AFFINITY LABELLING BY BROMOACETYLCHOLINE OF A CHARACTERISTIC SUBUNIT IN THE ACETYLCHOLINE RECEPTOR FROM MUSCLE AND *TORPEDO* ELECTRIC ORGAN

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1. Introduction

The nicotinic acetylcholine receptor protein (AChR) has been purified to homogeneity from cat denervated muscle [1] and shown to be an oligomer with a sedimentation coefficient of 9 S [2], containing 1 major protein subunit [3,4]. This purified protein exhibited similar properties to the native membrane-bound AChR, including its affinity for agonists and antagonists [4]. However, chemical confirmation has not been supplied for the common identity of the single polypeptide isolated and the ligand-binding unit in the intact muscle membrane. Such evidence can be obtained from appropriate affinity labelling. The alkylating affinity reagent bromoacetylcholamine is known from electrophysiological evidence to react irreversibly with the acetylcholine recognition site of the AChR of frog skeletal muscle following reduction [5]. Furthermore, bromoacetyl-[^{3}H]choline and 4-(*N*-maleimido)-[^{3}H]benzyltrimethylammonium iodide ([^{3}H]MBTA) specifically alkylate a single polypeptide of AChR from *Torpedo* electric organ [6,7] following the reduction of a disulphide in the vicinity of the ACh binding site [8]. In contrast, [^{3}H]MBTA has been reported to label 2 out of 5 polypeptide chains found in a purified AChR preparation from rat denervated muscle [9].

In this report, bromo-[^{3}H]acetylcholine (Br[^{3}H]-AcCh) of high specific activity is used to specifically label one major subunit from cat denervated muscle AChR in its membrane-bound, soluble and purified states. We also address the question whether this alkylated component from muscle is identical to the equivalent labelled subunit from *Torpedo marmorata* AChR.

2. Experimental

Bromo-[^{3}H]acetylcholine bromide (Br[^{3}H]AcCh) of high specific activity (1.7–2.7 Ci/mmol) was synthesised using bromo-[^{3}H]acetic acid, as will be described elsewhere. After purification by thin-layer chromatography, radiochemical purity was confirmed by ascending paper chromatography, and the specific activity ascertained by mass spectrometry. All manipulations were at 4°C unless stated otherwise. Crude membranes were prepared from fresh cat denervated leg muscles [1] and frozen *Torpedo marmorata* electric organ by homogenising 5–10 g tissue in 5 vol. buffer A (50 mM K-phosphate (pH 8.0) containing protease inhibitors [3]). The homogenate was treated with 10^{-3} M diisopropylfluorophosphate (DFP), filtered through glass wool and centrifuged at 30 000 × *g* for 20 min. The pellet was resuspended in 5 vol. buffer A followed by homogenisation and centrifugation. AChR was assayed and purified from cat denervated muscle and from *Torpedo* electric organ by a procedure modified from that in [3]. Sucrose density gradient centrifugation was used to isolate the 9 S form of the AChR from muscle crude extracts [2]; sucrose was removed by gel filtration in buffer B (0.2% Triton X-100–25 mM K-phosphate (pH 8.0) containing all the protease inhibitors except EDTA [3]).

The alkylation procedure was modified from that in [8]. For labelling crude membranes, these were suspended in buffer C (100 mM NaCl, 5 mM K-phosphate, 1 mM EDTA, 3 mM sodium azide, 10 mM Tris–HCl) at pH 8.0 and reduced for 45 min at 22°C with 0.3 mM dithiothreitol (DTT). The pH was adjusted to pH 7.0 with K-phosphate and the DTT concentration diluted to 0.2 mM with buffer C at pH 7.0. Alkylation was with 1 μM Br[^{3}H]AcCh for 3 min at 22°C and the reaction was stopped by dilution in buffer C adjusted to pH 7.0, containing 100 μM unlabelled BrAcCh. Non-specific labelling was determined by treating samples with 5 μM α-bungarotoxin, 10^{-4} M tubocurarine or 10^{-3} M carbamylcholine before alkylation. To label the 9 S AChR isolated from crude extract (see above), this was incubated with an excess of concanavalin-A–agarose (Con-A–agarose, from Sigma) in the presence of 10^{-3} M DFP, 10^{-2} M $MgCl_2$ and 3×10^{-3} M $CaCl_2$ for 3 h at 4°C. The resin was recovered by centrifugation and washed in buffer B. The AChR immobilised thus on Con-A–agarose was reduced in buffer B (1:1, v/v) containing 0.3 mM DTT for 45 min at 22°C. The pH was adjusted and the alkylation conducted as above. The reaction was stopped by dilution and centrifugation in buffer B at pH 7.0. To label the purified AChR, this was reduced with 0.3 mM DTT in 0.4 M K-phosphate (pH 7.4) containing 10^{-4} M DFP, the pH adjusted to pH 7.0 and the alkylation performed as above; the reaction was stopped by gel filtration.

