Functional expression of two neuronal nicotinic acetylcholine receptors from cDNA clones identifies a gene family

(neurotransmitter receptors/central nervous system/receptor pharmacology/*Xenopus* oocyte)

JIM BOULTER, JOHN CONNOLLY, EVAN DENERIS, DAN GOLDMAN, STEVEN HEINEMANN, AND JIM PATRICK*

The Molecular Neurobiology Laboratory, The Salk Institute, P.O. Box 85800, San Diego, CA 92138

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ABSTRACT A family of genes coding for proteins homologous to the α subunit of the muscle nicotinic acetylcholine receptor has been identified in the rat genome. These genes are transcribed in the central and peripheral nervous systems in areas known to contain functional nicotinic receptors. In this paper, we demonstrate that three of these genes, which we call α 3, α 4, and β 2, encode proteins that form functional nicotinic acetylcholine receptors when expressed in *Xenopus* oocytes. Oocytes expressing either α 3 or α 4 protein in combination with the β 2 protein produced a strong response to acetylcholine. Oocytes expressing only the α 4 protein gave a weak response to acetylcholine. These receptors are activated by acetylcholine and nicotine and are blocked by *Bungarus* toxin 3.1. They are not blocked by α -bungarotoxin, which blocks the muscle nicotinic acetylcholine receptor. Thus, the receptors formed by the α 3, α 4, and β 2 subunits are pharmacologically similar to the ganglionic-type neuronal nicotinic acetylcholine receptor. These results indicate that the α 3, α 4, and β 2 genes encode functional nicotinic acetylcholine receptor subunits that are expressed in the brain and peripheral nervous system.

It seems likely that complex brain functions, such as learning and memory, involve changes in the efficiency of synaptic transmission. One way in which synaptic efficiency might be modified is through a change in the availability or properties of neurotransmitter receptors in the postsynaptic membrane. Testing this idea, and understanding mechanisms that might accomplish such a modification, requires means of detecting and quantifying receptors at synapses in the central nervous system. However, the low abundance and great diversity of neurotransmitter receptors in the central nervous system have made their analysis difficult.

We therefore chose first to study neurotransmitter receptors at the more accessible neuromuscular junction and were able to obtain and express cDNA clones encoding the subunits of the muscle-type nicotinic acetylcholine receptor of the rat. We subsequently used these cDNA clones to identify homologous genes that code for acetylcholine receptor α subunits found in the central nervous system. This approach led to the isolation of two new cDNA clones (1, 2) that represent gene transcripts found in different regions of the brain and that encode proteins with the general structural features of muscle nicotinic acetylcholine receptor α subunits. We proposed that these genes, called α 3 and α 4, code for the α subunits of functional nicotinic acetylcholine receptors expressed in the central and peripheral nervous systems. We have tested this hypothesis and in this paper report that RNA transcribed from either the clone derived from the α 3 gene or the clone derived from the α 4 gene, in concert with RNA transcribed from a new

clone, PCX49, will direct the synthesis of functional neuronal nicotinic acetylcholine receptors in *Xenopus* oocytes.

MATERIALS AND METHODS

Isolation of Clone λ PCX49. Poly(A)⁺ RNA was isolated from adult rat hypothalamus and used as template for the synthesis of double-stranded cDNA by the method of Gubler and Hoffman (3). The double-stranded DNA was ligated into the *Eco*RI site of λ gt10. Approximately 5×10^5 plaques were screened at low stringency for hybridization with a radiolabeled probe prepared from clone λ PCA48 (encoding the rat α 3 gene product). One hybridizing clone, λ HYA5-1, contained an insert of approximately 1300 base pairs that showed nucleotide and deduced amino acid homology with clone λ PCA48; however, alignment of the deduced amino acid sequence with the λ PCA48-encoded protein suggested that clone λ HYA5-1 was not full-length. The cDNA insert from λ HYA5-1 was isolated, radiolabeled, and used for high-stringency screening of 10^6 plaques of a λ gt10 cDNA library prepared using poly(A)⁺ RNA obtained from the rat pheochromocytoma cell line PC12 (4). Approximately 50 strongly hybridizing plaques were obtained. One clone, λ PCX49, containing a cDNA insert of approximately 2200 base pairs, was shown to be identical to clone λ HYA5-1 except that its insert nucleotide sequence extended further in both the 5' and the 3' directions (5). The cDNA insert from clone λ PCX49 was ligated into the *Eco*RI site of the plasmid vector pSP65 immediately downstream of the bacteriophage SP6 promoter. This construct is shown in Fig. 2.

