SOLUBILIZATION FROM SKELETAL MUSCLE OF TWO COMPONENTS THAT SPECIFICALLY BIND α-BUNGAROTOXIN*

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SUMMARY - BuTX** binds irreversibly to two components of skeletal muscle, one with apparent molecular weight (by gel filtration) of 550,000 and one of 200,000. Only the former, thought to contain the acetylcholine receptor, is elevated in denervated muscle,about 20-fold at its maximum. After solubilization, these components retain their specific properties.

BuTX has been employed as an extremely specific blocking agent for the ACh*** receptor of skeletal muscle junctions (1-4) as well as that, in numerous other studies, of fish electric organs. In situ analyses (2), using ^{3}H-acetylated BuTX, have shown that there are 3 x 10^{7} and 6 x 10^{7} active centers of this receptor at each endplate of the mouse and rat diaphragm muscles respectively. However, we have recently found, somewhat surprisingly, that only about 50% of these sites are readily protected by d-tubocurarine (5). When muscle treated with radioactive BuTX is extracted by non-ionic detergent solutions, a labeled complex is removed (3,4), but the purification or characterization of the component(s) responsible has not been reported. We present here studies on the extraction and separation of the components of rodent skeletal muscle to which BuTX binds irreversibly.

Experimental

Materials and Animals: Adult male Sprague-Dawley rats and albino RR mice (20 g) were used. Denervation of rats was by removal of

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** BuTX, α-bungarotoxin

*** ACh, acetylcholine

a 5 mm section of the left phrenic nerve under ether anaesthesia. Triton X-100 and d-tubocurarine chloride pentahydrate were from Sigma, Soluene from Packard, sodium deoxycholate from Mann Research, digitonin from Calbiochem and lauryl dimethylamine oxide from Onyx Chem. Co., New Jersey. Ultrafiltration was on Millipore PSJM membranes. Chromatographically pure BuTX, and ^{3}H-acetylated BuTX, were prepared as described elsewhere (2). However, by further purification steps a homogeneous species, ^{3}H-monoacetyl-BuTX (^{3}H-BuTX), was obtained, with toxin activities unchanged from the native (unpublished results), and this was used in these experiments.

Liquid scintillation counting was in a dioxane-based fluid (6), or for Soluene solutions a toluene-based equivalent, at efficiencies of 40-45%.

Extraction of ^{3}H-BuTX-treated muscles. Rat diaphragms were removed with ribs attached, washed in Tyrode solution, and shaken continuously with ^{3}H-BuTX (1 µg/ml) at 27°C for 3 hr. The muscles were washed extensively (10 changes) with Tyrode and dissected into endplate-containing and endplate-free regions of approximately equal weights. Each was extracted at 27°C with continuous shaking for 3 hr, in 50 mM Tris-HCl, pH 8.0, containing 1.5% Triton X-100 (with variations in these conditions where noted), and gel filtered in the same medium. The residual muscle material was fully dissolved in Soluene at 60°C and counted. In other cases (Table I) the labeled dissected specimens were directly dissolved in Soluene and counted. Labeled mouse diaphragms were from mice injected intravenously with ^{3}H-BuTX (1µg/g) and were processed similarly.

Results

Uptake of ^{3}H-BuTX by Normal and Denervated Diaphragm. Treatment

Table I. Specific Binding of ^{3}H-BuTX in Normal and Denervated Diaphragm

Rats	Cpm per 100 mg		Relative uptake**	
	EP*	Non-EP		
Normal	1356	419	1	(4)
15-Day Denervated	14590	9070	18	(2)
22-Day Denervated	15254	5896	23	(3)

* EP = endplate-containing half of the muscle

** The uptake for denervated hemidiaphragm relative to the uptake for normal hemidiaphragm. Binding by the normal extra-junctional component (in peak III, Fig. 1) is first subtracted (EP less Non-EP Cpm), for normal muscle; for denervated, that contribution is then negligible (Fig 1C) and correction was not attempted. All values are means from the number of experiments given in parentheses.

of skeletal muscle with ^{3}H-BuTX (1μg/ml) for 3 hr saturates the endplate sites of irreversible binding (2, 5). In addition, the non-endplate zone of rat muscle is responsible for about 25% of the total muscle uptake (Table I). After the rat diaphragm is denervated, ^{3}H-BuTX binding increases many-fold (Table I), as has been previously reported with radioiodinated-BuTX (3,4), with the maximum increase at about 20 days (3). The binding in the half of the hemidiaphragm free of endplates is then greatly elevated, although in the endplate-containing half the increase is even greater (Table I).

