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VOLTAGE CLAMPING OF UNPARALYSED CUT RAT DIAPHRAGM FOR STUDY OF TRANSMITTER RELEASE

BY M. I. GLAVINOVIĆ*

From the Department of Anaesthesia Research, McGill University, 3655 Drummond Street, Montreal, Quebec, H3G 1Y6, Canada

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SUMMARY

1. As a result of the cutting procedure there is a decrease in the membrane potential, input resistance, and space constant of the rat diaphragm, and there is no contraction when the phrenic nerve is stimulated. While the reversal potential of end-plate currents in cut preparations appears normal, the size of miniature end-plate currents (m.e.p.c.s) is slightly decreased.

2. An increase in the external concentration of potassium from 2·5 to 10·0 mM results only in a minor change ($< 5\%$) in statistical parameters of transmitter release (m, n and p). The size of m.e.p.c. is also almost unchanged.

3. A decrease in temperature from 37 ° to 15 °C results in a decrease of m, n and p; however, the values are less than 25% smaller at 25 °C than at 37 °C. The size of m.e.p.c.s is very insensitive to changes in temperature.

4. As previously reported for the frog neuromuscular junction, changes in muscle membrane potential of cut and uncut rat diaphragm due to voltage clamping affect the frequency of m.e.p.c.s only in medium with raised external K concentration. The dependence of frequency of m.e.p.c.s on muscle membrane potential is remarkably similar in cut and uncut preparations. Evoked release in cut preparation in normal medium is not affected by the change in muscle membrane potential.

INTRODUCTION

Although the study of neuromuscular transmission at physiological levels of release has been an old problem it still remains largely unresolved. To prevent contraction of the muscle which naturally occurs as a result of nerve stimulation one has to use either curare or Mg^{2+}. Curare however, eliminates independent measurements of the quantal size. If a statistical analysis is used to estimate parameters of transmitter release one must assume a Poisson distribution of evoked responses. Alternatively, if a binomial distribution is assumed, either the probability of release or the available store has to be estimated from the decay of evoked responses in short tetanic trains. This, however, requires the assumption of zero replenishment, a constant probability of release and a constant capacity of the immediately available store to contain transmitter during short tetanic train, as well as the lack of any

* Present address: Department of Biophysics, University College London, Gower Street, London WC1E 6BT.

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presynaptic action of curare. Depression of evoked synaptic responses by increased Mg^{2+} in the medium leads to the alteration of the process of transmitter release. Moreover, owing to the non-linear summation of unitary potentials there are several uncertainties in the use of end-plate potentials as indicators of evoked transmitter release (Martin, 1955, 1976; Stevens, 1976).

The present investigations were performed in order to establish how well the voltage-clamped cut muscle preparation can serve as an adequate model for the study of neuromuscular transmission at physiological levels of release.

Although the cut muscle preparation, introduced fifteen years ago by Barstad (1962), offers the possibility of studying neuromuscular transmission in the absence of blocking agents, it has not been widely used. It was generally assumed that curarized preparations offer the same possibilities without requiring the apparently drastic procedure of cutting the muscle. However, more recent observations that curare probably has a significant presynaptic effect have somewhat changed this picture.

The cutting procedure leads to a decrease in input resistance and space constant as well as to depolarization of the muscle (Zolovick, Norman & Fedde, 1970; Hubbard & Wilson, 1973). Auerbach & Betz (1971) reported that clamping of the neuromuscular junction of the cut muscle preparations is not very satisfactory owing to increased noise which makes miniature end-plate currents (m.e.p.c.s) indistinguishable. Auerbach & Betz (1971) also claimed that because of the decreased space constant, positioning of micro-electrodes is necessarily less accurate and even small errors in positioning lead to large errors in estimates of parameters of transmitter release (m, n and p). The further the electrodes are from the neuromuscular junction, the more serious the errors become. However, it has already been noted (Hubbard & Wilson, 1973) that changes in input resistance and space constant due to cutting are not great.

Although the use of the voltage clamp eliminates uncertainties of non-linear summation of unitary potentials the problem arises as to how the clamping system changes the ionic environment around the nerve terminal. Takeuchi & Takeuchi (1961) have shown that some changes in ionic environment are not necessarily negligible, a significant accumulation or depletion of potassium being particularly likely; however, it has not been determined how and if accumulation of potassium around the nerve terminal would affect the process of transmitter release in the rat phrenic diaphragm preparation bathed in a normal medium.

METHODS

All experiments were done on the isolated left diaphragm of Sprague-Dawley (S-D) strain (240–250 g) male rats. The left hemidiaphragm together with about 2 cm of phrenic nerve was removed from the animal anaesthetized by ether. After cutting off connective tissue the muscle was placed thoracic side up in a small (4 ml.) Perspex chamber.

The standard Liley's (1956*a*) solution used in these experiments had the following composition (mM): NaCl, 137; KCl, 5·0; $CaCl_2$, 2·0; $MgCl_2$, 1·0; $NaHCO_3$, 24·0; NaH_2PO_4, 1·0; glucose, 11·0. The solutions were gassed with 95 % O_2 and 5 % CO_2, usually starting 1 hr before the experiment.

