Identification of Molluscan Nicotinic Acetylcholine Receptor (nAChR) Subunits Involved in Formation of Cation- and Anion-Selective nAChRs

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Acetylcholine (ACh) is a neurotransmitter commonly found in all animal species. It was shown to mediate fast excitatory and inhibitory neurotransmission in the molluscan CNS. Since early intracellular recordings, it was shown that the receptors mediating these currents belong to the family of neuronal nicotinic acetylcholine receptors and that they can be distinguished on the basis of their pharmacology. We previously identified 12 Lymnaea cDNAs that were predicted to encode ion channel subunits of the family of the neuronal nicotinic acetylcholine receptors. These Lymnaea nAChRs can be subdivided in groups according to the residues supposedly contributing to the selectivity of ion conductance. Functional analysis in Xenopus oocytes revealed that two types of subunits with predicted distinct ion selectivities form homopentameric nicotinic ACh receptor (nAChR) subtypes conducting either cations or anions. Phylogenetic analysis of the nAChR gene sequences suggests that molluscan anionic nAChRs probably evolved from cationic ancestors through amino acid substitutions in the ion channel pore, a mechanism different from acetylcholine-gated channels in other invertebrates.

Key words: Lymnaea stagnalis; cholinergic receptor; ion selectivity; Xenopus oocyte expression; mutation; evolution

Introduction

Nicotinic acetylcholine receptors (nAChRs) belong to the Cys-loop family of ligand-gated ion channels (LGICs), which also include the 5-HT3 receptors (5-HT3Rs), GABAA/γ receptors (GABARs), and glycine receptors (GlycRs), as well as the Zn2+-activated receptor (Le Novere and Changeux, 1995; Orteils and Lunt, 1995; Davies et al., 2003). nAChRs are heteropentameric or homopentameric complexes of homologous subunits that each consist of an N-terminal extracellular ligand-binding domain (LBD) and four transmembrane domains that contribute to the formation of a channel pore. These receptors are typically divided in excitatory, cation-conducting nAChRs and 5-HT3Rs and inhibitory anion-conducting GABARs and GlycRs, which are also distinct with respect to ligand specificity. Experiments with chimeric constructs of Cys-loop superfamly subunits have shown that LBD and channel pore can be considered independent functional entities, because they can be exchanged between receptors while keeping intact the intrareceptor conformational change that couples ligand binding to ion channel opening (Eisele et al., 1993; Bouzat et al., 2004). The important sequence homology of the second transmembrane domain between cationic and anionic receptors of Cys-loop family further suggested that the ion selectivity must be governed by a small group of residues that are lining the ion pore. This hypothesis was confirmed by site-directed mutagenesis and the conversion of ionic selectivity of a cationic into an anionic channel (Galzi et al., 1992). These observations have, since then, been extended to different receptor subtypes, including the 5-HT3, GABAA, and glycine receptors (Keramidas et al., 2000; Gunthorpe and Lummis, 2001; Jensen et al., 2002). In principle, the modular nature of ligand-gated ion channels, and the susceptibility to ion selectivity changes by limited mutations would predict the occurrence of naturally occurring recombination of ligand specificity and ion selectivity. Such events, however, do not seem to have occurred during mammalian evolution.

Molluscan species, however, are long known to display, in addition to excitatory, sodium-selective nAChRs, inhibitory chloride-selective nAChRs, suggesting that a natural recombination event of ligand specificity and ion selectivity has occurred during evolution of molluscan nAChRs (Tauc and Gerschenfeld, 1962; Vulfius et al., 1967, 2005; Kehoe, 1972a,b, 1976; Chemeris et al., 1982). Indeed, recently, acetylcholine-gated anionic channels have been characterized in Caenorhabditis elegans that are overall most similar to mammalian GABARs and GlycRs (Putenko et al., 2004). These data suggest evolutionary adaptation of the ligand-binding site toward cholinergic ligands.

Here, we determined the nature of molluscan nAChRs to ac-
quire an understanding of evolutionary mechanisms behind the development of selectivity for either ligand binding or ion selectivity in the nAChR family. In this study, we selected subgroups of Lymnaeae nAChRs (LnAChR) as putative anion- and cation-selective subunits based on amino acid sequences demonstrated previously to determine ion selectivity in mammalian nAChRs (Galzi et al., 1992). Functional expression of members of these subgroups in Xenopus oocytes revealed cation- and anion-selective channels. In contrast to the acetylcholine-gated channels in C. elegans, our data suggest that molluscan anion-selective nAChR subtypes have evolved from true cation-selective nAChRs by mutation of the ion channel pore.

