Accumulation of extracellular calcium at the endplate of mouse diaphragm after ecothiopate *in vitro*

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- 1 Experiments were carried out to investigate the accumulation from the extracellular medium of $^{45}\text{Ca}^{2+}$ by the endplate region of skeletal muscle.
- 2 Mouse diaphragm muscle was incubated in physiological saline labelled with ^{45}Ca at 37°C for periods of up to 1.5 h.
- 3 The muscle was divided into junctional and non-junctional portions and the Ca from the extracellular fluid accumulated at the endplate determined from the ^{45}Ca content of the portions.
- 4 The accumulation of extracellular Ca at the endplate region of muscles incubated in physiological saline alone was nil, but there was accumulation in the presence of the anticholinesterase ecothiopate iodide $0.5 \times 10^{-6} \text{ M}$ (ECO). Stimulation of the phrenic nerve at 0.02 Hz caused no further increase in accumulation, but reduced the amount of spontaneous fasciculation. In tetrodotoxin (TTX) 10^{-6} M , the accumulation was halved, and in 3.5 mM Mg^{2+} the accumulation was nil. Carbachol 10^{-4} M resulted in an accumulation of Ca similar to that in ECO.
- 5 It is concluded that there was an accumulation of extracellular Ca following excitation of the nerve by stimulation at a low frequency and during the spontaneous fasciculations, and about half of the accumulation of extracellular Ca after ECO in the experiments was due to the postsynaptic action of ACh released non-quantally from the nerve terminals.

Introduction

There is much evidence that anticholinesterases or cholinergic agonists provoke structural damage at the endplate region of skeletal muscle (see Salpeter *et al.*, 1982 for review) with the possibility that overloading with Ca may be a causative mechanism. As cholinergic agonists permit entry of Ca^{2+} through open endplate channels, it is possible that the necrotizing effect of anticholinesterases may be due to a prolongation and intensification of the postsynaptic action of acetylcholine (ACh) released quantally from the nerve terminals either spontaneously or evoked by action potentials following stimulation of the nerve and by that released non-quantally. Another contribution to an overloading of the endplate region with Ca may derive from the excitation of the nerve terminals during anticholinesterase-induced fasciculation. Thus it remains to be determined what is the contribution made by various events induced or modified by anticholinesterases to

the loading of the endplate with Ca^{2+} and perhaps to the genesis of localized necrosis.

It has already been established by Evans (1974) that carbachol caused uptake of Ca into the muscle at the endplate, as did physostigmine, but that stimulation of the nerve in the absence or in the presence of physostigmine caused no additional accumulation. This apparent lack of effect of stimulation is not in accord with the results of Ariens *et al.* (1969) that myopathy after anticholinesterases was enhanced by neural activity.

The following is an account of experiments undertaken to investigate the entry of Ca at the endplate after ecothiopate under conditions of low frequency stimulation similar to those in which prolonged localized contractions associated with prolonged endplate current (Burd & Ferry, 1987), fasciculations and enhanced synaptic currents have been observed, and to determine which of the various processes is important for the induction of myopathy. It is concluded that there was substantial accumulation of

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Ca at the endplate even in the absence of excitation of the nerve and attributable to spontaneous events and that there was an additional accumulation associated with excitation of the nerve.

Methods

Preparations of the phrenic nerve and diaphragm muscle were dissected from male albino mice of about 20 g weight after the animals were killed by a blow to the head and section of the cervical spinal cord. The preparations were maintained at $37 \pm 0.2^\circ\text{C}$ in a physiological saline of the following composition (mM): NaCl 137, NaH_2CO_3 12, CaCl_2 2, MgCl_2 1, KCl 5, NaH_2PO_4 1, dextrose 25 and gassed with 5% CO_2 in O_2 . In some experiments extra MgCl_2 was added without any other change.

Electrophysiological methods

Diaphragms were pinned horizontally to the Sylgard bottom of a Perspex bath and records were made of bioelectric potentials at the endplate region with a KCl-filled micropipette.

Determination of the accumulation of Ca

The method used to determine the Ca content of muscle was based on that of Evans (1974). The whole mouse diaphragm was pinned to Sylgard in the bottom of a petri dish and immersed in 25 ml of the physiological saline containing $^{45}\text{CaCl}_2$ made up from a stock solution of 0.8–1 mCi/mmol (Radiochemical Centre, Amersham), in amounts to give $3\text{--}8 \times 10^5$ c.p.m. ml^{-1} of physiological saline. The incubation medium thus contained 2 mM CaCl_2 plus about $0.5 \mu\text{M}$ $^{45}\text{CaCl}_2$ stock and is referred to as ^{45}Ca -saline.

