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FEBS Letters 342 (1994) 97–102

FEBS 13829

FEBS LETTERS

Cloning and expression of the vesamicol binding protein from the marine ray *Torpedo*

Homology with the putative vesicular acetylcholine transporter UNC-17 from *Caenorhabditis elegans*

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Received 26 January 1994; revised version received 16 February 1994

Abstract

Complementary DNA clones corresponding to a messenger RNA encoding a 56 kDa polypeptide have been obtained from *Torpedo marmorata* and *Torpedo ocellata* electric lobe libraries, by homology screening with a probe obtained from the putative acetylcholine transporter from the nematode *Caenorhabditis elegans*. The *Torpedo* proteins display approximately 50% overall identity to the *C. elegans unc-17* protein and 43% identity to the two vesicle monoamine transporters (VMAT1 and VMAT2). This family of proteins is highly conserved within 12 domains which potentially span the vesicle membrane, with little similarity within the putative intraluminal glycosylated loop and at the N- and C-termini. The ~ 3.0 kb mRNA species is specifically expressed in the brain and highly enriched in the electric lobe of *Torpedo*. The *Torpedo* protein, expressed in CV-1 fibroblast cells, possesses a high-affinity binding site for vesamicol (K_d = 6 nM), a drug which blocks in vitro and in vivo acetylcholine accumulation in cholinergic vesicles.

Key words: Vesicular acetylcholine transporter; Vesamicol; *Torpedo* electric lobe; *Caenorhabditis elegans* UNC-17

1. Introduction

Acetylcholine is an excitatory neurotransmitter at the skeletal neuromuscular junction, within the autonomic nervous system, and in the brain of vertebrates. In cholinergic nerve endings, acetylcholine is stored and concentrated in the synaptic vesicles [1–3]. The characteristics of acetylcholine transport have been well studied in isolated synaptic vesicles which can be prepared in high yield and purity from the electric organ of the marine ray *Torpedo* [1,4–10]. Acetylcholine transport and storage in this preparation depends on a transmembrane proton-electrochemical gradient maintained by an electrogenic vacuolar-type H^+-ATPase [9–12]. Chromaffin vesicle accumulation of biogenic amines has also been shown to depend on this gradient [13]. Thus, a common bioenergetic mechanism exists for accumulation of the positively charged neurotransmitters acetylcholine and biogenic amines in acidic intracellular organelles by proton exchange [14,15].

Vesamicol (±)*trans*-2-(4-phenylpiperidino) cyclohexanol (also known as AH5183) is a drug that blocks cholinergic neurotransmission by interfering with vesicular acetylcholine storage in vivo [16,17]. Its action involves inhibition of acetylcholine uptake into synaptic vesicles by binding to a single population of sites on the cholinergic vesicle [18,19]. Pharmacological studies on the interaction of acetylcholine and vesamicol with *Torpedo* vesicles and vesicle membrane preparations suggest that the vesamicol binding site is within the acetylcholine vesicular transporter [19,20], although a single polypeptide binding both vesamicol and acetylcholine has not yet been identified.

cDNAs encoding the vesicle monoamine transporters VMAT1 and VMAT2 have been cloned and expressed in heterologous mammalian cells. This has allowed unequivocal demonstration that high-affinity ATP-dependent uptake of biogenic amines and binding of specific

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SSDI 0014-5793(94)00214-G

inhibitors to the transporter are reconstituted by expression of a single polypeptide [21–25].

Recently, the cDNA corresponding to the gene responsible for the *unc-17* uncoordinated phenotype of *C. elegans* was cloned and sequenced [26]. The *unc-17* gene product (UNC-17) has been proposed as the vesicular acetylcholine transporter based on (i) its striking homology to the mammalian vesicle monoamine transporters, (ii) its expression in cholinergic nerve endings, and (iii) the observed defects in cholinergic skeletal muscle neurotransmission in *unc*-mutants consistent with the absence of vesicular cholinergic transport [26].

