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Functional immobilisation of the nicotinic acetylcholine receptor in tethered lipid membranes

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Abstract

The nicotinic acetylcholine receptor from *Torpedo* was immobilised in tethered membranes. Surface plasmon resonance was used to quantify the binding of ligands and antibodies to the receptor. The orientation and structural integrity of the surface-reconstituted receptor was probed using monoclonal antibodies, demonstrating that approximately 65% of the receptors present their ligand-binding site towards the lumen of the flow cell and that at least 85% of these receptors are structurally intact. The conformation of the receptor in tethered membranes was investigated with Fourier transform infrared spectroscopy and found to be practically identical to that of receptors reconstituted in lipid vesicles. The affinity of small receptor ligands was determined in a competition assay against a monoclonal antibody directed against the ligand-binding site which yielded dissociation constants in agreement with radioligand binding assays. The presented method for the functional immobilisation of the nicotinic acetylcholine receptor in tethered membranes might be generally applicable to other membrane proteins.

Keywords: Nicotinic acetylcholine receptor; Functional immobilisation; Tethered membranes; Ligand binding; Surface plasmon resonance; Fourier transform infrared spectroscopy

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1. Introduction

Investigating the interaction of ligands with their membrane-bound receptors by surface sensitive techniques is a method of choice for elucidating receptor function as well as for developing novel screening assays. In order to realise this approach, the receptor of interest has to be reconstituted into lipid bilayers tethered to a suitable sensor surface, which still is not a routine procedure, mainly due to the tendency of proteins to denature on the surface. In order to preserve the function of the immobilised receptor we exploit here a concept of tethered membranes leaving a hydrophilic layer between the membrane and the support, thus avoiding denaturation of extramembraneous parts of the protein. This 'decoupling' of the bilayer from the support was developed recently in our laboratory and will be applied here to an ionotropic receptor [1–3].

Surface-sensitive techniques, when one of the interaction partners, in our case a receptor protein, is immobilised on the surface, offer several advantages over bulk techniques for the study of biomolecular interactions. They efficiently discriminate between surface-bound and free ligands, allow efficient use of receptor protein as it is surface immobilised, and are compatible with microfluidics [4].

Here we study the immobilisation of the nicotinic acetylcholine receptor (nAchR) from *Torpedo*, not just because this receptor is available in large quantities from the electric organ of *Torpedo*, but rather because it is a representative example of a whole family of ligand-gated ion-channels [5], a very important class of receptors. Furthermore, because it has large, protruding extramembraneous domains, it allows to put the 'decoupled supported membrane' concept to the test. Moreover, a large body of experimental data and tools to study this receptor are available.

Numerous attempts have been made using different approaches to devise biosensors incorporating the nAchR.

The receptor has been immobilised on sensor surfaces in the absence of a membrane (or detergent) environment by physioisorption to glass [6–8] and polymers [9] or by inclusion in a polymer matrix [10]. Only Rogers et al. obtained pharmaceutically relevant data for antagonists, but they used rather harsh immobilisation conditions (pH 4). It was proposed that the nAchR needs to be in a membrane containing specific lipids to display native properties [8].

Next, it has been tried to immobilise the nAchR in a membrane mimetic environment. Neuronal nAchR subtypes have been immobilised on phosphatidylcholine monolayers covalently linked to beads ('immobilised artificial membrane particles') [11] or reconstituted in sterically entrapped membranes in Superdex beads [12]. In both cases, a chromatographic competition assay was used where the effect of unlabeled receptor ligands on the retention time of the radiolabelled agonist epibatidin was determined, and the affinity of receptor ligands obtained parallel those from radioligand-binding assays on cell membrane homogenates.

Some groups have pursued a free-standing membrane approach either on porous silicon [13] or micro-apertures [14,15]. Others have attempted to immobilise the nAchR in supported membranes on hydrophobized surfaces by Langmuir–Blodgett transfer on glass [16], by detergent dialysis on glass [17] or vesicle fusion to a platinum surface [18]. None of these reports presents data on the (functional) characterisation of the receptor, not to speak of its pharmacology. Recently, the nAchR was immobilised in a peptide-supported bilayer, and the binding of a monoclonal antibody and the snake toxin α-bungarotoxin (Bgtx) to the receptor was reported [19]. However, in that report no quantitative data are presented on the integrity and orientation of the immobilised receptor. Also, the binding of small receptor ligands has not been investigated.

An alternative, promising approach was recently described in which, not the receptor but a ligand, Bgtx, was immobilised [20]. The binding of nAchR reconstituted in sterically stabilised vesicles to surface-bound Bgtx was followed on-line by surface plasmon resonance spectroscopy (SPR), and the inhibition thereof by small receptor ligands was measured successfully.

