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EXTRACTION OF PERIPHERAL PROTEINS FROM NICOTINIC ACETYLCHOLINE RECEPTOR-ENRICHED MEMBRANES

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The solubilisation of membrane proteins from nicotinic acetylcholine receptor-enriched membranes from the electric organ of *Torpedo marmorata* was studied. Chaotropic ions were shown to be ineffective in extracting peripheral proteins from these membranes. Two different anhydrides, 2,3-dimethylmaleic and 3,4,5,6-tetrahydrophthalic anhydride, released certain peripheral membrane proteins but not the integral receptor protein. Treatment of membranes containing > 3 nmol α-bungarotoxin binding sites per mg protein with anhydride resulted in a 43 kDa polypeptide as the major constituent of the solubilised material. The nature of the 43 kDa polypeptide is discussed. Gentle anhydride treatment did not change the α-bungarotoxin and carbamoylcholine binding properties of the receptor.

Introduction

There are several methods available for preparing purified nicotinic acetylcholine receptor. Firstly, there are the affinity chromatographic methods of detergent solubilised receptor [1], with the major drawbacks that this receptor is unsuitable for ion-flux measurements and looses its high-affinity binding of agonists [2]. Secondly, the preparation of nicotinic acetylcholine receptor enriched membranes has been described, by methods using either centrifugation in sucrose density gradients [3–5] or liquid partitioning [6–8]. These membranes always contain, in addition to the four different receptor polypeptides, considerable amounts of one or possibly several unknown polypeptides with molecular weights of about 43000 [3–5,8,9]. Some high molecular weight material (M_r approx. 90000), suggested to be the large $(Na^+ + K^+)$-ATPase subunit [10] is occasionally present in variable amounts [5,11]. Recently it has been shown [12] that treatment of these membranes with alkali (NaOH at pH 11) releases the 43 kDa polypeptides without affecting any of the biochemical properties of the receptor. Lithium di-iodo salicylate has also been demonstrated to accomplish such an extraction [4].

Considerable interest has been focused on the function of the 43 kDa nonreceptor peptide [9,13,14]. In attempts to disclose this function several different extraction methods should be used so that artefactual interpretations may be avoided. The present work aims at the establishment of alternative methods, in particular such promoting the release of the peripheral membrane proteins without the disturbance of the receptor.

Materials and Methods

Electric organs from *Torpedo marmorata* were obtained deep-frozen from Station Biologique d'Arcachon, France, and stored at −90°C until use. The ^{125}I-α-bungarotoxin was from New Eng-

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land Nuclear. Ethidium bromide, carbamoylcholine chloride, citraconic anhydride and 2,3-dimethylmaleic anhydride (DMMA) were purchased from Sigma Chemical Co. Aldrich-Europe made the 3,4,5,6-tetrahydrophthalic anhydride (THPA) available. Electrophoresis supplies were obtained from BioRad Laboratories.

Preparation of receptor enriched membranes

The nicotinic acetylcholine receptor enriched membranes were prepared essentially as described by Saitoh and Changeux [15], including 1 mM EDTA and 0.1 mM PMSF in all solutions. In the final centrifugation on a continuous sucrose density gradient, 34–43% (w/w), the sample which was adjusted to 33% (w/w) was layered on top and the tubes were centrifuged for 15 h at 25000 rpm in a Beckman SW 28 rotor. The different membrane fractions were collected and assayed for neurotoxin binding. The maximal binding capacity ranged from 2 to 3.5 nmol/mg protein in the preparations. The acetylcholine receptor enriched membranes were stored in 10 mM sodium phosphate buffer, pH 7.4 and 50 mM NaCl at −90°C before use.

Assays

The neurotoxin binding assay, with Triton X-100 included during incubation, was described by Schmidt and Raftery [16]. Protein determinations were made according to Bradford [17] with bovine serum albumin as standard.

