COMPLETE PURIFICATION OF THE ACETYLCHOLINE RECEPTOR PROTEIN FROM MAMMALIAN MUSCLE

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1. Introduction

The receptor (ACh.R) for acetylcholine (ACh) has so far been obtained in apparently pure form only from fish electric organs (for reviews, see [1,2]). From vertebrate skeletal muscle, on the other hand, where ACh.R is essential for synaptic transmission and for denervation supersensitivity to ACh, no extensive purification has been reported. Muscle is a much poorer source of ACh.R, and even after its proliferation following denervation it is far below the level in the electric organ of *Torpedo*. For muscles, quantitation and localisation of ACh.R, by exploitation of its essentially irreversible binding of labelled α-bungarotoxin (BuTX), was initially described [3], and solubilisation of this ACh.R in detergents and some fractionation has been reported [4–7]. Subsequently [8], we reported upon a preparation, partly purified by affinity chromatography, of the form of ACh.R which is responsible for the extrajunctional ACh sensitivity of denervated mammalian muscle. We have further applied affinity chromatography in a more bio-specific form (in the sense in which this technique has been critically reviewed by Barry and O'Carra [9]), and describe here the complete purification of this muscle ACh.R.

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2. Materials and methods

2.1. *Receptor assay and analytical methods*

BuTX and [^{3}H]-acetylated-BuTX ([^{3}H]BuTX), and the assay method for ACh.R based upon its rapid, saturable binding of [^{3}H]BuTX, were all as described previously [8], as were materials not separately specified here. Protein was determined on 100 μl aliquots by Folin reaction [10], adapted where necessary for high sensitivity by reduction of the final volume of the reaction mixture to 0.2–0.4 ml and the use of micro cells; bovine serum albumin (Sigma) was used as the standard. Acetylcholinesterase (AChE) was determined by acetylthiocholine reaction [11], similarly adapted.

2.2. *Extraction of the ACh.R*

Chronically denervated cat leg muscles [8] were used as previously [8]: these are the mixed muscles in the lower hind leg supplied by all branches of the sciatic nerve, which was transected in the thigh about 4 weeks prior to sacrifice. The muscles were cut up finely and extracted (1 g per 2 ml medium) in 1.5% Triton X-100/50 mM K phosphate, pH 8, shaking in a bath at 5°C. Phenylmethanesulfonyl fluoride (10^{-4} M), benzethonium chloride (10^{-4} M) and EDTA (10^{-3} M) (all from Sigma) were present as protease inhibitors. The supernatant after centrifugation at 75 000 *g*, 20 min, was used without storage. All subsequent operations were at 5°C.

2.3. *Affinity chromatography*

Sepharose 4B was coupled, via two lengths of the arm $NH(CH_2)_3NH(CH_2)_3NHCO(CH_2)_2CO$-, to tri-

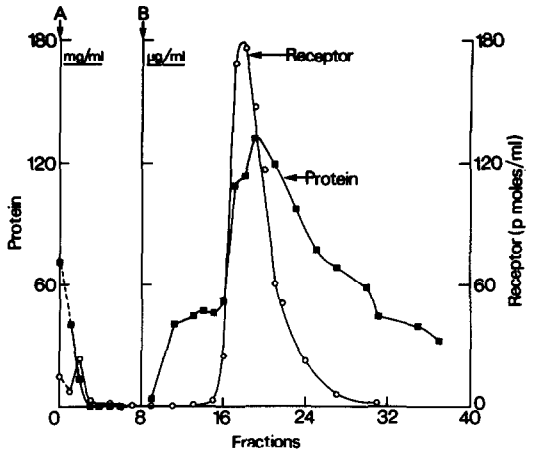

Fig.1. Affinity chromatography of receptor. The affinity column was equilibrated in 0.05 M K phosphate/0.2% Triton X-100, and washed after sample loading (A) with 0.05 M NaCl in that medium (60 ml). The receptor activity (○) and protein content (■), when read in the breakthrough volume (broken lines) and the washes (up to B), are expressed as though concentrated to 1 ml fractions from the larger volumes used. At B, 2.1 mM Flaxedil in the same NaCl medium was applied, and 1 ml fractions were collected. The first half of the peak was pooled for further purification. Note that the scale for protein is expanded 1000-fold after the Flaxedil elution commences.

methyl (*p*-aminophenyl) ammonium chloride by the method of Berman and Young [12]. A column (6 ml) was equilibrated in 50 mM K phosphate, pH 8/0.2% Triton X-100. The sample was applied and washed in the same medium, and eluted as shown in fig.1, using Flaxedil (May and Baker). When Flacedil and NaCl eluents were applied, these solutes were largely removed from the effluent by passing it (at 10 ml/hr) through hollow fibres of two Minibeaker dialyzers (BioRad Labs.) in series, outside which was pumped 0.2% Triton X-100 at 80 ml/min. Further details of the purification methods will be given in a full account elsewhere.