In the present work, we attempted to identify the UNC-17 homolog from *Torpedo*, a species in which the pharmacology of the vesicular acetylcholine transporter has been sufficiently well characterized to allow definitive analysis of the binding properties of the protein in a heterologous system.

2. Materials and methods

2.1. Cloning

A cDNA library from *Torpedo marmorata* electric lobe was constructed in bacteriophage λ vector (ZAP-cDNA synthesis kit, Stratagene). 6×10^5 plaques were plated, blotted onto duplicate nitrocellulose filters (Hybond N^+; Amersham) and screened with a random primed (Nonaprimer I labeling kit; Stratagene) ^{32}P-labeled *C. elegans unc-17* coding sequence (base 116 to 1,454; Genbank Accession No. L19621). Prehybridization (3 h) and hybridization (18 h; 10^6 cpm/ml) were performed at 45°C in a buffer containing 6 × SSPE; 20% deionized formamide; 2 × Denhardt's solution; 250 mg/ml herring sperm DNA (sheared and heat denatured). Non-specific hybridization was eliminated by three 15 min washes with 1 × SSPE; 0.1% SDS, at 45°C. Clones detected as positive through 3 successive platings were isolated as Bluescript II SK plasmids by in vivo excision with helper phage R408 (Stratagene).

A cDNA library was also constructed in a variation of the Okayama–Berg cDNA expression vector (pcdSP6/T7) from *Torpedo ocellata* (M.J. Brownstein, Laboratory of Cell Biology, NIMH). The library was subdivided (48 pools of 7,500 recombinants) and Southern blots of *Bam*HI restriction digests of plasmid prepared from overnight cultures were hybridized in a buffer containing 4 × SSC; 25% formamide; 5 × Denhardt's solution; 200 μg/ml tRNA, with a random-primed ^{32}P-labeled *C. elegans unc-17* coding sequence (base 206 to 1,291) at 45°C. The filters were washed in 3 × SSC; 0.1% SDS, at 60°C. Autoradiographs were analyzed using a BAS2000 phosphor-imaging system (Fuji Biomedical, Stamford, CT) after 12 h exposure. Pools of recombinants expressing longer hybridizing clones were then plated to isolate a single cDNA clone. Sequence analysis revealed this cDNA to lack a portion of the 5′ end including 20 N-terminal amino acids. The 5′ region of this cDNA was obtained by using the polymerase chain reaction on plasmid from the total cDNA library with a sense primer in the pcdSP6/T7 vector (GCCAGTGAATGGGTTGGAAA) and an antisense primer in the coding sequence of *T. ocellata* (CCGTTGGAAGGTATTGTGAT). This fragment (~ 400 bp) was then digested with *Pst*I which cuts in the vector and the cDNA portions of the PCR product, subcloned in pUC18 and sequenced.

2.2. Sequencing

The *Torpedo marmorata* cDNA was sequenced by using primers along both strands and Sequenase II according to the manufacturer instructions (USB, Amersham) and the *Taq* Dye Deoxy Terminator cycle sequencing kit (Applied Biosystems).

The *Torpedo ocellata* cDNA was sequenced by subcloning overlapping fragments into pUC18. DNA was amplified directly from individual colonies by PCR using primers flanking pUC18, purified on Magic PCR columns (Promega, Madison, WI), cycle sequenced using ABI dye primers, and analyzed on an ABI model 373A sequencer (Applied Biosystems, Inc., Foster City, CA). Sequence data were assembled using the Seqman program (DNASTAR, Madison, WI) and analyzed using DNA Strider [27]. Predicted transmembrane segments of the protein was estimated by hydropathy analysis [28].