Materials and Methods

Cloning of nAChR subunits. LnAChR subunits were identified as described previously (unpublished observations). In short, PCR products representing partial coding sequences of nAChR subunit mRNAs were generated using nested combinations of degenerate oligonucleotides on a number of Lymnaeae CNS-derived cDNA templates. Full-length sequences were obtained by a primer walking strategy on extended 5' and 3' sequences generated by PCR on a Lambda ZAPII (Stratagene, La Jolla, CA) cDNA library of the CNS of Lymnaea stagnalis. Final sequences were obtained by sequence from three independently generated PCR products generated on DT-primed cDNA of pooled Lymnaea CNS. For Xenopus oocyte expression, cDNAs of open reading frames were cloned into pcDNA3 (Invitrogen, San Diego, CA). Sequences of cloned products were checked by dideoxy-chain termination sequencing. GenBank accession numbers of the sequences are as follows: LnAChR A, DQ167344; LnAChR B, DQ167345; LnAChR C, DQ167346; LnAChR D, DQ167347; LnAChR E, DQ167348; LnAChR F, DQ167349; LnAChR G, DQ167350; LnAChR H, DQ167351; LnAChR I, DQ167352; LnAChR J, DQ167354; LnAChR K, DQ167353; and LnAChR L, DQ167355.

Oocyte preparation. Xenopus laevis oocytes were prepared, injected, and recorded as described previously (Bertrand et al., 1990). Once isolated, each cell was intranuclearly injected with 2 ng of expression vector cDNA. Oocytes were then placed in 96-well microtiter plates (Nunc, Naperville, IL) at 18°C. Barth's solution used to store the oocytes consisted of 88 mM NaCl, 1 mM KCl, 2.4 mM NaHCO3, 10 mM HEPES, 0.82 mM MgSO4, 0.33 mM Ca(NO3)2, 0.41 mM CaCl2, pH 7.4 adjusted with NaOH, and 100 U/ml penicillin.

Electrophysiology. Oocytes were used for experiments 2–4 d after cDNA injections. They were placed in the recess of a small chamber and continuously superfused with physiological medium. Solutions flowed at a rate of ~6 ml/min, and exchange was performed by the activation of computer-driven electromagnetic valves (type III; General Valve, Fairfield, NJ). Electrophysiological recordings were performed with a two-electrode voltage clamp (GeneClamp amplifier; Molecular Devices, Union City, CA). Electrodes were made out of borosilicate glass, pulled with a BB-CH-PC puller (Mecanex, Nyon, Switzerland), and filled with a filtered 3 mM KCl solution. Oocyte Ringer's solution was used in control experiments as a bath solution [(in mM) 82.5 NaCl, 2.5 KCl, 5 HEPS, 2.5 CaCl2, and 1 MgCl2], pH 7.4, adjusted with NaOH.

The ion selectivity of homomeric LnAChR A or B channels was investigated using extracellular dilution potential experiments. The nAChR-mediated currents were elicited using a near-saturating concentration of acetylcholine (200 μM) dissolved in the appropriate extracellular solution. Current–voltage (I–V) relationships were recorded in control and three diluted extracellular solutions, in sequence, for each oocyte. The following extracellular solutions were used (in mM): control, 80 NaCl, 2.5 KCl, 5 HEPS, 2.5 CaCl2, and 1 MgCl2; test dilution 1, 40 NaCl, 2.5 KCl, 5 HEPS, 2.5 CaCl2, and 1 MgCl2, and 74 sucrose; test dilution 2, 20 NaCl, 2.5 KCl, 0.2 CaCl2, 5 HEPS, and 110 sucrose; test dilution 3, 20 NaCl, 2.5 KCl, 0.2 CaCl2, 5 HEPS, and 127 sucrose (see Table 1). Sucrose was added to the diluted solutions to maintain osmolarity. The intracellular concentration of Na+ (10.1 ± 0.7 mEq/L), K+ (109.5 ± 3.3 mEq/L), and Cl− (37.7 ± 1.2 mEq/L) was taken from Costa et al. (1989). To minimize contamination by the endogenous calcium-activated chloride currents, all ion-selectivity experiments were performed in oocytes that had been incubated in BAPTA-AM (100 μM) for at least 5 h.