The carbamoylcholine binding assay was performed as previously described [18,19], with 1 μM ethidium bromide as a fluorescent probe in Hepes-Ringer buffer, pH 7.4. The change in ethidium fluorescence upon addition of carbamoylcholine was monitored in a Perkin-Elmer MPF 44 B spectrofluorometer. The excitation wavelength was 483 nm, slit 5 nm and the emission wavelength was 610 nm, slit 8 nm. A 430 nm emission filter was used. Carbamoylcholine was successively added, with intervals of 10–15 min, to the receptor membranes in the cuvette to ascertain equilibrium to be reached before the next addition.

Extraction of peripheral membrane proteins

Treatment of receptor-enriched membranes by chaotropic ions was accomplished by adding NaI or freshly made sodium trichloroacetate to a membrane suspension (1 mg protein/ml) in 50 mM sodium phosphate buffer, pH 7.4 at +4°C. The membrane suspension was incubated for 1 h before the material was centrifuged at maximal force in a Beckman Airfuge for 10 min. The pellet was washed three times in 50 mM sodium phosphate buffer, pH 7.4 including 0.15 M NaCl.

The release of proteins after exposure of the membranes to anhydride was studied by using a protein concentration of 0.2–0.5 mg/ml. The buffers used were 10 mM sodium phosphate, pH 7.4 (DMMA) or 10 mM Tris-HCl, pH 8.5 (THPA). The anhydrides were dissolved to 10% (w/v) in freshly distilled dioxane. An appropriate amount of that solution was transferred to a test tube where the dioxane was removed by a gentle stream of nitrogen before the protein was added. The pH was maintained at the stated pH values by careful additions of 0.1–1 M NaOH solution during 1 h. The sample was then centrifuged in a Beckman JA 20.1 rotor at 20000 rpm for 1 h, at +4°C. Both the pellet and supernatant were saved. The pellet was washed twice with 10 mM sodium phosphate buffer, pH 7.4 including 50 mM NaCl. The supernatant was dialyzed against pH 7.4 buffer including 150 mM KCl.

Electrophoresis

The protein samples were analyzed after solubilisation in 2.5% SDS and 0.1 M β-mercaptoethanol by SDS-polyacrylamide gel electrophoresis on 7.5% gels according to Laemmli [20]. The protein was stained by using the method of Fairbanks et al. [21].

Results

Generally the most effective methods for releasing peripheral membrane proteins are alkali extraction, chaotropic ions or anhydride treatment.

The chaotropic ions have successfully been used in the extraction of peripheral proteins especially from erythrocytes [22] and mitochondria [23]. An attempt was made to extract the peripheral proteins from the receptor-enriched membrane. Treatment with up to 1 M NaI or sodium trichloroacetate did, however, not release appreciable amounts of the 43 kDa polypeptides. Further, the

sodium trichloroacetate, in contrast to NaI, also did progressively reduce the α-toxin binding capacity of the receptor-containing membranes to zero.

Extraction of the 43 kDa polypeptide by anhydride

Citraconic anhydride, 2,3-dimethylmaleic anhydride and 3,4,5,6-tetrahydrophthalic anhydride [24–26] were then tested for the extraction of the 43 kDa polypeptides from receptor-enriched membranes.

In Fig. 1 the effects of the anhydrides on protein release from the membranes and on α-bungarotoxin binding capacity are shown. It is obvious that the tested anhydrides are able to release peripheral proteins. However, in our experiment THPA also caused a very marked reduction of the α-bungarotoxin binding to the receptor. The corresponding effect on the toxin binding of DMMA-treated membranes was small. There was even a small increase in the specific α-toxin binding at the lower anhydride concentrations, as should be expected when non-receptor proteins are released. No α-bungarotoxin binding was observed in the supernatant. At 4 mg DMMA/mg protein a partial inactivation was observed. Fig. 1 also reveals that the two anhydrides (THPA and DMMA) were equally efficient in the release of peripheral proteins. Citraconic anhydride, which was tested as a further alternative, was neither more gentle towards the receptor nor as efficient in extracting peripheral proteins as the other two anhydrides.

The reaction conditions were found to be optimal at less than a 1-h exposure to the anhydrides. At longer exposure the toxin binding capacity decreased slightly while no further gain in the amount of released protein was achieved.

