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RESEARCH PAPER

Positive modulation of the α9α10 nicotinic cholinergic receptor by ascorbic acid

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BACKGROUND AND PURPOSE
The activation of α9α10 nicotinic cholinergic receptors (nAChRs) present at the synapse between efferent olivocochlear fibres and cochlear hair cells can prevent acoustic trauma. Hence, pharmacological potentiators of these receptors could be useful therapeutically. In this work, we characterize ascorbic acid as a positive modulator of recombinant α9α10 nAChRs.

EXPERIMENTAL APPROACH
ACh-evoked responses were analysed under two-electrode voltage-clamp recordings in *Xenopus laevis* oocytes injected with α9 and α10 cRNAs.

KEY RESULTS
Ascorbic acid potentiated ACh responses in *X. laevis* oocytes expressing α9α10 (but not α4β2 or α7) nAChRs, in a concentration-dependent manner, with an effective concentration range of 1–30 mM. The compound did not affect the receptor's current–voltage profile nor its apparent affinity for ACh, but it significantly enhanced the maximal evoked currents (percentage of ACh maximal response, 240 ± 20%). This effect was specific for the L form of reduced ascorbic acid. Substitution of the extracellular cysteine residues present in loop C of the ACh binding site did not affect the potentiation. Ascorbic acid turned into a partial agonist of α9α10 nAChRs bearing a point mutation at the pore domain of the channel (TM2 V13′T mutant). A positive allosteric mechanism of action rather than an antioxidant effect of ascorbic acid is proposed.

CONCLUSIONS AND IMPLICATIONS
The present work describes one of the few agents that activates or potentiates α9α10 nAChRs and leads to new avenues for designing drugs with potential therapeutic use in inner ear disorders.

Abbreviations
5HT3, 5-hydroxytryptamine-3 receptors; ASC, ascorbic acid; DHA, D-iso-ASC, D-iso-ascorbic acid; DTNB, 5-5′-dithiobis-2-nitrobenzoic acid; DTT, dehidroascorbate dithiothreitol; nAChRs, nicotinic cholinergic receptors; PAMs, positive allosteric modulators; TM2, transmembrane region 2

Introduction

The exposure to overly loud sounds is a substantial and growing health burden in industrialized countries, leading to hearing loss and tinnitus (Vio and Holme, 2005). The activity of efferent inhibitory cholinergic fibres projecting from the brainstem and synapsing onto cochlear hair cells can ameliorate acoustic trauma in rodents (Maison *et al.*, 2002; Taranda *et al.*, 2009). This inhibitory control of auditory function is mediated by α9α10 nicotinic cholinergic receptors (nAChRs) coupled to SK2 Ca^{2+}-activated K^+ channels, both present at the postsynaptic membrane of cochlear hair cells

(Elgoyhen and Katz, 2012). Consequently, enhancing α9α10 receptor-mediated responses by means of a pharmacological positive modulator could have a potential therapeutic use in the prevention or treatment of noise induced hearing loss (Elgoyhen *et al.*, 2009).

The α9α10 nAChR is a very peculiar member of the nAChR family, since it displays a very distinct pharmacological profile that fits neither the muscarinic nor the nicotinic classification scheme of cholinergic receptors (Verbitsky *et al.*, 2000; Elgoyhen *et al.*, 2001). Moreover, it shares pharmacological properties with other members of the Cys-loop family of receptors, which includes $GABA_A$ and $GABA_C$, glycine, 5-hydroxytryptamine-3 ($5HT_3$) and some invertebrate anionic glutamate receptors (Rothlin *et al.*, 1999; 2003). Thus, this pharmacologically promiscuous receptor is sensitive to a wide variety of antagonists including glycinergic, gabaergic, serotonergic (Rothlin *et al.*, 1999; 2003) as well as nicotinic and muscarinic antagonists (Verbitsky *et al.*, 2000), ototoxic drugs like aminoglycoside antibiotics (Rothlin *et al.*, 2000) and quinine derivatives (Ballestero *et al.*, 2005), the NMDA antagonist neramexane (Plazas *et al.*, 2007), some conotoxins (Johnson *et al.*, 1995; McIntosh *et al.*, 2005; Ellison *et al.*, 2006) and opioid peptides and morphine (Lioudyno *et al.*, 2002). Furthermore, nicotine and other nicotinic agonists like cytisine and epibatidine behave as antagonists of the α9α10 nAChRs (Verbitsky *et al.*, 2000; Elgoyhen *et al.*, 2001). Despite this relatively large number of antagonists characterized for α9α10 receptors, only one pharmacological potentiator is known to date, ryanodine (Zorrilla de San Martin *et al.*, 2007), which is not suitable for use in therapeutics. Therefore, finding novel positive modulators of the α9α10 nAChR that can be used clinically would represent a significant breakthrough, with potential use in noise-induced acoustic trauma.