To prevent muscle contraction the 'cut muscle' preparation was used as described by Barstad (1962). The muscle was slightly stretched by pinning the tendon and parts of the ribs which remained attached to the diaphragm after dissection. The muscle fibres were carefully cut across their length, 4 mm on each side of the main intra-muscular nerve branch. To avoid nerve blockage due to the release of potassium which presumably occurs after the muscle is cut, low K^+ (2·5 mM) Liley's solution was used. In addition, the flow of solution through the bath was increased

during the cutting procedure, as well as for at least 10 min afterward. Nevertheless if too narrow a strip was cut, a conduction block appeared almost regularly in spite of the use of low K^+ medium and a fast flow of the bathing solution. Preparations with too wide a strip tended to contract and small abortive action potentials could be seen if intracellular recordings were attempted.

The temperature was continuously measured in the chamber, at the edge of the preparation with a thermistor probe. The thermistor was linked to a temperature control system which consisted of an error amplifier and a power amplifier with a zero to 15 V output (maximum current being 8 A), which served as a source of power for a Peltier device. The Peltier device was placed very close (3–5 cm) to the bathing chamber to improve the efficiency of temperature control.

Intracellular recordings were made with glass microcapillary electrodes filled with 1·5 M-potassium citrate and 1·5 M-potassium chloride in a volume ratio of 2 to 1. The resistances ranged from 3–15 MΩ, lower values being for current injecting electrodes. These were obtained by bevelling (Brown & Flaming, 1974).

The tips of two glass micro-electrodes were brought in close proximity (usually 2–10 μm) by two micromanipulators. This was checked in two planes by using two microscopes at right angles to each other. A piece of aluminum foil about 0·5 cm wide was placed between the micro-electrodes, the whole assembly being glued together with dental cement. Both electrodes could then be driven by a single micromanipulator, thus easing the procedure for impaling end-plates and maintaining the micro-electrodes inside the muscle fibre. The aluminum foil was connected to the ground and served to reduce capacitance coupling between voltage-recording and current-injecting electrodes.

In order to study changes in statistical parameters of transmitter release at different temperatures or potassium concentrations, the micro-electrodes had to be maintained at the same impalement site for 1 or 2 hr. This was not always easy and was really successful in only nine out of forty attempts. This required great attention in locating the end-plate as precisely as possible. The end-plate was identified by the following criteria: (1) the presence of nerve terminals visible under the microscope, (2) the largest amplitude of miniature end-plate potentials (m.e.p.p.s) (and, if stimulation was possible, of e.p.p.s) and (3) the shortest rising phase of e.p.c.s: rise times (10–90 %) of 300–400 μsec at −60 mV and at 23 °C were considered good. The aluminum foil which was introduced between the two electrodes (and grounded) proved to be absolutely essential to keep rise times short. From data obtained at junctions where these criteria were fulfilled, amplitude histograms of usually about 150 e.p.c.s were constructed. Similarly about sixty m.e.p.c.s were used to derive amplitude histograms.

In calculating the theoretical predictions for e.p.c. amplitude histograms, the average m.e.p.c. amplitude served as a measure of quantal size. In these calculations, variability of m.e.p.c.s was taken into account as well. The m.e.p.c.s were assumed to have a normal distribution. Giant m.e.p.c.s (those 2 or 3 times larger than the mean value of m.e.p.c.s) were not taken into account. The statistical parameters of transmitter release (m, n and p) were calculated as outlined by Miyamoto (1975) and the corresponding standard error of the mean as given by Robinson (1976). For all amplitude histograms the 'goodness of fit' of the binomial distribution to the observed distribution was determined by a χ^2 test.

The method of voltage clamping was in principle the same as used by other authors. The voltage clamp apparatus had a differential input head stage with a large input impedance and a gain of five. The signal was then applied at the summing point of the feed-back amplifiier. The feed-back amplifier had provision for proportional, derivative and integral control in different amounts, as well as variable gain. For further information about the properties of controllers having proportional, derivative and integral control, reference should be made to textbooks of control theory (for example, Ogata, 1970). The voltage clamp current was brought to the current-injecting electrode through a 1 MΩ resistor. The voltage drop across this resistor served as an indicator of the clamping current.

RESULTS

Properties of cut muscle preparations

Cutting the muscle (see Methods) seems a quite drastic procedure; thus an electrophysiological examination of the muscle properties before and after cutting is important in order to avoid possible misinterpretations.

