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FURTHER OBSERVATIONS ON THE DISTRIBUTION OF ACETYLCHOLINE-REACTIVE SITES IN SKELETAL MUSCLE

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It has been known since Langley's (1907) work that the sensitivity of a muscle to certain chemical stimuli shows striking differences along the fibre surface, the region of the neuromuscular junction being much more reactive than the rest of the fibre. Recently it has been found that marked changes can be produced in this distribution of chemoreceptive properties, for example, by complete or partial denervation and re-innervation of the muscle fibre (Ginetzinsky & Shamarina, 1942; Axelsson & Thesleff, 1959; Miledi, 1960*a*, *c*; Diamond & Miledi, 1962), and by direct muscle transection (Katz & Miledi, 1961*b*). While extending this work we came across several additional observations. One is the degree of variability to be found among apparently normal muscle fibres: although the sensitivity to acetylcholine (ACh) at the myoneural junction is much higher than elsewhere, the low level to which the sensitivity falls in the extra-junctional region varies in different fibres over more than a thousandfold range, between less than 1/500,000 and approximately 1/100 of the maximum at the end-plate. Another observation was that the muscle-tendon junction often shows a separate peak of ACh sensitivity, much lower than that at the end-plate, but well above that of the rest of the fibre. This is of interest because of the local concentration of ACh-esterase which has been reported at myoneural and muscle-tendon junctions by Couteaux (1953), Gerebtzoff (1954) and Schwarzacher (1960*b*).

METHODS

Most experiments were made on sartorius muscles of English *Rana temporaria*, at temperatures around 20° C. In a few experiments the sartorius muscles of *R. esculenta*, the rectus abdominis of *R. temporaria*, and the diaphragm and rectus abdominis of the rat were used.

Membrane potentials were recorded from superficial muscle fibres with an intracellular micro-electrode, and 'spot-sensitivities' to ACh were measured by iontophoretic application of the drug through an externally applied micropipette (see del Castillo & Katz, 1955; Miledi, 1960*a*). In some experiments twin pipettes were used, containing ACh in one pipette, and carbachol or edrophonium in the other (cf. del Castillo & Katz, 1957*a*; Katz & Thesleff, 1957*b*; Miledi, 1962).

At 'insensitive regions' it was necessary to verify that the drugs were released immediately on the surface of the appropriate muscle fibre and not, for instance, on a neighbouring fibre or on part of the adjoining tendon. This was ascertained by repeated testing, with the drug pipette exerting various degrees of pressure, and eventually penetrating and just being withdrawn from the fibre interior (the entry and withdrawal were signalled by the appearance, and disappearance, of an electrotonic potential; see del Castillo & Katz, 1955).

The steady 'braking' voltage on the drug pipette (del Castillo & Katz, 1955) was made just sufficient to prevent the appearance of a detectable depolarization when the tip of the pipette was close to a sensitive end-plate spot. The ACh test pulse was adjusted so as to produce a depolarization of one or several millivolts, though at regions of low sensitivity much smaller amplitudes had to be accepted. Both intensity and duration of the ACh pulse were varied according to the sensitivity of the region: durations ranged between about 1 msec for the most sensitive, and 0·35 sec for the least sensitive spots.

In devising a scale of ACh sensitivities a procedure similar to that described by Miledi (1960*a*) was adopted: the amplitude, in millivolts, of the transient membrane depolarization due to the ACh pulse was divided by the coulomb ($\times 10^{-9}$) quantity passed through the ACh pipette. In other words, if a pulse of '10^{-9} C' produced a depolarization of '10 mV', the 'sensitivity' was '10 units'.

As previously shown (Miledi, 1960*a*), the time course of an ACh potential becomes slower the lower the regional sensitivity. The reason for this is probably that at relatively insensitive spots the density of 'receptor' sites is low, and a larger membrane area must be acted upon. With the large pulses needed for this purpose the nearest receptor sites would necessarily become saturated, and more distant sites, activated after a longer diffusion time, make an increasingly important contribution to the potential change (see del Castillo & Katz, 1955). In view of such complicating factors our procedure gives only a rough measure of local sensitivity (or of the local density of ACh-reactive membrane sites, or 'receptors'). Theoretically it might perhaps have been better to measure maximum rates, rather than amplitudes of depolarization, but amplitudes are determined more conveniently and with greater accuracy.