Many drugs that have been tested as otoprotectants are antioxidant or related to antioxidant metabolism (Lynch and Kil, 2005). In this regard, it has been demonstrated that vitamin C, that is ascorbic acid (ASC), can prevent acoustic trauma in guinea pigs (McFadden *et al.*, 2005), but the underlying mechanism remains unclear. Several lines of evidence suggest that reducing agents scavenge free radicals produced during noise exposure (Le Prell *et al.*, 2007; Heinrich *et al.*, 2008), but other underlying mechanisms could account for this protectant effect. ASC is an endogenous antioxidant which is present in ~0.05 mM concentrations in plasma (Evans *et al.*, 1982), ~0.2 mM in CSF (Reiber *et al.*, 1993; Miele and Fillenz, 1996) and ~1 mM in whole brain (Spector and Johanson, 2006; Harrison and May, 2009). In certain tissues like the retina, concentrations of ASC are 100 times more concentrated than in plasma (Rose and Bode, 1991; Hediger, 2002). Particularly, in blood cells it reaches ~3 mM concentrations (Evans *et al.*, 1982) and in neurons 10 mM concentrations (Rice and Russo-Menna, 1998). Extracellular ASC levels can transiently rise substantially during neuronal activity (Bigelow *et al.*, 1984; Grunewald, 1993; Rebec and Pierce, 1994; Portugal *et al.*, 2009). It has been shown that ASC modulates the activity of several ion channels like NMDA glutamate receptors and T-type Ca^{2+} channels (Majewska *et al.*, 1990; Nelson *et al.*, 2007). Interestingly, in contrast to the effect of ASC on the above-mentioned channels where it decreases responses, it has been demonstrated recently that ASC potentiates responses through $GABA_A$ and $GABA_C$ receptors (Calero *et al.*, 2011). In the present work, we analysed the effect of ASC on other members of the Cys-loop family of receptors, namely the recombinant α7, α4β2 and α9α10 nAChRs. We show that ACh-evoked responses of α9α10 nAChRs expressed in *Xenopus* oocytes, but not α7 and α4β2, were potentiated by ASC in a concentration-dependent, stereo-specific, reversible and voltage-independent manner. The potentiating effect could involve an allosteric mechanism. The present results indicate that enhanced efferent activity to cochlear hair cells might account in part for the otoprotectant effect of ASC. In addition, they open the possibility of designing related compounds with improved properties for therapeutic use.

Methods

Expression of recombinant receptors in Xenopus laevis *oocytes*

For expression studies, α4, α7, α9, α10 and β2 rat nAChR subunits were subcloned into a modified pGEMHE vector. Capped cRNAs were *in vitro* transcribed from linearized plasmid DNA templates using RiboMAX™ Large Scale RNA Production System (Promega, Madison, WI). Mutant subunits were produced using Quick change XL II kit (Stratagene, La Jolla, CA). The maintenance of *X. laevis* and the preparation and cRNA injection of stage V and VI oocytes have been described in detail elsewhere (Verbitsky *et al.*, 2000). Typically, oocytes were injected with 50 nL of RNase-free water containing 0.01 to 1.0 ng of cRNA (at a 1:1 molar ratio when pairwise combined) and maintained in Barth's solution at 18°C. Electrophysiological recordings were performed 2 to 6 days after cRNA injection under two-electrode voltage clamp with an Oocyte Clamp OC-725B or C amplifier (Warner Instruments Corp., Hamden, CT). Recordings were filtered at a corner frequency of 10 Hz using a 900BT Tunable Active Filter (Frequency Devices Inc., Ottawa, IL). Data acquisition was performed using a Patch Panel PP-50 LAB/1 interphase (Warner Instruments Corp., Hamden, CT) at a rate of 10 points per second. Both voltage and current electrodes were filled with 3 M KCl and had resistances of ~1 MΩ. Data were analysed using Clampfit from the pClamp 6.1 software. During electrophysiological recordings, oocytes were continuously superfused (~15 $mL \cdot min^{-1}$) with normal frog saline composed of 115 mM NaCl, 2.5 mM KCl, 1.8 mM $CaCl_2$ and 10 mM HEPES buffer, pH 7.2. Acetylcholine, ASC, D-iso-ascorbic (D-iso-ASC) acid, dehidroascorbate (DHA), dithiothreitol (DTT) and 5-5′-dithiobis-2-nitrobenzoic acid (DTNB) were added to the perfusion solution for application. Unless otherwise indicated, the membrane potential was clamped to –70 mV. To minimize activation of the endogenous Ca^{2+}-sensitive chloride current (Elgoyhen *et al.*, 2001), all experiments were performed in oocytes incubated with the Ca^{2+} chelator BAPTA-AM (100 μM) for 3 h before electrophysiological recordings. Concentration–response curves were normalized to the maximal agonist response in each oocyte. The mean and SEM of peak current responses are represented. Agonist concentration–response curves were iteratively fitted, using Prism 5 software (GraphPad Software Inc., La Jolla, CA), with

the following equation: $I/I_{max} = A^{nH}/(A^{nH} + EC_{50}{}^{nH})$, where I is the peak inward current evoked by agonist at concentration A; I_{max} is the current evoked by the concentration of agonist eliciting a maximal response; EC_{50} is the concentration of agonist inducing half-maximal current response and nH is the Hill coefficient. Current–voltage (I–V) relationships were obtained by applying 2-s voltage ramps from –120 to –50 mV, 10 s after the peak response to 10 μM ACh from a holding potential (V_{hold}) of –70 mV. Leakage correction was performed by subtraction of the current–voltage curve obtained by the same voltage ramp protocol before the application of ACh. Data were analysed using Clampfit from the pClamp 6.1 software. Some of our data sets did not fit to a standard Gaussian distribution, tested using Kolmogorov–Smirnov, D'Agostino–Pearson or Shapiro–Wilk tests. Thus, statistical significance was evaluated using Mann–Whitney; $P < 0.05$ was considered significant. Correlation was evaluated using Pearson correlation test; $P < 0.05$ was considered significant.

