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# DESENSITIZATION IN THE INNERVATED AND IN THE CHRONICALLY DENERVATED SOLEUS MUSCLE OF THE MOUSE

MOIRA T. HALL, M.A. MALEQUE & R.M. WADSWORTH
Department of Physiology and Pharmacology, University of Strathclyde, Glasgow G1 1XW

1 A study was made of desensitization in chronically denervated and in normally innervated mouse soleus muscles.

2 Very high concentrations of acetylcholine produced small contractions of the innervated muscles; these were reduced in size when the addition was repeated 1 min after wash-out.

3 Desensitization in innervated muscles was receptor specific: contractions in response to KCl and caffeine were not reduced following acetylcholine, nor did KCl produce desensitization.

4 In chronically denervated muscles non-specific desensitization was observed if acetylcholine was added in the presence of low concentrations of acetylcholine or carbachol. Contractions to KCl but not to caffeine were reduced. KCl produced a similar kind of desensitization.

5 After washing out moderate or supramaximal concentrations from the chronically denervated muscle no desensitization was observed. However, 1 min after washout of very high concentrations the muscle was non-specifically desensitized.

6 It is concluded that relatively high concentrations of acetylcholine are required to produce specific desensitization in the soleus muscle. Chronically denervated muscles, being supersensitive, show non-specific loss of sensitivity with concentrations of acetylcholine too low to produce specific desensitization.

## Introduction

Katz & Thesleff (1957) observed a reduction in the depolarization of frog muscle endplates produced by acetylcholine when iontophoretically applied during a longer conditioning pulse of acetylcholine. They postulated that this was due to a change in the receptor properties on prolonged activation and proposed a cyclic model where the drug-receptor complex changes to a desensitized form which reverts only slowly to the active form. Using the chick biventer, Rang & Ritter (1970) provided evidence that desensitization involves receptor inactivation and mathematically analysed various possible mechanisms. They found that the rate constant of recovery from desensitization was the same for all agonists and this, in conjunction with their other data, was held to favour the cyclic model proposed by Katz & Thesleff (1957). Comparable experiments have not been performed in denervated muscle where receptors are formed *de novo* although desensitization is known to occur (Axelsson & Thesleff, 1959; Miledi, 1960a). After chronic denervation acetylcholine receptors can be demonstrated outside the endplate zone to which receptors in innervated fibres are confined (Axelsson & Thesleff, 1959). We have examined the desensitization process at these receptors in the chronically denervated mouse soleus muscle.

## Methods

Male mice weighing 20-30 g were anaesthetized with ether. The left sciatic nerve was aseptically sectioned in the popliteal space and about 1 cm removed. The wound was closed and the animals allowed to regain consciousness. After 7 to 12 days the animals were killed by a blow on the head and the soleus muscles were dissected from both legs. The denervated muscles weighed about 12% less than those from non-operated legs.

The muscles were suspended in 5 ml baths containing Krebs-Henseleit solution of the following composition (mM): $Na^+$ 143, $K^+$ 5.8, $Mg^{2+}$ 1.2, $Ca^{2+}$ 2.5, $H_2PO_4^-$ 1.2, $HCO_3^-$ 25, $Cl^-$ 128, $SO_4^{2-}$ 1.2, glucose 11.1, which was maintained at 37°C and bubbled with 5% $CO_2$ in $O_2$. Tension was measured with Grass FTO3 C or Nihon Kohden 58-IT isometric transducers and