In some experiments, the phrenic nerve of one hemidiaphragm was stimulated at 0.02 Hz via Ag wire electrodes, the other half of the diaphragm being unstimulated but still attached by the central tendon to the contracting hemidiaphragm.

The preparations were incubated under the various conditions described in Results and were rinsed in ^{45}Ca -free physiological saline, usually for 30 min and were then dehydrated in two changes of acetone over a minimum of 2 h. The diaphragm was cut into hemidiaphragms, the ribs removed and the muscle cut transversely about 1 mm on either side of the intramuscular branches of the phrenic nerve into a central junctional portion and two peripheral non-junctional portions (Hebb *et al.*, 1964). Immersion of the muscle in xylene made the nerve more visible.

The junctional portion and the nonjunctional portions from each hemidiaphragm were placed in different polythene scintillation vials which were loosely capped and placed for 30 min in a water bath at 60°C to drive off the xylene. The junctional portion and the nonjunctional portions were then weighed with a torsion balance (usually each was 1–2 mg). They were digested with 0.5 ml of concentrated HCl for 30 min at 60°C or overnight at room temperature. Absolute ethanol (2 ml) and 10 ml of either Toluene fluor (PPO 5 g, POPOP 0.3 g, toluene to 1 l) or Bray's fluor (PPO 4 g, POPOP 0.2 g, naphthalene 100 g, methanol 100 μl ; 1,4-dioxan to 1 l) were added to each vial and the radioactivity of the ^{45}Ca was determined by liquid scintillation counting at an average efficiency of 30%. Standards consisted of 0.1 ml of the ^{45}Ca -saline and were counted in the same way as the samples of muscle to allow calculation of Ca accumulation.

In order to calculate the accumulation of Ca at the endplate, it is necessary to take account of any Ca accumulated by the non-synaptic part of the muscle cell. It was assumed that such non-synaptic accumulation was evenly distributed along the length of the cell, and the amount of Ca in the non-synaptic part of the excised junctional portion was estimated as the product of the weight of the junctional portion (J_{wt}) and the accumulation of Ca per unit weight of the nonjunctional portion (NJ_{Ca}) of the same hemidiaphragm. Thus the accumulation of Ca at the endplate was calculated as the amount of Ca in the junctional portion ($J_{\text{Ca}} \times J_{\text{wt}}$) less the amount of Ca in the same weight of nonjunctional portion of the hemidiaphragm ($\text{NJ}_{\text{Ca}} \times J_{\text{wt}}$). The following equations describe the calculation of the accumulation of Ca at the endplate:

$$J_{\text{Ca}} = \frac{J_{\text{c.p.m.}}}{J_{\text{wt.}}} \times \frac{\text{Std}_{\text{Ca}}}{\text{Std}_{\text{c.p.m.}}} \text{ nmol mg}^{-1} \text{ dry wt.}$$

$$\text{NJ}_{\text{Ca}} = \frac{\text{NJ}_{\text{c.p.m.}}}{\text{NJ}_{\text{wt.}}} \times \frac{\text{Std}_{\text{Ca}}}{\text{Std}_{\text{c.p.m.}}} \text{ nmol mg}^{-1} \text{ dry wt.}$$

$$\text{EP}_{\text{Ca}} = \frac{J_{\text{wt.}}(J_{\text{Ca}} - \text{NJ}_{\text{Ca}})}{J_{\text{wt.}} + \text{NJ}_{\text{wt.}}} \text{ nmol mg}^{-1} \text{ total dry wt.}$$

where

J_{Ca} = accumulation of Ca in junctional portion (nmol mg^{-1}).

NJ_{Ca} = accumulation of Ca in nonjunctional portion (nmol mg^{-1}).

$J_{\text{c.p.m.}}$ = counts min^{-1} of junctional portion.

$\text{NJ}_{\text{c.p.m.}}$ = counts min^{-1} of nonjunctional portion.

$J_{\text{wt.}}$ = weight of junctional portion in mg.

$\text{NJ}_{\text{wt.}}$ = weight of nonjunctional portion in mg.

Std_{Ca} = nmol of Ca^{2+} in saline containing ^{45}Ca which was the standard with counts min^{-1} of $\text{Std}_{\text{c.p.m.}}$.

EP_{Ca} = accumulation of Ca at the endplate (nmol mg^{-1} total wt).

In preliminary experiments, as no difference was found in the accumulation of Ca in left and right hemidiaphragms, they were taken as equivalent.

Statistical methods

Usually each experiment was carried out on a group of hemidiaphragms and the mean and standard deviation (s.d.) of each group are reported in the text. Non-parametric two-tailed tests were used to estimate the probability of identity of groups of results, and when $P < 0.05$, a significant difference was reported. Unpaired data were subjected to the Mann-Whitney test and paired data to the Wilcoxon test.

