Proc. Natl. Acad. Sci. USA
Vol. 84, pp. 4318–4321, June 1987
Neurobiology

Expression of the α-bungarotoxin binding site of the nicotinic acetylcholine receptor by *Escherichia coli* transformants

(ligand overlays/colony blots)

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Communicated by George E. Palade, February 6, 1987 (received for review October 7, 1986)

ABSTRACT **Restriction fragments of DNA derived from a cDNA clone of the α subunit of the acetylcholine receptor were subcloned in *Escherichia coli* by using the *trpE* fusion vector, pATH2. Transformants expressing the amino acid sequences 166–315 or 166–200 are shown to produce a chimeric protein that bound α-bungarotoxin. Moreover, it is shown that sufficient amounts of toxin-binding proteins can be generated by individual colonies of bacteria. This provides a new approach for gene selection via functional expression—i.e., ligand overlays of colony blots.**

Whereas the nicotinic acetylcholine receptor (AcChoR) is probably the most extensively studied receptor today (for reviews, see refs. 1–3), the question of how ligand binding elicits its channel activity is still unanswered. One approach towards tackling this problem has been to concentrate on identifying the ligand binding site *per se*. Affinity alkylation of the AcChoR has led to the conclusion that the cholinergic binding site and the α-bungarotoxin (α-BTX) binding site are either identical or extremely close to one another and are situated near a cysteine residue of the α subunit (3). The application of recombinant DNA technology has provided the complete amino acid sequence of the receptor (4–6), and theoretical considerations as well as experimental data suggest that the binding site could be in the area of either cysteine-128 and cysteine-142 (4, 7–9) or cysteines-192/193 (10–13) of the α subunit. Protein blotting (14) has been found to be extremely useful in analyzing the toxin binding site (15, 16) and, in conjunction with anti-synthetic peptide antibodies (10, 13), strong evidence has been produced that indicates that the region beyond residue 153 is essential for toxin binding.

In this report a different approach has been adopted that brings additional support for this hypothesis. A cDNA fragment, corresponding to the amino acid sequence 166–200 of the α subunit, was cloned into an expression vector and used to transform *Escherichia coli*. The transformants were able to specifically bind α-BTX. These results not only demonstrate the importance of this region of the α subunit but also provide the basis for further experimentation towards understanding the mechanisms responsible for ligand-induced neurotransmission.

MATERIALS AND METHODS

Construction of Recombinant Plasmids. The *trpE* fusion vector pATH2 (17) was used. Restriction fragments of the plasmid p42, a cDNA clone of the α subunit of *Torpedo californica* AcChoR (18), were purified from 1% agarose gels. Preparative quantities of plasmids were obtained, and ligations and transformations of *E. coli* strain HB101 were performed by the methods of Maniatis *et al.* (19).

Induction of *E. coli* Transformants. *E. coli* HB101 cells were transformed with pATH2 or with constructs of pATH2 containing selected sequences of the AcChoR α-subunit cDNA. Cultures of these transformants were grown to stationary phase in M9 medium (19) supplemented with Casamino acids (0.5%), thiamine (10 μg/ml), tryptophan (20 μg/ml), and ampicillin (50 μg/ml). Such cells were then diluted 1:100 in the same M9 medium but lacking tryptophan and were allowed to reach OD_{600} = 0.2 at 33°C. Then 5 μg of 3-β-indoleacrylic acid per ml was added, and the cultures were continued for another 6–12 hr.

Gel Electrophoresis and Blot Analysis. After induction, cells were harvested, and aliquots (0.5 OD_{600} of cells) were solubilized in sample buffer [50 mM Tris·HCl, pH 6.8/25% (wt/vol) glycerol/2% (vol/vol) 2-mercaptoethanol/0.01% bromophenol blue] containing 1% sodium dodecyl sulfate. Complete solubilization was achieved by sonicating pelleted cells in an E/MC ultrasonic cleaner (model 250). The cell lysates were electrophoresed on 10% polyacrylamide gels, blotted onto nitrocellulose membrane filters, and probed with ^{125}I-labeled α-BTX (15). Proteolytic fragments of chimeric protein were produced essentially by the method of Cleveland *et al.* (20) as described (16).