Resting membrane potentials, recorded from ten junctions in the same preparations before and 1½ hr after cutting changed from −81·3 (S.E. ± 0·9) mV to −50·9 (S.E. + 0·7) mV. The relatively 'high' resting potentials (if compared with Liley's (1956*b*) data) were due to the low external K^+ concentration. The extent of depolarization depends on how close to the end-plate the muscle was cut. The nearer the cut the

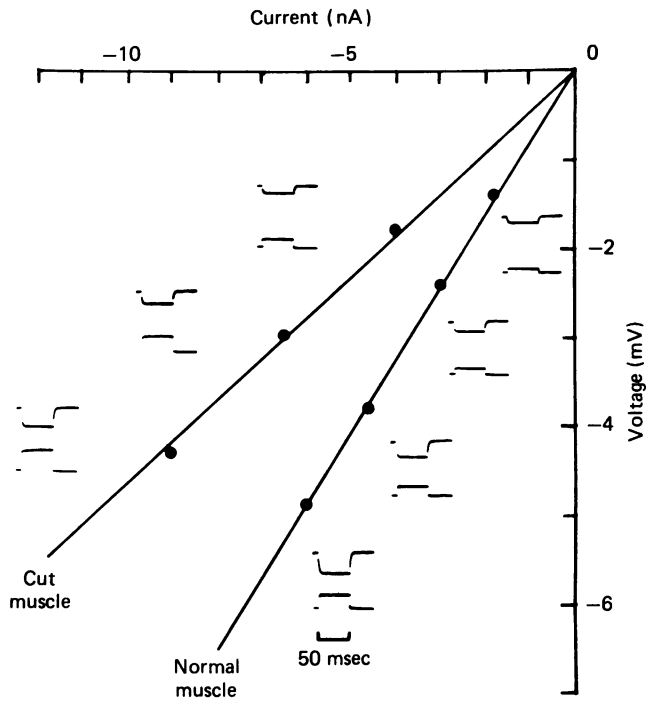

Fig. 1. Input resistance of normal and cut muscle, measured by 50 sec pulses of different intensities. The distance between the current injecting and recording electrode was 5–10 μm. Four current pulses and resulting electrotonic depolarizations were recorded before, and three after the cutting procedure. While the hyperpolarizing electronic potentials (upper traces) are shown as deflecting downwards, the current pulses (lower traces) are inverted.

more pronounced were the changes in the resting membrane potential. The values given here are from preparations where the cutting procedure was close to the end-plate and changes in resting potential were more pronounced.

Similarly the input resistance and the space constant of e.p.p.s decreased as the result of cutting. The input resistance was measured with two electrodes placed close to the end-plate and to each other. One was used to inject known amounts of current and the other measured electrotonic potentials. Since the distance between the electrodes was short ($< 10\,\mu m$), the potential measured by the second electrode is considered to represent the potential at the point of injection.

Several current pulses, together with the resulting electrotonic potentials, are shown in Fig. 1 (before and after cutting) together with the resulting V_m *vs.* I curves. The input resistance is calculated from the slope of $\Delta V_m/\Delta I$. Input resistances obtained from ten muscle fibres in the same preparation decreased after cutting from 710·0

(S.E. + 22·2) kΩ to 481·0 (S.E. ± 11·9) kΩ. Again the decrease in input resistance depends greatly on how narrow a strip of muscle is cut.

The space constant for the e.p.p.s was also measured. One electrode was placed as focally as possible in the end-plate and used as a reference, and the other was inserted at different points along the muscle to determine the corresponding size of the end-plate potential. When the space constant of normal muscle was measured, curare was applied to keep the e.p.p.s below firing threshold.

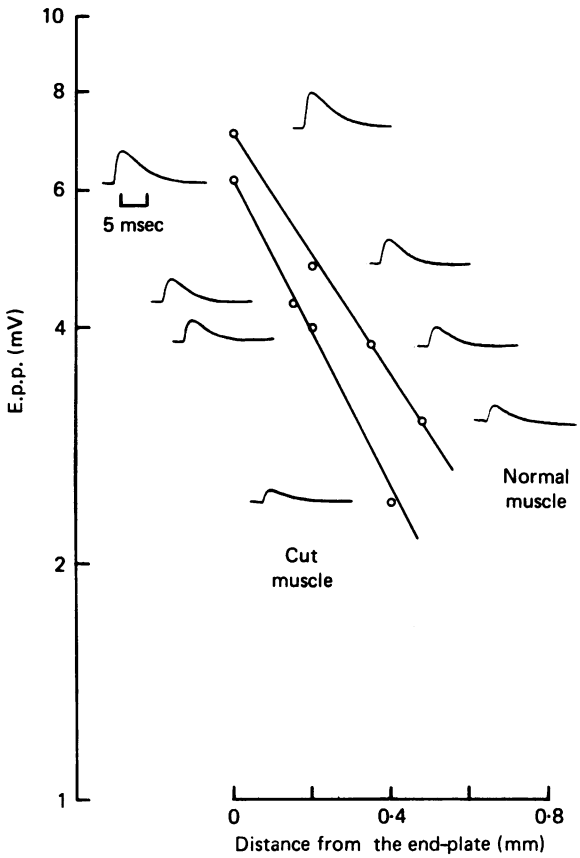

Fig. 2. Estimation of the space constant of the e.p.p.s of normal and cut muscle. The corresponding e.p.p. is shown together with each point. The points represent the average of ten such e.p.p.s.