The current–voltage plots were fitted to polynomials and the reversal potentials were read directly from the plots. Liquid junction potentials were corrected using JPCalc software (Barry, 1994). The reversal-potential data were then pooled, and the averaged reversal potential (Vrev) was plotted against ion activity and fitted to the Goldman–Hodgkin–Katz (GHK) equation: $V_{rev} = \frac{R T F}{F} \ln \left( \frac{d_{+}}{d_{-}} \right)$.
The extent of desensitization resulting from agonist exposure was determined again by a pulse of ACh after a 1–3 min washout period. The graph reflects agonist-induced peak currents expressed proportionally to currents elicited by a saturating dose of ACh.

The sensitivity of the LnAChR A homopentameric receptor expressed in Xenopus oocytes to nicotinic drugs. The LnAChR A cDNA reconstitutes a homopentameric receptor in Xenopus oocytes. A. Traces show currents elicited by pressure application of a low concentration of ACh and of high concentrations of glycine, GABA, 5-HT, and glutamate to oocytes expressing the LnAChR A subunit. Comparable results were observed on all oocytes tested (data not shown). B. ACh-evoked currents were recorded using a range of ligand concentrations (0.1–1000 μM). A dose–response curve was inferred from ACh-induced mean peak current values normalized to the maximum and minimum values and indicates an EC50 value of ~3 μM. C. The LnAChR A receptor is activated by the nicotinic agonists cytisine, choline, and nicotine. Before application of agonist, currents after a pulse of EC50 ACh was recorded to serve as a reference from oocytes expressing the LnAChR A subunit. The extent of desensitization resulting from agonist exposure was determined again by a pulse of ACh after a 1–3 min washout period. The graph reflects agonist-induced peak currents expressed proportionally to currents elicited by a saturating dose of ACh.

All drugs, apart from α-bungarotoxin (αBtx) and α-conotoxin-ImI (αCtx-ImI), were applied by pressure application. α-Bungarotoxin (Sigma, Basel, Switzerland), α-conotoxin-ImI (American Peptide, Sunnyvale, CA), methyllycaconitine (MLA), dihydro-β-erythroidine hydrobromide (DHβE), mecamylamine (Mec) (Sigma) were diluted to the indicated concentration in the control medium that contained 20 μg/ml bovine serum albumin to prevent adsorption of the toxin on the plastics.

nAChR constructs. For expression in Xenopus oocytes, cDNAs representing open reading frames of LnAChR subunits were PCR amplified, separated on agarose gels, and cloned into BamHI/Xhol-digested pcDNA3 (Invitrogen). Sequences of cloned products were checked by dyeoxy-chain termination sequencing.

Phylogenetic analysis. Protein alignments were performed with ClustalX (Thompson et al., 1997). Phylogenetic analysis was performed using the neighbor-joining method (Kisnura correction; gap positions included). Regions of weak alignment (corresponding to residues 1–24, 35–37, 188–192, and 324–452 of LnAChR A) were excluded from the analysis. Phylogenetic trees were midpoint rooted.

Real-time quantitative PCR. Total RNA was isolated from freshly dissected ganglia according to the method of Chromczynski and Sacchi (1987) using Trizol reagent (Invitrogen) that was followed by a DNase-I (10 U; Roche Diagnostics, Basel, Switzerland) treatment according to the instructions of the manufacturer, and a phenol/chloroform extraction and ethanol precipitation. cDNA was made using hexanucleotides (300 pmol) and 200 U of Moloney murine leukemia virus H–reverse transcriptase (Promega, Madison, WI). After cDNA synthesis, all samples were ethanol precipitated and dissolved in 60 μl of aquadest. Transcript-specific primers were designed using Primer Express software (Applied Biosystems, Foster City, CA): LnAChR A forward (fwd), 5′-gctaggaagctggaagttggtgatgc-3′; LnAChR A reverse (rev), 5′-gggaccacaacctgctctctgatc-3′; LnAChR B fwd, 5′-tcaccttgcttgacagtgttctc-3′; LnAChR B rev, 5′-ggcagcgcgtgatggtctgct-3′; Lymanea β-tubulin (Ltb) fwd, 5′-caagcactctgctcttgga-3′; Ltb rev, 5′-tgatttggcctctctgtaag-3′. Primers were checked for specificity in GenBank by basic local alignment search tool analysis. PCR amplification efficiency of each primer set was determined by using a repetitive dilution series of total CNS cDNA; all primer sets showed an efficiency ≥1.9. Quantitative PCR (qPCR) measurements were performed using an ABI PRISM 7700 (Applied Biosystems) sequence detection system. PCR conditions and SYBR Green reagents (Applied Biosystems) were used in 20 μl reactions and with transcript-specific primers (300 nM) and 0.5 μl cDNA template according to the Applied Biosystems guidelines (40 cycles; 94°C for 30 s; 58°C for 30 s; 72°C for 210 s). After PCRs, temperature dissociation curves were generated to confirm the specificity of generated PCR products. The threshold line to determine the cycle of threshold (Ct) value was set on 0.3 ΔRn (baseline-subtracted values), with curves reaching maximally 6–8 ΔRn, and baselines of –0.02 ΔRn. The obtained Ct values were used to calculate the relative level of gene expression (Ct norm) normalized to Ltb (GenBank accession number X15542). For gene x the Ct norm gene is calculated by: Ct gene = Ct Ltb. These values were transformed to yield

