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ACTION OF DETERGENTS ON COVALENTLY LABELLED, MEMBRANE BOUND MUSCARINIC ACETYLCHOLINRECEPTOR OF BOVINE NUCLEUS CAUDATUS

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SUMMARY

The muscarinic acetylcholinereceptor associated with highly purified neuronal membranes from bovine nucleus caudatus was covalently labelled with N-[2',3'-$^{3}H_2$]propyl-N-2-aziridiniumethylbenzilate. The influence of various detergents on binding activity of the membrane bound receptor and on solubilization of the labelled receptor was studied. A constituent part of the muscarinic acetylcholinereceptor, which is easily extracted by certain detergents without extracting the labelled protein, seems to be partially responsible for binding activity of the receptor protein.
Sodiumdodecylsulfate-gradient-gelelectrophoresis reveals that the labelled subunit has a molecular weight of 75 000.

INTRODUCTION

Difficulties in finding solubilizing agents not destroying the binding activity of solubilized and not solubilized muscarinic acetylcholinreceptor indicate the intimate association of this protein with the lipid membrane environment (1). Decrease in binding activity after treatment of crude neuronal membranes with phospholipases strongly support this finding (2). We were interested in the question, wether decrease or complete loss of binding activity on treatment of neuronal membranes with detergents is due to the action of a certain detergent on the receptor in the membrane bound state or is a consequence of converting the receptor from the membrane bound to the solubilized state.

METHODS

Digitonin and SPS-7,-11 and -15 were obtained from Serva (Heidelberg). The covalently labelling reagent of the m-AcChR[^{3}H]-PrBCM was synthesized and cyclizised according to lit. (5) as described in lit. (6).

ABBREVIATIONS

m-AcChR	muscarinic acetylcholinreceptor
[^{3}H]-PrBCM	N-[2',3'-$^{3}H_2$]propyl-N-2-aziridiniumethylbenzilate
SPS	Sucrose-palmitate-stearate
CTA	Cetyltrimethylammoniumbromide
SDS	Sodiumdodecylsulfate

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Labelling and extraction of neuronal membranes: Preparation and characterization of the neuronal membrane fractions $P_3^1/1$, $P_3^1/2$ and $P_3^1/3$ (subfractions of a washed and lysed "microsomal" P_3^1-fraction, numbers correspond to increasing density of the membranes; P_3^1: lysed P_3-fraction) is described in lit. (6).

Labelling conditions correspond to the conditions described for the filtration assay and were shown to give saturation (6).

Two equal samples of neuronal membranes were diluted with modified ringer solution (0,5 mM Ca^{2+}) to 100 ml (0,15 - 0,18 mg protein/ml), one containging 10^{-6} M atropine, the other containing no atropine, and incubated at 30° for 10 minutes. Both samples were then brought to a ^{3}H-PrBCM concentration of 5,7 x 10^{-9} M by adding 5 ml of 1,2 x 10^{-7} M ^{3}H-PrBCM. Labelling was stopped by adding 75 ml 40 mM $Na_2S_2O_3$, 20 mM $MgCl_2$ in modified ringer solution. Centrifugation was carried out at 100 000 g/60 minutes. The supernantant was discarded, the pellets carefully drained with paper, and rinsed twice with 5 ml 10 mM phosphate buffer pH 7,4. The appropriate pellets of the two samples were combined and rehomogenized to a protein concentration of 2 - 2,5 mg/ml. Aliquotes of the two membrane samples labelled in presence and in absence of atropine respectively were dotted on filter paper, combusted and the activity counted at an efficiency of 29 - 31 %.

Equal aliquotes of the two membrane samples labelled in presence and in absence of atropine were brought to the extraction conditions mentioned in table 1 by adding the detergent concerned in 10 mM phosphate buffer pH 7,4. After stirring for 3 hours at room temperature the mixtures were diluted to appropriate volumes and centrifuged at 200 000g_{max}/60 minutes. The percentage of specifically labelled m-AcChR extracted (respectively not extracted) was calculated from the amount of activity extracted (not extracted) from the membranes labelled in absence of atropine minus the amount of activity extracted (not extracted) from the membranes unspecifically labelled in presence of atropine, and is given in table 1 together with the percentage of protein extracted (not extracted).