Materials

The anticholinesterase ecothiopate iodide was made up from the commercially available Ecothiopate Eyedrops 0.25%, B.N.F., (Phospholine Eyedrops, Ayerst) which also contained potassium acetate to buffer the stock solution. The increase in C_K of the physiological saline due to this buffer was 60 nM which was considered negligible.

The ingredients of the physiological saline were BDH AR grade, and drugs were purchased from Sigma.

Results

The progress of accumulation of Ca

The accumulation of Ca in ^{45}Ca -saline alone The progress of accumulation of the ^{45}Ca in the junctional and nonjunctional portions and at the endplate was examined by incubating diaphragms in ^{45}Ca -saline for periods of 18, 30, 60 or 90 min before processing them as described in Methods. There was considerable variation between hemidiaphragms in the values of the accumulation of Ca in the junctional or in the nonjunctional portions (J_{Ca} and NJ_{Ca}), the coefficient of variation of these quantities in the group of 9 hemidiaphragms being 0.3–0.5. The values for the accumulation of Ca in the junctional portions (J_{Ca}) at various times were significantly different from each other except between 30 and 60 min,

Figure 1 Graph showing the progress of accumulation of Ca in portions of hemidiaphragms incubated in ^{45}Ca -saline, values are mean with s.d. shown by vertical bars (see Table 1). Note time-dependent accumulation in (■) junctional portions and in (□) non-junctional portions with no (◆) accumulation at the endplate.

and for the nonjunctional portions (NJ_{Ca}) between 60 and 90 min. These results indicate a progression accumulation of Ca with increasing time of incubation in ^{45}Ca for up to 60 min (Figure 1).

The calculation of the accumulation of Ca at the endplate of individual hemidiaphragms gave values for EP_{Ca} at the various times which were not significantly different from one another (Figures 1, 2). The mean values of EP_{Ca} are small and at 18, 30 and 60 min are due to small but just significant differences between J_{Ca} and NJ_{Ca} shown by the Wilcoxon test.

It is concluded that although there was a progressively increasing accumulation of Ca along the length of the muscle cells, there was no additional accumulation at the endplate.

The effect of ecothiopate on the accumulation of Ca

In this experiment, diaphragms were immersed at zero time in ^{45}Ca -saline containing ecothiopate (ECO) $0.5 \mu\text{M}$. Diaphragms exposed to ECO fasciculated, and had miniature endplate potentials (m.e.p.ps) of increased duration and amplitude. After incubation in ECO for 15, 30, 60 or 90 min, diaphragms were washed in ^{45}Ca -free physiological saline for 30 min and then prepared for assay of ^{45}Ca . Such diaphragms usually appeared swollen at the endplate region. At each time, the accumulation of Ca in the junctional portions (J_{Ca}) was significantly greater than that in the nonjunctional portions (NJ_{Ca}) of diaphragms. The accumulation of Ca

in the junctional portions (J_{Ca}) and in the non-junctional portions (NJ_{Ca}) increased throughout the 90 min. The values of J_{Ca} at each time were different from those at all other times, and the same for NJ_{Ca} .

Statistical testing of the nonjunctional accumulation of Ca in the presence and the absence of ECO showed no difference with incubation for up to 60 min, but at 90 min, the nonjunctional accumulation of Ca in the presence of ECO was greater than in its absence. This indicates that the early part of the progress of nonjunctional accumulation was independent of ECO. The junctional accumulations (J_{Ca}) at each time in ECO were significantly different from those in the absence of ECO.

The values of the accumulation of Ca at the endplate (EP_{Ca}) at 18, 30 and 60 min were significantly different from each other, and that at 90 min was not different from the values at 30 or 60 min indicating a progressive accumulation of Ca at the endplate until a plateau was reached at 60 min. With incubation in ECO, the EP_{Ca} at 18, 30, 60 and 90 min was significantly greater than that found at similar times in the absence of ECO. The results are shown in Table 1 and in Figure 2.

It is concluded that in the presence of ECO there was a progressive accumulation of Ca for up to 60 min in the nonsynaptic part of the cell which was not different from that found in the absence of ECO, and a progressive accumulation of Ca at the endplate which was different from the lack of accumulation in the absence of ECO (Figure 2).

