# ACETYLCHOLINE RECEPTOR ENRICHED MEMBRANES: ACETYLCHOLINE BINDING AND EXCITABILITY AFTER REDUCTION IN VITRO

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## 1. Introduction

The postsynaptic response of cholinergic synapses is strongly perturbed by incubation with dithiothreitol (DTT) [1,2]. The dose–response curve for the agonist acetylcholine is shifted to higher concentrations and its shape is changed from sigmoidal to hyperbolic. Labelling experiments with an –SH group directed affinity label suggested that this effect is due to a reduction of a disulfide bridge in one of the polypeptide chains of the acetylcholine receptor (AChR) [3]. But from these observations it is not clear if the dose–response curve reflects the concentration dependence of agonist binding and if by the reducing agent only the ligand binding to the receptor site of the postsynaptic membrane is affected or the ionophore, the ion transducing unit as well. In this report we show that the $^{22}Na$ efflux from receptor-rich membrane vesicles, an in vitro model system of postsynaptic events [4], prepared from the electric organ of *Torpedo californica* remains normal after treatment with the reducing agents β-mercaptoethanol or dithioerythritol (DTE) but that the dissociation constant for acetylcholine increases ten-fold. Similar to the response in vivo the cooperativity of the binding is lost.

## 2. Materials and methods

### 2.1. *Chemicals and reagents*

All compounds were of the highest commercially available purity. $^{22}Na$- and $^{3}H$-labelled acetylcholine were purchased from Amersham Buchler (Braunschweig, Germany). Sodium dodecyl sulfate, dithioerythritol, β-mercaptoethanol and all reagents for polyacrylamide gel electrophoresis were purchased from Serva (Heidelberg, FRG). Sucrose, ethylenediaminetetraacetate and all buffer substances were obtained from Merck (Darmstadt, FRG).

### 2.2. *Electric fish*

All experiments were performed with *Torpedo californica* obtained live from Biomarine Supply Inc., Venice, Calif. The animals were killed immediately before the preparation. Excess electric tissue was frozen with liquid nitrogen, stored at –60°C and used within four weeks.

### 2.3. *Receptor-rich membranes*

Membranes rich in acetylcholine receptor were prepared as follows. Electric tissue (60 g) from *Torpedo californica* are cut in small pieces and homogenized with a Waring blendor for 3 min at maximum speed in 120 ml 0.02 M sodium phosphate buffer, pH 7.4, containing 2 mM EDTA, 0.1 mM PMSF (phenylmethylsulfonylfluoride) and 0.4 M NaCl. This and all subsequent steps are performed at 2°C. The homogenate is centrifuged 90 min at 27 000 × *g* and the supernatant is discarded. The pellet is resuspended in 60 ml 0.02 M sodium phosphate buffer, pH 7.4, containing 2 mM EDTA and 0.1 mM PMSF with a Waring blendor at low speed (3 min). The suspension is centrifuged 90 min at 39 000 × *g*. The pellet is resuspended and centrifuged again and after this second wash the pellet is suspended in 25 ml of the same buffer. After 10 min centrifugation at 1000 × *g* each 5 ml of the supernatant are layered on top of 55 ml

of a continuous sucrose gradient (25–50% w/v sucrose in distilled water containing 0.02% $NaN_3$). Centrifugation is performed for 6 h at 59 000 × *g* in a Beckman SW 25 rotor. The preparations revealed on SDS–polyacrylamide gel electrophoresis predominantly the four bands [7] assigned to receptor protein.

2.4. *Sodium efflux*

$^{22}Na$ efflux was measured according to [5]. Receptor-rich microsacs from the combined peak fractions of the sucrose gradient were collected by 30 min centrifugation at 20 000 × *g* and suspended in Ringer's solution (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% $NaN_3$) to a final protein concentration of 5–10 mg/ml. The suspension was incubated overnight with 20 μCi/ml $^{22}Na$ at 6°C. The incubation mixture was diluted 140-fold in ice-cold 0.16 M KCl, 5 mM NaCl, 2 mM MgCl, 2 mM $CaCl_2$, 3 mM sodium phosphate buffer, pH 7.0, 0.02% $NaN_3$. Aliquots 1 ml were filtered through Millipore filters (0.45 μm pore width) at the time indicated in fig.1.

