Functional and structural analysis of acetylcholine receptor-rich membranes after negative staining

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Phosphotungstate (pH 7.4) used for negative staining of membranes from *Torpedo* electric tissue rich in acetylcholine receptor does not affect binding properties and cation permeability of the receptor and its ion channel. Uranyl salts, frequently used for negative staining, precipitate the receptor-rich membranes making measurements of ligand binding and ion-permeability regulation impossible. The gross ultrastructure in the two stains is not significantly different, but for future high-resolution electron microscopy aiming at visualizing structural details of functional receptor molecules it is necessary to resort to a stain preserving native and active receptor. Uranyl salts are not applicable for this purpose. The electron micrographs obtained with phosphotungstate reveal two distinct structures in the receptor-rich membrane: a closed ring ('doughnut') and an open ring ('horseshoe'), with a ratio of abundance of about 3:2.

Acetylcholine receptor *Electron microscopy* *Negative staining* *Image averaging*

1. INTRODUCTION

Since their first publication in 1973 [1,2] electron microscopic images of negatively stained nicotinic acetylcholine receptor (AChR) isolated from *Torpedo* electric tissue were obtained using mainly two stains, sodium phosphotungstate and uranyl acetate (or formiate). Initially these two stains were used in parallel and did not yield significantly different results. But with the advent of electron microscopy of ever higher resolution, achieved by improved techniques, including digital image analysis [3–5], it may become important to point out the basic difference between the stains applied: Uranyl salts have, at 1% in aqueous solution, a pH of 4.0 (uranyl acetate) or 3.9 (uranyl formiate) and precipitate at more physiological pH values. Sodium phosphotungstate on the other hand can be used in buffer solution at pH 7.4. The aim of high-resolution electron microscopy is to gain insight into the fine structure of the receptor protein, which is a pentameric protein [6] with an $\alpha_2,\beta,\gamma,\delta$-quaternary structure [7]. For these investigations it is important to ensure that the native structure is not affected by the sample preparation. Here we report that the main functions of the receptor, ligand binding and ion flux regulation, are well preserved in sodium phosphotungstate at pH 7.4, but that uranyl salts irreversibly inactivate both, although the gross ultrastructure seems not to be altered. A preliminary report of these results has been presented [8].

2. MATERIALS AND METHODS

2.1. *Receptor preparation*

Receptor-rich membrane fragments were prepared from *Torpedo californica* electric tissue according to [9] with the slight modifications described in [10].

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2.2. *Cation flux experiments*

Li^+-efflux from receptor-rich membrane vesicles was measured basically as in [11], with the exception that Li^+ was used instead of $^{22}Na^+$. Li^+ content within the vesicles, which were filtered from a membrane suspension at different time intervals after dilution with cellulose acetate filters, was determined by flame emission spectroscopy.

2.3. *[^{3}H]Acetylcholine binding*

Agonist binding in the presence or absence of the respective salt used for negative staining was determined by ultracentrifugation using an air fuge. The binding test was described in [12].

2.4. *Electron microscopy*

For electron microscopical preparation unfixed acetylcholine receptor-rich membranes were absorbed to 4 nm-thick carbon films [13] and negatively stained with 1% phosphotungstate dissolved in 20 mM phosphate buffer (pH 7.4). The films were picked up with Cu grids, air-dried, and visualized in a Philips EM 400 T at 100 kV. Instrumental magnifications of 60000 and 80000 were used.

For image analysis two different particle shapes of the receptor were taken with respect to the reconstruction of the final image. Images of 25 particles of each group were arranged as square arrays and averaged by means of optical diffraction methods (FAIRS) [14]. In combination with digital image processing (using the SEMPER-programme) the grey levels of the averaged two particle shapes were recorded to study the contrast variation dependent on the stain distribution within the particles.

