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Phylogenetic conservation of disulfide-linked, dimeric acetylcholine receptor pentamers in southern ocean electric rays

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Summary

Intact acetylcholine receptors have been purified on a novel affinity resin from three electric fish endemic to Australian waters. Their binding properties and morphology are compared with those of their northern hemisphere homolog, *Torpedo marmorata*. All four exhibit apparent dissociation constants, K_d, in the nanomolar range for the snake neurotoxin α-bungarotoxin and have a distinctive rosette-like appearance when viewed in negative stain under the electron microscope. Furthermore, these rosettes are paired, indicating that acetylcholine receptors from southern ocean electric fish exist as dimers, in the same fashion as their northern hemisphere counterparts. The cDNAs of the receptor's four subunits were sequenced from *Hypnos monopterigium* and the northern hemisphere counterpart, *Torpedo marmorata*, while cDNAs from only two subunits, α and δ, were able to be sequenced from *Narcine tasmaniensis*. The penultimate amino acid in the δ subunit of each of the newly sequenced fish species is a cysteine residue. Its conservation suggests that the mechanism for the observed dimerization of acetylcholine receptors is disulfide bond formation between the δ subunit of adjacent receptors, analogous to acetylcholine receptor dimers observed in other electric fish. It appears that this mechanism for receptor clustering is unique to acetylcholine receptors packed and organized in the specialized organs of electric fish. Alignment of the deduced protein sequences with the equivalent sequences from *Torpedo californica* and humans reveals a high degree of homology.

Key words: acetylcholine receptor, ligand-gated ion channel, electric fish, dimer, clustering, *Torpedo macneilli*, *Torpedo marmorata*, *Hypnos monopterigium*, *Narcine tasmaniensis*.

Introduction

Ligand-gated ion channels are responsible for rapid electrical signaling at the neuromuscular junction and in the central nervous system. In response to ligand binding, the protein undergoes conformational changes, transiently opening an integral, ion-selective pore and allowing ions to move spontaneously down their electrochemical gradient, thus altering the membrane potential. Members of this family include the cation-selective nicotinic acetylcholine and serotonin $5HT_3$ receptors, the anion-selective γ-aminobutyric acid type A and type C (GABA) and glycine receptors. It has been proposed, on the basis of sequence homology and structural modeling studies, that all members will have a three-dimensional fold and mechanism of activation in common.

The prototypal ligand-gated ion channel is the nicotinic acetylcholine receptor. This receptor was the first neurotransmitter receptor identified as a molecular entity (Noda et al., 1982, 1983) and the first to be isolated and purified in an active form (Miledi et al., 1971). This receptor was subsequently reconstituted in an artificial membrane system and shown to retain its physiological properties in a quantitative fashion (reviewed in Changeux et al., 1983; Conti-Tronconi and Raftery, 1982). It is also the only member of this family for which we have any direct structural information (Miyazawa et al., 1999; Unwin, 1993, 1995). Research into its structural and functional properties has benefited greatly from an abundant source of this receptor in the specialized (muscle-derived) electric organ of electric rays, where it is responsible for generating the electrical current used to stun its prey.

The acetylcholine receptor, affinity purified from the electric ray *Torpedo californica*, was shown by micro-sequence analysis to be a pentamer composed of four homologous subunits with a stoichiometry of two α subunits and one each of the β, γ and δ subunits (α2βγδ; Raftery et al., 1980). The density of the acetylcholine receptors is so high in the electric organ that they appear as an almost crystalline array and this allowed the use of electron microscopy to reveal the morphology of the receptor. By negative staining, distinctive

rosette-like structures were seen densely covering the postsynaptic membrane isolated from the electric organ. These same structures were observed with solubilized, purified acetylcholine receptors, validating their identification as acetylcholine receptor molecules (Chang et al., 1977). In contrast to neural and muscle forms of the acetylcholine receptor identified subsequently, the native receptor derived from the electric organ of specialized fish is a dimer, cross-linked by a disulfide bridge between δ subunits in adjacent pentamers (Hamilton et al., 1979).

Early investigators obtained the acetylcholine receptor from the electric rays *Torpedo californica*, *Torpedo marmorata*, *Torpedo nobiliana* and *Torpedo oscellata* or occasionally from the lessser known species the numbfish *Narcine entemedor* and *Narcine braziliensis* and the electric eel *Electrophorus electricus* (reviewed in Conti-Tronconi and Raftery, 1982). Indeed, it is the native receptor isolated from *T. marmorata* that has been used in combination with cryo-electron microscopy to derive direct structural information for this family of ligand-gated ion channels (Miyazawa et al., 1999; Unwin, 1993, 1995). Although *T. marmorata* has been used in such studies, its sequence data are incomplete. We report here the nucleotide sequence of the three uncharacterized subunits (β, γ and δ).

To date, all attempts to solve the acetylcholine receptor's structure at high resolution by X-ray diffraction have been unsuccessful, mainly due to the low quality of the crystals. This is a problem common to crystallographic studies of membrane proteins owing to the disorder caused by the necessity for detergent-based solvents and the inherent mobility of transmembrane α-helices. A number of membrane protein structures have been solved recently, many of which have employed the use of natural variation to select a suitable natural variant for X-ray crystallography (Chang and Roth, 2001; Chang et al., 1998; Doyle et al., 1998; Dutzler et al., 2002; Jiang et al., 2003). We have analyzed the neuromuscular acetylcholine receptor cDNAs and proteins sourced from three southern ocean electric fish that may provide alternative sources of protein for both biochemical and structural studies. The electric fish investigated in this study are members of three different families and all are endemic to Australian waters. The electric ray *Torpedo macneilli* is in the same family as *Torpedo californica* and *Torpedo marmorata*. The coffin ray, *Hypnos monopterigium*, is a member of the Hypnidae family, although it is sometimes given subfamily status within the family Torpedinidae. The numbfish *Narcine tasmaniensis* is the smallest of the electric rays and is a member of the family Narcinidae. Our results show that the acetylcholine receptor sourced from electric rays is highly conserved across these different families, and alignment of their deduced protein sequences with the equivalent human genes shows them to be a good model system for studying the human acetylcholine receptor at the neuromuscular junction. The large amount of sequence information available today further enforces the homologous nature of the subunits for any receptor in this family. Thus, in general, information obtained for one member is applicable to all members of the ligand-gated ion channel family.