The DMMA-extracted proteins were analyzed by SDS-gel electrophoresis (Fig. 2). It was apparent that several polypeptides were extracted, but the major component of the extract had a molecular weight of 43000. A polypeptide with

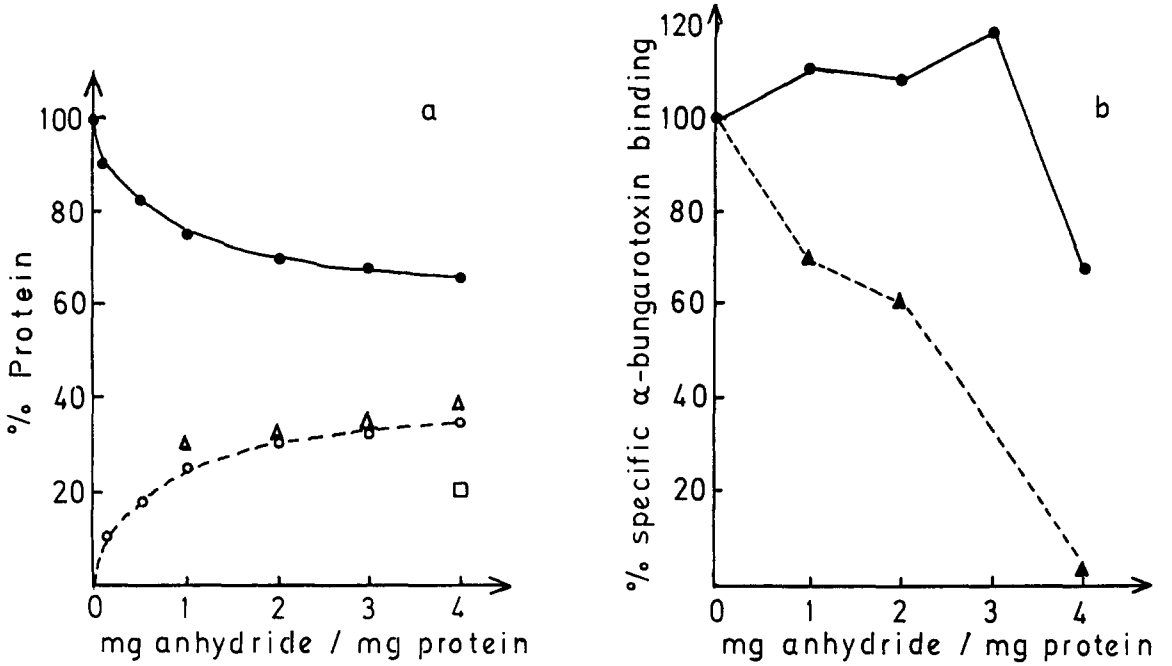

Fig. 1. (a) The release of membrane proteins from nicotinic acetylcholine receptor-enriched membranes was monitored after anhydride treatment. The protein concentration was 0.42 mg/ml and the specific α-bungarotoxin binding capacity was 3.0 nmol/mg protein at the start of the incubation. Protein in sedimentable fraction after DMMA treatment (●). Protein in supernatant fraction after DMMA treatment (○), after THPA treatment (△) and after citraconic anhydride treatment (□). (b) The effect of anhydride on the specific α-bungarotoxin binding capacity of receptor-enriched membranes. Incubation conditions as in (a). Specific α-bungarotoxin binding after DMMA treatment (●) and after THPA treatment (▲).