In another experiment, 6 hemidiaphragms were incubated in ^{45}Ca -saline and ECO for 30 min, then rinsed in physiological saline for a few seconds to remove any film or droplets of the incubation medium, then dehydrated in acetone. These diaphragms had a NJ_{Ca} of 2.99 ± 0.724 nmol mg^{-1} and a J_{Ca} of 3.95 ± 0.624 nmol mg^{-1} which were greater than those of preparations washed in ^{45}Ca -free saline for 30 min (Table 1). The EP_{Ca} was 0.334 ± 0.075 , which was not different from that of the washed preparations. It is concluded that the Ca

Figure 2 Graph showing progress of accumulation of Ca at the endplate EP_{Ca} in ^{45}Ca -saline alone ($\square$) and in that containing ecothiopate (ECO) 0.5 μM ($\blacklozenge$). Values are mean and s.d. Note progression of accumulation of EP_{Ca} over 60 min in ECO. Values of EP_{Ca} in the absence of ECO are from Figure 1.

which had accumulated at the endplate was not lost by washing for 30 min.

These experiments on the progress of accumulation of Ca have shown that the endplate accumulation (EP_{Ca}), which is the quantity of interest in this investigation, and which is derived from the difference between the junctional and nonjunctional accumulation is independent of the absolute Ca content of these portions.

The effect of nerve stimulation in ecothiopate on the accumulation of Ca

In previous experiments (Burd & Ferry, 1987), it was found that stimulation of the nerve at 0.02 Hz resulted in the appearance for about 15 min of prolonged contractions localized at the endplate and associated with prolonged endplate currents. To find

Table 1 The effect of the duration of incubation in $^{45}Ca^{2+}$ and ecothiopate (ECO) on the accumulation of Ca at the endplate (EP_{Ca}) and by the nonjunctional portion (NJ_{Ca}) and junctional portion (J_{Ca})

Incub (min)	EP_{Ca} (nmol mg^{-1})	NJ_{Ca} (nmol mg^{-1})	J_{Ca} (nmol mg^{-1})	n
15	0.162 $\pm$ 0.046	0.328 $\pm$ 0.081	0.657 $\pm$ 0.144	8
30	*0.531 $\pm$ 0.29	0.851 $\pm$ 0.418	1.86 $\pm$ 0.532	15
60	†1.17 $\pm$ 0.38	1.17 $\pm$ 0.23	*3.06 $\pm$ 0.546	7
90	*†0.836 $\pm$ 0.521	2.79 $\pm$ 0.41	*4.18 $\pm$ 1.07	7

Values are mean and s.d. of *n* hemidiaphragms. Mean values marked †, * are not different from other values so marked in the same column; unmarked values are significantly different from each other value ($P < 0.05$).

out if these prolonged localized contractions were associated with an increased accumulation of Ca, experiments were done on paired hemidiaphragms from the same mouse, one stimulated at 0.02 Hz, the other unstimulated, the duration of the exposure to ECO being 15 min.

In this experiment, diaphragms were incubated in ^{45}Ca for 15 min so that the tracer could permeate through the extracellular space before the addition of ECO.

Whole diaphragms were immersed in ^{45}Ca -saline at time -15 min and at -10 min, indirect stimulation at 0.02 Hz of one hemidiaphragm was begun, the other hemidiaphragm was not stimulated. At time 0 min, ECO was added and after incubation for a further 15 min, the diaphragms were placed unstimulated in unlabelled physiological saline to wash for 30 min, and then prepared for assay of ^{45}Ca . With the stimulated or unstimulated paired hemidiaphragms the Wilcoxon test showed the accumulation of Ca in the junctional portions (J_{Ca}) to be significantly greater than that in the nonjunctional portions (NJ_{Ca}). The accumulation of Ca at the endplate (EP_{Ca}) of stimulated hemidiaphragms was $0.334 \pm 0.075 \text{ nmol mg}^{-1}$, and when compared with the $0.213 \pm 0.016 \text{ nmol mg}^{-1}$ ($n = 6$) of unstimulated hemidiaphragms (Figure 3), the variability of the results was such that these groups were not significantly different.

It is concluded that the results did not support the hypothesis that stimulation of the nerve at 0.02 Hz in the presence of ECO increased the accumulation of Ca by the diaphragm above that found in the presence of ECO without stimulation. Even though

Figure 3 Histogram showing the mean (columns) and s.d. (vertical bars) of the accumulation of Ca at the endplate (EP_{Ca}) in hemidiaphragms incubated in ^{45}Ca -saline with ecothiopate (ECO) for 15 min and those stimulated in ECO. For further details see text.

there was no significant effect of stimulation, because of the variability of the small number of observations and the difference between the mean values, perhaps the stimulated EP_{Ca} may not be the same as the unstimulated EP_{Ca} .

