2/CO_2 gas mixture). On the following day the tissues were set up in the experimental chamber and m.e.p.c.s were recorded from six to ten cells in the presence of MECh; subsequently 40 mM-K^+ and 0·1 mM-choline or 0·1 mM-MECh was added to the bathing solution for 90 min, after which m.e.p.c.s were again recorded. Two such experiments are shown in Table 2. Addition of 40 mM-K^+ plus 0·1 mM-MECh produced shortening of the m.e.p.c.s, the ratio $\tau_{\text{final}}/\tau_{\text{initial}}$ being 0·61 (i.e. close to value of 0·58 of $\tau_{\text{AMECh}}/\tau_{\text{ACh}}$); in contrast, if choline was present during the K^+ treatment, there was no change in the τ value, showing that ACh and not AMECh was present in the spontaneously released quanta, even after prolonged incubation with MECh. On the other hand, if muscles were first loaded with AMECh by exposing them to 40 mM-K^+ in the presence of MECh for 60–90 min, transferred to normal Krebs solution containing MECh for measurement of the m.e.p.c.s, then transferred to normal Krebs solution containing 0·1 mM-choline, the m.e.p.c.s progressively lengthened towards normal (Fig. 1*B*; Table 3). In six such experiments addition of 0·1 mM-choline for 4 hr caused the mean value of $\tau_{\text{m.e.p.c.}}$ to increase from $0{\cdot}98 \pm 0{\cdot}06$ msec to $1{\cdot}40 \pm 0{\cdot}97$ msec. Since complete replacement of AMECh by ACh lengthens $\tau_{\text{m.e.p.c.}}$ by a factor 1·72, the lengthening occurring during 4 hr incubation in choline solution represents approximately 62 % replacement of AMECh by ACh.

TABLE 2. Effect of overnight incubation in the presence of choline or MECh on the characteristics of m.e.p.c.s

Pre-treatment	Overnight treatment	Initial $\tau_{m.e.p.c.}$ (msec)	Incubation	Final $\tau_{m.e.p.c.}$ (msec)	$\frac{\tau_{final}}{\tau_{initial}}$
—	0·1 mM-MECh	1·58 ± 0·06	40 mM-K^+; 0·1 mM-MECh	0·96 ± 0·04	0·61
—	0·1 mM-MECh	1·50 ± 0·07	40 mM-K^+; 0·1 mM-choline	1·48 ± 0·07	0·98
40 mM-K^+; 0·1 mM-MECh	0·1 mM-choline	1·60 ± 0·07	40 mM-K^+; 0·1 mM-MECh	0·86 ± 0·03	0·54
40 mM-K^+; 0·1 mM-MECh	0·1 mM-choline	1·72 ± 0·05	40 mM-K^+; 0·1 mM-choline	1·77 ± 0·06	1·03

TABLE 3. Transmitter exchange in resting muscle

		Initial			Final			
Pre-treatment	Number of muscles	$\tau_{m.e.p.c.}$ (msec) (*a*)	m.e.p.c. amplitude (nA) (*b*)	Recovery solution	$\tau_{m.e.p.c.}$ (msec) (*c*)	m.e.p.c. amplitude (nA) (*d*)	*c*/*a*	*d*/*b*
MECh	6	0·95 ± 0·04	1·63 ± 0·1	MECh	1·06 ± 0·06	2·23 ± 0·13	1·12 ± 0·05	1·38 ± 0·05
MECh	6	0·98 ± 0·06	1·57 ± 0·08	Choline	1·40 ± 0·07	2·63 ± 0·08	1·44 ± 0·06	1·69 ± 0·07
MECh (overnight)*	6	1·15 ± 0·04	2·02 ± 0·1	Choline	1·23 ± 0·05	2·51 ± 0·12	1·08 ± 0·04	1·25 ± 0·06
MECh	3	1·13 ± 0·05	1·62 ± 0·1	No precursor	1·36 ± 0·03	2·32 ± 0·03	1·21 ± 0·05	1·44 ± 0·1
Choline	3	1·71 ± 0·29	3·40 ± 0·08	MECh	1·84 ± 0·28	2·96 ± 0·11	1·09 ± 0·05	0·87 ± 0·02
Choline	4	1·71 ± 0·11	3·14 ± 0·07	Choline	1·77 ± 0·13	3·53 ± 0·04	1·03 ± 0·02	1·13 ± 0·03

The muscles were initially bathed for 60–90 min in a solution containing 40 mM-K^+ plus the transmitter precursor shown in the first column at 0·1 mM concentration. The K^+ concentration was then reduced to normal and m.e.p.c.s were recorded ('initial' values). The muscles were then transferred for 4–5 hr to the 'recovery solution' containing the transmitter precursor indicated (0·1 mM) and a further series of m.e.p.c. recordings was made ('final' values).

* In these experiments the muscles were first bathed in 40 mM-K^+ plus 0·1 mM-MECh for 90 min, then left in normal Krebs' solution containing 0·1 mM-MECh overnight (20 hr) before the initial m.e.p.c. recordings were made.

If the muscles were incubated for long enough in choline, AMECh was replaced completely by ACh. Table 2 shows the result of two experiments in which, after incorporation of the false transmitter, the tissues were incubated for 18–20 hr in 0·1 mM-choline. After this period of time, the m.e.p.c.s possessed the electrophysiological characteristics of pure ACh quanta, judged by the fact that further treatment with K^+ plus choline produced no increase in the τ values, whereas K^+ plus MECh shortened the m.e.p.c.s, the ratio $\tau_{final}/\tau_{initial}$ being 0·54.

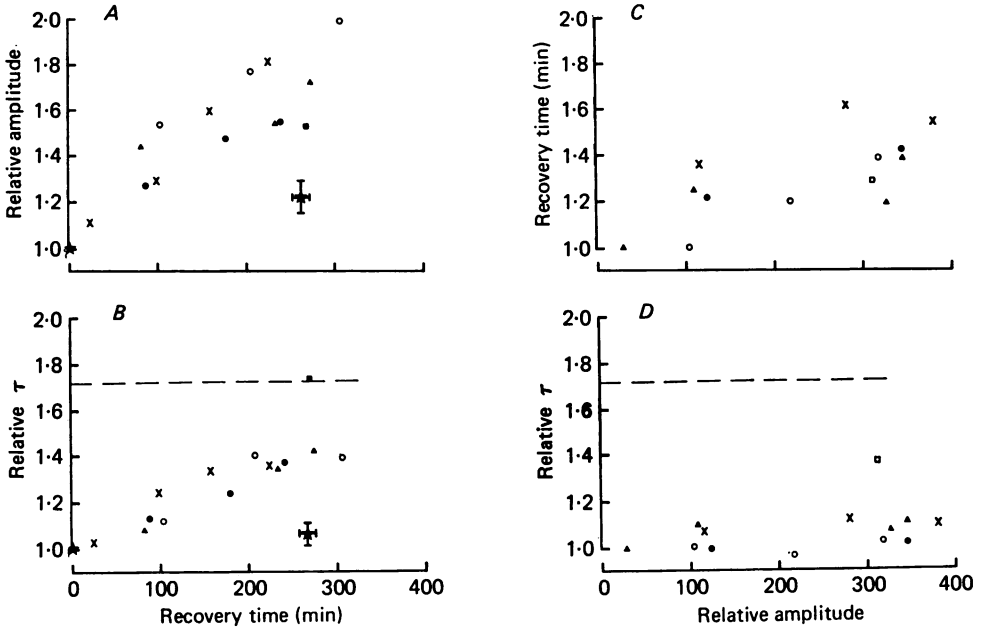

Fig. 1. The effect of choline and MECh on the amplitude and time constant of decay (τ) of m.e.p.c.s recorded from resting muscles loaded with AMECh. Diaphragms were first incubated in 40 mM-K^+ and 0·1 mM-MECh for 90 min and then in normal solution containing 0·1 mM-MECh, at which time control measurements were made. In *A* and *B* MECh was replaced by 0·1 mM-choline at time 0. The symbol with error bars indicates those experiments in which, after the loading procedure, the muscles were bathed for about 22 hr in MECh before choline was added. In *C* and *D* 40 mM-K^+ was removed at time 0 and MECh was present in the bathing solution throughout the experiments. Note that there was an increase in τ (showing the presence of ACh in the released quanta) only when choline was added immediately after the loading procedure (*B*). The different symbols represent separate experiments.

