EVIDENCE FOR STRUCTURAL HETEROGENEITY IN THE ALPHA SUBUNIT OF ACHRS FROM ASTERIAS FORBESI AND NEREIS VIRENS: IMMUNO- AND SOUTHERN BLOTTING STUDIES.

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Previous work in our laboratory has focused on comparative functional studies of neuronal acetylcholine receptors (AChRs) from the starfish, Asterias forbesi, the nematode, Nereis virens, and the jellyfish, Cyanea capillata. These studies have highlighted a significant degree of functional diversity in the AChRs in each organism (e.g. Welsford et al., Bull. MDIBL 33: 107-109, 1994). The logical extension of such functional studies is to begin the structural characterization of these AChRs. Some of the more obvious structural questions to be addressed at the outset include: what is the subunit stoichiometry of the AChRs from each species?; and how similar are the subunit sequences between the AChRs of varying species? We have begun to address these questions by conducting immunoblotting and Southern blotting analyses of the AChRs from two of the species from which functional data have been gathered, Asterias forbesi and Nereis virens.

Specimens of Nereis and Asterias were collected locally at the MDIBL. mRNA was isolated from the CNS of each species using an mRNA Isolation Kit from Stratagene Inc. mRNA was analyzed for purity and degradation using standard techniques including agarose-formaldehyde gel electrophoresis, spectrophotometry (OD_{280} v.s. OD_{260}) and using a spot blot kit for nucleic acids (InVitrogen Inc.). During our experiments, mRNA degradation during analysis and storage was a serious problem. Because of this, mRNA was subjected to reverse transcriptase PCR (RT-PCR) using Tth DNA Polymerase (Promega Inc.) in a Perkin-Elmer Model 480 thermal cycler to produce cDNA and sequence similarities between species were assessed using Southern blotting. An oligo dT 15-mer (Promega Inc.) was used in the RT-PCR for both first strand synthesis (i.e. "upstream" priming) and amplification of the cDNA reverse transcript (i.e. "downstream" priming). Following amplification, the concentration of cDNA was determined by UV spectrophotometry and spot blotting as described above. Approximately 1 ug of cDNA from each species was run on an 1.6% TBE-agarose gel at 25V for 10-16 h, depurinated, denatured, neutralized and transferred onto charge-modified nylon membrane (MSI Inc.) using positive pressure (Stratagene Posiblotter). Following transfer, membranes were dried at 200^0 C for 1 h. The probes used for Southern blotting were full-length clones for the α, β, γ and δ subunits of the Torpedo californica electroplax AChR (obtained from Dr. M. White at the Medical College of Pennsylvania). Probes were random-prime labeled with DIG-dUTP (Genius Kit, Boehringer Mannheim) and labeling efficiency was determined using spot-blotting. Since the degree of sequence homology between the vertebrate AChR probes used and the AChRs from the species under study was not known, hybridization stringency was determined empirically. Optimal signal was achieved when membranes were hybridized using 5X SSC (0.7 M NaCl, 0.075 M Na_3-citrate-$2H_2O$, pH 7.0). Higher stringencies (i.e. lower salt concentrations) significantly reduced hybridization reaction product while lower stringencies (i.e. higher salt concentrations) resulted in very high background signal.

Membranes were prehybridized for 12 h at 42^0 C in a standard prehybridization solution (5X SSC, 1.0% (w/v) blocking reagent supplied with Genius Kit, 0.1% N-laurylsarcosine and 0.02% sodium dodecyl sulfate (SDS)). Following this, membranes were hybridized for 12 h at 42^{0}C with 10 ng/mL labelled probes (diluted in standard prehybridization mix) for 12 h at 42^0 C. Probe binding was assessed colorometrically using an alkaline phosphatase reaction. Controls for probe specificity included hybridization of labeled cDNA with non-labeled cDNA prior to application to the membrane and running unlabelled insert cDNAs as control lanes in the Southern blot.