SDS-gradient-gelelectrophoresis: Acrylamide-gradient-gels (3 % - 23 %) were prepared in tubes of 6 - 7 mm diameter corresponding to a method described in lit. (7). The buffer system used was 0,1 M borate/acetate pH 8,5, containing 0,1 % SDS. Membranes labelled in absence of atropine or protein extracts prepared from such membranes, brought to a suitable protein concentration, were incubated at 90° for 5 minutes with 1 % SDS, 1 % mercaptoethanol. 100 - 200 µl of the denatured protein solutions containing 50 - 150 µg protein and 2500 - 6000 dpm were layered on top of a gradient gel and electrophorized at 40 V, 7 - 8 mA/gel for 22 hours. Preliminary experiments had shown, that during staining and destaining of the gels for protein only little of the activity applied to the gel was lost. The gels were stained (0,1 % Coomassie brilliant blue for 15 hours, destaining with methanol, acetic acid, water 1:1, 5:17,5; 2 days), and scanned at 560 nm. The stained bands as well as the unstained intermediate spaces were dissected, considering the diagram of the scanner, in a way that the thickness of the slices could be taken from the scanner-diagram. The gel slices were dried on filter paper and combusted. For graphical representation the activity measured was standardized to a gel slice thickness of 1 mm and plotted as dpm/mm against the migration distance, the actual gel slice thickness indicated by the bars. Electrophoresis experiments with membranes labelled in presence of atropine had shown that only 50 % of the activity applied was recovered (70 - 80 % in the case of membranes labelled in absence of atropine), most of the activity being accumulated in a band at the same migration distance as in the experiments with membranes labelled in absence of atropine. Calculations of molecular weights were done using oligomers of bovine serum albumin and ovalbumin.

RESULTS AND DISCUSSION

The membranes chosen for extraction were the highly purified neuronal membrane fractions of potential postsynaptic origin, differing in their enrichment of Na^+K^+-ATPase, Acetylcholinesterase and m-AcChR (6). As a consequence of their careful and tedious preparation these membranes do not contain as much myelin and other lipid material as can be expected from the membranes used in lit. (1,2). The extraction experiments carried out with these membranes could not reveal marked differences in solubilization of the m-AcChR from different membrane fractions reflecting their different lipid content.

The intimate association of the m-AcChR with its membrane environment is shown by the data presented in table 1. Using different kinds of detergents the labelled [^{3}H]-PrBCM-binding-site of the receptor is extracted only nearly to the same extent as protein is extracted from the membranes. The data available up to now did not show, wether the binding capacity of the m-AcChR is destroyed under the influence of a certain detergent as a consequence of solubilizing the binding site or in spite of the fact that the binding site remains membrane bound. It is known that after exposure of neuronal membranes to Triton X 100 with concentrations higher than 0,05 % no binding activity of the receptor is retained, neither among the proteins solubilized nor in the remaining pellet (1,6). The data in table 1 demonstrate that even under the relatively drastic extraction conditions applied only 66 % of the [^{3}H]-PrBCM binding sites were extracted (this value dropped for about 10 % on extraction at 4^o). So it turns out, that under the influence of Triton X 100 the binding activity is lost not only if the binding site becomes solublized but even if it remains membrane bound. The same considerations are suitable to anionic and cationic detergents like sodium deoxycholate or Cetyltrimethylammoniumbromide, the latter leading to an irreversible loss of binding capacity at concentrations above 10^{-3} M (2), conditions under which only little extraction of the [^{3}H]-PrBCM-binding-site takes place (see table 1). These results might leed to the conclusion, that an extractable lipid-like constituent of the membrane is

Table 1: Extraction of neuronal membranes containing ^{3}HPrBCM labelled mAcChR with different detergents.

