Ca^{2+}-dependent inactivation of acetylcholine receptors by an endogenous transglutaminase

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The nicotinic acetylcholine receptor (nAChR) from *Torpedo californica* and *T. marmorata* electric tissue polymerises irreversibly when DTE and Ca^{2+} are added to receptor-rich membranes. The polymerisation is time-dependent and complete within 3 h at 30°C. It can be completely prevented by EGTA or the transglutaminase inhibitor cystamine. Transglutaminase activity can also be monitored with the exogenous substrates [^{3}H]putrescine and dimethylcasein. This assay can also be inhibited by EGTA or cystamine.

Nicotinic acetylcholine receptor *Ca^{2+}* *Transglutaminase* *Receptor regulation*

1. INTRODUCTION

Transglutaminases (EC 2.3.2.13) are enzymes cross-linking certain proteins covalently by γ-glutamyl-ϵ-lysyl side chain bridges [1]. It has been suggested that protein cross-linking may be essential for receptor-mediated endocytosis of some protein and peptide hormones [2] and down-regulation of membrane receptors [3]. According to this model, receptors would cluster after ligand binding, move to coated pits where they are internalised via coated vesicles after covalent cross-linking by a Ca^{2+}/SH-dependent transglutaminase [2]. Here, we report a transglutaminase activity present in membranes rich in nicotinic acetylcholine receptor (nAChR) prepared from the electric organ of *Torpedo californica*. We observed that this receptor polymerises in vitro in the presence of thiol compounds (dithiothreitol), a process inhibited by EGTA or the transglutaminase inhibitor cystamine. Polymerisation is accompanied by receptor inactivation ([^{3}H]acetylcholine binding). A transglutaminase could be extracted from the receptor-rich membranes which may be involved in receptor regulation.

2. MATERIALS AND METHODS

2.1. *Materials*

T. californica electric tissue was purchased N_2-frozen from Pacific Bio-Marine Laboratories (Venice, CA). The tissue was stored at −75°C. [^{3}H]Putrescine (28.8 Ci/mmol) was purchased from New England Nuclear (Dreieich). [^{3}H]Acetylcholine (67 mCi/mmol) was purchased from Amersham (Braunschweig). Dimethylcasein was a product of Sigma (München). Cystamine was purchased from Sigma (München). All other reagents were of the highest commercially available purity.

2.2. *Receptor-rich membranes*

Membranes rich in nAChR were prepared from *T. californica* (or *T. marmorata* which gave the same results) according to [4] with a sucrose density gradient centrifugation (25–50% sucrose in H_2O) as the final purification step. The membranes usually contained 2500–3500 nmol/g ^{125}I-α-bungarotoxin-binding sites.

2.3. *Transglutaminase assay*

Transglutaminase activity was determined according to [5] measuring the incorporation of

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[^{3}H]putrescine into dimethylcasein. The assay was performed in 50 mM Tris-HCl, pH 8.1.

2.4. *Acetylcholine binding*

Binding of [^{3}H]acetylcholine to receptor-rich membranes was determined by ultracentrifugation. The centrifugation mixture contained 0.015 mg/ml membrane protein in Ringer's solution (pH 7.4). To inactivate traces of acetylcholinesterase the receptor-rich membranes were pretreated for 30 min with 10^{-4} M eserine. Centrifugation was performed for 10 min with an airfuge at room temperature. [^{3}H]Acetylcholine concentration (Amersham, 67 mCi/mmol) was varied between 3.6×10^{-8} and 3.6×10^{-7} M. 50 μl of the supernatant were removed after centrifugation for [^{3}H]acetylcholine determination (liquid scintillation counting).

2.5. *Receptor polymerisation and inactivation*

All further experimental details are contained in the figure legends documenting the respective experiment.

3. RESULTS

Incubation of receptor-rich membranes with 1 mM dithiothreitol (DTE) at 30°C resulted in a time-dependent polymerisation of the membrane proteins present as evidenced by SDS-polyacrylamide gel electrophoresis (fig.1). After 1 h incubation a significant part of the membrane protein did not enter the 10% acrylamide gel and after prolonged incubation increasing amounts did not even enter the 3% sample gel. All proteins were affected: not only the 4 polypeptide chains α, β, γ and δ of the nAChR, but also the peripheral 43 kDa protein [6], the minor components visible on the gel and the protein with an apparent molecular mass of 100 kDa presumed to be an ATPase located in non-receptor membranes present as an impurity in AChR-rich membrane preparations [7].

Very little protein cross-linking was observed in the absence of DTE, even after 24 h incubation at 30°C (fig.2A). The complete polymerisation observed in the presence of 0.8 mM DTE (fig.2B) could be prevented by 0.8 mM cystamine (fig.2C), and EDTA (fig.2D). The EDTA effect could be partially overcome by a 10-fold excess of Ca^{2+} (not shown).