2.4. *Gel electrophoresis*

A system based on that specified by Hyden and Langer [13] was used, but employing 5% polyacrylamide in the running gel (0.5 × 9 cm) at pH 9, and 2.5% in the stacking gel at pH 7.5. 0.1% Triton X-100 was in all media; 4 mA/tube was applied.

3. Results

3.1. *Isolation of ACh.R from muscle*

Cat denervated lower leg muscles were extracted in 1.5% Triton X-100, until solubilization had occurred (usually overnight) of at least 80% of the total receptor content of the muscle.

The ACh.R solution was fractionated on a column either of Sepharose 6B or of Ultragel AcA 22, in 0.2% Triton X-100/10 mM phosphate (pH 8), to yield a single peak of receptor activity, centred in the zone corresponding to apparent molecular weights of 400 000–500 000 (as illustrated previously [7]). Most of the protein present emerged after this peak. About the first two-thirds of this ACh.R peak was pooled, concentrated by ultrafiltration on a Millipore PSJM membrane, and at once applied to the affinity column.

As shown in fig.1, the great bulk of the protein present was removed in the non-absorbed or salt-eluted fractions from this column, but essentially all the receptor was retained (unless the column was overloaded). When no more protein could be removed by the salt wash given, 2.1 mM Flaxedil (gallamine triethiodide) solution was used to achieve a bio-specific [9] elution of ACh.R. This eluent also displaced a small amount of other protein. The use of NaCl gradients, or of *d*-tubocurarine (*d*-TC) or carbachol as alternative ligands, even at much higher concentrations gave poor purifications or yields. Almost all the Flaxedil was removed by continuous flow dialysis, and correction was made for the effect of residual ligand in the assays after performing the latter at several dilutions of the effluent sample.

Final separation from extraneous protein was made by ion-exchange chromatography on DEAE-Sephadex (Whatman, A-25). Details are shown in fig.2. It is seen that a gradient of NaCl separates the ACh.R from the residual protein, which either is not bound to this ion-exchanger (broken line in fig.2 profile) or is only slightly retarded (first protein peak), or is displaced at a higher NaCl concentration than elutes the ACh.R. Pure ACh.R was taken from the central fractions of the peak of activity.

Troughout, we take 1 nmol of ACh.R as that amount which binds 1 nmol of [^{3}H]BuTX when the plateau of specific uptake in the assay [8] is reached. In the crude extract, each gram of denervated muscle

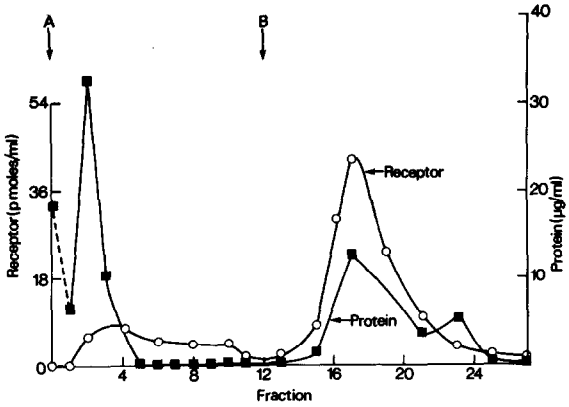

Fig.2. Ion-exchange chromatography of receptor. A column (0.7 x 2 cm) of DEAE-Sephadex was equilibrated in 25 mM K phosphate (pH 8)/0.1% Triton X-100, and was washed after sample loading (A) with 0.1 M NaCl in that medium (35 ml). Notation up to point B is as given for fig.1. At B, a linear gradient in the same medium, from 0.1 to 1.2 M NaCl (10 ml of each) was applied, with continuous flow dialysis of the effluent (as for the affinity column) against 0.1% Triton X-100, and 1 ml fractions were collected. Fractions 16–18 were taken to give the final product.

