ACR-26: a novel nicotinic receptor subunit of parasitic nematodes

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Abstract

Nematode nicotinic acetylcholine receptors are the targets for many effective anthelmintics, including those recently introduced into the market. We have identified a novel nicotinic receptor subunit sequence, *acr-26*, that is expressed in all the animal parasitic nematodes we examined from clades III, IV and V, but is not present in the genomes of *Trichinella spiralis*, *Caenorhabditis elegans*, *Pristionchus pacificus* and *Meloidogyne spp.* In *Ascaris suum*, ACR-26 is expressed on muscle cells isolated from the head, but not from the mid-body region. Sequence comparisons with other vertebrate and nematode subunits suggested that ACR-26 may be capable of forming a functional homomeric receptor; when *acr-26* cRNA was injected into *Xenopus* oocytes along with *X. laevis ric-3* cRNA we occasionally observed the formation of acetylcholine- and nicotine-sensitive channels. The unreliable expression of ACR-26 *in vitro* may suggest that additional subunits or chaperones may be required for efficient formation of the functional receptors. ACR-26 may represent a novel target for the development of cholinergic anthelmintics specific for animal parasites.

Keywords: anthelmintic, filaria, immunofluorescence, nicotinic receptor

1. Introduction

Control of parasitic nematode infections continues to rely on the use of chemical anthelmintics. Many of these compounds act at ion channels, including the nicotinic acetylcholine receptors (nAChRs) found at the nematode neuromuscular junction, on pharyngeal muscle and within the central nervous system [1,2,3,4]. Indeed, the two most recently introduced veterinary anthelmintics, monepantel [5] and derquantel [6], as well as tribendimidine, proposed for use against human infections [7], all act at nicotinic receptors [8,9,10]. Nematodes possess many nAChR genes, and these vary considerably between species, with several parasites having fewer than the model organism, *Caenorhabditis elegans* [11]. From *in vitro* studies that have reconstituted levamisole-sensitive receptors [12,13,14] it is clear than the subunit composition and pharmacology of the neuromuscular nAChR are different between parasites and *C. elegans*, and this has led us to search for novel nAChR sequences in the parasitic nematode, *Ascaris suum.* Such novel receptors may have potential as drug targets. This large worm was selected for these studies because the physiology and pharmacology of many of its nAChR can be studied *ex vivo* [15,16], and because the distribution of the receptor subunits can be studied in dissected tissues and on disassociated muscle cells.

We report here the identification and expression of a nAChR subunit sequence, *acr-26*, that is present in *A. suum* and orthologues of which are widely distributed in animal parasitic nematodes, but are absent from the genomes of several free-living and plant parasitic species.

2 Materials and Methods

2.1 Parasite Material

Adult *A. suum* were a kind gift of Prof A. Maule (Queen's University, Belfast) and Dr Richard Martin (Iowa State University). *Haemonchus contortus* L3 larvae of the drug sensitive ISE isolate were kindly supplied by Dr Philip Skuce (Moredun Institute).

2.2 Molecular cloning

An EST sequence (Accession number FE918510), derived from *A. suum* and showing significant identity to known nAChR subunit cDNAs, was identified in the database. 5′ and 3′ RACE reactions (primers; 5′ RACE, SL1 (GGTTTAATTACCCAAGTTTGAG) and ACR26-RV3 (AACGTTTATCGTCAACACCTG); 3′ RACE, anchor (GACCACGCGTATCGATGTCGAC) and ACR26-FW2 (TAATTATGTTGTGTCGGGTG) were carried out as described previously [12,17] to amplify the rest of the cDNA sequence. These partial products were cloned into pGEM-Teasy and sequenced. Specific primers (forward – ATGATGGCAACTCGTCGG; reverse – TTAATGCAGACCATATAAAGAC) were used to amplify a full-length sequence from *A. suum* cDNA (made from RNA extracted from the head region); this sequence was also cloned into pGEM-Teasy and sequenced. The sequence was deposited in the database under the Accession Number GU135625. An essentially identical procedure was used to amplify a full-length cDNA from *Haemonchus contortus*, which was deposited under Accession number EU006791.

In order to search for related sequences in cDNA from other species (*Cooperia oncophora, Ostertagia ostertagi* and *Teladorsagia circumcincta*) degenerate oligonucleotide primers were

designed based on the aligned sequences from *A. suum* and *H. contortus* and used to amplify partial sequences from the target organisms.

