Br. J. Pharmacol. (1988), **94**, 968–976

Effect of soluble factors from nerve and muscle on α-bungarotoxin binding sites in isolated sarcolemmal membranes of the rat

Alan J. Harborne & [1]Margaret E. Smith

Department of Physiology, Medical School, University of Birmingham, Birmingham B15 2TJ

1 Sarcolemmal membranes were isolated from normal and from denervated muscles and the specific binding of [^{125}I]-α-bungarotoxin to the membranes was determined.

2 Cytosol prepared from either slow (soleus) or fast (extensor digitorum longus) muscle increased the toxin binding. Similar effects were seen with cytosol from muscles which had been denervated 5 to 7 days previously and with cytosol from sciatic nerve. A purified preparation of a bacterial phospholipase C also produced this effect.

3 Bathing medium in which normal or denervated muscles had been incubated, increased the toxin binding to normal membranes, but the normal bathing medium decreased the binding to membranes prepared from denervated muscles.

4 The possibility that pre-existing latent acetylcholine receptors can be activated by intracellular factors released from muscles is discussed.

Introduction

The endplate area of a normally innervated adult mammalian skeletal muscle is extremely sensitive to the neuromuscular transmitter, acetylcholine (ACh), as there is a high density of ACh receptors at this location. The extrajunctional surface membrane of most adult mammalian muscles is normally insensitive to ACh, but slow muscle fibres exhibit detectable sensitivity over the entire surface membrane (Albuquerque & Thesleff, 1968). Following section of the motor nerve, however, the entire muscle membrane of both fast and slow muscles becomes hypersensitive, due largely to an increase in the synthesis of ACh receptors and their subsequent incorporation into the extrajunctional membrane (Fambrough, 1970). Both contractile activity and neurotrophic factors have been implicated in the regulation of the receptors (for a discussion see Gordon *et al.*, 1976). In previous work it was shown that incubation of intact normal muscles with cytosol prepared from either normal or denervated muscles resulted in an increase in the amplitude of the contracture and membrane depolarization responses of the muscles to ACh. Purified preparations of phospholipase C exerted a similar effect (Watson *et al.*, 1976; Harborne *et al.*, 1978; 1987). The ACh responses were also increased following incubation of the intact muscles with medium in which denervated muscles had previously been incubated (Harborne *et al.*, 1984). It is possible therefore that factors which contribute to the development of membrane hypersensitivity are released from denervated muscles to exert an action on the external face of the membrane.

An increase in the physiological response to ACh could be due to an inhibition of the activity of acetylcholinesterase, or a change in the electrical properties of the membrane or an increase in the number of available ACh receptors. In this laboratory it was recently shown that the number of binding sites for α-bungarotoxin (α-BTX) can be increased on isolated sarcolemmal membranes by incubation of the membranes with phospholipase C (Adham *et al.*, 1986). Other workers (Rehm & Betz, 1981), using chick retina membranes, have also described the unmasking of cryptic ACh receptors by a phospholipase C preparation. Since this toxin binds almost irreversibly to the nicotinic receptor on skeletal muscle (Chang & Lee, 1963) the increase on sarcolemmal membranes was probably due to exposure of pre-existing ACh receptors. In the present experiments an attempt was made to show directly whether the previously demonstrated increase in the ACh responses, caused by endogenous soluble

[1] Author for correspondence.

factors (Harborne *et al.*, 1984), could be due to an exposure of ACh receptors. The number of receptor sites was assessed by measuring the binding of [^{125}I]-α-BTX to isolated sarcolemmal membranes, and the effect of cytosol prepared from fast or slow normal muscles, or from denervated muscles, or from nerve, on the toxin binding to the membranes was investigated. In order to see whether denervation hypersensitivity could be partly explained by a greater release of the factors in denervated muscles than in normal muscles, bathing medium in which normal or denervated muscles had been incubated was tested for its effect on the binding of a α-BTX to sarcolemmal membranes. A brief account of part of this work has already been published (Adham *et al.*, 1987).