Extraction and Separation of Receptor-Toxin Complex. Four detergents, non-ionic or ionic, when used for the extraction of toxin-labeled whole diaphragms at 27°C, gave about the same results (Table II). Homogenization of the labeled muscle prior to or during extraction was not employed, since it was found to release a great deal more protein into the extract. The extraction

Table II. Extraction of ^{3}H-BuTX-Binding Components from Whole Mouse Diaphragm.

Detergent		No. of Animals	% Total ^{3}H Solubilized*
Deoxycholate	(1%)	2	71
Digitonin	(1%)	2	80
Lauryl dimethylamine oxide	(1.5%)	2	71
Triton X-100	(1.5%)	4	76
(1.5% homogenized)		2	85
	(0.25%)	2	33
	(1.5%)	2	78**
	(1.0%)	1	47**
	(0.5%)	1	46**
	(0.25%)	1	43**
	(0.1%)	1	13**

* This represents the sum of the components of peaks I and II (Fig. 1A) since only a relatively small amount of free ^{3}H-BuTX was shown (by gel filtration) to be present in the extracts.

** Extracted for 17 hr at 4°C. The other extractions were for 3 hr at 27°C.

reached about 80% when the whole muscle was used, with negligible increase in this percentage with homogenization (Table II). Decreases in Triton concentration, temperature (4°-27°C range) and duration of extraction all lower the yield of solubilized complex. Extraction at 4°C for longer periods was best, since this greatly reduced the total protein simultaneously extracted. With the rat diaphragm, because of its thickness, longer extraction periods were necessary.

When mouse or rat diaphragm was labeled with ^{3}H-BuTX (1 µg/ml)