2.3 Immunofluorescence

A specific goat antiserum (Sigma-Genosys, USA) was raised against a synthetic multiple antigenic peptide, EIDGTATDEQKLLHLL, (Alta Biociences, UK) corresponding to the N-terminus of the mature ACR-26 polypeptide, essentially as described [12]. IgG was isolated from the serum by affinity chromatography over a CPG column to which the antigenic peptide had been immobilised, and the purified antibody used in immunofluorescence experiments in dissociated muscle cells isolated from the body wall and head regions. Adult *A. suum* were kindly provided by Prof, A. Maule (Queen's University, Belfast, UK) and were shipped and stored in Ascaris Ringer Solution (4mM NaCl, 5.9mM $CaCl_2$, 4.9mM $MgCl_2$, 5mM Tris-HCl pH7.4, 125mM sodium acetate, 24.5mM KCl). They were used within 24hrs of their arrival. The worms were pinned out on a dissection tray and injected at 3cm intervals with 5mg/ml collagenase 1A in ARS. After 2 hrs at 37° the cuticle was cut longitudinally and pinned flat at the head end. Disassociated head and muscle cells were removed independently with a Pasteur pipette and fixed in 5% (v/v) formaldehyde in ARS for 9hrs at 4°. The cells were washed three times in 0.1% (v/v) Triton X-100 in phosphate-buffered saline (PBS). The affinity-purified anti-ACR-26 was applied at a 1:200 dilution in PBS and the cells incubated with gentle agitation for 40hrs at 4°. Control cells were incubated with purified control goat IgG under the same conditions. The cells were washed three times in Triton X-100/PBS as before and then an FITC-conjugated rabbit anti-goat IgG (Sigma, Poole, UK, catalogue number F7367), diluted 1:200 in PBS, added for 4hrs at 4°. The cells were washed three times in Triton X-100/PBS before being

mounted in Mowiol 4-88 reagent (Polysciences, Inc, USA) and observed under a Zeiss LSM510 confocal microscope.

2.4 Functional Expression

The *acr-26* cDNA was subcloned into the BglII and SpeI sites of the pT7TS vector, which was linearised and transcribed into cRNA using the mMessage mMachine T7 kit (Ambion). The cRNA was injected into defolliculated *Xenopus* oocytes along with cRNA encoding the *X. laevis* orthologue of RIC-3 (Bennett et al., unpublished), which were screened for acetylcholine-gated channels as described [12].

3 Results

3.1 Cloning of acr-26 cDNA from parasitic nematodes

We successfully amplified a full-length cDNA encoding the novel nAChR subunit from *A. suum*, extending the EST sequence (Accession number FE918510) that had already been deposited in the database. We compared the sequence of the *A. suum* subunit with the other nAChR subunits from both *A. suum* and *C. elegans* (Figure 1). The results showed that the new subunit was not orthologous to any of those from *C. elegans* and we therefore named the new sequence *acr-26* to distinguish it from them. In order to determine whether *acr-26* was confined to *A. suum* or was present in other parasitic nematodes, we searched the partial genome sequence of *H. contortus* for similar sequences and used the results of that search to amplify a full-length orthologous sequence from this clade V parasite. Alignments of the Asu-ACR-26 and Hco-ACR-26 sequences (Figure 2) showed that they were very similar, and would almost overlap if both were plotted on the tree shown in Figure 1. Both subunits shared key amino-acid residues in loops that form the agonist binding sites with vertebrate α7 subunits, especially in the complementary loop D normally provided by β-subunits in heteromeric receptors, suggesting that ACR-26 may be able to form a homomeric nAChR [18]. Further BLAST searches (Table 1) revealed that *acr-26* like sequences are present in the filarial parasites *Brugia malayi*, *Dirofilaria immitis*, *Loa loa* and *Wuchereria bancrofti*, and *Strongyloides ratti*, but not in *Trichinella spiralis*, the plant parasitic *Meloidogyne spp.* or in the free living *Pristionchus pacificus*. When we searched other invertebrate phyla for ACR-26-like sequences, the best hits were with the nAChR subunits G and D from *Lymnaea stagnalis* [19], which shared 67% and 61% amino-acid identity with Asu-ACR-26, respectively. In order to investigate whether or not the gene is present in other trichostrongylid nematodes of economic importance, we amplified and sequenced partial *acr-26*

cDNAs from *C. oncophora*, *O. ostertagi* and *T. circumcincta*. The partial clones were sequenced and proved to possess high levels of identity to Hco-ACR-26, showing that this subunit is also expressed in these parasites (Figure 3). They are deposited in the sequence database under Accession numbers JN966888, JN966889 and JN966890.

3.2 Distribution of ACR-26 in Ascaris suum

In order to determine where in the parasite ACR-26 was expressed, we raised an antiserum against a synthetic peptide corresponding to the predicted N-terminal sequence of the mature polypeptide. Antibodies purified from this antiserum recognized an HA-tagged version of ACR-26 when this was expressed in mammalian cells, and when examined under confocal microscopy the anti-ACR-26 immunofluorescence completely overlapped with that produced by an anti-HA antibody (data not shown). We applied the purified anti-ACR-26 antibody to isolated muscle cells, derived both from the mid-body region and from the head. No specific staining of the body wall muscles was observed (Fig 4), but immunoreactivity was detected on the surface of the head muscle cells. This was consistent with the cloning of the *acr-26* cDNA from RNA isolated from the head region of the worm. No fluorescence was observed when preparations were treated with a control goat IgG.

