Photoaffinity labeling of acetylcholine receptor in millisecond time scale

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Photoaffinity labeling of acetylcholine receptors can be performed with a time resolution allowing to discriminate reaction sites within the receptor protein in its different functional states. This is achieved by a combination of a stopped-flow apparatus with a high energy pulse laser. The photoaffinity label used is the lipophilic cation [^{3}H]TPMP$^+$ which has been shown to be a non-competitive antagonist and a specific ion channel blocker. AChR in its resting (channel closed) and active (channel open) state incorporates the label mainly into the α-polypeptide chain of the receptor. Only several hundred milliseconds after mixing AChR with agonist labeling of δ-chains becomes significant.

Acetylcholine receptor *Photoaffinity labeling* *Ion channel* *TPMP$^+$*

1. INTRODUCTION

Photoaffinity labeling of the nicotinic acetylcholine receptor (AChR) has been successfully applied for the identification of agonist and antagonist binding sites [1,2] within the protein complex. In addition to these effector sites binding sites for non-competitive blockers (NCBs) have been characterized primarily by binding studies with reversible ligands [3,4]. NCBs are especially interesting compounds because there is evidence that they exert their blocking effect at least partly by direct interaction with the ion channel of the AChR [5,6]. At present there is no clear evidence as to the structural correlate of this functional subunit, the ion channel of the AChR. The AChR from *Torpedo* electric tissue has been shown to consist of 5 polypeptide chains (stoichiometry: α_2, β, γ, δ [7]) and a quaternary structure model has been proposed [8]. Several components of the protein complex have been implicated at times in the ion translocation process; recently, evidence was presented that the central cavity surrounded by the 5 polypeptide chains may comprise the ion channel [6] and the δ chain was postulated to contain the NCB binding site or at least part of it [9].

The disadvantage of photoaffinity labeling was so far that all the experimental procedures involved, e.g., mixing of AChR with the photoaffinity ligand and irradiation, are much slower than the physiological events comprising AChR activity. Especially transitions from the resting state (channel closed) to the activated state (channel open) occurs very rapidly (<ms), and channel open times are in the ms time scale [10]. To trap even transient receptor states of brief half life (in the order of several ms) we combined a stopped-flow apparatus with a high energy ns pulse laser. This set-up allowed us to induce a photoreaction between AChR and a NCB within ms after mixing the receptor with, for example, an agonist. As a photosensitive NCB we used [^{3}H]TPMP$^+$, previously shown by us [11,12] to be a specific blocker of the AChR ion channel and a useful tool for monitoring structural transitions related to AChR activity.

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2. MATERIALS AND METHODS

2.1. *Preparation of membrane fragments from* Torpedo *electric tissue*

Membrane vesicles from *Torpedo marmorata* and *Torpedo californica* were prepared as in [13], recovered from sucrose gradients by dilution in H_2O (5-fold) and sedimentation (35000 × *g*, 90 min). Membrane fragments were then resuspended in sodium Ringer solution and left for equilibration overnight in ice. Protein was determined as in [14]. Vesicles were then diluted to a protein concentration of 1 mg/ml with N_2-deaerated sodium Ringer solution. Specific activity of AChR was 1500–2500 nmol/g α-[^{125}I]bungarotoxin (NEN) binding sites.

2.2. *Rapid photoaffinity labeling*

AQ-switched Nd:YAG DCR-2A laser (Quanta-Ray) at wavelength 266 nm (4-fold the primary frequency) and in single pulse mode was used. The light beam was focussed by a 300-mm lens and triggered by a self-built variable delay-pulse-generator which was connected to the stop-switch of a stopped-flow apparatus. Pulse energy was 15 mJ, pulse duration 4–5 ns.