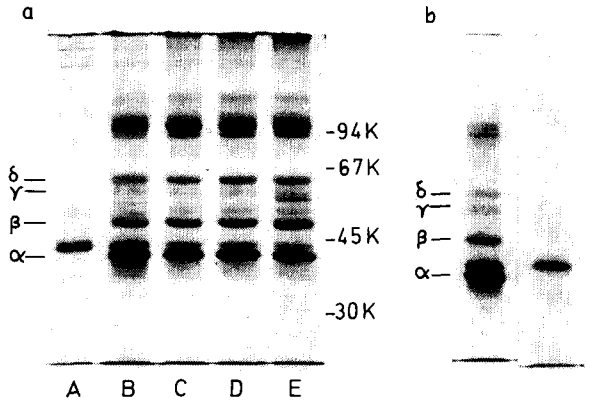

Fig. 2. SDS-polyacrylamide gel electrophoresis of polypeptides from nicotinic acetylcholine receptor-enriched membranes after treatment with 2,3-dimethylmaleic anhydride (DMMA). (a) The protein concentration was 0.42 mg/ml. In lane A 13 μg of supernatant protein were applied after treating the membranes with 4 mg DMMA/mg protein. The sedimentable fraction was applied, 20 μg/well, after anhydride treatment at the following concentrations: lane B–E, 0, 1, 2 and 3 mg DMMA/mg protein. (b) Receptor membranes isolated from fresh fish were used. The protein concentration was 0.33 mg/ml. The right gel shows the supernatant fraction (15 μg protein) after treating the membranes with 3 mg DMMA/mg protein. The left gel shows the sedimentable fraction of untreated receptor (35 μg of protein).

this molecular weight was also the major component of earlier described alkali extractions of receptor-enriched membranes [4,9,12,13].

A considerable amount of the 43 kDa component was released by the treatment with 3 mg DMMA/mg protein at all occasions. The exact amount was difficult to evaluate from the electrophoretograms because of possible differences in staining behaviour between the samples. No receptor polypeptides were seen in the supernatant after anhydride treatment.

The quality of receptor-enriched membranes after anhydride treatment

An essential property of the receptor is its ability to undergo an agonist-induced transition from a low to a high-affinity agonist binding state. A carbamoylcholine binding assay was used to test this property in the anhydride-extracted membranes. A marked time dependence of the binding demanded exposure of the receptor from all the preparations for at least 10 min in order to reach equilibrium. Fig. 3 shows a Scatchard plot of the high-affinity carbamoylcholine binding, revealing dissociation constants (K_d) for binding to untreated and DMMA-treated membranes of 72