Materials and methods

Sequencing of acetylcholine receptor cDNAs from northern and southern ocean electric rays

The four acetylcholine receptor subunit cDNAs were sequenced from four electric rays, three that inhabit the southern ocean (*Torpedo macneilli* Whitley 1932, *H. monopterigium* Shaw 1795 and *N. tasmaniensis* Richardson 1841) and one from the North Atlantic (*Torpedo marmorata* Risso 1810). Electric organ tissue (1 cm^3) was flash frozen upon dissection and stored at –70°C. Total RNA was isolated from frozen tissue first by grinding the tissue using a mortar and pestle and then further homogenizing it through a QIAshredder column (Qiagen, Germantown, MD, USA). Total RNA was extracted from this homogenate using the RNeasy mini kit (Qiagen). Specific acetylcholine receptor subunit cDNAs were amplified from the purified RNA by RT-PCR, essentially according to manufacturer's instructions (Access RT-PCR kit; Progen Industries, Darra, Queensland, Australia). Specific subunit cDNAs were amplified using primers whose design was based on the known acetylcholine receptor gene sequences from *T. californica* (numbering refers to published *T. californica* nucleotide sequences, accession numbers J00963, J00964, J00966 and J00965 for α, β, γ and δ, respectively); α, 5′-atgattctgtgcagttatt-3′ (187–206) and 5′-ttagccctcttgactgagt-3′ (1572–1554); β, 5′-atggaggacgtgaggag-gatg-3′ (44–65) and 5′-ttagctcggagcgtttccctctga-3′ (1702–1679); γ, 5′-atggtactgacacttctcct-3′ (32–52) and 5′-ttacggg-acgtatttcctagg-3′ (1552–1532); δ, 5′-atggggaacattcattttgtt-3′ (458–479) and 5′-ttagataggcacatgaataca-3′ (2170–2150). All the forward primers begin at the ATG start codon of the protein coding sequence, hence the first seven amino acids of the signal sequence have not been determined. The reverse primers for the α and γ genes bind within the coding sequence, and hence the last six residues of these subunits have not been determined. However, in the case of the β and δ subunits, the reverse primer binds just outside the translated region, hence all amino acids in the mature proteins were determined.

RT-PCR products were subcloned into the pGEM-T Easy vector (Promega, Madison, WI, USA) and two or three isolates from each of the four subunit-specific cDNA clones were sequenced on both the positive and negative strands. Automated sequencing was performed using BigDye3 (ABI, Foster City, CA, USA) sequencing mix and run on an ABI377 DNA Sequencer. Sequence data were analyzed using MacVector software, and sequence alignments created in ClustalW. All sequences have been deposited in GenBank (accession numbers: betaAChRTmar AY472103; gammaAChRTmar AY472104; deltaAChRTmar AY472105; alphaAChRHmon AY472106; betaAChRHmon AY472107; gammaAChRHmon AY472108; deltaAChRHmon AY472109; alphaAChRNtas AY472110; deltaAChRNtas AY472111). The *T. marmorata* α subunit sequence has

previously been published (accession no. J00963; Noda et al., 1982) and its sequencing here serves as a positive control for the RNA extraction and RT-PCR procedures.

Synthesis of the TDAC affinity ligand

4,7,10-trioxa-1-trityl,13-tridecanediamine (compound 1)

To 170 g of 4,7,10-trioxa-1,13-tridecanediamine (0.77 moles) dissolved in 500 ml of tetra-hydro furan (THF) was added 15.6 g of triethylamine (0.154 moles). To this solution was added 25 g (0.077 moles) of trityl bromide dissolved in 250 ml of THF *via* a drop funnel over a 1 h period. The solution was allowed to stir for 4 h and solvent removed *in vacuo*. The remaining liquid was dissolved in EtOAc and washed with 3×500 ml of water, and the organic phase was filtered through anhydrous magnesium sulfate and dried *in vacuo*. The resultant product gave a single spot on thin layer chromatography (TLC) at an Rf of ~0.2 in 80:20 IPA:EtOAc. The yield was 95%.

4,7,10-trioxa-1-trityl,13-carboxyethyl-2-bromide-tridecanediamine (compound 2)

Compound 1 (33.91 g/0.0733 moles) was dissolved in 300 ml of dry Et_2O and cooled to 0°C. 5.8 g (0.0733 moles) of pyridine was added dropwise and then 13.6 g (0.0733 moles) of ClC(O)Et-Br was added dropwise with stirring. After the completion of this addition, the solution was allowed to stir for 1 h and warm to room temperature. The ether solution was extracted with 500 ml of water and washed with an additional 3×500 ml of water. The organic layer was filtered through anhydrous magnesium sulfate, and solvent was removed *in vacuo*. The product showed a single spot by TLC and was used directly in the next step.

4,7,10-trioxa-1-trityl,13-carboxyethyl-2-trimethylamine-tridecanediamine (compound 3)

Compound 2 was dissolved in toluene (200 ml), and 10 ml of liquid trimethylamine was bubbled through the solution *via* a cannular. The reaction mixture immediately turned cloudy as a white precipitate appeared. The reaction was allowed to stir overnight and solvent removed *in vacuo*. The resultant syrup was dissolved in MeOH, a slurry was made with silica gel and the excess MeOH was removed *in vacuo*. The silica slurry was washed with EtOAc to remove all excess starting material and then extracted with EtOH in a soxhlet extractor. Yield was 98% from compound 2.