Materials

All drugs were obtained from Sigma-Aldrich (St. Louis, MO). ACh chloride was dissolved in distilled water as 100 mM stocks and stored aliquoted at –20°C. BAPTA-AM was stored at –20°C as aliquots of a 100 mM solution in dimethyl sulfoxide (DMSO), thawed and diluted 1000-fold into Barth's solution shortly before incubation of the oocytes. ASC, D-iso-ASC, DHA and DTT solutions in Ringer's saline were freshly prepared immediately before application. The pH was adjusted with NaOH when required. When NaOH was added to solutions to adjust the pH, Na^+ concentration was adjusted to remain at 115 mM to avoid changes in the ion's driving force. ASC, D-iso-ASC and DHA solutions were always covered to avoid exposure to light. DTT solutions were prepared in the presence of 10 mM tricine in order to avoid any interference of zinc traces (Pan *et al.*, 2000). DTNB was dissolved in DMSO to reach a 250 mM concentration and then diluted 1000-fold in frog saline immediately before application. All studies involving animals are reported in accordance with the ARRIVE guidelines for reporting experiments involving animals (McGrath *et al.*, 2010). All experimental protocols were carried out in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals (NIH Publications no. 80-23) revised 1978. The drugs and receptors names mentioned in the manuscript conform to BJP's *Guide to Receptors and Channels* (Alexander *et al.*, 2011).

Molecular homology modelling

Homology models for the extracellular domain of the rat α9α10 receptor were constructed using Modeller 9v8 software (Sali and Blundell, 1993), considering the known stoichiometry (Plazas *et al.*, 2005b) and an alternate subunit assembly, using the *Aplysia californica* AChBP apo form crystallography (2W8E.pdb) as a template.

Results

Effect of ASC on responses to ACh of recombinant α9α10 nAChRs

Figure 1A shows representative responses evoked by the reported EC_{50} concentration of ACh, 10 μM (Elgoyhen *et al.*, 2001), in *X. laevis* oocytes expressing rat α9α10 nAChRs. The co-application of 3 mM ASC potentiated responses to the agonist. Potentiation was reversible since initial control responses to ACh were recovered after washing the oocytes with frog saline for 3 min. Pre-incubation with ASC for 5 min did not further modify its potentiating effect (data not shown). In addition, ASC *per se* did not have an effect on α9α10 expressing oocytes. Figure 1B shows a scatter plot to depict that the potentiating effect of ASC was quite variable. This variability was not correlated with the expression level of the receptor in oocytes ($P = 0.1139$, Pearson test). The mean magnitude of potentiation by 3 mM ASC, expressed as the percentage of the response to 10 μM ACh, was 165 ± 6% (n = 77, eight frogs). As shown in Figure 1C, 3 mM ASC did not change the macroscopic current decay kinetics of responses to 100 μM acetylcholine, assessed evaluating the relation between the current amplitude at 20 s after reaching the maximum (I_{20}) and current amplitude at the peak (I_{20}/I_{max} control: 66 ± 8%; plus ASC: 60 ± 10, $n = 4$, two frogs; $P = 0.99$ Mann–Whitney test). A possible intracellular effect was ruled out as the injection of oocytes with ASC (to reach a final 3 mM concentration) did not produce a significant effect on ACh evoked currents (Figure S1).

In order to test if ASC also potentiates other nAChRs, the effect was analysed on α4β2 and α7 nAChRs (Figure 2). Responses to an EC_{50} concentration of ACh was not modified

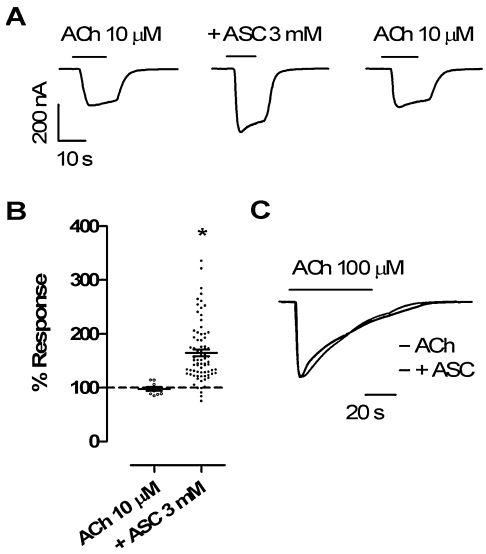

Figure 1

Effect of ASC on responses to ACh of recombinant α9α10 nAChRs. (A) Responses evoked by ACh either alone, plus 3 mM ASC or after a 3 min wash, in oocytes expressing α9α10 nAChRs (representative of n = 77, eight frogs). (B) Scatter plot displaying the experimental variability of the responses to the application of 10 μM ACh alone (n = 10, two frogs) or co-applied with 3 mM ASC (n = 77, eight frogs). Peak current values are shown, expressed as the percentage of the peak control current elicited by 10 μM ACh alone. The mean and SEM for each group is shown in black; *$P < 0.0001$ Mann–Whitney test. (C) Representative responses (of n = 4 repetitions, two frogs), scaled to the same peak amplitude, evoked by a 1 min application of 100 μM ACh either alone or co-applied with 3 mM ASC, in oocytes expressing α9α10 nAChRs.

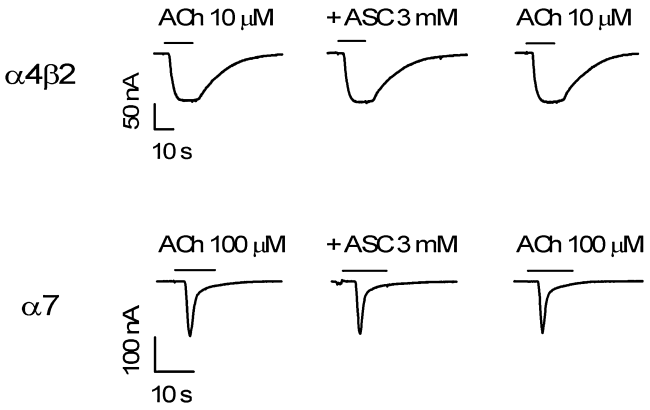

Figure 2

Effect of ASC on responses to ACh of recombinant α4β2 or α7 nAChRs. Responses evoked by ACh either alone, plus 3 mM ASC or after a 3 min wash, in oocytes expressing α4β2 or α7 nAChRs (representative of $n = 15$, three frogs; $n = 16$, three frogs respectively).