We present here a method which allows the

rapid and reproducible immobilisation of a membrane protein, nAchR from *Torpedo*, in decoupled supported lipid bilayer membranes. It is based on the fusion of receptor-containing vesicles to a gold surface. These vesicles contain lipids suitable for the functional reconstitution of the receptor as well as thiolipids, specially conceived phospholipids comprising a hydrophilic polyethyleneglycol spacer and a terminal thiol moiety in the lipid headgroup [1]. These thiolipids allow both stable anchoring of the bilayer on the gold surface through S–Au bonds and decoupling it from the surface via the long hydrophilic spacer creating a water film between the support and the bilayer. These thiolipids have been recently used for the formation of micropatterned lipid bilayers on gold. The G protein-coupled receptor rhodopsin was integrated into these tethered lipid bilayers exhibiting its full functional activity by interacting with its G protein transducin as demonstrated by SPR microscopy [2,3]. SPR is also used in the present work.

The functional integrity, orientation and ligand binding activity of the immobilised nAchR were investigated by measuring the specific binding of receptor ligands [the agonist carbamoylcholine (CBC) and the antagonist Bgtx] and conformational-specific monoclonal antibodies (mAb) directed against epitopes localised either in the extracellular or in the cytoplasmic domains of the nAchR (Fig. 1). Using Fourier transform infrared spectroscopy (FTIR) it is demonstrated that the structure of the nAchR is preserved upon surface immobilisation.

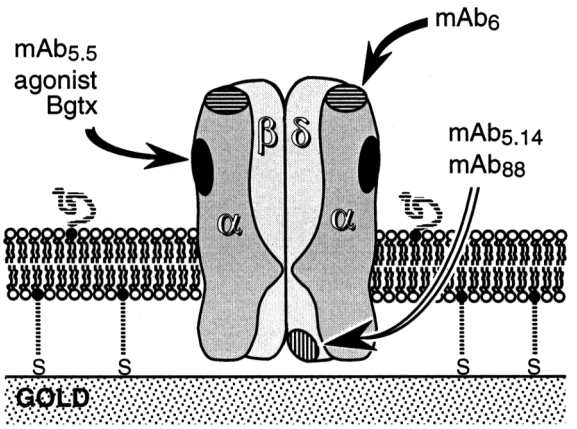

Fig. 1. nAchR reconstituted in a tethered bilayer: determining receptor orientation and functionality. Upon right-side-out presentation of the receptor only probes directed against the extracellular domains of the receptor will bind (Bgtx, $mAb_{5.5}$ and mAb_6), whereas mAb's directed against intracellular epitopes will not bind ($mAb_{5.14}$ and mAb_{88}). The structural integrity of the right-side-out immobilised receptors is tested by comparing the binding of the conformationally sensitve $mAb_{5.5}$ and mAb_6 with that of Bgtx, which binds to both native and denatured receptors.

2. Materials and methods

2.1. Materials

nAchR-rich membranes [21] in water at a total protein concentration of 1.5–2 mg/ml (Bradford test) were used without further purification. The production of the monoclonal antibodies mAb_6 is described elsewhere [22,23]. $mAb_{5.14}$ and $mAb_{5.5}$ were a generous gift from S. Fuchs (Weizmann Institute, Rehovot, Israel) [24,25]. Mab_{88} was purchased from ABR (Golden, CO, USA). Rabbit immunoglobin G (IgG) and 3-[(3-cholamidopropyl)dimethylammonio]-1-propane-sulfonate (CHAPS) were from Fluka (Buchs, Switzerland), cholesterol from Serva (Heidelberg, Germany), soy bean lecithin Type II from Sigma, biotinylated α-bungarotoxin (biot-Bgtx) from Molecular Probes, streptavidin and CBC from Calbiochem. *N*-[propionyl-^{3}H]-labeled Bgtx (55 Ci/mole) was obtained from Amersham (Buckinghamshire, UK). Antibodies were dialysed twice against 1000 volumes of 10 mM HEPES, 500 mM NaCl, 3 mM NaN_3 (pH 7.4) (Buffer A); whereas receptor ligands were directly dissolved in the same buffer.

Plain gold Biacore chips (Sensor Chip J, Prototype) were purchased from Biosensor AB (Uppsala, Sweden). The thiolipid [*N*-(14′-mercapto-1′,11′-dioxo-3′,6′,9′-trioxa-12′-azatetradecyl)-2-oleoyl-1-palmitoyl-*sn*-glycero-3-phosphatidyl-ethanolamine] was synthesised as described elsewhere [2]. All other chemicals were of the highest quality available from regular sources.