Methods

Animals and surgical procedure

Adult female Sprague-Dawley rats were used throughout the study. Animals of 200 to 250 g body weight were used for the preparation of sarcolemmal membranes and animals of 150 g body weight were used for organ bath experiments. Denervations were performed 5 to 7 days before the experiment by unilateral section of the sciatic nerve high in the thigh. The anaesthetic used was diethyl ether and asceptic precautions were maintained throughout.

Isolation of sarcolemmal membranes

Sarcolemmal membranes were prepared by a modification of the method of Boegman *et al.* (1970) as described previously (Shute & Smith, 1985a), using mixed hindlimb muscles of the rat. The final membrane pellet was suspended in distilled water.

Characterization of membranes

Electron microscopy The membrane pellet was fixed in 2.5% glutaraldehyde in phosphate buffer (0.2 M, pH 7.2) for 1.5 h and then in 1% osmium tetroxide in phosphate buffer for 1 h. The pellet was then washed for 15 min in 70% ethanol, followed by 90% ethanol for 10 min and twice in absolute ethanol for 10 min, once in dried absolute ethanol for 10 min, twice in propylene oxide for 10 min, and once in the propylene oxide and resin (1:1 by vol.) mixture of Mollenhauer (1964) for 1 h. The pellet was then vacuum embedded in the resin. Ultrathin sections were taken, stained in 30% uranyl acetate in methanol and in Reynold's lead citrate. The grids were then examined in a Jeol 100cx electron microscope.

Enzyme and analytical determinations Lactate dehydrogenase (EC 1.1.1.27) was determined by the method of Wroblewski & La Due (1955), acid phosphomonoesterase (EC 3.1.3.2) by the method of McLaughlin *et al.* (1974) and succinate dehydrogenase (EC 1.3.99.1) by the method of Pennington (1961). Na^+/K^+-stimulated Mg^{2+}-dependent ATPase (EC 3.6.1.3) and Ca^{2+}-dependent ATPase (EC 3.6.1.3.) were assayed according to Boegman *et al.* (1970) and Wan & Boegman (1980), respectively. RNA and DNA were determined by the methods of Schneider (1957) and Burton (1956), respectively. Protein was measured by the method of Lowry *et al.* (1951).

α-Bungarotoxin binding The binding of [^{125}I]-α-BTX to the membranes was determined by a modification of the millipore filtration method of Tipnis & Malhotra (1979), as described previously (Adham *et al.*, 1986). The non-specific adsorption of [^{125}I]-α-BTX to the millipore filters (Type HA, 0.45 μ dia) was reduced by preincubation of the filters with 312.5 nM unlabelled α-BTX. Specific [^{125}I]-α-BTX binding sites were also present on the millipore filters. Attempts to block these sites with unlabelled (non-iodinated) α-BTX were abandoned as the sites became available again during the washing procedure at the end of the incubation (see below) resulting in binding of the labelled toxin to the filters. However, the specific sites on the filters as well as the membranes could be effectively blocked by treatment with (+)-tubocurarine (0.75 mM) which competes with [^{125}I]-α-BTX for the specific receptor sites and each filter was pretreated with (+)-tubocurarine (0.75 mM), as described previously (Adham *et al.*, 1986).

Sarcolemmal membranes (30 μg protein ml^{-1}, equivalent to approximately 0.1 g wet weight of muscle) were incubated for 30 min at 37°C with [^{125}I]-αBTX (12.5 nM, 0.05–0.10 Ci ml^{-1}) and 0.01% bovine serum albumin in Krebs-Henseleit solution, in a total volume of 1.0 ml. Non-specific binding was determined by including for each incubation, a control containing 0.75 mM (+)-tubocurarine. Incubations were performed in triplicate, and the replicates showed good agreement. After the incubation the mixture was filtered rapidly on millipore filters under partial vacuum and each filter was washed with 5 × 10 ml volumes of fresh Krebs-Henseleit solution. The radioactivity on the filter and adhering membrane was measured using an ICN Gamma Set counter at 40% efficiency. The non-specific binding of the toxin varied between 30% and 40% of the

total binding. The specific binding of [^{125}I]-α-BTX to the membranes had reached saturation by 15 min incubation with membranes prepared from normal muscles, and by 30 min incubation with membranes prepared from denervated muscles. Previous work (Adham *et al.*, 1986) showed that the maximum specific binding to the membranes was achieved with concentrations of the toxin in excess of 3 nM.