Conditions of extraction; extracted membrane fraction	Atropine sensitive activity[1] (% of activity applied) in the supernatant	pellet	Protein[1] (% of protein applied) in the supernatant	pellet
1 mM Phosphate, 1 mM EDTA; P_3^1	16 ± 1	82 ± 1	10 ± 2	88 ± 2
2 M NaCl; $P_3^1/3$	13 ± 2	86 ± 1	14 ± 2	86 ± 2
1 % SPS 7; P_3^1	10 ± 1	88 ± 1	5 ± 1	-
1 % SPS 11; P_3^1	11 ± 1	88 ± 1	7 ± 1	-
1 % SPS 15; $P_3^1/1$ and $P_3^1/2$	53 ± 3	45 ± 1	36 ± 3	58 ± 3
0,5 % SPS 15; $P_3^1/3$	40 ± 1	59 ± 1	32 ± 1	68 ± 1
1 % Digitonin; $P_3^1/3$	60 ± 5	38 ± 5	49 ± 3	54 ± 5
0,5 % Digitonin; $P_3^1/2$	53 ± 4	48 ± 5	40 ± 4	62 ± 4
0,1 % Digitonin; $P_3^1/2$	9 ± 1	86 ± 3	11	88 ± 1
0,5 % Sodiumdeoxycholate; $P_3^1/2$	90	11	69	23
0,1 % Sodiumdeoxycholate; $P_3^1/2$	15	76	23	73
0,5 % Sodiumtaurocholate; $P_3^1/2$	56	45	51	55
0,5 % CTA; $P_3^1/2$	77	24	85	10
0,1 % CTA (4×10^{-3} M); $P_3^1/2$	25	74	39	60
1 % Triton X 100; $P_3^1/1$ and $P_3^1/2$	66 ± 4	34 ± 4	65 ± 2	32 ± 3
0,1 % Triton X 100; $P_3^1/1$ and $P_3^1/2$	66 ± 3	30 ± 2	42 ± 2	57 ± 3
0,02 % Triton X 100; $P_3^1/3$	9	91	8	92

[1] the given values are means of 2 - 3 experiments; values without deviation depend on one extraction experiment only

partially responsible for binding, as can also be supposed on the basis of some experiments in which binding capacity was partially restored by adding certain lipids after treatment of membranes with phospholipases and Cetyltrimethylammoniumbromide at concentrations lower than 10^{-3} M (2).

The intention to solubilize the m-AcChR with as much as possible binding capacity retained must therefore take into account that the constituent essential for binding activity, and present in the membrane, but lost on exposure to one of the usual detergents, must be compensated by a molecule which could fullfil the needed requirements for preserving binding activity. Digitonin seems to be a molecule, which at least partially can take the place of this essential constituent, as was demonstrated by the experiments in which 20 % of the original binding capacity could be retained in the solubilized state (1,3). In our hands the used charge of digitonin turned out to be nearly as effective in extracting the labelled [^{3}H]-PrBCM-binding-site as Triton X 100. This would mean, that with digitonin too some binding capacity is lost on solubilization, reflecting the fact that not all of the binding sites are solubilized with their binding activity retained.

The question arose, wether other detergents which resemble digitonin in so far that the hydrophilic part of the molecule contains a carbohydrate moiety could substitute the expensive digitonin. The extractions with the mixed fatty acid esters of sucrose which are rather inexpensive chemicals demonstrated that SPS-15, the most soluble of these mild detergents, is nearly as effective as digitonin in extracting the [^{3}H]-PrBCM-binding-site.

The data in table 1 also definitely confirm our assumption that the m-AcChR from bovine nucleus caudatus cannot be solubilized by treatment with 2 M NaCl (6). The atropine sensitive activity released on exposure to 2 M NaCl is only in the same order of magnitude as if NaCl is absent. A solubilizing effect of NaCl leading to a 60 % solubilization (8) is therefore not established and we cannot assume a binding site anchored only by ionic forces to the membrane.

