Functional characterization of mongoose nicotinic acetylcholine receptor α-subunit: resistance to α-bungarotoxin and high sensitivity to acetylcholine

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Abstract The mongoose is resistant to snake neurotoxins. The mongoose muscle nicotinic acetylcholine receptor (AChR) α-subunit contains a number of mutations in the ligand-binding domain and exhibits poor binding of α-bungarotoxin (α-BTX). We characterized the functional properties of a hybrid (α-mongoose/βγδ-rat) AChR. Hybrid AChRs, expressed in *Xenopus* oocytes, respond to acetylcholine with depolarizing current, the mean maximal amplitude of which was greater than that mediated by the rat AChR. The IC_{50} of α-BTX to the hybrid AChR was 200-fold greater than that of the rat, suggesting much lower affinity for the toxin. Hybrid AChRs exhibited an apparent higher rate of desensitization and higher affinity for ACh (EC_{50} 1.3 vs. 23.3 μM for the rat AChR). Hence, changes in the ligand-binding domain of AChR not only affect the binding properties of the receptor, but also result in marked changes in the characteristics of the current.

Key words: Acetylcholine receptor; Mongoose; α-Bungarotoxin; Acetylcholine; *Xenopus* oocyte

1. Introduction

The muscle acetylcholine receptor (AChR) is a ligand-gated ion channel that mediates synaptic transmission at the neuromuscular junction. When AChR is activated by binding ACh, the pore opens to allow a transient depolarizing current due to the entry of mainly Na^+ ions. The AChR is a hetero-pentamer composed of four subunits exhibiting a molar stoichiometry of $\alpha_2\beta\gamma\delta$ [1,2]. The α-subunit contains the ligand-binding site. Snake α-toxins (e.g. α-bungarotoxin, α-BTX), potent antagonists of the nicotinic AChRs, bind to isolated α-subunits, their fragments, or peptides that include the binding domain [3–11]. The major domain involved in the binding of ACh and α-BTX is in close proximity to two tandem cysteine residues, 192–193, which are conserved in all α-subunits of the known nicotinic receptors.

The mongoose, snakes and hedgehogs are resistant to snake α-toxins. To investigate the molecular basis of this resistance, we have previously cloned the binding domain of the AChR α-subunit of these species [7,8,10], and recently the entire mongoose α-subunit [9]. We have shown that the mongoose α-subunit (α_M) contains four substitutions within the binding domain (at positions 187, 189, 194, 197) which markedly reduce the α_M ability to bind α-BTX in vitro [7–9,11]. Two additional substitutions (at positions 112 and 153) were identified as well [7,9].

To complement these molecular studies, we have expressed the α_M together with the β-, γ-, and δ-subunits of the rat, as well as the entire rat AChR, in *Xenopus* oocytes. The functional characteristics of the hybrid receptor were compared to those of the rat AChR. Here, we demonstrate that the hybrid receptor possesses higher sensitivity to ACh, markedly lower affinity for α-BTX, greater mean maximal current amplitude and a greater apparent desensitization.

2. Materials and methods

2.1. Subcloning of the mongoose AChR α-subunit

Rat AChR subunit cDNA clones inserted in a derivative of the pSP64T vector were kindly provided by Dr. V. Witzemann (Max Planck Institute, Heidelberg) [12]. The cDNAs inserted in this vector are flanked by the 3′ and 5′ untranslated regions of the *Xenopus* α-globin gene. The mongoose AChR α-subunit cDNA was subcloned into the modified pSP64T vector by PCR. The entire subcloned insert was sequenced using the DNA sequencing facility at the Weizmann Institute of Science.

2.2. cRNA synthesis

The AChR subunit cDNAs that were subcloned in the pSP64T vector were transcribed in vitro, essentially as described by Witzemann et al. [12]. cRNA quality was monitored by 1% agarose gel analysis and its concentration was determined by UV spectrophotometry.