The effect of cytosol or other factors was determined, unless otherwise stated, by preincubation of the membranes with the cytosol or medium for 7 min at 37°C in a volume of 0.8 ml. The protease inhibitors, leupeptin, pepstatin and aprotinin, in a concentration of 25 $\mu g\,ml^{-1}$ in each case, were also present when the effect of unpurified cytosol, or medium in which muscles had been incubated was examined, but the protease inhibitors were not included when the effect of the purified preparation of phospholipase C or partially purified cytosol was examined. The action of the enzyme or cytosol was stopped by addition of chloroquine sulphate to give a final concentration of 15 mM. This concentration of the drug was shown to prevent the action of cytosol and phospholipase C (see later) and under the conditions of the experiment had no effect on the toxin binding. Chloroquine sulphate has been shown to inhibit a number of hydrolytic enzymes including phospholipase C (Matsuzawa & Hostetler, 1980; Shute & Smith, 1985b). After 5 min incubation with the drug [^{125}I]-α-BTX was added to the mixture and the binding was determined as described above.

Preparation of cytosol

Hindlimb muscles were dissected out and as much fat, nerve and connective tissue as possible was removed. The tissue was minced in ice-cold Krebs-Henseleit solution and homogenized for 4 × 15 s intervals using a Polytron homogeniser (type PT 20 Od, Kinematica Gmbh) at setting 6. The homogenate was filtered through muslin and the filtrate was centrifuged at 104,000 *g* for 60 min (MSE Superspeed 50 centrifuge). Nerve cytosol was prepared in a similar way from sections of the sciatic nerves removed from the thigh regions. In some experiments cytosol was prepared from the individual soleus or extensor digitorum longus (EDL) muscles. The former is a slow muscle and the latter is a fast muscle.

In some instances the cytosols were fractionated between 30% and 50% saturation with ammonium sulphate. The precipitate obtained was resuspended in Krebs-Henseleit solution and dialysed for 2 × 2 h periods, against 2.0 litres of fresh Krebs-Henseleit solution containing 5 mM cysteine. The latter had previously been shown to stabilize the active factors and the phospholipase C activity.

Incubation of muscles to collect released substances

Normal or denervated muscles were incubated in Krebs-Henseleit solution in an organ bath with continuous oxygenation of the solution. Six muscles, three soleus and three EDL, were secured at their tendons to each organ bath. The muscles were washed with six bath volumes of Krebs-Henseleit solution before the incubation which was for 1 h at room temperature. In some experiments, the muscles were stimulated directly at supramaximum intensity at the beginning and end of the incubation period to monitor their viability. The method has been described in detail previously (Harborne & Smith, 1982). The bathing medium was collected after the incubation and dialysed against distilled water for 2 h at 4°C, and then freeze-dried in order to concentrate it. Before use, the solids were resuspended in water (one tenth of the original volume) and the insoluble material was removed using a bench centrifuge. Krebs-Henseleit solution treated in the same way was used as a control.

Materials

Unlabelled α-BTX was obtained from Boehringer-Mannheim Ltd and [^{125}I]-α-BTX from Amersham Ltd. (+)-Tubocurarine, leupeptin, pepstatin, aprotinin and phospholipase C (Type I, ex *Clostridium perfringens*, approximately 20 u mg^{-1} protein) were purchased from Sigma Chemical Co. and chloroquine sulphate was obtained from May & Baker Ltd.

Results

Characterization of sarcolemmal membranes

Sarcolemmal membranes were prepared from normal and from 5 to 7 day denervated muscles. Electron microscopy of the membrane pellets from both normal and denervated muscles indicated a high degree of purity. The single membrane profiles were rounded in the preparation obtained from normal muscles but more elongated in the preparation obtained from denervated muscles. This difference in appearance could be related to the known differences in composition between membranes from normal and denervated muscles. Both preparations were characterized using marker substances. The purification of the sarcolemmal membranes was followed by determining their activity of Na^+/K^+-stimulated Mg^{2+}-dependent ATPase and their capacity to bind [^{125}I]-α-BTX, both of which are