4,7,10-trioxa-1,13-carboxyethyl-2-trimethylamine-tridecanediamine (TDAC)

The trityl group was removed by treatment with toluene sulfonic acid in EtOH and monitoring with TLC. The product was extracted into water and washed with $CHCl_3$ to remove

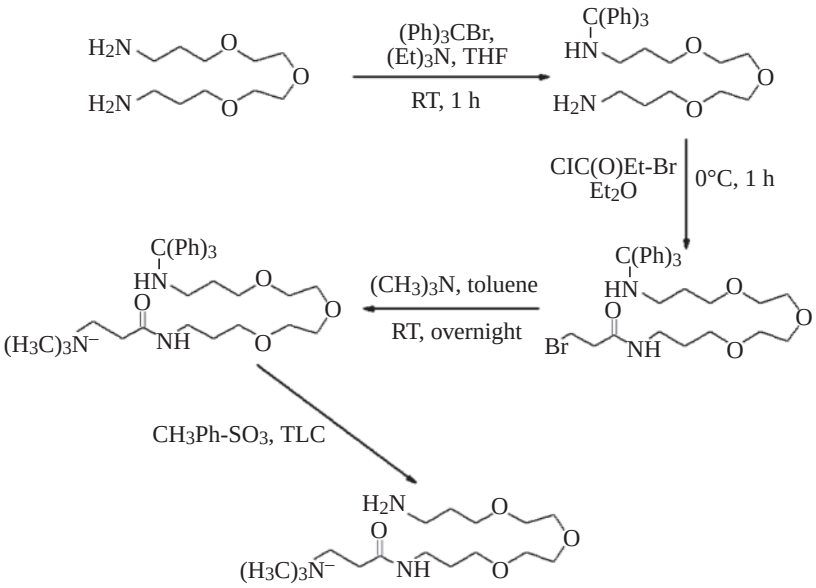

Fig. 1. Synthesis of 4,7,10-trioxa-1,13-carboxyethyl-2-trimethylamine-tridecanediaamine (TDAC).

trityl-OH and then lyophilized to yield essentially pure TDAC (Fig. 1). Yield was 99%. The product showed a M^{2+} mass of 174.60 compared with the expected 174.65. The lyophilized product was used directly in coupling to *N*-hydroxysuccinimide-activated Sepharose fast flow (NHS-FF; Amersham Pharmacia Biotech, Piscataway, NJ, USA), as per the manufacturer's instructions, to produce the TDAC affinity column.

Purification of the acetylcholine receptor using a novel ligand affinity column

The acetylcholine receptor was purified from the electric organ of the three southern ocean electric rays (*T. macneilli*, *H. monopterigium* and *N. tasmaniensis*) and from *T. marmorata* from the North Atlantic. Each tissue was treated identically for comparative purposes. Frozen tissue (50 g) was thawed in 150 ml of ice-cold homogenization buffer (buffer A: 20 mmol l^{-1} Na phosphate, pH 7.4, 400 mmol l^{-1} NaCl). The tissue was homogenized in a blender (high speed, 2 min), and insoluble material removed by centrifugation at 6000 ***g*** for 10 min. The supernatant was filtered through cheese-cloth, 75 μl of a protease inhibitor cocktail was added (stock solution: 1.0 mg ml^{-1} leupeptin, 1.0 mg ml^{-1} pepstatin, 50 mg ml^{-1} PMSF) and then re-centrifuged to pellet the membranes (150 000 ***g***, Ti45 rotor, 30 min, 4°C). Membrane pellets were weighed and resuspended in 10× vol./mass buffer B (20 mmol l^{-1} Na phosphate, pH 7.4, 80 mmol l^{-1} NaCl) plus protease inhibitors (1.0 μl ml^{-1}). Triton X-100 was added to a final concentration of 1.5% and the solution left stirring on ice for 60 min. Solubilized protein was recovered following centrifugation in a Ti45 rotor at 150 000 ***g*** for 30 min at 4°C, and following a twofold dilution with water the supernatant was loaded directly onto the TDAC affinity column (4.0 ml). The chromatography was performed at 4°C on an Akta

Explorer Chromatography workstation (Amersham Pharmacia Biotech). Buffers for the chromatography contained 0.2 mmol l^{-1} Brij35 instead of Triton X-100, enabling detection of protein by absorbance at 215 and 280 nm. Bound receptor was eluted from the TDAC resin using an NaCl gradient from 0.04 mol l^{-1} to 1.0 mol l^{-1} over 15 column volumes. Buffer A: 20 mmol l^{-1} Na phosphate, pH 7.4, 0.2 mmol l^{-1} Brij35. Buffer B: 20 mmol l^{-1} Na phosphate, pH 7.4, 0.2 mmol l^{-1} Brij35, 1.6 mol l^{-1} NaCl. Using a flow rate of 4.0 ml min^{-1}, the chromatography step was completed within 80 min.

Protein eluted from the TDAC column was diluted fourfold in buffer containing 20 mmol l^{-1} Na phosphate, pH 7.4, 0.2 mmol l^{-1} Brij35 and applied to a 1.0 ml ResourceQ anion exchange column (Amersham Pharmacia Biotech). Protein was eluted from this column using a 0.04–1.0 mol l^{-1} NaCl gradient over 40 column volumes generated with the same buffers used for the TDAC affinity chromatography and run at 4.0 ml min^{-1}. Gel permeation chromatography was performed on a Superdex200 column and run at 0.2 ml min^{-1} in buffer containing 20 mmol l^{-1} Na phosphate, pH 7.4, 0.2 mmol l^{-1} Brij35, 80 mmol l^{-1} NaCl. The system was calibrated using the gel permeation protein standard markers from BioRad (Hercules, CA, USA), which contain a mixture of proteins ranging in molecular mass from 1.35 to 670 kDa.