Ligand Overlays of Colony Blots. For the selection of toxin binding transformants, a different colony blot (21) selection assay was developed. Transformed cells were grown on LB agar plates supplemented with ampicillin (50 μg/ml) (19). Replicas of the plates were grown on nitrocellulose membrane discs on agar plates of M9 medium as described above but without tryptophan and pretreated with 100 μg of 3-β-indoleacrylic acid per plate. Thus, starvation for tryptophan in the presence of 3-β-indoleacrylic acid selectively induces the biosynthesis of *trpE* gene product or its fusion proteins (17). The bacteria were grown at 33°C overnight under these conditions. Then they were lysed for 0.5–1 hr at 25°C by placing the filters onto Whatman blotting paper saturated with a 5% solution of sodium dodecyl sulfate in water. Afterwards the filters were electroblotted for 1 hr in 15.6 mM Tris/120 mM glycine with the bacterial surface of the filter facing the cathode. The filter discs then were quenched in 1% hemoglobin in phosphate-buffered saline (pH 7.4) and overlayed with ^{125}I-labeled α-BTX as described (15). There was no apparent need for DNase digestion.

RESULTS AND DISCUSSION

The p42 cDNA clone is a derivative of clone 2D8, which contains most of the α subunit of *T. californica* AcChoR (18). A 1.2-kilobase (kb) *Pst* I fragment was isolated from this clone, which was further digested with *Xho* II. In this manner, a fragment corresponding to the amino acid se-

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Abbreviations: α-BTX, α-bungarotoxin; AcChoR, acetylcholine receptor.

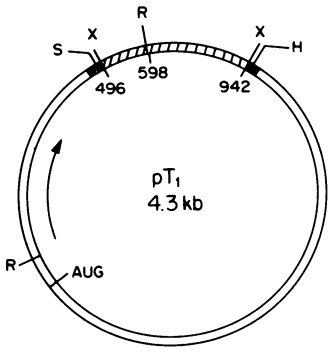

FIG. 1. A diagrammatic representation of pT_1 (not drawn to scale). The *Xho* II (X) fragment (hatched; 446 base pairs) derived from the p42 cDNA clone (18) was inserted into the *Bam*HI site of the polylinker of pATH2 (shaded areas) (17). Partial digest with *Eco*RV (R) at the site within the insert allowed the construction of pR_1 and pO_1 by subsequently cutting with *Hin*dIII (H) or *Sma* I (S), respectively, and allowing the vector to ligate on itself. The numbers represent the nucleotide residue numbers of the α subunit of the AcChoR according to Noda *et al.* (4).

quence 166–315 of the α subunit was produced and inserted into the *Bam*HI site of the pATH2 expression vector (17). Two clones of transformed *E. coli* were selected: one that contained the insert in proper orientation (designated pT_1), and another that contained the same but in the reversed orientation (designated pT_2). pT_1 (Fig. 1) was used as the source for subsequent constructs. Partial *Eco*RV digests of pT_1 were performed, and linear plasmids were isolated. These were further digested with either *Sma* I or *Hin*dIII and ligated to produce two additional constructs corresponding to the amino acid sequences 166–200 (designated pR_1) and 201–315 (designated pO_1).

Total-cell lysates from transformants of each of the four constructs, grown under inductive conditions, were separated by sodium dodecyl sulfate/polyacrylamide gel electrophoresis. The gels were either stained or were blotted and overlayed with ^{125}I-labeled α-BTX. The major peptide of *E. coli* transformed with pATH2 and induced for expression was about M_r 35,000 (Fig. 2, arrowhead). This peptide shifts to an apparent M_r of ≈45,000 in pT_1, whereas no such change is