Three different rat monoclonal antibodies were obtained from American Type Tissue and Cell Culture Inc. (ATCC) and used in the immunoblotting experiments: mAb 35, which recognizes the main immunogenic region (MIR; i.e. the region associated with myasthenia gravis) of the α subunit of the mammalian neuronal nAChR (Whiting and Lindstrom, Biochemistry 25: 2082-2093, 1986a; Whiting and Lindstrom, J. Neurosci. 6: 3061-1069, 1986b), mAb 64 which recognizes the α subunit of mammalian neuronal and muscle nAChRs but not the MIR (Schoepfer et al., Neuron 1: 241-248, 1988; Schoepfer et al., Neuron 5: 35-48, 1990), and mAb 270 which recognizes the structural subunit (probably β) of chicken and mammalian neuronal nAChRs which does not bind α-bungarotoxin (Whiting and Lindstrom Proc. Nat. Acad. Sci. USA 84: 595-599, 1987; Whiting et al., J. Neurosci. 3: 4005-4016, 1987). mAbs were obtained by purifying hybridoma media using gel-filtration chromatography (Bio-Rad Econo System). Nervous tissue was dissected from freshly collected organisms on ice and placed into a sterile glass homogenizer. Tissue was emulsified in sterile PBS^{++} (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4 7 H_2O, 1.4 mM KH_2PO_4, pH 7.4, with 0.1 % w/v phenylmethyl-sulfonylfluoride (PMSF) to inhibit serine proteases and 0.1 % w/v dithiothreitol (DTT) as an anti-oxidant) and loaded into the wells of a dot-blot apparatus (BioRad Inc.) which contained a prewashed charge-modified nylon membrane. The membrane was incubated for 12 h at 4^0 C, washed twice with PTB5 (1X PBS with 0.02% TWEEN 20 and 5% w/v BSA added). mAbs were diluted 1:100 in PTB1 (PBS with 0.02% TWEEN 20 and 1% BSA added) and applied either alone or in the presence of curare and alpha-bungarotoxin. Membranes were incubated in primary antibody for 12-16 h at room temperature and secondary antibody (HRP-labeled goat anti-rat IgG (heavy and light chain); Southern Biotechnology Associates Inc.) was applied for 8 h at room temperature (1: 1500 dilution). The membrane was removed from the dot blot apparatus, washed several times in PTB1 and reacted with 4-chloro-1-napthol and H_2O_2. Reacted blots were quantified using an optical densitometer (Hoeffer Inc.) and data were analyzed using ANOVAs followed by post-hoc comparisons. Positive controls included immunoblotting mammalian CNS tissue extracts (mouse), and presorbing the mAbs with mouse extracts prior to immunoblotting. Negative controls included deleting primary and secondary antibodies and immunoblotting extracts from human leukemia (HL-60) cells which are known not to contain neuronal AChRs.

Figure 1 shows a sample Southern Blot on cDNA from Asterias and Nereis using probes directed against either α (Fig. 1A), β (Fig. 1B), γ (Fig. 1C) or δ (Fig. 1D) subunit. The only significant reaction product obtained in such blots was with the α probe. The specificity of hybridization was supported by the finding that probes for each

subunit hybridized specifically to their own complementary sequence (e.g. α to α only; lane 4 in Figs. 1A-D) and prehybridization of labeled probes with high concentrations (100 µg) of unlabeled complementary probes blocked hybridization (data not shown). The calculated size of α cDNA fragment labeled in each marine species was slightly smaller than that expected in a vertebrate (i.e. 2100 MW), being 1900 for Asterias and only about 1800 for Nereis (Fig. 1A). This difference in molecular weight could represent true differences between species in the size of the alpha subunit gene, differences in 3' or 5' non-coding regions of the gene or could be due to differences in reverse transcriptase and/or amplification efficacy during the PCR. Further experimentation, including direct analyses of mRNA (i.e. Northern blotting) will be required to distinguish between these alternatives. However, the fact that the α probe did hybridize with cDNA from both Asterias and Nereis argues that there is sufficient sequence conservation (or convergence) between the vertebrate, Torpedo and these divergent invertebrate species to allow hybridization at moderate stringency. Since cDNA probes directed against subunit sequences other than α did not specifically react with either species, these data could indicate that AChRs from the species under study are essentially made up of one type of subunit and/or the sequence divergence of other putative subunits is greater than that seen in α. Alternatively, these differences could be due to differences in PCR efficacy between varying subunits.