2.3. Functional expression of AChRs in oocytes

Oocytes were dissected from hypothermia-anesthetized *Xenopus laevis* frogs, defolliculated for 2–3 h with 2 mg/ml collagenase (type IA, Sigma) in Ca^{2+}-free modified frog Ringer's solution (in mM, 107 NaCl, 2.5 KCl, 1 $MgCl_2$, 1 $NaHPO_4$, 5 HEPES, pH 7.6) and then transferred to the same solution containing 1 mM Ca^{2+}, 10 mg/l penicillin G, 10 mg/l streptomycin sulfate, 2.5 mM pyruvate, and 0.5 mM theophylline [13]. Stage V oocytes were injected with a mixture containing 4 ng of rat β-, γ-, δ-subunits and 8 ng of either rat or mongoose α-subunit cRNA. Oocytes were maintained for 3 days at 19–21°C in the modified Ringer's solution with a daily change. Responses were recorded under a two-electrode voltage clamp at a holding potential of −60 mV [14]. Recordings were performed either in the modified Ringer's solution without additions or in ND96 solution (96 mM NaCl, 2 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM HEPES, pH 7.4). We measured responses to direct bath application of 1 ml solution of different ACh concentrations in the presence of 1 μM atropine [13]. For the α-BTX inhibition curve experiments, oocytes were incubated in different concentrations of the toxin for 20 min prior to recording. These recordings were done at approximately the calculated EC_{50} values, 10 and 1 μM ACh for the rat and the mongoose/rat hybrid AChR, respectively.

2.4. Analysis of data

All results are presented as means ± S.E.M. of the indicated number

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of experiments. n designates the number of oocytes assayed for each condition, while N is the number of different experiments performed. Values for I_{max}, EC_{50}, IC_{50} and Hill coefficients were obtained using the Grafit software with simple weighting.

3. Results

cRNAs encoding the α_M and the rat βγδ-subunits, or cRNAs encoding the rat αβγδ-subunits were expressed in *Xenopus* oocytes. Whole cell current recordings under two-electrode voltage clamp confirmed the functional expression of both the hybrid and the rat AChRs. Dose response to ACh demonstrated that the amplitudes of the currents evoked by the stimulation of the hybrid AChRs were greater than those observed for the rat AChRs at all ACh concentrations (Fig. 1, Table 1). Moreover, the hybrid AChR exhibited higher apparent sensitivity to ACh than the rat receptor (EC_{50}s 1.3 ± 0.3 vs. 23.3 ± 2.1 μM, respectively). Tomaselli et al. [15] found similar values (11.0 ± 0.7 μM) for mouse AChR expressed in oocytes.

To compare the interaction of α-BTX with the hybrid and the rat AChRs, we measured the effect of different concentrations of α-BTX on ACh-evoked currents. In order to compare the inhibition by α-BTX at approximately the EC_{50} for ACh, the concentrations of ACh used in these experiments were 1 and 10 μM for the hybrid and the rat AChR, respectively. Under these conditions, the IC_{50} of α-BTX for the hybrid AChRs was 80.4 ± 15.7 vs. 0.4 ± 0.1 nM for the rat AChR (Fig. 2, Table 1). Thus, the apparent affinity of α-BTX for the hybrid receptor was 200-fold lower than that for the rat AChR.

We have compared the kinetic characteristics of responses of the wild type rat AChR with that of the mongoose/rat hybrid. We measured the half-time of current activation ($t_{1/2on}$), the half-time of inactivation ($t_{1/2off}$) and the time to peak (ttp). The $t_{1/2on}$ and ttp values were similar for both receptors. There was a significant difference, however, in the kinetics of current decay. The decay of the current mediated by the hybrid receptor was faster than that of the rat ($t_{1/2off}$ values of 6.5 ± 0.8 vs. 9.4 ± 1.5 s, $n = 7$ and 9, respectively, $P \leq 0.05$). To test whether the dissociation of the agonist was involved in the kinetics of decay, we conducted similar experiments, except that ACh was added to the bath during continuous and rapid perfusion. Under these conditions, the agonist was washed out very rapidly after its addition. Contrary to the prediction that the rapid removal of the agonist will accelerate the decay of the response, this method yielded slightly longer (by approximately 1.5 s) $t_{1/2off}$ values for the hybrid and the wild type AChR (7.9 ± 0.4 vs. 10.9 ± 0.6 s, $n = 42$ and 39, respectively, $P \leq 0.05$), suggesting rapid desensitization in the continuous presence of the agonist. The ttp values increased for both receptors significantly, suggesting that rapid desensitization shortened the ttp under the no-wash condition (5.0 ± 0.5 to 10.9 ± 0.6 s and 5.2 ± 1.1 to 8.1 ± 1.2 s for the hybrid and the rat AChRs, respectively). The increase was greater for the hybrid AChRs. These results are presented in Table 2. Assuming that the kinetics of the current reflect the desensitization of the AChR, it appears that the mongoose/rat hybrid is desensitized more rapidly.