$$P_{ACh} = \frac{P_{ACh,0} + P_{ACh,1} + P_{ACh,2}}{P_{ACh,0} + P_{ACh,1} + P_{ACh,2}}$$

where $R$ is the Gas constant, $T$ is temperature in Kelvin, $F$ is Faraday’s constant, $P_{ACh,0}$ is the permeability of the ion, and $\alpha_{i,j}$ is the activity of the ion in the extracellular (superscript $o$) and intracellular (superscript $i$) solutions. All data are expressed as mean ± SEM.
linear expression levels for each sample by the following: 2E – Ct. The relative gene expression levels of the transcripts from replicate measurements were calculated from normalized linear gene expression levels and expressed as mean ± SEM. With qPCR, the expression of all LnAChR subunits was detected in all CNS preparations with the highest Ct value (lowest abundance) measured at least six cycles (factor 32) from the Ct value of the no-template control.

In situ hybridization. In situ hybridization (ISH) was performed as described previously (Koert et al., 2001). Briefly, *Lymnaea* CNSs were frozen at –80°C, and 14 μm cryostat sections were mounted on SuperFrost slides (Menzel, Braunschweig, Germany). The sections were air dried and fixed in 1% paraformaldehyde and 1% acetic acid for 1 h. Sections were pretreated with 1.49% triethanolamine and 0.25% acetic acid, pH 8.0, and were passed through a graded series of alcohol (70–100%). Specific [32P]UTP-labeled RNA probes were transcribed from linearized cDNA (200 ng) corresponding to stretches of coding sequence of LnAChR A (nucleotides 1039–1380) and B (nucleotides 1096–1470).

### Results

#### Predicted ion selectivity of LnAChR subunits

In another work, we identified 12 neuronal nAChR (LnAChR) subunits from the freshwater snail *L. stagnalis* that possibly include subunits for cationic and anionic nAChR subtypes (our unpublished observations). The presence of a proline residue at the inner mouth of transmembrane domain 2 (TM2) is being thought of to determine anion selectivity, whereas the presence of a Ser-Gly-Glu motif at the corresponding position correlates to cation selectivity (Fig. 1) (Galzi et al., 1992; Keramidas et al., 2001; Jensen et al., 2002). Of the identified LnAChR subunits, the LnAChR B, F, I, and K subunits possess proline residues in the TM1/TM2-joining loop, whereas LnAChR C, D, E, and G display the Ser-Gly-Glu residue motif. The LnAChR A, H, and J subunits all possess a Glu residue on a position corresponding to E237 (rat α7 nAChR), demonstrated to be an important factor for cation selectivity (Keramidas et al., 2002; Wotring et al., 2003; Jensen et al., 2005b).

Based on these findings, we predict, therefore, that LnAChR B, F, I, and K subunits participate in anion-selective nAChRs, whereas LnAChR A, C, D, E, G, H, and J participate in cation-selective nAChRs.

#### Functional expression of nAChRs composed of LnAChR A or LnAChR B

To determine the contribution of LnAChR subunits to functional receptors, we expressed LnAChR subunits in *X. laevis* oocytes.

### Table 1. Ionic composition of the solutions used for determination of the reversal potential

<table>
<thead>
<tr>
<th>Estimated intracellular solution</th>
<th>Extracellular solutions</th>
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<tr>
<td>Control</td>
<td>Test dilution 1</td>
</tr>
<tr>
<td>[Na⁺]i = 10.1 ± 0.7 mEq</td>
<td>[Na⁺]o = 80 mM</td>
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<tr>
<td>(+ 2.0 mEq Na⁺ from NaOH)</td>
<td>+ 2.0 mEq Na⁺ from NaOH</td>
</tr>
<tr>
<td>(sucrose) = 74 mM</td>
<td>(sucrose) = 110 mM</td>
</tr>
<tr>
<td>[K⁺]i = 109.5 ± 3.3 mEq</td>
<td>[K⁺]o = 2.5 mM</td>
</tr>
<tr>
<td>(Cl⁻)o = 37.7 ± 1.2 mEq</td>
<td>(Cl⁻)o = 42.9 mM</td>
</tr>
<tr>
<td>(Ca²⁺)o = 0.2 mM</td>
<td>(Ca²⁺)o = 0.2 mM</td>
</tr>
<tr>
<td>(Mg²⁺)o = 0 mM</td>
<td>(Mg²⁺)o = 0 mM</td>
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From Costa et al. (1989).