2.3. Northern analysis

Poly(A)$^+$ RNA was purified by the guanidinium isothiocyanate/cesium chloride method followed by oligo-dT cellulose chromatography [29]. Poly(A)$^+$ RNA (6 μg per lane) was dissolved in 20 μl of a solution containing 2.2 M formaldehyde; 50% formamide; 20 mM MOPS; 5 mM sodium acetate; 1 mM EDTA (pH 7.0); 0.025% Bromophenol blue, and denatured at 60°C for 10 min. The RNA was electrophoresed in 1.0% agarose gels containing 2.2 M formaldehyde; 20 mM MOPS; 5 mM sodium acetate; 1 mM EDTA. After transfer to nitrocellulose membranes (Hybond C extra, Amersham) the RNA blots were baked at 80°C (3 h), prehybridized (3 h) and then hybridized with random primed ^{32}P-labeled *Torpedo marmorata* cDNA probe (10^6 cpm/ml) in a buffer containing 6 × SSPE; 50% formamide; 1 × Denhardt's solution; 250 μg/ml salmon sperm DNA (sheared and heat denatured) for 18 h at 45°C. The filters were washed in 0.1 × SSC; 0.1% SDS at 60°C and exposed to X-ray film with an intensifying screen at −70°C.

2.4. In situ hybridization histochemistry

Torpedo brains were removed and rapidly cut into coronal and sagittal pieces. The tissue was fixed overnight at room temperature in 100 mM sodium-phosphate buffer (pH 7.4), 300 mM NaCl, 4% paraformaldehyde and subsequently transferred to phosphate buffer containing 20% sucrose for at least 24 h at 4°C. Tissue pieces were then frozen in chilled isopentane on dry ice and 10 μm sections were cut on a Leitz cryostat, mounted on gelatin coated slides and stored at −70°C until use. An oligodeoxynucleotide (45-mer; GAGCATGTTGTCCAATAACATTGCTATGCACACGATGACAAGCAG) complementary to the coding region of *Torpedo marmorata* was synthesized on an Applied Biosystems 380A DNA synthesizer and labeled to a specific activity of $0.5–1 \times 10^9$ cpm/μg using terminal deoxynucleotidyl transferase (Boehringer) and [α-^{35}S]dATP. Sections were also hybridized with the full length *Torpedo marmorata* cDNA labeled by random-priming using [α-^{33}P]dATP. The sections were thawed at room temperature, fixed in 2% paraformaldehyde; 150 mM phosphate buffer (pH 7.4), rinsed twice with phosphate buffer, dehydrated in ethanol solution and air-dried. After prehybridation (3 h) in buffer containing 6 × SSPE; 1 × Denhardt's solution; 250 μg/ml salmon sperm DNA (sheared and heat denatured), hybridization was performed in buffer containing 4 × SSC; 50% formamide; 1 × Denhardt's solution; 250 μg/ml tRNA; 500 μg/ml salmon sperm DNA; 10 mM dithiothreitol and labeled probe (10^6 cpm/slide) for 16 h at 40°C in a humid chamber. Slides were washed in 0.1 × SSPE at 40°C, air dried and exposed to Hyperfilm β-max (Amersham) for 4 days at −70°C.

2.5. Recombinant vaccinia virus infection and transfection of CV-1 cells

Functional expression of cDNA's was performed using the vaccinia virus/bacteriophage T7 hybrid system [30,31] which enables high level expression of vesicle membrane proteins. The cDNA clones used were prepared as follows. The *Torpedo marmorata* (*Xho*I–*Not*I) and *unc-17* (*Eco*RV–*Xba*I) full-length inserts were subcloned into pcDNAI/Amp (Invitrogen). The human VMAT1 and VMAT2 clones were obtained from a human pheochromocytoma cDNA library in pcdm7/Amp (Erickson et al., unpublished). Monkey kidney fibroblasts (CV-1 cells) were cultured in Dulbecco's modified Eagle medium containing 10% fetal bovine serum, penicillin (100 U/ml), streptomycin (100 mg/ml) and glutamine (4 mM). Cells were plated at 2×10^6 per plate (10 cm) and infected the following day with a recombinant vaccinia virus encoding bacteriophage T7 RNA polymerase at a multiplicity of infection of 10. After 30 min the cells were transfected with plasmid DNA (1 μg/ml) by using Transfectace (10 μg/ml, Bethesda Research Laboratories). After 16 h the cells were washed with phosphate-buffered saline, harvested and homogenized (Dounce, type B pestle) in buffer containing 0.1 M sucrose; 10 mM Tris (pH 7.4); 5 mM EDTA; 6 μg/ml leupeptin; 10 μg/ml pepstatin; 1 mM phenylmethanesulfonyl fluoride; 5 μg/ml aprotinin, at 4°C. The suspension was centrifuged at 3,000 rpm for 10 min and the supernatant was centrifuged at 100,000 × g for 60 min. Membrane pellets were stored at −70°C until use.