2.5. *Binding experiment*

Binding of [$^3$H]acetylcholine was determined by 30 min centrifugation at 100 000 × *g*, 4°C of a membrane suspension containing 0.1 mg/ml protein in Ringer's solution. Acetylcholine esterase activity was inhibited by incubation with 0.1 mM eserine.

## 3. Results and discussion

Figure 1 shows the efflux of $^{22}Na$ from AChR enriched membrane vesicles from *Torpedo californica*. As described before [4] it can be stimulated by carbamylcholine, a cholinergic agonist and the amplitude of the response decreases after treatment of the membrane with DTE. β-Mercaptoethanol is less potent than DTE but both reagents show qualitatively the same effect. (With microsacs prepared from *Torpedo marmorata* 10 mM β-mercaptoethanol did not suppress the response to carbamylcholine [5].) Within 10 min incubation time DTE abolished the excitability of the membrane by 0.01 mM carbamylcholine virtually completely.

This effect can be overcome by higher agonist concentrations: Increasing the carbamylcholine concentration to 0.1 mM (fig.1B) results in about the same stimulation of $^{22}Na$ efflux from the reduced microsacs as compared with 0.01 mM carbamylcholine and the native vesicles. This indicates that

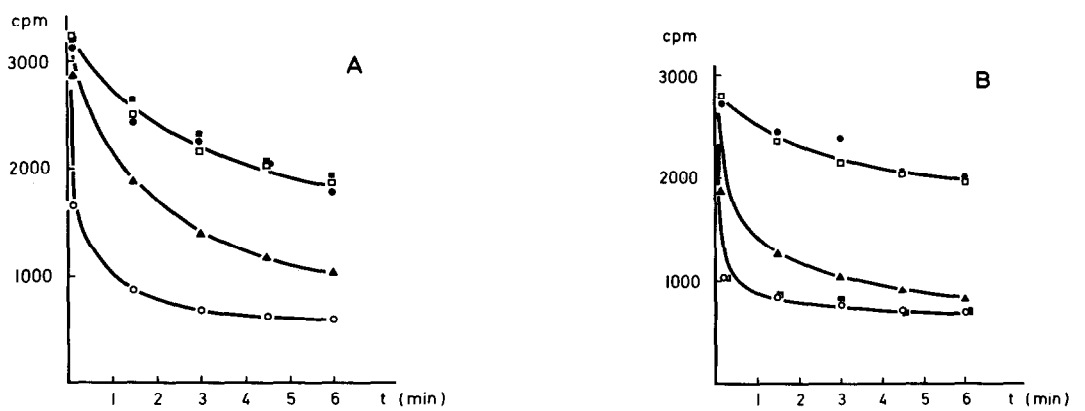


Fig.1(A). Influence of reducing agents on $^{22}Na$ efflux from receptor-rich microsacs. Residual cpm in 1 ml microsac suspension: (□–□–□) Control, untreated membranes (○–○–○) stimulation of $^{22}Na$ efflux by 0.01 mM carbamylcholine. (■–■–■) Control, membranes preincubated with 5 mM DTE for 30 min at room temperature. (●–●–●) Stimulation of the DTE-reduced membranes with 0.01 mM carbamylcholine. (▲–▲–▲) Stimulation of membranes reduced by 30 min preincubation with 20 mM β-mercaptoethanol. (B). Stimulation of DTE-reduced membranes with increasing carbamylcholine concentrations. (□–□–□) Control, untreated membranes. (○–○–○) Stimulation of $^{22}Na^+$ efflux from untreated membranes with 0.01 mM carbamylcholine. (●–●–●) Stimulation of $^{22}Na^+$ efflux from DTE-reduced membranes with 0.01 mM carbamylcholine. (▲–▲–▲) Stimulation of DTE-reduced membranes with 0.1 mM carbamylcholine. (■–■–■) Stimulation of DTE-reduced membranes with 1 mM carbamylcholines.

reduction lowers the affinity of the AChR for the agonist but does not affect the ionophore.