properties peculiar to the plasma membranes. The values for specific binding of [^{125}I]-α-BTX to membranes isolated from normal and denervated muscles were 609 ± 53 ($n = 11$) and 4568 ± 1177 ($n = 6$) fmol mg^{-1} membrane protein, respectively. The binding to the latter was always considerably (approximately 7 to 8 times) higher than that seen with the normal membranes, although the values were extremely variable in the case of the membranes from denervated muscles. Assessment of contamination of the membrane preparations, by determination of lactate dehydrogenase, acid phosphomonesterase, Ca^{2+}-dependent ATPase, RNA and DNA, showed negligible contamination by cytosol, lysosomes, smooth and rough sarcoplasmic reticulum and nuclei, respectively, for preparations from either normal or denervated muscles. Determination of succinate dehydrogenase indicated that the contamination by mitochondria represented less than 4.0% of the total in the original homogenate.

Effect of phospholipase C and muscle cytosols on [^{125}I]-α-bungarotoxin binding to normal sarcolemmal membranes

In the absence of protease inhibitors the binding of [^{125}I]-α-BTX to normal membranes was reduced following incubation with cytosol prepared from hindlimb muscles. However, when protease inhibitors were included in the incubation medium the cytosol was seen to increase the number of toxin binding sites on the membranes. The inhibitors included were leupeptin which inhibits cathepsin B and Ca^{2+}-activated neutral protease (Ikezawa *et al.*, 1971; Sugita *et al.*, 1980), and pepstatin and aprotinin, both of which inhibit cathepsin D (Morishima *et al.*, 1970; Werle, 1972). The protease inhibitors themselves had no effect on the toxin binding in the absence of cytosol when they were present in concentrations up to 30 μg ml^{-1}. Figure 1 shows the effect of cytosol from EDL muscles on the binding to normal sarcolemmal membranes. An appreciable increase in the number of binding sites was seen after incubation of the membranes with the cytosol. When membranes were incubated in the absence of cytosol no increase in the toxin binding was seen. Figure 1 also shows the effect of purified phospholipase C on the binding of [^{125}I]-α-BTX to normal membranes. In the latter case inclusion of protease inhibitors with phospholipase C in the incubation mixtures, did not increase the binding and they were therefore omitted.

Figure 2a shows the effect of different concentrations of cytosol, prepared from soleus or EDL muscles, on the toxin binding to normal membranes. The cytosol prepared from soleus muscle was more effective than that from EDL muscles. Table 1 shows

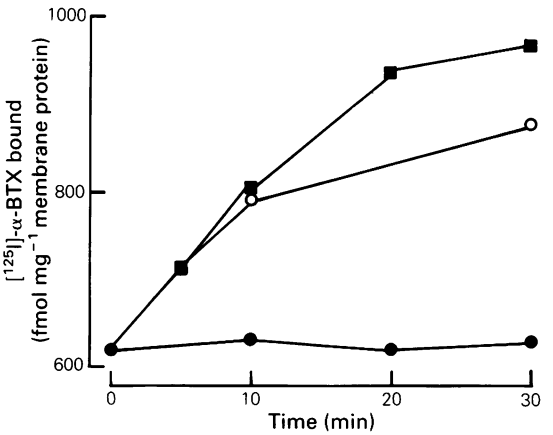

Figure 1 Effect of preincubation with cytosol prepared from extensor digitorum longus muscles (○), phospholipase C (■) or Krebs-Henseleit solution (●) on the binding of [^{125}I]-α-bungarotoxin (α-BTX) to normal sarcolemmal membranes; the cytosol was present in a concentration of 30 μg protein ml^{-1} and the phospholipase C was present in a concentration of 2.5 mu ml^{-1}, where 1 unit = 1 μmol phosphatidylcholine hydrolysed min^{-1}. The action of the soluble factors was stopped by addition of chloroquine sulphate (15 mM) to the incubation. The subsequent incubation with the toxin (12.5 nM) was for 30 min at 37°C.