3.3 ACR-26 forms a functional nicotinic receptor

The amino-acid sequence of ACR-26, specifically the conservation of residues in the loops forming the ligand-binding site with those present in vertebrate α7 subunits (Fig 1), suggested that it may be able to form a functional nAChR when expressed as a homomer. We therefore injected *Xenopus* oocytes with *Asu-acr-26* or *Hco-acr-26* cRNA and attempted to detect the

formation of Ach- and nicotine-sensitive channels. Expression of Asu-ACR-26 nAChRs was sporadic and unreliable, but on occasion channels were detected in response to the application of Ach and nicotine (Fig 5). These channels were extremely sensitive to Ach, with concentrations >100μM producing maximal responses; the unreliable expression of this receptor makes an accurate estimate of the EC_{50} for Ach very difficult but it was between 10 and 100nM. For nicotine the EC_{50} was 25μM (95% confidence limits 15-42μM), with a Hill coefficient of 1.66 ±1.29. Since ACR-26 is expressed on some muscle cells (Fig 4), it is possible that it co-assembles with other muscle nAChR subunits *in vivo*, but attempts to improve the reliability and reproducibility of *in vitro* ACR-26 expression by co-expression with *Asu-unc-29* or *Asu-unc-38* cRNAs were unsuccessful. No functional channels were detected in oocytes injected with *Hco-acr-26* cRNA.

4 Discussion

Nematodes encode a rich variety of nAChR and these continue to be exploited as effective targets for the development of new anthelmintic drugs [5,6,7]. We report here that many animal parasitic species possess a new gene, *acr-26*, that is not present in *C. elegans*, several other free living species or the *Meloidogyne* genus of plant parasites. In *A. suum*, the ACR-26 subunit is expressed in head, but not body-wall, muscle cells, and is capable of forming a homomeric receptor – though expression of this receptor is unreliable, which might indicate that further subunits are required for full activity *in vivo*. Attempts to express ACR-26 homomers from a second parasitic species, *H. contortus*, were unsuccessful. It would be interesting to add ACR-26 to the reconstituted *H. contortus* nAChR recently reported by Boulin et al. [13]. The pharmacology of the homomeric receptor, if it reflects that of native ACR-26 containing nAChR, would appear to be distinct from those previously reported for reconstituted levamisole receptors [12,13,14]. The ACR-26 channels were extremely sensitive to Ach, and the EC_{50} of between 10 and 100nM was 1-2 orders of magnitude less than that of the *A. suum* UNC-29/UNC-38 receptor (~1μM) [12] – compare the responses to 10 and 100nm Ach with those to 10 and 30μM nicotine in Figure 5. If this reflects the pharmacology of native ACR-26 containing receptors on head muscle cells, it suggests that they may mediate responses to lower levels of cholinergic signaling than the previously characterized levamisole-sensitive receptors [12-14]. This novel pharmacology might enable it to be developed as a target for compounds that are effective against nematode parasites, but are less dangerous to free-living species in the environment.

The expression of ACR-26 on *Ascaris* head but not body-wall, muscles, is distinct from that of other nicotinic subunits, such as UNC-29 and UNC-38, that are found on both muscle types [12]

and suggests that it may have a specific function there. It is tempting to relate the function of ACR-26 to the more complex movements in the nematode head - body-wall muscle permits only dorsal-ventral bends, whereas the head can also move laterally – but it is difficult to explain why the subunit would only be present in parasitic and not free-living species. We have as yet no information on the distribution of ACR-26 in nematodes other than *Ascaris*. The evolutionary history of this gene is interesting; it is conserved in animal parasitic species of multiple clades [19], though not clade I, implying that it pre-dates their appearance, but is absent from free-living species of clade V and plant parasitic species of clade IV. Analysis of nematode phylogenetics has led to the conclusion that animal parasitism evolved multiple times [20,21,22,23], which is on the face of it hard to reconcile with a gene that is specifically associated with parasitic species, even if, as suggested by van Mengen et al [23], convergent evolution seems to be a feature of the Nematoda. It is probably more likely that *acr-26* has been lost in the free-living and plant parasitic species; this, together with its specific expression in head muscles, raises interesting questions about its likely function. Further developments in functional genetics methods for parasitic nematodes [24,25,26] may allow us to understand that function better. The high level of amino-acid identity between ACR-26 and the molluscan D and G subunits [19] is interesting; these subunits form a small out-group on the phylogenetic tree of mollusc nAChR and are expressed only at low levels in the CNS. No functional expression of either could be detected in *Xenopus* oocytes [27]. ACR-26 may thus be a member of a small group of invertebrate nAChR subunits whose function has yet to be determined.

Acknowledgements

We would like to thank Aaron Maule (Queen's University, Belfast) and Richard Martin (Iowa State University) for their generosity in supplying adult *Ascaris suum.* This work was funded by an Industrial Partnership Grant from BBSRC (UK) and Pfizer Animal Health.