Fig. 2 shows several e.p.p.s which decline in amplitude with increasing distance from the end-plate (before and after cutting), together with the resulting e.p.p. *vs.* distance (*d*) plot on semi-logarithmic co-ordinates. The space constant is calculated from the slope of the curve. The e.p.p. space constants, determined from ten junctions in the same preparation, decreased due to cutting from 661·5 (S.E. ± 23·6) μm to 429·5 (S.E. ± 13·0) μm.

Three estimates of the true e.p.c. reversal potential in cut muscle gave values from −7 to 0 mV. The reversal potential was not estimated in normal muscle because

extrapolation could give quite inaccurate estimates (see the Discussion of Dionne & Stevens, 1975; Mallart, Dreyer & Peper, 1976).

The values obtained for the cut muscle are close to the published values obtained at the frog neuromuscular junction (~ -15 mV, Takeuchi & Takeuchi, 1960; ~ -5 mV, Kordaš, 1969; ~ 0 mV, Magleby & Stevens, 1972). Although the decrease in membrane potential indicates that cutting alters the internal ionic composition, the reversal potential appears normal.

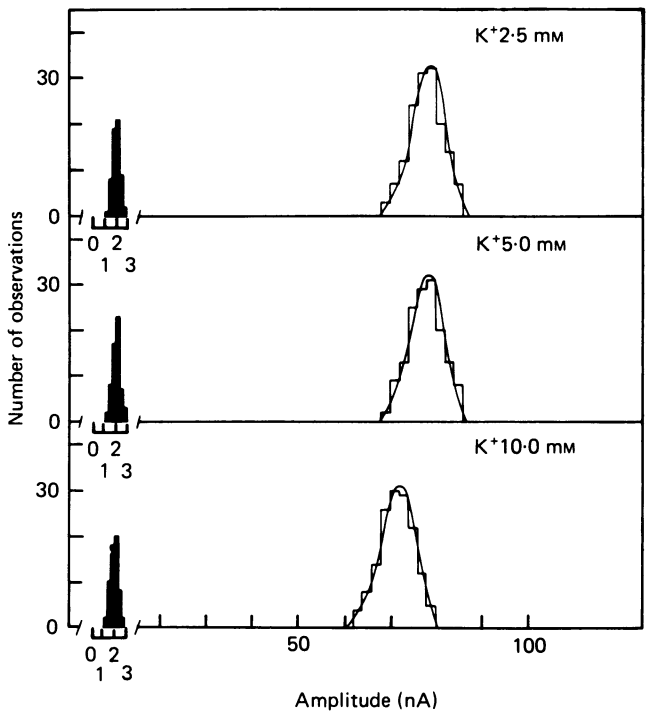

Fig. 3. Quantal contents of e.p.c.s and m.e.p.c.s from one end-plate in Ringer solution containing three different concentrations of K^+: *A*, 2·5 mM; *B*, 5·0 mM; *C*, 10·0 mM. Open histograms for e.p.c.s filled histograms for m.e.p.c.s. The curves are the corresponding binomial predictions assuming that the amplitudes of the quantal units are distributed with the same mean and variance as the m.e.p.c.s. The values of statistical parameters are: 37·50, 37·56, 35·03 for m; 39·89, 39·96, 37·67 for n; and 0·94, 0·94 and 0·93 for p, respectively.

The data were calculated from the measurement of 150 e.p.c.s and sixty m.e.p.c.s recorded during continuous stimulation at 0·5 Hz. The values of χ^2 for each of the histograms were 2·4, 3·1 and 0·5 and the corresponding P values $> 0{\cdot}7$, 0·5 and 0·99.

Size of m.e.p.c.s

M.e.p.c.s were examined in the same muscle before and after cutting. Distributions of sixty m.e.p.c. amplitudes from each of ten different junctions in three different preparations were compared with the same number of m.e.p.c.s from the same three preparations after cutting. For this comparison all end-plates were clamped at -55 mV. The coefficient of variation of individual distributions varied between 10 and 25 % of the mean and did not change greatly after the cutting procedure. M.e.p.c.s

with values 2, 3 or more times larger than the mean value were not taken into account. Mean sizes of m.e.p.c.s in normal muscle were from 1·9 to 2·4 nA with an over-all mean value of 2·11 nA. In cut muscle, the mean values were from 1·65 to 2·24 with an over-all mean value of 1·88 nA. The m.e.p.c.s therefore decreased by approximately 10%.

TABLE 1. M.e.p.c.s, e.p.c.s and statistical parameters of release m, n and p obtained in three different cut diaphragms at three external K^+ concentrations (2·5 mM, 5·0 mM and 10·1 mM) in otherwise normal Ringer solution at 23 °C. The phrenic nerve was stimulated at 1 Hz. At least sixty m.e.p.c.s and 150 e.p.c.s were used for each case. The statistical parameters of release were calculated as outlined in Miyamoto (1975) and the corresponding standard errors as in Robinson (1976)