If muscles loaded with AMECh were soaked in a medium containing 0·1 mM-MECh, no appreciable lengthening of the m.e.p.c.s occurred during the subsequent 4 hr (Fig. 1*D*; Table 3). The mean change in τ in six muscles was from 0·95 msec to 1·06 msec; in five of the muscles there was no significant change, and we conclude that no appreciable restoration of ACh stores occurs when the muscle is soaked in MECh at rest. If, after incorporation of AMECh, the muscles were soaked for 4 hr in normal Krebs solution containing neither choline nor MECh, a partial restoration of ACh stores occurred (Table 3), the increase in τ corresponding to a 29 % replacement of AMECh by ACh.

In control experiments in which the initial phase of transmitter exchange by raised K^+ concentration was carried out in the presence of 0·1 mM-choline, so that no incorporation of false transmitter took place, the m.e.p.c.s were not shortened, and a further 4 hr period of incubation in 0·1 mM-choline had no effect on the m.e.p.c. duration (Table 3).

These experiments show that replacement of AMECh by ACh occurs at an appreciable rate in resting muscle exposed to choline and also, though more slowly, when no exogenous choline is supplied. The reverse process does not occur, however, since no detectable incorporation of AMECh occurred in resting muscle. In freshly dissected muscles the m.e.p.p. frequency was $3{\cdot}53 \pm 0{\cdot}66$ Hz (mean ± S.E. of mean from fifty cells in five muscles) and after overnight incubation it had decreased to $1{\cdot}37 \pm 0{\cdot}13$ Hz (forty cells from four muscles). Using the mean of these two values, 2·45 Hz, as the over-all spontaneous rate of release, we calculate that about 176,000 quanta were released over the 20 hr, which is 65 % of the total store size estimated by Elmqvist & Quastel (1965).

The effects of these various manoeuvres on m.e.p.c. amplitude were also studied. In the control experiments where choline was present both during the initial phase of increased turnover and during the later resting period, the mean m.e.p.c. amplitude increased by 13 % during the 4 hr resting period (Table 3). Various authors have reported a decline in m.e.p.p. amplitude following nerve stimulation (Jones & Kwanbunbumpen, 1970) so the small increase seen probably represents the recovery from this effect. In muscles exposed to MECh during both the initial phase of increased turnover and the later 4 hr resting period, a greater (38 %) increase in m.e.p.c. amplitude occurred (Table 3 and Fig. 1*C*). In both cases the change in m.e.p.c. amplitude occurred without any appreciable change in the composition of the transmitter being released, as judged by the m.e.p.c. time course, so it appears that the transmitter content of a single quantum may be depleted by a period of stimulated release to a greater extent when the transmitter is AMECh than when it is ACh, possibly because the maximum rate of transmitter synthesis is lower for AMECh than for ACh.

A lower rate of synthesis of AMECh than of ACh might partly explain the finding that ACh can replace AMECh in resting nerve terminals but not vice versa. If stimulation in the presence of MECh causes not only a replacement of ACh by AMECh but also a decrease in the total transmitter store (because of the slowness of AMECh synthesis), the incorporation of ACh during incubation in choline-containing medium at rest may largely represent the replenishment of the depleted store rather than replacement of AMECh by ACh. To test this possibility we soaked muscles overnight in MECh-containing medium following the usual period of stimulation in the presence of MECh and then measured the effect of 4 hr exposure to 0·1 mM-choline. The result (Table 3 and Fig. 1*A* and *B*) was that little or no replacement of AMECh by ACh occurred, and there was a much smaller increase in m.e.p.c. amplitude than occurred when choline was added a short time after stimulation in the presence of MECh. This suggests that if the preparation is given sufficient time to replenish its transmitter stores fully with AMECh, ACh is not rapidly incorporated into the stores at rest during incubation with choline.

Exchange of transmitter during nerve stimulation and exposure to high-potassium Krebs solution

The rate of transmitter depeletion in hemicholinium-treated rat muscle during nerve stimulation was studied by Elmqvist & Quastel (1965) who found that, after an interval during which no decline in transmitter output occurred, the amplitudes of e.p.p.s and m.e.p.p.s declined exponentially. From these experiments they estimated the total store of releasable transmitter per junction to be equivalent to about $2{\cdot}7 \times 10^5$ quanta. We have measured the time course of AMECh incorporation when transmitter output was increased in the presence of MECh in four different ways, namely (1) nerve stimulation in curarized preparations, (2) nerve stimulation in Mg^{2+} blocked preparations, (3) nerve stimulation in unblocked preparations and (4) exposure to 20 mM-K^+.

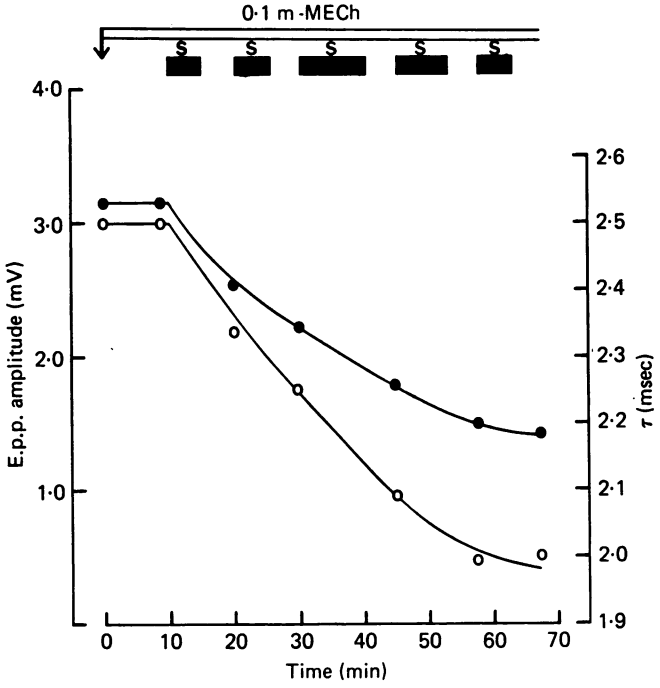

Fig. 2. The effect of MECh on the amplitude (●) and time constant of decay (○) of e.p.p.s in a curarized rat phrenic nerve–diaphragm preparation. At the solid bars marked S the frequency of nerve stimulation was increased from 0·1 to 3 Hz.