The laser was used in combination with a stopped-flow apparatus (Sigma Instrumente GmbH, Berlin) modified for our purpose. A special thermoresistant mixing chamber from German silver was constructed (dead time 2.4 ms at 3.2 bar pressure). Irradiated samples could be removed with a Hamilton air-locked syringe. Dead volume was 8 μl, reaction chamber volume 22 μl; diameter of the chamber was 2.4 mm and the beam path through the reaction mixture 5 mm. Distance from the laser was chosen as to allow full irradiation of the chamber. For composition of the reaction mixtures see legend to fig.3.

2.3. *Slow photoaffinity labeling*

The procedure was described in [12]. Analysis of photoaffinity-labeled proteins was by SDS-polyacrylamide gel electrophoresis (10% acrylamide) as in [15] and fluorography as in [12]. For the experiment shown in fig.2 the protein band corresponding to the δ-polypeptide chain was cut out of the stained gel, solubilized and counted with Omnifluor/Toluol/Protosol (3%) in a scintillation counter.

3. RESULTS AND DISCUSSION

Photoaffinity labeling of receptor-rich membranes under steady state conditions is shown in fig.1. In the absence of any further cholinergic receptor irradiation of the AChR–[^{3}H]TPMP$^+$ complex yields incorporation of radioactivity mainly into the α-polypeptide chains. In the presence of the agonist carbamoylcholine a dramatic change of the labeling pattern can be

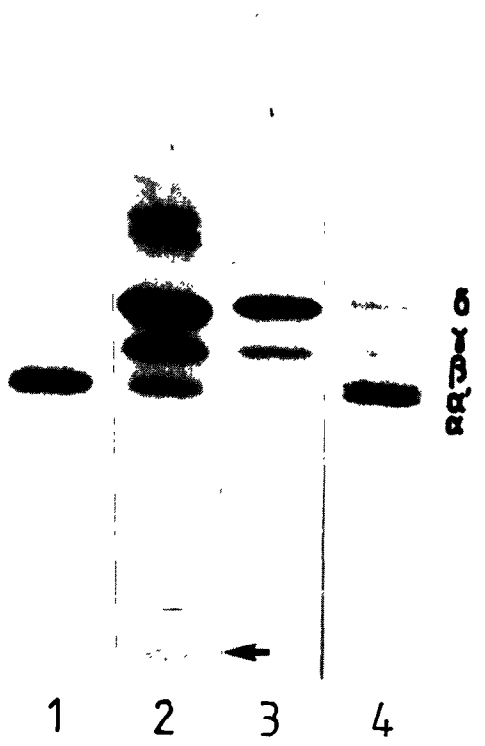

Fig.1. Photoaffinity labeling of acetylcholine receptor under equilibrium conditions. Eighteen μl of receptor-rich membranes (1 mg/ml protein), in Ringer solution, were mixed with 6 μl [^{3}H]TPMP$^+$ (1 μM, about 2 μCi) and 12 μl of a solution of cholinergic effector (3×10^{-4} M in Ringer solution. The mixture was incubated for 5 min at room temperature and subsequently irradiated with a mercury-vapour lamp. Irradiation time was 2 min at 4°C. For further experimental details see section 2. The figure shows fluorograms of the fixed electrophoresis gels. (1) Resting receptor (buffer was added instead of cholinergic effector), (2) receptor membranes, pre-equilibrated with 10^{-4} M carbamoylcholine, (3) as (2) but with 10^{-4} M D-tubocurarine instead of the agonist, (4) Coomassie blue-stained polyacrylamide gel of the receptor protein.

observed: in addition to α-chains now β- and most conspicuously δ-polypeptide chains were photoaffinity labeled (the additional doublet visible in the autoradiogram above δ may represent dimerisation products created by cross-linking of polypeptide chains during UV irradiation. These products have not been further analyzed). A qualitatively similar labeling pattern was obtained when the agonist was replaced by the antagonist D-tubocurarine; but quantitative differences are obvious: the AChR picked up considerably less label, and the high-M_r doublet did not show up. In all our photoaffinity experiments using [^{3}H]TPMP$^+$ very little radioactivity was found in the membrane lipids (running around the dye front of the gel, see arrow). Since most NCBs exert part of their effects through the protein–lipid interface and also bind non-specifically to the lipid phase, our finding confirms previous evidence that [^{3}H]TPMP$^+$ acts as a true channel blocker binding to a single specific site close to the channel similar to NCBs as phencyclidine and histrionicotoxin.