### Figure 3.

Sensitivity of the LnAChR B homopentameric receptor expressed in *Xenopus* oocytes to nicotinic drugs. A–D. The analyses, as shown in Figure 2, were applied to LnAChR B receptors expressed in *Xenopus* oocytes. B, The dose–response curve of LnAChR B receptors for ACh reveals a biphasic pattern of binding, with the high-sensitivity component (H) of −0.5 μM and a low-sensitivity component (L) of −17 μM, max. Maximum error bars represent the SEM.
oocytes. As a first step, LnAChR subunits were expressed individually, allowing only homopentameric nAChR subtypes. Expression of functional receptors was observed with LnAChR A and B subunits.

The LnAChR A subunit is expressed as a rapidly desensitizing receptor that is activated by ACh (4 μM), but not by GABA, glycine, glutamate, or serotonin (Fig. 2A). This receptor displays an EC_{50} value of ~3 μM for ACh and a Hill coefficient of 1.1 (Fig. 2B). Determination of the pharmacological profile of this receptor revealed that, as is the case for the α7 receptor of vertebrates, a high concentration of choline (1 mM) is also able to activate the receptor (Fig. 2C). The typical nicotinic agonists nicotine and cyanide potently activate the LnAChR A receptor, the former inducing an irreversible state of desensitization (tested up to several minutes). ACh-evoked activation was partially blocked by a 10 μM concentration of the competitive inhibitor DHβE or the broad-spectrum noncompetitive inhibitor Mec (Fig. 2D). The selective blocker of vertebrate α7 nAChRs, MLA, yielded no specific inhibition of the ACh-evoked currents. Although insensitive to MLA, the LnAChR A receptor was markedly blocked by the competitive inhibitor αBtx, with properties resembling those of the vertebrate α7 receptors (see Fig. 4A). Moreover, the αCtx-ImI completely blocked receptor activation.

Expression of the LnAChR B yielded functional receptors that, as for LnAChR A, were activated by ACh but not by GABA, glycine, glutamate, or serotonin (Fig. 3A). The rate of desensitization of the LnAChR B receptor, however, is considerably lower than that of LnAChR A. Determination of the ACh dose–response curve revealed that LnAChR B receptors display a strongly biphasic sensitivity, as observed for the heteromeric αβ2 receptors of vertebrates (Covernton and Connolly, 2000; Buisson and Bertrand, 2001). The high-affinity component is characterized by an EC_{50} value of 0.5 μM and a Hill coefficient of 0.88, whereas the low-affinity component has an EC_{50} value of 17 μM and a Hill coefficient of 1.5 (Fig. 3B). Determination of the pharmacological profile of the agonists revealed that, as for LnAChR A, these receptors can be activated by choline, cyanide, or nicotine (Fig. 3C). Interestingly, choline and nicotine are equipotent in activating the receptor. The LnAChR B receptor was fully blocked by the competitive nicotinic antagonist DHβE but not by the noncompetitive antagonist Mec (Fig. 3D). In contrast to the LnAChR A, both MLA (Fig. 3D) and αBtx (Fig. 4B) markedly inhibited the receptor, but only a small recovery from αBtx blockade could be observed after a 3 min wash, suggesting that this receptor is more sensitive to the snake toxin than the LnAChR A. In contrast, the LnAChR B receptors was insensitive to the αCtx-ImI (Fig. 4B).

Together, these data support classification of LnAChR A and B receptors as acetylcholine receptors of the nicotinic type, albeit with different pharmacological and biophysical characteristics.