Curarized muscles

Muscle fibres were impaled with a single micro-electrode in the region of the fine nerve terminals and were considered acceptable for use in an experiment if the rise time of the e.p.p. was less than 2 msec and if the membrane potential remained close (within 5 mV) to the value obtained on initial penetration (usually -70 to -80 mV). A typical experiment is shown in Fig. 2. In normal Krebs solution, when nerve stimulation was increased from 0·1 to 3 Hz, there was a rapid reduction in the e.p.p. amplitude followed by a somewhat slower decline; after 5 min at 3 Hz the e.p.p. amplitude at 3 Hz was about one third of the value obtained with the lower

stimulation rate. On return to 0·1 Hz nerve stimulation, the e.p.p. amplitude recovered to the pre-stimulation value within 5–15 min. This procedure produced no change in the time constant of decay of the e.p.p. After 0·1 mM-MECh was added to the bathing solution, the effects on the e.p.p. amplitude and time course of successive 5 or 10 min periods of stimulation at 3 Hz were measured. After each period of stimulation at 3 Hz, the frequency was reduced to 0·1 Hz for 2–5 min before the e.p.p.s were measured. This was done because the larger e.p.p.s at 0·1 Hz made measurement of the decay constants more accurate. It can be seen from Fig. 2 that as little as 5 min stimulation at 3 Hz in the presence of 0·1 mM-MECh reduced both the amplitude and time constant of decay of the e.p.p.s, showing that AMECh was being released. The reduction in the e.p.p. duration was half-maximal after about 10 min stimulation and achieved its maximum value after 25–30 min stimulation.

It was of interest to correlate the incorporation of AMECh with the total amount of transmitter released. It is not possible to measure directly the quantal content (m) of e.p.p.s in curarized preparations since the m.e.p.p.s are not discernible, and the method used involved the measurement of e.p.c.s by voltage clamp in unblocked muscles. M.e.p.c.s were recorded from muscle cells at a clamped membrane potential of -80 or -100 mV and then a single e.p.c. was evoked by stimulating the phrenic nerve with a single shock, and the quantal content was calculated from

$$m = \frac{\text{e.p.c. amplitude}}{\text{mean m.e.p.c. amplitude}}.$$

An example of an e.p.c. is shown in Fig. 3 with an m.e.p.c. from the same cell. The rise times of e.p.c.s were always longer than those of m.e.p.c.s, and in one experiment where systematic measurements were made the mean rise time of the m.e.p.c.s was $0{\cdot}37 \pm 0{\cdot}02$ msec (ten cells) compared to a mean of $0{\cdot}72 \pm 0{\cdot}02$ msec for the e.p.c.s. On occasion there was an inflexion in the rising phase of e.p.c.s and these records were not used for calculation of m as it was assumed that there was poor voltage control of the end-plate membrane. The ratio of $\tau_{\text{e.p.c.}}/\tau_{\text{m.e.p.c.}}$ was always close to 1·00. The average quantal content of the e.p.c. evoked by a single stimulus was 134 ± 6 (mean $\pm$ S.E. from sixty-three cells in ten muscles). The amplitude of the e.p.p. in the curarized rat diaphragm muscle does not increase at frequencies lower than 0·1 Hz stimulation, so we conclude that 134 quanta contribute to an e.p.p. at 0·1 Hz. To calculate the quantal output during stimulation at higher frequency we made two assumptions: (1) that the reduction in e.p.p. amplitude seen when increasing nerve stimulation from 0·1 to 3 Hz is due solely to a decrease in quantal content and (2) that neither tubocurarine nor MECh, at the concentrations used, affect the quantal content at 0·1 or 3 Hz. The frequency of spontaneously occurring m.e.p.p.s increases during nerve stimulation (del Castillo & Katz, 1954) but it is impossible to record m.e.p.p.s in curarized muscles; we therefore used a value for m.e.p.p. frequency estimated from Mg^{2+}-blocked preparations. In these experiments stimulation at 10 Hz increased the m.e.p.p. frequency to 30 ± 5 Hz (mean $\pm$ S.E. in twenty cells). This is likely to be an over-estimate for the m.e.p.p. frequency during stimulation at 3 Hz. However in the absence of a more reliable value we have allowed for spontaneous release at 30 quanta per sec in calculating the total transmitter release during stimulation at 3 Hz. The mean release rate in five experiments was

151 ± 5 quanta per sec (Table 4). In the experiment shown in Fig. 2, the half-way stage of the incorporation of AMECh occurred after about 95,000 quanta had been released and there was little further shortening of the e.p.p. after about 250,000 quanta had been released. The results for all of the experiments on curarized muscles are shown in Table 4. In these experiments the fraction of AMECh in the released transmitter increased in a roughly exponential manner when expressed as a function of the total number of quanta released after the addition of MECh to the bathing

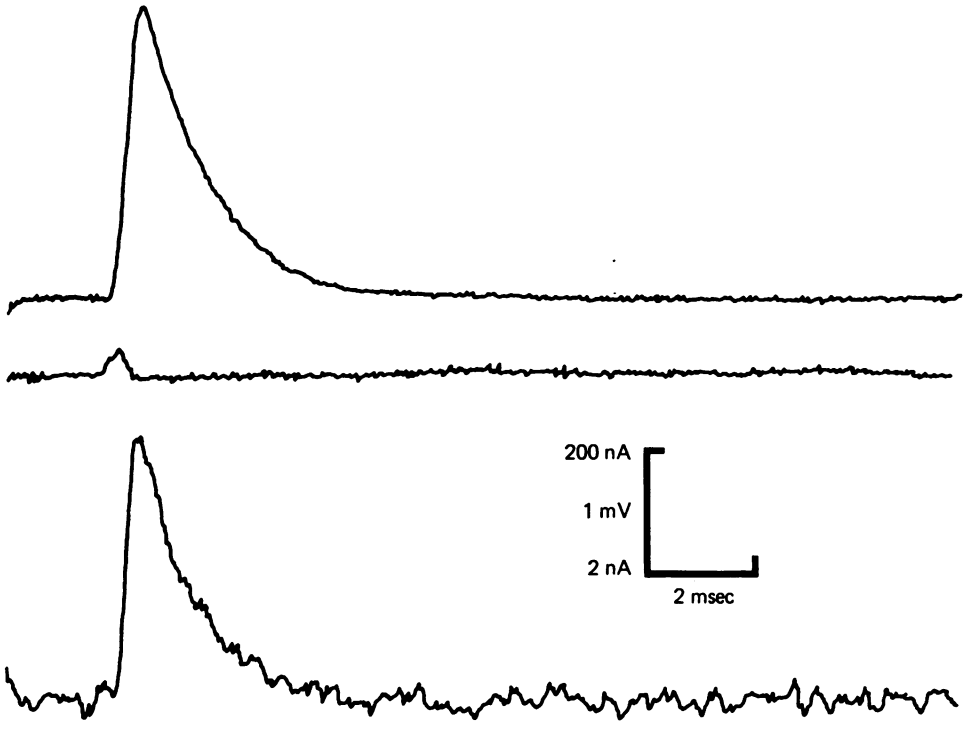

Fig. 3. Examples of an e.p.c. (upper trace) and an m.e.p.c (lower trace) recorded from the same cell. The middle trace shows the voltage control during the recording of the e.p.c. Inward current is represented as an upward deflexion.

solution. This could be interpreted in terms of a model in which the newly synthesized transmitter consists entirely of AMECh which is incorporated into a pool consisting of a fixed number of quanta of releasable transmitter. The number of quanta in this pool would be given by the number of quanta which have to be released in order to bring the fraction of AMECh to 0·63 $(1 - 1/e)$. The mean size of the releaseable pool calculated in this way is $133{,}000 \pm 20{,}000$ (five experiments). If this value does represent the total transmitter store size, then the same value should be found irrespective of the conditions under which release is evoked.