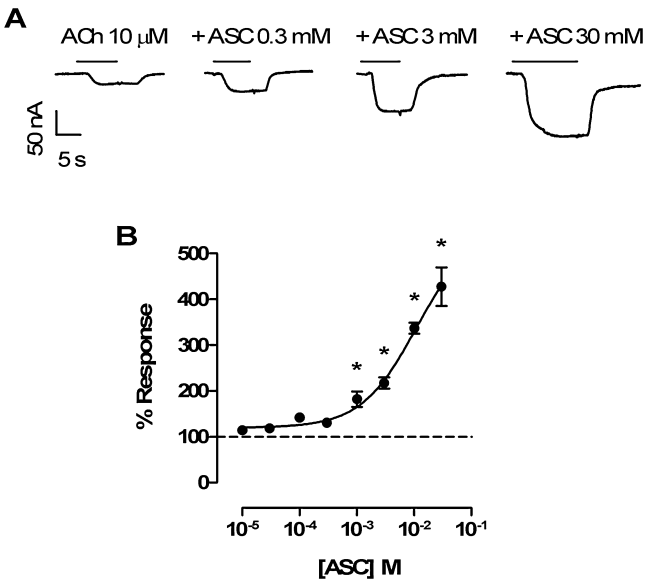

Figure 3

Concentration-dependent effect of ASC on the recombinant α9α10 nAChR. (A) Representative traces of responses evoked by 10 μM ACh either alone or co-applied with increasing concentrations of ASC in oocytes injected with α9 and α10 cRNAs. (B) Concentration–response curve constructed by performing co-applications of 10 μM ACh and increasing concentrations of ASC. Peak current values are shown, expressed as the percentage of the peak control current elicited by 10 μM ACh alone. The mean and SEM of 4 to 13 experiments per group, using oocytes from four frogs, are shown. *$P < 0.05$ Mann–Whitney test.

by the co-application of 3 mM ASC in the case of α4β2 (percentage of 10 μM ACh response, 102 ± 4%, $n = 15$, three frogs) and α7 nAChRs (percentage of 100 μM ACh response, 105 ± 4%, $n = 16$, three frogs). A 10 mM concentration of ASC inhibited responses to ACh of α4β2 and α7 nAChRs (Figure S2).

As shown in Figure 3, the potentiating effect of ASC on ACh-evoked currents through α9α10 receptors was concentration-dependent. Figure 3B shows a concentration–response curve for the potentiating effect of ASC performed at 10 μM ACh ($n = 4$–13 per point, four frogs). Potentiation was significant at 1 mM or higher concentrations of ASC and did not saturate at the maximal concentration tested (30 mM). To rule out a possible osmotic effect of applying ASC solutions of such concentrations we applied 3, 10 and 30 mM ASC, without ACh, and observed no change in the leak currents (data not shown). In addition, the application of sucrose solutions isoosmotical to 3, 10 or 30 mM ASC solutions, produced no effect on the leak currents and did not potentiate ACh responses (data not shown). To further characterize this potentiating effect, concentration–response curves to ACh were performed in the presence or absence of 3 mM ASC. As shown in Figure 4A, ASC produced a significant increase in the agonist maximal response (percentage of maximal response, 240 ± 20%, $n = 6$, three frogs) and no change in the EC_{50} (control, 16 ± 1 μM; 3 mM, ASC 18 ± 1 μM; $n = 5$–12, three frogs) or Hill coefficient (control, 1.05 ± 0.05; 3 mM ASC, 0.8 ± 0.1; $n = 5$–12, three frogs). In addition, the effect was not voltage-dependent (Figure 4B). Thus, potentiation was equally observed at depolarized (percentage of ACh response at +40 mV: 130 ± 14%, $n = 10$, two frogs) and hyperpolarized (percentage of ACh response at –90 mV: 137 ± 10%, $n = 10$, two frogs) potentials, and there was no change in the reversal potential (control: –5 ± 2 mV; 3 mM ASC, –7 ± 3 mV, $n = 10$, two frogs).

We tested two structurally related analogs: DHA, the oxidized product of ASC, which lacks antioxidant activity, and the stereoisomer D-iso-ASC, which has the same antioxidant activity as ASC (Figure 5A). In α9α10 expressing oocytes, neither 3 mM DHA nor 3 mM D-iso-ASC potentiated responses evoked by 10 μM ACh. In fact, at ACh concentrations lower than 10 μM, they both inhibited α9α10 receptor currents (Figure 5B). Inhibition was only observed at concentrations of ACh lower than 10 μM. To test if the presence of D-iso-ASC could influence the effect of ASC, as they are very similar molecules, we co-applied both compounds at a 3 mM concentration with ACh, using an agonist concentration at which D-iso-ASC produced no effect *per se* (10 μM, Figure 5B). Potentiation was not significantly different in the presence or absence of 3 mM D-iso-ASC (percentage of ACh response: 3 mM ASC, 131 ± 8%; +3 mM D-iso-ASC, 130 ± 11%; $n = 6$, three frogs; $P = 0.8182$, Mann–Whitney; data not shown). Taken together, these results indicate that the potentiation effect is exclusive of the reduced L form of ASC.