However, the results are that the accumulation of Ca^{2+} at the endplate after stimulation was not different from that in the absence of stimulation. It is possible that this accumulation is not caused by action potentials in the nerve, or that the accumulation depends on the aggregate of evoked and spontaneous neural activity. Perhaps in these experiments the aggregate was similar in stimulated and unstimulated preparations because stimulation had changed the amount of spontaneous activity (see below). Therefore, to distinguish between the accumulation of Ca consequent upon spontaneous excitation of the nerves and that due to other mechanisms of the spontaneous release of transmitter, experiments were done in which action potentials in nerve and muscle were prevented by treatment with tetrodotoxin.

The effect of tetrodotoxin on the accumulation of Ca after ecothiopate

In this experiment three groups each of three diaphragms were used and incubated for 15 min in ^{45}Ca -saline to load the extracellular space and for a further 30 min in ^{45}Ca -saline only, or with added ECO, or in ^{45}Ca -saline and 10^{-6} M tetrodotoxin (TTX) throughout with ECO added for the final 30 min.

Those diaphragms incubated in ^{45}Ca -saline or in ^{45}Ca -saline with TTX and ECO did not fasciculate. Those incubated in ECO fasciculated and had a swollen endplate region at the end of the incubation. Some of the diaphragms in TTX and ECO were swollen at the endplate, but most seemed normal to the naked eye.

It was found that the accumulation at the endplate (EP_{Ca}) in ^{45}Ca -saline only was $-0.147 \pm 0.103 \text{ nmol mg}^{-1}$, in ^{45}Ca -saline and ECO was $0.361 \pm 0.157 \text{ nmol mg}^{-1}$ and in ^{45}Ca -saline, ECO and TTX was $0.13 \pm 0.061 \text{ nmol mg}^{-1}$ ($n = 6$). Each group was significantly different from each of the others, see Figure 4. The value obtained with incubation in ECO for 30 min was not different from that found in the experiments on the progress of accumulation and shown in Table 1.

It is concluded that TTX prevented about half the accumulation of Ca at the endplate and also prevented the fasciculation. Thus it seems that the fasciculation after ECO due to spontaneous excitation of the nerves (Ferry, 1988) may have been responsible for about half of the accumulation of Ca.

Figure 4 Histogram showing the mean (columns) and s.d. (vertical bars) of the accumulation of Ca at the endplate (EP_{Ca}) incubated for 30 min in ⁴⁵Ca-saline only, with added ecothiopate (ECO) and with ECO and tetrodotoxin (TTX). For further details see text.

Another mechanism which may have contributed to the accumulation at the endplate is the postsynaptic action of ACh released spontaneously either quantally or nonquantally.

The effect of carbachol on the accumulation of Ca

Although Evans (1974) reported an accumulation of Ca at the endplate after incubation with carbachol it was decided to repeat these experiments under the conditions in this laboratory. In these experiments, unstimulated diaphragms were incubated for 30 min in ⁴⁵Ca-saline containing carbachol 10⁻⁴ M. They were then washed in ⁴⁵Ca-free saline and prepared for assay of ⁴⁵Ca. These diaphragms were swollen in the endplate region. In five hemidiaphragms there was an accumulation of Ca at the endplate of 0.649 ± 0.142 nmol mg⁻¹ which was significantly different from that found after incubation in physiological saline alone, and was not different from that found after incubation in ECO for the same time and without stimulation of the nerve (Table 1).

It is concluded that carbachol caused an accumulation of Ca at the endplate. This was about one third of that reported by Evans (1974) in his experiments with 5 mM [Ca²⁺]_o. In our experiments the [Ca²⁺]_o was 2 mM. Evans showed that in 1 mM ACh, the accumulation of Ca at the endplate in 2 mM [Ca²⁺]_o was 0.36 of that in 5 mM. Thus our results are consistent with those published by Evans.

In another experiment with carbachol, ECO was included in the solution used to wash the diaphragms. The accumulation of Ca at the endplate in this experiment was 0.664 ± 0.337 nmol mg⁻¹ and

was not different from that found when the washing solution did not include ECO.

It is concluded that as ECO did not affect the EP_{Ca} of preparations preloaded by incubation in carbachol, it did not affect the efflux of Ca²⁺. Thus the accumulation of ⁴⁵Ca in ECO was not due to prevention of efflux by ECO.

The effect of indirect stimulation on fasciculations after ecothiopate

In the experiments with ECO and with TTX, the accumulation of Ca at the endplate could be fractionated into that part due to a TTX-labile fasciculation and that due to a TTX-resistant process, possibly the action of spontaneously released m.e.p.s and non-quantal ACh. One explanation of the finding that there was no difference in the accumulation of Ca at the endplate between stimulated and unstimulated preparations might be that in both preparations a similar spontaneous release of ACh caused a similar accumulation, and that in the stimulated preparation the aggregate of the fasciculations and nerve stimulation caused accumulation no different from the unstimulated preparation due to the fasciculations only. This might happen if stimulation inhibited fasciculation so that the reduction of accumulation due to this inhibition was equivalent to the gain attributable to stimulation of the nerve, resulting in no net change in the component of EP_{Ca} due to excitation of the nerve.

