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RECONSTITUTION OF ACTIVE ACETYLCHOLINE RECEPTOR BY HYBRIDISATION OF BINDING SITE-BLOCKED WITH ION CHANNEL-BLOCKED ACETYLCHOLINE RECEPTOR PROTEIN

WERNER SCHIEBLER and FERDINAND HUCHO

Freie Universität Berlin, Fachbereich Chemie, Fabeckstrasse 34, 1000 Berlin 33 (Germany)

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Summary

The nicotinic acetylcholine receptor regulates the ion permeability of the postsynaptic membrane. This report presents evidence that the transmitter binding site and the ion channel may be located on distinct subunits. By hybridisation of receptor complexes, in which the transmitter binding site was blocked with complexes in which the ion channel was irreversibly inhibited, we reconstituted active acetylcholine receptor complexes. The reconstituted system was similar to the native receptor in its ability to regulate the ion permeability of lipid vesicles in response to nicotinic cholinergic effectors

Cell surface receptors convey biosignals by various mechanisms from the outside, through the cell membrane, to the inside of the cell. In some cases they regulate an enzymatic activity located in the membrane producing a compound which is released into the cytoplasm as a 'second messenger'. Hormone receptor-adenylate cyclase systems belong to this class. In other cases the receptor regulates the ion permeability of the membrane, thereby affecting the membrane potential directly. Certain neurotransmitter receptors, especially the nicotinic acetylcholine receptor, appear to belong to this class. For the hormone-regulated adenylate cyclase, it has been shown that receptor and enzyme are distinct molecules which form a complex in the membrane, but which can be separated by methods of cell biology [1] and biochemistry [2]. The same adenylate cyclase appears to form varying complexes with different hormones, an observation which led to the formulation of the 'mobile receptor' concept [3, 4]. It is unknown, however, whether an analogous relationship exists between neurotransmitter receptors and their

ion channels. In this report we present evidence that in the case of the nicotinic acetylcholine receptor from the electric organ of *Torpedo californica*, the transmitter binding site and the ion channel are not necessarily on the same polypeptide chain. By hybridisation of receptor complexes, in which the transmitter binding site is blocked with complexes in which the ion channel is irreversibly inhibited, we were able to reconstitute active acetylcholine receptor complexes. The reconstituted system was similar to the native receptor in its ability to regulate the ion permeability of lipid vesicles in response to nicotinic cholinergic effectors.

The hybridisation experiment was performed with membrane fragments enriched in acetylcholine receptor prepared from the electric organs of *Torpedo californica* as described in Ref. 5 with the slight modifications described in Ref. 6. One sample of the receptor-enriched membranes was treated with α-bungarotoxin, a strong competitive inhibitor of the transmitter binding site in nicotinic acetylcholine receptors. Another sample of this preparation was treated with quinacrine mustard (for experimental details see the legend to the Fig. 1). Quinacrine has been shown to act upon the postsynaptic membrane of nicotinic cholinergic synapses as a local anaesthetic [7], characterized by its blocking effect on the ion translocation through the membrane without competitively inhibiting transmitter binding. We have shown that the alkylating derivative, quinacrine mustard, irreversibly blocks the ion permeability response to agonists in receptor-enriched membrane microsacs [8]. These modified membranes still contained at least 50% of their high-affinity binding sites for [^{3}H]acetylcholine.

Both these modified samples were dissolved in detergent supplemented with egg lecithin, separately at first for the controls, jointly later for the hybridisation experiment. Reconstitution of the detergent-solubilized membranes to functional liposomal receptor-membrane systems was performed by column chromatography with Sephadex G-50 as described before [6]. Receptor function was measured by determining the $^{22}Na^+$ efflux from reconstituted vesicles by the Millipore filtration assay developed by Popot et al. [9]. Maximum efflux was determined by adding gramicidin to the vesicles (open squares in Fig. 1).