Fig.2 shows the dose-dependence of the stimulation of covalent δ-chain labeling by carbamoylcholine. The curve is very similar to the one obtained previously for the reversible [^{3}H]TPMP$^+$ binding to AChR stimulated by carbamoylcholine. It correlates well with receptor occupancy by the agonist assuming the receptor to be in its high affinity (desensitised) state. This indicates that the stimulated photolabeling of δ-chains parallels AChR–[^{3}H]TPMP$^+$ complex formation.

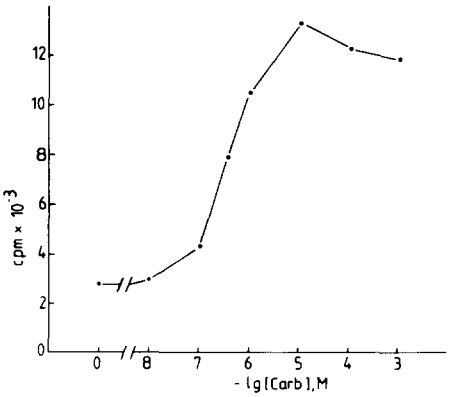

Fig.2. Dose-response curve of carbamoylcholine stimulated [^{3}H]TPMP$^+$ photolabeling of receptor protein. Experimental conditions were the same as in the experiment shown in fig.1. The ordinate shows cpm ^{3}H incorporated into the δ-band, determined by cutting it out of the stained electrophoresis gel.

Similar results with other NCBs have been interpreted as desensitization being the reason for the observed shift of the predominant labeling from α- to δ-polypeptide chains. The main site of the photoaffinity labeling therefore might be used as a monitor of the functional state of the AChR. For elucidating the molecular mechanism of, for example, receptor activation (channel opening) and desensitization (channel closing, transition to a high affinity state) it would be useful to achieve photoaffinity labeling with a time resolution, comparable to the physiological events. Channel opening is a very rapid event (<ms), the kinetics of which cannot be resolved at present by any method available. But the open channel has a half life of several milliseconds [10] and the desensitization rate too is slow enough to be accessible to kinetic analysis (actually a rapid and a slow desensitization can be discriminated, occurring in the 10–100 ms and in the s time range, respectively).

Fig.3 shows the photolabeling patterns of AChR with the laser triggered at different time lapses after mixing AChR–[^{3}H]TPMP$^+$ with carbamoylcholine in the stopped-flow apparatus. Obviously, labeling of the δ-polypeptide chain becomes significant several hundred milliseconds after mixing. Clearly activated AChR (channel open) yields the same labeling pattern as resting receptor. Predominantly α-polypeptide chains are labeled. Appearance of radioactivity in δ-chains seems to be too slow to account for rapid desensitization and too fast for the slow process. For final conclusions in this respect a more detailed kinetic analysis is necessary. At present we can conclude safely that the α-chains are the primary target of the photoreaction with our NCB with the receptor in its resting state and its active state. Channel opening does not change the labeling pattern. Of course this does not exclude the possibility that different sites within the primary structure of the α-chains react in absence and presence of agonist.