An experiment was done to investigate the effect on the fasciculations of stimulation of the nerve in three preparations. In these preparations, contractions at 0.1 Hz were recorded, the output of the force transducer being stored on an analogue storage oscilloscope with a 10 s sweep time. Several sweeps were superimposed, then ECO was added. Fasciculations appeared about 3 min later and persisted for about 30 min. In Figure 5 it can be seen that, although the discharge was irregular, after stimulation of the nerve there tended to be fewer spontaneous twitches. It is concluded that stimulation of the nerve decreased the overall amount of fasciculation. The reduction was not quantified so it is not possible to equate this reduction in fasciculatory synaptic activity with that extra activity due to stimulation.

The effect of elevated [Mg²⁺] on the accumulation of Ca after ecothiopate

The ECO-induced changes in the contractile response to nerve stimulation i.e. enhancement of the twitch due to repetitive firing of the muscle cells, a prolonged contraction at the endplate and fascicu-

Figure 5 Mouse phrenic nerve-diaphragm preparation set up to display isometric tension on a storage oscilloscope, a.c.-coupled to minimize baseline drift, sweep time 95 s. Each trace is of several superimposed sweeps. Calibration 1 g. (a) Trace of 3 superimposed twitches elicited at 0.01 Hz. No ecothiopate (ECO). (b) Trace of 3 superimposed sweeps from 200 s to 500 s after ECO, no stimulation. Note spontaneous fasciculations. (c) Trace of 5 superimposed sweeps from 500 s to 1000 s after ECO with fasciculations and contractions evoked at 0.01 Hz. Note the prolonged localized contraction recorded differentiated as an elevated then depressed baseline, at which time there was a small reduction of the fasciculations. (d) Trace of 4 superimposed sweeps from 1000 s to 1400 s after ECO. Note the clear reduction of fasciculations after stimulation. (e) Trace of 9 superimposed sweeps from 1400 s to 2300 s after ECO, no stimulation. Note fairly even distribution of a generally reduced fasciculatory activity.

lation were not seen in experiments in which the saline contained 3.5 mM Mg^{2+} (Burd & Ferry, 1987), but m.e.p.ps were increased in amplitude and duration.

The effect of this concentration of Mg^{2+} on the accumulation of Ca after ECO was investigated. Five hemidiaphragms were incubated in 3.5 mM Mg^{2+} with ECO for 30 min, then they were prepared for assay of ^{45}Ca . It was found that the endplate

accumulation of Ca (EP_{Ca}) was -0.003 ± 0.132 nmol mg^{-1} which was no different from that found in control preparations incubated in ^{45}Ca -saline for 30 min and was significantly less than that of preparations exposed to ECO in the physiological saline containing 1 mM Mg^{2+} (Table 1).

It is concluded that elevation of $[Mg^{2+}]_o$ prevented the accumulation of Ca at the endplate expected in the presence of ECO which exerted its anticholinesterase effect.

Discussion

There can be little doubt that there was accumulation of Ca derived from the bathing medium at the endplate after ECO, but our experiments do not provide direct evidence of the location of this accumulated Ca, which does not wash out and could have been sequestered by both presynaptic and postsynaptic structures. It is considered that the Ca may be sequestered intracellularly in the subsynaptic region of the muscle cells for the following reasons. If the Ca accumulated at the endplate were to have been sequestered by extracellularly-accessible structures, then it would be expected that such accumulation would have occurred in the absence of ECO, which is not the case. The role of ECO seems to be to make the site of Ca sequestration accessible from the extracellular space. Muscles incubated in ECO or in carbachol have a swollen endplate region. On microscopical examination, such regions show a considerable hypercontraction of the subsynaptic sarcomeres with swollen sarcoplasmic reticulum and mitochondria, an appearance attributable to Ca overload (Cullen & Ferry, 1982) with apparently normal nerve terminals.