Mg^{2+}-blocked muscles

At the neuromuscular junction transmitter release can be depressed by elevating the Mg^{2+} concentration or lowering the Ca^{2+} concentration. It has been demonstrated in sympathetic ganglia (Collier & Ilson, 1977) and in the guinea-pig myenteric plexus (Pert & Snyder, 1974) that choline uptake during stimulation is a Ca^{2+}-dependent

process, and in addition Collier & Ilson (1977) demonstrated that high Mg^{2+} concentration does not reduce choline uptake. We therefore used high Mg^{2+} rather than low Ca^{2+} to depress transmitter release in order to avoid any possible interference with choline uptake. It was usually necessary to increase the Mg^{2+} concentration to 20 mM to block the muscle twitch, and this depressed the amplitude of m.e.p.p.s to between

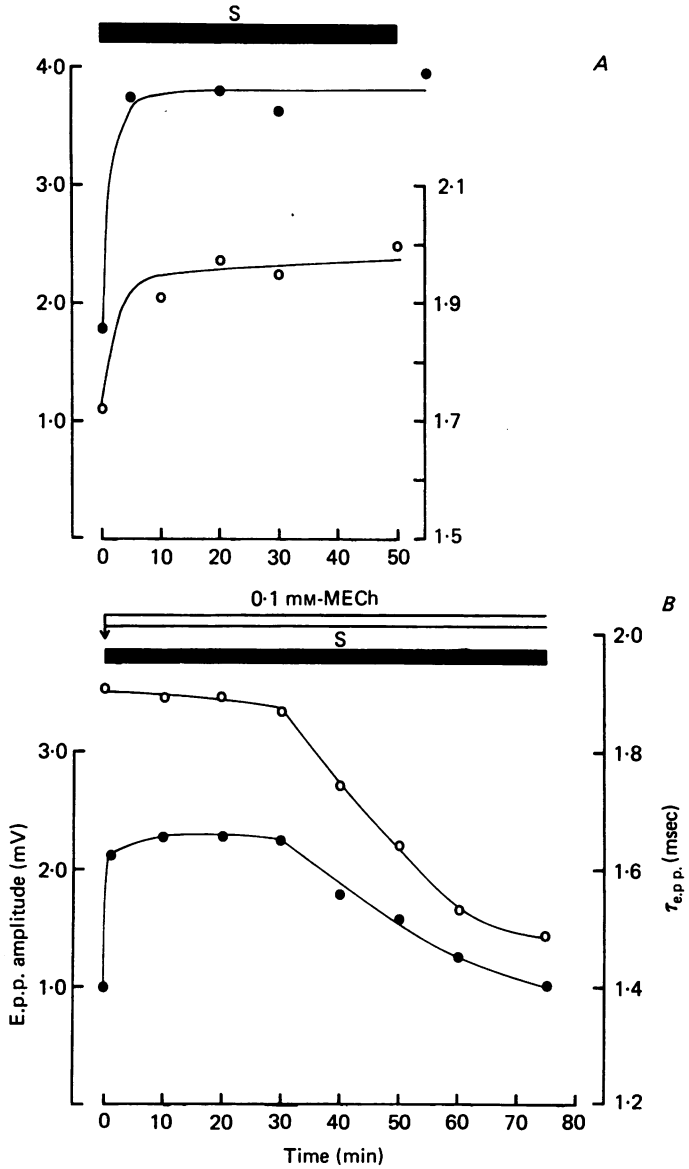

Fig. 4. The effect of nerve stimulation in the absence (*A*) and in the presence (*B*) of MECh on the amplitude (●) and time constant of decay (○) of e.p.p.s in an Mg^{2+}-blocked diaphragm. At the bars marked S the frequency of nerve stimulation was increased from 0·1 to 10 Hz. Note that there was no reduction in the amplitude or τ during the initial 30 min period of 10 Hz nerve stimulation in the presence of MECh.

0·2 and 0·5 mV and, as it was desirable to measure the quantal content of the e.p.p.s directly, cells with relatively large m.e.p.p.s were selected for the experiments. Nerve stimulation at 10 Hz achieved a release rate of 141 ± 17 quanta sec^{-1} (mean ± S.E. in eleven cells, see Table 4) which was close to the rate of release in curarized muscle stimulated at 3 Hz.

A control experiment in which the phrenic nerve was stimulated at 10 Hz in Krebs solution containing 20 mM-Mg^{2+} (no MECh present) is shown in Fig. 4*A*. When the frequency of nerve stimulation was increased from 0·1 to 10 Hz the e.p.p. amplitude increased from 1·8 to 3·8 mV within 5 min owing to facilitation (del Castillo & Katz, 1954).

In some control experiments slight lengthening of the e.p.p. occurred during stimulation, and occasionally there was a small decrease (10 % or less) in the m.e.p.p amplitude. These effects were small and inconsistent, and were not taken into account in measuring the effect of MECh.

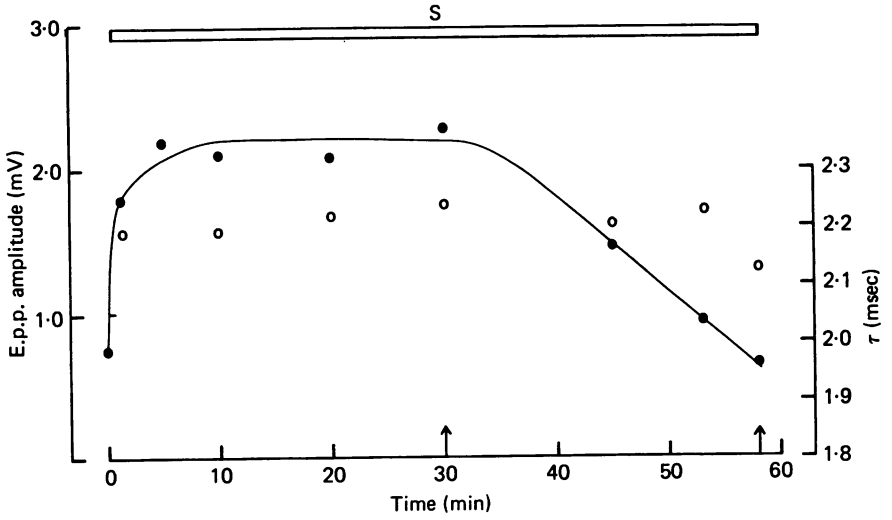

Fig. 5. The effect of hemicholinium on the amplitude (●) and time constant of decay (○) of e.p.p.s in an Mg^{2+}-blocked preparation. 10^{-5} M-HC-3 was present throughout the experiment and at the bar marked S the frequency of nerve stimulation was increased from 0·1 to 10 Hz. At the first arrow, representing the point at which the amplitude began to decline, 179,000 quanta had been released and at the second arrow, representing 63 % decline, 346,000 quanta had been released.