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Figure Legends

Figure 1. Tree of *C. elegans* and *A. suum* nAChR subunits. A maximum likelihood neighbour joining bootstrapped tree of translated nAChR sequences drawn with Geneious Pro 5.4. Sequences of *C. elegans* nAChR subunits are shown in black. *A. suum* nAChR subunit sequences identified in the transcriptome [29] are shown in red, and are named after their *C. elegans* orthologue, with the exception of ACR-26 (red box), which has no orthologue. All orthologue pairings gave bootstrap values of 100.

Figure 2. Alignment of nematode ACR-26 sequences with vertebrate α7. An alignment of the ACR-26 polypeptides from *A. suum* and *H. contortus* with the murine α7 nAChR subunit was made with Clustal (http://www.ebi.ac.uk/Tools/msa/clustalw2/). Amino-acid residues conserved between the two ACR-26 subunits, and α7, are shown in bold. The yellow shading indicates the residues predicted to form the loops of the ligand-biding site, and the grey shading shows the predicted membrane-spanning regions. The underlined residues are those that were used to raise an antibody against the *A. suum* subunit.

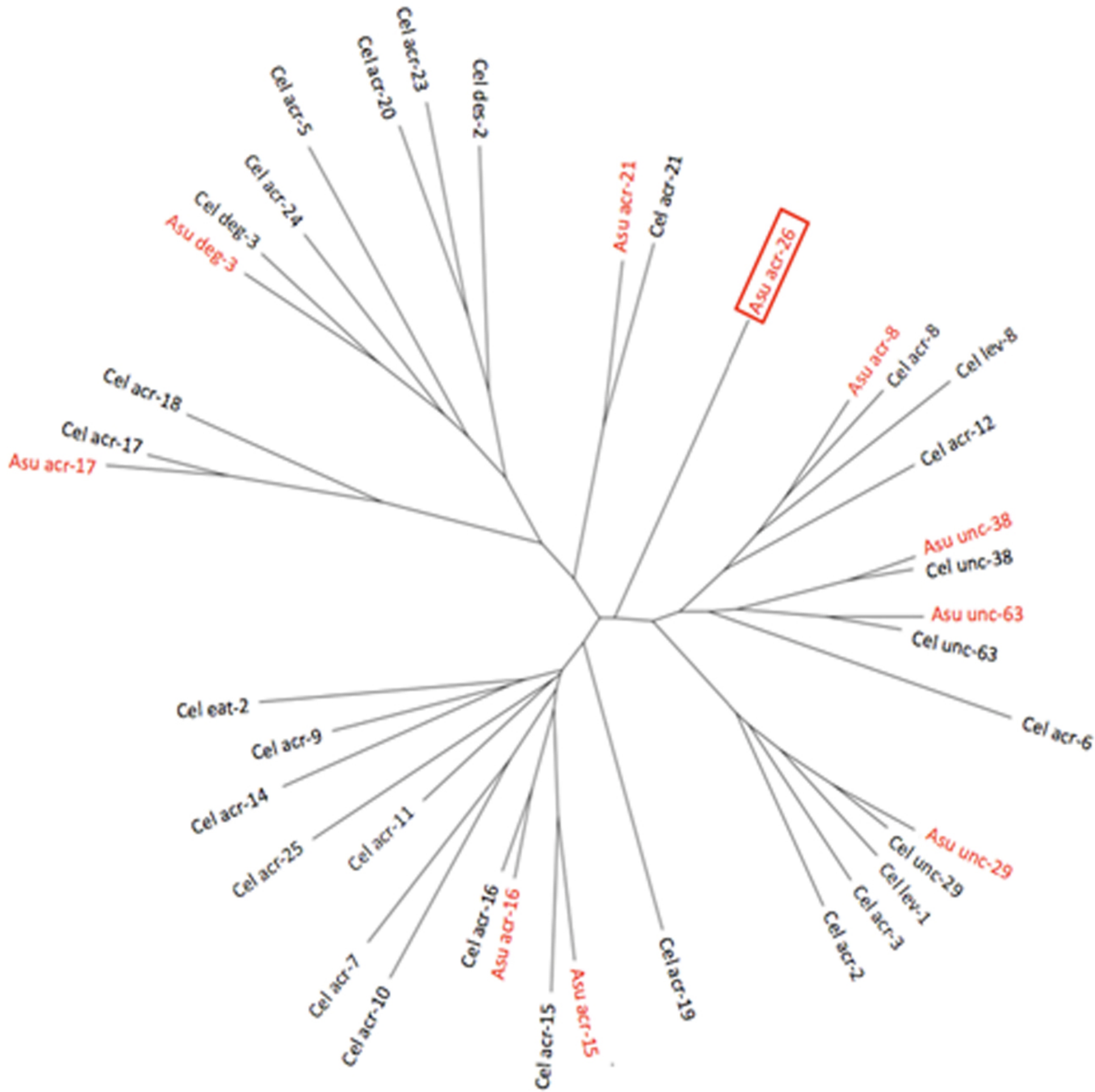
Figure 3. Partial *acr-26* sequences from nematodes of medical and agricultural importance. Full-length amino-acid sequences of ACR-26 from *A. suum*, *B. malayi* and *H. contortus* were aligned with the translated partial sequences from *O. ostertagi*, *C. oncophora* and *T. circumcincta* obtained from PCR reactions using degenerate primers designed to conserved regions.