2.2. Reconstitution of nAchR in thiolipid vesicles

Receptor-rich membranes were brought to a concentration of 0.5 mg/ml in a final solution of 44 mM CHAPS, 10 mM HEPES, 60 mM NaCl, 3 mM NaN_3 (pH 7.4) containing 0.16 mg cholesterol, 0.16 mg thiolopid and 1.28 mg soy bean lecithin, and incubated for 2 h at 4°C under mild agitation. Detergent was removed by dialysis for 72 h with a buffer exchange after 48 h in a Slide-A-lyzer (molecular weight cut-off 10 kDa, Pierce, Rockford, IL, USA) against 2000 volumes of Buffer A. The vesicles thus obtained were frozen immediately and stored at −80°C. Before use, vesicles were thawed, centrifuged for 10 min at $10\,000 \times g$ at 4°C and the supernatant was diluted five times with Buffer A.

Cryo-electron microscopy showed that the diameter of the vesicles ranged from 300 to 500 nm (not shown).

2.3. SPR measurements — instrumentation

A Biacore 1000 (Biosensor AB) was used to monitor the formation of supported membranes, and the binding of antibodies and ligands to the nAchR in these supported membranes. This instrument uses SPR to detect changes in the optical properties at the surface of a thin film of gold (approx. 50 nm) deposited on a glass slide. The slide is fixed in a flow cell with the gold surface facing the cell's interior. A liquid handling system controls the delivery of sample solutions over the sensor surface. When binding to the surface occurs (for instance formation of tethered membranes or antibody binding to the receptor), the refractive index in the vicinity of the gold surface changes in relation to the surface concentration of adsorbed molecules leading to a shift of the surface plasmon resonance angle. This shift of the resonance angle is measured by the SPR instrument and expressed as 'resonance units' (RU). Under the present conditions, 1000 RU corresponds an angle shift of 0.1° or a layer thickness of 9.1 Å, assuming a refractive index $n = 1.45$ for the layers studied [26]. Further details of SPR instrumentation and methods of analysis have been described elsewhere [4,27].

2.4. SPR measurements

2.4.1. Formation of nAchR-containing supported membranes

Clean gold chips were purged with 70 μl of the vesicle suspension at a flow rate of 20 μl/min until no further change in the SPR signal was observed (generally 30 min). The tethered membrane thus formed was washed thoroughly with Buffer A at a flow rate of 100 μl/min, and the layer thickness was measured subsequently.

The quality of the supported membranes was determined by incubating the layers with solutions in Buffer A of BSA (0.1 or 1 mg/ml) or polyclonal rabbit IgG of increasing concentrations at a flow rate of 12 μl/min.

2.4.2. Analyte binding

Before the injection of analytes, non-specific interaction sites were blocked by incubating the nAchR-containing tethered membranes first for 10 min in a flow of 12 μl/min of 2.2 μM polyclonal rabbit IgG solution, followed by washing with Buffer A at a flow rate of 100 μl/min.

Then, the analyte (mAb or biot-Bgtx) was passed through the flow cell with a 12 μl/min flow rate until no further change in the SPR signal was observed (usually within 70 μl). The cell was rinsed with Buffer A (1 μl/min) and the shift of the resonance angle was measured. To accurately quantify the binding of biot-Bgtx, a second binding reaction was performed as an amplification step using 70 μl 3.3 μM streptavidin at a 12 μl/min flow rate.

The binding constants of the monoclonal antibodies were determined by incubation of the same nAchR-containing tethered membrane using increasing concentrations of antibodies. The binding constant of biot-Bgtx was measured on different nAchR-containing lipid layers, since a second binding step with streptavidin was used as an amplification step.

2.4.3. Competition experiments

The affinity of the receptor ligands CBC or biot-Bgtx was determined by their concentration-dependent ability to inhibit the binding of $mAb_{5.5}$,

which has been shown elsewhere to inhibit ligand binding [25].

An aliquot of the desired final concentration of CBC (70 μl) was injected into the cell (12 μl/min) and the flow was stopped for 40 min. After rinsing with buffer (1 μl/min), the surface was incubated with 120 μl of $mAb_{5.5}$ (1.1 μM) and the same concentration of CBC. As final layer thickness we used that value measured when no further change in the SPR signal occurred (approx. 20 min).

Biot-Bgtx binding was measured using 120 μl of a solution of $mAb_{5.5}$ (1.1 μM) containing increasing concentrations of biot-Bgtx, which were flown (12 μl/min) over the tethered membrane until no further change in signal occurred (20–40 min), then the cell was rinsed with buffer (1 μl/min) and the final signal measured.