Other workers have shown that Ca^{2+} enters the endplate of vertebrate skeletal muscle as a consequence of agonist action on the endplate channels (Jenkinson & Nicholls, 1961; Takeuchi, 1963; Evans, 1974; Miledi *et al.*, 1980). In our experiments with ECO the agonist is probably ACh released from the nerve terminals. The lack of effect of ECO on the washout of ^{45}Ca suggests it did not open channels permeable to Ca^{2+} . In the presence of an anticholinesterase the ACh acting on the endplate channels may have been released in several ways. The spontaneous release of ACh includes that released quantally which causes m.e.p.ps and that released non-quantally to cause a small depolarization of the endplate region equivalent to that produced by about 10^{-8} M ACh in the frog (Katz & Miledi, 1977), 10^{-7} M in mice (Vyskočil *et al.*, 1983) and 6×10^{-9} M in rats (Smith, 1984). In unstimulated tissues, the amount released non-quantally from the nerve is about ten times greater than that released quantally (Miledi *et al.*, 1983). The evoked release is quantal

and gives rise to the endplate potentials; included in this category is the ACh released by excitation of the nerve terminal during the fasciculations which arise after ECO. The non-quantal release of ACh is not dependent on Ca^{2+} and is not affected by TTX (Vizi & Vyskočil, 1979; Doležal & Tuček, 1983; Stanley & Drachman, 1983) and is from the cytoplasm of the nerve (Molenaar *et al.*, 1987).

The experiments with TTX showed that about half of the EP_{Ca} persisted and was associated with a local hypercontraction of the cell. This suggests that the m.e.p.ps and the non-quantal release together could account for about half of the accumulation of Ca at the endplate, the other half presumably being due to transmitter action following excitation of the nerve.

The experiments with stimulation of the nerve indicate that the accumulation at the endplate (EP_{Ca}) in preparations which were fasciculating and were stimulated was not different from those which only fasciculated. Taken with the experiments which showed a reduction of fasciculation after stimulation of the nerve it may be concluded that the responses evoked at 0.02 Hz which usually were associated with prolonged localized contractions at the endplate were responsible for some of the accumulation of Ca although this cannot be quantified. This conclusion is supported by experiments with more vigorous stimulation of the nerve by trains of 5 pulses at 50 Hz every 5 s in which the Ca content of the endplate assayed by atomic absorption spectrophotometry was elevated 5 min after ECO (Ferry, unpublished).

The result of the experiments with 3.5 mM $[\text{Mg}^{2+}]_0$ was that the accumulation of Ca at the endplate was reduced to zero. Half of the reduction is attributable to the prevention of fasciculation, as in the experiments with TTX. The remainder of the reduction of Ca accumulation may be due to prevention of the effects of ACh released non-quantally and quantally, by competition between Mg^{2+} and Ca^{2+} in the synaptic cleft reducing Ca^{2+} entry through the opened endplate channels (Adams *et al.*, 1986). In this regard, Jenkinson & Nicholls (1961) and Takeuchi (1963) reported abolition by Mg^{2+} of the local contracture normally found after ACh. Evans (1974) found that an increased $[\text{Mg}^{2+}]_0$ reduced the

accumulation of Ca in the junctional region. Because ACh is released nonquantally in larger amounts than that causing m.e.p.ps, despite the increased frequency of the latter (Laskowski & Dettbarn, 1975), it may be that the nonquantal release of ACh is the most important spontaneous process in the accumulation of Ca at the endplate.

The accumulation at the endplate EP_{Ca} in 10^{-4} M carbachol is similar to that found after ECO, which suggests that the carbachol was about twice as effective as the non-quantally released ACh in loading the endplate region with Ca, even though the concentration of carbachol was 10^3 – 10^4 times that estimated for non-quantally released ACh. This inconsistency may be explained if the effect of carbachol were to have been limited by desensitization of the endplate cholinergic receptors. In our experiments with ECO, m.e.p.ps were present, but the m.e.p.ps of rat diaphragm 'promptly disappeared' in the presence of 10^{-4} M carbachol (Creese & Mitchell, 1981), and the endplate current due to carbachol applied to guinea-pig diaphragm declined rapidly over the first 5 min (Creese *et al.*, 1987). Furthermore, Bray *et al.* (1982) found that m.e.p.ps were unaffected by 10^{-7} M carbachol but reduced by 10^{-6} M. Thus it is considered that the accumulation of Ca after carbachol was limited by desensitization and the accumulation due to non-quantal release of ACh after ECO was not limited to the same extent.

To conclude, although stimulation of the nerve after ECO caused an additional accumulation of Ca at the endplate which may or may not have been involved in the prolonged contraction at the endplate in our experiments with a very low frequency (0.02 Hz) of stimulation, the action of non-quantally released ACh is responsible for about half of the total accumulation at the endplate.

It may be that this accumulation of Ca is associated with the structural changes at the endplate in anticholinesterase-induced myopathy, in particular that after ECO reported briefly by Cullen & Ferry (1982).

