FACTORS REGULATING THE SUSCEPTIBILITY OF THE ACETYLCHOLINE RECEPTOR PROTEIN TO HEAT INACTIVATION

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1. Introduction

It is now well established that, in skeletal muscle, the acetylcholine receptor (AChR) which is localized at the endplate (junctional AChR) differs from the receptor distributed outside the endplate (extra-junctional AChR) by: isoelectric point [1], mean channel open time [2–4], immunological reactivity [5,6] and metabolic stability. The extrajunctional AChR is degraded in vivo with a half-time of ~20 h, whereas the degradation of the junctional AChR occurs ⩾10-times slower [7–9]. At the molecular level, these differences might be accounted for either by different primary structures, or by the existence of several forms of the AChR which would derive from each other, for instance, as a result of either a covalent modification [10] or of an association with different membrane components (reviewed [11]). In order to investigate this question, we have been concerned in the past few years with the factors, or conditions, which may lead to a modification of the structural properties of the AChR protein in the membrane, using the AChR from fish electric organ as a model system [12–14].

The AChR-rich membranes prepared from the electric organ of *Torpedo marmorata* contain two major polypeptide chains of app. mol. wt 40 000 (40 k) and 43 000 (43 k) and two minor ones of app. mol. wt 50 000 (50 k) and 66 000 (66 k) [15]. Unambiguously, the 40 k chain carries the acetylcholine binding site. The significance of the 43 k chain is still rather unclear. This polypeptide can be removed from the AChR-rich membranes by pH 11 treatment without changing most of the known functional properties of the AChR protein [16]. It does not comigrate with *Torpedo* actin and its amino acid composition does not resemble that of any known cytoskeletal protein [17,18] but [19].

Here we report experiments carried out on the susceptibility to heat inactivation of AChR from *T. marmorata.* They reveal that this property may change as a consequence of intrinsic or extrinsic modifications of the membrane-bound AChR and therefore can be used as a criterion to reveal minor differences in the structure of the AChR molecule or of its close environment.

2. Materials and methods

2.1. *AChR-rich membrane fragments from* Torpedo marmorata: *Treatment by alkaline and acid pH*

AChR-rich membrane fragments were prepared essentially by the method in [15] as modified [20]. The alkaline pH treatment of the membranes was done as follows: the membrane fragments resuspended in distilled water at 0.25 mg protein/ml were supplemented with 0.1 M NaOH to the desired pH, allowed to stand at room temperature for 60 min, centrifuged in a Beckman airfuge for 2 min and the pellet resuspended in *Torpedo* physiological saline solution. The supernatants were neutralized with 0.1 M HCl and used as the source of the 43 k polypeptide. The acid pH treatment of the membranes was performed with a 0.5 mg protein/ml suspension of membrane fragments and 0.1 M acetate or Tris–HCl buffer at 0°C for 10 h; membranes were then washed as above.

2.2. *Labelling of the 43 k polypeptide with* ^{35}S

The 43 k polypeptide was labelled with the diazonium salt of [^{35}S]sulfanilic acid, synthesized by the procedure in [21]. The supernatant resulting from the pH 11 treatment of receptor enriched membranes containing about 0.06 mg 43 k protein/ml was adjusted to pH 9.1 with 0.05 M NaCl–0.025 M borate buffer, added to tubes containing the diazonium salt of sulfanilic acid giving 7×10^{-6} M final conc., allowed to stand at 4°C for 10 min, then dialyzed extensively against reaction buffer.

2.3. *Enzymatic treatments of the AChR*

AChR-rich membranes were solubilized with 1% Triton X-100 at 0.5 mg protein/ml. The solution was centrifuged and the supernatant adjusted to pH 7.8 with 0.1 M Tris–HCl, and incubated with 1 unit/ml of alkaline phosphatase from *Escherichia coli* (type II-R, Sigma) at 4°C for 1 h. The treatment with 200 units/ml of phospholipase A_2 from porcine pancreas was done under the same conditions except that the incubation was carried out at 37°C and that 2 mM $CaCl_2$ was added to the incubation mixture. The treatment with a mixture of exoglycosidases and endoglycosidases from *Streptococcus pneumoniae* [22] was performed at 37°C and for 1 h. The enzymes used here did not contain detectable amount of protease activity.

