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Functional properties of nicotinic acetylcholine receptor subunits expressed in various combinations

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The four kinds of subunits of the *Torpedo californica* nicotinic acetylcholine receptor have been produced in various combinations by injecting *Xenopus* oocytes with the corresponding subunit-specific mRNAs synthesized by transcription in vitro of the cloned cDNAs. Functional analysis suggests that association of the α-subunit with either the γ- or the δ-subunit is a prerequisite for generating the conformation necessary for agonist binding. The acetylcholine receptor devoid of either the β-, γ- or δ-subunit exhibits weak channel activity.

Nicotinic acetylcholine receptor; cDNA expression; α-Bungarotoxin binding; Agonist binding; Channel activity; (*Xenopus oocyte*)

1. INTRODUCTION

The nicotinic acetylcholine receptor from *Torpedo* electroplax consists of four kinds of subunits assembled in a molar stoichiometry of $\alpha_2\beta\gamma\delta$ [1–3]. The pentameric complex contains both the binding site for ACh and the ionic channel. Primary structures of the four subunits of the *T.californica* AChR, deduced from cDNA sequences [4–7], exhibit conspicuous amino acid sequence homology, suggesting that these polypeptides are oriented in a pseudosymmetric fashion across the membrane. Electron image analysis has shown that subunits of the AChR lie at pentagonally symmetrical positions around the channel over a large fraction of their length [8]. Because AChR subunits are tightly associated and dissociate only by denaturation, it was difficult to examine the functional roles of the individual subunits. This issue has been facilitated by the use of an expression system in which the formation of functional AChR is directed by the cloned subunit cDNAs [9–14]. Thus it has been shown that at least the δ-subunit is involved in the gating of the channel [12] and that replacement of the γ-subunit by the ϵ-subunit is responsible for the functional alteration of the AChR during muscle development [13]. In the present investigation, *T.californica* AChR subunits in all possible combinations have been produced in *Xenopus* oocytes, and their functional properties have been analysed. The results obtained suggest that either the γ- or the δ-subunit, when combined with the α-subunit, contributes to generating the conformation necessary for agonist binding. They also show that the combination of the α-subunit with any two of the β-, γ- and δ-subunits elicits low channel activity.

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Abbreviations: ACh, acetylcholine; AChR, acetylcholine receptor; α-BTX, α-bungarotoxin

2. MATERIALS AND METHODS

mRNAs specific for the α-, β-, γ- and δ-subunits of the *T. californica* AChR were synthesized by

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transcription in vitro of the respective cDNAs [10,14]. *X. laevis* oocytes were injected with various combinations of the α-, β-, γ- and δ-subunit-specific mRNAs (final concentrations 100, 50, 50 and 50 ng/μl, respectively; average volume injected per oocyte, about 40 nl) and then incubated at 19°C for 2–3 days in modified Barth's medium [15] containing 0.1 mg/ml cefmenoxime (Takeda) and 50 U/ml nystatin (Serva).

For labelling translation products, injected oocytes were incubated for 2 days in the presence of L-[^{35}S]methionine (spec. act. 1000–1330 Ci/mmol; final concentration 1.0 mCi/ml). Cell extracts were prepared from 15–30 oocytes as in [9], except that 10 KIU/ml aprotinin, 20 μM leupeptin, 20 μM antipain and 5 mM *N*-ethylmaleimide were added as protease inhibitors. The AChR subunits were immunoprecipitated as in [9], except that carrier AChR was omitted and that monoclonal antibodies mAb6, mAb111, mAb168 and mAb166 [16], and protein A-Sepharose coated with rabbit anti-rat immunoglobulin G were used. The resulting immunoprecipitates were analysed by electrophoresis on 0.1% SDS/10% polyacrylamide gels [17], followed by fluorography [18]. *T. californica* electroplax AChR was purified by α-toxin affinity chromatography as in [19].