K^+ (mM)	Expt. no.	Membrane potential (mV)	Mean m.e.p.c. (nA)	Mean e.p.c. (nA)	Quantal content (± S.E.)	Available store (± S.E.) (quanta)	Probability of release (± S.E.)
2·5	1	−55	2·08	77·08	37·50 ± 0·75	39·89 ± 2·45	0·94 ± 0·03
	2	−65	2·43	82·53	33·96 ± 0·68	36·91 ± 2·17	0·92 ± 0·04
	3	−60	2·27	89·26	39·32 ± 0·97	43·69 ± 3·45	0·90 ± 0·03
5·0	1	−55	2·07	77·75	37·56 ± 0·80	39·96 ± 2·61	0·94 ± 0·03
	2	−65	2·41	81·77	33·93 ± 0·69	37·29 ± 2·33	0·91 ± 0·04
	3	−60	2·25	88·02	39·12 ± 0·98	43·46 ± 3·23	0·90 ± 0·04
10·0	1	−55	2·04	71·47	35·03 ± 0·79	37·36 ± 2·43	0·93 ± 0·03
	2	−65	2·36	70·53	29·89 ± 0·65	33·58 ± 1·98	0·89 ± 0·03
	3	−60	2·21	79·25	35·86 ± 0·88	41·22 ± 3·07	0·87 ± 0·04

Some change can be expected since the time from dissection to recording is considerably longer in the case of the cut muscle preparation (usually 2 hr) than for normal preparations, and since choline was not present in the medium the vesicular content is likely to decline. The results, however, indicate that a change in sensitivity of post-synaptic membrane to the transmitter is unlikely.

Influence of potassium concentration in the bathing solution on transmitter release

As noted in the Introduction, the most likely change in the ionic environment around the nerve terminal due to the voltage clamping of the end-plate is a change in external K^+ concentration. The following represents an attempt to see how the parameters of transmitter release depend on extracellular potassium.

The statistical analysis was performed as outlined in the Methods. Amplitude histograms for both m.e.p.c.s and e.p.c.s at three different levels of K^+ are shown in Fig. 3. The calculated binomial distribution fitted the experimentally observed responses well. In Table 1 the values of parameters of transmitter release from three other preparations are given. The size of m.e.p.c.s was very little ($< 5\%$) affected by the fourfold increase of K^+ concentration, but the average e.p.c.s decreased more (10–20%), as did the quantal content (5–15%). This seems to be the result of a reduction in the available store (5–10%), although the probability of release also decreased to a small extent ($< 5\%$). An extensive discussion about the validity of estimates of statistical parameters of transmitter release at cut rat diaphragm neuromuscular junction is given in the following paper (Glavinović, 1979*a*).

Influence of temperature on statistical parameters of transmitter release

Four e.p.c. and corresponding m.e.p.c. histograms from the same junction at four different temperatures are shown in Fig. 4, with statistical parameters of transmitter release given in the legend of the same figure. The values of m.e.p.c.s, e.p.c.s and

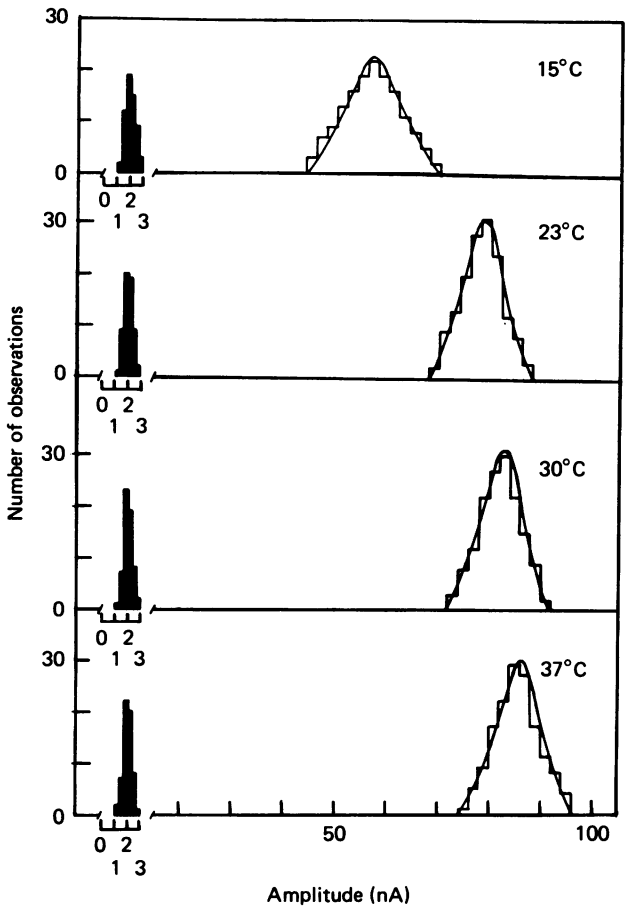

Fig. 4. Amplitude histograms of m.e.p.c.s and e.p.c.s from the same neuromuscular junction at four different temperatures (15, 23, 30 and 37 °C). Open histograms for e.p.c.s; filled histograms for m.e.p.c.s. The corresponding binomial predictions are given by the curves, assuming that the amplitudes of the quantal units are distributed with the same mean and variance as the m.e.p.c.s. The values of statistical parameters were: 27·91, 37·90, 39·86, 41·92 for m; 36·34, 40·88, 42·91, 45·91 for n; 0·768, 0·927, 0·929 and 0·913 for p, respectively.