Equilibrium binding of ^{125}I-α-bungarotoxin to detergent-purified acetylcholine receptors

Radio-ligand binding experiments were carried out essentially as described previously (Schmidt and Raftery, 1973), using the rapid filtration method through anionic DE-81 filters to bind receptors and wash away unbound ligand. Purified receptor was diluted into buffer containing 20 mmol l^{-1} Na phosphate, pH 7.4, 80 mmol l^{-1} NaCl, 1.5% Triton X-100 and used in binding studies at a final concentration of 6.6 μg ml^{-1}. Each of the four different receptor preparations was tested at eight toxin concentrations ranging from 0.5 to 75.0 nmol l^{-1} α-bungarotoxin (α-Bgt). Unlabeled toxin was diluted to a final concentration of 250 nmol l^{-1} and spiked with 3-[^{125}I]iodotyrosyl-α-bungarotoxin (Amersham Pharmacia Biotech; specific activity 74 TBq mmol l^{-1}). Each concentration was tested in triplicate and the assay repeated. To determine the level of non-specific binding arising from the filters or the protein, no protein was added or a 100-fold excess of cold α-Bgt was added, respectively. No significant difference was seen in the level of counts between these two experiments, indicating that, at the protein concentration used, non-specific binding was due to the filters. Specific binding was calculated by subtracting non-specific binding from the total binding for each radio-ligand concentration tested. The data were fitted to the equation for a single binding site by least square non-linear regression using the program Prism™ (version 1.03; GraphPad Software, San Diego, CA, USA). The equation used was: $Y=B_{max}\times X/(K_d+X)$, where B_{max} is the maximum binding, X is the concentration of α-Bgt and K_d is the dissociation constant.

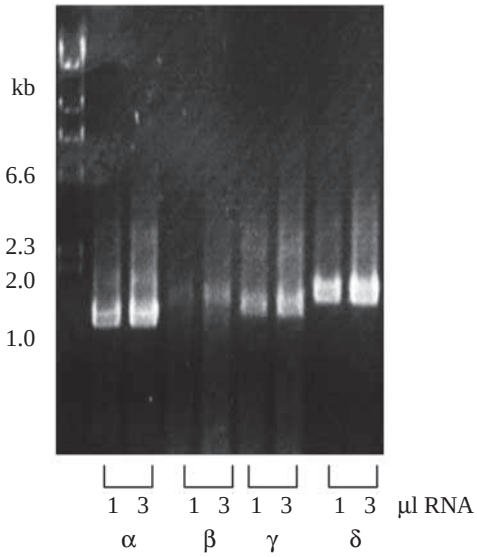

Fig. 2. RT-PCR products generated using specific primers for acetylcholine receptor genes amplified from *H. monopterigium*. RNA isolated from the electric organ of the coffin ray was reverse transcribed and amplified using primers specific for either the α, β, γ or δ genes of the acetylcholine receptor. Two RNA concentrations were tested with each primer pair, and RT-PCR products were run on a 0.8% agarose gel and stained with ethidium bromide. Lambda DNA cut with *Eco*R1 and *Hin*dIII was used to estimate the size of the DNA fragments (left-hand lane).

Electron microscopy

Copper grids (TAAB, 400 mesh) on a formvar film were coated with a thin layer of carbon and glow discharged for 1–2 min immediately prior to use. Protein samples (2–5 μl) were added to the carbon-coated grids and stained with 3% uranyl acetate. Samples were viewed using a Hitachi 7100 electron microscope operating at 75 kV. Specimens were viewed under the electron microscope at 30 000–40 000× magnification. Images of the TDAC affinity-purified material were acquired using an SIS Megview III Widefield CCD camera and saved as 16-bit images at 100 000–150 000× magnification using the software analySIS (Soft Imaging Systems, Munster, Germany).

Protein detection and quantification

Protein components were identified and their purity checked on sodium dodecyl sulfate–polyacrylamide (SDS–PAGE) gels. Samples were run on NuPAGE 4–12% Bis-Tris polyacrylamide gels (Invitrogen, Carlsbad, CA, USA) using MES (morphino-ethane sulfonic acid) as the buffer. Each sample was diluted twofold in electrophoresis sample buffer (5% SDS, 500 mmol l^{-1} dithiothreitol, 6 mol l^{-1} urea, 62 mmol l^{-1} Tris, pH 6.8, 10% glycerol, 0.002% bromophenol blue) and left at 37°C for ~2 h prior to electrophoresis. Gels were stained in Coomassie brilliant blue (R350).

Protein concentrations were determined using the Pierce BCA protein assay (Pierce, Rockford, IL, USA) in accordance

Table 1. *Amino acid sequence identity between acetylcholine receptor subunits from different species*

		T. californica				Human				
Species	Subunit	α	β	γ	δ	α	β	γ*	ε*	δ
T. marmorata	α	**99**	39	32	33	**78**	34	31	27	33
	β	39	**96**	37	34	38	**54**	39	35	38
	γ	32	35	**97**	46	32	38	**54**	**53**	47
	δ	32	34	46	**98**	32	35	42	39	**57**
H. monopterigium	α	**98**	39	32	33	**79**	34	30	27	33
	β	38	**88**	38	36	38	**54**	40	35	39
	γ	30	38	**94**	46	32	39	**53**	**53**	46
	δ	32	34	45	**95**	32	35	43	39	**58**
N. tasmaniensis	α	**96**	39	32	32	**78**	34	30	27	33
	δ	32	34	46	**98**	32	35	42	39	**57**

Values represent the percentage identity at the amino acid sequence level between the acetylcholine receptor subunits from *T. marmorata*, *H. monopterigium* and *N. tasmaniensis*, compared with the equivalent proteins from *T. californica* and humans.

*The acetylcholine receptor γ and ε subunits, present at the fetal and adult human neuromuscular junction, respectively, are equally homologous to the γ subunit in electric fish, representing a hybrid of the two.

with the manufacturer's instructions and employed bovine serum albumin (BSA) as the standard.

Results

Sequencing of acetylcholine receptor cDNAs from electric rays

The primers listed in the Materials and methods were used to amplify acetylcholine receptor cDNAs from electric rays. RT-PCR products were readily generated from RNA purified from tissue isolated from the electric organ of freshly killed fish whose tissue had been either flash frozen in liquid nitrogen or immersed in RNA-Later (Ambion, Austin, TX, USA) and then flash frozen. These methods were used for *H. monopterigium* and *T. marmorata*, respectively (Fig. 2). Tissue from the remaining two southern ocean electric rays was extracted from dead specimens packed in ice, approximately 12–18 h post capture. Using the same RNA isolation and RT-PCR procedures it was not possible to amplify any products from tissue extracted from *T. macneilli* specimens. However, two of the four acetylcholine receptor cDNAs could be amplified from the numbfish *N. tasmaniensis* after increasing the $MgSO_4$ concentration. No other changes to the reaction conditions, e.g. altering annealing temperatures or using random hexa-peptide primers, resulted in PCR products and it was concluded that RNA degradation following the fish's death was most likely to be the reason.