This assumption is supported by binding studies (fig.2). We found a significant decrease of the binding of [$^3$H]acetylcholine to the receptor-enriched membranes after reduction. The dissociation constant increased from $1.4 \times 10^{-8}$ to $1.0 \times 10^{-7}$. In contrast to other authors [6] we obtained cooperative binding with a Hill coefficient $n = 1.7$, which decreases after reduction to about 1.0. This result parallels the alteration of the dose–response curve in vivo.

Partial reduction of the membrane vesicles, e.g., by 30 min incubation with mercaptoethanol (fig.2) appears to yield a mixed population of completely reduced and completely unreduced molecules. The Scatchard plot indicates the presence of high affinity and low affinity binding sites the former possibly still showing positive cooperativity.

The disulfide bridge involved was localized in the α-polypeptide chain of the *Torpedo* receptor (mol. wt 40 000) [3] which also contains the ligand binding sites [3,7]. In addition to this bridge we demonstrated the presence of an inter-polypeptide chain disulfide bridge between two of the δ-chains (mol. wt 68 000) the function of which is still unknown [8]. Under the conditions of the experiment in fig.2 the interchain disulfide bridge is completely cleaved (B. A. Suarez-Isla, unpublished observation). Since the ionic flow was not influenced to a significant degree this group appears not to be essential for the ion-transfer mechanism.

Ionic channels in excitable membrane consist of two functional units: a gating unit which regulates the opening and the closing of the channel and an ionophore which contains the selectivity filter and determines which ion can pass the channel at what maximum rate. It is not known if these functional units are different molecular structures. At the post-synaptic membrane gating is achieved by a transmitter molecule which binds to the acetylcholine receptor. Evidence is accumulating [9–12] that the receptor molecules are not identical with the ionophore. This evidence derives from the differential influence of certain toxins on the two functional units. Our experiments also support a model where the functional units of this channel are on separate molecular sites.

## Acknowledgements

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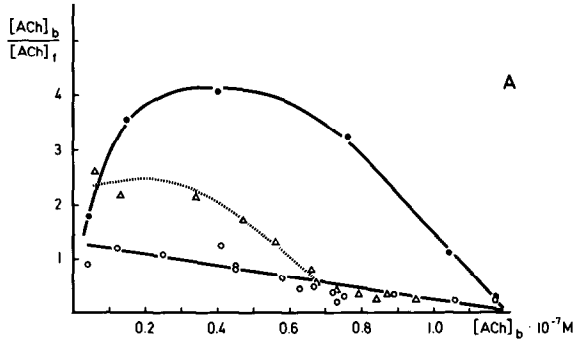


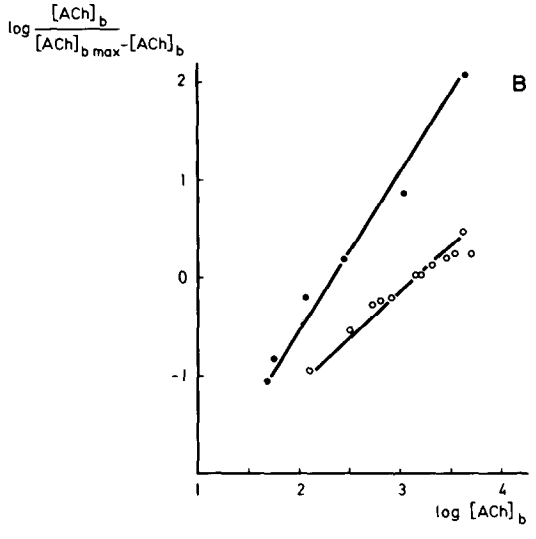


Fig.2(A). Scatchard plot of [$^3$H]acetylcholine binding to receptor-rich microsacs. Binding was determined by centrifugation at 4°C. (●–●–●) Untreated membranes. (○–○–○) Membranes reduced by 2 h preincubation with 5 mM DTE at room temperature. (△–△–△) Membranes reduced by 30 min preincubation with 20 mM β-mercaptoethanol at room temperature. (B). Hill plot of the data of fig.2(A). The Hill coefficient $n = 1.7$ for the untreated membranes decreases to $n = 1.0$ for the DTE-reduced membranes. (●–●–●) Untreated membranes. (○–○–○) DTE-reduced membranes.

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