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A STUDY OF THE MUSCARINIC RECEPTOR BY GEL ELECTROPHORESIS

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1 Muscarinic receptors from the brain of rat, guinea-pig and frog have been labelled with tritiated propylbenzilylcholine mustard ([^{3}H]-PrBCM).

2 After solubilisation and sodium dodecyl sulphate (SDS)-polyacrylamide gel electrophoresis a single radiolabelled peak was seen which was completely suppressed when labelling was carried out in the presence of 10^{-6} M atropine. The results suggest the presence of a single polypeptide chain with a molecular weight of ~80,000.

3 In the case of intestinal smooth muscle multiple labelled peaks were found. Precautions against proteolysis led to the identification of a major peak with a molecular weight in the region of 80,000.

Introduction

We have shown previously that the muscarinic receptor in brain behaves as a homogeneous entity in its binding parameters for antagonists (Hulme, Birdsall, Burgen & Mehta, 1978), but shows heterogeneity when tested by agonist binding (Birdsall, Burgen & Hulme, 1978a). The heterogeneity requires the presence of at least three populations of sites whose proportions vary in different parts of the brain (Hulme, Birdsall & Burgen, unpublished observations). We have suggested that a possible explanation for these findings is that there is in reality only a single class of receptor subunit that binds the ligands but that the energetics of the agonist-induced conformational change are modified by different membrane environments or by coupling of subunits to various different effector units (Birdsall, Burgen & Hulme, 1978b). One way of distinguishing this situation from one in which there are structurally different ligand binding receptor units is to label the receptor binding site and see if the labelled units appear as single or multiple bands in protein separation procedures. We have previously shown that the aziridinium ion of [^{3}H]-propylbenzilylcholine mustard ([^{3}H]-PrBCM) is a highly specific covalent affinity label for the muscarinic receptor and that it labels the receptor in a uniform manner like reversible antagonists and does not discriminate between the sub-types revealed by agonist binding (Burgen, Hiley & Young, 1974; Birdsall *et al.*, 1978a). Receptor thus prelabelled in brain homogenates or purified membrane preparations provides suitable material for examination of receptor homogeneity or heterogeneity by electrophoretic methods.

Methods

Subcellular fractions

Brain A crude synaptosome fraction from brain was prepared as described previously (Hulme *et al.*, 1978) by differential centrifugation of a homogenate prepared in 0.32 M sucrose. The microsomal fraction was prepared by centrifugation of the 10,000 *g* supernatant at 100,000 *g* for 60 min. Synaptic membrane fractions were prepared as described previously (Birdsall *et al.*, 1978a).

Smooth muscle A microsome fraction was prepared from smooth muscle of the guinea-pig ileum as described by Fewtrell & Rang (1973). Homogenization was carried out with a Sorvall 'Omnimixer' at full speed in 15 s bursts, separated by 2 min of cooling in ice. Total homogenization time was 2 min. When necessary, 1 mM phenylmethylsulphonyl fluoride (PMSF) was present throughout the isolation procedure.

Labelling of subcellular fractions

The subcellular fraction was resuspended to a concentration of 0.1 to 0.3 mg/ml protein (corresponding to a concentration of muscarinic antagonist binding sites of 0.2 to 0.6 × 10^{-9} M, Hulme *et al.*, 1978) in Krebs-Henseleit solution, or 10 mM sodium phosphate buffer, pH 7.0. After preincubation for 15 min at 30°C cyclised [^{3}H]-PrBCM (sp. act. 35 Ci/mmol) was added to a final concentration which was routinely 2.5 × 10^{-9} M, although higher concentrations

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were sometimes used for specific purposes. After 15 min incubation at 30°C the incubation mixture was rapidly cooled to 0°C and the membrane fraction was recovered by centrifugation at 100,000 *g* for 30 min at 0°C. The pellet was resuspended to its original volume in ice-cold buffer, and recentrifuged. This washing procedure was repeated. The pellet could then be frozen in dry ice/isopropanol and stored at −70°C, until required or solubilized immediately. The choice of buffer in which labelling was carried out did not affect the level of labelling obtained, or the distribution of radioactivity on sodium dodecyl sulphate (SDS)-gels. For measurement of non-specific (non-receptor) binding of [^{3}H]-PrBCM, 10^{-6} M unlabelled atropine was included in the incubation medium. In one set of experiments with smooth muscle microsomes, 1 mM PMSF was added to the incubation medium (10 mM sodium phosphate) without altering the level of labelling.