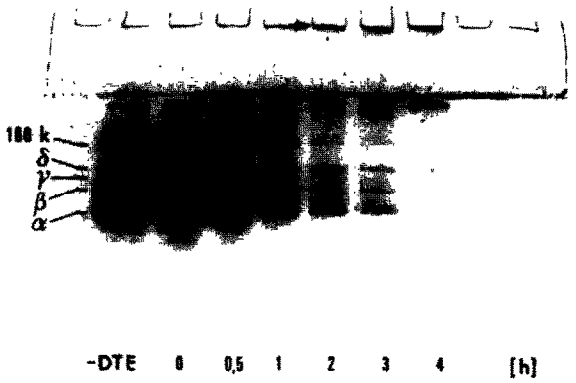

Fig.1. Time-dependent polymerisation of membrane proteins in an AChR-rich membrane preparation from *T. californica* electric tissue. A total of 6 ml incubation mixture contained: 0.03 mg/ml AChR-rich membranes, 50 mM Tris-HCl, pH 8.4, 2 mM DTE. Incubation was at 30°C. At the time indicated 1 ml aliquots were removed, centrifuged for 10 min at 10000 × *g* and the pellet dissolved in 0.1 ml of 1% SDS in sample gel buffer [13]. SDS-polyacrylamide gel electrophoresis was performed on 10% polyacrylamide gel, with a 3% sample gel [13]; α, α', β, γ, δ are the bands representing AChR polypeptide chains. 100 K represents probably an ATPase. The rapid disappearance of the γ-band (cf. control track showing AChR incubated without DTE with track 0 h incubation time in the presence of 2 mM DTE) is probably due to Ca^{2+}/SH-dependent proteolysis by a membrane-bound protease [14]. The arrows indicate protein not entering the lower gel or the upper gel, respectively.

Since Ca^{2+}- and thiol-dependent protein polymerisation which can be inhibited by cystamine hinted at the involvement of a transglutaminase, we looked for this enzyme activity [5]. Incorporation of [^{3}H]putrescine into *N,N*-dimethylcasein was catalysed by receptor-rich membranes (fig.3) and by membranes solubilized in 2% Triton X-100 (not shown, activity was lower in Triton). The catalysis showed the characteristics of a transglutaminase: it required the presence of DTE and was inhibited by EGTA and by cystamine. [^{3}H]Putrescine could not be incorporated into the intact receptor-rich membranes, nor into Triton-solubilized AChR. Neither the AChR-polymerisation nor the transglutaminase assay with the exogenous substrates could be affected by cholinergic agonists (0.1 mM carbamoylcholine) or antagonists (0.1 mM hexamethonium).

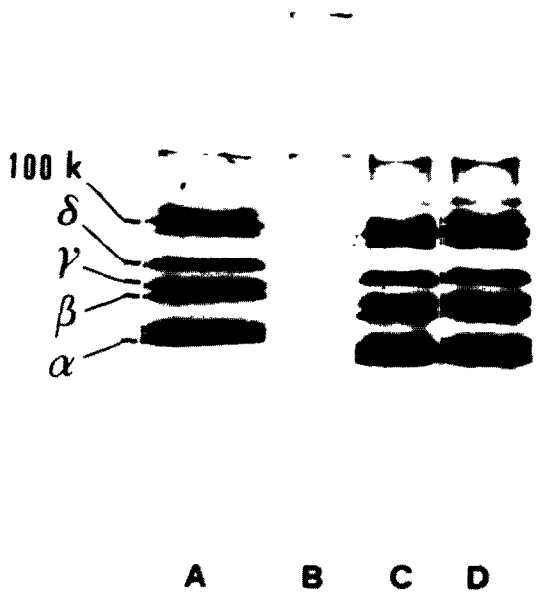

Fig.2. Membrane protein polymerisation in the presence of various effectors. AChR-rich membranes from *T. marmorata* electric tissue in 50 mM Tris-HCl, pH 8.4, on SDS-polyacrylamide gels [13] after 3 h incubation without effector (A), with 0.8 mM DTE (B), with 0.8 mM DTE and 0.8 mM cystamine (C), with 0.8 mM DTE and 0.8 mM EDTA (D). Cystamine (C) and EDTA (D) are effective inhibitors of the DTE-dependent protein polymerisation; the membrane proteins show electrophoresis patterns comparable to the control (A). Protein concentration in all incubation mixtures was 0.05 mg/ml. For all other experimental conditions see legend to fig.1. 20 μg protein were applied to each electrophoresis track.