2.6. [^{3}H]Vesamicol binding assay

Vesamicol binding was performed as described previously with minor modifications [32]. Briefly, membranes were thawed, suspended at 0.5–1 mg/ml and incubated under gentle shaking for 45 min at room temperature in the presence of 50 mM sodium-phosphate buffer (pH 7.4), 2 mM chaps and unlabeled vesamicol (Research Biochemicals Incorporated) or vehicle, and various concentrations of L-[^{3}H]vesamicol (49 Ci/mmol, New England Nuclear). Bound [^{3}H]vesamicol was measured by vacuum filtration on glass fiber filters. Non-specific binding in the presence of 30 μM of unlabeled ligand was subtracted from the total binding. Protein concentration was determined by the method of Lowry [33] in the presence of 1% SDS, with bovine serum albumin as a standard.

3. Results

3.1. Sequence analysis of the Torpedo cDNAs

Torpedo marmorata and *ocellata* cDNA libraries were screened under reduced stringency with a probe derived from the coding sequence of *Caenorhabditis elegans unc-17*. The nucleic acid sequence of the *ocellata* cDNA (Genbank Accession no. UO5339) predicts an open reading frame of 1,533 base pairs using as the initiation codon the first ATG after an in-frame termination codon. The initiation codon in the *marmorata* cDNA sequence (Genbank Accession No. UO5591) is not as clearly defined and has been taken to be the ATG homologous to the *ocellata* initiation codon. This choice of initiation codon is consistent with the position of the initiation codon in a homologous rat cDNA (unpublished data). The predicted *Torpedo* proteins are both 511 amino acids with a molecular mass of approximately 56 kDa. The amino acid conservation between *Torpedo* species is 98%. The *Torpedo* proteins are 50% identical to UNC-17 and 43% identical to the rat vesicle monoamine transporters VMAT1 and VMAT2. Hydrophobicity analysis predicted 12 transmembrane domains (TM 1-12). The highest sequence conservation between this family of proteins occurs within these assigned TM domains. TM domains 1, 4, 5 and 11 display the highest conservation between *Torpedo* and *C. elegans* with approximately 90% identity. *Torpedo* and *C. elegans* proteins are more similar to each other in TM domains 4 and 5 than they are to VMATs. TM domains 1, 2 and 11 of *Torpedo* are approximately 65% identical with VMATs.

The greatest divergence between the sequences of *Torpedo*, UNC-17 and the two forms of VMAT occurs in the large luminal loop located between the first two transmembrane domains and in the cytoplasmic N- and C-termini of these proteins (< 10% identity). Three po-