Solubilization of subcellular fractions

Subcellular fractions were solubilized at a concentration of 1 mg protein/ml in 20 mM sodium phosphate buffer containing 1% w/v SDS, and 1% v/v 2-mercaptoethanol, pH 7.0. Samples were usually heated at 100°C (boiling water bath) for 3 min, then incubated at 50°C for 2 h. In the case of the brain subcellular fractions, milder solubilization conditions were also explored; omission of heating to 100°C altered neither the staining profile nor the distribution of radioactivity in the gels. After solubilization, samples were centrifuged for 60 min at 100,000 *g*. More than 95% of the radioactivity remained in the supernatant, which was dialysed for 15 h at room temperature against 10 mM sodium phosphate/0.1% w/v SDS containing 0.1% 2-mercaptoethanol, when electrophoresis was to be carried out according to the method of Weber & Osborn (1969), or against 0.1 M Tris-Cl, 0.1% 2-mercaptoethanol, 0.1% SDS before use of the discontinuous system of Laemmli (Laemmli, 1970; Dewald, Dulaney & Touster, 1974).

Labelling and solubilization of smooth muscle strips

Intact strips of longitudinal smooth muscle from the guinea-pig ileum were prepared and labelled with [^{3}H]-PrBCM (10^{-8} M) as described by Burgen *et al.* (1974). The labelled strips were plunged for 3 min into boiling 10 mM sodium phosphate buffer (0.02 to 0.04 g tissue/ml), containing 1 mM PMSF, 1% v/v β-mercaptoethanol and various concentrations of SDS. Incubation was continued at 50°C for 2 h. Insoluble material was removed, and the extract dialysed as described above.

Chemical modification of extracted proteins

In some experiments carboxamidomethylation of the initial extract was carried out before dialysis. Iodoacetamide (0.2 mmol) was added to each 1 ml of extract, at room temperature. The pH of the reaction mixture was maintained between 9 and 10 by the addition of concentrated NaOH solution. After 30 min, the reaction was stopped by addition of 1% v/v 2-mercaptoethanol, and the reaction mixture dialysed before electrophoresis as described above.

Gel electrophoresis

Disc gel electrophoresis was carried out according to the method of Weber & Osborn (1969) or according to the method of Laemmli (1970) as described by Dewald, *et al.* (1974), with the addition of 0.1% SDS to the electrophoresis buffer, and to the gel solutions. Gels had a diameter of 0.5 cm and a length of 7 cm. Gel composition was 2.7%C/7%T. The tracking dye (bromophenol blue) was allowed to migrate about 6 cm. Gels were either stained and destained as described by Weber & Osborn (1969) or cut into 1 mm slices and incubated with 0.2 ml of 50% H_2O_2 at 40°C in closed scintillation vials overnight, or until dissolved. Radioactivity was estimated by liquid scintillation counting after the addition of 10 ml of a Triton-based scintillator. Recovery of radioactivity from the gels was 70 to 80%. Counting efficiency was 30%. β-Galactosidase (*E. coli*), catalase (beef liver), lactate dehydrogenase (rabbit muscle) and lysozyme (all from Sigma) were used as molecular weight markers.