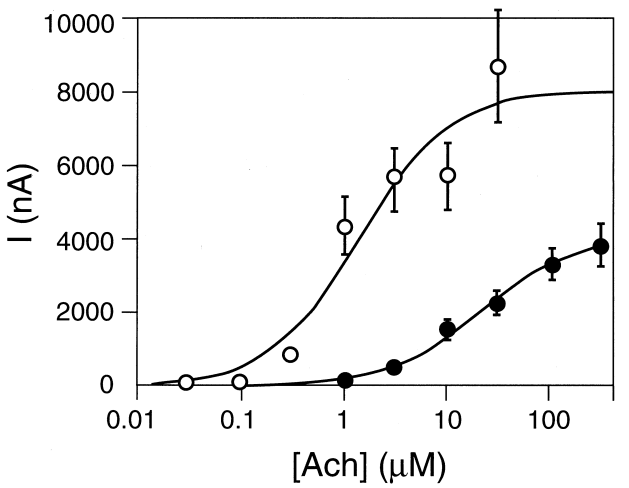

Fig. 1. ACh dose-response curves for mongoose/rat hybrid receptor and for rat AChR. Oocytes were injected with mongoose α- together with rat β-, γ-, and δ-subunit cRNAs (mongoose/rat hybrid) or with rat α-, β-, γ-, and δ-subunit cRNAs and assayed after 3 days. ACh at the indicated concentrations was added and the peak current at a holding potential of 60 mV was recorded. The points represent the mean ± S.E.M. ($n > 10$, $N > 4$) current amplitudes for the mongoose/rat hybrid (○) and rat (●) AChR, respectively. Calculated Hill coefficient values were 0.93 and 1.02 for the hybrid and the rat AChR-mediated responses, respectively.

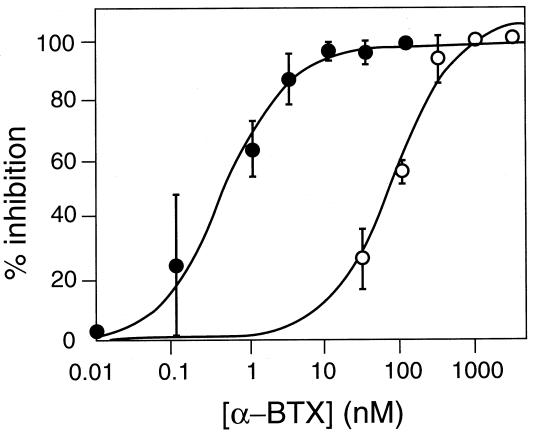

Fig. 2. Inhibition of AChR current by α-BTX in oocytes. Oocytes were injected with AChR subunit cRNAs from either rat or mongoose/rat hybrid and assayed after 3 days. Cells were exposed for 20 min to the indicated concentrations of α-BTX. Responses to 10 and 1 μM ACh (for the rat and the hybrid receptor, respectively) were measured. The results are presented as mean ± S.E.M. ($n > 10$, $N > 4$) percent inhibition when compared to controls without α-BTX. Mongoose/rat hybrid AChR (○); rat (●).

4. Discussion

The present study was aimed at investigating the effects of the molecular changes in the α_M subunit on the functional properties of the entire receptor. Co-expression of the α_M subunit with the other rat subunits in oocytes resulted in a functional AChR and could be compared to the rat AChR expressed in the same system. The mongoose/rat hybrid receptor channels exhibited in oocytes a higher sensitivity for ACh, higher current amplitudes, lower apparent affinity for α-BTX, similar $t_{1/2on}$ and ttp and markedly faster rate of desensitization than the wild type rat AChR. Moreover, under the condition of rapid ACh washout, the slight increase in $t_{1/2off}$

Table 1
Response characteristics of oocytes expressing AChRs

cRNA injected	EC_{50} (μM)	I_{max} (μA)	IC_{50} (nM)
$\alpha_M\beta\gamma\delta_R$	1.3 ± 0.3	8.1 ± 0.9	80.4 ± 15.7
$\alpha\beta\gamma\delta_R$	23.3 ± 2.1	4.0 ± 0.1	0.4 ± 0.1

The calculated values of EC_{50} and I_{max} for ACh, and IC_{50} for α-BTX, were obtained from fitting of data presented in Figs. 1 and 2. All results are presented as means ± S.E.M. obtained from the fitting procedure.