3. RESULTS

Fig.1 shows Scatchard plots of [^{3}H]acetylcholine binding to receptor-rich membrane vesicles in the absence and presence of 1% phosphotungstate dissolved in 20 mM phosphate buffer (pH 7.4). Shape and slope of the curves as well as the number of binding sites appear not to be affected significantly by phosphotungstate. On the other hand, 1% uranyl acetate precipitated the AChR-rich membranes, making binding studies by ultracentrifugation impossible.

Besides agonist recognition and binding the other important function of AChR is regulation of the ion permeability of the receptor-containing membrane. Fig.2 shows that this function is not influenced to a significant extent by 1% phosphotungstate at pH 7.4. Leakage of the resting membrane and excitability by 10^{-5} M carbamoylcholine are similar in the presence and absence of phosphotungstate. These functions could not be tested in the presence of uranyl

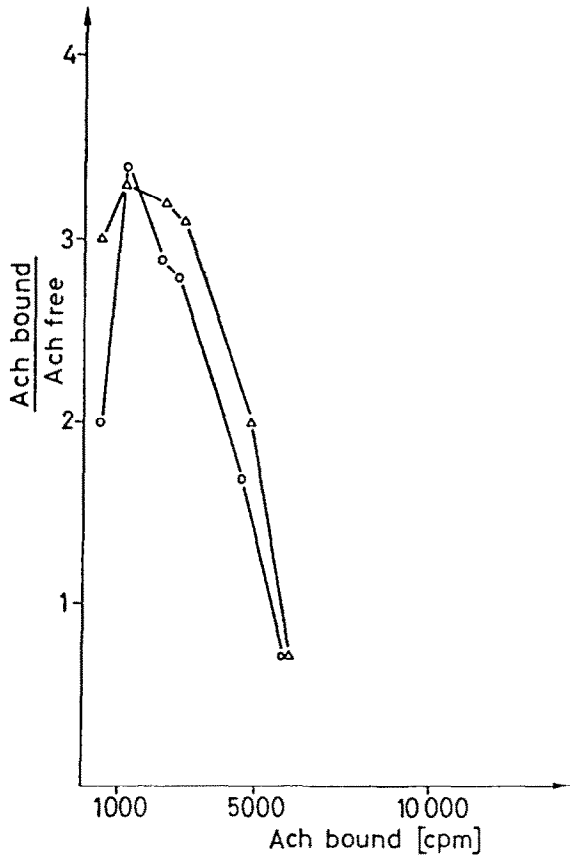

Fig.1. Binding of [^{3}H]acetylcholine to receptor-rich membrane vesicles from *Torpedo californica*. Scatchard plot of binding in absence (○—○) or presence (△—△) of 1% phosphotungstate in 20 mM phosphate buffer (pH 7.4). Varying [^{3}H]acetylcholine concentrations were added to a suspension of 0.1 mg/ml AChR protein preincubated with 10^{-4} M eserine to block acetylcholinesterase. After 15 min at room temperature 165-μl aliquots of this suspension were centrifuged for 30 min in a Beckman airfuge [12]. With uranyl acetate AChR precipitated and ligand binding could not be measured.