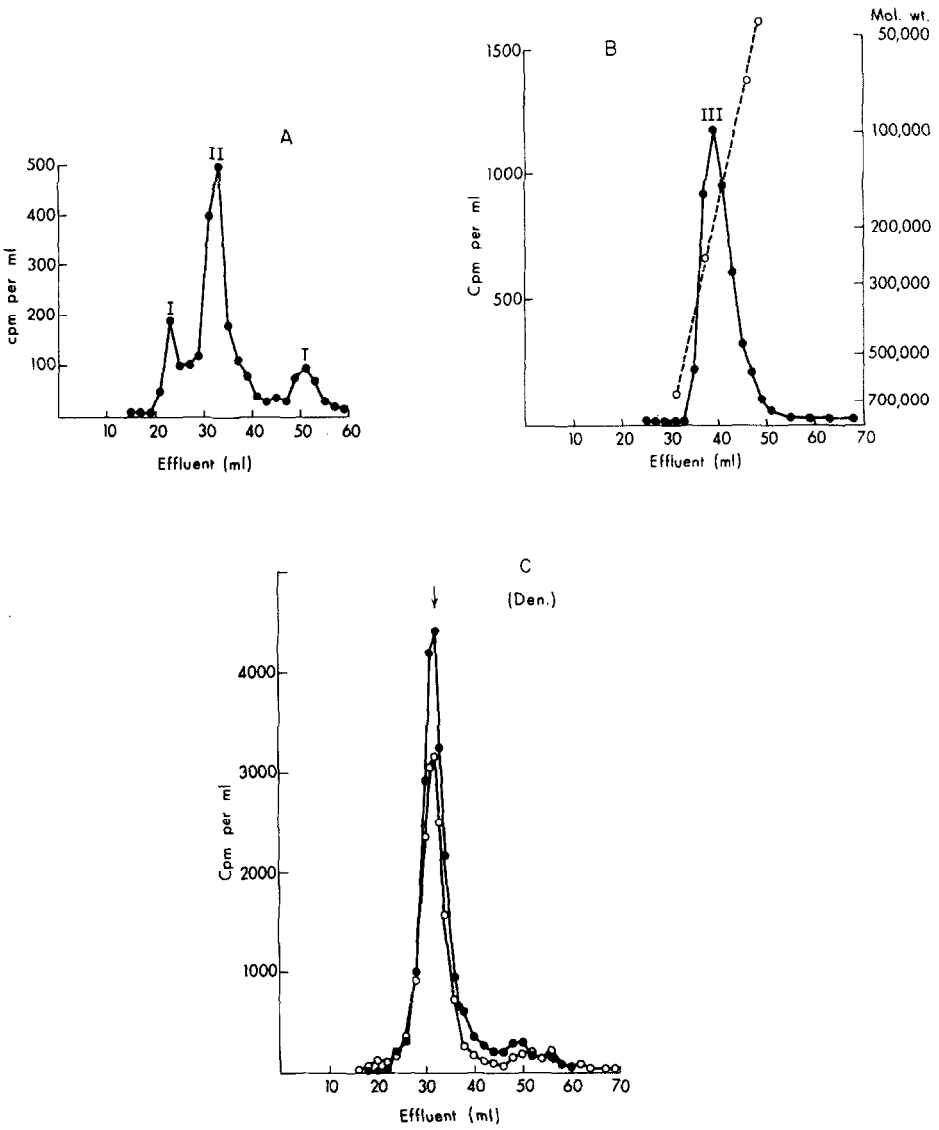

Fig. 1. Gel Filtration of the ^{3}H-BuTX-Binding Components of Diaphragm Muscle. The muscles were extracted with 10-20 times their weight of 1.5% Triton X-100/50 mM Tris-HCl, pH 8.0 and the soluble extracts were gel filtered on a Sepharose 6B column (1 x 68 cm; 1 ml fractions) in the same medium and counted. A: Mouse diaphragms were labeled in vivo with ^{3}H-BuTX (1 µg/g body wt.). T is the elution position of free ^{3}H-BuTX. B: Mouse diaphragms were first treated in vivo with unlabeled toxin (3 µg/g body wt.) and then treated in vitro with ^{3}H-BuTX (10 µg/ml). The broken line shows a plot of log molecular weight against elution volume of standard protein markers (ovalbumin, serum albumin, catalase and thyroglobulin). C: 22 days denervated rat diaphragms were labeled in vitro with ^{3}H-BuTX (1 µg/ml). Extracts of endplate-containing (●) and endplate-free (○) portions of the muscle were gel filtered. The arrow is at the elution position of the ^{3}H-BuTX-receptor complex from normal muscle.

and extracted in 1.5% Triton X-100, the major toxin-bound component present was shown on gel filtration (peak II in Fig. 1A) to have an apparent molecular weight of 550,000. A small peak of a toxin-binding component of much higher molecular weight could also be found (peak I).

When a 10-fold higher concentration of ^{3}H-BuTX is used, binding then becomes prominent throughout the muscle (5), i.e. in areas without endplates. When such a muscle was extracted and chromatographed, the extract gave rise to a third labeled peak, with an apparent M_w of about 200,000. This latter peak could be obtained in isolation by first blocking the specific endplate sites with a lethal *in vivo* dose of unlabeled BuTX, and then exposing the washed muscle *in vitro* to ^{3}H-BuTX (10μg/ml, 3 hr, 27°C). On gel filtration, the extract of this muscle now showed only peak III (Fig. 1B). Hence, the latter is formed by a toxin-binding component from non-endplate regions of the muscle, which becomes more heavily labeled at higher toxin concentrations.

Receptors in Denervated Muscle. Upon similar extraction of ^{3}H-BuTX-labeled denervated muscle, the major portion of the radioactivity appeared on gel filtration in a single peak, with the same elution volume as the specific endplate component; this profile was the same from both endplate-containing and endplate-free regions (Fig. 1C). The non-endplate (peak III) component was still present, but since the endplate type of complex (peak II) is elevated about 20-fold after denervation, its amount is now very much greater than that of the peak III component.