3. Results and discussion

3.1. *Alkaline treatment increases the susceptibility to heat inactivation of the membrane-bound AChR*

As reported [16] alkaline treatment of AChR-rich membranes caused the release of the 43 k polypeptide from the membrane (fig.1B,C). This release took place, as a sharp transition, at pH 10.4–10.8 under conditions where little inactivation of the AChR sites, as determined by following the ability to bind ^{125}I-labelled α-bungarotoxin, occurred. It is now well established that the alkaline treatment does not significantly alter the allosteric transitions of the AChR protein and their regulation by non-competitive blocking agents such as local anesthetics [16,23]. In order to test if other molecular properties of the receptor protein were modified by the treatment, the extent of heat inactivation of the AChR

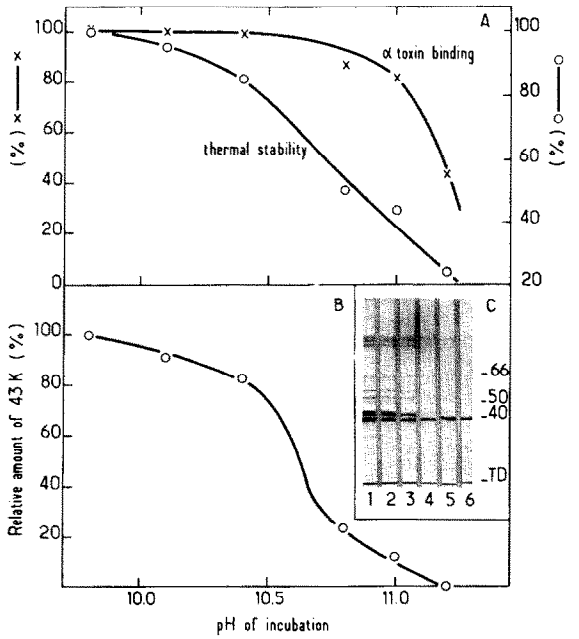

Fig.1. Effect of alkaline pH treatment of AChR-rich membranes on the release of the 43 k polypeptide, on ^{125}I-labelled α-bungarotoxin binding and on the thermal stability of the AChR. (A) AChR-rich membranes were exposed to the indicated pH values in section 2 and centrifuged. The ^{125}I-labelled α-bungarotoxin binding and heat inactivation of the AChR were determined on the resuspended pellet. ^{125}I-labelled α-bungarotoxin binding was determined by measuring the retention of the ^{125}I-labelled α-bungarotoxin–membrane complex on Millipore Filters (HA-0.45) and expressed as the percentage of the value for untreated membranes. The heat inactivation was determined by following the residual ^{125}I-labelled α-bungarotoxin binding of the membranes after 7 min incubation at 60°C, and is expressed as the percentage of the value for untreated membranes. (B) Release of the 43 k polypeptide from AChR-rich membranes as a function of pH. The quantification of the 43 k polypeptide was performed by weighing the tracing paper of a scan of Coomassie stained SDS–polyacrylamide gels presented in (C). (C) Protein pattern of the membranes treated at: (1) pH 9.7; (2) pH 10.1; (3) pH 10.4; (4) pH 10.8; (5) pH 11.0; (6) pH 11.2.

protein at 60°C was determined (see legend of fig.1) after alkaline treatment and neutralization of the membrane suspension. Figure 1A shows that, as a consequence of the alkaline treatment, the loss of ^{125}I-labelled α-bungarotoxin binding sites at 60°C increased markedly and that the enhanced susceptibility to inactivation grossly paralleled the release of the