As was pointed out by Miledi (1960*a*), the less sensitive a region, the faster and more pronounced is the 'desensitization' which results from repetitive application of ACh pulses. This may again be, at least to some extent, a consequence of the large doses needed at such regions, for the speed of desensitization of any given site greatly increases with the concentration of the depolarizing drug (Katz & Thesleff, 1957*a*). This is an important point to remember, for it makes necessary the use of longer intervals between successive test pulses when one is studying regions of relatively low sensitivity.

RESULTS

ACh receptors at the muscle-tendon junction

In view of the curious localization of ACh-esterase at muscle-tendon junctions, it was of interest to see whether this region had appreciable sensitivity to ACh. Previous attempts to obtain an ACh depolarization at the pelvic end of the frog sartorius had given negative results (Miledi, 1960*b*; Schwarzacher, 1960*b*; Katz & Miledi, 1961*a*). However, in the course of the present more extensive work we observed occasionally a small response due to application of a large ACh pulse at the extreme end of a muscle fibre. Pursuing this further, a great deal of variation was found between different fibres, but on carefully applying the drug micro-

pipette to the cleaned terminal portion of the fibre the majority (214 out of 249 fibres) gave a detectable depolarization.

Examples are shown in Fig. 1, and the distribution of sensitivities at

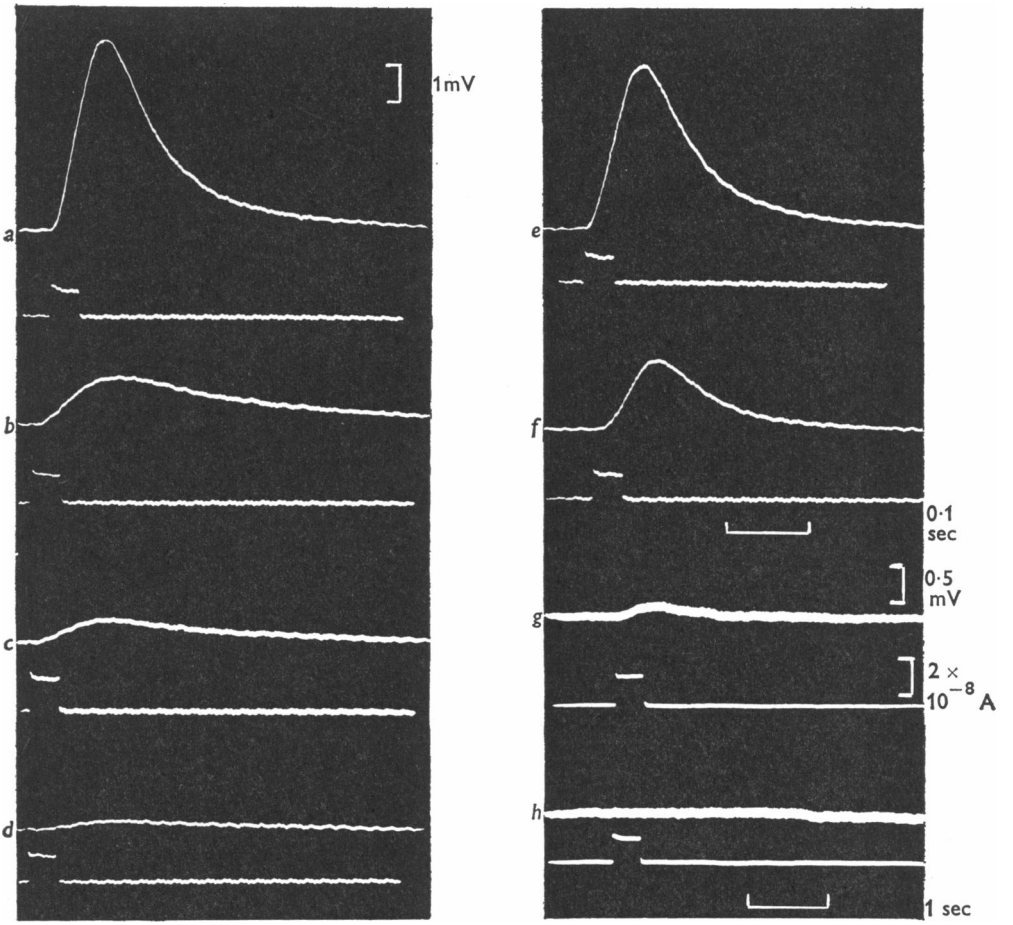

Fig. 1. Acetylcholine potentials recorded at a muscle-tendon junction. Frog sartorius, intracellular recording. Lower trace of each pair shows iontophoretic current pulse. The ACh pipette was placed at the following distances (μ) from the pelvic tendon-junction; *a*, *e* and *f*, two spots at 10; *b*, 75; *c*, 300; *d*, 500; *g*, 750; *h*, 1200. The recording micro-electrode was inserted close to the tendon junction for records *a*–*e*; it was then re-inserted at a distance of 1230 μ for records *f*–*h*. 1 mV and 0·1 sec scales apply to records *a*–*f*, the 0·5 mV and 1 sec scales to *g* and *h*.