Results

Figure 1 shows the results obtained with these methods on a microsome preparation from rat cortex. A single labelled peak was seen which contained essentially all of the specifically labelled protein. This peak was entirely absent when the labelling procedure was carried out in the presence of 10^{-6} M atropine which would be expected to reduce specific labelling by a factor of 1000. Virtually identical electrophoretic profiles were obtained when crude synaptosomes, synaptic membrane fractions, microsomes and crude post-nuclear preparations were labelled with [^{3}H]-PrBCM and analysed by SDS-polyacrylamide gel electrophoresis (PAGE). Staining of the gels with Coomassie blue revealed a sharp pattern of stained bands showing the high resolution of the method. Calibration of the gels gave an estimated molecular weight for the receptor of 83,200 ± 2,500 (Table 1).

The radioactivity cannot be associated in any confident way with any of the stained bands because of

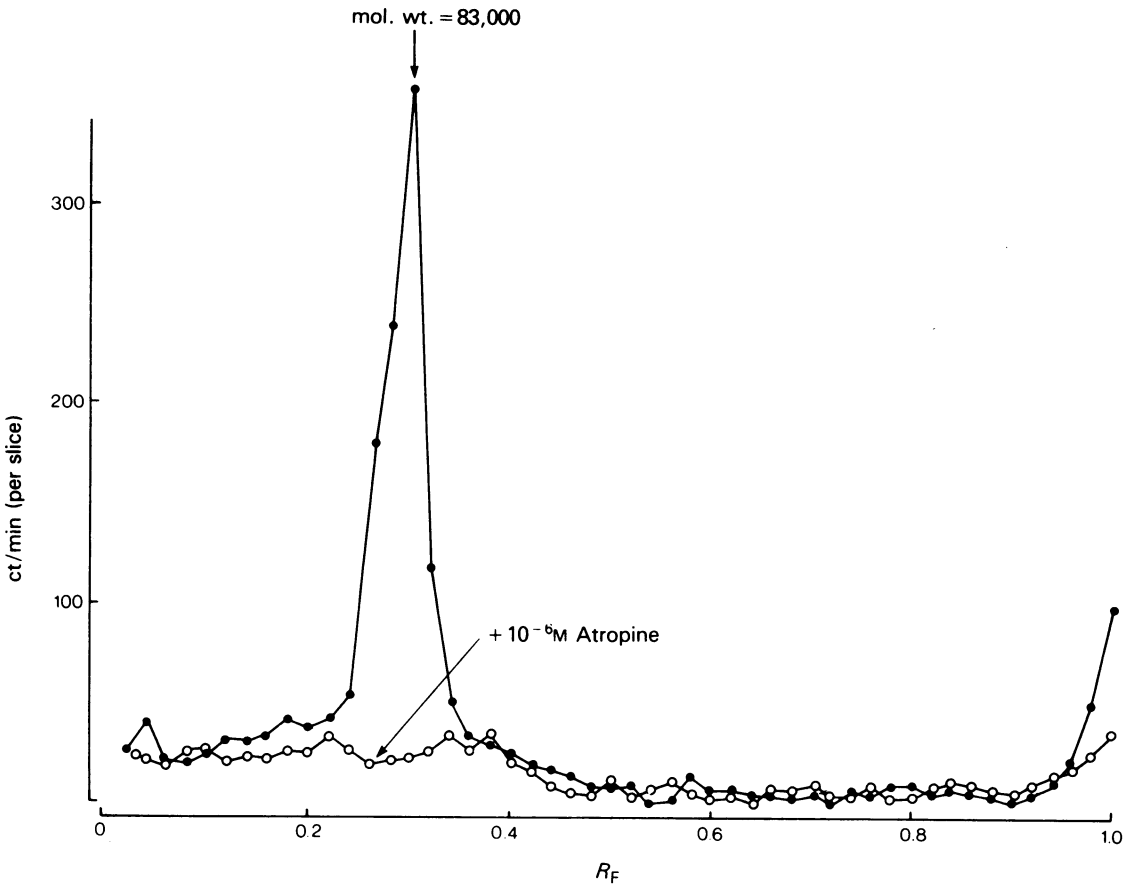

Figure 1 Sodium dodecyl sulphate-polyacrylamide gel electrophoresis of [^{3}H]-propylbenzilylcholine mustard ([^{3}H]-PrBCM)-labelled microsome preparation from rat brain. Total incorporation of [^{3}H]-PrBCM was 1.4 nmol/g protein when labelling was carried out in the absence of atropine and 0.46 nmol/g protein when labelling was conducted in the presence of 10^{-6} M atropine; 56 μg protein was applied to gels corresponding to 1840 ct/min (●, no atropine) and 600 ct/min (○, 10^{-6} M atropine). Overall recovery of radioactivity was 82%. Recovery between fractions 12 and 17 corresponded to 81% of the specific binding.

the low abundance of the receptor. If we take the mol. wt. of 83,200 and the specific labelling density in this preparation of 1.4 nmol/g protein then receptor protein accounted for only 0.012% of the total protein and would not yield a visible band.