Figure 4. Immunostaining of *A. suum* muscle cells with anti-ACR-26 antibody. Muscle cells were isolated from either *A. suum* body wall muscle or from the 2 cm most anterior region (in front of the nerve ring), fixed and stained. A) A representative head muscle cell in a negative control condition, treated with control goat IgG and anti-goat IgG FITC. In the experimental condition cells were treated with affinity purified anti-ACR-26 and anti-goat IgG FITC. B) A representative body wall muscle cell treated with the anti-ACR-26 antibody. C) A representative head muscle cell demonstrating a strong positive signal. D). Close-up of the muscle arm after anti-ACR-26 staining on head muscle. Confocal image of head muscle stained with affinity-purified anti-ACR-26 antibody. Part of the arm is out of the plane of the image.

Figure 5. Asu-ACR-26 is capable of forming a functional nAChR in *Xenopus* oocytes. A) Example dose-dependent responses to applied ACh. B) Dose response curve for nicotine at the ACR-26 receptor. C) Example dose-dependent responses to applied nicotine.

Table 1. Distribution of *acr-26* sequences in nematode species.

Clade[19]	Trophic ecology	Species	*Acr-26*?	Length of known coding sequence and reference numbers	Source
I	Vertebrate parasite	*Trichinella spiralis*	No	-	BLAST completed genome project [2~~6~~8]
III	Vertebrate parasite	*Ascaris suum*	Yes	Full-length (1533 bp), GenBank: GU135625	EST [2~~7~~9], this manuscript.
III	Vertebrate parasite	*Brugia malayi*	Yes	Full-length (1542 bp), NCBI: XM_001901191	BLAST completed genome project[~~28~~30]
III	Vertebrate parasite	*Loa loa*	Yes	Full-length (1428 bp), NCBI: XM_003140235 (LOAG_04698)	BLAST completed genome project (http://www.broadinstitute.org/annotation/genome/filarial_worms/Info.html)
III	Vertebrate parasite	*Wuchereria bancrofti*	Yes	Partial (630 bp), Broad Institute: WUBG_13499	BLAST incomplete genome project (http://www.broadinstitute.org/annotation/genome/filarial_worms/Info.html)
III	Vertebrate parasite	*Dirofilaria immitis*	Yes	Partial (577 bp), EST cluster: DIC00454	EST and completed genome project [~~29,~~ ~~30~~,31,32]
IV	Phytoparasite	*Meloidogyne incognita*	No	-	BLAST completed genome project [3~~1~~3]
IV	Phytoparasite	*Meloidogyne hapla*	No	-	BLAST completed genome project [3~~2~~4]