The results of an experiment in which 0·1 mM-MECh was added to the bathing solution is shown in Fig. 4*B*. After the initial increase in e.p.p. amplitude, caused by facilitation when the frequency was increased to 10 Hz, there was no change in the e.p.p. amplitude or time course for about 30 min, indicating that no AMECh was released. After this plateau period there was a progressive reduction in both the amplitude and duration of e.p.p.s and incorporation of AMECh appeared to be near completion after 300,000 quanta had been released. In ten out of eleven such experiments there was a marked lag between starting stimulation at 10 Hz and a reduction in $\tau_{e.p.p.}$ or e.p.p. amplitude, and we carried out three experiments to see if this feature was apparent when ACh synthesis was inhibited by 10^{-5} M-HC-3 rather than

MECh. The results of one of these experiments is shown in Fig. 5, and it can be seen, as with MECh, that there was a long plateau during which 179,000 quanta were released before the amplitude of the e.p.p. was reduced. Consequently the lag period appears to be a characteristic of the experimental conditions used, since in the experiments on curarized muscles there was usually an immediate incorporation of AMECh, as seen from a reduction in the $\tau_{e.p.p.}$ soon after 3 Hz nerve stimulation commenced (sometimes within 1 min; see Fig. 1 of Colquhoun *et al.* 1977). One explanation for the 'lag period' is that there might be a significant pool of intraneuronal choline, which permits ACh synthesis to continue for an appreciable period while the muscle is soaked in MECh. We tested this possibility by incubating resting muscles in 0·1 mM-MECh for 4 hr prior to nerve stimulation in order to replace any choline by MECh. In three such experiments there was still a marked lag between the start of nerve stimulation and a reduction in the $\tau_{e.p.p.}$ (see Table 4). In this group of experiments the exchange of AMECh for ACh followed a markedly non-exponential time course, so the simple interpretation of the kinetics of exchange in terms of admixture of newly formed transmitter with a fixed presynaptic pool, which appeared to account for the results on curarized muscles, is not appropriate. For comparison, however, we calculated the number of quanta that had to be released in order to bring about 63 % incorporation of AMECh. The mean in eleven experiments was 246,000 ± 39,000 quanta.

Unblocked preparations

In these experiments m.e.p.c.s were recorded from 6–8 fibres in the presence of 0·1 mM-MECh and the phrenic nerve was then stimulated at 3 Hz for 15–30 min. A further series of m.e.p.c.s was then recorded, and the nerve stimulated for a further period until the maximal shortening of the m.e.p.c.s was achieved. The results of eleven such experiments are shown in Fig. 6. In control experiments in which MECh was omitted, there was usually no change in $\tau_{m.e.p.c.}$ with similar periods of stimulation. Occasionally, in both control and MECh experiments, a marked lengthening of the m.e.p.c.s occurred after stimulation. Three MECh experiments have been omitted from Fig. 6 for this reason; in each case the m.e.p.c.s measured after 15 min stimulation were 10–20 % longer than the controls, though a further period of stimulation caused the normal shortening. We have not investigated this sporadic effect further.

The time course of exchange in these experiments was roughly exponential, as found for e.p.p.s in curarized muscle; there was certainly no long lag period. The time taken for 63 % exchange was about 29 min. Assuming the same total rate of release as was determined for the experiments on curarized muscle (151 quanta sec^{-1}), the number of quanta released in 29 min is 263,000 (Table 4). It is interesting and unexpected that this figure is twice as great as the corresponding estimate of the exchangeable store size obtained from measurements of e.p.p.s in curarized muscle, a result which suggests that at intermediate stages of incorporation of AMECh there can be a marked difference between the composition of the transmitter released by nerve stimulation and that released spontaneously.

A single experiment was carried out in the presence of neostigmine (3×10^{-6} M). The m.e.p.c.s were, of course, much longer than normal, but the ratio $\tau_{normal}/\tau_{false}$ is

TABLE 4. Transmitter exchange as a function of the number of quanta released in stimulated muscles

Type of experiment	Inhibitor	Fibre	Parameter measured	Release rate (quanta sec^{-1})	Q_0 (quanta $\times 10^{-3}$)	Q_e (quanta $\times 10^{-3}$)	$Q_e - Q_0$ (quanta $\times 10^{-3}$)
Muscles blocked with tubocurarine, 3 Hz stimulation	MECh 0·1 mM	1	$\tau_{e.p.p.}$	160	< 19	107	107
		2		138	< 41	209	209
		3		167	< 50	137	137
		4		145	< 44	107	107
		5		146	18	107	89
Mean ± S.E. of mean				151 ± 5		133 ± 20	130 ± 21
Muscles blocked with tubocurarine, 3 Hz stimulation	TECh 0·1 mM	6	e.p.p. amplitude	As above	—	128	
		7			—	75	
	HC-3 0·01 mM	8			—	72	
Unblocked muscles, 3 Hz stimulation	MECh 0·1 mM	Calculated from Fig. 9	$\tau_{m.e.p.c.}$	As above	< 136	263	263
Mg^{2+}-blocked muscles, 10 Hz stimulation	MECh 0·1 mM	9	$\tau_{e.p.p.}$	67	121	193	672
		10		150	45	207	162
		11		162	< 41	154	154
		12		88	50	127	77
		13		237	71	206	135
		14		123	37	85	48
		15		230	241	482	241
		16		134	161	338	177
		*17		160	192	335	143
		*18		138	248	431	183
		*19		62	74	152	78
Mean ± S.E. of mean				141 ± 17	113 ± 26	246 ± 39	134 ± 18
Mg^{2+}-blocked muscles, 10 Hz stimulation	HC-3 0·01 mM	20	e.p.p. amplitude	99	229	337	108
		21		74	281	510	229
		22		164	89	182	93
Mean ± S.E. of mean				112 ± 27	200 ± 57	343 ± 95	143 ± 43
Release evoked with 20 mM-K^+ in unblocked muscles	MECh 0·1 mM	23	$\tau_{m.e.p.c.}$	100	260	525	265
		24		68	530	—	—
		25		102	140	378	238
		26		50	< 175	251	251
Mean ± S.E. of mean				80 ± 13	232 ± 113	385 ± 79	251 ± 8

Q_0 is the number of quanta released before there was a reduction in the τ of synaptic potentials or currents.
Q_e is the number of quanta released when the reduction of τ of synaptic potentials or currents was 63% $(1 - 1/e)$ complete.
* Experiments in which muscles were incubated in 0·1 mM-MECh for 4 hr before starting nerve stimulation.

unaffected by neostigmine (Colquhoun *et al.* 1977). The rate of exchange of AMECh for ACh in this experiment appeared to be similar to that found in the absence of neostigmine, which suggests that the recapture of choline from hydrolysed ACh does not appreciably retard the uptake and incorporation of MECh.

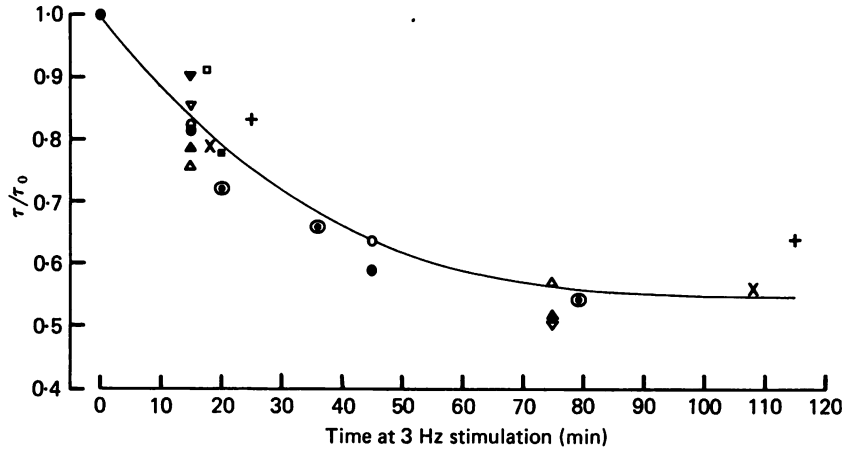

Fig. 6. Time course of the effect of MECh on the time constant of decay (τ) of m.e.p.c.s recorded from unblocked muscles. The mean values were estimated from at least six cells prior to nerve stimulation (τ_0) or after the appropriate amount of 3 Hz stimulation (τ). The results were taken from eleven separate experiments as denoted by the different symbols and ⊙ indicates the experiment in which 3 μM-neostigmine was present. The continuous line was drawn by eye.