**Ion selectivity of functionally expressed LnAChR A and B receptors**

The successful reconstitution of LnAChR A and B receptors allowed determination of their ion selectivity. For this, I–V relationships were determined for LnAChR A and B receptors under different external ionic conditions. The ion charge selectivity of LnAChR A and B receptors was determined using dilution potential experiments. I–V relationships were recorded in a series of extracellular solutions in which Na\(^+\) and Cl\(^-\) ions were diluted (see Materials and Methods). The experimental strategy was designed to reveal whether the channels preferred anions (Cl\(^-\)) or cations (Na\(^+\) and K\(^+\)). The concentrations of potentially permeant divalent cations (Ca\(^{2+}\) and Mg\(^{2+}\)) were kept constant, as was the concentration of K\(^+\). Examples of these experiments are shown in Figure 5 (left, LnAChR A; right, LnAChR B). For the LnAChR A channels, when extracellular NaCl is diluted, the reversal potential shifts in a leftward direction, toward the equilibrium potential for Na\(^+\) (E_{N} = −92.1 mV) (Fig. 5A,B). In dilution experiments, such as those illustrated by these results, the equilibrium potential for sodium ions progressively shifts toward zero, whereas the equilibrium potential for chloride shifts toward the right in the positive values. The leftward shift observed for the LnAChR A receptors indicates that this channel is mainly influenced by Na\(^+\), making these channels predominantly cation selective. Plotting the reversal potential for all of the solutions tested versus the corresponding extracellular Na\(^+\) concentration and fitting the data to the GHK equation yielded a chloride/sodium permeability ratio (P_{Cl}/P_{Na}) of 0.005 ± 0.001 (Fig. 5C).

The best fit was achieved by fixing the potassium/sodium permeability ratio (P_{K}/P_{Na}) to 1.1. From our results, it can be inferred that the LnAChR A channels are ~200-fold more selective for cations over anions and exhibit no significant selectivity between Na\(^+\) and K\(^+\).

Similar experiments were performed on homomeric LnAChR B channels. In contrast to the LnAChR A channels, when extracellular NaCl was diluted, the reversal potential shifted in the direction of the equilibrium potential for Cl\(^-\) ions (Fig. 5D,E), suggesting predominantly anion-selective channels. Plotting the
The expression of LnAChR A and B subunits in the Lymnaea CNS
The expression of the LnAChR A and B, cationic and anionic receptors, respectively, in the central ring ganglia of the CNS was analyzed using real-time qPCR and ISH. qPCR showed that the LnAChR A subunit is most abundantly expressed in the cerebral ganglia, whereas lower levels of expression were detected in the pedal and buccal ganglia (Fig. 7A). ISH labeled few unidentified neurons in the cerebral, buccal and pedal ganglia, whereas little positive neurons were observed in other ganglia; the labeling intensity of the latter was considerably lower (Fig. 7B). In contrast to LnAChR A, the LnAChR B subunit is expressed at highest levels in the buccal ganglia and at lower levels in the pedal ganglia (Fig. 7C). This distribution was confirmed by ISH that showed clusters of labeled neurons in the buccal ganglia, as well as some labeled neurons in the pedal ganglia and sporadic positive neurons in the other ganglia (Fig. 7D).

Discussion
Based on molecular features of the TM1–TM2-joining loop demonstrated to influence ion selectivity, we defined groups within the identified family of LnAChR subunits presumed to participate in anion-selective (LnAChR B, F, I, and K) and cation-selective (LnAChR A, C, D, E, G, H, and J) nAChRs, respectively.
Figure 6. Sequence comparison of ligand-binding domains of LnAChR subunits and other members of the Cys-loop family of LGICs. A, B. LGICs phylogenetic comparison was performed on separate protein-sequence alignments of LBD (A) and ion channel (B) domains of invertebrate and human Cys-loop subunits. Indicated are transmitter phenotypes and ion selectivity of human subunits groups, respectively (dashed boxes). Human subunits are indicated in italics. ACH, Nicotinic acetylcholine receptor; GABA, GABA receptor A; GLU, glutamate receptor; HIS, histamine receptor; cl, chloride channel; Exp, Exp-1 gene product; Mod, MOD-1 gene product; Ceal, C. elegans; drme, D. melanogaster; homa, Homo sapiens; lst, L. stagnalis. GenBank accession numbers for the sequences are as follows: 5HTmod1cael, N_741580; 5HT3Ahosa, AAH4453; 5HT3Bhosa, AAH46990; ACH1hosa, NP_000742; GLUcl1drme, AAH74980; GABA1apca, AF467899; ACH2haosa, NP_000070; ACH3haosa, NP_000735; ACH4haosa, NP_061028; GABA1hosa, NP_000738; ACH6hosa, NP_000742; GABA1hosa, NP_000797; GABA2hosa, NP_000070; GABA3hosa, NP_002033; GABA4hosa, NP_000803; GABA5hosa, NP_495229; GABA6hosa, NP_775807; GABA7hosa, NP_002031; GABA8hosa, NP_061028; GABA9hosa, NP_147357; GLUcl1cael, AAAS0786; GLUC1drme, A1112210.