The first control experiment was aimed to show that, under the conditions of the reconstitution experiment, the bungarotoxin block is virtually irreversible (top frame of Fig. 1, labelled T for toxin). The time-dependent $^{22}Na^+$ efflux from vesicles reconstituted with toxin-blocked receptor could not be stimulated by 0.1 mM carbamoylcholine although considerable amounts of radioactivity could be released with gramicidin. A similar result was obtained in the second control experiments with quinacrine mustard-blocked receptor (middle frame of the figure, labelled Q): the $^{22}Na^+$ efflux from vesicles reconstituted with mustard-blocked receptor could also not be stimulated by 0.1 mM carbamoylcholine (in this experiment unstimulated leakage of $^{22}Na^+$ was higher as compared to the T-experiment: this increased leakage was consistently observed with quinacrine-modified membranes [8] and may be due to the increase of lipid fluidity caused by the local anaesthetic).

Reconstitution of a 1:1 mixture of toxin-blocked and quinacrine mustard-blocked receptor-rich membranes resulted in vesicles in which the $^{22}Na^+$

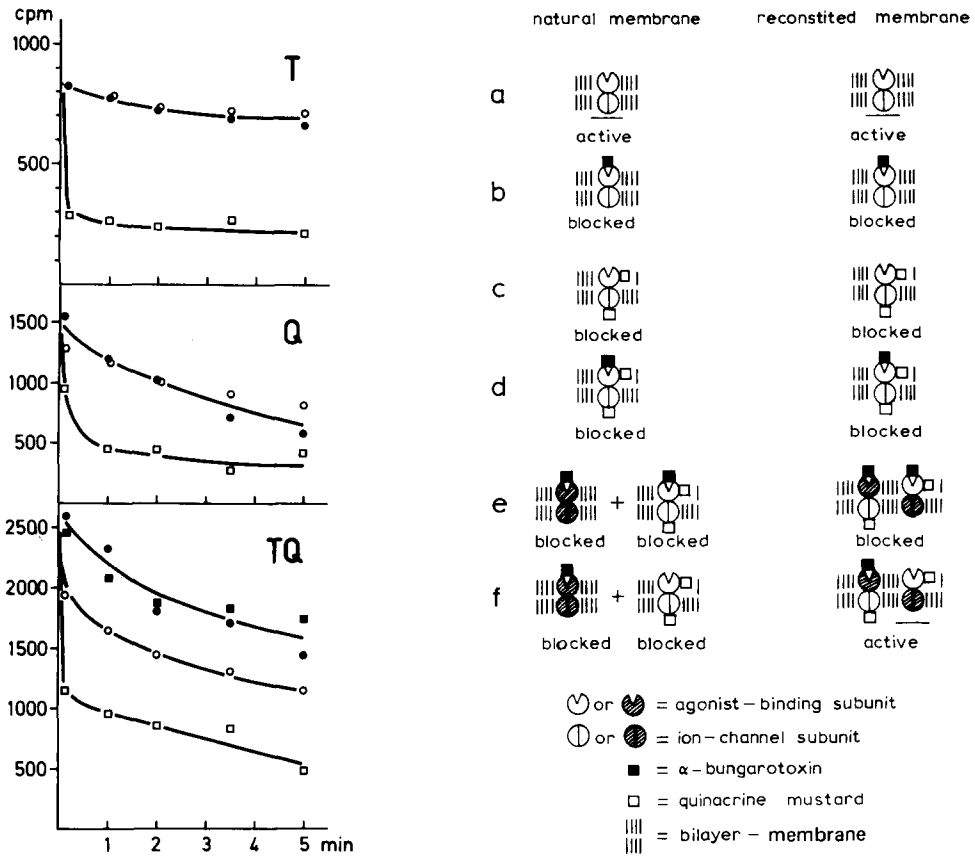

Fig. 1. $^{22}Na^+$ efflux from reconstituted acetylcholine receptor containing lipid vesicles. Reconstitution of acetylcholine receptor function by hybridisation of α-bungarotoxin-blocked and quinacrine mustard-blocked receptor-rich membranes. T, receptor-rich membranes were blocked with α-bungarotoxin before reconstitution (competitive antagonist). No agonist-stimulated $^{22}Na^+$ efflux was observed from the reconstituted receptor vesicles containing the α-bungarotoxin-blocked acetylcholine receptor. Q, receptor-rich membranes were blocked with the covalently reacting local anaesthetic, quinacrine mustard, before reconstitution (noncompetitive antagonist). No agonist-stimulated efflux was observed with lipid vesicles containing the quinacrine mustard-blocked acetylcholine receptor. TQ, α-bungarotoxin- and quinacrine mustard-blocked receptor-rich membranes are combined before reconsitution. As reconstitution. An efflux can be stimulated with $1 \cdot 10^{-4}$ M carbamoylcholine when the α-bungarotoxin- and quinacrine mustard-blocked receptor-rich membranes were combined before reconstitution. As with the natural membranes, this efflux can be blocked with $1 \cdot 10^{-4}$ M toxin from *Naja naja siamensis*. In all of the three experiments gramicidin A, an ion pore-forming oligopeptide, causes efflux of $^{22}Na^+$ indicating that bilayer vesicles have been formed. The acetylcholine receptor function was measured by the $^{22}Na^+$ efflux method described in Ref. 9 with the following modifications: Ringer's solution was used for both the incubation mixture and the dilution medium. At the time indicated, aliquots of the dilution medium were removed and filtered through