K^+-evoked release

In a fourth series of experiments, transmitter release was evoked by increasing the concentration of K^+ to 20 mM, which produced a maximum release rate of about 100 quanta sec^{-1}. This concentration of K^+ depolarized the muscle fibres by about 30 mV and caused the m.e.p.p.s to become shorter by increasing the membrane conductance. To record m.e.p.p.s under standard conditions, we therefore changed the solution back to normal at intervals and recorded m.e.p.p.s after the membrane potential had recovered. Fig. 7*A* shows a control experiment in which 20 mM-K^+ solution was tested without any MECh. Immediately the K^+ was added to perfusion fluid, both the amplitude and duration of m.e.p.p.s were reduced and reached steady values within 10 min. On removing the extra K^+, the m.e.p.p.s returned to normal and repeated administrations of K^+ could be carried out without affecting the time course of m.e.p.p.s. However the amplitudes of m.e.p.p.s were usually slightly reduced; for example the m.e.p.p. amplitude in the experiment of Fig. 7*A* was reduced from 1·14 to 0·99 mV by the end of the experiment. The change in $\tau_{m.e.p.p.}$ was used as an indicator of the fraction of AMECh in the released transmitter. An example of an experiment in which 0·1 mM-MECh was added to the bathing fluid is shown in Fig. 7*B*. As with the Mg^{2+} experiments, there was (in three out of four experiments) a clear lag period when no AMECh was released. In the experiment of Fig. 7*B*, 260,000 quanta were released at a mean rate of 100 Hz in the presence of MECh before there was any discernible reduction in $\tau_{m.e.p.p.}$. After this intial lag there was

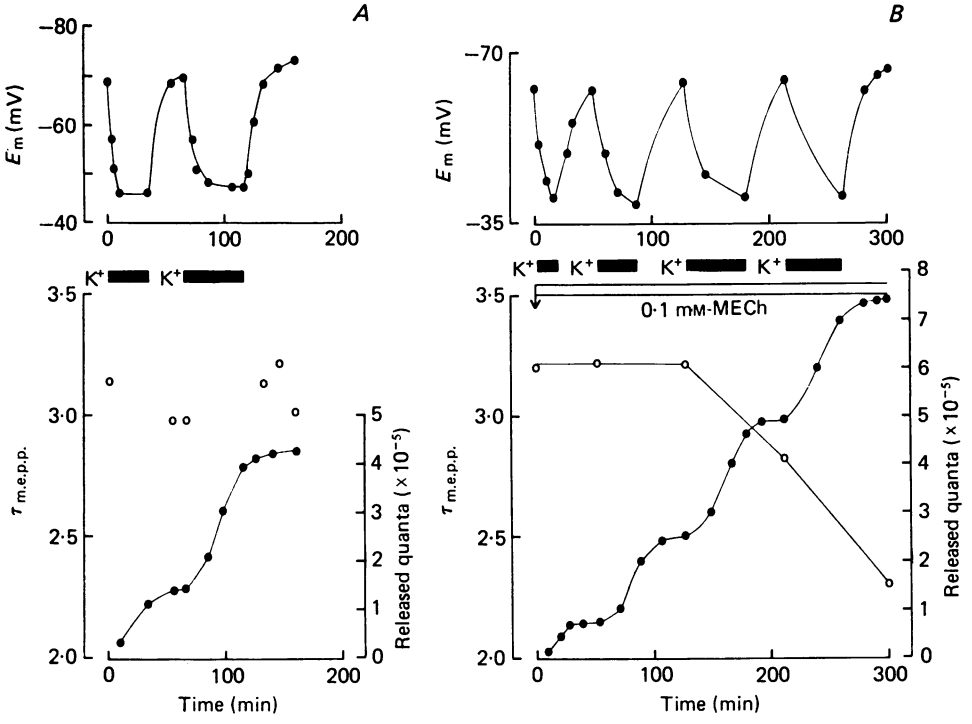

Fig. 7. Effect of MECh on the time constant of decay (○) of m.e.p.p.s after incubation in solution of high K^+ concentration. At the solid bars the K^+ concentration was increased to 20 mM in the absence (*A*) or presence (*B*) of 0·1 mM-MECh. Note that there was a lag period in *B* when there was no reduction in $\tau_{m.e.p.p.}$, even though 260,000 quanta had been released. In the lower graphs the filled symbols show the number of quanta released.

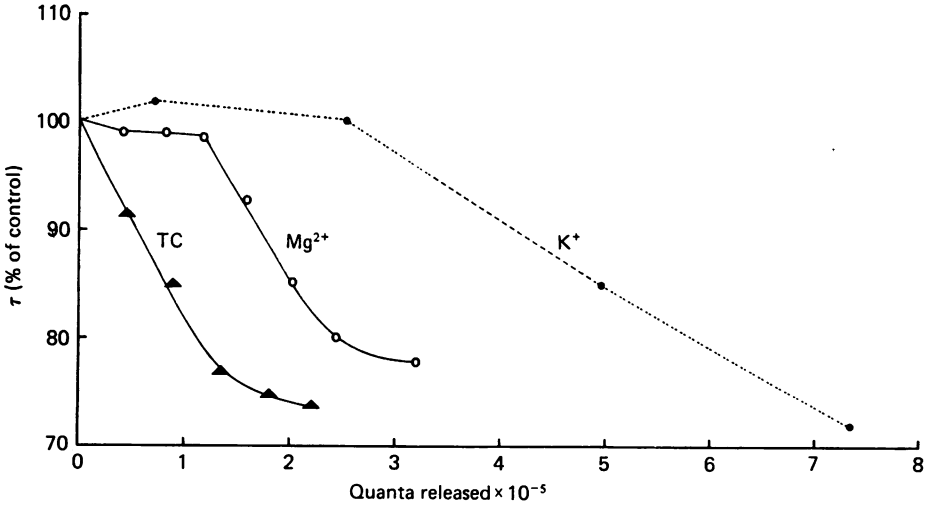

Fig. 8. Incorporation of AMECh into the released transmitter as a function of the number of quanta released by three different experimental techniques. TC, e.p.p.s recorded in curarized muscle; Mg^{2+}, e.p.p.s in Mg^{2+}-blocked muscle; K^+, m.e.p.p.s in K^+-stimulated muscle. Each curve represents a single experiment.

a progressive decline in $\tau_{m.e.p.p.}$ and after about 700,000 quanta were released the quanta appeared to contain only AMECh. In three experiments, the mean number of quanta released at the time when 63 % conversion to AMECh occurred was 385,000 ± 79,000 quanta. These estimates were only approximate, since relatively few experimental points could be obtained from each muscle.

It is evident that with K^+-induced release, more quanta have to be released to incorporate AMECh than with nerve stimulation. For direct comparison, Fig. 8 shows the results of three individual experiments, the shortening of the e.p.p. or m.e.p.p. being shown as a function of the total number of quanta released.