the 249 muscle-tendon junctions is seen in Fig. 2. To include the 35 cases in which no response was recorded (blank columns) the assumption was made that the 'true' sensitivity was 0·5 log. unit (about 3 times) below

the limit of detection in the particular case (this limit depended on electrode noise in the recording circuit and on the limited current which could be passed through the ACh pipette). Values of sensitivities were scattered over a very wide range, between 10^{-4} and 10 units (1 unit = 1 mV/10^{-9} C). The mean of the log. distribution in Fig. 2 was $3{\cdot}9 \times 10^{-2}$. This value is about 10,000 times lower than the peak sensitivity which one frequently obtains when carefully exploring a superficially located nerve-muscle junction.

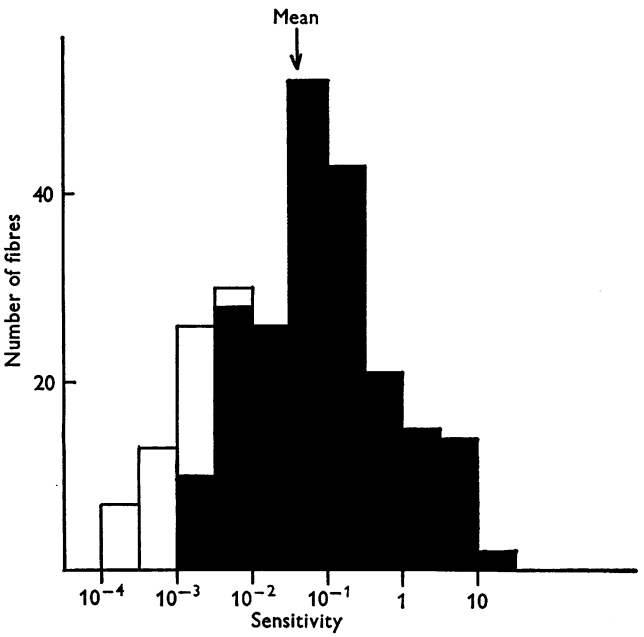

Fig. 2. Histogram showing the distribution of ACh sensitivities at the muscle-tendon junctions of 249 frog sartorius fibres; semi-log scale.

In many fibres the ACh sensitivity showed a sharp peak at the tip of the muscle-tendon junction and rapidly fell below detectable levels, within 1 mm from the junction. Figure 3 shows the spatial distribution at one of the most sensitive muscle-tendon junctions which we encountered. The gradient was almost as steep as that around an end-plate focus (Miledi, 1962), the sensitivity falling to 1/10 in about 0·3 mm. In some fibres even sharper gradients were seen; in others, although there was a local maximum at the extreme end of the fibre, the sensitivity remained at a detectable level over the whole fibre length which could be examined (see Miledi, 1962; and Table 1).