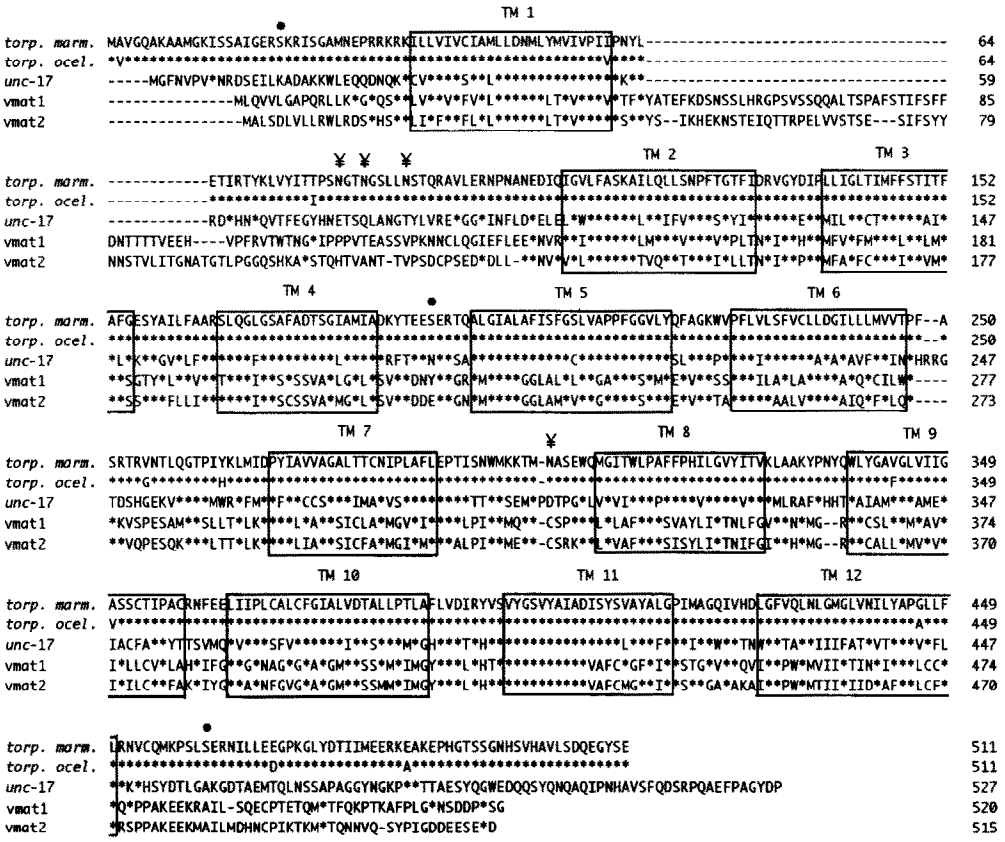

Fig. 1. Predicted amino acid sequence (single-letter code) of the *Torpedo marmorata* and *ocellata* proteins. (•) positions where the UNC-17 and the rat vesicle monoamine transporters VMAT1 and VMAT2 are identical to the *Torpedo marmorata* sequence. Gaps (−) are introduced to maximize alignment. Putative transmembrane domains (TM) 1–12 are boxed. (¥) potential N-linked glycosylation sites. (●) Potential protein kinase C phosphorylation sites.

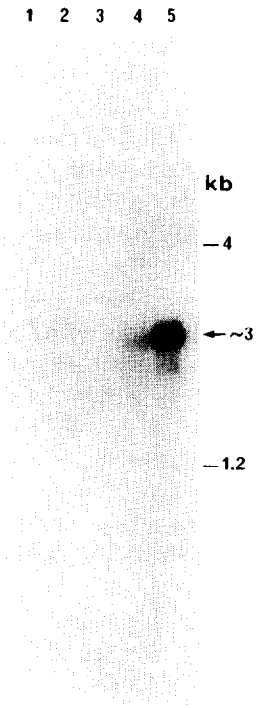

Fig. 2. Northern hybridization analysis of mRNA (6 μg) from different tissues of *Torpedo marmorata*: Lane 1, kidney; 2, liver; 3, electric organ; 4 and 5, electric lobe. Lanes 4 and 5 are different samples of RNA which underwent one or two cycles of purification on oligo dT-cellulose resin. Blots were washed under stringent conditions and a ~3 kb mRNA transcript was detected only in the electric lobe. Exposure time was approximately 4 days at −70°C with an intensifying screen.

tential sites for N-linked glycosylation for the *Torpedo* proteins are located within this luminal loop and one exists between TM domains 7 and 8. Three potential sites for phosphorylation by protein kinase C are located on the cytoplasmic face of the *Torpedo* proteins.

Within the assigned TM domains of the *Torpedo* proteins are located several charged amino acid residues. An aspartic acid residue is found in TM domains 1, 4, 6, 10 and 11 and a lysine residue is located in TM domain 2. Aspartic acids in TM 1, 6, 10 and 11 and lysine in TM domain 2 are conserved between these proteins, UNC-17 and both VMAT1 and VMAT2. The *Torpedo* and *C. elegans* proteins contain an additional aspartic acid residue in TM domain 4 which is not found in VMATs.