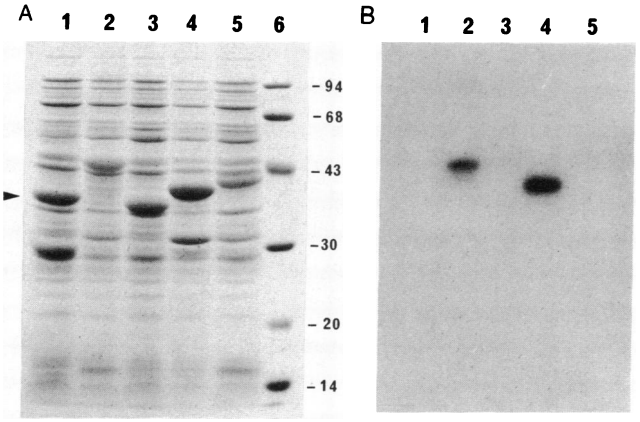

FIG. 2. Analysis of total cell homogenates of transformed *E. coli*. Samples (0.5 OD_{600}) of *E. coli* cultures induced for expression were solubilized in 1% sodium dodecyl sulfate and resolved on 10% polyacrylamide gels. The gels were either stained with Coomassie brilliant blue (*A*) or were blotted and overlayed with ^{125}I-labeled α-BTX (*B*; an autoradiogram of such a blot). *E. coli* were transformed with pATH2 (lanes 1), pT_1 (lanes 2), pT_2 (lanes 3), pR_1 (lanes 4), or pO_1 (lanes 5). Lane 6 contains molecular weight standards shown × 10^{-3}. The arrowhead indicates the position of the major polypeptide of pATH2.

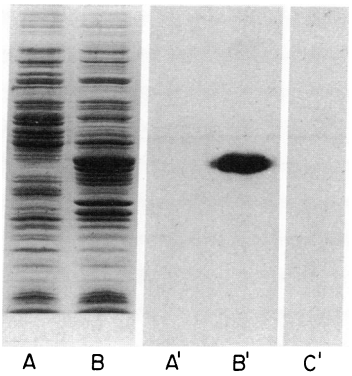

FIG. 3. Analysis of pR_1 transformants. pR_1 cells were grown in LB medium (lanes A and A′) or in M9 medium under inductive conditions (lanes B, B′, and C′). Cell lysates were then resolved on 10% polyacrylamide gels and either stained with Coomassie brilliant blue (lanes A and B) or blotted and overlayed with ^{125}I-labeled α-BTX (lane A′–C′). For pharmacological characterization of the complex formed, the blot of lane C′ was coincubated with *d*-tubocurrarine (1 mM).

observed for pT_2. pR_1 contains a unique peptide slightly larger than that of pATH2, and the size of the chimeric protein of pO_1 is intermediate between those of pR_1 and pT_1. From the blot analysis, it is apparent that only the pT_1 and pR_1 chimeras bind toxin. These results demonstrate clearly that the area of 166–200 of the α subunit is necessary for toxin binding (see also refs. 12 and 13). However, it is insufficient to establish the high-affinity binding site of the native receptor.

Further demonstration of the specificity of this binding is demonstrated in Fig. 3. The pR_1 clone was grown under inductive or noninductive conditions. Blots from such cells show that starvation for tryptophan, and thus induction of the *trp* promoter of the plasmid, is obligatory to obtain any signal whatsoever (this induction is dependent on the presence of 3-β-indoleacrylic acid). Moreover, the binding of α-BTX could be inhibited by *d*-tubocurrarine. As has been found for the isolated α subunit (15), the IC_{50} for this competition was 0.5 mM.

When the chimeric proteins of pT_1 and pR_1 are subjected

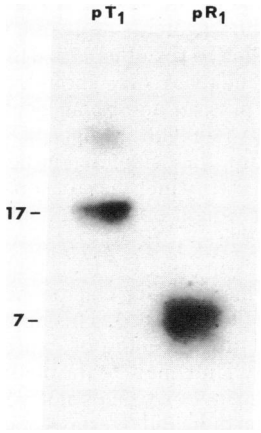

FIG. 4. *S. aureus* V_8 protease release of functional binding sites from *E. coli* chimeras. The chimeric proteins of pT_1 and pR_1 were excised out of 10% polyacrylamide gels and subjected to *S. aureus* V_8 protease cleavage in 15% polyacrylamide gels as described (16). The proteolyzed fragments were then blotted onto nitrocellulose membrane filters, which then were overlayed with ^{125}I-labeled α-BTX. An autoradiogram of such an experiment is shown above, with molecular weight shown × 10^{-3}.