The validity of this prediction is supported by demonstrating the cation and anion selectivity of functionally expressed nAChRs composed of LnAChR A or B subunits, respectively. The definition of LnAChR B, F, I, and K as a presumed anion-selective subgroup is paralleled by a high overall sequence relatedness of these LnAChR subunits (our unpublished observations). Therefore, the LnAChR B, F, I, and K represent a group of related subunits that are constituents of anion-selective nAChRs.

In view of their pharmacological properties, the LnAChR A and LnAChR B receptors display similarities to the vertebrate α7 and α9 subunit-containing nAChRs. First, like α7 and α9 nAChRs, LnAChR A and B are functional homopentameric receptors when expressed in oocytes (Couturier et al., 1990; Elgoyhen et al., 1994; Gerzanich et al., 1994). Second, receptors composed of LnAChR A or B can be activated by choline, a weak but selective agonist of α7 and α9 homopentameric nAChRs (Papke et al., 1996; Alkondon et al., 1997; Verbitsky et al., 2000). Third, receptors composed of LnAChR A and B are sensitive to αBtx, a selective agonist of α7- and α9-containing receptors in the vertebrate CNS (Schoepfer et al., 1990; Elgoyhen et al., 1994; Orr-Urtreger et al., 1997; Elgoyhen et al., 2001). Also, the LnAChR A receptor shows sensitivity to αCtx-Iml, and the LnAChR B receptor is selectively blocked by nanomolar concentrations of MLA, both properties that are attributed specifically to α7- and α9-containing nAChRs (Ward et al., 1990; Alkondon et al., 1992; Johnson et al., 1995; Palma et al., 1996). Pharmacological similarity of native molluscan nAChRs to vertebrate α7, α8, and α9 subunits has been noted in previous studies (Kehoe and McIntosh, 1998; Vulfius et al., 2005).

Unlike in the vertebrate nervous system, cholinergic transmission in the molluscan CNS is known to be mediated by cation- and anion-selective nAChRs (Tauc and Gerschenfeld, 1962; Vulfius et al., 1967; Kehoe, 1972a,b, 1976; Chemeris et al., 1982). Kehoe and McIntosh (1998) demonstrated the expression of two different anion-selective nAChR subtypes by identified cells in the buccal ganglia of Aplysia. In line with this, Vulfius et al. (2005) reported the expression of three distinct anion-selective nAChR subtypes by identified Lymnaea neurons. In addition, Woodin et al. (2002) showed, using whole-cell recordings on cultured Lymnaea soma–soma synapses, that, of the excitatory and inhibitory nAChR type(s) expressed by the right pedal dorsal 1 (RPedD1) neuron, the former can be selectively blocked by the cationic pore blocker Mec, which suggests the coexpression of cation- and anion-selective nAChRs by RPedD1. In addition to ion-selectivity differences of the channels, Woodin et al. (2002) described different properties of the ligand-binding sites of excitatory and inhibitory nAChRs expressed by RPedD1, as indicated by the distinct action of the competitive antagonists hexamethonium and MLA on excitatory and inhibitory nAChRs, respectively. In line with these data, the LnAChR A and B receptors display similar differential effects toward Mec and MLA application.

The LnAChR A and the LnAChR B subunits both display distinct patterns of expression characterized by a small number of expressing neurons in specific subsets of ganglia. The nonoverlapping expression pattern suggests that LnAChR A and B mediate each distinct task that is linked to a ganglion-specific function. For instance, in the buccal ganglia, a well characterized neuronal network involved in execution and regulation of feeding behavior resides (Benjamin and Elliott, 1989). Identified neurons of this
feeding network were shown to functionally express inhibitory nAChRs (Yeoman et al., 1993). The predominant expression of LnAChR B in the buccal ganglia matches this observation and justifies additional investigation into the involvement of LnAChR B in the feeding network.