The data were calculated from the measurement of 150 e.p.c.s and sixty m.e.p.c.s collected during continuous stimulation at 0·5 Hz for 15 °C and 1 Hz for 23, 30 and 37 °C. The values of χ^2 for each of the histograms were 2·9, 1·7, 3·2, 1·8 and the corresponding P values were $> 0{\cdot}90$, 0·90, 0·70 and 0·95.

statistical parameters of transmitter release at four different temperatures from two other junctions are given in Table 2.

There are several points of interest. As the temperature was lowered below 23 °C, the quantal content markedly decreased, mainly owing to a reduction in the prob-

TABLE 2. Mean values of m.e.p.c.s, e.p.c.s and statistical parameters of release m, n and p from two junctions at four different temperatures (15, 23, 30 and 37 °C) in normal Ringer solution. The nerve was stimulated at 0·5 Hz at 15 °C and 1 Hz at other temperatures. Sixty m.e.p.c.s and 150 e.p.c.s were analysed for each case. The statistical parameters of release were calculated as outlined in Miyamoto (1975) and the corresponding standard errors as in Robinson (1976)

Temp. (°C)	Membrane potential (mV)	Mean m.e.p.c. (nA)	Mean e.p.c. (nA)	Quantal content (± S.E.) (quanta)	Available store (± S.E.)	Probability of release (± S.E.)
15	−60	2·35	84·07	35·77 ± 0·99	55·03 ± 2·93	0·65 ± 0·04
	−55	2·17	68·95	31·77 ± 0·87	44·13 ± 2·57	0·72 ± 0·03
23	−60	2·45	125·23	51·11 ± 1·38	60·85 ± 3·17	0·84 ± 0·04
	−55	2·35	97·77	41·60 ± 1·43	53·33 ± 2·95	0·78 ± 0·04
30	−60	2·50	137·29	54·92 ± 1·59	61·71 ± 3·19	0·89 ± 0·04
	−55	2·35	108·35	46·11 ± 1·47	53·00 ± 2·87	0·87 ± 0·05
37	−60	2·55	158·35	62·12 ± 1·69	66·80 ± 3·47	0·93 ± 0·04
	−55	2·35	138·17	58·80 ± 1·61	64·62 ± 3·39	0·91 ± 0·04

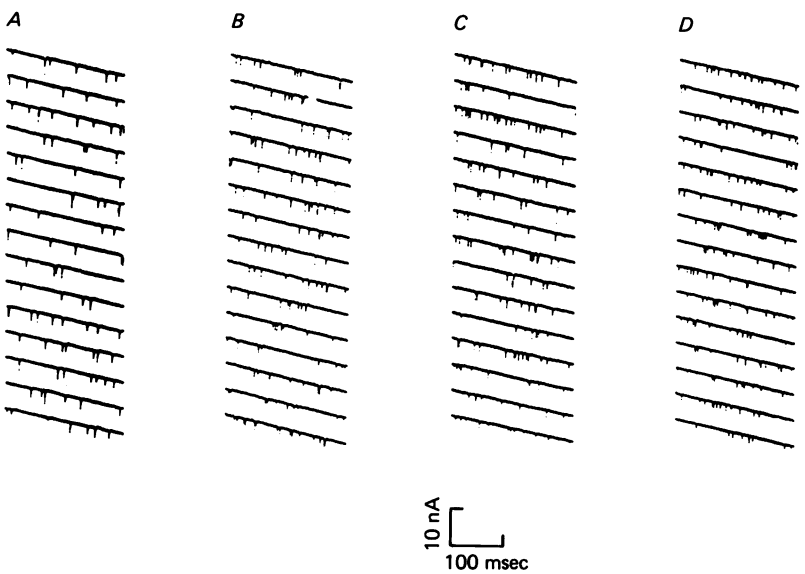

Fig. 5. Effect of clamping end-plate membrane of the uncut rat diaphragm at different potential levels (A, −90 mV; B, −80 mV; C, −65 mV, D, −50 mV) on the frequency of m.e.p.c.s. The experiments were performed in the presence of 17·5 mM-K^+ Ringer solution.

ability of release. As the temperature was raised above 23 °C toward the physiological level (37 °C) there was a somewhat smaller increase in quantal content, brought about by an increase in the available store. The size of m.e.p.c.s did not change significantly in the range of temperatures (15–37 °C).

Influence of post-synaptic membrane potential on synaptic transmission

As Takeuchi & Takeuchi (1961) reported from their experiments on the frog neuromuscular junction, the application of voltage clamping probably changes

considerably the ionic environment around the nerve terminal. The following tests were performed to determine if this is equally true in the case of cut and uncut rat diaphragm and to see if this changes the process of spontaneous and evoked release.