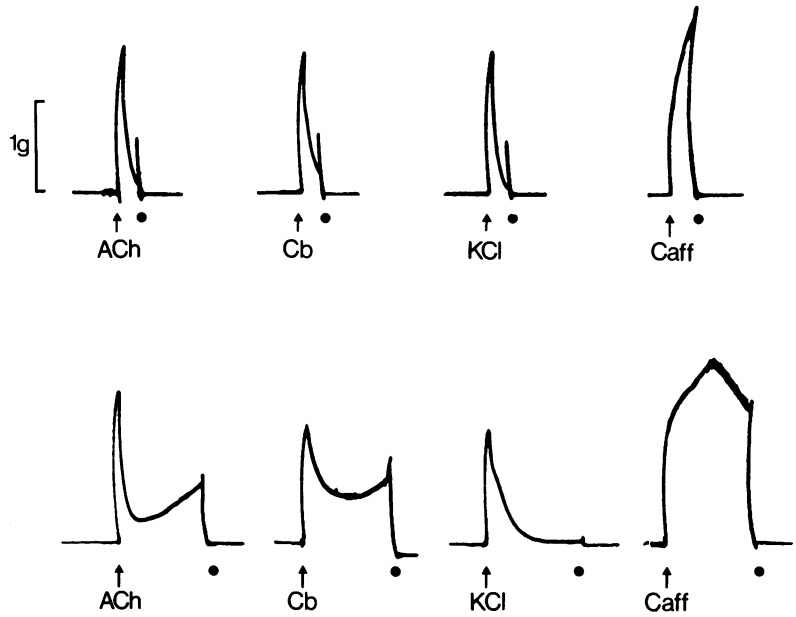


**Figure 1** Contractions of the chronically denervated mouse soleus muscle obtained by the addition of acetylcholine $5.5 \times 10^{-6}$ M (ACh), carbachol $5.5 \times 10^{-6}$ M (Cb), potassium chloride 100 mM (KCl) or caffeine 26 mM (Caff). The drugs were washed out (•) after 1 min (top row) or after 4 min (bottom row).

recorded on a Grass 7B polygraph. The resting tension was adjusted to 1 gram.

The drugs used were acetylcholine chloride (Sigma), caffeine citrate (Evans), carbachol chloride (BDH). The concentrations quoted in the text are the final bath concentrations.

## Results

### *Chronically denervated*

Acetylcholine in concentrations greater than $5.5 \times 10^{-7}$ M produced contractions of the denervated mouse soleus. The contraction reached a maximum in about 6 s and tension then returned towards the baseline (Figure 1). If the contact time was 4 min, a second phase of the contraction was normally seen (Figure 1). In some preparations the second phase became more prominent during the course of the experiment, although in 4 out of 15 experiments it was absent. In two preparations the second phase was greater than the first. With higher concentrations of acetylcholine, the second phase was found to develop more rapidly. A wash contraction was normally produced after rinsing out acetylcholine (Figure 1).

Contractions in response to carbachol (in concentrations greater than $5.5 \times 10^{-7}$ M) and potassium chloride (KCl) (10-150 mM) were similar in form to those in response to acetylcholine, except that KCl did not produce a second phase on prolonged exposure (Figure 1). Contractions to caffeine ($2.6 \times 10^{-3}$—$2.6 \times 10^{-2}$ M) developed more slowly, but the tension was maintained during 4 min contact time (Figure 1).

Dose-response curves were constructed for acetylcholine, carbachol, caffeine and KCl (Figure 2). The acetylcholine and carbachol curves were parallel, acetylcholine being 2.7 times more potent than carbachol. The 50% maximum contraction (determined by inspection of the graph) required $7.6 \times 10^{-7}$ M acetylcholine. The slopes of the caffeine and KCl dose-response curves were greater than the acetylcholine or carbachol curves. The maximum contraction produced by caffeine was significantly greater than that produced by acetylcholine or carbachol ($P < 0.01$).