Solubilization of Receptor Component. The endplate-rich region of rat diaphragm muscle was extracted (with continuous shaking) in 1.5% Triton 50 mM Tris-HCl, pH 8 (2 hr, 4°C), *without* toxin treatment. After concentration at 4°C on a membrane ultrafilter,

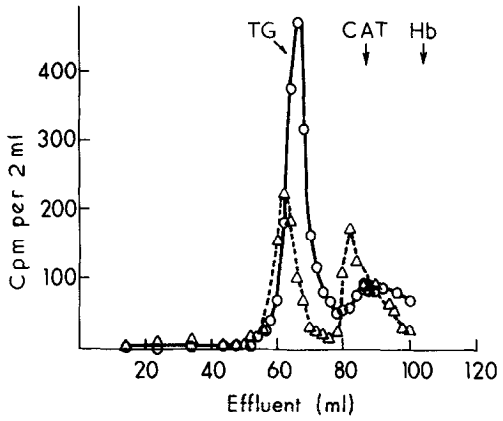

Fig. 2. Gel Filtration of ^{3}H-BuTX Binding Components of Rat Diaphragm Labeled after Solubilization. The diaphragms were extracted, ultrafiltered, and the extracts labeled in solution (see Results). Gel filtration was on a Sepharose 6B column (1.5 x 87 cm) in 0.2% Triton X-100/50 mM Tris-HCl, pH 8.0. Portions of the 2 ml fractions were counted. Half of the extract was incubated with d-tubocurarine (50 µg/ml) for 15 min prior to the reaction with ^{3}H-BuTX (Δ), while the remainder (O) was treated with ^{3}H-BuTX only. All treatments here were at 4°C. The arrows show the elution position (on the column used for the control sample) of marker proteins (TG, thyroglobulin; CAT, catalase; Hb hemoglobin).

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the material was reacted in solution with ^{3}H-BuTX (0.25 µg/ml, 1 hr) and gel-filtered (Fig. 2). The endplate-specific component gave rise to peak II, with the same mobility as before. Peak III was also present and showed the same mobility as the peak III (Fig. 1B) previously attributed to the extra-junctional binding component. Peak I material was sometimes present also.

The solubilized material was also reacted similarly with ^{3}H-BuTX in the presence of d-tubocurarine (50 µg/ml), and again fractionated (Fig. 2). Distinct protection from the BuTX reaction was exerted thus, but the peak III component did not appear to be protected.

No acetylcholinesterase activity (7) was detectable in the Triton X-100 extracts of muscle before or after concentration.

Discussion

The present results show, firstly, that in mammalian skeletal

muscle, BuTX binds essentially irreversibly to two different macromolecular species. The first of these, giving rise to peak II in these gel filtrations, is present at the endplates only, and gives a labeled complex with a Stokes' radius corresponding to M_w (apparent) of 550,000 in Triton X-100 (0.2% - 1.5%) solution. A complex of about the same apparent size has been found in BuTX-treated electroplax preparations (8,9) and has been interpreted as having, after correction for detergent binding, a true M_w of 360,000 (10), a conclusion which is likely to hold true for the muscle material, too. The second binding component (peak III) is extra-junctional and its complex has M_w (apparent) of 200,000. This component is not associated with the ACh receptor, and has very little affinity for d-tubocurarine (Fig. 2).

In some experiments, a third BuTX-binding component was observed in extracts of labeled muscle (peak I in Fig. 1A) and also in muscle extracts labeled after solubilization. Raftery et al. (9) observed, in Triton X-100 extracts of submaximally-labeled Torpedo electroplax, a BuTX-labeled component which displayed a very high molecular weight on gel filtration. This material would correspond to our peak I. However, we noted that the peak I material was only present when relatively high temperatures (25°-37°C) were employed for the extraction of the muscle and the reaction with ^{3}H-BuTX.

We have also examined the roughly 20-fold increase in BuTX binding that occurs in denervated muscle at the peak time (around 20 days) after nerve section. This is due to an increased content of a component of the same apparent size as the specific endplate (peak II) component from normal muscle. It is not yet known if it is identical with the latter, but it is import-

ant to note that the large denervation-produced increase in toxin binding, and in extra-junctional ACh sensitivity (11), is not due to an increase in the amount of the extra-junctional type of BuTX-binding site that is found in normal muscle fibers.

Finally, it is significant that a component retaining the properties of the ACh receptor can be obtained in soluble form from extracted muscle. This extends the observation of Berg et al. (4) on this system. The specific properties so far shown here in solution are the irreversible reaction with BuTX and the binding of d-tubocurarine.

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