Alignment of the northern hemisphere *T. marmorata* and southern hemisphere *H. monopterigium* and *N. tasmaniensis* acetylcholine receptor protein sequences with the sequences previously determined from the related electric ray *T. californica* and the equivalent human sequences was performed using the program ClustalW. Table 1 displays the resultant sequence identity scores. Across the fish species, each corresponding subunit is highly conserved (94–99% identity). The only subunit that departs slightly from this trend is the β subunit from *H. monopterigium*, in which the sequence identity falls to 88% compared with the equivalent subunit from *T. californica*. The identity between non-equivalent pairs of subunits from the electric fish sequences ranges from 32 to 46%.

The degree of sequence conservation between the non-equivalent subunit pairs is similar when this comparison is made with the subunits from human muscle; for example, *T. marmorata* α and *T. californica* δ, 33% identity; *T. marmorata* α and human δ, 33% identity. Comparison of the corresponding subunits from fish and humans reveals a striking conservation of the α subunits (~78%), which is significantly higher than that seen between the other pairs of equivalent subunits (53–58%). Finally, of note is the slightly higher sequence identity observed between γ and δ subunits from the different species (around 46% identity). This trend is seen in all species comparisons and reflects their divergence, in evolutionary terms, from an intermediate species (Ortells and Lunt, 1995). It should be noted that many of the substitutions between corresponding and between non-equivalent subunits are conservative, thus tending to further relate the polypeptides.

The natural variation in the acetylcholine receptor observed between species may provide information about structural and functional constraints placed on the amino acid sequences of these receptors. In order to view the distribution of amino acid changes, Table 2 groups these differences relative to *T. californica* into those that lie in the extracellular or pore-forming domains of the receptor. Using the atomic structure of the analogous acetylcholine binding protein (Brejc et al., 2001) as the framework, the amino acid differences have been grouped into those that lie in the β-strands and α-helices or the loops of the extracellular domain. The distribution of those that lie in the transmembrane α-helices as opposed to the

Table 2. *Classification of the amino acid differences in the mature subunits of the acetylcholine receptor into domains and then further into secondary structure elements when compared with* T. californica *subunits*

Amino acid differences relative to *T. californica*						
			Extracellular domain		Pore-forming domain	
Species	Subunit	Total amino acid changes	β-strands	Loops/ α-helices	Transmembrane α-helices	Loops
T. marmorata	α	6	1	0	3	2
	β	18	3	8	7	0
	γ	12	1	4	5	2
	δ	13	5	2	4	2
H. monopterigium	α	7	4	0	0	3
	β	51	9	17	13	12
	γ	27	4	8	3	12
	δ	23	7	6	8	2
N. tasmaniensis	α	16	8	7	1	0
	δ	13	5	2	4	2

intervening loops of the pore-forming domain is based on the latest structural information from cryo-electron microscopic images of the acetylcholine receptor isolated from *T. marmorata* (Miyazawa et al., 2003). When these data are viewed in this context, it is apparent that the amino acid sequence differences are uniformly distributed between both the secondary structure elements and between the two domains. As might be expected for members of the same genus, the number of amino acid differences between equivalent *T. marmorata* and *T. californica* proteins is low. For example, there are just six differences in the α subunits and all are conservative amino acid changes (except G254V) resulting from single nucleotide changes (S66N, G254V, I315V, D342N, V347L, S448C). The greatest number of differences observed with respect to *T. californica* is in the β subunit from *H. monopterigium*, which carries 51 amino acid changes. Overall, the majority of amino acid differences observed between *T. californica* and the newly sequenced electric ray subunits are conservative in nature, including those that fall within the ligand-binding loops and the transmembrane α-helices. On the other hand, of the residues that are conserved, it is interesting to note that the penultimate residue in the δ subunit, a cysteine residue, is present in all electric ray species sequenced to date.

Affinity purification of functional, native acetylcholine receptors

The membrane-containing fraction isolated by differential sedimentation from the fish electric organs was resuspended in buffer containing Triton X-100, and the resulting solubilized protein was run through an affinity column specific for intact acetylcholine receptors (Fig. 3A). A salt gradient (0.4–1.0 mol l^{-1} NaCl) was used to elute bound receptors from the affinity resin, and a single peak was observed. This peak contained receptor that was 90–95% pure as judged from Coomassie-stained gels run under reducing conditions (Fig. 4). The assignment of protein bands corresponding to each of the four acetylcholine subunits was confirmed using specific antibodies in western blots (Tierney and Unwin, 2000; data not shown). As expected, the migration pattern of the four subunits was identical to that determined previously for the acetylcholine receptor subunits isolated from the torpedo ray (reviewed in Conti-Tronconi and Raftery, 1982). The protein band migrating between the α and β subunits most likely represents the α subunit, as it is often seen to run as a doublet when purified from native sources. The only major contaminating protein in the affinity-purified material of all the fish preparations is most likely one of the subunits from a Na^+/K^+-ATPase. This rationale was based on its electrophoretic mobility (Fig. 4, migrating just below the 98 kDa marker) and its reported contamination of all other preparations using the electric organ as its source of receptor protein (e.g. Waser et al., 1989). This protein was removed effectively on a Q-sepharose anion exchange resin, where it eluted later than the receptor, as shown in Fig. 3B. The purified receptor fraction ran as a single peak on a Superdex200 gel permeation column, suggesting that the fraction is homogeneous (Fig. 3C). Its elution, close to the void volume of the column (670 kDa molecular mass marker), was consistent with the native receptor being a dimer.