Construction of Expressible Clone PCA48E(3). Clone λ PCA48, as described (1), has an inverted repeat sequence located at its 5' end that contains ATG sequences coding for methionine residues that are not in the same reading frame as the mature protein. Since these sequences might generate inappropriate translation start sites, we cut the λ PCA48 cDNA insert at the 5' *Sst* I site (nucleotide 147), removed the 4-base overhang with mung bean nuclease, digested the DNA with *Eco*RI, and purified the resulting blunt-ended *Eco*RI fragment by electrophoresis in a low-melting-point agarose gel. This fragment, containing 76 nucleotides of 5' untranslated sequence and the coding sequence for a complete signal peptide and the entire mature protein, was subcloned between the *Sma* I and *Eco*RI sites of the plasmid vector pSP64. The construct, PCA48E(3), is shown in Fig. 2.

Construction of Expressible Clone HYA23-1E(1). Clone λ HYA23-1 (corresponding to the α 4-1 gene transcript) lacks a translation initiator methionine codon at the 5' end of the protein-coding region (2). To render it suitable for expression studies, we synthesized two complementary oligodeoxynucleotides (5' AATTGGCCATGGTGA 3' and 5' AGCTTCACCATGGCC 3') that, when annealed, form a linker with an *Eco*RI-compatible end, a *Hind*III-compatible

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*To whom reprint requests should be addressed.

end, and an internal ATG codon. Sequences flanking the ATG codon conform to the eukaryotic translation-initiation consensus sequence (6). The annealed oligonucleotides were ligated to the full-length *EcoRI* fragment obtained from clone λ HYA23-1, digested with *HindIII*, and subcloned into the *HindIII* site of the plasmid vector pSP64. The construct, HYA23-1E(1), is shown in Fig. 2.

In Vitro Synthesis of RNA for Oocyte Injections. Plasmid DNA for each construct illustrated in Fig. 2 was linearized with restriction enzymes that cleave at the 3' end of each clone. These DNAs were used as template for the *in vitro* synthesis of diguanosine triphosphate-capped RNA transcripts by bacteriophage SP6 RNA polymerase (7).

Xenopus laevis Oocyte Injections. Oocytes were removed from anesthetized, mature female *X. laevis* (*Xenopus* I, Madison, WI) and treated with collagenase type II (Sigma) at 1 mg/ml for 2 hr at room temperature. The oocytes were dissected free of ovarian epithelium and follicle cells, injected with *in vitro*-synthesized RNAs (0.5–5 ng per oocyte) in a total volume of 50 nl of H₂O, and incubated in Barth's saline (8) at 20°C until needed.

Electrophysiology. Individual oocytes were placed in a groove in the base of a narrow Perspex chamber (0.5-ml volume) through which solutions can be perfused at up to 40 ml/min. Drugs were applied by adding them to the perfusing solution and subsequently washing them out with control solution. Control solution contained 115 mM NaCl, 1.8 mM CaCl₂, 2.5 mM KCl, 10 mM Hepes (pH 7.2), and 1 μ M atropine. Voltage recordings were made using the bridge circuit of the Dagan 8500 voltage-clamp unit. Micropipettes were filled with 3 M KCl. Electrophysiological recordings were made at room temperature (20–25°C), 2–7 days after injection of the oocytes. Bovine serum albumin (0.1 mg/ml) was added to test solutions to prevent nonspecific binding of toxins. Oocytes with resting potentials less than –30 mV were rejected from these studies.