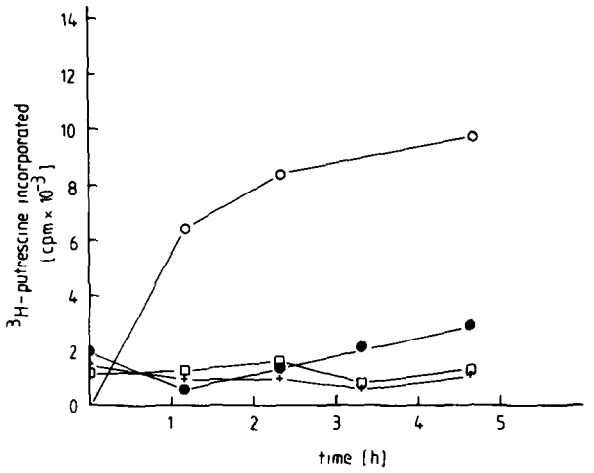

Fig.3. Transglutaminase activity in AChR-rich membranes, as measured by [^{3}H]putrescine incorporation into dimethylcasein [5]. Membranes from *T. californica* electric tissue incubated with [^{3}H]putrescine and dimethylcasein in the absence of any effector (○—○) or presence of 10 mM EGTA (+—+). Complete inhibition of transglutaminase activity was also achieved by 1 mM cystamine (not shown). A small but significant transglutaminase activity is present in the dimethylcasein substrate (●—●); these values, obtained by incubating [^{3}H]putrescine with dimethylcasein in the absence of AChR-rich membranes, have been subtracted as blanks. In the presence of EGTA this transglutaminase activity of dimethylcasein disappears (□—□).

The transglutaminase activity can be separated from the AChR. After solubilization of receptor-rich membranes with 4% cholate most of the enzyme activity together with the AChR remained in the supernatant after 30 min centrifugation at 45000 × *g*. Ion-exchange chromatography of this detergent extract (DE 52, 50 mM Tris-HCl, pH 7.4, 1% cholate, 0–0.5 M NaCl) yielded one peak of AChR free of transglutaminase activity and another of transglutaminase activity free of AChR. This indicates that the enzymatic activity and receptor cross-linking/inactivation is not an intrinsic property of the receptor protein but resides in a protein which can be extracted from the membranes.

Incubation of receptor-rich membranes under conditions favourable for cross-linking (DTE, no EGTA) causes rapid inhibition of [^{3}H]acetylcholine binding to the membrane-bound receptor (fig.4). This effect is distinguishable from the lowering of AChR affinity by DTE observed in [8] which had been investigated in the presence of EDTA preventing polymerisation.

4. DISCUSSION

Ca^{2+} is a major regulator of synaptic transmission. Besides its participation in transmitter release [9] it has been shown to modulate postsynaptic responses. The nicotinic acetylcholine receptor is desensitized more rapidly in the presence of Ca^{2+} [10] and here we show that it inactivates AChR irreversibly. This may be a further step in the down-regulation of this receptor. The Ca^{2+} involved may come from the presynaptic side where it is released together with the transmitter and from the

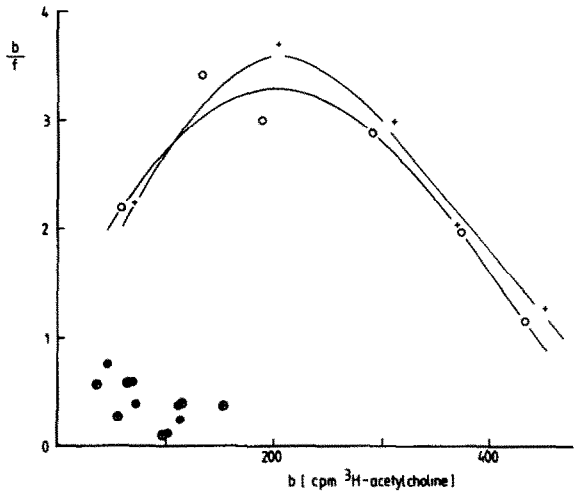

Fig.4. Inhibition of [^{3}H]acetylcholine binding to AChR-rich membranes by Ca^{2+}/SH-dependent protein polymerisation. Scatchard plot of binding data. [^{3}H]Acetylcholine binding to membranes after 1 h (⊕—⊕) and 3 h (●—●) incubation in the presence of 1 mM DTE. Control: incubation in absence of DTE for 1 h (+—+) and 3 h (○—○) prior to the binding experiment. The convex Scatchard plots are due to positive cooperativity of acetylcholine binding as observed before by several authors.

postsynaptic membrane itself where it is present in large amounts and from where it is released by the agonist [11].

Both these Ca^{2+} sources are extracellular with respect to the postsynaptic membrane. However, the ion channel regulated by the AChR is also permeable for Ca^{2+} [12]. Therefore, the transglutaminase-catalysed cross-linking of the receptor molecules could take place on either side of the postsynaptic membrane. At present we do not know the orientation of the enzyme or the site of the receptor side-chains involved.

Of course one has to take into account that the extracellular Ca^{2+} concentration is high (about 2 mM) and probably not affected very much by additional Ca^{2+} contributed by various steps of chemical transmission. Nevertheless, one cannot exclude that locally significant concentration changes take place.

At present we are investigating whether the Ca^{2+}-dependent cross-linking and inactivation of AChR also occurs in cells, e.g. in myoblasts. We believe the reaction described here could be involved in synapse plasticity in vivo.

Furthermore, the observed AChR inactivation is of practical importance. Even without addition of Ca^{2+} and DTE very slow receptor polymerisation and inactivation occur. For experiments of prolonged duration like crystallization attempts [12] this might be a source of problems or artifacts.

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