2.4.4. FTIR measurements

Infrared measurements were recorded using a Bruker IFS 28 spectrometer, accumulating for each sample 500 scans at a resolution of 1 cm^{-1} with Boxcar apodization and two levels of zero-filling. Spectra were corrected for baseline, atmospheric water vapour bands and smoothed with the Savitzky Golay algorithm.

For transmission spectra, the reconstituted receptor preparations were dialysed extensively against Buffer A in D_2O without azide, and measured between CaF_2 windows with a 50-μm lead spacer. Attenuated total reflection (ATR) measurements were performed with gold-covered ZnSe crystals, produced as described elsewhere [28]. nAchR, reconstituted in thiolipid-containing vesicles, was allowed to interact with the gold surface until a stable FTIR signal was obtained, then the overstanding solution was replaced by Buffer A in D_2O without azide.

2.4.5. [^{3}H] Bgtx binding assay

Routinely, samples containing 1–3 nM nAchR were incubated for 60 min at room temperature in 10 mM HEPES (pH 7.4) with [^{3}H]-Bgtx in a final volume of 100 μl. The incubation was terminated by rapid filtration through Whatman GF/B filters [presoaked for 15 min in 0.5% (w/v) polyethylenimine] followed by two washes with 3 ml of ice-cold 10 mM HEPES (pH 7.4). Filters were transferred into scintillation vials, and 4 ml Ultima Gold™ (Packard, Meridan, CT, USA) was added. The radioactivity was measured in a Tri-Carb 2200CA liquid scintillation counter (Packard) and corrected for quenching and counting efficiency.

The total number of ligand-binding sites was determined by incubation with seven concentrations of [^{3}H] Bgtx ranging from 10 pM to 10 nM. The binding affinity of nAchR to different pharmacologically active compounds was determined by inclusion of increasing concentrations of the agonist (CBC) and antagonist (Bgtx) in the standard binding assay containing 0.4–0.8 nM [^{3}H]-Bgtx. Non-specific binding was determined in the presence of 50 μM Bgtx. All experiments were performed in triplicate.

2.4.6. Curve-fitting

Dissociation constants, total number of binding sites, concentrations of half inhibition and Hill coefficients were evaluated from experimental binding isotherms by curve-fitting with an iterative Levenberg–Marquardt algorithm minimising chi-square using the software package Igor 3.0 (WaveMetrics Inc., Lake Oswego, USA).

3. Results and discussion

For the development of a reliable protocol of a ligand-binding assay to the nAchR, a controlled formation of tethered membranes is a prerequisite. Therefore, we first characterised the surface-immobilised receptor: (i) by investigating the binding of four different monoclonal antibodies, each directed to a different epitope on the receptor, using SPR; (ii) by measuring the specific binding of two ligands to the receptor again by SPR; and (iii) by investigating the conformation of the receptor in the tethered lipid membrane using FTIR spectroscopy.

A typical antibody-binding experiment is shown in Fig. 2. A solution of nAchR reconstituted in thiolipid-containing vesicles is injected into the flow cell, and a rapid increase in the SPR response is observed. In approximately 4 min 95% of the final layer was formed. Eventually, a layer

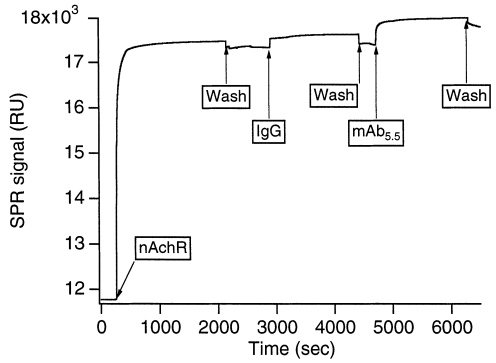

Fig. 2. Formation of an nAchR-containing tethered membrane and subsequent binding of a monoclonal antibody. A bare gold surface was purged with a solution of thiolipid vesicles comprising reconstituted nAchR, resulting in the rapid formation of a tethered membrane (5540 RU, or 5.1 nm). Injection of a 2.2 μM IgG solution resulted in a small increase of the SPR response (65 RU, or 0.05 nm) demonstrating the quality of the layer with respect to non-specific protein binding. Injection of 2.2 μM $mAb_{5.5}$ yielded a large response (385 RU, or 0.37 nm) indicating the presentation of the ligand-binding domain towards the bulk flowing solution. Buffer washes in between the injections of the different antibody solutions were necessary in order to re-establish the refractive index of the buffer and thus enabling accurate measurement of the amount of the adsorbed mass, as well as removal of loosely bound material. A representative experiment is shown.