^{125}I-α-bungarotoxin (α-BTX) binding activity in cell extracts, prepared from 20–60 oocytes, was measured by the antibody precipitation assay [9,20] using a mixture of the four subunit-specific monoclonal antibodies mentioned above; incubation was carried out at room temperature for 18 h in the presence of 7 nM ^{125}I-α-BTX (spec. act. 200–250 Ci/mmol). The effects of increasing concentrations of carbamylcholine on the ^{125}I-α-BTX binding activity were examined as in [9]. The ^{125}I-α-BTX binding activity on the cell surface was measured using 10 oocytes per assay as in [12], except that 7 nM toxin was used.

Electrophysiological measurements were performed at 22–24°C in a solution containing 115 mM NaCl, 2.5 mM KCl, 1 mM $MgCl_2$, 10 mM Hepes-NaOH (pH 7.2) and 1 μM atropine. After removal of the follicular cell layer as in [12], oocytes were held in a recording chamber of 0.2 ml volume through which the solution was superfused continuously at a rate of 2.5 ml/min. ACh responses were recorded with a conventional two-micropipette voltage clamp; two pipettes were filled with 3 M potassium acetate. Whole-cell currents activated by bath application of ACh for approx. 25 s were measured at −70 mV membrane potential. For estimating reversal potentials of ACh-activated currents, ACh was applied iontophoretically.

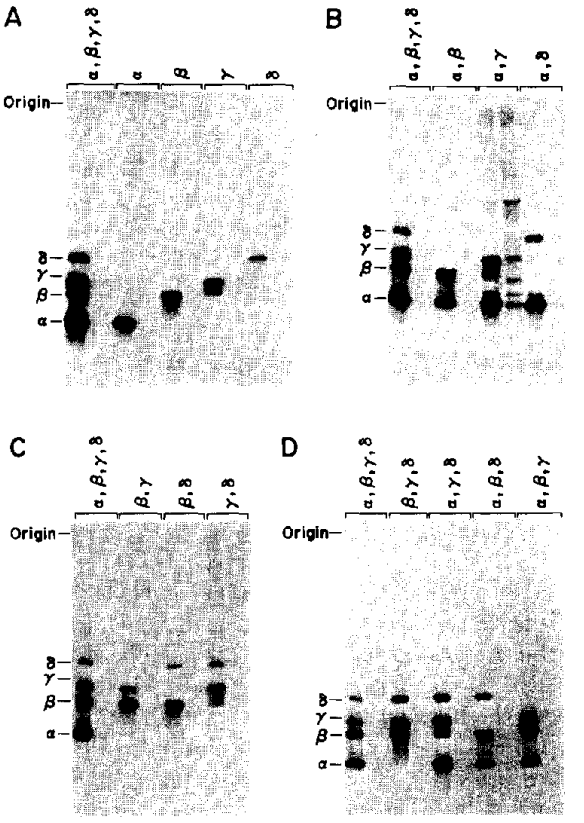

Fig.1 (A–D) Fluorograms of electrophoretic analysis of polypeptides formed by translation of AChR subunit-specific mRNAs in various combinations. For each sample, two lanes bracketed together are presented, of which the left lane shows the immunoprecipitate derived from the oocytes injected with the indicated combination of subunit-specific mRNAs (corresponding to 0.4 oocyte) and the right lane a control immunoprecipitate obtained in the presence of an excess of the purified *T. californica* AChR. The positions to which the α-, β-, γ- and δ-subunits of the purified *T. californica* AChR migrated are indicated. The γ-subunit formed in oocytes migrated faster than that of the purified native AChR; this may be due to a difference in glycosylation or other processing events in vivo or to proteolytic modification during the isolation procedure [1,9,10].