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS–PAGE) was performed by the method in [10] adapted for 10% acrylamide slabs (13 × 10 cm); samples were extracted and denatured at 80°C in 2% SDS–1% mercaptoethanol in sample buffer. Protein staining was as in [3]. Gels were prepared for fluorography as in [11] or sliced into 1 or 2 mm sections and counted.

3. Results

Crude membrane preparations from cat denervated muscle bound ~2 nmol α-[^{3}H]bungarotoxin/g protein. Reduction of these membranes by DTT and alkylation with Br[^{3}H]AcCh, followed by SDS–PAGE gave one major peak of radioactivity corresponding to app. mol. wt 43 000–44 000 together with minor

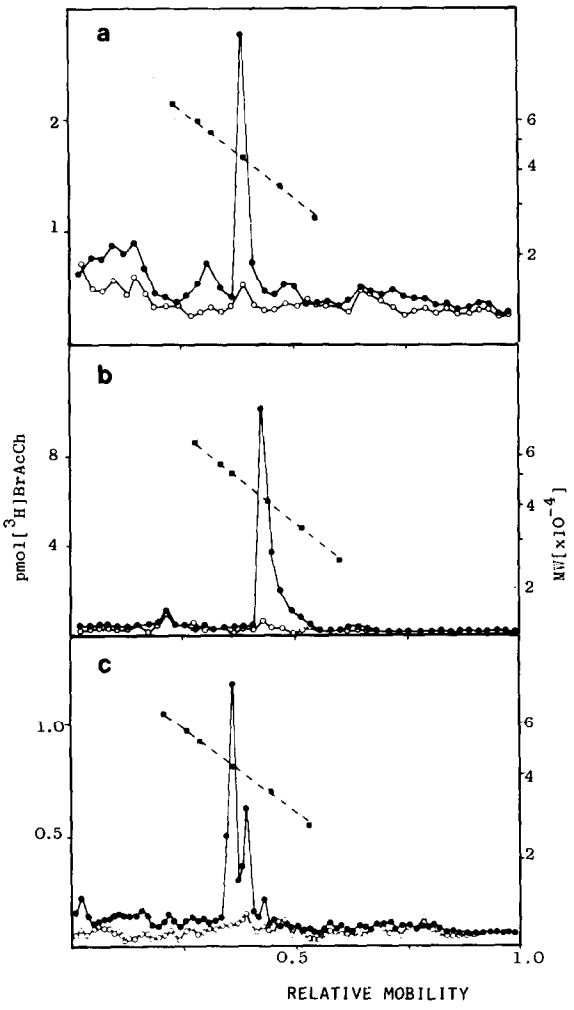

Fig.1. SDS–PAGE analysis of Br[^{3}H]AcCh alkylated AChR from cat denervated muscle. Gel tracks were stained for protein or sequentially sliced into 2 mm (a,b) or 1 mm (c) sections and counted as in section 2. (•) Alkylation of (a) muscle crude membranes, (b) 9 S fractions of Triton X-100 extract of muscle labelled after immobilisation on Con-A–agarose and (c) purified AChR. Labelling is expressed as pmol Br[^{3}H]AcCh/gel slice located by mobility relative to bromophenol blue (~10 cm). (○) Alkylation of samples pretreated with α-bungarotoxin. (▪) Calibration of molecular weights of standard proteins according to mobility relative to bromophenol blue (3 determinations). The proteins were bovine serum albumin, catalase, glutamate dehydrogenase, ovalbumin, lactate dehydrogenase and α-chymotrypsinogen A.