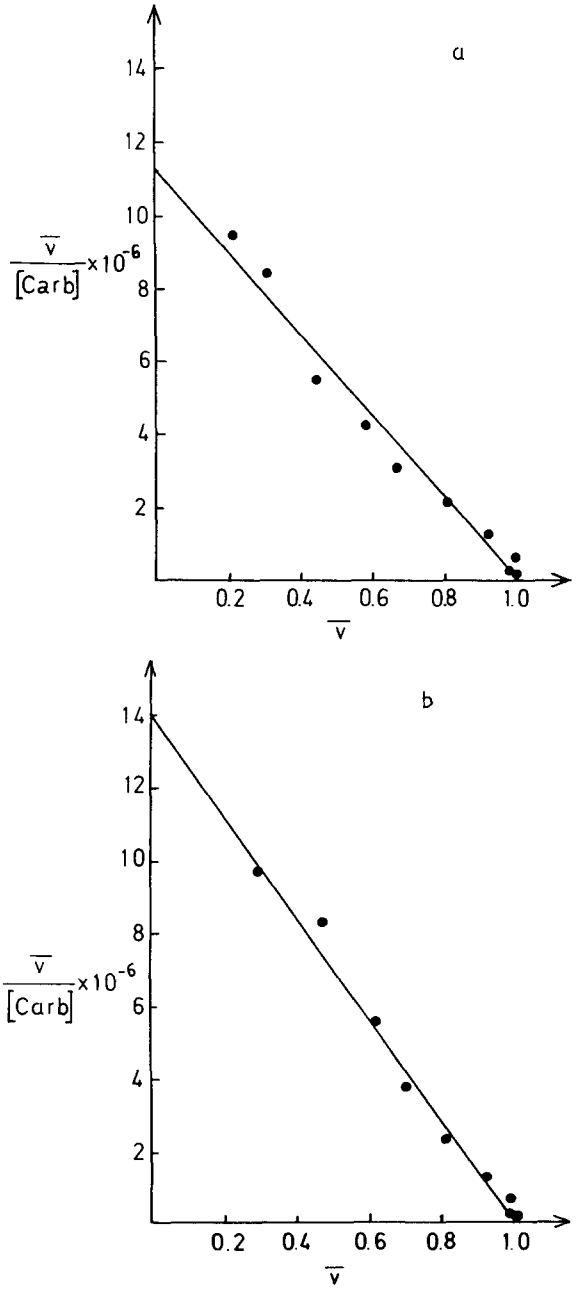

Fig. 3. Scatchard plots of carbamoylcholine binding to nicotinic acetylcholine receptor-enriched membranes. (a) 0.27 μM α-bungarotoxin binding sites, membranes treated with 3 mg DMMA/mg protein. (b) 0.31 μM α-bungarotoxin binding sites, untreated membranes. [Carb] is the unbound carbamoylcholine concentration and $\bar{v}$ is the fractional occupational ratio.

(± 14) nM and 89 (± 16) nM, respectively. These figures were based on the assumption that the number of α-toxin binding sites equals the number of carbamoylcholine sites [19]. This method did not reveal any difference in carbamoylcholine binding to the different receptor preparations. The K_d values are comparable to a value reported for the receptor from *Torpedo californica* [19] where the same binding assay was used.

Discussion

The usefulness of two classes of agents, chaotropic substances and anhydrides, respectively, for the solubilisation of peripheral membrane proteins was studied on acetylcholine receptor-enriched membranes. The chaotropic agents could not release any of the polypeptides of the receptor-enriched membranes, not even a very strong chaotropic ion such as trichloroacetate was able to do this. The forces involved in anchoring peripheral polypeptides to this receptor membrane are apparently stronger or of a different kind than those in mitochondria or erythrocyte membranes. If actin or some related protein was present these should have been expected to be released by this treatment [27]. As this was not observed such proteins are unlikely to occur in significant amounts (cf. Ref. 28). Anhydrides do react with nucleophiles, in proteins preferentially with the ε-amino group of lysine, creating an amide bond and thereby changing a positive charge to a negative one. As a consequence, a treatment of a lipid-protein complex such as a membrane with anhydrides will occasionally release some proteins (and lipids) from the membrane.

As shown in this paper, an anhydride treatment may successfully be used to solubilise some peripheral membrane proteins, especially a 43 kDa polypeptide, from the nicotinic acetylcholine receptor enriched membranes. The solubilising power of the anhydrides was dependent on the relative amounts of anhydride and protein. The extraction was maximal within an hour, at room temperature. The two most effective anhydrides tested were THPA and DMMA, both known to yield reversible products with proteins. This was evident from the amino-group content as, after an initial decrease, this became larger than that for the untreated controls after one week storage in the refrigerator at neutral pH (data not shown).

The optimal conditions for the depletion of peripheral proteins with DMMA were treatment at the most for 1 h at an amount of 3 mg/mg of protein at a protein concentration of 0.4 mg/ml. This yields membranes considerably depleted of peripheral proteins. It was essential not to use a higher protein concentration than that given above if the α-toxin or agonist binding capacity should be retained. The negative effects appearing at non-optimal conditions are probably due to an acylation of some less reactive but crucial nucleophilic group(s) in the receptor. These negative effects were more pronounced with THPA than with DMMA.

Several other reports have suggested a heterogeniety in the 43 kDa polypeptide [9,29,30], elicited as a doublet on SDS-gel electrophoretograms. At least a partial explanation is that the 43 kDa chain is microheterogenous [15,29]. Saitoh and Changeux [15] suggested that this polypeptide is phosphorylated to different extents. The functional role of the 43 kDa chains is, however, presently unknown. Suggestions have been made that these might be proteins with a structural function [9,13,29]. Another possibility is that a protein kinase or a part of such might be contained in the 43 kDa fraction. As yet unpublished results from this laboratory show that a fraction enriched in 43 kDa polypeptides from the anhydride solubilised proteins has a protein kinase activity. A 43 kDa protein has previously also been shown to bind ATP [31], a requirement for a protein kinase. This suggestion does not exclude the previous ones, as the possibility remains that the 43 kDa fraction is heterogenous. The method described in this paper for anhydride treatment of nicotinic acetylcholine receptor containing membranes appears useful as a substitute for and a complement to the alkali treatment of such membranes, as well as for studies of the peripheral proteins.