3. RESULTS AND DISCUSSION

The mRNAs specific for the α-, β-, γ- and δ-subunits of the *T. californica* AChR, singly or in various combinations, were injected into *Xenopus* oocytes. The amounts of the individual AChR subunits formed were estimated by labelling the polypeptides with [^{35}S]methionine and isolating them by immunoprecipitation, followed by SDS-polyacrylamide gel electrophoresis. Densitometric scanning of resulting fluorograms (fig.1) showed that individual subunit-specific mRNAs, alone or in any combination, directed the synthesis of corresponding subunits in sufficient amounts (at least about 40% of the amounts observed for the complete AChR synthesized by injecting the four subunit-specific mRNAs) to allow the assay of functional parameters for the AChR. We previously observed that much smaller concentrations of certain subunits were found in *Xenopus* oocytes when one of the four subunits was lacking [9]. The presence of relatively large quantities of the individual subunits observed in the present study, even when only one kind of subunit-specific mRNA was injected, can apparently be ascribed to the use of pure subunit-specific mRNAs instead of poly(A)$^+$ RNA from COS cells transfected with AChR subunit cDNAs [9].

The oocytes were next tested for ^{125}I-α-BTX binding activity (table 1). The cell extracts from oocytes injected with the α-subunit-specific mRNA, alone or in combination with other subunit-specific mRNAs, clearly exhibited α-BTX binding activity, whereas no activity was detected in the cell extracts from oocytes injected with any combination of mRNAs without the α-subunit-specific mRNA. This extends our previous results [9] and is consistent with the finding that the isolated α-subunit alone is capable of binding α-BTX [21–25]. However, the α-BTX binding activi-

Table 1

Functional properties of AChR subunits expressed in various combinations

AChR subunit-specific mRNAs injected	^{125}I-α-BTX binding (fmol per oocyte)		Whole-cell current (nA)	
	Surface	Extract	1 μM ACh	10 μM ACh
$\alpha, \beta, \gamma, \delta$	13.4 ± 3.3 (10)	54.6 ± 29.1 (10)	132 ± 60 (60/60)	3680 ± 1510 (60/60)
α	0.1 ± 0.02 (3)	0.6 ± 0.4 (3)	ND (0/20)	ND (0/20)
β	ND (3)	ND (3)	ND (0/20)	ND (0/20)
γ	ND (3)	ND (3)	ND (0/20)	ND (0/20)
δ	ND (3)	ND (3)	ND (0/20)	ND (0/20)
α, β	0.2 ± 0.04 (3)	0.5 ± 0.07 (3)	ND (0/20)	ND (0/20)
α, γ	0.3 ± 0.1 (3)	14.3 ± 13.4 (3)	ND (0/20)	ND (0/20)
α, δ	0.3 ± 0.1 (3)	4.4 ± 2.7 (3)	ND (0/20)	ND (0/20)
β, γ	ND (3)	ND (3)	ND (0/20)	ND (0/20)
β, δ	ND (3)	ND (3)	ND (0/20)	ND (0/20)
γ, δ	ND (3)	ND (3)	ND (0/20)	ND (0/20)
α, β, γ	4.8 ± 1.2 (6)	37.5 ± 30.0 (6)	13 ± 9 (29/30)	364 ± 233 (30/30)
α, β, δ	0.5 ± 0.5 (6)	5.5 ± 3.0 (6)	0.4 ± 1 (3/30)	16 ± 21 (25/30)
α, γ, δ	0.3 ± 0.2 (6)	18.0 ± 12.1 (6)	ND (0/30)	3 ± 4 (14/30)
β, γ, δ	ND (6)	ND (6)	ND (0/30)	ND (0/30)

The oocytes injected as specified were incubated for 2 days before being tested for ^{125}I-α-BTX binding activity or response to ACh. Data are given as means ± SD. Numbers in parentheses indicate the number of experiments for ^{125}I-α-BTX binding activity or the number of responsive oocytes/number of oocytes tested for ACh response. Our detectable limits were ~0.1 fmol per oocyte for ^{125}I-α-BTX binding activity on the cell surface, ~0.01 fmol per oocyte for ^{125}I-α-BTX binding activity in the cell extract and ~3 nA for ACh response. The means for ACh-activated currents were calculated by taking the value for unresponsive oocytes as zero. ND, not detectable. 1 of the 5 unresponsive oocytes injected with the α-, β- and δ-subunit-specific mRNAs and 10 of the 16 unresponsive oocytes injected with the α-, γ- and δ-subunit-specific mRNAs responded to 100 μM ACh