A few tests were made to check whether the pharmacological properties

at the muscle-tendon junction differ qualitatively from those at the end-plate. Curare (D-tubocurarine chloride) greatly reduced the ACh sensitivity in concentrations of 10^{-6} and above. Tubocurarine chloride in a concentration of 10^{-6} g/ml. applied to the pelvic end of a sartorius fibre of *Rana esculenta*, depressed the ACh sensitivity to 1/8, which is not far off the curare effect on end-plates of normal or denervated sartorius muscles (see Jenkinson, 1960).

When a potent anticholinesterase was applied (neostigmine methylsulphate, 10^{-6} g/ml., for about 30 min, or edrophonium chloride by local iontophoresis), the ACh effect showed no or only slight potentiation;

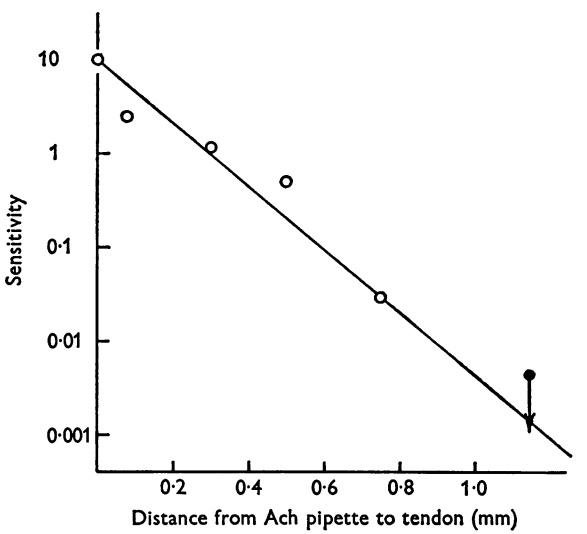

Fig. 3. Gradient of ACh sensitivity at muscle-tendon junction; same muscle fibre as in Fig. 1; semi-log scale.

the maximum increase, after iontophoretic pulse application of edrophonium amounted to about 20%, while often the ACh potential was reduced after a large dose of edrophonium. This behaviour contrasts with the striking potentiation obtained at the end-plate (Katz & Thesleff, 1957*b*; Miledi, 1962), but resembles the behaviour of extra-junctional receptor sites (Miledi, 1962), where cholinesterase seems to be either absent or much less effective in removing ACh from the receptors. The very small size of the potentiation found at the muscle-tendon junction could be interpreted in various ways: it may be due to there being only relatively low local concentrations of cholinesterase, or to less effective alignment between receptive and hydrolytic sites. There is also the obvious possibility that the large doses of ACh which are required at a region of

TABLE 1. ACh sensitivity at pelvic region of eight sartorius muscles: the 'means' are geometric mean values.