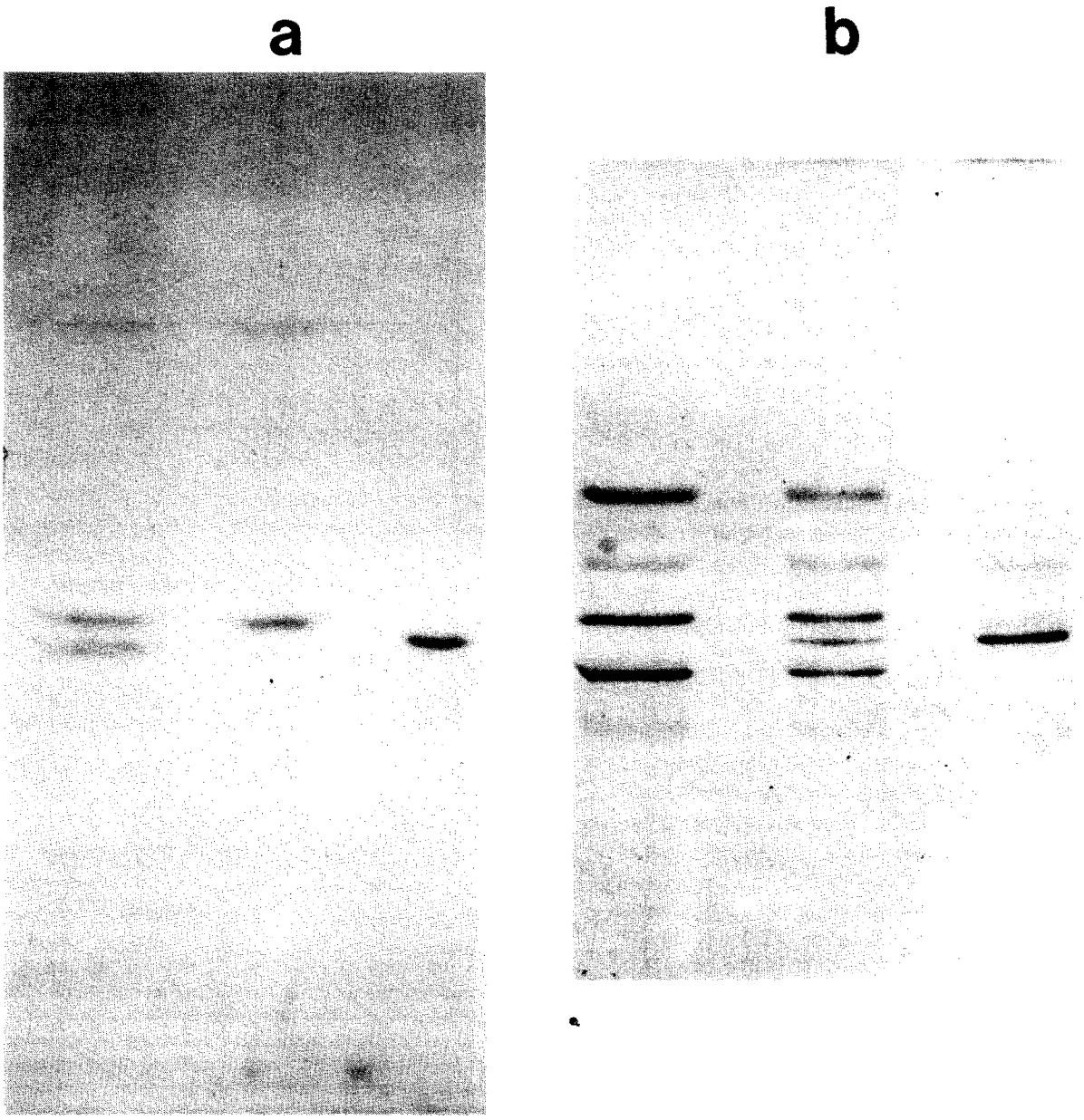

Fig.2. SDS–PAGE of muscle and *Torpedo* AChR. (a) Fluorogram of SDS–PAGE of Br[^{3}H]AcCh alkylated AChR of crude membranes from cat denervated muscle and *Torpedo* electric organ. Electrophoresis and fluorography were as in section 2. Prelashed Fuji RX film was exposed at −70°C for 7 days. Tracks left to right; (1) a mixture of *Torpedo* and muscle AChR; (2) muscle AChR; (3) *Torpedo* AChR. (b) SDS–PAGE and protein-staining of purified AChR from cat denervated muscle and *Torpedo* electric organ. Tracks left to right: (1) *Torpedo* AChR; (2) *Torpedo* and muscle AChR mixed; (3) muscle AChR.

levels of background radioactivity which varied in position between preparations (fig.1a). In another preparation where the radioactivity in SDS–PAGE was visualised by fluorography (fig.2a), the minor components of fig.1a were absent. Only the major peak was absent in samples treated with α-bungarotoxin before alkylation, and this corresponds to the single protein subunit of the AChR purified from the same tissue [3]. The ability of Con-A to bind AChR was used to immobilize the native 9 S form of AChR [4]. The immobilised complex proved to be stable under the conditions of reduction and alkylation. Analysis of alkylated samples after extraction from Con-A–agarose in SDS showed a single specifically-labelled component of mol. wt 43 000 as before, with negligible non-specific labelling (fig.1b). Reduction and alkylation of Con-A–agarose with similar extraction, showed no radioactivity on SDS–PAGE.