Conversion of ion selectivity by molecular changes

In vitro mutagenesis studies (Galzi et al., 1992; Corringer et al., 1999; Gunthorpe and Lummis, 2001) suggested that anion selectivity is conferred by (1) insertion of a proline in the TM1–TM2-joining region, (2) substitution of Glu to Ala at position +1 relative to this proline (the intermediate charged ring), and (3) substitution of Val to Thr at position +15 (Fig. 1). Because anion-selective vertebrate LGICs and Lymnaea nAChRs have evolved along distinct evolutionary paths (for >600 million years), the convergent evolution of a proline positioned at the cytoplasmic border of TM2 supports the importance of this residue in establishing anion selectivity. However, the indifferent length of the TM1–TM2-joining region in anion-selective and cation-selective LnAChR subunits suggests that anion selectivity by itself does not require the insertion of the proline but that substitution is allowed as well. In addition, cationic LnAChR subunits contain negatively charged Glu residues in the intermediate ring (Jensen et al., 2002, 2005b; Keramidas et al., 2002), whereas anionic LnAChR subunits possess uncharged Ala residues (Galzi et al., 1992; Corringer et al., 1999; Gunthorpe and Lummis, 2001). Our data, therefore, confirm the importance of negatively charged residues in the intermediate ring for cation selectivity and the removal of these charges for anion selectivity, with Ala as the preferred substituent. Moreover, several charged or polar rings in the LGIC pore are known to influence ion permeation (Fig. 1), but mounting evidence suggests that discrimination between cations and anions is influenced by the intermediate charged ring (for review, see Keramidas et al., 2004; Jensen et al., 2005a). The similar residue properties of cation- and anion-selective LnAChR subunits in all but the intermediate ring support this notion. Last, none of the anion-selective LnAChR subunits show a Thr residue at position +15, indicating that other adaptive changes that mimic the Val-to-Thr substitution are allowed. Together, comparison of anion- and cation-selective LnAChR subunits suggests that presence of a proline residue in the TM1–TM2-joining region in combination with charge removal in the intermediate ring are preferred for the cation-to-anionic conversion of LGICs.

Although our data are supportive of an important role of a proline residue insertion at the border of TM2 in determining anion selectivity, alternative molecular mechanisms might exist. For instance, in the chloride-conducting GABAR of β3/α2/γ2 stoichiometry, only the α2 and γ2 subunits possess prolines in the TM1–TM2-joining region, whereas β3 does not. Jensen et al. (2002) demonstrated that the ion selectivity of these heteropentameric GABARs can only be converted by introduction of the Ser-Gly-Glu motif of the TM1–TM2-joining region of the β3 subunit, demonstrating that cation selectivity can be induced despite the presence of prolines in α2 and γ2 subunits. These findings hint at subunit-specific differences in dominance over ion selectivity, which might only become apparent in heteropentameric receptors.

Evolutionary perspective

A number of invertebrate species are known to express LGIC types that cross the ligand specificity/ion selectivity boundaries as present in vertebrates (Cully et al., 1994, 1996; Horoszok et al., 2001; Gisselmann et al., 2002; Beg and Jorgensen, 2003; Putrenko et al., 2004). Our findings show that anionic nAChRs in L. stagnalis represent true nAChRs that have acquired the ability to conduct anions by specific mutations in the ion channel domain. This is different from Acc 1 and Acc 2, found in C. elegans (Putrenko et al., 2004). These receptor subunits are highly similar to vertebrate GABA or glycine receptor subunits and lack the characteristic conserved residues of the ligand-binding site of nAChRs. As such, the C. elegans receptors are also not sensitive to nicotine. Of interest to our study is the C. elegans excitatory GABAR EXP-1, of which phylogenetic analysis suggests that it has evolved along an analogous, but opposite, evolutionary track (Fig. 6) (Beg and Jorgensen, 2003). In fact, most convertant LGICs, such as the C. elegans serotonin-gated (Ranganathan et al., 2000) Drosophila melanogaster histamine-gated chloride channels (Gisselmann et al., 2002, 2004) and the invertebrate inhibitory glutamate receptors (Cully et al., 1994, 1996; Horoszok et al., 2001), appear to originate from mutations in the LBD that induced changes in ligand specificity. This shows that mutations in either LBD or ion channel pore domains functionally contributed to LGIC subtype diversity in invertebrates. None of these receptor types, however, seem to have originated from recombination of LBD and ion channel domains.
Given the limited number of neurons, an increase in functional diversity of LGICs might serve to enhance the information handling capacity of the molluscan CNS. In molluscan species, fast synaptic chemical transmission of a single interneuron can have different effects (i.e., excitation, inhibition, or biphasic modulation) on the different postsynaptic partners (Wachtel and Kandel, 1971; Syed et al., 1990; Yeoman et al., 1993). Therefore, LGIC diversity in the invertebrate CNS might prevent the requirement for multiple interneurons, which each use a different LGIC to combine distinct ligand binding and channel specificities. Nature 366:479–483.


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References


Bhandal NS, Ramsey RL, Harvey RJ, Darlison MG, Usherwood PN (1995) Choline is a selective agonist of alpha7 nicotinic acetylcholine receptor expressed in Xenopus oocytes from a cloned cDNA. Invert Neurosci 1:267–272.