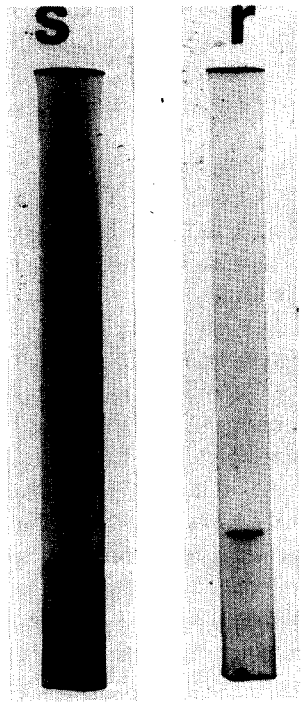

Fig.3. Gel electrophoresis of the final ACh.R product (r), stained with Amido-Schwartz. Origin at top; s=human serum albumin (p*I* = 4.9) as reference, run for one-half the period used for the ACh.R tube. In shorter runs of ACh.R, where the tracking dye had not migrated to the end, it was checked that no faster components were detectable. In absorbance scanning the band appeared homogeneous.

gave rise to about 80 pmol of ACh.R. Recovery in the entire peak of ACh.R on the gel filtration column was about 100% but, as noted, about one-third of this was discarded. Recovery in the peak in bio-specific chromatography was 62% of the applied receptor. Ignoring all overlap fractions that were discarded at the three chromatographic steps, the final recovery of the pure component was 14% of the ACh.R in the original crude extract. The overall purification typically achieved with respect to the crude extract is 3800-fold.

3.2. *Criteria for the purity of the ACh.R*

The first criterion to be considered is specific activity. The final product had in the usual case a value of 3 500 nmol/g protein, and in the best case so far realized 6000 nmol/g. The protein content was found using an adaptation of the Folin-Lowry method, based upon the arbitrary use of serum albumin as a standard. It has been reported that the pure electroplaque receptor shows values in this same range for its specific activity (reviewed in Refs. [1,2]), but that they increase by about 33% when the protein estimation is made, more correctly, by amino acid analysis [14]. The muscle ACh.R has a specific activity, therefore, in the same range as the purest preparations reported of the electroplaque ACh.R, being 8000 nmoles/g if thus corrected, in the purest sample obtained.

Secondly, gel electrophoresis (in non-denaturing medium) was applied for detection of impurities. In the final product only a single band of protein was was detectable (fig.3). Running of the gels for shorter and for longer periods, to look for components running off rapidly or barely separated from the main component, did not yield any other bands.

Thirdly, ACh.E was undetectable in the pure ACh.R. The ACh.R fractions, even at the early stage of the peak of activity on the first gel column used, contained no ACh.E activity except at the lower trailing edge of the ACh.R peak on that column, a region which was discarded, so that ACh.E was found to be totally ab-

sent later. On the basis that the muscle ACh.E has the specific activity of pure electroplaque ACh.E [15], the limits of assay showed that the final ACh.R product contained less than 0.0004% of active ACh.E.

3.3. *Properties of the pure ACh.R*

The pure ACh.R was stable for several weeks at 4°C (in the presence of 0.01% Na azide), losing only a few per cent of its activity then. This is in contrast to the considerable instability of the muscle ACh.R at early stages of the isolation. The material upon complete hydrolysis in 6N HCl and analysis gave a typical amino acid content, which will be reported elsewhere, confirming its protein identity.

The molecular weight as estimated by gel filtration [7] (on Sepharose 6B in 0.2% Triton X-100) is 430 000. This is only on the basis of Stokes' radius comparisons, with globular proteins as standards. It has been shown that a similar value is obtained for electroplaque ACh.R in such gel filtration conditions and this is an overestimate of the molecular weight, due to the binding of detergent, the true value being derived by other methods being about 250 000 [16]. Further analysis of the molecular weight of the muscle ACh.R will be reported elsewhere, but it is clear that the size of this protein is similar to that of electroplaque ACh.R.

Specific binding of cholinergic ligands to the ACh.R was demonstrated by their ability to retard the reaction with the toxin. The initial rates of [^{3}H]Butx reaction at 25°C in the presence of various concentrations of a given ligand, above and below its estimated affinity constant (K_D) for the ACh.R, were used for this purpose, as described previously [8] for less pure muscle ACh.R. In the presence of saturating concentrations of *d*-tubocurarine (*d*-TC) (10^{-4}M) or of ACh (10^{-3}M) the reaction with [^{3}H] BuTX (2 - 4 nM) was suppressed, the initial rate (over 30 min) being 6% (*d*-TC) or 0% (ACh) of the normal initial rate. This is to be compared with the 10–15% of the toxin-binding sites that were found incapable of binding these ligands in less pure and less protected preparations [8].

Values of K_D for the pure ACh.R with various ligands, determined by the protection method, will be reported elsewhere [17]. They are quite similar to those reported for the pure electroplaque ACh.R [1,2], with high affinity ($K_D < 10^{-7}$M) sites for ACh, and strong binding of *d*-TC, carbamylcholine and decamethonium.