Table 1 Effect of cytosol from normal and denervated soleus and extensor digitorum longus (EDL) muscles on the binding of [^{125}I]-α-bungarotoxin (α-BTX) to normal sarcolemmal membranes

Cytosol	[^{125}I]-α-BTX bound (fmol mg^{-1} membrane protein)	%	P
None	609 ± 53 (11)	100	
Boiled soleus	581 ± 74 (3)	95	NS
Normal soleus	2029 ± 417 (7)	333	<0.01
Normal EDL	835 ± 71 (4)	137	<0.03
Denervated soleus	916 ± 132 (5)	150	<0.01
Denervated EDL	992 ± 157 (5)	163	<0.01
Normal soleus AS fraction	1358 ± 101 (4)	230	<0.01

Fifty μg cytosol protein was present in the incubation mixture in each case, except for the fraction purified by ammonium sulphate (AS) fractionation, when 10 μg cytosol protein was present. Each value is given as the mean ± s.e.mean with the number of experiments in parentheses. *P*, the probability, was determined by use of Student's *t* test.

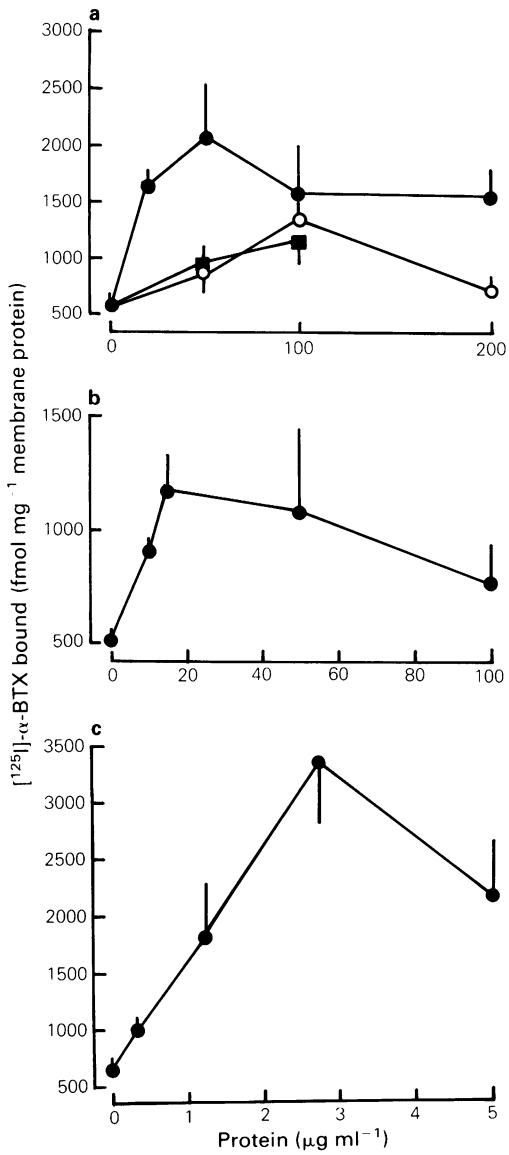

Figure 2 Effect of (a) cytosol from (●) normal soleus, (○) normal extensor digitorum longus (EDL), and (■) denervated soleus muscles, (b) nerve cytosol, (c) bathing medium in which normal muscles had been incubated, on the binding of [^{125}I]-α-bungarotoxin (α-BTX) to isolated normal sarcolemmal membranes. Preincubation with cytosol or bathing mediun (in which normal muscles had been incubated) was for 7 min at 37°C and the reaction was stopped by addition of chloroquine sulphate. Incubation with the labelled toxin was for 30 min at 37°C. Each value is given as the mean and vertical lines represent s.e.mean of at least 4 experiments in each case.

that when it was present in the optimum concentration (50 μg protein ml^{-1}) the soleus cytosol produced an approximately 3 fold increase in the number of binding sites.

Figure 2a also shows the effect of cytosol prepared from 5 to 7 day-denervated soleus muscles on the toxin binding to normal membranes. The binding was increased by incubation with the cytosol, but the cytosol prepared from denervated soleus muscle was not as effective as that prepared from normal soleus muscle. Table 1 compares the effect of cytosols prepared from normal and denervated soleus and EDL muscles. The effect of cytosol prepared from denervated soleus muscles was not significantly different from that of cytosol from denervated EDL muscles or that from normal EDL muscles, in the concentration used.