The receptors isolated from each of the four fish species behaved identically on the chromatography system, with the only difference being in the amount of protein purified. Starting with the same amount of electric organ (i.e. 50 g), the largest quantity of purified receptor was isolated from *T. marmorata* (6.9 mg) followed by *T. macneilli* (4.0 mg) and *H. monopterigium* (4.0 mg); interestingly, the lowest yield was from the smallest specimen, *N. tasmaniensis* (3.0 mg). The binding capacity of the resin was not exceeded in any of the purification runs as judged by the lack of immunoreactive protein in the unbound and wash fractions when examined by western blotting using antisera specific for the α subunit (data not shown).

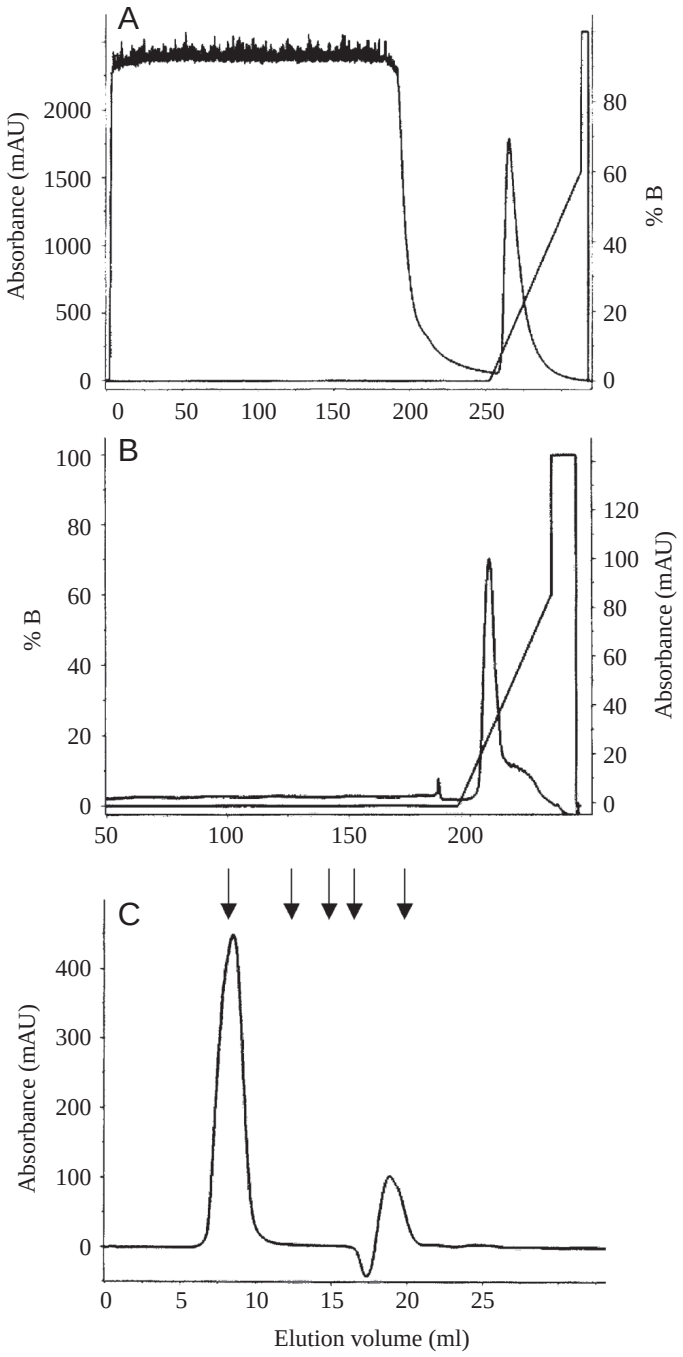

Fig. 3. Purification of the acetylcholine receptor from the numb fish *N. tasmaniensis*. (A) The membrane fraction from the electric organ was treated with 1.5% Triton X-100, and solubilized material applied to the TDAC affinity column. Bound protein was eluted from the column using an increasing salt gradient. (B) The major contaminant (~98 kDa) could be purified away from the acetylcholine receptor on an anion exchange column (ResourceQ), where it eluted later in the NaCl gradient. (C) The purified receptor ran close to the void volume on a Superdex200 gel permeation column (670 kDa), consistent with the receptor maintaining its dimeric form under the purification conditions used. Arrows indicate the elution positions of molecular mass markers used to calibrate the column: 670, 158, 44, 17 and 1.35 kDa. Absorbance was measured at 215 nm in A and C and at 280 nm in B.

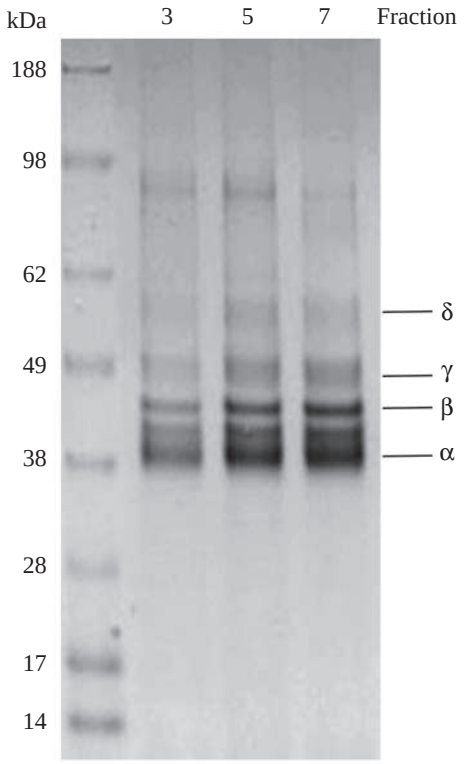

Fig. 4. SDS–PAGE of the acetylcholine receptor purified from the numb fish *N. tasmaniensis* on the TDAC affinity column. Fractions 3, 5 and 7, correlating to the peak absorbance in Fig. 3A, were run on a 4–12% Bis-Tris NuPAGE gel and stained with Coomassie. The protein bands corresponding to the four subunits comprising the acetylcholine receptor are indicated on the right. The protein running between the α and β subunits is mostly likely the α subunit, which is known to run as a doublet.