Desensitization, as described by Rang & Ritter (1970), could not be obtained in the chronically denervated mouse soleus muscle. No effect at all was observed 1 or 3 min after washout of submaximal ($5.5 \times 10^{-6}$—$5.5 \times 10^{-5}$ M) or even supramaximal ($5.5 \times 10^{-5}$—$2.8 \times 10^{-4}$ M) con-

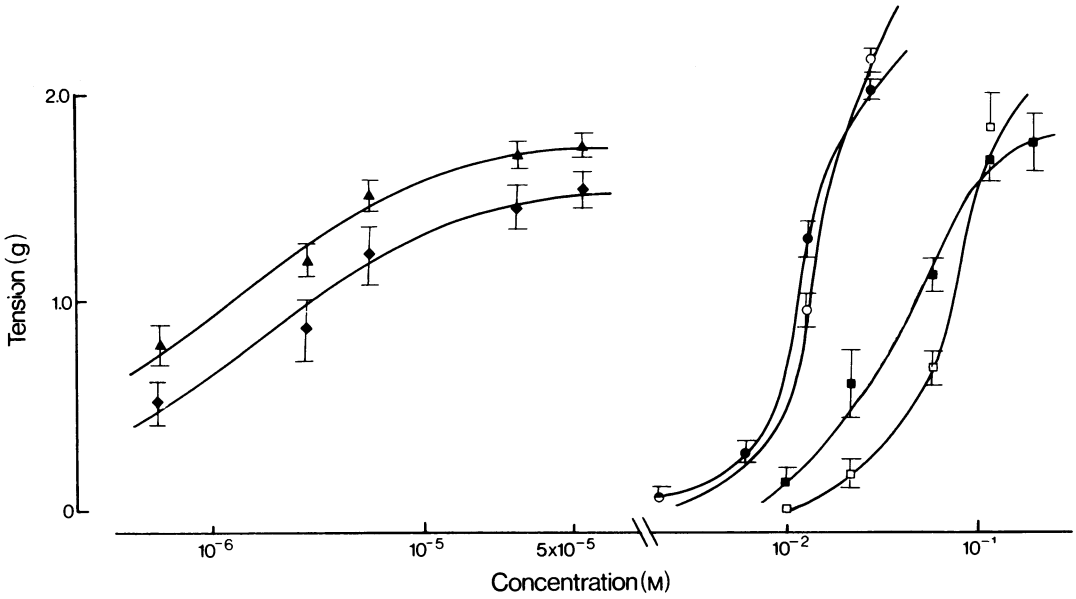


**Figure 2** Concentration-response curves obtained using the chronically denervated (closed symbols) and the innervated (open symbols) mouse soleus muscles. Contractions were produced by acetylcholine (▲), carbachol (◆), caffeine (●) or potassium chloride (■) in the denervated preparations and by caffeine (○) or potassium (□) in innervated preparations. Each point is the mean of 6 observations; vertical bars show s.e. mean.