ty in the cell extract containing the α-subunit alone was much lower than expected from the concentration of the subunit polypeptide present (about 40% of the α-subunit concentration for the complete AChR). Most of the α-subunit molecules formed have apparently not acquired the proper conformation required for α-BTX binding. The presence of an α-subunit species that does not bind α-BTX has been reported [26,27]. The combination of the α-subunit with either the γ- or the δ-subunit or with any two of the β-, γ- and δ-subunits increased the α-BTX binding activity markedly, whereas the combination of the α-subunit with the β-subunit failed to do so. These results suggest that the γ- or the δ-subunit contributes to generating the correct conformation of the α-subunit necessary for α-BTX binding. The apparent binding affinity for ^{125}I-α-BTX of the α-subunit, alone or in combination with one or two other subunits, was lower than that of the complete AChR (see the legend to fig.2). Table 1 also shows that the combination of the α-, β- and γ-subunits elicited a fairly high α-BTX binding activity on the surface of oocytes, whereas other combinations exhibiting α-BTX binding activity in the cell extract evoked only a little toxin binding activity on the cell surface. This suggests that the proper assembly of AChR subunits is important for them to be transported efficiently onto the cell surface.

The effect of carbamylcholine on the ^{125}I-α-BTX binding activity of the α-subunit or of the α-subunit combined with the β-subunit was indistinguishable from that of NaCl as a control (fig.2A). In contrast, the ^{125}I-α-BTX binding activities of the combined α- and γ-subunits and the α- and δ-subunits (fig.2A) as well as of the combined α-, β- and γ-subunits, the α-, β- and δ-subunits and the α-, γ- and δ-subunits (fig.2B) were clearly suppressed by carbamylcholine. The apparent binding affinities for the agonist of these combined subunits were much lower than that of the complete AChR (see the legend to fig.2). These results suggest that association of the α-subunit