Equilibrium binding of purified acetylcholine receptors to ^{125}I-α-bungarotoxin

Although the acetylcholine receptor was purified on an agonist affinity resin, the binding properties of the detergent-purified receptors were tested further in α-bungarotoxin equilibrium binding assays in order to obtain dissociation constants for comparison with alternative purification procedures and with receptors from different species. Fig. 5 shows the results of a typical binding experiment in which the line represents the best fit of the data to the equation for a single binding site. The detergent-solubilized and affinity-purified acetylcholine receptors all exhibited values for K_d in the nanomolar range for the snake neurotoxin. The dissociation constants derived from the data for the individual species were not significantly different from each other (Table 3). Dissociation constants for α-bungarotoxin in the low nanomolar range have been reported previously for *T. californica* receptors assayed *in situ* from membrane preparations (Chang et al., 1977; Vandlen et al., 1976), detergent-purified preparations (Waser et al., 1989) and recombinantly expressed acetylcholine receptors (Sine, 1997).

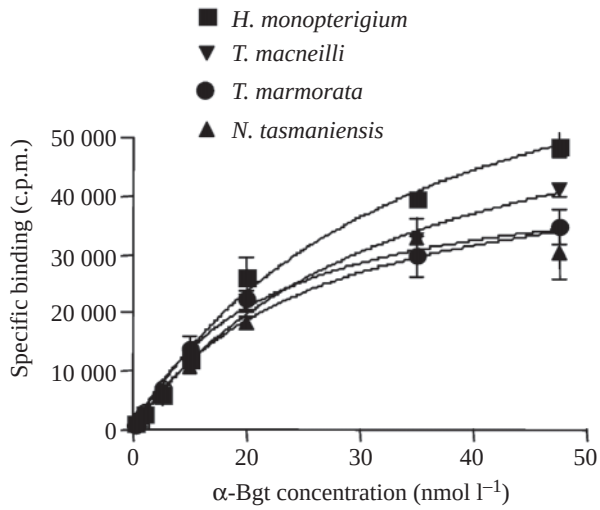

Fig. 5. A rapid filtration method through DE-81 filters was used to measure the binding of ^{125}I-α-bungarotoxin (α-Bgt) to affinity-purified acetylcholine receptors from electric rays at equilibrium. The data presented are from a typical experiment where the specific binding is plotted against increasing α-Bgt concentrations. The dissociation constants calculated from the equation for a single binding site were not significantly different in the four electric rays tested, ranging between 21 and 53 nmol l^{-1}. Error bars represent S.E.M.

Electron microscopy of acetylcholine receptors purified from fish electric organs

The detergent-solubilized and affinity-purified acetylcholine receptors from the electric rays were viewed in negative stain under the electron microscope. All exhibited the distinctive rosette-like structure with a central stain-filled pore that is typical of ligand-gated ion channels (Brisson and Unwin, 1985). Furthermore, the acetylcholine receptors isolated not only from *T. marmorata* but also those isolated from all three southern ocean fish species remained as dimers when purified under these conditions (Fig. 6).

Discussion

The acetylcholine receptor subunits from *T. marmorata* and its southern ocean counterparts *H. monopterigium* and *N. tasmaniensis* show a high degree of conservation at the amino acid sequence level with all other known acetylcholine receptor subunit sequences. The infrequent amino acid differences observed between equivalent subunits in the taxa sampled were randomly distributed throughout the molecule and outside the known critical sequence elements. The greatest degree of conservation is observed between the α subunit sequences, which is perhaps not surprising as these subunits are the major participants in both ligand binding and in the transmission of this signal to the pore domain (Miyazawa et al., 2003; Unwin et al., 2002). The molecular basis for acetylcholine binding by the receptor has been investigated extensively using mutagenesis in conjunction with measurements of ligand binding or receptor function (reviewed by Corringer et al., 2000; Prince and Sine, 1997). Such studies lead to the formation of a multi-segment binding site model (Galzi and Changeux, 1995) in which residues from the α subunits form the principle binding segments, including most of the docking site of the quaternary ammonium group of acetylcholine (Zhong et al., 1998). Detailed structural information has been obtained from cryo-electron microscopy studies of the receptor from *T. marmorata* imaged in the closed (unbound) and open (bound) states (Miyazawa et al., 2003; Unwin et al., 2002). The structural change that occurs upon ligand binding, which entails a switch in conformation of the two α subunits, is proposed to act as the trigger that opens the gate in the membrane-spanning pore. Hence, the α subunits may have evolved under tighter functional constraint than the β, γ and δ subunits, which do not appear to move significantly upon agonist binding. The similarity in the binding affinities for α-bungarotoxin, as measured in purified acetylcholine receptors isolated from the four electric fish, is concordant with the high level of sequence homology displayed by the α subunits.

Table 3. *Affinity constants for ^{125}I-α-bungarotoxin binding by purified acetylcholine receptors from different species*

Species	K_d (nmol l^{-1})
T. marmorata	21±4
T. macneilli	40±6
H. monopterigium	53±15
N. tasmaniensis	28±7

Average results are expressed as the mean ± S.E.M. of six determinations. The K_d values were not significantly different from each other (P<0.05), calculated using a paired Student's *t*-test.

A cholinergic affinity resin was used to purify intact acetylcholine receptors following their solubilization from the membrane fraction of the fish's electric organ. The resin constitutes a chemically modified carbamylcholine molecule, in which the ester has been substituted by an amide, thus eliminating the problem associated with hydrolysis of this ester bond. Early attempts to affinity purify the receptor using carbamylcholine as the immobilized ligand were hampered by this problem (Vandlen et al., 1976). The hydrolysis of the carbamylcholine off this resin was attributed to the actions of acetylcholine esterase, which is known to hydrolyse carbamylcholine and is present in significant amounts in the detergent extracts from electric fishes. The interaction between the immobilised TDAC ligand and the acetylcholine receptor, while being specific, is relatively easy to disrupt with an increasing NaCl gradient (160–320 mmol l^{-1} NaCl; Fig. 3A). It has long been established that the binding of ligands to the acetylcholine receptor is sensitive to the presence of cations (Akk and Auerbach, 1996; Schmidt and Raftery, 1974). Therefore, it is perhaps not surprising that the receptor's interaction with the immobilised TDAC ligand is sensitive to the NaCl concentration. Indeed, the NaCl concentration must be optimised in order to ensure that the TDAC resin acts as an affinity column and not as a general anion exchange column.