	Muscle-tendon junction			2–5 mm from pelvic end		
		Sensitivity			Sensitivity	
Number of experiment	No. of fibres	Mean	Range	No. of fibres	Mean	Range
1	10	3·1	$8{\cdot}7\times10^{-1}$ to 10	7	$3{\cdot}8\times10^{-2}$	9×10^{-3} to $1{\cdot}5\times10^{-1}$
2	5	2·3	$7{\cdot}9\times10^{-1}$ to 9·1	15	$2{\cdot}2\times10^{-1}$	10^{-1} to $4{\cdot}9\times10^{-1}$
3	10	$7{\cdot}7\times10^{-1}$	$9{\cdot}4\times10^{-2}$ to 3·6	5	$2{\cdot}4\times10^{-1}$	10^{-1} to $4{\cdot}2\times10^{-1}$
4	5	$5{\cdot}3\times10^{-1}$	3×10^{-1} to $7{\cdot}8\times10^{-1}$	8	$9{\cdot}3\times10^{-3}$	$3{\cdot}9\times10^{-3}$ to $1{\cdot}5\times10^{-2}$
5	10	$2{\cdot}1\times10^{-1}$	$6{\cdot}3\times10^{-3}$ to 1·3	9	$<1{\cdot}6\times10^{-3}$	$<9{\cdot}4\times10^{-4}$ to $4{\cdot}8\times10^{-3}$
6	5	$1{\cdot}4\times10^{-1}$	$7{\cdot}5\times10^{-2}$ to 3×10^{-1}	5	$<7{\cdot}8\times10^{-3}$	$<5{\cdot}8\times10^{-3}$ to $1{\cdot}3\times10^{-2}$
7	10	$5{\cdot}7\times10^{-2}$	$7{\cdot}5\times10^{-3}$ to $1{\cdot}7\times10^{-1}$	10	$4{\cdot}2\times10^{-2}$	$1{\cdot}3\times10^{-2}$ to $1{\cdot}3\times10^{-1}$
8	10	$7{\cdot}5\times10^{-3}$	$<2\times10^{-3}$ to 10^{-2}	5		all $<4{\cdot}3\times10^{-3}$

Note: Peak sensitivities at end-plate ranged up to 670, depending largely on the chance of locating highly sensitive spots near nerve endings. Highest sensitivities observed at muscle-tendon junction, 10; at 2–5 mm from tendon, 5×10^{-1}.

low sensitivity tend to saturate the enzyme, and that hydrolysis is therefore less effective. These problems will be discussed in a separate paper by Miledi.

In an experiment in which carbachol (a stable analogue of ACh) and ACh were applied for comparison from a twin pipette, the relative sensitivity to carbachol was approximately 1/10–1/20 of the ACh sensitivity. The same ratio was obtained at 'extra-junctional' receptor sites near the end-plates, while at focal end-plate sites the ratio was nearer 1:5. These observations are consistent with the finding that hydrolysis of ACh at the

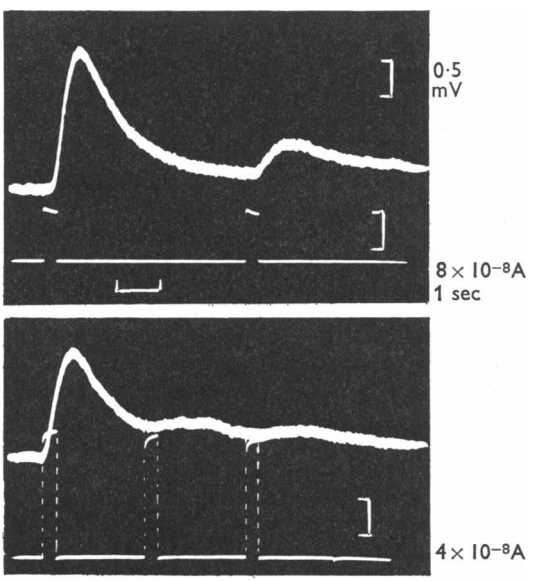

Fig. 4. Examples of desensitization following ACh pulses applied at two muscle-tendon junctions.

muscle-tendon junction (and at non-focal receptor sites) is relatively ineffective. They also show that the pharmacological discrimination of the muscle-tendon receptors between ACh and carbachol is similar to that at other typical 'cholinoceptive' sites (see Welsh & Taub, 1948; del Castillo & Katz, 1957*b*).

The ACh responses showed rapid desensitization (Fig. 4). In view of the large doses applied, this was to be expected; it does not necessarily follow that the receptors in this region are more liable to inactivation by a given dose of the drug than are end-plate receptors.

These pharmacological comparisons are incomplete, but they suggest that there are no important differences in the chemical specificity of the receptors at different regions of the muscle fibre, and that the observed

gradients of sensitivity along the fibre may be merely a function of differences in the local surface density of the receptor molecules.