Acknowledgements

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References

1 Karlsson, E., Heilbronn, E. and Widlund, L. (1972) FEBS Lett. 28, 107–111
2 Heidmann, T., Sobel, A. and Changeux, J.-P. (1980) Biochem. Biophys. Res. Commun. 93, 127–133
3 Sobel, A., Weber, M. and Changeux, J.-P. (1977) Eur. J. Biochem. 80, 215–224
4 Elliot, J., Blanchard, S.G., Wu, W., Miller, J., Strader, C.D., Hartig, P., Moore, H.-P., Racs, J. and Raftery, M.A. (1980) Biochem. J. 185, 667–677
5 Jeng, A., St. John, P.A. and Cohen, J.B. (1981) Biochim. Biophys. Acta 646, 411–421
6 Hartman, A. and Heilbronn, E. (1978) Biochim. Biophys. Acta 513, 382–394
7 Flanagan, S.D., Barondes, S.H. and Taylor, P. (1976) J. Biol. Chem. 251, 858–865
8 Johansson, G., Gysin, R. and Flanagan, S.D. (1981) J. Biol. Chem. 256, 9126–9135
9 Barrantes, F.J. (1982) J. Cell Biol. 92, 60–68
10 Elfman, L., Heilbronn, E. and Jørgensen, P.L. (1982) Biochim. Biophys. Acta 693, 273–279
11 Saitoh, T., Oswald, R., Wennogle, L.P. and Changeux, J.-P. (1980) FEBS Lett. 116, 30–36
12 Neubig, R.R., Kordel, E.K., Boyd, N.D. and Cohen, J.B. (1979) Proc. Natl. Acad. Sci. U.S.A. 76, 690–694
13 Cartaud, J., Sobel, A., Rousselet, A., Devaux, P.F. and Changeux, J.-P. (1981) J. Cell Biol. 90, 418–426
14 Froehner, S.C., Gulbrandsen, V., Hyman, C., Jeng, A., Neubig, R.R. and Cohen, J.B. (1981) Proc. Natl. Acad. Sci. U.S.A. 78, 5230–5234
15 Saitoh, T. and Changeux, J.-P. (1980) Eur. J. Biochem. 105, 51–62
16 Schmidt, J. and Raftery, M.A. (1973) Anal. Biochem. 52, 349–354
17 Bradford, M.M. (1976) Anal. Biochem. 72, 248–254
18 Schimerlik, M.I., Quast, U. and Raftery, M.A. (1979) Biochemistry 18, 1884–1890
19 Dunn, S.M.J., Blanchard, S.G. and Raftery, M.A. (1980) Biochemistry 19, 5645–5652
20 Laemmli, U.K. (1970) Nature 227, 680–685
21 Fairbanks, G., Steck, T.L. and Wallach, D.F.H. (1971) Biochemistry 10, 2606–2616
22 Kahlenberg, A. (1976) J. Biol. Chem. 251, 1582–1590
23 Hanstein, W.G., Davis, K.A. and Hatefi, Y. (1971) Arch. Biochem. Biophys. 147, 534–544
24 Steck, T.L. and Yu, J. (1973) J. Supramol. Struct. 1, 220–232
25 Vadlamudi, B.P., Dzandu, J.D. and Larway, P.F. (1975) Biochem. Biophys. Res. Commun. 64, 64–71
26 Howlett, G.J. and Wardrop, A.J. (1978) Arch. Biochem. Biophys. 188, 429–437
27 Trotter, J.A., Foerder, B.A. and Keller, J.M. (1978) J. Cell Sci. 31, 361–392
28 Strader, C.D., Lazarides, E. and Raftery, M.A. (1980) Biochem. Biophys. Res. Commun. 92, 365–373
29 Gysin, R., Wirth, M. and Flanagan, S. (1981) J. Biol. Chem. 256, 11373–11376
30 Barrantes, F.J., Neugebauer, D.-C. and Zingsheim, H.P. (1980) FEBS Lett. 112, 73–78
31 Gordon, A.S., Guillory, R.J., Diamond, I. and Hucho, F. (1979) FEBS Lett. 108, 37–39