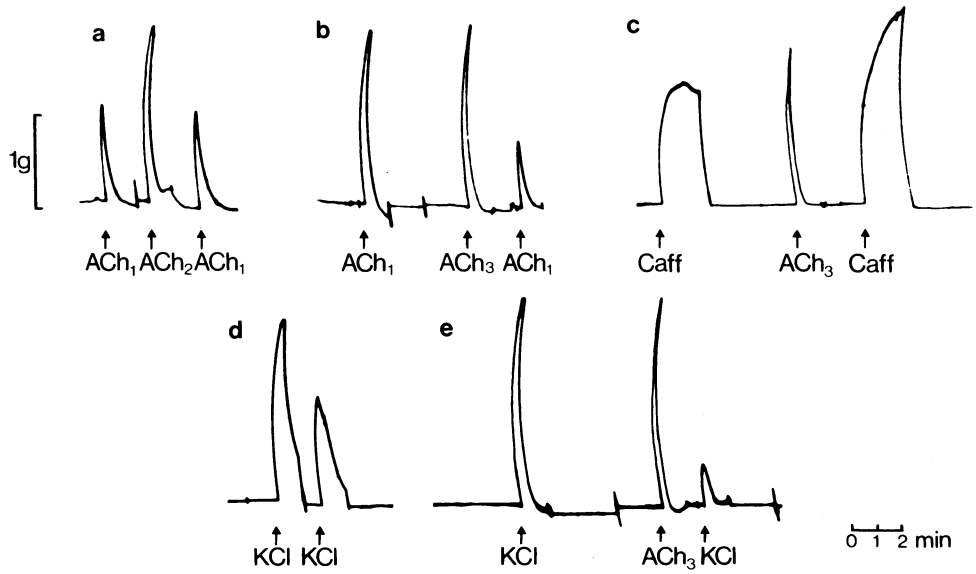


**Figure 3** Contractions of the chronically denervated mouse soleus muscle. The drugs were added at the points indicated by the arrows and in each case were washed out after 1 minute. (a) A control response ($ACh_1$) was obtained with acetylcholine ($5.5 \times 10^{-6}$ M). After a 10 min rest (during which the recording was stopped), acetylcholine $2.8 \times 10^{-4}$ M ($ACh_2$) was added and 1 min after washing out this high dose another test addition of acetylcholine ($5.5 \times 10^{-6}$ M) was made ($ACh_1$). (b) The first response is a control, obtained with acetylcholine $5.5 \times 10^{-6}$ M ($ACh_1$). The preparation was rested and then a very high dose of acetylcholine given ($ACh_3$), $5.5 \times 10^{-4}$ M). One minute after washout, the test dose was repeated ($ACh_1$). (c) Caffeine, $1.3 \times 10^{-2}$ M (Caff) was given first as a control response and then repeated 1 min after washing out acetylcholine, $5.5 \times 10^{-4}$ M ($ACh_3$). (d) Some reduction was seen in the response to a second dose of KCl (100 mM) when applied 1 min after washing out the first dose. (e) The KCl test response (KCl, 100 mM) gave a reduced contraction when added 1 min after washing out acetylcholine ($ACh_3$, $5.5 \times 10^{-4}$ M).

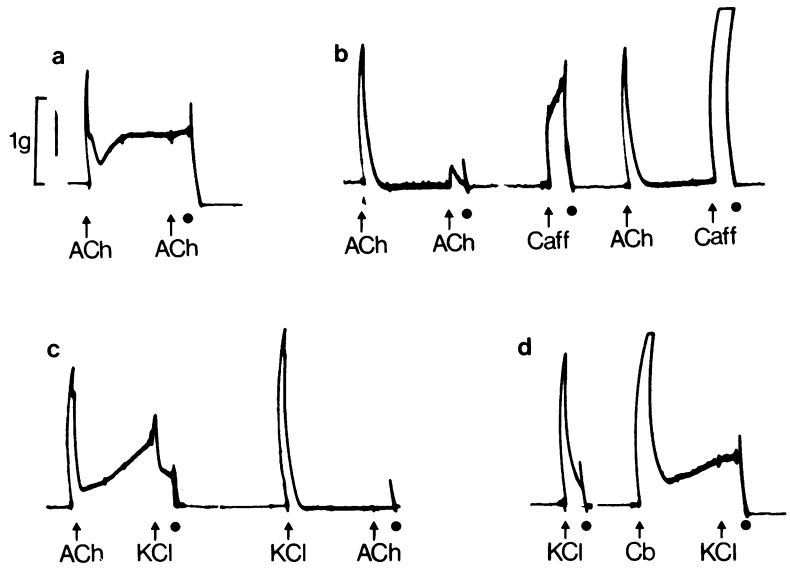


**Figure 4** Contractions of four different chronically denervated mouse soleus muscles. Drugs were added at the arrows. At the dots, the preparations were washed and rested for 10 minutes. (a) Acetylcholine 5.5 x $10^{-6}$ M caused a contraction (ACh). An identical addition repeated 4 min after the first and during the second phase of the contraction produced no effect. (b) In this preparation the contraction produced by acetylcholine (ACh) (5.5 x $10^{-6}$ M) had no second phase. A second addition made 4 min after the first produced a small contraction only. A control response to caffeine, 13 mM (Caff) was obtained and this was augmented 4 min after the addition of acetylcholine (5.5 x $10^{-6}$ M). (c) Potassium chloride, 100 mM (KCl) was added during the second phase of the acetylcholine (ACh, 5.5 x $10^{-6}$ M) contraction in this preparation. It produced a biphasic response consisting of a smaller contraction than KCl added alone and this was immediately followed by relaxation. Acetylcholine (5.5 x $10^{-6}$ M) was inactive when added in the presence of KCl 100 mM. (d) In this preparation, KCl (50 mM) caused no effect when added during the second phase of the contraction produced by carbachol (Cb), (5.5 x $10^{-6}$ M). A similar result could also be obtained when KCl was added during the second phase of acetylcholine contractions.