Properties of the cytosol factors

The active factors could be partially purified from soleus muscle cytosol by fractionation of the cytosol between 30 and 50% saturation with ammonium sulphate (Table 1). This procedure increased the specific activity of the active factor by 3 to 4 fold compared to the unpurified soleus muscle cytosol. This observation is consistent with the active factor being a protein molecule. The addition of protease inhibitors to the incubation with this partially purified cytosol fraction was not necessary for an increase in toxin binding to be seen, indicating that the proteolytic factors which reduced the binding in the absence of the inhibitors when the unpurified cytosol was used had been removed by the fractionation procedure. It seems unlikely therefore that the active factor is a proteolytic enzyme.

When soleus muscle cytosol was boiled for 3 min before being added to the incubation mixture, its effect on the toxin binding to normal membranes was abolished (Table 1). This observation is consistent with the active factor in the cytosol being an enzyme. Furthermore, the action of the cytosol to increase the binding was abolished by including chloroquine sulphate (15-mM) in the preincubation medium (4 experiments). This drug inhibits the soluble phospholipase C of skeletal muscle (Shute & Smith, 1985b) as well as a number of other hydrolytic enzymes (Matsuzawa & Hostetler, 1980).

Effect of nerve cytosol

Figure 2b shows the effect of nerve cytosol, which had been fractionated between 30% and 50% saturation with ammonium sulphate, on the binding of [^{125}I]-α-BTX to normal membranes. The nerve cytosol increased the binding by approximately 2

fold when present in a concentration of 50 μg of the partially purified cytosol protein.

Effect of factors released from intact muscles on the toxin binding to normal membranes

Intact normal soleus and EDL muscles were incubated in Krebs-Henseleit solution in an organ bath for 1 h and the bathing medium, containing the substances which had leaked from muscles during the incubation, was added to normal sarcolemmal membranes to assess its effect on the toxin binding. Figure 2c shows the effect of different concentrations of the medium on the specific binding. The binding was increased 2 to 3 fold at the optimum concentration of the medium (2.5 μg protein ml^{-1}).

In order to see whether denervated muscles are leakier in this respect than normal muscles, 5 to 7 day-denervated muscles were incubated in the same way as the normal muscles and the bathing medium, containing factors which had leaked from the muscles, was tested for its effect on the toxin binding to normal membranes. This medium was more potent than the medium obtained after incubation of normal muscles in increasing the toxin binding. The normal muscles released 336 ± 126 (mean ± s.e. mean, $n = 6$) $\times 10^3$ units of activity per organ bath during the 1 h incubation period and the denervated muscles released 696 ± 210 ($n = 6$) $\times 10^3$ units per organ bath, where 1 unit is defined as an increase in binding of 1 fmol mg^{-1} membrane protein. The denervated muscles had atrophied to some extent and their wet weight was approximately 18% less than that of the normal muscles. The release of the factor g^{-1} wet weight of muscle was 173 ± 30 ($n = 6$) $\times 10^3$ units for normal muscles, and 287 ± 56 ($n = 6$) $\times 10^3$ units for denervated muscles. Thus the denervated muscles released significantly more of the factor than the normal muscles ($P < 0.05$) on this basis. However, the specific activity of the released factor from normal and denervated muscles was similar; the specific binding of the toxin was increased by 804 ± 247 fmol mg^{-1} membrane protein by 1 μg protein released from normal muscles and by 786 ± 285 fmol mg^{-1} membrane protein by 1 μg protein released from denervated muscles. Thus there was no selective release of the factor from the denervated muscles.

Effect of released factors on the specific binding of α-BTX to membranes prepared from denervated muscles

In order to see whether membranes prepared from denervated muscles were, like normal membranes, also susceptible to the action of the soluble factors, sarcolemmal membranes were prepared from 5–7 day-denervated rats and incubated with bathing medium containing the released factor. Incubation of these membranes with medium obtained after incubation of denervated muscles caused significant increases in the binding. Incubation with 5 μg released protein ml^{-1} or 10 μg released protein ml^{-1} increased the binding to $203 \pm 40\%$ ($n = 6$, $P < 0.01$) and $227 \pm 37\%$ ($n = 5$, $P < 0.01$), respectively. However, medium obtained after incubation of normal muscles caused a significant decrease in the toxin binding to membranes from denervated muscles. Incubation with 5 μg released protein ml^{-1} or 10 μg released protein ml^{-1} reduced the binding to $50.5 \pm 18\%$ ($n = 4$, $P < 0.05$) and $53 \pm 21\%$ ($n = 4$, $P < 0.05$) respectively. Incubation of the membranes with Krebs-Henseleit solution alone had no significant effect on the toxin binding to the membranes prepared from denervated muscles.