Three end-plates of the cut and normal muscle were clamped at several membrane potentials and the m.e.p.c.s were monitored. The frequency of these was determined

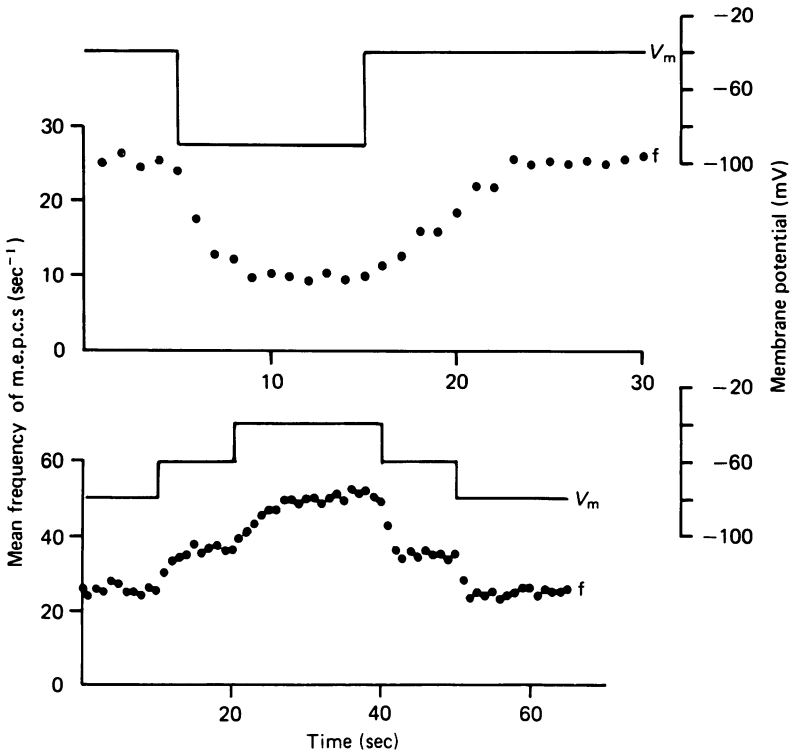

Fig. 6. The time course of the change in the frequency of m.e.p.c.s in an uncut preparation, in 17·5 mM-K^+ Ringer solution, following sudden changes in the holding potential of the muscle fibre. Each point represents mean frequency during 1 sec.

at each stage during this procedure, the membrane potential being held at each level for a minimum of 20 sec. This was done at each neuromuscular junction at four different K^+ concentrations of the medium (2·5, 5·0, 10·0, 17·5 mM). At low or normal levels (2·5 and 5·0 mM) the frequency of m.e.p.c.s did not vary with the post-synaptic membrane potential, either in uncut or in cut diaphragms. However when the extracellular concentrations were raised to 10·0 and 17·5 mM, the frequency of m.e.p.c.s showed a clear dependence on post-synaptic membrane potantial.

M.e.p.c.s from the uncut rat diaphragm junction at four different post-synaptic membrane potentials in 17·5 mM-K^+ Ringer solution are shown in Fig. 5. Depolarization of the muscle membrane results in an increase of m.e.p.c. frequency, the opposite being true with hyperpolarization. It is important to note that it takes 5–10 sec for the frequency of m.e.p.c.s to reach a new steady state after the onset of hyperpolarization. A similar slow or even slower change in frequency of m.e.p.c.s was observed when the post-synaptic membrane was depolarized (Fig. 6). The steady state fre-

quencies of m.e.p.c.s of (*a*) uncut and (*b*) cut muscle diaphragm, as they depend on the post-synaptic membrane potential, are shown in Fig. 7. In both cases the frequency of m.e.p.c.s depended in a similar fashion on membrane potential and on the K^+ concentration in the Ringer solutions, as can be seen from Fig. 7 (and as was observed in two other cases). These observations are in agreement with the findings of Takeuchi & Takeuchi (1961) in frog neuromuscular junctions.

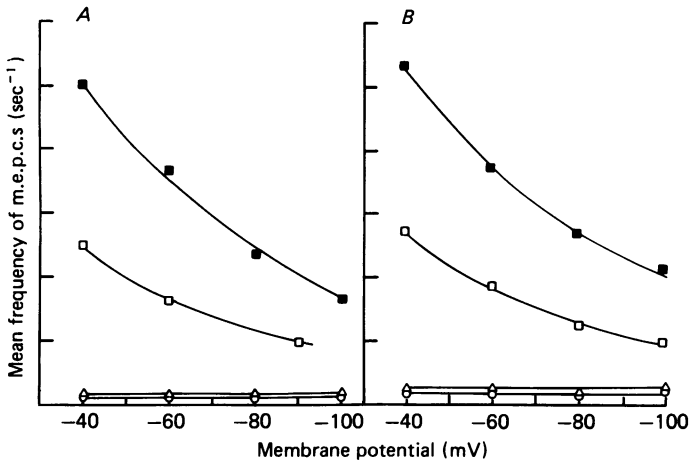

Fig. 7. Effect of changes in post-synaptic membrane potential (held by voltage clamp) of the (*A*) uncut (*B*) cut preparation on the frequency of m.e.p.c.s in four different K^+ concentrations: open circles at 2·5 mM, open triangles at 5·0 mM, open squares at 14·5 mM and filled squares at 17·5 mM.