centrations. Figure 3a demonstrates this lack of desensitization 1 min after washing out a conditioning concentration of acetylcholine (2.8 x $10^{-4}$ M) when the response to a test addition of acetylcholine (5.5 x $10^{-6}$ M) was unaltered. With very high concentrations of acetylcholine or carbachol (5.5 x $10^{-4}$ M and in some cases also with 2.8 x $10^{-4}$ M) desensitization was observed but was found to be non-specific. One minute after washout of very high concentrations of acetylcholine or carbachol, test responses to acetylcholine or carbachol were reduced (Figure 3b) as were responses to KCl, 100 mM (Figure 3e). This desensitization at very high conditioning concentrations of acetylcholine or carbachol was therefore not specifically related to a single receptor type. Nevertheless, caffeine contractions were not reduced by very high conditioning concentrations of acetylcholine or carbachol (Figure 3c). Further evidence that this desensitization was non-specific was obtained from the observation that high concentrations of KCl also reduced the size of test responses to KCl (Figure 3d), acetylcholine and carbachol, but not to caffeine.

In another series of experiments, conditioning concentrations of acetylcholine (5.5 x $10^{-6}$ M) or carbachol (5.5 x $10^{-6}$ M) were added and, after 4 min without washing, a test dose was administered. Test responses to acetylcholine (5.5 x $10^{-6}$ M), carbachol (5.5 x $10^{-6}$ M) and KCl (50 mM) were reduced, indicating non-specific desensitization (Figure 4). Test additions of acetylcholine or carbachol were ineffective when superimposed on the second phase of the acetylcholine or carbachol contraction (Figure 4a). However, when no second phase was present the test addition did cause a contraction, though it was much reduced in size (Figure 4b). Test additions of KCl (50 mM) added in the presence of either acetylcholine or carbachol (5.5 x $10^{-6}$ M) in some experiments had no effect (Figure 4d) and

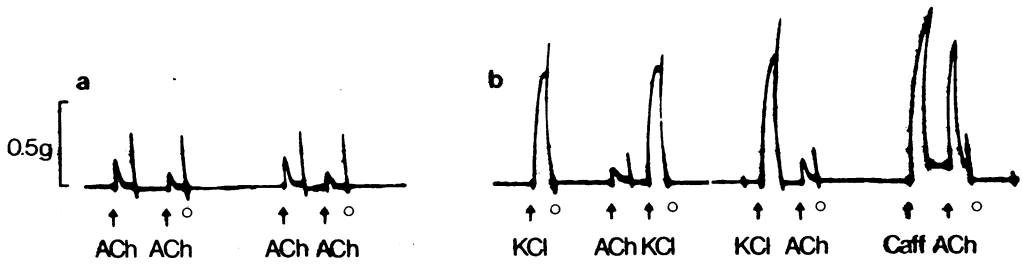


**Figure 5** Contractions of the normally innervated mouse soleus preparation. Drugs were added at the arrows and washed out after 1 minute. The preparation was rested for 10 min at the points marked thus: ○. (a) Acetylcholine $5.5 \times 10^{-3}$ M caused a small contraction (ACh). When repeated 1 min after washing out, the contraction was reduced. This sequence was repeated. (b) A control response to potassium chloride, 50 mM (KCl) was obtained. The size of the KCl contraction was not reduced when added 1 min after washing out acetylcholine, $5.5 \times 10^{-2}$ M (ACh). Also acetylcholine ($5.5 \times 10^{-2}$ M) gave a contraction of control size when added 1 min after washing out potassium (50 mM). However, added 1 min after rinsing out caffeine (13 mM), the response to acetylcholine, $5.5 \times 10^{-2}$ M (ACh) was considerably augmented.