The log R_F of the peak was linearly dependent on total acrylamide concentration and its free electrophoretic mobility was similar to that of standard soluble proteins (Figure 2). There is thus no anomalous behaviour on SDS-PAGE making it unlikely that the receptor has a large carbohydrate content (Banker & Cotman, 1972). Carboxamidomethylation with iodoacetamide did not change the apparent molecular Cotman, 1972). Carboxamidomethylation with iodacetamide did not change the apparent molecular weight of the receptor peak nor introduce heterogeneity. Membrane preparations from the rat cerebellum and hippocampus, two areas with patterns of

Table 1 Mol. wt. of [^{3}H]-propylbenzilylcholine mustard-binding subunit of muscarinic receptor

	Mol. wt. ± s.e. mean
Rat brain	83,200 ± 2,500 (6*)
Guinea-pig brain	83,200 ± 6,000 (3)
Guinea-pig ileum	77,600 ± 2,000 (4)
Frog brain	80,000

* Number of independent experiments.

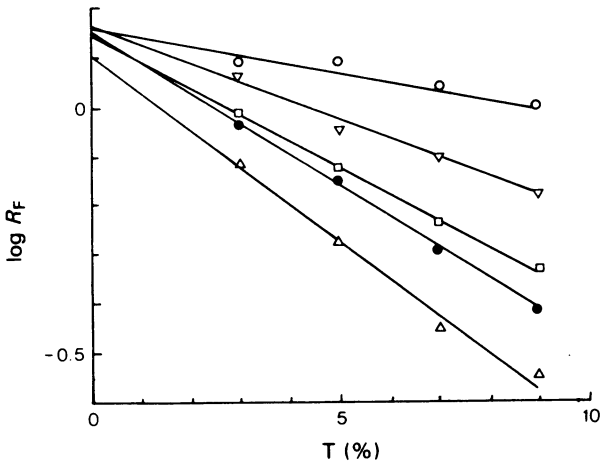

Figure 2 Ferguson plot comparing the [^{3}H]-propylbenzilylcholine mustard ([^{3}H]-PrBCM) labelled polypeptide with a series of standard proteins: (△) β-galactosidase; (□) catalase; (▽) lactate dehydrogenase; (○) lysozyme; (●) [^{3}H]-PrBCM-labelled polypeptide. Points were fitted to the equation

$$\log(R_f) = \log(\mathrm{Mo}) - K_R.\mathrm{T}$$

where R_F is the mobility relative to tracking dye, Mo is the free electrophoretic mobility, K_R is the retardation coefficient and T is the acrylamide concentration. Values of Mo did not differ significantly. Values of K_R were β-galactosidase: 0.075; catalase: 0.055; muscarinic receptor: 0.065; lactate dehydrogenase: 0.041; lysozyme: 0.018.

agonist affinity different from that seen in the cortex (Hulme, Birdsall & Burgen, unpublished observations) again showed the same electrophoretic profile.