Contribution of redox sensitive extracellular residues to the potentiation by ASC

Potentiation by ASC could derive from at least two different mechanisms: redox modulation or the binding to an allosteric potentiating site. In an attempt to gain insight into the mechanism underlying the potentiating effect of ASC, we first tested whether the α9α10 nAChR is sensitive to modulation by redox agents. Co-application of 10 μM ACh together with the reducing agent DTT (2 mM) potentiated α9α10 receptor currents (percentage of control response, 230 ± 30%, $n = 24$, four frogs). On the other hand, co-application

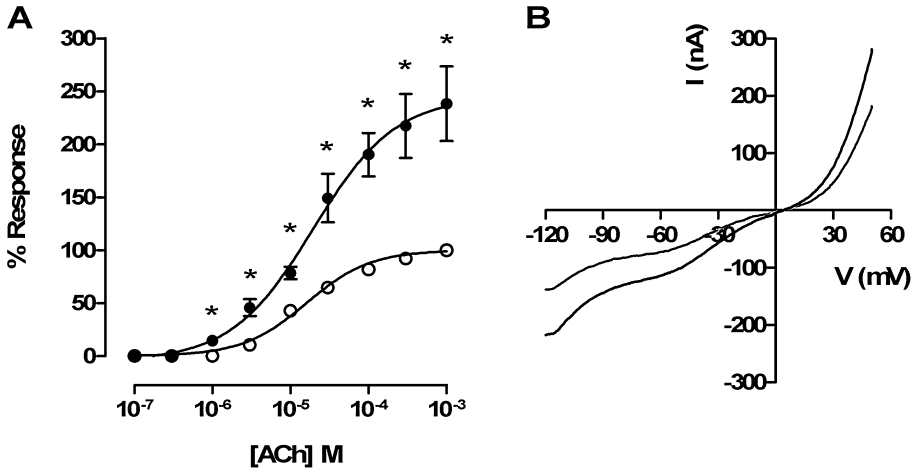

Figure 4

Characterization of the potentiating effect of ASC on recombinant α9α10 nAChRs. (A) Concentration–response curves to ACh performed either alone or in the presence of 3 mM ASC (filled circles). Peak current values were normalized and referred to the maximal peak response to ACh (1 mM). The mean and SEM of 5 to 12 experiments per group, using oocytes from three frogs, are shown. *, significantly different form control, $P < 0.05$ Mann–Whitney test. (B) Representative ($n = 10$, 2 frogs) I–V curve obtained in the presence of 10 μM ACh either alone (grey) or co-applied with 3 mM ASC (black). A 2 s voltage ramp from –120 to 50 mV was applied at the plateau phase of the response obtained in α9α10 expressing oocytes clamped at –70 mV. Currents were leak-corrected by subtracting the response to the same voltage ramp obtained prior to the application of ACh.

of 10 μM ACh and the oxidizing agent DTNB (250 μM) inhibited α9α10 receptor currents (percentage of inhibition, 33 ± 4%, n = 17, three frogs). Figure 6A shows representative responses, and Figure 6B shows quantification of results. As previously observed with ASC, potentiation by DTT was very variable. On the other hand, the degree of inhibition by DTNB was quite reproducible (Figure 6C). The effects of DTT and DTNB were readily reversible after 3 min wash of the oocytes with frog saline solution. Neither, 2 mM DTT nor 250 μM DTNB elicited responses in α9α10 injected oocytes (data not shown). These results indicate that the α9α10 is sensitive to redox modulation.

Cysteine residues are subject to rapid and reversible redox modification in physiological conditions, which makes them the most likely candidate target for a redox mechanism of modulation (Klomsiri *et al.*, 2011). In addition, there is evidence showing that ASC is able to reduce disulfide bridges *in vitro* (Landino *et al.*, 2006). The α9 and α10 subunits have four highly conserved extracellular cysteines, two consecutive ones (C192 and C193, *Torpedo marmorata* α numbering) in loop C, characteristic of the principal component of the binding site of α subunits and two additional ones that form the Cys-loop disulfide bond characteristic of the family (C128 and C142, *T. marmorata* α numbering). We analysed the effect of ASC in oocytes expressing mutant α9 and α10 subunits with the two consecutive Cys residues in loop C substituted by serine. Mutant α9α10 CC192/193SS receptors responded to ACh with an EC_{50} of 983 ± 84 μM and a Hill coefficient of 1.1 ± 0.2 (n = 7, three frogs) (Figure 7A, B). Responses to ACh were potentiated by 3 mM ASC. As shown in Figure 7B, and as previously seen for wild-type receptors (Figure 4A), ASC produced a significant increase in the agonist maximal response (percentage of maximal response, 160 ± 10%, n = 6, three frogs) and no change in the EC_{50} (798 ± 44, n = 6, three frogs) or Hill coefficient (1.2 ± 0.2, n = 6, three frogs). In addition, the effect was not voltage-dependent (Figure 7C). Thus, potentiation was equally observed at depolarized (percentage of ACh response at +40 mV: 160 ± 20%, n = 6, two frogs) and hyperpolarized (percentage of ACh response at –90 mV: 155 ± 16%, n = 6, two frogs) potentials, and there was no change in the reversal potential (control: –8 ± 2 mV; 3 mM ASC, –13 ± 3 mV, n = 6, two frogs). These results indicate that a direct effect of ASC on the contiguous CC192/193 does not contribute to its potentiating effect. Consistently, the presence of the cysteine to serine substitutions in only α9 or α10 single mutant receptors did not abolish the potentiation by ASC (Figure S3).