IV	Vertebrate parasite	*Strongyloides ratti*	Yes	Sanger Institute, pathogen_RATTI_Contig 74886	BLAST genome project (http://www.sanger.ac.uk/resources/downloads/helminths/strongyloides-ratti.html)
V	Free-living bacterivore	*Caenorhabditis elegans*	No	-	BLAST completed genome project [3~~3~~5]
V	Free-living omnivore	*Pristionchus pacificus*	No	-	BLAST completed genome project [3~~4~~6]
V	Vertebrate parasite	*Cooperia oncophora*	Yes	Partial (639 bp) GenBank: JN966889	Cloning – this manuscript
V	Vertebrate parasite	*Haemonchus contortus*	Yes	Full-length (1573 bp), GenBank: EU006791	BLAST incomplete genome project (http://www.sanger.ac.uk/resources/downloads/helminths/haemonchus-contortus.html), Cloning – this manuscript
V	Vertebrate parasite	*Ostertagia ostertagi*	Yes	Partial (597 bp) GenBank: JN966890	Cloning – this manuscript
V	Vertebrate parasite	*Teladorsagia circumcincta*	Yes	Partial (708 bp) GenBank: JN966888	Cloning – this manuscript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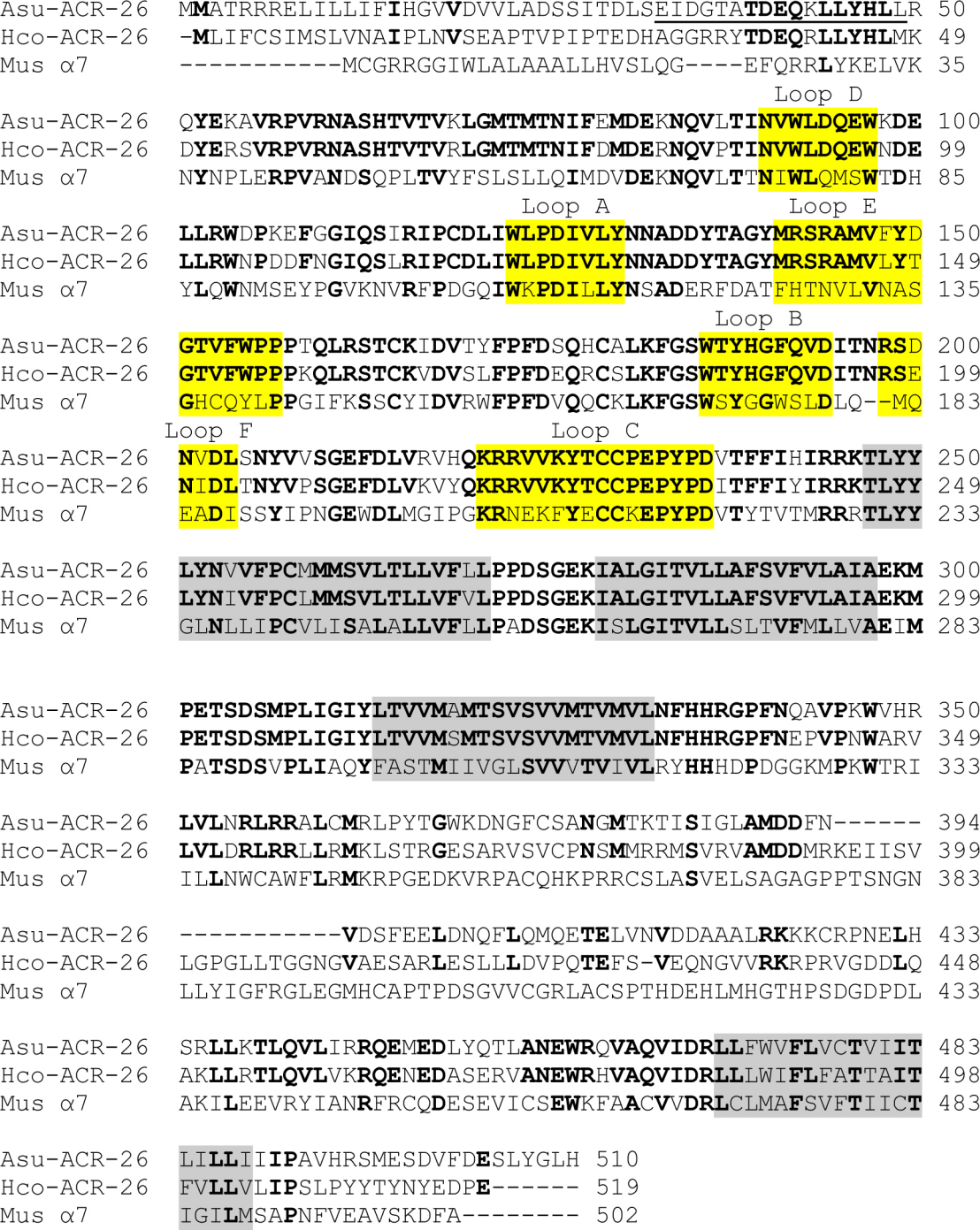
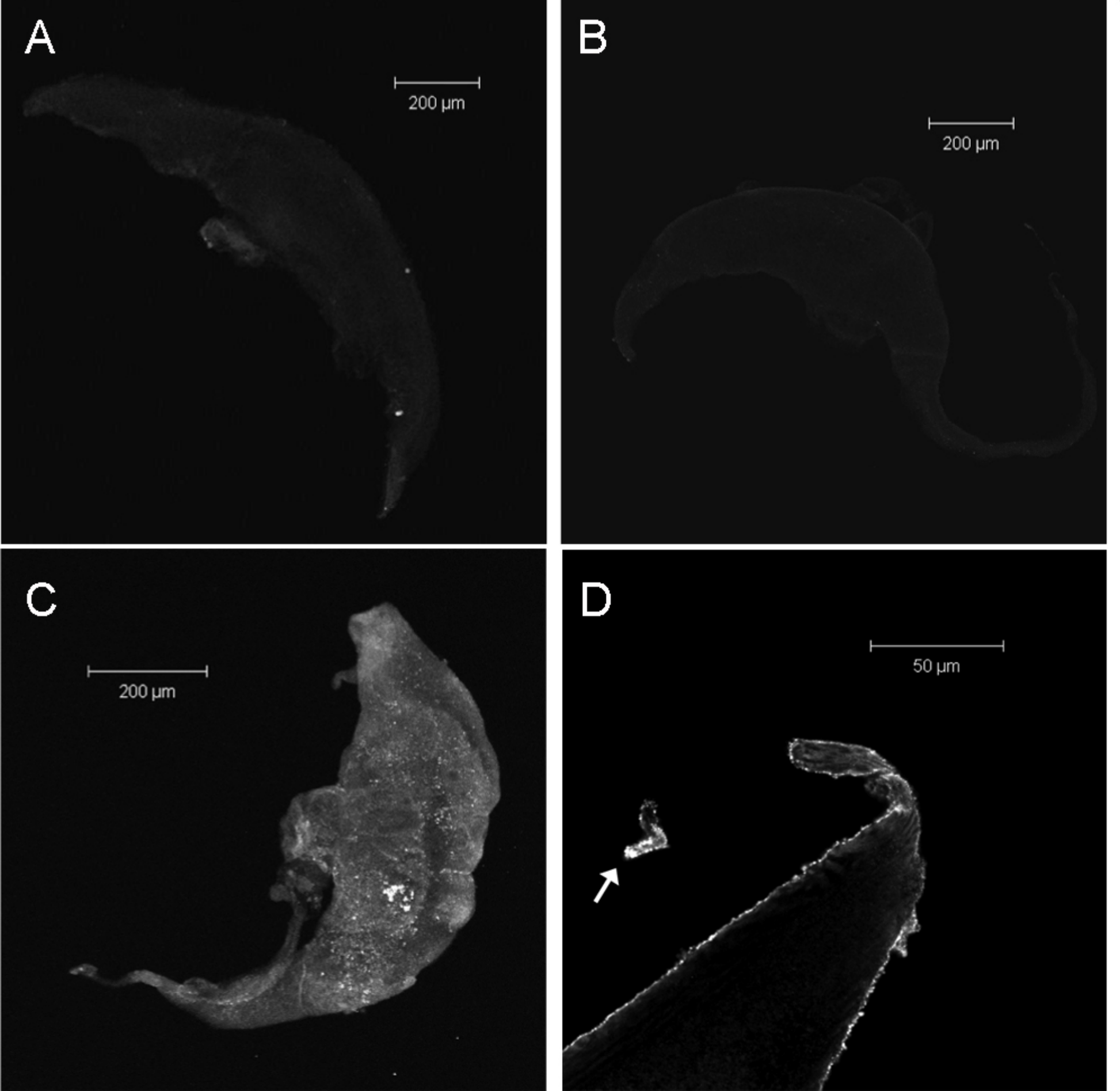
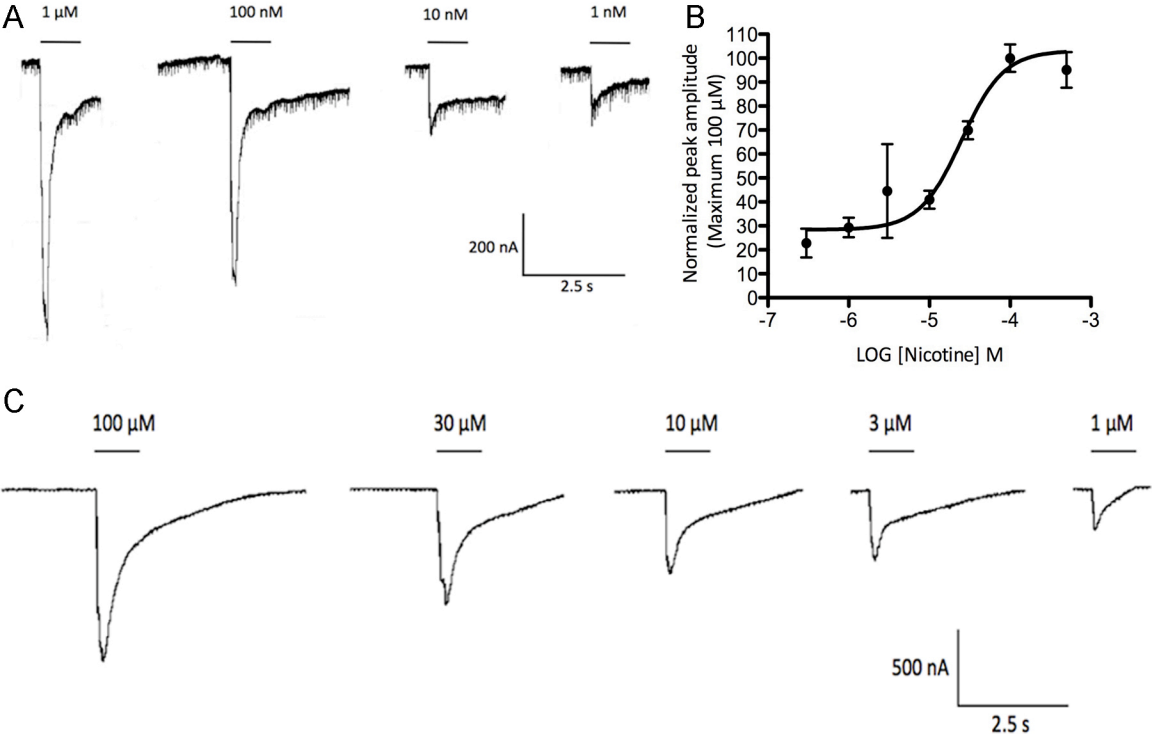
Cel acr-19