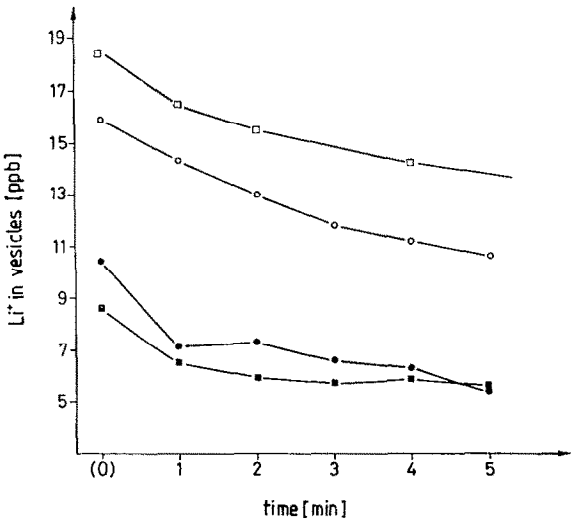

Fig.2. Li^+-efflux from receptor-rich membrane vesicles. Efflux in the absence (□—□) or presence (○—○) of 1% phosphotungstate. Closed symbols: Efflux stimulated by 10^{-5} M carbamoylcholine; open symbols: leakage of Li^+ from unstimulated vesicles. Assay conditions: AChR-rich membrane vesicles (5 mg/ml, 20 mM phosphate buffer, pH 7.4) were incubated overnight with 0.3 M LiCl (4°C). For efflux measurements 50 μl of this suspension were diluted into 7 ml 20 mM phosphate buffer (pH 7.4). At the time indicated 1-ml aliquots of this incubation mixture were filtered by suction through a cellulose acetate filter. The filters were washed 4 times with 5 ml ice-cold buffer; subsequently they were extracted for several hours in a vial with 3 ml of 1% Triton. The Li^+ content was determined by flame emission spectroscopy. With uranyl acetate (1%, pH 4.0) AChR precipitated and ion flux could not be measured.

acetate, because at the pH of this salt the receptor denatured and precipitated.

Figs.3 and 4 show electron micrographs of receptor-rich membranes negatively stained with 1% phosphotungstate in 20 mM phosphate buffer (pH 7.4). The gross appearance of the surface structure is very similar to previously published images obtained with uranyl salts as the negative stain