in others produced a small rise in tension followed by inhibition of the second phase of contraction (Figure 4c). Contractions in response to caffeine ($1.3 \times 10^{-2}$ M), on the other hand, were augmented when caffeine was added in the continuing presence of acetylcholine ($5.5 \times 10^{-6}$ M) (Figure 4b). A similar kind of non-specific desensitization was produced by KCl (50 mM), which reduced responses to acetylcholine (Figure 4c) to carbachol or to KCl, but not to caffeine.

*Innervated*

In innervated mouse soleus muscles, taken either from the contralateral (non-operated) leg or from intact animals, caffeine and KCl produced contractions that were similar in form to those obtained in denervated preparations, except that the KCl contractions were slightly better sustained. The dose-response curves produced by caffeine and KCl were similar in denervated and in innervated muscles (Figure 2).

Acetylcholine or carbachol in concentrations greater than $5.5 \times 10^{-4}$ M usually gave small contractions although these were rather variable in size even in a single preparation. In 8 preparations the mean contraction produced by $5.5 \times 10^{-3}$ M acetylcholine was $0.09 \pm 0.01$ g and by $5.5 \times 10^{-2}$ M acetylcholine was $0.13 \pm 0.02$ gram. When a second addition of acetylcholine or carbachol was made 1 min after washout of a control addition of either agonist, then the response was reduced or almost abolished (Figure 5). The mean control contraction produced by $5.5 \times 10^{-2}$ M acetylcholine in 10 preparations was $0.14 \pm 0.01$ g and on repetition 1 min after washing was $0.01 \pm 0.01$ gram. On the other hand, contractions to caffeine and KCl were of control size when additions were made 1 min after washing out acetylcholine. Contractions to acetylcholine were of normal size when added after washout of KCl (50 mM) and were augmented after washout of caffeine ($1.3 \times 10^{-2}$ M).

## Discussion

In innervated preparations, desensitization was observed when test additions of acetylcholine or carbachol were made 1 min after washout of a previous dose. Since the KCl contractions were normal at this time, this desensitization appears to be a property of the receptors themselves, and resembles the receptor specific desensitization observed at the frog motor endplate (Katz & Thesleff, 1957; Nastuk, Manthey & Gissen, 1966) in the chick biventer cervicis muscle and leech dorsal muscle (Rang & Ritter, 1970) and cultured skeletal muscle (Harvey & Dryden, 1974a). This conclusion is supported by the observation that following a contraction to KCl, the sensitivity to acetylcholine was not reduced, and therefore this type of desensitization does not follow the action of all agonists. Also, the sensitivity to acetylcholine was not reduced following the action of caffeine and in fact was found to be augmented. This is probably the result of caffeine reducing the rate of calcium re-uptake by the sarcoplasmic reticulum (Weber & Herz, 1968).

In contrast to these results, a similar procedure applied to the chronically denervated mouse soleus muscle produced no desensitization

at all when moderate to large doses were used. However, desensitization was observed following washout of very high concentrations of carbachol or acetylcholine ($2.8-5.5 \times 10^{-4}$ M) and this appeared to be different in type from that observed in the innervated preparations. Test responses to KCl were reduced as well as those to acetylcholine and carbachol, and moreover it was found that KCl could itself cause desensitization. This desensitization was therefore non-specific in the sense that it was not limited to drugs acting at the acetylcholine receptor. Thus the desensitization demonstrated here in the denervated soleus muscle of the mouse differs from that studied by Freeman & Turner (1972), who demonstrated, in the denervated rat diaphragm, cross desensitization among some nicotinic agonists but not between acetylcholine and carbachol. Following pretreatment with acetylcholine, the muscle was not desensitized to caffeine and therefore it is likely that the non-specific desensitization process involves a change in the electrical or permeability characteristics of the plasma membrane as caffeine is considered to act intracellularly at the level of the sarcoplasmic reticulum (Ogawa, 1970).