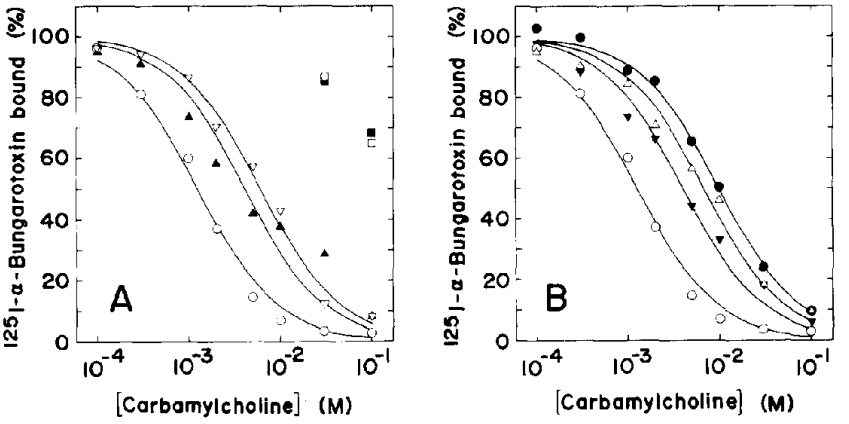

Fig.2. Effect of carbamylcholine on ^{125}I-α-BTX binding activity in extracts from oocytes injected with AChR subunit-specific mRNAs in various combinations. (A) Extracts from oocytes injected with the α-, β-, γ- and δ-subunit-specific mRNAs (○); α-subunit-specific mRNA (□); α- and β-subunit-specific mRNAs (■); α- and γ-subunit-specific mRNAs (▽); or α- and δ-subunit-specific mRNAs (▲). (B) Extracts from oocytes injected with the α-, β-, γ- and δ-subunit-specific mRNAs (○) (the same data as in A); α-, β- and γ-subunit-specific mRNAs (●); α-, β- and δ-subunit-specific mRNAs (△); or α-, γ- and δ-subunit-specific mRNAs (▼). In the presence of 30 mM and 100 mM NaCl, the ^{125}I-α-BTX binding activities of the α-subunit were 80% and 67% and those of the α-subunit combined with the β-subunit were 83% and 61% of the control value, respectively. The theoretical curves have been drawn by nonlinear least-squares analysis according to the equation $y = IC_{50}/(x + IC_{50})$. The apparent dissociation constants for carbamylcholine (K_d) were calculated by the equation $K_d = IC_{50}/(1 + [^{125}\text{I-}\alpha\text{-BTX}]/K_d^*)$, where K_d^* is the apparent dissociation constant for ^{125}I-α-BTX estimated by Scatchard analysis: $\alpha, \beta, \gamma, \delta$, $K_d = 0.03$ mM, $K_d^* = 0.2$ nM; α, $K_d^* = 1.5$ nM; α,β, $K_d^* = 1.7$ nM; α,γ, $K_d = 0.6$ mM, $K_d^* = 0.8$ nM; α,δ, $K_d = 0.5$ mM, $K_d^* = 0.9$nM; α,β,γ, $K_d = 1.2$ mM, $K_d^* = 1.0$ nM; α,β,δ $K_d = 0.8$ mM, $K_d^* = 1.0$ nM; α,γ,δ, $K_d = 0.3$ mM, $K_d^* = 0.6$ nM.

with either the γ- or the δ-subunit is a prerequisite for generating the conformation necessary for agonist binding and that all four subunits are required for forming the agonist binding site with normal affinity. This is consistent with the presence in vivo of an α-subunit species whose binding to α-BTX is not inhibited by cholinergic ligands [28].

ACh responses of oocytes injected with various combinations of AChR subunit-specific mRNAs were measured under voltage clamp at -70 mV membrane potential by application of 1 μM and 10 μM ACh (table 1). All oocytes injected with the α-, β-, γ- and δ-subunit-specific mRNAs responded to ACh. The combination of the α-, β and γ-subunits or of the α-, β- and δ-subunits also yielded functional AChR, although these combinations elicited much smaller currents than did the complete AChR. This confirms our previous observation that 2–3% of the oocytes provided with these subunit combinations responded to ACh [9]; the use of pure subunit-specific mRNAs in the present study markedly increased the number of responsive oocytes. None of the 30 oocytes injected with the α-, γ- and δ-subunit-specific mRNAs showed a detectable current in response to 1 μM ACh, but 14 of them responded to 10 μM ACh. No response was detected for oocytes injected with other combinations of subunit-specific mRNAs even when 100 μM ACh was applied. The ACh responses observed for oocytes implanted with all but the β-, γ- or δ-subunit were blocked by (+)tubocurarine, a specific antagonist for the nicotinic AChR. The reversal potential of the ACh response of oocytes implanted with the combined α-, β- and γ-subunits was -2.8 ± 2.4 mV (mean $\pm$ SD, $n = 11$), being essentially the same as that observed for the complete AChR (-3.9 ± 2.9 mV, $n = 11$). Thus our results show that all four subunits of the *T. californica* AChR are required for the formation of fully responsive AChR, but that the AChR devoid of either the β-, γ- or δ-subunit can function to some extent. It is possible that the missing subunit is replaced by another subunit. The lower AChR channel activities of oocytes implanted with all but the β-, γ- or δ-subunit may at least partly be accounted for by the lower densities of AChR molecules expressed on the cell surface and by the lower affinities for the agonist (see above). The single-channel properties of the AChR may also be affected by the absence of the β-, γ- or δ-subunit. In this context, we have recently observed that the conductance and gating properties of aberrant AChR channels formed in oocytes injected with the bovine α-, β- and δ-subunit-specific mRNAs are markedly different from those of the bovine AChR channels produced by injecting the three mRNAs in combination with the γ- or the ϵ-subunit-specific mRNA [13].

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