RESULTS

We have isolated and sequenced two cDNA clones that encode proteins homologous to the α subunit of the muscle nicotinic acetylcholine receptor. These clones represent transcripts from two of what now appears to be a family of genes that encode the ligand-binding subunits of a family of nicotinic acetylcholine receptors. One clone, PCA48, was

isolated from a cDNA library prepared from the PC12 cell line and represents a transcript of the alpha3 gene (1). Another clone, HYA23-1, was isolated from a cDNA library prepared from rat hypothalamus and represents a transcript of the alpha4 gene (2). In addition, a genomic clone containing an alpha2 gene has been isolated (9). These genes are expressed in the central nervous system, and we propose that the encoded proteins comprise the ligand-binding subunits of a family of neuronal acetylcholine receptors.

The sequences of the proteins corresponding to gene alpha1 (expressed in muscle) and genes alpha3 and alpha4 (expressed in neurons) are shown aligned in Fig. 1. The similarities among the protein sequences are evident in the several conserved sequences, including those defining the hydrophobic regions thought to form membrane-spanning helices (10–12). The asterisks indicate two contiguous cysteines that are found in each sequence. The equivalent cysteines in the α subunit of the receptor from *Torpedo* electric organ can be labeled with affinity-labeling reagents (13). These cysteines are found in all muscle-type α subunits but not in muscle-type β , γ , or δ subunits. Their presence in each of the sequences shown in Fig. 1 suggests that these proteins all contain an acetylcholine binding site. Because of the overall sequence homology and the conserved cysteines, we have proposed that the alpha3 and alpha4 gene products are the ligand-binding subunits of the neuronal nicotinic acetylcholine receptors and, by analogy with the muscle nicotinic acetylcholine receptor, have called them the α subunits.

We tested the idea that these clones encode receptor subunits by injecting *Xenopus* oocytes with RNA transcribed from them and assaying the oocytes electrophysiologically for the appearance of functional acetylcholine receptors. Since, by analogy with the muscle nicotinic acetylcholine receptor, we expected that a functional neuronal nicotinic receptor might require more than one type of subunit, we searched for clones encoding additional receptor subunits. Our search (see *Materials and Methods*) yielded clone PCX49, which was placed in plasmid pSP65 downstream of the SP6 promoter. This construct, along with the constructs PCA48E(3) and HYA23-1E(1), is shown in Fig. 2. The protein encoded by clone PCX49 shows about 50% sequence homology with nicotinic acetylcholine receptor α subunits. It also has features common to the α subunits, such as the four hydrophobic sequences proposed to constitute membrane-

FIG. 1. Comparison of deduced amino acid sequences (standard one-letter symbols) of the mouse muscle (alpha1) and two neuronal (alpha3 and alpha4) nicotinic acetylcholine receptor α subunits. The two asterisks indicate the cysteine residues at positions 192 and 193 that are thought to be close to the acetylcholine binding site. The molecular weights of the unglycosylated mature alpha1, alpha3, and alpha4 subunits are 55,085, 54,723, and 67,124, respectively.

FIG. 2. Restriction maps of the expressible cDNA clones encoding neuronal α subunits derived from the alpha3 gene [PCA48E(3)] and the alpha4 gene [HYA23-1E(1)] and the clone PCX49 derived from the beta2 gene. These clones were constructed as described in *Materials and Methods*. SP6 refers to the SP6 promoter, and the hatched areas indicate the multiple cloning site.

spanning domains. However, in contrast to the α subunits, it lacks the cysteines thought to contribute to the acetylcholine binding site (5). Because, as described below, the protein encoded by clone PCX49 acts synergistically with the neuronal alpha gene products to form functional nicotinic acetylcholine receptors, and because it constitutes a second class of neuronal receptor subunits, we have called it a β subunit. By analogy with the α subunit nomenclature, we have called the gene encoding this protein beta2.