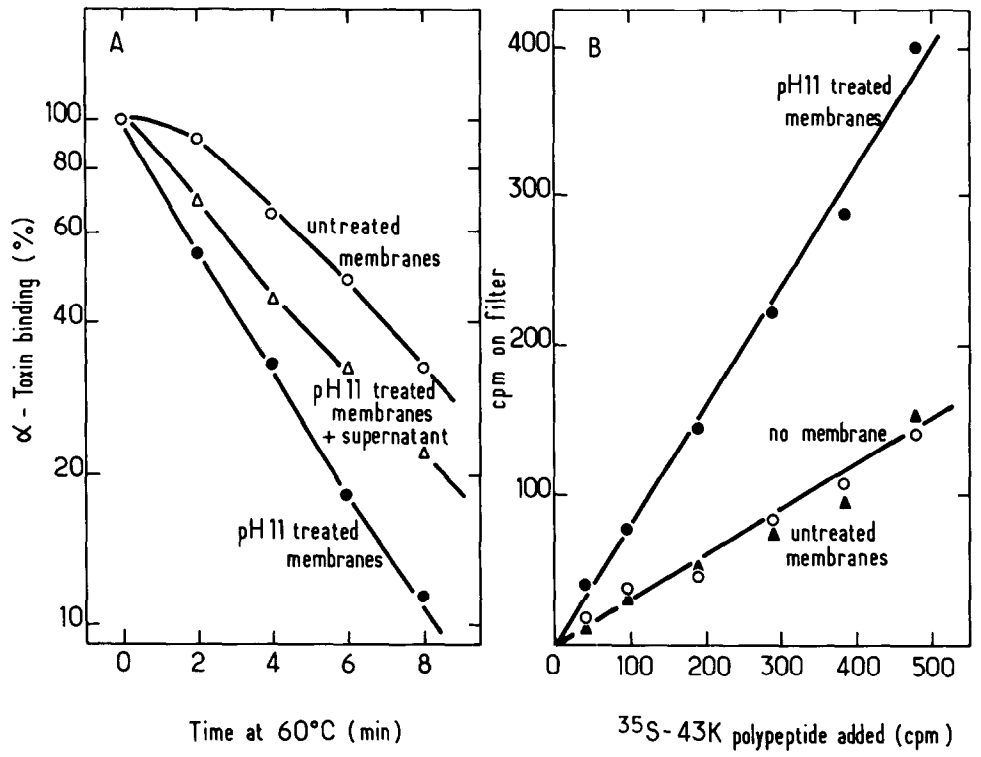

Fig.2. Reassociation of the 43 k polypeptide to alkaline-treated AChR-rich membranes. (A) AChR-rich membranes treated at pH 11 as in section 2 were centrifuged, suspended either in 10^{-3} M NaOH (pH 11) (●——●) or in its own supernatant (△——△) containing the 43 k polypeptide, and neutralized with 0.1 M HCl. After 10 h, the membranes were spun down, resuspended in *Torpedo* physiological saline solution and the time course of heat inactivation of α-bungarotoxin binding determined as in fig.1. The time course of heat inactivation of untreated membranes, is also presented (○——○). (B) Various amounts of ^{35}S-labelled 43 k polypeptide (~250 cpm/μg) were added to test tubes containing either alkaline-treated membranes (●——●), or untreated control membranes (○——○), both containing 40 pmol α-bungarotoxin sites, or to tubes without membranes (▲——▲). Tubes were incubated at room temperature for 10 h. Membrane-associated radioactivity was determined by filtering the samples through Millipore filters (HA-0.45), drying the filters, and counting in Toluene–PPO scintillator with a liquid scintillation counter.

43 k polypeptide. Conversely, the thermal stability was partially regained when a supernatant containing the 43 k protein was mixed with alkaline-treated membranes, and subsequently neutralized (fig.2A). Under the same conditions the binding of ^{35}S-labelled 43 k polypeptide was shown to occur with the alkaline-treated membranes, but not with native, untreated membranes (fig.2B). The simplest interpretation of this data is that the association of some factor, presumably the 43 k protein, with the AChR-rich membranes stabilizes the AChR protein against heat inactivation.

3.2. *Acid pH treatment increases the susceptibility to heat inactivation of the membrane-bound AChR*

Mild acid pH treatment, contrary to the exposure to alkaline pH, does not significantly modify the polypeptide chain composition of the AChR-rich membranes [24]. Nevertheless, incubation of the AChR-rich membranes at an acid pH, where little, if any, loss of α-toxin binding sites occurred, caused a marked increase in susceptibility to heat inactivation (fig.3). For example, exposure of the AChR-rich membranes at pH 5.5 for 5 days did not result in any significant loss of ^{125}I-labelled α-bungarotoxin

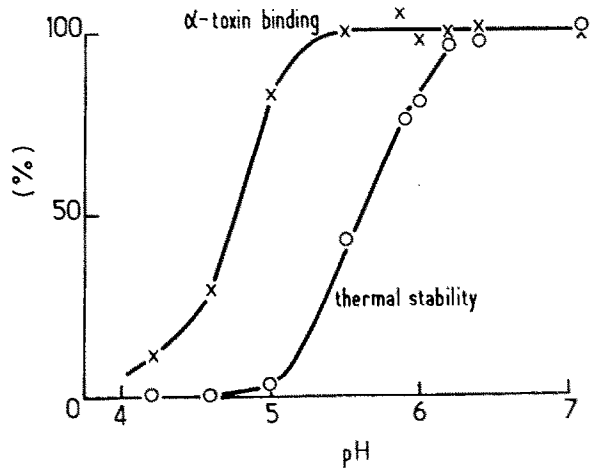

Fig.3. Effect of acid pH treatment of AChR-rich membranes on ^{125}I-labelled α-bungarotoxin binding and on the thermal stability of the AChR. AChR-rich membranes were exposed to the indicated pH as in section 2. ^{125}I-labelled α-bungarotoxin binding and thermal stability of AChR were determined as in fig.1.