Discussion

[^{125}I]-α-BTX was shown to bind specifically to sarcolemmal membranes prepared from either normal or denervated muscles. The values obtained for the specific binding were similar to those found by Tipnis & Malhotra (1979) who used a similar method for the preparation of the membranes. The mean specific toxin binding to the membranes prepared from denervated muscles was 7 to 8 times higher than that observed with membranes prepared from normal muscle. This is in agreement with the findings of Hartzell & Fambrough (1972) who demonstrated a 6 to 7 fold increase in binding to membranes from 5 to 7 day-denervated muscles compared to those from normal muscles.

Incubation of normal membranes with the cytosol preparations or medium in which muscles had been incubated resulted in a dramatic increase in the number of specific α-BTX binding sites. It appears therefore that considerable numbers of 'hidden' specific toxin binding sites exist in normal muscle membranes. Previous work has shown that muscle cytosol (Watson *et al.*, 1976; Harborne *et al.*, 1978) and nerve cytosol increased the contracture (Harborne *et al.*, 1987) and membrane depolarization (A.W. Turner & M.E. Smith, unpublished) responses to ACh in intact skeletal muscles *in vitro*. Furthermore, treatment of muscles in which the responses had previously been blocked using unlabelled α-BTX, with cytosol or with phospholipase C, enabled ACh responses to be detected subsequently in these muscles (Harborne *et al.*, 1978). As α-BTX binds specifically to the ACh receptors it seems likely that at least some of the new binding sites exposed

by cytosol or medium on the isolated membranes represent the functional ACh receptors. It is not possible to say whether all of the new specific toxin binding sites represent such receptors, since, in the present experiments both faces of the membranes are exposed to the action of the cytosol or released factors, whereas only the sites on the external face are likely to be involved in transmitter-receptor interaction. At high concentrations the factors were less effective in increasing the binding. This may have been due to degradation of the receptors or other membrane components by hydrolases present in the extracts.

It is well established that *de novo* synthesis of receptors occurs following denervation (for a review, see Fambrough, 1970) and this is probably the major mechanism whereby ACh sensitivity in denervated muscles increases. The ACh sensitivity of hindlimb skeletal muscles increases after denervation to a maximum at around 6 to 8 days (Gordon *et al.*, 1976; Almon & Appel, 1976; Harborne & Smith, 1982), although the α-BTX binding sites and ACh sensitivity in rat diaphragm are still increasing up to at least 14 days (Hartzell & Fambrough, 1972). If latent ACh receptors are present in large numbers in normal membranes and can be exposed *in vivo* by an intracellular factor released from the muscles, then this action of the factor could be a mechanism whereby receptors are exposed after they have been incorporated into the plasma membranes. Indeed, it has been speculated (Gordon *et al.*, 1976) that exposure of ACh receptors already present in the membrane may promote the incorporation of newly synthesized receptors into the membrane. In the present experiments incubation medium from denervated muscles also increased the toxin binding to membranes from 5–7 day-denervated muscles. Thus, pre-existing cryptic membrane toxin binding sites can also be exposed or activated in the muscle membranes at this time after denervation.

Muscles contain a variety of cell types apart from muscle fibres, including nerve terminals and a variety of mononuclear cells. The factors which increased the toxin binding could have originated from a cell type other than muscle fibres. Since nerve cytosol also increased the toxin binding to isolated membranes, it is possible that factors originating from the nerve terminals could also activate the receptors. Nerve terminals degenerate after denervation, but the possibility exists that the degenerating nerves release these factors.