It has been shown that mutations of the cysteine residues that form the Cys-loop disulfide bond lead to non-functional or non-expressing nAChRs in *X. laevis* oocytes (Dunckley *et al.*, 2003). Therefore, mutating these residues was not an option and the participation of the cysteine residues of the Cys-loop to the potentiating effect of ASC was not tested.

The transmembrane 2 mutation V13′T converts ASC from a potentiator to an agonist

Gill *et al.* (2011) have shown that TQS, an allosteric potentiator of α7 nAChRs, becomes an agonist of mutant L9′T α7 receptors. Figure 8A shows representative responses evoked by 3 mM ASC and control responses to a saturating (3 μM) ACh in *X. laevis* oocytes expressing rat α9 and α10 subunits bearing the V13′T mutation, which produces similar but more pronounced effects than those of the L9′T mutation (Plazas *et al.*, 2005a). ASC elicited inward currents in a concentration-dependent manner, with a maximal effect that was 20 ± 4% (n = 5, two frogs) of that observed with a saturating 3 μM ACh concentration (Figure 8A, B). On the contrary, in the presence of 3 mM D-iso-ASC, outward currents were observed which were 14 ± 3% (n = 6, two frogs) of currents elicited by 3 μM ACh (Figure 8C). In addition, 2 mM

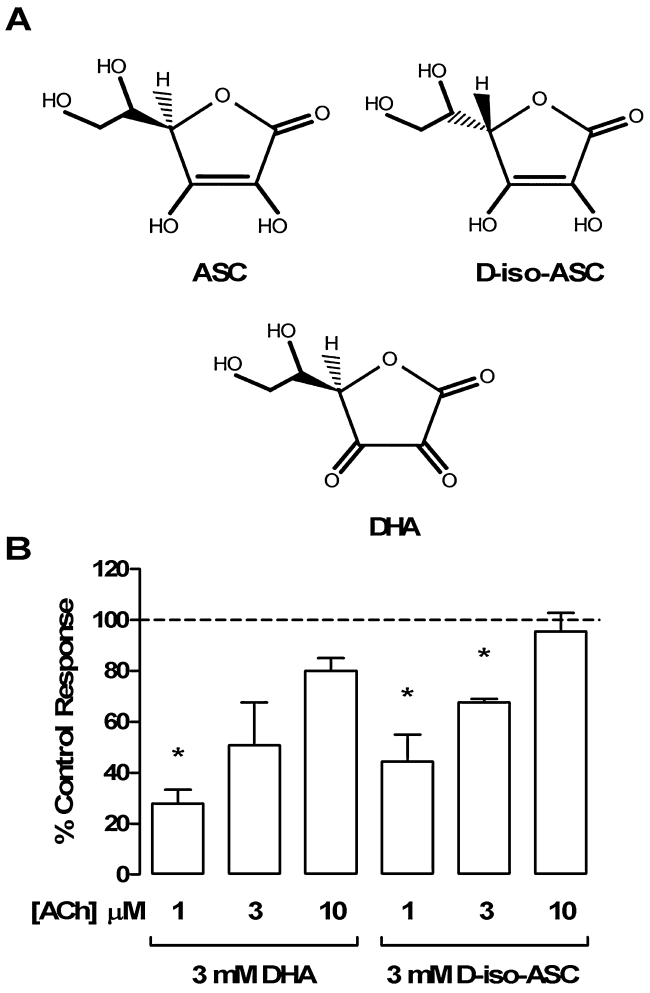

Figure 5

Effect of different ASC analogs on responses to ACh of recombinant α9α10 nAChRs. (A) Chemical structure of L- ASC, DHA and D-iso-ASC. (B) Quantification of the effect of 3 mM DHA or D-iso-ASC on currents evoked by increasing concentrations of ACh. Peak current values obtained by the co-application of ACh and each of the compounds were normalized and referred to the control peak response to ACh for each ACh concentration. The mean and SEM of 3 to 8 experiments per group, using oocytes from two frogs, are shown. *$P < 0.05$ Mann–Whitney test.

DTT also elicited outward currents (percentage of ACh 3 μM response; 23 ± 4, $n = 6$, two frogs). Taken together, these results suggest that an allosteric potentiation mechanism rather than a redox effect might contribute to the action of ASC on α9α10 nAChRs.

Contribution of extracellular arginine residues to the potentiation by ASC

The ASC interaction site has been characterized in a number of proteins (Pande and Myer, 1980; Burmeister *et al.*, 2000; Sharp *et al.*, 2003; Mishra *et al.*, 2009). In all of these, arginine directly interacts with ASC. Consequently, we searched for extracellular arginine residues in α9 and α10 subunits. To narrow down the number of candidate arginine residues, we focused on those present at, or close to, domains related to receptor gating (Bartos *et al.*, 2009). To this end, we constructed molecular homology models for the extracellular domain of the α9α10 receptor, considering the known stoichiometry (Plazas *et al.*, 2005b) and an alternate subunit assembly, using the *Aplysia californica* AChBP apo form (2W8E.pdb) as a template. Of the many extracellular arginine residues present in α9 and α10 subunits, we focused on two (Figure 9A), one close to the ACh binding site in loop D (R57) and another one at the β1-β2 loop (R46). Other arginine residues were either not exposed at the surface of the molecule, which would make them inaccessible to ASC in solution, or were not located in sites of the molecule known to be related to channel gating. To analyse the influence of these residues on the effect of ASC, we substituted them to alanine to produce mutant α9 and α10 subunits. Figure 9B shows that currents elicited by two different concentrations of ACh in R46A mutant α9α10 nAChRs were potentiated by co-applied 3 mM ASC (percentage of 10 μM ACh response, 179 ± 9%, $n = 7$, three frogs). Similar results were obtained with R57A mutant receptors (Figure 9C, percentage of 100 μM ACh response, 152 ± 5%, $n = 4$, two frogs). Thus, arginine residues R46 and R57 are most likely not involved in the effect of ASC.