Asu-ACR-26 MMATRRRELILLIFIHGVVDVVLADSSITDLSEIDGTATDEQKLLYHLLR 50
Hco-ACR-26 -MLIFCSIMSLVNAIPLNVSEAPTVPIPTEDHAGGRRYTDEQRLLYHLMK 49
Mus α7 -----------MCGRRGGIWLALAAALLHVSLQG----EFQRRLYKELVK 35
Loop D
Asu-ACR-26 QYEKAVRPVRNASHTVTVKLGMTMTNIFEMDEKNQVLTINVWLDQEWKDE 100
Hco-ACR-26 DYERSVRPVRNASHTVTVRLGMTMTNIFDMDERNQVPTINVWLDQEWNDE 99
Mus α7 NYNPLERPVANDSQPLTVYFSLSLLQIMDVDEKNQVLTTNIWLQMSWTDH 85
Loop A
Loop E
Asu-ACR-26 LLRWDPKEFGGIQSIRIPCDLIWLPDIVLYNNADDYTAGYMRSRAMVFYD 150
Hco-ACR-26 LLRWNPDDFNGIQSLRIPCDLIWLPDIVLYNNADDYTAGYMRSRAMVLYT 149
Mus α7 YLQWNMSEYPGVKNVRFPDGQIWKPDILLYNSADERFDATFHTNVLVNAS 135
Loop B
Asu-ACR-26 GTVFWPPPTQLRSTCKIDVTYFPFDSQHCALKFGSWTYHGFQVDITNRSD 200
Hco-ACR-26 GTVFWPPPKQLRSTCKVDVSLFPFDEQRCSLKFGSWTYHGFQVDITNRSE 199
Mus α7 GHCQYLPPGIFKSSCYIDVRWFPFDVQQCKLKFGSWSYGGWSLDLQ--MQ 183
Loop F
Loop C
Asu-ACR-26 NVDLSNYVVSGEFDLVRVHQKRRVVKYTCCPEPYPDVTFFIHIRRKTLYY 250
Hco-ACR-26 NIDLTNYVPSGEFDLVKVYQKRRVVKYTCCPEPYPDITFFIYIRRKTLYY 249
Mus α7 EADISSYIPNGEWDLMGIPGKRNEKFYECCKEPYPDVTYTVTMRRRTLYY 233
Asu-ACR-26 LYNVVFPCMMMSVLTLLVFLLPPDSGEKIALGITVLLAFSVFVLAIAEKM 300
Hco-ACR-26 LYNIVFPCLMMSVLTLLVFVLPPDSGEKIALGITVLLAFSVFVLAIAEKM 299
Mus α7 GLNLLIPCVLISALALLVFLLPADSGEKISLGITVLLSLTVFMLLVAEIM 283
Asu-ACR-26 PETSDSMPLIGIYLTVVMAMTSVSVVMTVMVLNFHHRGPFNQAVPKWVHR 350
Hco-ACR-26 PETSDSMPLIGIYLTVVMSMTSVSVVMTVMVLNFHHRGPFNEPVPNWARV 349
Mus α7 PATSDSVPLIAQYFASTMIIVGLSVVVTVIVLRYHHHDPDGGKMPKWTRI 333
Asu-ACR-26 LVLNRLRRALCMRLPYTGWKDNGFCSANGMTKTISIGLAMDDFN------ 394
Hco-ACR-26 LVLDRLRRLLRMKLSTRGESARVSVCPNSMMRRMSVRVAMDDMRKEIISV 399
Mus α7 ILLNWCAWFLRMKRPGEDKVRPACQHKPRRCSLASVELSAGAGPPTSNGN 383
Asu-ACR-26 -----------VDSFEELDNQFLQMQETELVNVDDAAALRKKKCRPNELH 433
Hco-ACR-26 LGPGLLTGGNGVAESARLESLLLDVPQTEFS-VEQNGVVRKRPRVGDDLQ 448
Mus α7 LLYIGFRGLEGMHCAPTPDSGVVCGRLACSPTHDEHLMHGTHPSDGDPDL 433
Asu-ACR-26 SRLLKTLQVLIRRQEMEDLYQTLANEWRQVAQVIDRLLFWVFLVCTVIIT 483
Hco-ACR-26 AKLLRTLQVLVKRQENEDASERVANEWRHVAQVIDRLLLWIFLFATTAIT 498
Mus α7 AKILEEVRYIANRFRCQDESEVICSEWKFAACVVDRLCLMAFSVFTIICT 483
Asu-ACR-26 LILLIIIPAVHRSMESDVFDESLYGLH 510
Hco-ACR-26 FVLLVLIPSLPYYTYNYEDPE------ 519
Mus α7 IGILMSAPNFVEAVSKDFA-------- 502


Loop A
Loop E
Loop E
Loop B
Loop F
Loop C
TM1
TM2


A
200 µm
B
200 µm
C
200 µm
D
50 µm


A
1 µM
100 nM
10 nM
1 nM
200 nA
2.5 s
B
Normalized peak amplitude
(Maximum 100 µM)
110
100
90
80
70
60
50
40
30
20
10
0
-7
-6
-5
-4
-3
LOG [Nicotine] M
C
100 µM
30 µM
10 µM
3 µM
1 µM
500 nA
2.5 s