Millipore filters (0.45 μm pore-width, 0.05 mg protein each). The amount of $^{22}Na^+$ retained on the Millipore filter in lipid vesicles is plotted against time after dilution of the $^{22}Na^+$-equilibrated vesicle suspension. ●——●, Efflux of $^{22}Na^+$ from reconstituted receptor-vesicles in the absence of agonist. ○——○, Efflux in the presence of $1 \cdot 10^{-4}$ M carbamoylcholine. □——□, Efflux from the reconstituted receptor vesicles preincubated with 100 μg/ml gramicidin A (15 min). ■——■, Efflux after preincubation with $1 \cdot 10^{-4}$ M toxin from *Naja naja siamensis* (30 min) and addition of $1 \cdot 10^{-4}$ M carbamoylcholine.
Experimental details: acetylcholine receptor-rich membranes in 0.1 M NaCl/0.01 M Tris-HCl buffer (pH 7.3)/0.02% NaN_3 (4—5 mg protein, specific binding activity approx. 1 nM/mg) prepared from the electric organs of *Torpedo californica* as described in Ref. 6 were either incubated with a 2-fold molar excess of α-bungarotoxin or 20—60 μM quinacrine mustard, a covalent reacting local anaesthetic [8]. After 40 min incubation at room temperature the membranes were washed and centrifuged three times

efflux could be stimulated by 0.1 mM carbamoylcholine (bottom frame, labelled TQ) and 0.1 mM acetylcholine (not shown). This stimulation (open circles) was prevented by preincubation of the reconstituted TQ-vesicles with *Naja naja siamensis* toxin, another competitive inhibitor of nicotinic acetylcholine receptors (closed squares).

The simplest explanation of this result would be based on the following model (Fig. 2): the nicotinic acetylcholine receptor of the *Torpedo* membrane is composed of two types of subunit, one carrying the transmitter binding site (being the receptor 'sensu stricto'), the other translocating the cations (being the ion channel, ionophore). In the T-experiment of Fig. 1 only the binding subunits are blocked (Fig. 2b), in the Q-experiment all the ion channel subunits are blocked but at least 50% of the binding subunits are still active [8] (Fig. 2c). In the TQ-experiment these binding subunits form functional receptor-ion channel complexes with the active ion channel subunits of the T-sample (Fig. 2f).

One alternative possibility cannot be excluded: the receptor could be composed of identical polypeptide chains, each chain carrying both a transmitter binding site and an ion channel. In our reconstitution experiment a complementation could have occurred, the unblocked binding site of the one chain interacting with the unblocked ion channel of the other. In this case our result shows an exchange of these identical polypeptide chains and one would have to postulate a dimer or oligomer as the functionally active unit. A receptor dimer cross-linked by a disulfide bridge has been found, but no evidence exists that this dimerization is a prerequisite for activity. In our ex-