3.2. Tissue-specific mRNA expression

A ~ 3.0 kb mRNA species was identified in poly(A)$^+$ RNA from the electric lobe of *Torpedo* brain by Northern blot analysis (Fig. 2). Hybridization to the *Torpedo* cDNA was not observed in the electric organ, liver, or kidney. In situ hybridization histochemistry of *Torpedo* brain sections clearly showed intense labeling of the electric lobe (Fig. 3).

3.3. [^{3}H]Vesamicol binding

L-[^{3}H]vesamicol binding to membranes of *Torpedo marmorata*, *C. elegans unc-17*, human VMAT1 or VMAT2 transfected cells is shown in Fig. 4. Analysis of binding revealed affinity constants (K_d) of 6.4 ± 0.4 nM for *Torpedo* and 124.2 ± 7.9 nM for UNC-17. The specific binding was not different in membranes prepared from mock transfected cells (455.1 ± 75.2 nM) (data not shown) and those from VMAT1 or VMAT2 (430.1 ± 97.7 nM) expressing cells. While the infection and transfection efficiency of these experiments were not measured the apparent difference in the number of binding site between *Torpedo* and *C. elegans* may correspond to the efficiency of transfection, protein stability, or possibly the expression in different membrane compartments.

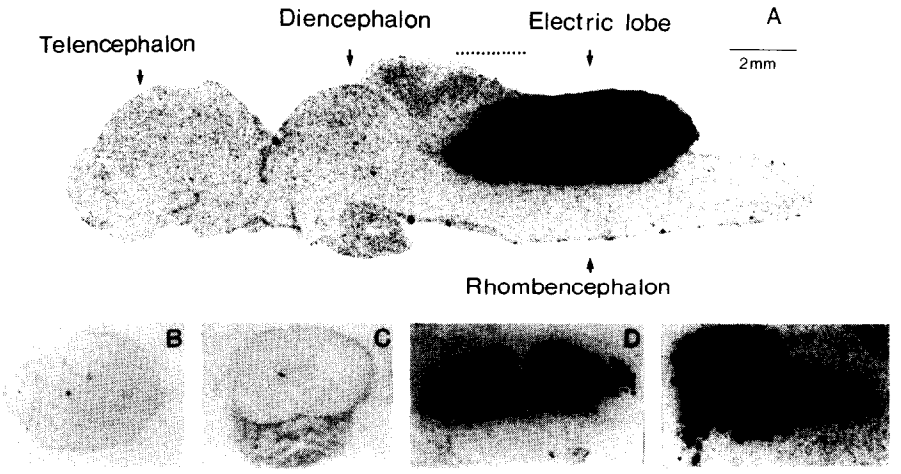

Fig. 3. Representative autoradiograms of in situ hybridization in the brain of *Torpedo marmorata*. (A) Coronal sections hybridized with an ^{35}S-labeled 45-mer oligonucleotide probe. Sagittal sections were hybridized with a ^{33}P-labeled cDNA probe and included telencephalon (B); mesencephalon (C); electric lobe and rhombencephalon (D); and (E) enlarged view of labeled electroneurons on a sagittal section. Levels are indicated on panel A.