We have shown that it is possible to label selectively the high and low affinity receptor binding sites by agonist protection (Birdsall *et al.*, 1978a). When this was done, once again no distinction could be made in electrophoretic behaviour.

Experiments with microsome preparations from guinea-pig cerebral cortex and with a P_2 fraction from the frog forebrain yielded essentially the same results, a single radioactive peak with an apparent mol. wt. close to 80,000 (Table 1).

Smooth muscle

Microsome fractions were prepared from guinea-pig ileum longitudinal muscle strips and labelled with [^{3}H]-PrBCM to a level of 1 to 2 nmol/g protein. The main peak detected on electrophoresis (Figure 3a) had an apparent mol. wt. of 60,000 and corresponded to about 40% of the specific radioactivity. There was also a smaller peak at 80,000 and some labelled material of lower mol. wt.; little radiolabelled material was retained in the top of the gel. All these peaks were suppressed by inclusion of atropine when reacting the membranes with [^{3}H]-PrBCM. This heterogeneity of specific labelling was highly suggestive of proteolysis of the receptor due to liberated proteases such as is well known to be a problem in attempting to isolate the nicotinic receptor from skeletal muscle (Dolly & Barnard, 1975). Addition of PMSF (1 mM) during labelling confirmed this by increasing the labelling in the higher mol. wt. peak but did not change the pattern to a single peak (Figure 3b). A considerable proportion (about 40%) of the radioactivity was now retained in the top 2 mm of the gel. A drastic solution was adopted. Intact strips of longitudinal smooth muscle from the guinea-pig ileum were labelled with [^{3}H]-PrBCM and then plunged into buffer containing 4% SDS, 1% mercaptoethanol and 1 mM PMSF, maintained at 100°C (cf. Methods). Ninety percent of the label went into solution but the contractile proteins remained undissolved. Electrophoresis of the soluble fraction now revealed only one major peak at about 76,000 mol. wt. However, this peak accounted for only 26% of the specifically bound radioactivity, the remainder being in the upper part of the gel. The staining pattern of these gels showed considerable streaking in the high mol. wt. region. Carboxamidomethylation of the extract improved the fraction under the 80,000 peak to 47% (Figure 3c) but there remained evidence of high mol. wt. aggregates. Simultaneously conducted electrophoresis experiments revealed no detectable differences between the electrophoretic mobilities of the [^{3}H]-PrBCM-labelled peaks obtained from rat cortex membrane preparations, and intact longitudinal muscle strips from the guinea-pig ileum.

It is interesting that we have seen no evidence of proteolysed fragments of receptor in preparations from the cerebral cortex, but some proteolysis occurred in membranes from the cerebellum.

Discussion

SDS-PAGE is a method that separates proteins according to their apparent molecular weights. Deviations from true mol. wt. occur due to variations in binding of SDS and to assymetry factors. Nevertheless, the present results indicate that the receptor component in brain that binds antagonists has a mol. wt. of about 80,000 and that this is similar in rat, guinea-pig and frog. There seems to be a single labelled peak in these preparations and this shows that if there is heterogeneity in the receptor component it does not involve proteins that differ in mol. wt. within the resolution of this method (~3000 daltons). We cannot exclude the possibility of hetero-