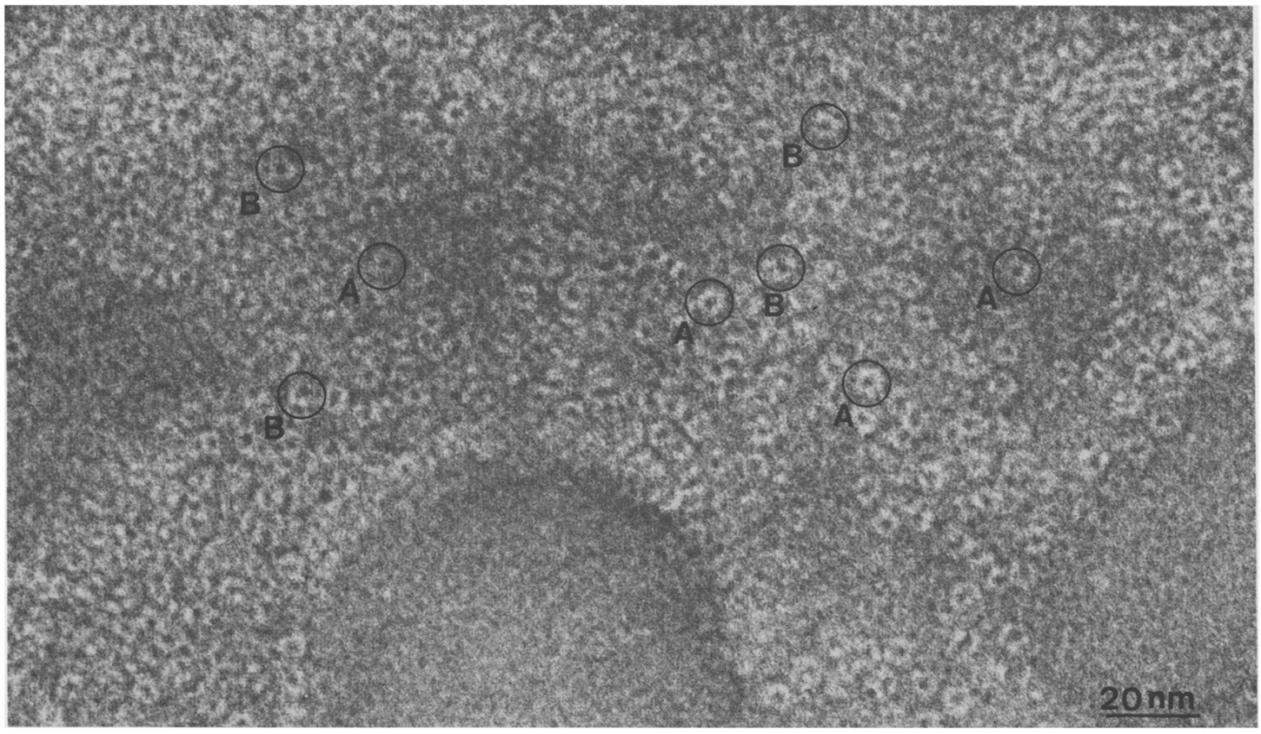

Fig.3. Acetylcholine receptor-rich membrane negatively stained with 1% phosphotungstate in 20 mM phosphate buffer (pH 7.4). At least two different particle shapes are seen: particles with a closed-ring ('doughnut') structure (A) and particles with an open-ring structure ('horseshoe') (B).

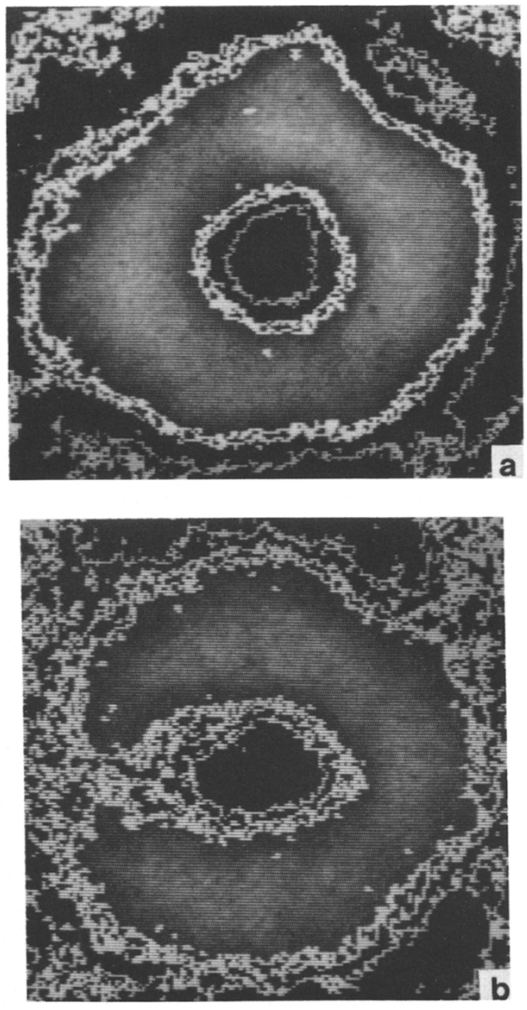

Fig.4. Half-tone images of averaged A (a) and B (b) particle forms of the acetylcholine receptor with contour lines added.

[4,5]. In the latter as well as in our images (fig.3) we discovered two populations of protein molecules believed to represent AChR. Fig.4 shows an image analysis of the closed-ring and horseshoe-shaped structures. Occurrence of the two is roughly 3:2 in favor of the closed-ring; this ratio is not significantly affected by desensitizing concentrations of carbamoylcholine (10^{-3} M).

4. DISCUSSION

Although the overall appearance of membrane-bound AChR is not significantly different with the two negative stains, the functional difference is striking: with uranyl acetate the AChR is completely inactivated, while with sodium phosphotungstate binding and ion flux properties are unchanged under the conditions of our assays. This is not surprising since it had been shown previously that α-bungarotoxin [15] and acetylcholine [12] binding to AChR decreases steeply below pH 6. Involvement in ligand binding of a dissociating group with a pK of about 6 had been deduced from such pH-dependencies [15]. We observed that at pH 4 this inhibition of ligand binding becomes irreversible. Taking this into account it is surprising that α-bungarotoxin bound to AChR has been identified by image averaging of electron micrographs obtained from AChR–toxin complexes negatively stained with uranyl formiate [5].