Table 4 summarizes the results obtained in the different types of experiment. The initial lag period, following addition of MECh, during which no AMECh is released, is denoted by the number of quanta, Q_0. The number of quanta corresponding to the point at which 63 % of the released transmitter is AMECh is denoted by Q_e. The difference between these two, $Q_e - Q_0$, is a measure of the rate at which AMECh incorporation occurs once it has begun to be released. It can be seen from Table 4 that the length of the lag period varies markedly, being virtually zero in the experiments on curarized muscle, 113,000 in Mg^{2+}-treated muscles and about 232,000 (with great individual variations) in the experiments in which release was evoked by increased K^+ concentration. $Q_e - Q_0$ was, however, about the same in the experiments in curarized and Mg^{2+}-blocked muscle, but rather greater in the experiments with K^+-evoked release.

One possible explanation of the difference in the rate of AMECh incorporation is that ACh synthesis is sustained by the recapture of choline produced by ACh hydrolysis, and that the efficacy of this process is, for some reason, greater under the conditions of the high Mg^{2+} experiments than in curarized muscles. This was tested by carrying out similar experiments in the presence of hemicholinium (10^{-5} M) instead of MECh. Hemicholinium is known to inhibit ACh synthesis by inhibiting choline uptake (Schuberth, Sundwall, Sörbo & Lindell, 1966; Marchbanks, 1968; Guyenet, Lefresne, Rossier, Beaujouan & Glowinski, 1973), and the reduction in the amplitude of e.p.p.s or m.e.p.p.s can be used to follow the process of depletion of transmitter (cf. Elmqvist & Quastel, 1965). The results in Table 4 show that the same difference between curarized and Mg^{2+}-treated muscle is present, so it is unlikely that differences in choline recapture are involved.

DISCUSSION

The present experiments were done to elucidate some details of the process of transmitter turnover at motor nerve terminals. The usual technique for studies of this kind is to use radioactive choline and to follow its incorporation by biochemical techniques (Potter, 1970; Collier & MacIntosh, 1969; see Marchbanks, 1977). The use of electrophysiological recording provides better time resolution than can be achieved by labelling experiments; it also has advantages for studies on skeletal muscle in which the amount of transmitter released is inconveniently small for biochemical work. The succeeding paper (Large & Rang, 1978) reports on the transmitter composition of individual quanta, which could not be measured chemically.

Comparison of choline and MECh; transmitter exchange in resting muscle

It is evident from these results that the handling of MECh by nerve terminals differs in some respects from that of choline. Thus normal muscles, containing stored ACh, did not release any AMECh even after they had been soaked for up to 24 hr in MECh, whereas muscles loaded with AMECh reverted to ACh if choline was added to the medium. The ability of the muscle to revert to ACh was much greater if the choline was added shortly after the muscle had been loaded with AMECh than if the muscle was soaked in MECh for a long time before changing the medium to one containing choline. Moreover, if MECh was in the perfusion solution during the few hours after the initial loading procedure (40 mM-K^+ plus MECh) there was no lengthening of the m.e.p.c.s, which indicates that the ACh in the released quanta was synthesized from choline added to the bathing fluid rather than from an intracellular source of choline. This suggests that the nerve terminals may be unable to synthesize AMECh as fast as they can synthesize ACh, so that after release has been evoked in the presence of MECh, the total transmitter stores are somewhat depleted. In support of this we find that the m.e.p.c. amplitude, which is a measure of the amount of transmitter contained in each quantum, increases steadily for at least 4 hr in muscles bathed in MECh solution after a period of rapid transmitter release evoked by 40 mM-K^+ in the presence of MECh, whereas in the control experiment done in the presence of choline the m.e.p.c. amplitude changes very little (Table 3). Of the possible rate-limiting processes in transmitter synthesis the most likely are (*a*) choline uptake, (*b*) acetylation of choline and (*c*) packaging of transmitter into vesicles. Our results suggest that one or more of these processes is considerably slower for MECh than for choline. Studies with labelled MECh have not so far revealed large differences between MECh and choline. Thus, in the cat superior cervical ganglion Collier *et al.* (1976) found that similar amounts of labelled AMECh and labelled ACh were formed from the appropriate precursor. Also, in brain synaptosomes Barker & Mittag (1975) showed that there was only about a twofold difference in the rates of uptake and of acetylation of MECh when compared with choline. Another possibility is that ACh and AMECh are incorporated into vesicles at different rates. This would be consistent with a recent observation by von Schwarzenfeld (1977) who measured the ratio of ACh to acetylpyrrolidinecholine in synaptosomes and in purified synaptic vesicles from guinea-pig cerebral cortex after a mixture of labelled choline and labelled pyrrolidinecholine had been administered. He found a greater ratio of ACh:acetylpyrrolidinecholine in the vesicles than in the synaptosomes, suggesting that vesicular uptake favours ACh. A reduced rate of synthesis or vesicular uptake of AMECh, compared with ACh, would account for the gradual increase in the amplitude of the false m.e.p.c. which occurs shortly after the initial loading procedure.

The failure of MECh to be incorporated into the transmitter store during long incubations at rest, in spite of a continuous spontaneous release of ACh (including, probably, a continuous 'molecular leakage' not associated with discrete synaptic potentials; Katz & Miledi, 1977; Vyskocil & Illes, 1977) suggests that the nerve terminals can draw in a source of endogenous choline for ACh synthesis at rest. This is known to occur in ganglia and brain slices incubated in choline-free media

(for references see MacIntosh & Collier, 1976) but it is interesting that this source, which probably involves phospholipid break-down, is used in preference to an abundant exogenous supply of MECh.

Transmitter exchange during evoked release

The results obtained, summarized in Fig. 8 and in Table 4, show that the exchange of ACh for AMECh in the releaseable store of transmitter cannot be accounted for simply in terms of a single homogeneous pool of transmitter of fixed size into which the newly synthesized transmitter becomes incorporated. This simple model for transmitter exchange would predict that the changeover of the released transmitter from ACh to AMECh, when synthesis is suddenly switched from choline to MECh, should occur exponentially as a function of the amount of transmitter released, with the 'characteristic point' when $1/e$ of the released transmitter consists of ACh corresponding to the point at which the amount of transmitter released is equal to the size of the presynaptic store. In the simplest situation the exchange would follow this pattern irrespective of the rate of release or of the mechanism by which it was evoked. The results differed sharply from these predictions in two important respects: (*a*) the exchange was, in general, not exponential; this was particularly marked when release was evoked by nerve stimulation in the presence of a raised Mg^{2+} concentration, or by increasing the K^+ concentration, when a prolonged lag period, during which (in the Mg^{2+} blocked muscles) an average of $1{\cdot}1 \times 10^5$ quanta were released before any AMECh release was detected, (*b*) the rate of exchange varied markedly with the method used to evoke release, the 'characteristic release' being on average $1{\cdot}3 \times 10^5$ quanta in the experiments on curarized muscle, $2{\cdot}4 \times 10^5$ quanta with Mg^{2+}-blocked muscle and about $3{\cdot}8 \times 10^5$ quanta (though highly variable) in the experiments on K^+-evoked release.