Cytosol prepared from normal soleus muscles was more potent than that from normal EDL muscles in increasing the toxin binding. The slow soleus muscles *in vivo* exhibit appreciable numbers of extrajunctional receptors, whilst the fast EDL muscles are largely insensitive to ACh on the extrajunctional regions. It is possible, therefore, that the difference in membrane chemosensitivity in the two muscles is related to the different concentrations of the active factors in the muscles. Denervated muscles, on the other hand, which are hypersensitive to ACh and respond to the transmitter in concentrations several orders of magnitude lower than those which elicit responses in normal muscles, did not exhibit higher activities of the active factor. However, the release of the factor from denervated muscles was greater than the release from normal muscles during the incubation procedures used in these experiments. The concentration of the factor in the extracellular fluid surrounding the muscle fibres *in vivo* could therefore be higher for denervated muscles. It is also possible that normal muscles release a substance which obscures the receptors, since the number of binding sites on membranes from denervated muscles was reduced after incubation of the membranes with medium containing factors released from normal muscles, whilst that obtained after incubation of denervated muscles increased the binding. It is interesting in this respect that previous work (Harborne *et al.*, 1984) showed that a substance which inhibits the effect of the factor which increased the ACh contracture responses was released from normal muscles. It is not clear why normal and denervated membranes are different in this respect.

The nature of the factor in the cytosol and incubation media which increases the specific α-BTX binding is unknown. However it was heat-labile, the activity being abolished by boiling. Moreover the activity was inhibited by chloroquine, a drug which inhibits a number of enzymes, such as phospholipases A and C (Matsuzawa & Hostetler, 1980), including the soluble phospholipase C in skeletal muscle (Shute & Smith, 1985b), and proteases (Romstedt *et al.*, 1983). Previous work has shown that pepsin and phospholipase C, but not phospholipase A, trypsin, collagenase or certain bacterial proteases, increased the ACh responses of intact muscles (Watson *et al.*, 1976) and that both phospholipase C and proteolytic enzymes are present in the bathing medium obtained after incubation of normal or denervated muscles (Harborne *et al.*, 1984). However, inhibitors of cathepsins and Ca^{2+}-activated protease were included in the incubations with unpurified cytosol. It seems unlikely, therefore, that the active factor is a protease. Bacterial phospholipases exhibited a similar action to the endogenous factors in increasing the α-BTX binding to the membranes. In addition soluble phospholipase C activity is endogenous to both muscle (Shute & Smith, 1984) and nerve (e.g. Hirasawa *et al.*, 1982). The activities of phospholipase C in various cytosol preparations have been studied previously in this laboratory, and some correlation between the

enzyme specific activities and the ability of these preparations to increase α-BTX binding to sarcolemmal membranes is evident from the findings. Thus soleus cytosol, which was considerably more effective than EDL cytosol in increasing the toxin binding, exhibited an approximately three fold higher specific activity of the enzyme than EDL cytosol (Shute & Smith, 1984); cytosols prepared from 5–7 day denervated (mixed hind limb) muscle and from normally innervated muscle showed no significant difference in their ability to increase the toxin binding or in their specific activities of the enzyme (Harborne *et al.*, 1984). Furthermore, both the enzyme and the active factor could be purified by fractionation of cytosol between 30 and 50% saturation with ammonium sulphate (Harborne *et al.*, 1987). Finally, phospholipase C was released from either normal or denervated muscles incubated in Krebs-Henseleit solution under the conditions used in the present study (Harborne *et al.*, 1984). It is interesting also, in view of the fact that ACh receptors are normally restricted to the endplate region of muscles, that recent work in this laboratory has shown that both phospholipase C (Smith *et al.*, 1988) and a factor which increases the binding of α-BTX to isolated sarcolemmal membranes (Adham & Smith, 1987) are released upon stimulation of the phrenic nerve in an isolated nerve-diaphragm preparation. Thus phospholipase C is one possible candidate for the mediation of the effect of cytosol on the toxin binding to isolated membranes. Attempts are currently being made to purify the active factor in order to identify it.

We are grateful to the National Fund for Research into Crippling Diseases, the Wellcome Trust, the Medical Research Council and the SmithKline Foundation for supporting this work. Thanks are due to Mrs L. Tompkins for performing the electron microscopy.

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(Received January 6, 1988
Revised March 9, 1988
Accepted March 11, 1988)