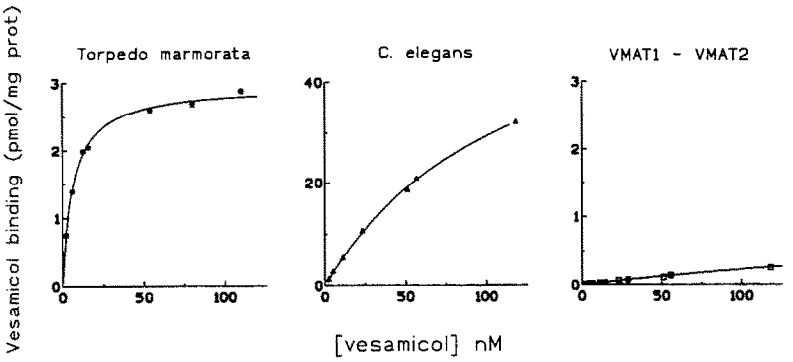

Fig. 4. L-[^{3}H]Vesamicol binding to membranes of *Torpedo marmorata*, *C. elegans unc*-17, human VMAT1 or VMAT2 transfected fibroblasts. Specific binding was measured as described in section 2. Data are the mean of duplicate determinations and the experiments were repeated once with similar results. ■, VMAT1; □, VMAT2.

4. Discussion

The electric lobe of the *Torpedo* abundantly expresses a messenger RNA encoding a protein with a high degree of homology to UNC-17, the putative vesicular acetylcholine transporter from *C. elegans* which was used to obtain the *Torpedo* cDNA by low-stringency screening of two *Torpedo* electric lobe cDNA libraries. This protein and UNC-17 possess significant homology to VMAT1 and VMAT2, mammalian proteins that mediate biogenic amine transport into acidic intracellular organelles via a proton electrochemical gradient. Sequence homology between members of this family exhibits strongest conservation within the transmembrane domains thought to be critical for substrate transport. In particular, there is absolute conservation of aspartic acid residues in the assigned transmembrane regions 1, 6, 10 and 11 which may be involved in binding of the cationic amines transported in cholinergic and aromatic aminergic secretory vesicles [24]. The vesicular transporters represent a distinct class of proteins found in membrane-bounded organelles that sustain proton gradients, and differ from the family of the neurotransmitter transporters that are found on plasma membranes (for review, see [34]).

Expression of the *Torpedo* protein in mammalian fibroblasts confers high-affinity vesamicol binding to membranes isolated from transfected cells similar to that described on synaptic vesicles (7–20 nM([19,35]. While the binding of vesamicol to synaptic vesicles from *C. elegans* has never been reported, UNC-17 expressed separately in the same heterologous system demonstrated specific vesamicol binding as well, with lower affinity (~124 nM) than that of *Torpedo*. It is tempting to speculate that amino acid substitutions at crucial points of the vesamicol binding site might explain the differences in binding affinity between the two species.

The mechanism of vesamicol inhibition of vesicular acetylcholine transport in vitro is not yet clearly understood. The inhibition constant of the transport of acetylcholine is similar to the dissociation constant of the drug, suggesting that binding and inhibition are directly related. However, inhibition is of the mixed non-competetive type [19,36] indicating that vesamicol does not bind to the acetylcholine uptake site. Two models to account for this data have been proposed by Parsons and co-workers [37]: (i) vesamicol binds to an allosteric site on the vesicular acetylcholine transporter or (ii) vesamicol binds to a different protein that acts by an unknown mechanism to inhibit acetylcholine uptake. The present work is the first direct demonstration that the vesamicol binding protein belongs to the family of the vesicular neurotransmitter transporters.

We conclude on the basis of these observations that the *Torpedo* cDNAs described here and *unc-17* gene encode the vesicular acetylcholine transporter/high-affinity vesamicol binding protein. Protein expression within a membrane fraction in a heterologous cell system with preservation of high-affinity vesamicol binding suggests that unequivocal demonstration of the role this molecule in acetylcholine transport via a proton electrochemical gradient in this system should ultimately be possible. This will provide a means of establishing the cellular basis for vesicular accumulation of the highly evolutionarily conserved neurotransmitter acetylcholine.

Acknowledgements: We would like to thank Dr. M. Israël and Dr. M. Brownstein for their advice and support. We are thankful to the Genethon and Ph. Millasseau (Evry, France) for their help in the sequencing of the *Torpedo marmorata* clone. We gratefully acknowledge L. Théraulaz and R. Charré for skillful technical assistance. This work was supported in part by a grant from DRET (No. 92/175) and by the Institut de Formation Supérieure Biomédicale (fellowship to H.V.).

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