of 5.1 nm is obtained, which corresponds nicely to the expected average thickness of a lipid bilayer with 0.09 mol% nAchR. To test the quality of the layer, and also to block non-specific binding sites, polyclonal rabbit IgG was injected, which is known to adsorb on hydrophobic surfaces. The non-specific binding of the IgG led to a layer of approximately 0.05 nm average thickness, corresponding to less than 1% coverage of the surface with IgG. In many experiments the amount of non-specifically adsorbed IgG to tethered lipid membranes ranged from 0 to 80 RUs, demonstrating both the reproducibility of layer formation and the quality of the final supported membrane. When the $mAb_{5.5}$, directed against the ligand-binding site was injected a final signal of 385 RUs was observed, corresponding to a layer of 0.37 nm average thickness.

3.1. Influence of receptor concentration in the thiolipid vesicles on the quality of the tethered membranes

The receptor was reconstituted into tethered membranes by self-assembly of thiolipid vesicles comprising different amounts of receptor. The receptor content in the vesicles did not substantially affect the thickness of the formed tethered membranes; in all cases the self-assembled layers had thicknesses between 4500 and 5500 RU (Table 1). This indicates that independent of the initial receptor content, the same amount of material is deposited on the sensor surface. However, the initial composition of the vesicle solution had a marked effect on the extent of the non-specific binding of IgG. A high binding was observed to layers made from vesicles with a low nAchR content (0.002 and 0.04%), whereas layers formed from vesicles containing 0.09 mol% nAchR were almost perfect with respect to the extremely low non-specific binding observed.

The amount of nAchR presenting their binding site towards the flow cell was probed with mAb_6. This quantity was not very dependent on the nAchR content of the vesicles; from 0.002 to

Table 1
Specific and non-specific antibody binding to tethered membranes produced from vesicles containing different amounts of nAchR

nAchR content in vesicles (mol%)[a]	Non-specific binding of rabbit IgG (RU)[b]	Specific binding of mAb_6 (RU)[c]
0.002	600–700	500
0.04	100–200	800–850
0.09	0–80	800–850

[a] The mol% of nAchR indicated correspond to the composition of the lipid–protein–detergent solution before dialysis.
[b] After purging the sensor surface with a solution of 2.2 μM IgG.
[c] After purging the sensor surface with a solution of 2.2 μM mAb_6.

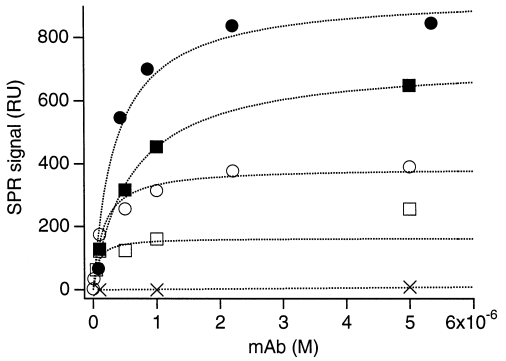

Fig. 3. Binding of mAb's to tethered membranes. Supported membranes containing nAchR were incubated with a solution of the particular mAb of the increasing concentrations to determine the specific binding: (●) mAb_6, directed against the major immunogenic region, (○) $mAb_{5.5}$, against the ligand-binding site, (■) $mAb_{5.14}$ and (□) mAb_{88} directed against cytosolic domains of the α and δ or γ-subunits, respectively. (x) Non-specific binding of the polyclonal rabbit IgG. The data are well fitted by Langmuir binding isotherms (dotted lines) yielding dissociation constants of 0.4 ± 0.1, 0.16 ± 0.07, 0.60 ± 0.08 and 0.072 ± 0.004 μM and maximal binding of 940 ± 80, 390 ± 20, 723 ± 32 and 163 ± 34 RU for mAb_6, $mAb_{5.5}$, $mAb_{5.14}$ and mAb_{88}, respectively. The non-specific binding of rabbit IgG to the tethered bilayer was extremely low: at 5 μM, the highest concentration tested, a binding signal of only 9 RU was observed.

0.04% the amount increased by approximately 60%, whereas an additional increase in receptor content in the vesicles did not enhance the binding of mAb_6 to the tethered membrane.

Therefore, all further tethered membranes were formed using vesicles containing 0.09 mol% of nAchR, as these vesicles yielded the highest signal-to-background ratio.

3.2. Binding of mAb's to the nAchR in tethered membranes

The orientation (ligand-binding site oriented towards the bulk buffer vs. ligand-binding site oriented towards the support) and the structural integrity (native vs. denatured) of the nAchR in tethered membranes were investigated using antibodies directed against different epitopes on the receptor (Figs. 1 and 3), as well as with Bgtx (Figs. 1 and 4).