In chronically denervated muscles, non-specific desensitization was also observed following much lower concentrations of acetylcholine and carbachol if the test drug was added without washing out the desensitizing concentration. This was normally done 4 min after the first addition, at which time with acetylcholine or carbachol there was a second contraction. No increase in tension could be obtained with acetylcholine or carbachol, and potassium either had no effect or caused a relaxation. These experiments reveal a non-specific desensitization having properties similar to the desensitization observed 1 min after washout of very high concentrations.

In the soleus muscle of the rat, the greater acetylcholine sensitivity occurs near the endplate and at the muscle-tendon junction; the remainder of the fibre has a detectable, though very low responsiveness (Miledi & Zelená, 1966; Albuquerque & Thesleff, 1968). After denervation, the entire fibre exhibits uniformly high sensitivity, comparable with that found in denervated fast muscles (Albuquerque & Thesleff, 1968). Assuming that the mouse is similar to the rat, the supersensitivity of the denervated soleus is due to an increase in receptor density in non-endplate regions and it can safely be assumed that contractions obtained in denervated muscles are initiated by interaction with these new receptors. Contractions obtained with high concentrations of acetylcholine in innervated soleus muscles might be caused by an action at receptors of the myoneural or muscle-tendon junctions or at the low density receptors covering the remainder of the muscle fibre. It is likely that it is the endplate receptors that are predominantly activated in these experiments, since similar small responses can be obtained in normal mouse diaphragms (M.A. Maleque, unpublished experiments) which are only sensitive to acetylcholine at the endplate (Miledi, 1960b; Hartzell & Fambrough, 1972).

Although desensitization was observed in chronically denervated and in innervated muscles, only in the latter was it found to be receptor specific. We have considered three explanations for this: (1) the properties of individual receptors in the two preparations may be fundamentally different, (2) specific desensitization may require intimate association of receptors with acetylcholinesterase or (3) the concentration of agonist may determine the type of desensitization produced. The first explanation seems to be ruled out since most comparative studies have revealed a striking similarity between receptors in the vicinity of the innervated endplate and in non-endplate regions of chronically denervated muscle (Axelsson & Thesleff, 1959; Freeman & Turner, 1969). Only quantitative differences have been demonstrated in sensitivity to antagonists (Jenkinson, 1960; Beránek & Vyskočil, 1967; Vyskočil & Beránek, 1968; Miledi & Potter, 1971; Lapa, Albuquerque & Daly, 1974) and in the acetylcholine reversal potential (Feltz & Mallart, 1971). Since receptor specific desensitization has been observed in cultured skeletal muscle (Harvey & Dryden, 1974a) which has no functional cholinesterase (Kano & Shimada, 1971; Harris, Marshall & Wilson, 1973; Harvey & Dryden, 1974b) the deficiency of cholinesterase in denervated muscle (Axelsson & Thesleff, 1959; Miledi, 1962; for review see Koelle, 1963) cannot be directly linked to the type of desensitization observed. We favour the third explanation: specific desensitization occurs only where the concentration of agonist is high, that is in those preparations that are insensitive. If a response is obtained with low doses, then it is impossible to achieve sufficient concentration to produce specific desensitization without causing such massive activation that non-specific changes occur, resulting in loss of sensitivity to a variety of agonists. This hypothesis is consistent with the Katz & Thesleff (1957) model for specific desensitization:

$$\begin{array}{ccc} A + R & \rightleftharpoons & AR \\ & \uparrow \text{ slow } \downarrow \text{ slow} & \\ A + R' & \rightleftharpoons & AR' \end{array}$$

A high concentration of agonist activates a greater fraction of receptors and thus drives a

greater proportion of them into the desensitized configuration. Experimental evidence for greater desensitization at higher agonist concentrations has been obtained by Katz & Thesleff (1957), Miledi (1960a), Katz & Miledi (1964), Nastuk *et al.* (1966) and Harvey & Dryden (1974a).

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