Table 2
Kinetic characteristics of ACh responses in oocytes expressing AChRs

cRNA injected	Without wash			With wash		
	$t_{1/2on}$ (s)	$t_{1/2off}$ (s)	ttp (s)	$t_{1/2on}$ (s)	$t_{1/2off}$ (s)	ttp (s)
$\alpha_M\beta\gamma\delta_R$	1.5 ± 0.0	6.5 ± 0.8*	5.0 ± 0.5**	2.3 ± 0.4	7.9 ± 0.4*	10.9 ± 0.6
$\alpha\beta\gamma\delta_R$	2.2 ± 0.4	9.4 ± 1.5	5.2 ± 1.1**	2.1 ± 0.3	10.9 ± 0.6	8.1 ± 1.2

Oocytes expressing the hybrid or rat AChRs were challenged with 1 mM ACh, either continuously (without wash) or transiently (with wash). The kinetic parameters ($t_{1/2on}$, $t_{1/2off}$ and ttp) were calculated as means ± S.E.M. of results obtained with at least three different oocytes.
*$P \leq 0.05$ when compared to the respective values of rat AChR. **$P \leq 0.05$ when compared to the respective values with wash.

(by 1.5 s) and the significant increase in the ttp (particularly for the hybrid AChR) suggest that prolonged exposure to ACh promotes desensitization. This desensitization appears to be more prominent for the hybrid AChR. The oocyte assay, precluding rapid drug application and utilizing a relatively slow whole cell clamp, is not best suited to investigate rapid desensitization phenomena. This hypothesis should be confirmed in a patch clamp setup.

Assuming, however, that these properties faithfully reflect the characteristics of the native mongoose channel, two questions can be posed. What is the molecular basis for these phenomena and what are the physiological implications to the mongoose?

The sites of altered amino acid residues are not involved in the pore-forming domain of the α-subunit and cannot simply explain the increased currents. It is thus likely that the substitutions in the extracellular segment of the mongoose α-subunit are directly related to the decreased affinity of α-BTX and the increased affinity for ACh, while the increased current represents most probably an indirect effect.

As to the physiological significance of the behavior of the hybrid channel, one can easily speculate that it endows the mongoose with a much better nicotinic system to cope with snake bites. On the one hand, the affinity for α-BTX is markedly decreased, while the affinity for ACh is increased. This gives a much better chance for ACh to compete with α-BTX for the receptor under equilibrium binding conditions. This advantage is even greater under non-equilibrium conditions, the conditions usually observed in the nicotinic synapse, where the average time that ACh is in a bound state is approximately 2 ms. Under these conditions, the rate constant of association of a small molecule will be diffusion controlled, while that of α-BTX will be much less. The increased affinity for ACh will prolong the time of its binding to the receptor and prevent the slower binding of α-BTX. The apparent increased rate of desensitization may be an artifact of the experimental system due to the non-physiological time of exposure to ACh. On the other hand, it may be physiologically significant for the mongoose. Only very rapid kinetic patch clamp experiments may resolve this question.

Sine et al. reported a mutation (Gly-153 → Ser) in the human α-subunit cloned from patients suffering from a slow channel congenital myasthenic syndrome [16]. Introduction of this mutation into the mouse α-subunit resulted in AChR (similar to that of the mongoose/rat hybrid) which exhibited a markedly higher affinity for ACh and greater desensitization. Sine et al. proposed that these properties might underlie the muscle weakness found in myasthenic patients [16]. Since the mongoose is not myasthenic, it is possible that the other modifications at the mongoose α-subunit or in the other subunits, as yet uncloned, modify the channel properties to prevent myasthenia. In conclusion, our findings from studying the mongoose/rat AChR hybrid help explain the mongoose toxin resistance in molecular and functional terms.

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