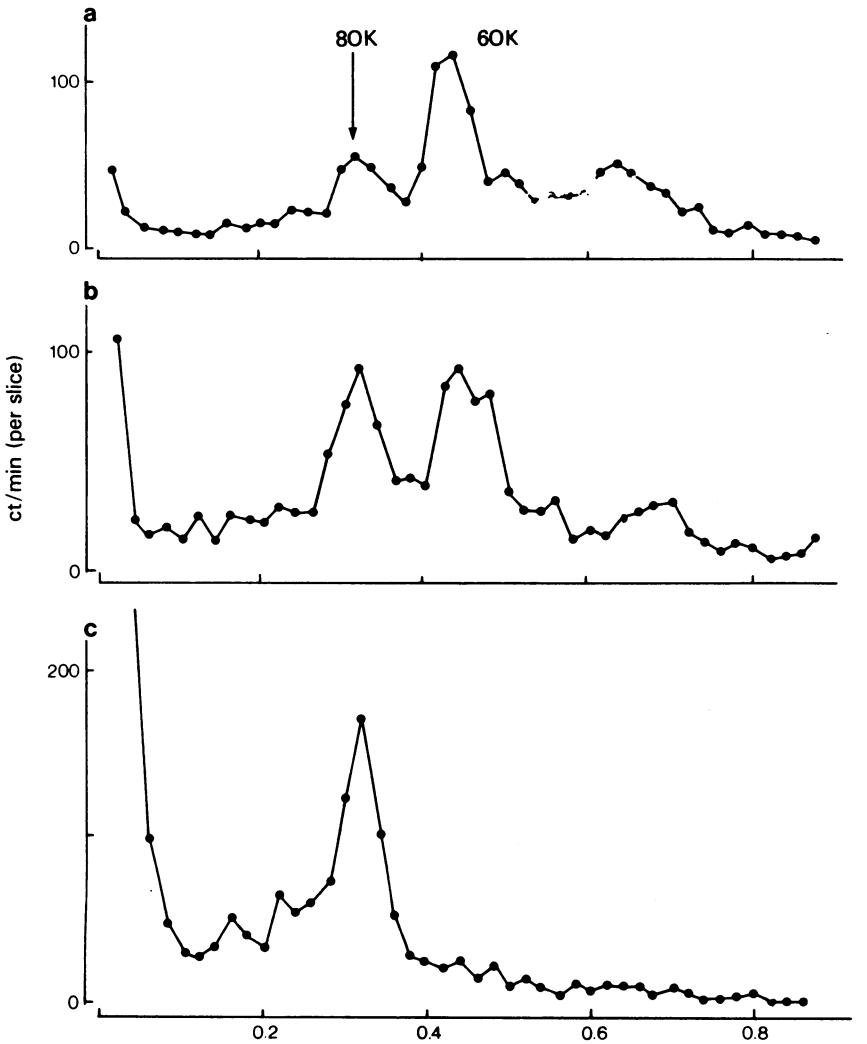

Figure 3 Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) of [^{3}H]-propylbenzilylcholine mustard [^{3}H]-PrBCM-labelled polypeptide from guinea-pig ileum smooth muscle preparations. (a) Microsome fraction prepared and labelled in the absence of proteolytic inhibitors; (b) microsome fraction prepared and labelled in the presence of 1 mM phenylmethylsulphonyl fluoride (PMSF); (c) intact longitudinal muscle strips labelled with [^{3}H]-PrBCM and extracted directly with buffer containing 4% SDS and 1mM PMSF. The extract was carboxamidomethylated. Incorporation of [^{3}H]-PrBCM was about 1 nmol/g protein in (a) and (b) and 44 pmol/g wet weight tissue in (c); 80 to 90% of the label was atropine-sensitive. Loading was 2000 to 3000 ct/min per gel. Recoveries were 60 to 80%.

geneity due to amino acid substitutions without overall change in the size of the polypeptide.

All we can say is that there is a further piece of evidence consistent with but not proving the idea that the receptor polypeptide itself is homogeneous and that the heterogeneity in binding properties is due to associated elements not investigated in the present experiments (Birdsall *et al.*, 1978b).

The results in smooth muscle are still not very satisfactory. Fewtrell & Rang (1973) found a pattern not dissimilar to that which we show in Figure 3a and also found smearing of protein bands in the gel.

Our results show that the low mol. wt. peaks found by these authors were due to proteolysis, a notoriously difficult problem with membrane preparations from muscle. There seems little doubt that the receptor from smooth muscle has a similar mol. wt. to that from brain. However, it differs in its tendency to aggregate; this may well be due to associated proteins extracted with the receptor.

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