The presentation of the N-terminal extracellular ligand binding domain towards the bulk medium of the flow cell was quantified using two mAb's. The first one was mAb_6, which is directed against the major immunogenic region involved in the disease *Myasthenia gravis*, corresponding to the amino acids 67–76 of the α-subunit of the nAchR from *Torpedo*, which are located at the apex of the extracellular domain of the protein [29]. MAb_6 is specific for the non-denatured form of the receptor [22,23]. The second antibody used was $mAb_{5.5}$ which has been shown to compete with cholinergic ligands like CBC and Bgtx, for receptor binding. $MAb_{5.5}$ is directed against a conformational epitope [25,30]. These two mAb's thus served to probe the native receptor presented 'right-side-out'. The exposure of the cytoplasmic site of the nAchR towards the bulk medium of the flow cell was quantified with $mAb_{5.14}$ and mAb_{88}. The former is directed against an epitope in the cytoplasmic part of α-subunits [24], whereas the latter is specific for a cytoplasmic epitope on the δ- and γ-subunits [31]. These two mAb's recognise both the native and denatured form of the nAchR [24,31].

Upon purging the immobilised receptor with increasing concentrations of the four different

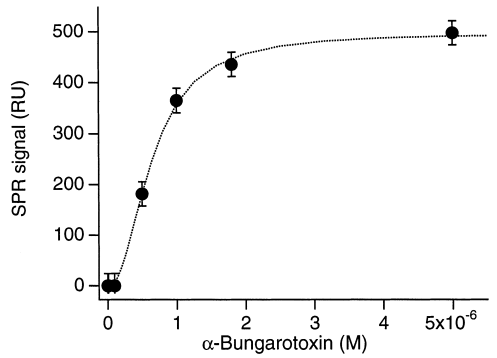

Fig. 4. Binding of Bgtx to nAchR in tethered membranes. nAchR-containing tethered membranes were incubated with the indicated concentrations of biot-Bgtx. To amplify the signal of toxin binding, the layers were subsequently purged with a 3.3 μM streptavidin solution. The experimental binding curve (average of three measurements with standard deviations indicated by the error bars) had a sigmoidal appearance, and could be fitted well by the Hill equation yielding a dissociation constant of 0.63 ± 0.03 μM with a Hill coefficient of 2.1 ± 0.2 and a maximal binding amplitude of 500 ± 10 RU.

mAb's, saturable binding with submicromolar dissociation constants is observed (Fig. 3). The excellent quality of the tethered membranes is reflected by the extremely low binding of non-specific rabbit IgG. Even at the highest concentration used, only 9 RU was observed for the non-specific adsorption, as compared to 935 and 163 RU for the specific adsorption of mAb_6 and mAb_{88}, respectively.

3.3. Binding of Bgtx to the nAchR in tethered membranes

Bgtx is known to bind to the native as well as to denatured nAchR, and is thus a useful probe to detect the total amount of receptor incorporated right-side-out.

Due to the relatively low molecular weight of Bgtx (8.4 kDa), we used a biotinylated derivative, biot-Bgtx, in order to profit from a 7.5-fold amplification of the signal upon subsequent streptavidin (60 kDa) binding. Increasing the Bgtx concentration, the signal increased in a sigmoidal manner to level off at approximately 2 μM (Fig. 4). The experimental binding curve was well fitted assuming a co-operative binding yielding a dissociation constant of 0.6 μM and a Hill coefficient of 2.1, indicating that two molecules of Bgtx bind per receptor, as has been observed before (for a review see [32]).

The results obtained (Figs. 3 and 4) are summarised in Tables 2 and 3.

3.4. Orientation and integrity of the nAchR in tethered membranes

We first derived from these results the ratio of the receptor incorporated right-side-out to the total receptor in the following way: biot-Bgtx reveals the presence of the receptors having their extracellular domain oriented towards the bulk of the flow cell, whereas mAb_{88} and $mAb_{5.14}$ probe the receptors in the opposite orientation. Assuming a 1:1 binding stoichiometry for Bgtx and $mAb_{5.14}$ and a 1:3 stoichiometry in the case of mAb_{88} [31], 60–69% of nAchR is incorporated presenting its binding site towards the bulk of the flow cell.

The percentage of native nAchR among the properly oriented receptors, was derived by comparing maximal binding amplitudes of the antibody mAb_6 to that of Bgtx, as this antibody recognises conformational epitopes on the native receptor [22,23] whereas Bgtx is known to bind both native [32] and denatured [33] nAchR. Fragments of mAb_6 [29] and Bgtx [32] have been shown to bind to the nAchR in a 2:1 stoichiometry. One can assume that for the complete mAb_6 this stoichiometry is an upper limit. Therefore, at least 85% of the receptors oriented with the ligand-binding site towards the bulk of the flow cell are functional.