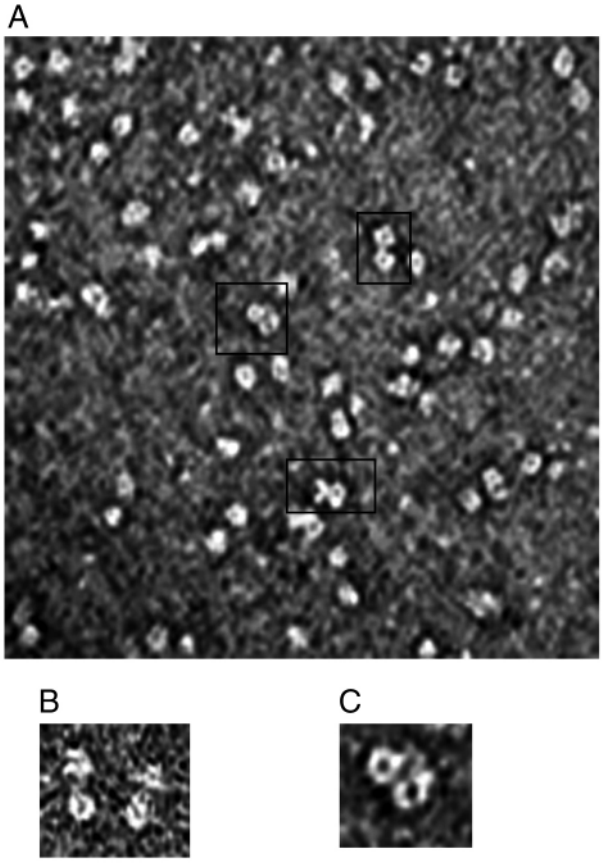

Fig. 6. Electron microscopic images of purified acetylcholine receptors from electric rays show them to be dimers. The affinity-purified acetylcholine receptor from *H. monopterigium* was viewed under the electron microscope in negative stain and is shown in A. Receptor dimers are evident in this (boxes in A) and all other acetylcholine receptor preparations isolated on the TDAC affinity column: (B) *T. marmorata*; (C) *N. tasmaniensis*.

Affinity chromatography of the acetylcholine receptor has traditionally employed either reversibly binding, acetylcholine analogues or the snake venom α-toxins immobilized to an inert support (e.g. Chang et al., 1977; Sobel and Changeux, 1977; Vandlen et al., 1976; Waser et al., 1989). However, the purification of the acetylcholine receptor proteins has been incomplete, the recovery of the receptors from the affinity columns was not satisfactory or the affinity matrices were hydrolyzable. Previous purification procedures have also encountered problems associated with proteolysis of the subunits and with the reduction of the inter-pentamer disulfide bond that leads to the presence of significant amounts of monomeric receptor. Both of these problems were exacerbated in many of the reports because of the length of time it took to perform the chromatography under manual conditions. An important advantage of our procedure is its automation, which in turn minimizes the time and controls the temperature during chromatography. Proteolysis was not evident in any of our receptor preparations as judged from western blotting. Furthermore, dimeric receptors were isolated under the conditions used as illustrated by electron microscopy and implied by the homogeneity of the purified receptor run on gel permeation chromatography. The acetylcholine receptors purified to date do not readily form crystals that diffract at high resolution. Native acetylcholine receptors obtained from southern ocean electric rays and rapidly purified on the TDAC affinity resin offer alternative sources from which to attempt crystallization trials.

The formation of the receptor dimer in *T. californica* is known to be the result of a disulfide bond forming between the penultimate cysteine residue of δ subunits in adjacent pentamers (Hamilton et al., 1979). This cysteine residue is conserved in all species examined here and it would appear that this mechanism of receptor pairing is present also in the southern ocean electric rays, suggesting that dimerization through a cysteine residue is a property of all acetylcholine receptors found in the electric organs of specialized fish. Functioning of the nicotinic synapse requires not only the intrinsic ion-gating properties of the acetylcholine receptors but also a critical density of acetylcholine receptor molecules (reviewed in Sanes and Lichtman, 2001). Cytoskeletal anchoring and clustering of receptor molecules in the plasma membrane occurs for all members of the ligand-gated ion channel family (reviewed in Colledge and Froehner, 1998). Their specialized organization and concentration are initiated through interactions with receptor-specific cytoplasmic proteins such as rapsyn, gephyrin and $GABA_A$ receptor associating protein (GABARAP). It is the interaction of these proteins with microtubules that anchors receptors at specific sites in the membrane. In addition to these constraining mechanisms, limiting the lateral diffusion of ligand-gated ion channels in the membrane, there is increasing evidence of a physical interaction between individual receptors. The clustering that occurs *via* such protein–protein interactions has been shown to occur between intracellular domains of $GABA_A$ receptor pentamers and has effects on both channel kinetics (Chen et al., 2000) and channel conductance (Everitt et al., 2004). The physiological role of the unique, additional clustering mechanism of acetylcholine receptors in the electric organ *via* an inter-pentamer disulfide bond remains to be elucidated. It may act, however, to stabilize receptor clusters, thereby facilitating synchronous gating of the ion channels, and provide a physical mechanism for regulating information flow by enhancing receptor–receptor cross-talk.

Phylogenetically, the acetylcholine receptor is highly conserved across species, whether it be stacked in the electric organs of fish or clustered at the neuromuscular junctions of vertebrates, including humans. The sequencing and functional characterization of the abundant acetylcholine receptors from both southern and northern hemisphere electric rays described here shows them to be a good model system for the pharmacologically important human receptor found at the neuromuscular junction.

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