Several methods for in vitro kinetic investigations in the time scale of the physiological events are now available: Authors in [16] investigated the reversible interaction of a fluorescent agonist with the receptor, authors in [17] developed a method

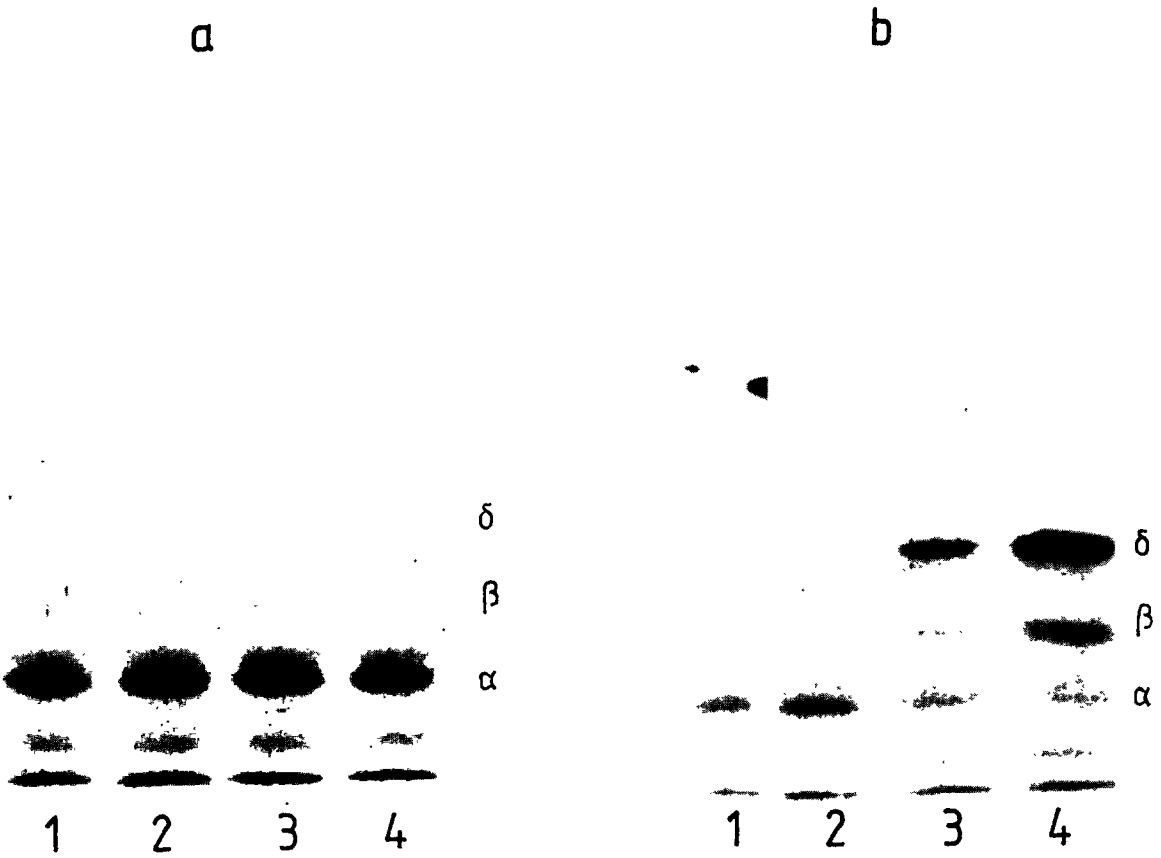

Fig.3. Rapid laser-flash photolabeling of acetylcholine receptor with [^{3}H]TPMP$^+$; comparison of labeling in presence and absence of agonist. All concentrations were as in fig.1. The irradiated volume was 22 μl (for details see section 2). The laser was triggered several times after mixing of receptor-rich membranes with [^{3}H]TPMP$^+$ and buffer (a) or carbamoylcholine (b). Delay times were: (1) zero (dead time of the stopped-flow apparatus was 2.4 ms); (2) 25 ms; (3) 250 ms; (4) 2500 ms. The figure shows fluorograms of SDS-polyacrylamide gels of the irradiated receptor protein.

for rapid measurements of cation flux through receptor-rich membranes, and here we add a rapid covalent labeling method for non-competitive agonists. Together with the patch clamp technique in [10], appropriate tools are now available for elucidating important functional mechanisms of the nicotinic acetylcholine receptor.

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