The 'lag period', observed with Mg^{2+}-blocked muscles, during which no exchange occurred, might have been due to an endogenous store of choline which had to be used up before AMECh began to be synthesized. A similar phenomenon is evident in many of Elmqvist & Quastel's (1965) experiments where normal transmitter output continued for a period of time after HC-3 had been added. We found, however, that prolonged pre-incubation of muscles in the presence of MECh, which would be expected to remove any stored choline (Potter, 1970) and replace it with MECh, made no difference to the rate of exchange during nerve stimulation (Table 4). A second possibility is that the efficiency of nerve terminals in recapturing the choline released when ACh is hydrolysed (Collier & MacIntosh, 1969; Potter, 1970; Bennett & McLachlan, 1972; Collier & Katz, 1974) varies under the different experimental conditions, being highly efficient in the muscles stimulated at 10 Hz in the presence of Mg^{2+} but much less so in curarized muscles stimulated at 3 Hz. We found, however, that much the same results were obtained when we measured failure of transmitter output in the presence of HC-3 (Table 4) in which choline re-uptake was responsible for the delay in AMECh incorporation in Mg^{2+}-blocked muscles. This should not be taken to imply that choline re-uptake is unimportant in sustaining ACh synthesis in normal muscle, because the presence of 0·1 mM-MECh would be expected to inhibit choline uptake greatly in our experiments (Collier *et al.* 1976). It seems most likely, therefore, that the lag period, seen with Mg^{2+}-blocked muscles, reflects some

kind of compartmentation within the nerve terminal such that the newly formed AMECh enters into a store from which it is not at first released, as though the newly formed transmitter goes to the back of the queue of quanta awaiting release rather than mixing at random. This suggestion runs counter to much biochemical evidence suggesting that newly synthesized ACh is released preferentially in response to nerve stimulation or increased K^+ concentration (cat superior cervical ganglion (Collier, 1969); rat diaphragm (Potter, 1970); Torpedo electric organ (Dunant, Grautron, Israël, Lesbats & Manaranche, 1972); rat brain slices (Molenaar & Polak, 1976)). This evidence is, however, very scanty for skeletal muscle, and it is possible that much of the transmitter that is released into the bathing fluid has nothing to do with synaptic release. Thus, it has long been realized that the resting release of ACh far exceeds the amount contributed by the quanta detected as spontaneous m.e.p.p.s (see MacIntosh & Collier, 1976). Furthermore, stimulation in the presence of HC-3 reduces the ACh content of rat diaphragm by 77% or about 0·7 n-mole/g (Potter, 1970). The number of quanta that are released before exhaustion under the same conditions is $2{\cdot}7 \times 10^5$ per junction (Elmqvist & Quastel, 1965). Assuming 10^4 molecules of ACh per quantum (Kuffler & Yoshikami, 1975) and 3×10^4 junctions per g muscle, the amount of ACh attributable to this quantal release is only 0·13 n-mole/g. In the rat diaphragm, therefore, much of the ACh released from the muscle during stimulation may have nothing to do with the transmitter released in quanta, and the conclusion that newly formed ACh is released preferentially may not apply to the synaptically released ACh.

The difference in the pattern of exchange produced by nerve stimulation of curarized muscle and Mg^{2+}-treated muscle was unexpected. The conclusion that transmitter exchange occurs more quickly (in relation to quantal output) in curarized than in Mg^{2+}-treated muscle obviously depends on the validity of our estimate for the quantal content of e.p.p.s in curarized muscle. On the basis of voltage-clamp experiments on unblocked muscles we have calculated that 134 quanta are released by a single stimulus at 20 °C. This is somewhat lower than most estimates of the quantal content of e.p.p.s in curarized mammalian muscle based on the variance method, which range roughly from 100 to 300 (see Ginsborg & Jenkinson, 1976). The statistics for the release process may over-estimate quantal content (Wilson, 1977) and we are therefore inclined to trust the direct estimate. One possibility is that tubocurarine inhibits release, particularly during repetitive stimulation (Hubbard & Wilson, 1973). The evidence for this is controversial (see Auerback & Betz, 1971; Beani, Bianchi & Ledda, 1964); if it occurs then our method of calculation of quantal release during repetitive stimulation would lead to an over-estimate of the rate of release in curarized muscle, and an underestimate of the release in unblocked muscle. The difference between the rates of exchange in curarized and Mg^{2+}-blocked muscles would therefore be even greater than we have concluded, and the difference between the rate of appearance of AMECh in evoked and spontaneously released quanta (Fig. 9) would also be greater. If Elmqvist & Quastel's (1965) estimate of $2{\cdot}7 \times 10^5$ as the total number of releaseable quanta in rat diaphragm is correct, our results suggest that in Mg^{2+}-blocked muscle, or with K^+ evoked release, the time taken for conversion of the released transmitter to AMECh corresponds to the time taken for the whole of the ACh store to be released. In curarized muscle, it appears that the transmitter

being released is converted to AMECh well before all of the ACh stores have been exhausted, which is in agreement with the usual model of a readily releaseable pool of transmitter which only slowly exchanges with a larger 'depot' of transmitter (Birks & MacIntosh, 1961). Further experiments will be needed to measure the rate of exchange between the readily releaseable pool (about $1{\cdot}3 \times 10^5$ quanta or half of the total in our experiments) and the depot.

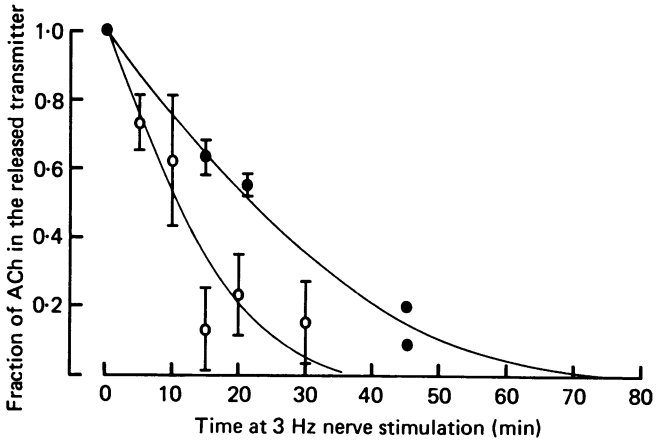

Fig. 9. Comparison of exchange of AMECh for ACh in nerve-evoked (○) and spontaneously released (●) quanta. In parallel experiments the phrenic nerve was stimulated at 3 Hz; the results for the nerve-evoked quanta were estimated from measurement of e.p.p.s in curarized muscle, while m.e.p.c.s were recorded from unblocked muscles to estimate the incorporation of AMECh into spontaneously released quanta. The continuous lines were drawn by eye.

Differences between spontaneous and evoked transmitter release

When the nerve is stimulated in the presence of MECh, AMECh begins to appear in transmitter released by nerve stimulation appreciably sooner than it appears in spontaneously released quanta (Fig. 9). Thus, after 15 min. stimulation at 3 Hz, the evoked e.p.p.s represent 65 % AMECh, whereas the spontaneous m.e.p.c.s represent only 36 % AMECh. There is other evidence that spontaneously released transmitter giving rise to miniature synaptic potentials can come from a different source from the transmitter released by nerve impulses. McLachlan (1975), working on the sympathetic ganglion, found that nerve stimulation in the presence of HC-3 caused the evoked synaptic potentials to decline more quickly than the spontaneous miniature potentials. Dennis & Miledi (1974) found that at early stages of regeneration in frog muscle after a crush injury to the motor nerve, the mean amplitude of spontaneous m.e.p.p.s was smaller than that of the quanta contributing to evoked end-plate potentials. Further work will be needed to test the possibility that the different pools of transmitter responsible for the spontaneous and evoked release are related to the compartmentation suggested by the experiments on the kinetics of AMECh incorporation.

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