Evoked transmitter release was similarly tested at different muscle membrane potentials in normal K^+ (5·0 mM), in three cut rat diaphragms. The post-synaptic membrane potentials were varied between -40 and -100 mV. No consistent change in quantal content could be observed in either case. Therefore, these results do support the idea that, although the procedure of voltage clamping changes the ionic environment around the nerve terminal, it does not affect seriously the process of transmitter release when the muscle (cut or uncut) is kept in normal Ringer solution.

DISCUSSION

This study was performed in order to find out whether the results obtained from voltage-clamped cut muscle preparations represent the physiological process of transmitter release. Although the cut muscle eliminates the need for blocking agents, the question is how the procedure itself affects transmitter release or the possibility of clamping the end-plate. Similarly, although the voltage clamp technique eliminates the uncertainties of the non-linear summation of unitary potentials, it may alter significantly the environment around the nerve terminal (Takeuchi & Takeuchi, 1961).

Several factors could cause the fall of resting potential after cutting: (*a*) change in intracellular ionic composition, (*b*) decrease of the G_K/G_{Na} ratio and (*c*) muscle changes from long (or infinite) cable to short cable with short-circuited ends. How-

ever, the estimates of the reversal potential of the cut muscle give values which are very close to zero and therefore similar to those previously obtained in uncut muscles (Takeuchi & Takeuchi, 1960; Kordaš, 1969; Magleby & Stevens, 1972). This indicates that at least around the end-plate there is no great change in the intracellular ionic composition.

The input resistance and space constant were both reduced, especially when the muscle was cut relatively close to the end-plate (as in the examples given in the Results). These changes are in good agreement with those observed by Zolovick *et al.* (1970) and Hubbard & Wilson (1973).

These alterations in membrane properties, however, did not preclude effective voltage clamping of the end-plate as Auerbach & Betz (1971) suggested. Whenever the necessary precautions were taken in locating end-plates (see Methods) the clamping was adequate, as indirect evidence also strongly suggests (Glavinović, 1979*a*). The level of noise, though higher than in non-cut muscle, was still low enough so that m.e.p.c.s could be clearly distinguished.

The present experiments and the experiments in accompanying papers (Glavinović, 1979*a*, *b*) were performed mostly at room temperature (23 °C), because the preparations remain in good shape for longer periods of time (often several hours). Since the junction normally operates at 37 °C, the question arises whether the quantal content is subject to much variation when the temperature is lowered from 37 to 23 °C. Although the quantal content falls with the temperature, its value at 23 °C is usually only about 25 % lower than at 37 °C. A qualitatively similar dependence of synaptic output on temperature was observed by Takeuchi (1958) at the frog neuromuscular junction, by Morris & Krnjević (1976) in the cuneate nucleus of the cat, and by Weight & Erulkar (1976) at the squid giant synapse.

As reported by Takeuchi & Takeuchi (1961), voltage clamping can itself change the ionic environment around the nerve terminal of the frog, hyperpolarization of the end-plate leading probably to a depletion of extracellular K^+ and depolarization to its accumulation. The present results confirm that similar changes take place in the cut and uncut rat diaphragm. These variations in external K^+ are not negligible over the maximal range of post-synaptic membrane potentials studied in the present experiments (from -40 to -100 mV). However, in normal Ringer solutions and at normal levels of release, they do not alter significantly either the spontaneous or evoked release. These results are not surprising if the dependence of the quantal content on external K^+ is taken into account. As the present results indicate, a rise in concentration from 2·5 to 10·0 mM results in a decrease in quantal content of less than 5 %. These results stand in contrast to those of Takeuchi & Takeuchi (1961) and Branisteanu, Miyamoto & Volle (1976) which indicated an *increase* in quantal content when external K^+ was raised from 2·5 to 10·0 mM. However, their experiments were performed on Mg^{2+}-depressed junctions, which may well behave differently.

On this basis one can conclude that the voltage-clamped cut rat diaphragm at room temperature is a good model for the study of neuromuscular transmission at normal levels of transmitter release since: (*a*) no paralyzing agents are needed, (*b*) although the cutting procedure seems drastic, when a wide strip of muscle is left around the nerve terminals, the changes in the electrophysiological properties of the muscle membrane (membrane potential, input impedance, space constant, reversal

potential) are not great and the clamping of the neuromuscular junction is adequate; neither does there seem to be any significant change in the sensitivity of the post-synaptic membrane to the transmitter, (*c*) a lowered temperature (23 °C instead of 37 °C) did not change transmitter release drastically and the size of m.e.p.c.s was practically unaffected, (*d*) although voltage clamping may itself change the ionic environment around the nerve terminal, the changes which do occur are not sufficient to affect transmitter release in normal medium. In the light of the fact that large changes in extracellular K^+ (from 2·5 to 10·0 mM) change statistical parameters of transmitter release by less than 5% and leave the size of m.e.p.c.s practically unaffected, the lack of effect of the clamping procedure on the process of transmitter release, at normal levels of transmitter release, is not surprising.

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