The maximal binding amplitude of $mAb_{5.5}$ is significantly lower compared to that of mAb_6, suggesting that a large part of the $mAb_{5.5}$ epitope, the ligand-binding site, is not accessible to this antibody.

Table 2
Binding of mAb's and Bgtx to the nAchR in tethered membranes

Binding partner	Target site	Conformation sensitivity	Maximal binding (RU)
biot-Bgtx	Binding site, extracellular	No	1102 ± 26[a]
mAb_6	Residues α67 to α76, extracellular	Yes	940 ± 80
$mAb_{5.5}$	Binding site, extracellular	Yes	390 ± 20
$mAb_{5.14}$	Intracellular loop of α-subunit	No	723 ± 32
mAb_{88}	Intracellular loop of δ/γ-subunits	No	163 ± 34

[a] The maximal binding of biot-Bgtx is 501 RU; this value was multiplied by 2.2 to compensate for the difference in molecular weight, allowing direct comparison with the maximal amplitudes obtained from antibody binding.

Table 3
Comparison of binding data

	Biacore pK_d	Biacore pK_i vs. $mAb_{5.5}$	[^{3}H]-Bgtx binding assay pK[a]
[^{3}H] Bgtx			9.34 ± 0.14
Biot-Bgtx	6.19 ± 0.02	7.22 ± 0.14	7.46 ± 0.43
mAb_6	6.44 ± 0.13		
$mAb_{5.5}$	6.80 ± 0.11		6.22 ± 0.14
$mAb_{5.14}$	6.22 ± 0.06		
mAb_{88}	7.14 ± 0.35		
CBC		2.64 ± 0.02	2.70 ± 0.22

[a] The dissociation constant of [^{3}H] Bgtx was measured by a saturation ligand-binding assay; the affinity of the unlabeled ligands was measured in a competition assay with 0.4–0.8 nM [^{3}H]-Bgtx.

3.5. Conformation of nAchR reconstituted in tethered membranes

Direct immobilisation of proteins on metal surfaces might induce denaturation. Here we used FTIR spectroscopy in order to determine the conformation of the nAchR in tethered membranes on gold surfaces and reconstituted in lipid vesicles for comparison (Fig. 5).

In the Amide I spectral region of the nAchR in vesicles a broad band with a maximum at approximately 1645 cm^{-1} was observed, which is in good agreement with the spectra reported elsewhere [34]. The corresponding spectrum of the receptor in tethered membranes is almost superimposable to that of receptors in vesicles with a minor additional component at higher wavenumbers. Similar higher wavenumber spectral components have been observed on metal surfaces [35–37]. In summary, the spectra for the two nAchR preparations indicate that the secondary structure of the receptor in tethered membranes is practically identical to that in lipid vesicles.

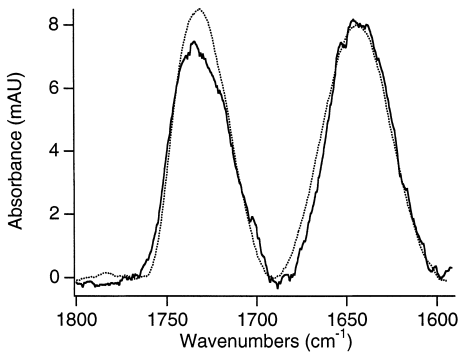

Fig. 5. Conformation of the nAchR in tethered membranes measured by FTIR. Spectra of the nAchR in tethered membranes (dotted line) and in vesicles (solid line) showing the phospholipid carbonyl group (maximum 1732 cm^{-1}) and Amide I region (maximum 1645 cm^{-1}). The structure of the nAchR in the tethered membranes was determined upon layer formation on a gold-coated ZnSe element by the surface sensitive attenuated total reflection FTIR technique, and compared with the transmission absorbance spectrum of the nAchR reconstituted in lipid vesicles used for the formation of the tethered membrane. Spectra were normalised to the intensity of the Amide I band in order to facilitate comparison.

Moreover, the intensity ratio between of the Amide I and the carbonyl bands of the two spectra is identical within 2%, suggesting that during self-assembly there has not been a preferential enrichment of phospholipid over receptor, or vice versa, on the surface.