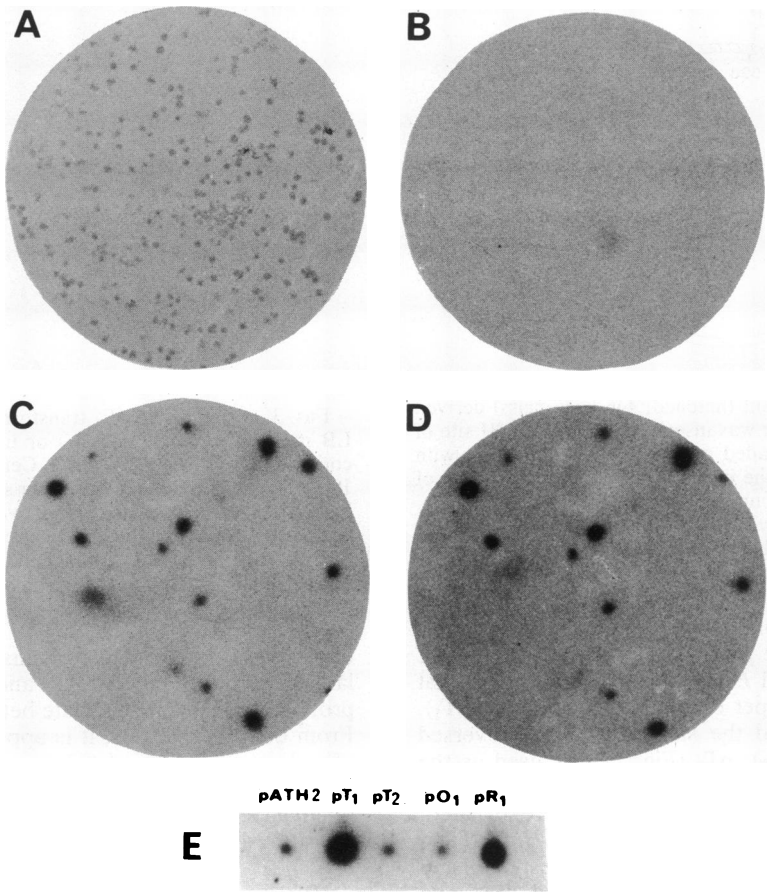

FIG. 5. Ligand overlays of colony blots. *E. coli* HB101 cells transformed with pATH2 or pT_1 were mixed at a ratio of 10:1 and plated on LB/ampicillin plates at 150–200 colonies per plate (*A*). Three replicas of such a plate were prepared, grown either on LB/ampicillin (*B*) or on M9 medium/3-β-indoleacrylic acid as described (*C* and *D*). The replicas were then processed for ligand overlay and autoradiographed (6 hr). Note that positive clones are only detected on the plates that have undergone induction (*C* and *D*) and that both identical replicas give the same result. Further demonstration of the selectivity of this assay is provided in *E*. Transformants of pATH2, pT_1, pT_2, pO_1, and pR_1 were grown on nitrocellulose membrane discs under inductive conditions as described. This autoradiogram is appreciably overexposed (36 hr) to emphasize that only pT_1 and pR_1 bind toxin.

to *Staphylococcus aureus* V_8 protease digestion, peptide fragments of apparent M_r values 17,000 and 7000, respectively, are produced (Fig. 4). Because the first V_8 cleavage site after residue 166 is Glu-172, it is reasonable to assume that these proteolytic fragments represent sequences of the α subunit corresponding to residues 173–315 and 173–200. The actual size of the cleaved pR_1 should correspond to the sequence 173–200 plus an additional peptide, Ser-Leu-Ser-Met-Ile-Ser-Ser-Cys-Gln-Thr, which is derived from the polylinker. Therefore, the calculated molecular mass for this fragment is 4540 Da, which is reasonably close to the apparent molecular weight observed, considering the quality of resolution in this area of the gel.

Especially interesting is the fact that the selection of α-BTX binding transformants can be performed by toxin overlays of colony blots (Fig. 5). This approach is extremely useful as it allows the screening of expression libraries for functional peptides. Thus, new toxin-binding proteins can be isolated from organisms or tissues not yet analyzed. Moreover, the application of ligand overlays of colony blots should enable the identification of totally novel receptors.

Barkas *et al.* (22) have recently demonstrated similar results regarding the area responsible for toxin binding in mouse AcChoR.

The author thanks Dr. Marc Ballivet for providing the p42 clone and a preprint of ref. 22; Dr. Ben-Zion Shilo for his critical comments, advice, and support; Yoav Eshel for his help in preparing Figs. 3 and 5; and Jean-Michel Lebleautte for technical assistance. This research was funded by Keren Yeda and a grant from the Leo and Julia Forschheimer Center for Molecular Genetics at the Weizmann Institute of Science.

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