Discussion and conclusions

The present work shows that ASC at high micromolar-low millimolar concentrations potentiates ACh-elicited responses in *Xenopus* oocytes expressing the α9α10 nAChR. To the best of our knowledge, this is the first report of a potentiating effect of ASC on a nAChR and adds to the previously described action of this compound on $GABA_A$ and $GABA_C$ receptors (Calero *et al.*, 2011), members also of the Cys-loop family of ligand-gated ion channels (Thompson *et al.*, 2010). The effect is not a broad one over nAChRs in general, since α4β2 and α7 receptors were not subject to potentiation. The effective potentiating concentration of ASC on the α9α10 nAChRs is similar to that described for $GABA_A$ and $GABA_C$ receptors and within the concentration that can be attained in neuronal tissues. Thus, this endogenous redox agent is highly concentrated in several regions of the CNS and is accumulated in neurons and glial cells by specific transporters (Harrison and May, 2009; Corti *et al.*, 2010). It is present at high micromolar concentrations in the cerebrospinal fluid and millimolar concentrations within cells (Evans *et al.*, 1982; Reiber *et al.*, 1993; Miele and Fillenz, 1996; Rice and Russo-Menna, 1998; Spector and Johanson, 2006), and in neurons, the concentration may reach as high as 10 mM (Rice and Russo-Menna, 1998; Rice, 2000). In certain tissues like the retina, concentrations of ASC are 100 times more concentrated than in plasma (Rose and Bode, 1991; Hediger, 2002). Moreover, the extracellular concentration of ASC can transiently undergo substantial increases during neuronal activity under physiological or pathological conditions (Bigelow *et al.*, 1984; Grunewald, 1993; Rebec and Pierce, 1994; Portugal *et al.*, 2009). Neither the concentration of ASC in cochlear fluids nor within hair cells, nor the expression of ASC transporters in the cochlea has been investigated. However, it is likely that, as reported for other tissues, micro

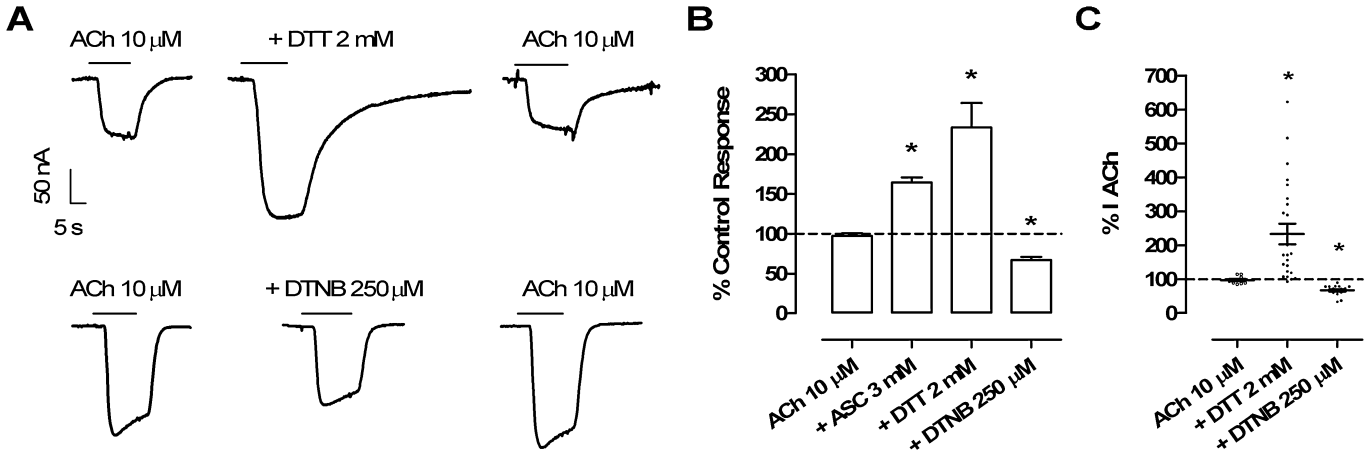

Figure 6

Effect of reducing and oxidizing agents on ACh-evoked currents. (A) Representative traces of responses evoked by the co-application of 10 μM ACh and 2 mM DTT (upper panel, $n = 24$, four frogs) or 250 μM DTNB (lower panel, $n = 17$, three frogs). DTT was co-applied with 10 mM tricine to rule out an effect of Zn^{2+} traces. (B) Quantification of the effect of DTT and DTNB on currents evoked by 10 μM ACh. Effect of ASC was included for the sake of comparison. Peak current values were normalized and referred to the peak response to 10 μM ACh. The mean and SEM of 6 to 24 experiments per group, using oocytes from at least two frogs, are shown. *$P < 0.05$ Mann–Whitney test. (C) Scatter plot displaying the experimental variability of the responses to the application of 10 μM ACh alone ($n = 10$, two frogs), co-applied with 2 mM DTT ($n = 24$, four frogs) or 250 μM DTNB ($n = 17$, three frogs). Peak current values are shown, expressed as the percentage of the peak control current elicited by 10 μM ACh alone. The mean and SEM for each group is shown in black. *$P < 0.05$ Mann–Whitney test.

to millimolar concentrations of ASC are present in or around hair cells or might be attained under pathophysiological conditions such as trauma.