We synthesized RNA corresponding to the alpha3, alpha4, and beta2 genes and injected it into *Xenopus* oocytes either singularly or in pairwise combinations. Injected oocytes were incubated for 2–7 days and those which expressed functional nicotinic acetylcholine receptors were identified by testing for depolarizations in response to perfused acetylcholine. The voltage traces in Fig. 3 (see lines A and B) show that the combination of the beta2 subunit with either the alpha3 or the alpha4 subunits resulted in depolarizing responses to acetyl-

choline. Since we observed no response to acetylcholine in oocytes injected only with RNA encoding the beta2 subunit, these results show that both the alpha3 and the alpha4 subunits contribute to the formation of a nicotinic cholinergic acetylcholine receptor. We tested the idea that the beta2 subunit was required for the appearance of a functional receptor by injecting oocytes with only the alpha3 transcript. We detected no response to acetylcholine in these oocytes. In contrast, we did find cholinergic responses in oocytes injected with RNA corresponding to the alpha4 gene. However, as seen in Fig. 3, line C, these responses were weak, even in the presence of high concentrations of acetylcholine. The results of these experiments, summarized in Table 1, show that functional acetylcholine receptors can be formed with the beta2 subunit in combination with either the alpha3 or the alpha4 subunit. The alpha4 subunit alone will also form a functional receptor, but neither the alpha3 nor the beta2 subunit alone will do so.

The receptors constituted from these clones are cholinergic, since they are activated by acetylcholine. We demonstrated that they are nicotinic by showing depolarizing responses to nicotine (see Fig. 3). However, there are nicotinic receptors on both muscle and neurons, and these receptors have different pharmacological properties. We determined that the receptors formed from these clones are of the neuronal type by testing their sensitivity to toxins. Activation of acetylcholine receptors at the neuromuscular junction is blocked by the neurotoxin α -bungarotoxin, whereas acetylcholine receptors on PC12 cells (14), rat cervical ganglia (15), and chick sympathetic ganglia (16) are resistant

FIG. 3. Voltage traces obtained from five different *Xenopus* oocytes injected with RNA derived from the neuronal alpha and beta genes. The RNA combinations injected are shown on the left and representative responses to applied acetylcholine and nicotine are shown on the right. RNA and oocytes were prepared and injected as described in *Materials and Methods* and recordings made 2–7 days later.

Table 1. Requirements for functional expression

RNA injected	No. of oocytes tested	No. of oocytes positive
alpha3	30	0
alpha4	30	10
beta2	21	0
alpha3 + beta2	50	46
alpha4 + beta2	49	48
No injection	21	0
Sham injection	21	0

Two to seven days after injection with RNA, oocytes were tested for responses to acetylcholine. Each test included a maximal concentration of 1 mM acetylcholine. Detection of a reproducible depolarization greater than a noise level of +1 mV was considered to be a positive response. These data represent the results of experiments conducted over a period of 4 months with more than six different lots of RNA for the injections.

FIG. 4. Effect of two different neurotoxins on the activation by acetylcholine (ACh) of two neuronal nicotinic acetylcholine receptor subtypes. The voltage tracing on the left shows the response before application of the toxin and the voltage tracing on the right shows the response following a brief washing and a 30-min incubation with α -bungarotoxin (α -Bgt) or *Bungarus* toxin 3.1 (α -Bgt 3.1).