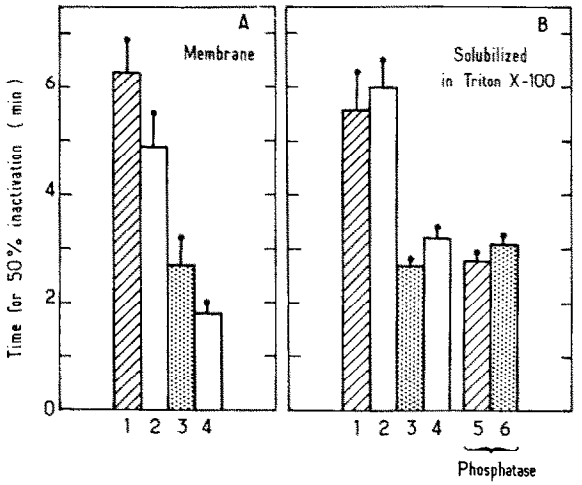

Fig.4. Effect of various treatments of the AChR-rich membranes on the thermal stability of the AChR. (A) The time for a 50% decrease of ^{125}I-labelled α-bungarotoxin binding at 60°C was determined with AChR-rich membranes: (1) without treatment; (2) after treatment at pH 11; (3) after treatment at pH 5.5; (4) after treatment at pH 5.5, then at pH 11. Bar length = standard deviation. (B) The time for 50% decrease of ^{125}I-labelled α-bungarotoxin binding at 49°C was determined by a DEAE filter assay [29] on 1% Triton X-100 solubilized AChR from the same AChR-rich membranes as for: (1) A-1; (2) A-2; (3) A-3; (4) A-4; (5) untreated membranes were solubilized in 1% Triton X-100 and treated with 1 unit/ml of alkaline phosphatase as in section 2; (6) pH 5.5-treated membranes were solubilized in 1% Triton X-100 and treated with 1 unit/ml of alkaline phosphatase as in section 2.

binding, but the rate of inactivation of the AChR protein at 60°C increased by a factor of ~5. When alkaline-treated membranes were submitted to the acid treatment, an increased susceptibility to heat inactivation was still noticed (fig.4A). In other words, the effects of alkaline and acid treatments on the thermal stability of the membrane-bound AChR were additive and therefore did not affect the same membrane target.

3.3. *The increased susceptibility to heat inactivation caused by exposure of the AChR-rich membranes to acid pH remains after solubilization of the membrane by Triton X-100*

The effects of the considered pH treatments may be interpreted on the basis of a change of the local environment of the receptor protein in the membrane and (or) of an intrinsic modification of the receptor molecule. To distinguish between these alternatives the thermal stability of the receptor protein was investigated after dissolution by Triton X-100 of the acid or alkaline-treated membranes. As shown in fig.4B the receptor protein solubilized from alkaline-treated membranes exhibited the same thermal stability as the receptor protein extracted from native membranes. This was not unexpected, since Triton X-100 is known to dissociate the 43 k and the AChR protein [17]. On the other hand, in Triton X-100 extracts of acid pH-treated membranes, the AChR was still more susceptible to heat inactivation than in similar extracts of native membranes. These results are consistent with the interpretation that the effect of alkaline-treatment results from the elimination of the 43 k polypeptide rather than from an intrinsic modification of the receptor protein. In fact, this last possibility must be considered in the case of the acid pH treatment.

3.4. *Effect of various enzymatic treatments on the susceptibility of the AChR to heat inactivation*

Since lipids and polysaccharides might still be associated with the AChR after dissolution by Triton

X-100, the effect of enzymes known to attack these molecules was tested on the Triton X-100 solubilized AChR-receptor. Though phospholipase A_2 treatment was reported to alter the binding properties of the AChR protein [25], it did not cause any significant change in the thermal stability of the AChR. A mixture of exo- and endoglycosidases, which was effective in modifying the electrophoretic mobility of the 66 k polypeptide, did not affect the thermal stability of the AChR. On the other hand, treatment of the Triton X-100 extracted AChR with *Escherichia coli* alkaline phosphatase, which is known to dephosphorylate phosphoproteins [26,27], increased its susceptibility to heat inactivation (fig.4B). Interestingly, the phosphatase was no longer effective when the AChR was extracted from acid pH treated membranes (fig.4B). This suggests that the exposure to acid pH and the phosphatase treatment have the same effect, i.e., cause a dephosphorylation of a phosphopeptide(s) present in the preparation of soluble AChR protein and, as a consequence, increase the susceptibility to heat inactivation of the AChR protein.

4. Conclusion

These results demonstrate that factors, or conditions, which do not significantly modify the binding of α-bungarotoxin to the membrane-bound AChR may modify its thermal stability. Among them, one may distinguish between 'extrinsic' factors, like the 43 k protein, present in the close environment, or directly associated with the AChR in the membrane, and 'intrinsic' modifications of the receptor molecule such as a phosphorylation [14,28].

These experiments further show that the determination of the thermal stability of the AChR in vitro might be a useful method to investigate the discrete differences of structure existing between extra- and subsynaptic AChR and to study their evolution during synapse formation.

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