Purified AChR showed in protein staining after SDS–PAGE, a major subunit of app. mol. wt 43 000 together with a minor band of mol. wt 38 000. Radioactive analysis gave a peak of labelling at mol. wt 43 000 together with specific labelling, to a much lower extent, of a discrete peak at mol. wt 38 000 (fig.1c). The latter corresponds to the minor glycoprotein subunit observed in a small number of purified preparations of muscle AChR [3] which was sugggested to be a proteolytic product of the major polypeptide. It should be noted that in fresh, crude membranes immediately alkylated, the mol. wt 38 000 component was not present (fig.1a,2a). Analysis of other purified preparations of AChR alkylated after immobilisation on Con-A–agarose, gave an identical pattern of labelling which was totally abolished by α-bungarotoxin or D-tubocurarine or carbamylcholine. All of these observations support the assignment of the mol. wt 38 000 subunit as a proteolytic product of a native mol. wt 43 000 subunit containing the ACh-binding site.

Reduction and alkylation of crude membranes prepared to spec. act. 200 nmol α-bungarotoxin bound/g protein from *Torpedo* electric organ gave a single peak of radioactivity on SDS–PAGE in agreement with [6,7]. When the labelled *Torpedo* membranes were mixed with labelled muscle membranes and analysed in the standard gel system [10], the two specifically labelled subunits could not be readily separated. However, comparison of the protein subunit composition of AChR purified from these two sources in polyacrylamide gels with a lower concentration of *NN'*-methylene bisacrylamide (0.08% instead of the standard 0.27%; see [10]) demonstrated that the α-subunit (i.e., the smallest) of *Torpedo* and the single subunit of muscle AChR could be separated (fig.2b). The apparent molecular weights thus found were 43 000 and 46 000, but we do not attribute significance to the absolute values because the muscle receptor varies in apparent size between two electrophoretic systems. However, the separation of the two species in one system is significant. Analysis of *Torpedo* and muscle membranes specifically labelled with Br[^{3}H]AcCh gave a similar separation of the ACh-binding subunits of the two species in this latter system (fig.2a).

4. Discussion

This is the first report of the application of Br[^{3}H]AcCh as an affinity reagent for the AChR of skeletal muscle. Br[^{3}H]AcCh has been synthesised in our laboratory to a specific activity considerably higher than previously reported, and the incorporation of tritium into the acetyl moiety using bromo-[^{3}H]-acetic acid confers an advantage upon the reagent for protein structural studies, compared to that synthesised using [^{3}H]choline [6,7].

Specific labelling of a single, major polypeptide is observed in preparations of widely different purity from crude membranes to purified AChR, and this labelling is blocked by nicotinic agonists and antagonists. In addition, we have found that the 9 S oligomer may be bound to Con-A–agarose and manipulated through the alkylation procedure to give the same labelling of the AChR subunit. All the results confirm that the single subunit found in the muscle receptor carries the ACh binding site of the AChR oligomer. The molecular weight of the alkylated purified muscle AChR using a standard gel system agrees with that obtained in an identical analysis of AChR from crude membranes, but differs slightly from that previously reported from this laboratory for purified AChR using a less extensive set of standards, and actin as an internal marker [3]. SDS–PAGE using a lower bisacrylamide concentration can resolve the alkylated subunits of *Torpedo* and cat muscle

crude membranes, and a similar separation was obtained using purified material (fig.2a,b). The exact reason for variation of relative electrophoretic mobility with changes in gel composition is unclear, but parameters such as carbohydrate content and SDS-distribution may account for the observed separation. Thus, despite the great similarities between AChR of *Torpedo* and muscle [4], this clear dissimilarity, together with the purification by the same procedure of AChR with different protein subunit composition from the two tissues, is noteworthy.

The reported presence [9] of 2 major and 3 minor subunits in a purified AChR preparation from rat denervated muscle is in contrast to the single polypeptide observed in cat denervated muscle AChR [3]. In addition, equal labelling with [^{3}H]MBTA was reported for 2 subunits of mol. wt 49 000 and 45 000 from rat purified AChR although the 49 000 peak, is curiously, not a prominent component in the protein subunit pattern for this preparation [9]. It might be proposed that these two labelled polypeptides would correspond to the major subunit (mol. wt 43 000) and its putative degradation product (mol. wt 38 000) in purified AChR from cat denervated muscle. However, we disregard this possibility since the alkylation by Br[^{3}H]AcCh of a single, major polypeptide in crude membranes, together with the observation of a similar protein-stained subunit of purified AChR establishes the existence of a single size of ACh binding polypeptide in the AChR oligomer of cat denervated muscle.

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