to this toxin. The neuronal nicotinic acetylcholine receptors on PC12 and ciliary ganglia are, however, blocked by toxin 3.1 (17), which is a minor component in the venom of *Bungarus multicinctus*. We tested the sensitivity of the nicotinic acetylcholine receptors comprised of the beta2 subunit and either the alpha3 or the alpha4 subunit for their sensitivity to these toxins. The voltage traces in Fig. 4 and the data summarized in Table 2 show that receptors formed with beta2 and either the alpha3 (Fig. 4, lines A and B) or the alpha4 (lines C and D) subunit are resistant to α -bungarotoxin but are blocked by toxin 3.1. This is in contrast to the nicotinic receptor derived from clones encoding the mouse muscle receptor subunits, which is blocked by α -bungarotoxin under these conditions (data not shown). These results are consistent with the observation that the nicotinic receptor on the PC12 cell line, the source of clones PCX49 (beta2) and PCA48 (alpha3), is resistant to α -bungarotoxin and sensitive to toxin 3.1. The results also show that these neuronal nicotinic acetylcholine receptors, which are expressed in the brain, are resistant to α -bungarotoxin.

Table 2. Effects of neurotoxins

RNA injected	n	AcCho, μ M	Toxin	Before toxin		After toxin	
				RP, mV	Δ , mV	RP, mV	Δ , mV
alpha3 + beta2	4	10	α -Bgt	66.8 $\pm$ 4.1	25.4 $\pm$ 3.3	71.9 $\pm$ 4.1	24.8 $\pm$ 3.3
	3	5	α -Bgt 3.1	76.3 $\pm$ 2.3	24.0 $\pm$ 1.7	77.3 $\pm$ 1.7	4.1 $\pm$ 0.4
alpha4 + beta2	4	10	α -Bgt	70.1 $\pm$ 2.6	35.4 $\pm$ 4.7	72.4 $\pm$ 3.5	32.7 $\pm$ 6.4
	3	5	α -Bgt 3.1	69.3 $\pm$ 3.8	21.7 $\pm$ 3.5	75.6 $\pm$ 2.4	0.8 $\pm$ 0.3

Oocytes were injected with RNA and tested for depolarizing responses. The depolarizations (Δ) from the corresponding resting potential (RP) produced by the perfusion of acetylcholine (AcCho) were measured before and after a 30-min incubation with either 0.1 μ M α -bungarotoxin (α -Bgt) or 0.1 μ M toxin 3.1 (α -Bgt 3.1). Values presented are the averages ($\pm$ SEM) of experiments with n oocytes.

DISCUSSION

In previous papers (1, 2), we reported the nucleotide and deduced amino acid sequences of two cDNA clones that we proposed were derived from two members of a family of genes encoding the α subunits of neuronal nicotinic acetylcholine receptors. We based this proposal upon two observations. First, the proteins encoded by these clones show considerable homology with the α subunits of muscle nicotinic acetylcholine receptors, including the cysteines (residues 192 and 193) shown to be close to the acetylcholine binding site. Second, the genes encoding these proteins are transcribed in parts of the brain known to have nicotine binding sites (18). For example, the medial habenula contains transcripts for both the alpha3 and the alpha4 genes and is known to have neurons with nicotinic acetylcholine receptors (19). In this paper we show that these clones each encode α subunits that, in combination with the β subunit encoded by clone PCX49, will form functional nicotinic acetylcholine receptors. Furthermore, we show that the receptors thus constituted have pharmacological characteristics of ganglionic nicotinic acetylcholine receptors; they are resistant to α -bungarotoxin and sensitive to toxin 3.1.