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References

- ADAMS, D.J., DWYER, T.M. & HILLE, B. (1986). The permeability of endplate channels to monovalent and divalent metal cations. *J. Gen. Physiol.*, **75**, 493–510.
- ARIENS, A.T., MEETER, E., WOLTHIUS, O.L. & VAN BENTHEM, R.M.J. (1969). Reversible necrosis at the endplate region in striated muscles of the rat poisoned with cholinesterase inhibitors. *Experientia*, **25**, 57–59.
- BRAY, J.J., FORREST, J.W. & HUBBARD, J.I. (1982). Evidence for the role of non-quantal acetylcholine in the maintenance of the membrane potential of rat skeletal muscle. *J. Physiol.*, **326**, 285–296.
- BURD, P.F. & FERRY, C.B. (1987). A prolonged contraction at the end-plate region of rats and mice after anticholinesterases *in vitro*. *J. Physiol.*, **390**, 429–440.
- CREESE, R. & MITCHELL, L.D. (1981). Spontaneous recovery from depolarizing drugs in rat diaphragm. *J. Physiol.*, **313**, 173–186.
- CREESE, R., HEAD, S.D. & JENKINSON, D.F. (1987). The role

- of the sodium pump during the prolonged endplate current in diaphragm. *J. Physiol.*, **384**, 377–404.
- CULLEN, M.J. & FERRY, C.B. (1982). Ultrastructural effects of an elevated calcium ion concentration in skeletal muscle fibres. An experimental myopathy at the neuromuscular junction induced by an anticholinesterase. *Fifth International Congress on Neuromuscular Diseases*.
- DOLEŽAL, V. & TUČEK, S. (1983). The synthesis and release of acetylcholine in normal and denervated rat diaphragms during incubation *in vitro*. *J. Physiol.*, **334**, 461–474.
- EVANS, R.H. (1974). The entry of labelled calcium into the innervated region of the mouse diaphragm muscle. *J. Physiol.*, **240**, 517–534.
- FERRY, C.B. (1988). The origin of the anticholinesterase-induced repetitive activity of the phrenic nerve-diaphragm preparation of the rat *in vitro*. *Br. J. Pharmacol.*, **94**, 169–179.
- HEBB, C.O., KRNJIVIĆ, K. & SILVER, A. (1964). Acetylcholine and choline acetyltransferase in the diaphragm of the rat. *J. Physiol.*, **171**, 504–513.
- JENKINSON, D.H. & NICHOLLS, J.G. (1961). Contractures and permeability changes produced by acetylcholine in depolarized denervated muscle. *J. Physiol.*, **159**, 111–127.
- KATZ, B. & MILEDI, R. (1977). Transmitter leakage from nerve terminals. *Proc. R. Soc. B*, **196**, 59–72.
- LASKOWSKI, M.B. & DETTBARN, W.D. (1975). Presynaptic effects of neuromuscular cholinesterase inhibition. *J. Pharmacol. Exp. Ther.*, **194**, 351–361.
- MILEDI, R., MOLENAAR, P.C. & POLAK, R.L. (1983). Electrophysiological and chemical determination of acetylcholine release at the frog neuromuscular junction. *J. Physiol.*, **334**, 245–254.
- MILEDI, R., PARKER, I. & SCHALOW, G. (1980). Transmitter induced calcium entry across the postsynaptic membrane at frog endplate measured using Arsenazo III. *J. Physiol.*, **300**, 197–212.
- MOLENAAR, P.C., OEN, B.S., POLAK, R.L. & VAN DER LAAKEN, A.L. (1987). Surplus acetylcholine and acetylcholine release in rat diaphragm. *J. Physiol.*, **385**, 147–168.
- SALPETER, M.M., LEONARD, J.P. & KASPRZAK, H. (1982). Agonist-induced postsynaptic myopathy. *Neurosci. Comm.*, **1**, 73–83.
- SMITH, D.O. (1984). Acetylcholine storage, release and leakage at the neuromuscular junction of mature adult and aged mice. *J. Physiol.*, **347**, 161–176.
- STANLEY, E.F. & DRACHMAN, D.B. (1983). Botulinum toxin blocks quantal but not non-quantal release of acetylcholine at the neuromuscular junction. *Brain Res.*, **261**, 172–175.
- TAKEUCHI, N. (1963). Effects of calcium on the conductance change of the end-plate membrane during the action of transmitter. *J. Physiol.*, **167**, 141–155.
- VIZI, E.S. & VYSKOČIL, F. (1979). Changes in total and quantal release of acetylcholine in mouse diaphragm during activation and inhibition of membrane ATPase. *J. Physiol.*, **286**, 1–14.
- VYSKOČIL, F., NIKOLSKY, E. & EDWARDS, C. (1983). An analysis of the mechanism underlying the non-quantal release of acetylcholine at the mouse neuromuscular junction. *Neuroscience*, **9**, 429–435.

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