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(30 min at 20 000 × *g* with dilution in 20 ml of the same buffer as above). Egg lecithin (2 g/l) and sodium cholate (1.5%, w/v) were added to the labelled membranes (final protein concentration approx. 4 g/l in a volume of 1.4 ml).
For the hybridisation experiment, 0.7 ml of α-bungarotoxin- and 0.7 ml of quinacrine mustard-blocked receptor membranes were combined (control experiments were performed with 1.4 ml of labelled receptor membranes). This mixture was filtered through a Sephadex G-50 column (4° C, flow rate 7 ml/h, column size 30 × 1 cm, 2.2 g Sephadex G-50 medium equilibrated overnight in the same buffer as above). The fractions containing receptor lipid vesicles were dialysed for 14 h at 4° C against 0.16 M NaCl/5 mM KCl/2 mM $MgCl_2$ /2 mM $CaCl_2$ /3 mM sodium phosphate buffer (pH 7.0)/0.02% NaN_3 according to the reconstitution procedure described in Ref. 6. The receptor vesicles were finally incubated with $^{22}Na^+$ (10—40 μCi/ml, 24 h).

Fig. 2. Summary of the reconstitution experiments explained in a simple model of the acetylcholine receptor. (a) Reconstitution of the natural receptor-rich membranes without modification of the acetylcholine receptor [6] yields active receptor complexes. (b) Reconstitution of α-bungarotoxin-blocked receptor-rich membranes. Only the agonist-binding subunit of the receptor is labelled: blocked receptor complexes are obtained (see also Fig. 1, T). (c) Reconstitution of quinacrine mustard-blocked receptor-rich membranes. The ion channel subunit is labelled. Because of the nonspecificity of quinacrine mustard the binding subunit could be labelled too, but high affinity binding of acetylcholine is still possible [8]: blocked receptor complexes are obtained (see also Fig. 1, Q). (d) Reconstitution of receptor-rich membranes blocked by both α-bungarotoxin and quinacrine mustard ('ternary' complex). Blocked receptor complexes are obtained. (e) Reconstitution of a 1:1 mixture of α-bungarotoxin-blocked and α-bungarotoxin/quinacrine mustard-blocked receptor-rich membranes. Blocked receptor complexes are obtained. (f) Reconstitution of a 1:1 mixture of α-bungarotoxin-blocked and quinacrine mustard-blocked receptor-rich membranes. Active receptor complexes are obtained (see Fig. 1, TQ). From experiments a—d one can conclude that in f an exchange of subunits has taken place. The multiple labelling ('ternary' complex) does not cause a removal of the inhibitors thereby activating again the receptor complex (d, e). The active receptor complex in f could still be labelled by quinacrine mustard at its binding subunit and may, therefore, not resemble exactly the natural receptor complex in a.

periment no disulfide cleaving agent is present which would allow a separation and recombination of such identical polypeptide chains.

Several further alternatives were investigated and ruled out. The stimulated efflux is not due to a dilution of the respective inhibitors by the 1:1 mixing of the samples in the hybridisation experiment. Doubling the inhibitor concentrations gave the same result. It is also not due to a removal of bungaro toxin by formation of 'ternary' complexes with quinacrine mustard in the TQ-experiment. Addition of quinacrine mustard to receptor complexes already blocked by α-bungarotoxin has no influence on the blocking effect (Fig 2d, e).

The stimulated efflux from the reconstituted vesicles does not reach the maximum value obtained with gramicidin. It varies from 20 to 40% of this value. This could be due to low efficiency of the subunit exchange process during reconstitution. A similar only partial efflux was obtained with native receptor-enriched vesicles and was attributed to desensitization caused by the agonist [10]. Preincubation of our reconstituted vesicles with 0.1 mM carbamoylcholine before the efflux assay reduced the amount of radioactivity released even further, indicating that the receptor may indeed be still subject to desensitization.

Distinct molecular entities for the acetylcholine receptor and its ion channel have been postulated before [11]. This structure would be especially economical where acetylcholine receptors regulate different functions (as has been observed in *Aplysia* neurons [13]). We have shown that, at least under our reconstitution conditions, an exchange of receptor subunits can occur. If the binding site and ion channel are located on different polypeptide chains it should be possible to separate the two functional units physically. In this case our reconstitution method using irreversibly labelled acetylcholine receptors should provide a useful assay especially for the ion channel subunit.

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