Other laboratories have begun biochemical studies on neuronal nicotinic acetylcholine receptors. Hanke and Breer (20) found that the locust neuronal acetylcholine receptor can be reconstituted from a purified protein preparation that forms a single band upon NaDodSO₄/polyacrylamide gel electrophoresis. A clone encoding a protein with sequence homology to the rat alpha3 subunit but lacking the cysteines characteristic of the α subunits, and therefore similar to the beta2 subunit we have used in these expression studies, was isolated from a *Drosophila* cDNA library (21). Whiting and Lindstrom (22) identified bands in NaDodSO₄/polyacrylamide gels following precipitation of brain extracts with anti-nicotinic acetylcholine receptor antibodies and showed that some of these bands are labeled with the receptor affinity-labeling reagent 4-(N-maleimido)benzyltrimethylammonium. These bands may correspond to the proteins encoded by the clones we used in these expression studies. A chicken gene homologous to the rat alpha3 gene was isolated and sequenced by Ballivet and coworkers (9); in addition, they found clones encoding a protein that appears to be the product of an alpha2 gene (9), which they propose will contribute to the formation of yet another neuronal nicotinic receptor subtype.

Our present results show that neuronal nicotinic acetylcholine receptors differ from muscle nicotinic receptors in that they can be constituted from only two different gene products. In all experiments reported to date, nicotinic acetylcholine receptors have been formed with $\alpha\beta\gamma\delta$, $\alpha\beta\gamma$, $\alpha\beta\delta$, or $\alpha\gamma\delta$ -subunit RNAs, but not with any pairwise combinations (23). In contrast, both the alpha3 and alpha4 neuronal receptors can be constituted with only two different types of polypeptide chains, one derived from the specific alpha gene and one derived from the beta2 gene. The fact that these combinations function in the oocyte does not, however,

address the issue of the subunit structure of these two neuronal nicotinic acetylcholine receptors *in vivo*.

We did not detect a functional acetylcholine receptor when we injected only the alpha3 transcript. However, addition of beta2 transcripts to alpha3 transcripts resulted in the appearance of a functional neuronal nicotinic acetylcholine receptor. Although other explanations are conceivable, the simplest interpretation seems to be that the beta2 subunit joins the alpha3 subunit in the formation of a heterooligomer. The experiments described here did not address the issue of the number of subunits that might comprise this heterooligomer. However, the single-channel conductances of the muscle and neuronal (24, 25) acetylcholine receptors suggest that the channels are similar, and the homologous hydrophobic domains suggest that both receptors are formed by a similar arrangement of membrane-spanning regions. We propose therefore, by analogy to the nicotinic acetylcholine receptor of the *Torpedo* electric organ, that the functional neuronal receptor is a pentamer, presumably with two alpha chains.

Although the alpha4 subunit is capable of forming an acetylcholine receptor with no added subunits, it produces a more robust response in combination with the beta2 subunit. We also note that we have used only one of the possible alpha4 subunits. At least two different transcripts of the alpha4 gene are made (2), presumably by alternative splicing, and we have studied only the alpha4 product encoded by clone HYA23-1E(1). The different alpha4 subunits may be functionally distinct and interact with as-yet-undiscovered subunits. Again, however, we propose that the alpha4 receptor constituted in the oocyte is either a homooligomer composed of five alpha4 subunits or a pentameric heterooligomer composed of alpha4 and beta2 subunits.

The alpha3 and alpha4 genes are transcribed in different parts of the central nervous system, yet both the alpha3 and alpha4 subunits interact functionally with the beta2 subunit in our assay. Since the clone encoding the beta2 subunit (PCX49) and the clone encoding the alpha3 subunit (PCA48) are both derived from PC12 RNA, the cell must make these two transcripts. Therefore, there is clear opportunity for these proteins to assemble into a nicotinic receptor *in vivo* in this cell line. It is not known whether the beta2 gene is transcribed in a cell that also contains alpha4 transcripts. However, since we have shown that both the alpha3 and alpha4 subunits can be constituted with the beta2 subunit to form a functional neuronal nicotinic acetylcholine receptor, it is possible that different regions in the brain synthesize receptors with different alpha subunits and share the beta2 subunit. Since the alpha3 and alpha4 subunits differ in their cytoplasmic domains, they may contribute, in different parts of the brain, different regulatory capacities to receptors containing the beta2 subunit. Alternatively, additional, as-yet-unidentified subunits may exist.

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