At present work is underway in several laboratories to extend the limit of resolution of electron microscopy beyond the overall shape of protein molecules. Authors have shown that details of the folding, e.g., helical structures crossing the membrane and even structural changes correlated with the function of a protein [17], can be visualized. The nicotinic acetylcholine receptor in its membrane-bound state as a model for ion channel-coupled receptors is being investigated with similar aims. For future high-resolution electron microscopy, physiological staining conditions (or no stain at all) are highly desirable.

The two shapes of AChR observed (fig.4), the closed ring ('doughnut') and the open ring ('horseshoe') are not related to the staining technique; they are detectable with uranyl acetate and sodium phosphotungstate as negative stains. Actually, we observe them both in many of the published electron microscopic pictures. Apparently, in selecting appropriate molecules for high-resolution analysis there existed a bias for the open [5,18] or closed [19] ring structure. Along the edges of the AChR-rich vesicles we had previously observed pairs of protrusions [10] which we interpreted as side views of AChR molecules. These structures have been confirmed by others (e.g. [18]). From these investigations and from nearest-neighbour analysis of the polypeptide chains within the AChR protein complex a quaternary structure model has been deduced, which again is compatible with the horseshoe structure [20]. At

present there is no evidence that the two structures represent different functional states of the AChR.

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REFERENCES

[1] Nickel, E. and Potter, L.T. (1973) Brain Research 57, 508–517.
[2] Cartaud, J., Benedetti, E.L., Cohen, J.B., Meunier, J.-C. and Changeux, J.-P. (1973) FEBS Lett. 33, 109–113.
[3] Zingsheim, H.-P., Neugebauer, D.-C., Barrantes, F.J. and Frank, J. (1980) Proc. Natl. Acad. Sci. USA 77, 952–956.
[4] Kistler, J. and Stroud, R.M. (1981) Proc. Natl. Acad. Sci. USA 78, 3678–3682.
[5] Zingsheim, H.-P., Barrantes, F.J., Frank, J., Hänicke, W. and Neugebauer, D.-C. (1982) Nature 299, 81–84.
[6] Hucho, F., Bandini, G. and Suárez-Isla, B.A. (1978) Eur. J. Biochem. 83, 335–340.
[7] Reynolds, J. and Karlin, A. (1978) Biochemistry 17, 2035–2038.
[8] Reinhardt, S., Tesche, B., Schmiady, H. and Hucho, F. (1983) Proceedings of the Congress of Electronmicroscopy, Amsterdam.
[9] Duguid, J.R. and Raftery, M.A. (1973) Biochemistry 12, 3593–3597.
[10] Schiebler, W. and Hucho, F. (1978) Eur. J. Biochem. 85, 55–63.
[11] Popot, J.L., Sugiyama, H. and Changeux, J.-P. (1976) J. Mol. Biol. 106, 469–483.
[12] Muhn, P. and Hucho, F. (1983) Biochemistry 22, 421–425.
[13] Martelli, S., Urban, J. and Tesche, B. (1983) Thin Solid Films 105, 49–60.
[14] Ottensmeyer, F.P., Andrew, J.W., Basett-Jones, D.P., Chan, A.S.K. and Hewitt, J. (1977) J. Microsc. 109, 259–268.
[15] Schmidt, J. and Raftery, M.A. (1974) J. Neurochem. 23, 617–622.
[16] Henderson, R. and Unwin, P.N.T. (1975) Nature 257, 28–32.
[17] Unwin, P.N.T. and Zampigki, G. (1980) Nature 283, 545–549.
[18] Zingsheim, H.-P., Neugebauer, D.-Ch., Frank, J., Hänicke, J. and Barrantes, F.J. (1982) EMBO J. 1, 541–547.
[19] Karlin, A. (1980) in: The Cell Surface and Neuronal Function (Cotman, C.W., Poste, G. and Nicolson, G.L. eds) Elsevier, Amsterdam, New York.
[20] Hucho, F. (1981) Trends Biochem. Sci. 6, 242–244.