Other muscles. Most of the present experiments were done during December 1960–April 1961, on sartorius muscles of *Rana temporaria*. A few checks were made during the summer months with similar results. The variation in sensitivity between different specimens is probably even greater than is apparent from the present results, because in previous experiments (done during the summer months and using the same technique), we generally failed to find any sensitivity outside the innervation zone (cf. Miledi, 1960*b*). As we extended our experiments to other muscles additional variants were seen. Thus, muscle fibres of the rectus abdominis were found to have smaller gradients of sensitivity between the peaks at the end-plates and the minima at an intermediate position between end-plate and tendon. The fibres tested were probably 'twitch fibres' because in all of them spike potentials could be obtained by a sufficient dose of ACh or by mechanical injury with the micro-electrode. A distribution of ACh sensitivity similar to that seen in the rectus abdominis was found in the sartorius muscle of a toad, the level being about 0·8 (0·8 mV/10^{-9} C) at a position 3 mm from the pelvic tendon, while the sensitivity at the tendon end rose to about 9. In the rat no responses to ACh were detected outside the 'circumjunctional' zone in diaphragm muscle fibres, confirming earlier observations (Miledi, 1960*b*), but in the rectus abdominis four out of twenty-two fibres showed a sensitivity to ACh at the myotendinous junction ranging from 10^{-2} to 1 unit.

DISCUSSION

The present experiments give some information on the degree of variability in the spread and occurrence of ACh sensitivity beyond the region of the nerve-muscle junction. The 'extra-junctional' sensitivity is low compared with the peak at the end-plate, but within the low-level range there are variations of several orders of magnitude. It seemed possible that some of the variations at the muscle-tendon junction might have arisen from extraneous factors, such as a connective tissue or tendinous barrier which might more or less shield the receptive sites. We tried to overcome this by cleaning the muscle surface and by exerting varying pressure with the drug pipette. In one experiment a solution of collagenase was applied which after 2 hr caused the muscle to separate from its ligated tendons. None of these procedures reduced the scatter of the measurements. At present we remain ignorant of the factors which control the chemoreceptor properties of the muscle surface. A particularly striking example of the lability of this property is shown by the development of

supersensitivity which follows mechanical injury (Katz & Miledi, 1961*b*, 1964).

The occurrence of a separate low peak of sensitivity at the tendon end of the fibre (and of histochemically detectable ACh-esterase) is a curious phenomenon which may find an explanation when its developmental origin has been explored. One of the structural peculiarities of the muscle-tendon junction is the formation of deep folds in the muscle membrane which, similarly to the post-junctional folds at the end-plate, appear to be the sites at which ACh-esterase is concentrated (Edwards, Ruska, de Souza Santos & Vallejo-Freire, 1956; Couteaux, 1958; Schwarzacher, 1960*a*). There is, however, no obvious relation between this feature and the increased local sensitivity to ACh, for the membrane folds are restricted to the extreme tip of the muscle fibre (within 30 μ or less), while the ACh-sensitivity extends with a gradient over a few hundred microns. The situation resembles to some extent that at the end-plate, where ACh sensitivity is detectable well beyond the region of the junctional folds, in contrast with the more sharply restricted localization of ACh-esterase (Miledi, 1960*b*, 1962).

The question may be raised whether focal concentrations of cholin-esterase and of ACh sensitivity, away from the adult end-plate, could be the residue of an embryological site of innervation. However, in the foetal rat diaphragm no myoneural junctions were found except in the middle of the fibres (Diamond & Miledi, 1962), while cholinesterase is demonstrable at myotendinous junctions of the adult diaphragm.

SUMMARY

1. Extensive measurements were made of the low-level sensitivity to acetylcholine (ACh) obtained outside end-plate regions in normal frog sartorius muscle. Intracellular recording and iontophoretic micro-application of ACh were employed.

2. In many fibres the ACh sensitivity was found to reach a maximum at the muscle-tendon junction. This maximum was very low (frequently less than 1/1000) compared with the peak sensitivity at the end-plate, but much higher than the barely detectable sensitivity in other nerve-free zones.

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