3.6. Competition of receptor ligands vs. $mAb_{5.5}$ for nAchR binding

Due to their low molecular mass, the binding of cholinergic ligands to the nAchR cannot be measured directly by SPR. Therefore, we performed a competition assay using $mAb_{5.5}$, an antibody known to compete with ligands for receptor binding [25]. As represented in Fig. 6, increasing concentrations of both the agonist CBC and the antagonist Bgtx inhibited the binding of the $mAb_{5.5}$, with concentrations of half-inhibition of 18 mM and 0.5 μM, respectively. The binding data obtained in this study are summarised in Table 3.

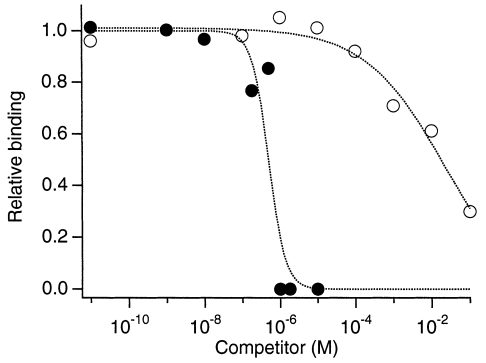

Fig. 6. Competition between $mAb_{5.5}$ and receptor ligands for binding to the nAchR in tethered membranes. The inhibition of the binding to the immobilized nAchR of the ligand-binding site directed $mAb_{5.5}$ by CBC (○) and Bgtx (●) was determined by co-incubating the receptor in tethered membranes with 1.1 μM $mAb_{5.5}$ in the presence of the indicated inhibitor concentrations. The concentrations of half-inhibition were 0.5 μM and 18 mM for Bgtx and CBC, respectively. The dotted lines serve to guide the eye.

The affinity of [^{3}H]-Bgtx for the nAchR in receptor-enriched membranes determined here corresponds well to the reported dissociation constant of 0.15 nM [25]. The lower affinity for the receptor of biot-Bgtx as compared to Bgtx seems to be due to the modification with biotin, as is suggested by the radioligand and $mAb_{5.5}$ competition assays. The reduced affinity of biot-Bgtx for the surface immobilised nAchR found in the direct assay as compared to the competition assay with $mAb_{5.5}$ might be due to sterical hindrance for the streptavidin to reach the receptor-bound biot-Bgtx.

The dissociation constant of approximately 2 mM of CBC for the receptor measured by its ability to inhibit [^{3}H]-Bgtx binding is identical to the one obtained with the $mAb_{5.5}$ competition assay with the nAchR in tethered membranes, showing that the surface-immobilised receptor displays wild-type-like ligand binding. Published values for the affinity of CBC for the receptor are very diverse and range from 0.5 [38] up to 500 μM [25]; this latter value being determined from the inhibition of $mAb_{5.5}$ binding to Triton X-100 solubilised nAchR.

The affinity of the binding of $mAb_{5.5}$ to the nAchR in tethered membranes is virtually identical to that for the binding to the receptor in the original receptor-enriched membranes, suggesting that in both cases the receptor is presented in a comparable manner. The determined dissociation constants of the different monoclonal antibodies employed range from 70 to 600 nM. The blood of patients suffering from the autoimmune disease *Myasthenia gravis* contains anti-nAchR antibodies, ranging in concentration from 4.5 to 110 nM, whereas the blood of healthy or control patients contains only 0.4 nM of these antibodies [39]. The sensitivity of the SPR detection of antibody binding to the nAchR in tethered membranes would enable to measure anti-nAchR antibody concentrations as low as 10–40 nM depending on their affinities. Therefore, this assay might have potential to screen the serum of patients for the presence of autoimmune nAchR antibodies.

4. Summarising conclusion

We have presented a method to functionally immobilise the nAchR in tethered membranes in a controlled and reproducible manner. The formed tethered bilayer is of excellent quality as judged from the extremely low non-specific binding of a non-specific IgG. The orientation and structural integrity of the immobilised receptor have been investigated, demonstrating that the immobilised nAchR displays a native structure and is mainly oriented with its ligand-binding site towards the bulk of the flow cell. Ligand-binding studies to the receptor in tethered membranes delivered affinities in agreement with data obtained from radioligand competition assays. The presented method for the immobilisation of the nAchR using tethered membranes should be generally applicable to other membrane proteins [40].

5. Nomenclature

CBC: Carbamoylcholine
nAchR: Nicotinic acetylcholine receptor (from *Torpedo*)
Bgtx: α-Bungarotoxin
biot-Bgtx: Biotinylated α-bungarotoxin

Hepes: 4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid
CHAPS: 3-[(3-Cholamidopropyl)dimethylammonio]-1-propane-sulfonate
mAb: Monoclonal antibody
IgG: Immunoglobin G
RU: Resonance units
BSA: Bovine serum albumin
FTIR: Fourier transform infrared spectroscopy
SPR: Surface plasmon resonance spectroscopy

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