4. Discussion

ACh.R from denervated mammalian muscle can be solubilized in dilute solutions of Triton X-100 or other detergents [7] and can be completely purified by a combination of gel filtration, biospecific chromatography and ion-exchange chromatography. The final product is a homogeneous protein. It has a specific receptor activity similar to that of the pure ACh.R from electroplaques.

The properties of the protein finally obtained that categorized it as an ACh receptor include the rapidity and specificity of the reaction with BuTX, and the high affinities for a typical spectrum of nicotinic cholinergic ligands. It has already been shown [8] on this ACh.R in less pure form that non-nicotinic ligands (e.g. atropine, pilocarpine, choline) exhibit only low affinities for this component, whereas the K_D values for nicotinic ligands there are in a series consistent with their pharmacological effectiveness.

The receptor purified here is that from denervated muscle. The endplate ACh.R of innervated muscle may not be identical. A small difference in iso-ionic points between soluble forms of these two types has been reported [18]; differences in blocking by *d*-TC in intact muscle in the two states have been analyzed [19], but these are not major. Others have found [20] no difference between ACh.R in homogenates of normal and of denervated muscle in their corresponding affinities for a series of nicotinic agonists and antagonits. The known physiological differences between normal and denervated muscle could, alternatively, arise from a difference in the associated ion translocation modulator in the two cases. However, isolation of the endplate ACh.R, although this is much less abundant, would be feasible by the method used here, and a comparison will be presented elsewhere.

The information so far obtained on the muscle ACh.R is sufficient to indicate a close relationship to that from electroplaques of various sources, beyond the level [1,2] at which the latter may differ between species. The α-neurotoxin reactivity, detergent solubility, nicotinic ligand binding, and molecular weight are essentially similar in each case. It appears, from the specific activities obtained, that an ACh-binding unit of the order of 100 000–150 000 daltons per α-toxin-binding site is the common basis of the nicotinic ACh.R from these diverse sources.

Acknowledgements

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References

[1] Meunier, J. C., Sealock, R., Olsen, R. and Changeux, J. P. (1974) Eur. J. Biochem. 45, 371–394.
[2] Karlin, A. (1974) Life Sciences 14, 1385–1415.
[3] Barnard, E. A., Wieckowski, J. and Chiu, T. H. (1971) Nature 234, 207–211.
[4] Miledi, R. and Potter, L. T. (1971) Nature 233, 599–601.
[5] Berg, D. K., Hall, R. B., Sargeant, P. B., Williamson, P. and Hall, Z. W. (1972) Proc. Nat. Acad. Sci. U.S.A. 69, 147–151.
[6] Barnard, E. A., Chiu, T. H., Dolly, J. O. and Porter, C. W. (1973) Trans. Amer. Soc. Neurochem. 4, 49.
[7] Chiu, T. H., Dolly, J. O. and Barnard, E. A. (1973) Biochem. Biophys. Res. Commun. 51, 205–213.
[8] Dolly, J. O. and Barnard, E. A. (1974) FEBS Lett. 46, 145–148.
[9] Barry, S. and O'Carra, P. (1973) Biochem, J. 135, 595–607.
[10] Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) J. Biol. Chem. 193, 265–275.
[11] Ellman, G. L., Courtney, K. D., Andres, V. and Featherstone, R. N. (1961) Biochem. Pharm. 7, 88–95.
[12] Berman, J. B. and Young, M. (1971) Proc. Natl. Acad. Sci. U.S.A. 68, 395–398.
[13] Hyden, H. and Langer, P. W. (1971) in New Techniques in Amino Acid, Peptide and Protein Analysis (A. Niederwieser and G. Patali, eds.) Ann Arbor Sci. Publ. Inc. 271–290.
[14] Eldefrawi, M. E. and Eldefrawi, A. T. (1973) Arch. Biochem. Biophys. 159, 362–373.
[15] Rosenberry, T. L., Chang, H. W. and Chen, Y. T. (1972) J. Biol. Chem. 247, 1555–1565.
[16] Hucho, F. and Changeux, J. P. (1974) FEBS Lett. 38, 11–15.
[17] Barnard, E. A. and Dolly, J. O. (1975) Proc. 6 th Internat. Cong. Pharmacol., Helsinki (in press).
[18] Brockes, J. P. and Hall, Z. W. (1975) Biophys. J. 15, 332a.
[19] Chiu, T. H., Lapa, A. J., Barnard, E. A. and Albuquerque, E. X. (1974) Exp. Neurol. 43, 399–413.
[20] Alper, R., Lowy, J. and Schmidt, J. (1974) FEBS Lett. 48, 130–132.