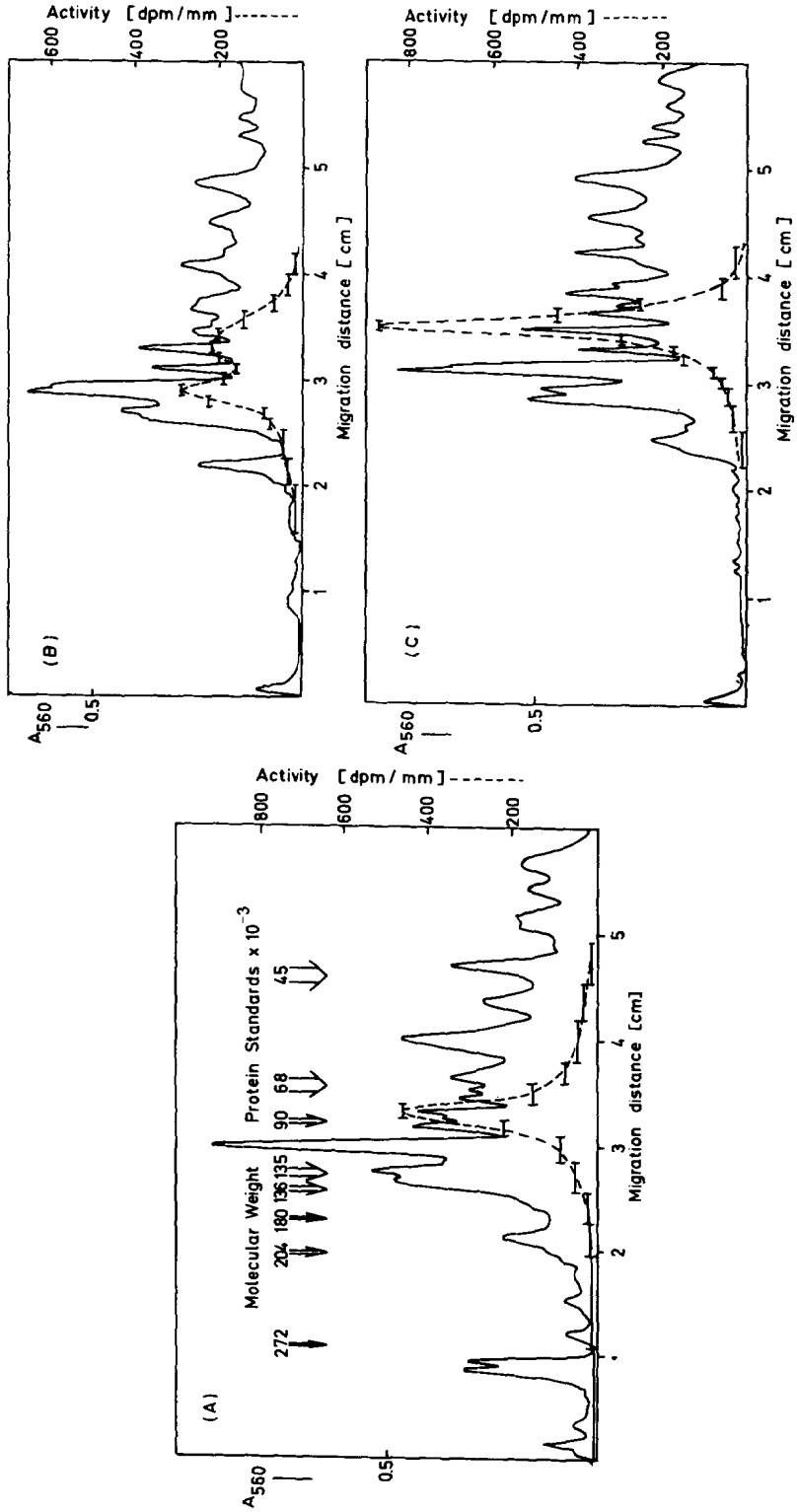

Fig. 1: Densitometer tracings at 560 nm (——) and distribution of activity (- - - -) in SDS-polyacrylamid-gradient-gelelectrophoretograms (3 % - 23 %) of [^{3}H]-PrBCM-labelled neuronal membranes and extracts from such membranes, electrophoresed under the conditions described in Methods.

(A) [^{3}H]-PrBCM-labelled P_3^1/2-membranes; 40 µg protein; 2750 dpm.

(B) 1 % digitonin extract from [^{3}H]-PrBCM-labelled P_3^1/2-membranes; 40 µg protein; 2750 dpm.

(C) 1 % SPS-15 extract from [^{3}H]-PrBCM-labelled P_3^1/2-membranes; 40 µg protein; 3800 dpm.

SDS-gradient-gelelectrophoresis gives clear resolution of numerous protein species present in the different membranes and extracts. Some more detailed information about the protein species which contain the labelled binding site could therefore be obtained.

From the diagrams shown in figure 1, and from other diagrams not shown, a molecular weight of 75 000 ± 10 000 was calculated for the smallest protein subunit bearing the tritiated label. In some detergent extracts, as well as in the corresponding detergent treated pellets, a second labelled protein species with an approximate molecular weight of 115 000 ± 15 000 could be detected, for example in the case of digitonin (figure 1 b) and in the case of Triton X 100 (not shown) but not in the case of SPS-15 (figure 1 c). It has to be investigated, wether this second labelled protein is a dimer only, or wether its occurence has something to do with a characteristic influence of certain detergents on the m-AcChR.

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