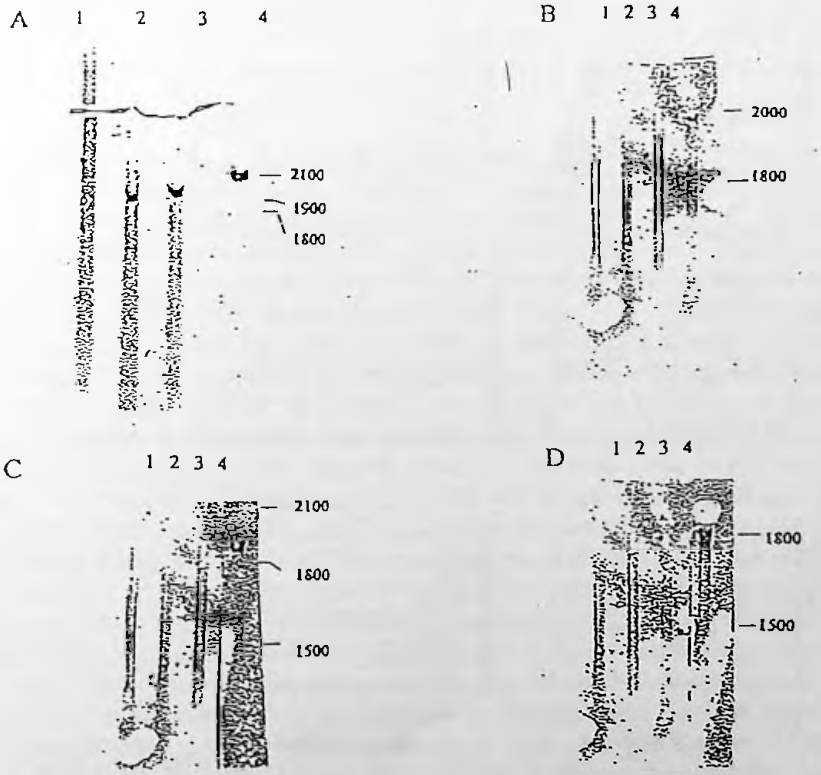

Fig. 1. Southern Blots of cDNA using labelled probes corresponding to the α subunit (A), the β subunit (B), the γ subunit (C) and the δ subunit (D) of Torpedo electroplax AChR. In each blot, lane 1= 0 cDNA, 2= Nereis, 3 = Asterias and 4= unlabelled cDNA probe.

The three antibodies used in immunoblotting reacted with extracts from mouse CNS but not with extracts from HL-60 cells ($F_{1,25} = 181.26$; $p < 0.001$). Binding of mAb 270 to CNS homogenates of both Asterias and Nereis was inhibited by α-bungarotoxin, but not curare, in a dose-dependent manner ($F_{5,69} = 3.11$; $p = 0.0136$ followed by Sheffe post hoc tests at 0.05 criterion; Fig. 3A, B). Binding of mAb 35 and 64 was unaffected by the presence of either blocker ($F_{5,69} = 1.23$; $p = 0.876$; Fig. 3C-F). These data argue that Asterias and Nereis may possess CNS sequences that are similar to the α subunit found in vertebrates (in that both mAb 35 and mAb 64 bound to the homogenates) and that the competitive binding properties of this subunit may be similar to that seen in vertebrates. In addition, these data argue for the presence of a sequence in both invertebrate species which is recognized by an antibody directed against the structural subunit of the vertebrate AChR. This is interesting in that this putative subunit was not recognized by the cDNA probes for vertebrate structural subunits. In addition, such a putative structural subunit may exhibit altered binding properties from those seen in vertebrates (in that antibody binding is affected by α-bungarotoxin). Further experimentation, including more definitive Western blotting analyses is ongoing.

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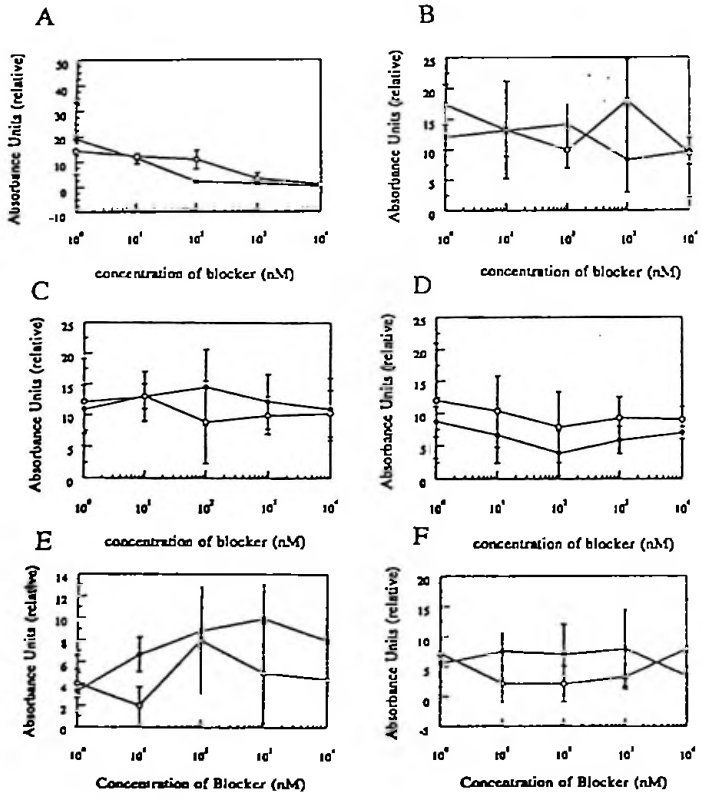

Fig. 3. Summary graphs of competitive binding studies using dot blots. In each figure, dark circles correspond to Asterias and open circles, Nereis. A = mAb 270 with α-bungarotoxin, b = mAb 270 with curare, C = mAb 35 with α-bungarotoxin, D = mAb 35 with curare, E = mAb 64 with α–bungarotoxin, F = mAb 64 with curare.