The fast onset, fast offset and reversible character of the effect of ASC on α9α10 nAChRs receptors suggest that it is not due to its entry into the cells. This is in agreement with the fact that pre-incubation with ASC did not enhance the potentiating effect (data not shown). Moreover, oocytes do not express ASC transporters (SCVT1 and SCVT2) (Dyer *et al.*, 1994); and, being a hydrophilic compound, ASC does not cross the lipid bilayer. In addition, the injection of ASC into oocytes did not modify ACh-evoked currents. Based on this evidence an extracellular superficial site of action is likely. One possible explanation for the observed action of ASC is via an antioxidant effect. It has been reported that nAChRs are subject to redox modulation. Thus, DTT reduces the maximal binding of nicotine or the binding affinity of α-bungarotoxin in total rat brain preparations (Stitzel *et al.*, 1988) and the responses of nAChRs present in PC12 cultured cells (Leprince, 1983). In addition, reduction by DTT followed by alkylation in the presence of NEM results in nAChRs with decreased open times and single-channel currents in BC3H-11 cells (Bouzat *et al.*, 1991). The observation that DTT potentiated and DTNB inhibited currents through the α9α10 nAChR indicates that this receptor is also subject to redox modulation, albeit in the opposite direction to that reported for other nAChRs and similar to that described for $GABA_A$ and $GABA_C$ receptors (Amato *et al.*, 1999; Pan *et al.*, 2000; Calero and Calvo, 2008). Previous studies have suggested that cysteine residues are commonly involved in the redox modulation of various ion channels (Ruiz-Gomez *et al.*, 1991; Ruppersberg *et al.*, 1991; Sullivan *et al.*, 1994; Chu *et al.*, 2006). The fact that the potentiating effect of ASC was still observed in the α9α10 CC192/193SS mutant receptor precludes the participation of these residues in a possible redox effect of ASC. The observation that DHA, the oxidized product of ASC, did not potentiate but decreased the amplitude of responses to low concentrations of ACh could speak towards an antioxidant effect of ASC over the α9α10 nAChR. However, one cannot exclude the possibility that both ASC and DHA are binding to different sites within the receptor and producing opposite effects (e.g. a competitive antagonism in the case of DHA and a positive allosteric modulation in the case of ASC). Moreover, the –OH groups only present in ASC might be required for recognition of a putative binding site within the receptor. The observation that the potentiating effect of ASC was stereoselective since D-iso-ASC, ASC's stereoisomer with equal antioxidant activity, did not potentiate responses to ACh might speak towards the existence of a binding site for the compound resulting in a positive allosteric mechanism of action, as reported for several novel compounds acting upon other nAChRs (Iorga *et al.*, 2006; Bertrand *et al.*, 2008; Young *et al.*, 2008; Arias *et al.*, 2011; Gill *et al.*, 2011). Although the potentiating effect of ASC was not inhibited by 3 mM D-iso-ASC, it is possible that they both bind to the same site but with different affinities, and that very high millimolar D-iso-ASC concentrations are needed in order to overcome the effect of ASC.

Positive allosteric modulators (PAMs) have a long and successful clinical track record exemplified by the benzodiazepine family of drugs (e.g. diazepam), which function as allosteric modulators of $GABA_A$ receptors (Bateson, 2004). In recent years, positive allosteric modulators (PAMs) mostly of α7 and α4β2 nAChRs have emerged. These compounds reinforce the endogenous cholinergic neurotransmission without directly stimulating the target receptors (Bertrand and Gopalakrishnan, 2007; Arias, 2010). PAMs do not bind to the ACh orthosteric binding sites but allosterically enhance the

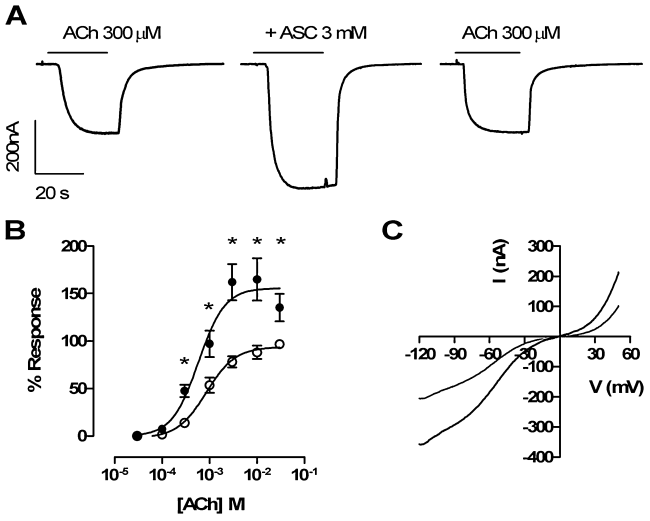

Figure 7

Role of extracellular Cys residues in the potentiation by ASC. (A) Responses evoked by ACh either alone, plus 3 mM ASC or after a 3 min wash, in oocytes expressing mutant α9 and α10 subunits with CC 192/193 SS substitutions (*Torpedo* α1 numbering) (n = 6, three frogs). (B) Concentration–response curves to ACh either alone or in the presence of 3 mM ASC (black circles) obtained in oocytes expressing mutant α9 and α10 subunits with CC 192/193 SS substitutions. Peak current values were normalized and referred to the maximal peak response to ACh. The mean and SEM of 6 to 7 experiments, using oocytes from three frogs, per group, are shown. *P < 0.05, Mann–Whitney test. (C) Representative (n = 6, two frogs) I–V curve obtained in the presence of 1 mM ACh either alone (grey) or co-applied with 3 mM ASC (black). A 2 s voltage ramp from –120 to 50 mV was applied at the plateau phase of the ACh response obtained in oocytes expressing CC 192/193 SS mutant α9α10 nAChRs clamped at –70 mV. Currents were leak-corrected by subtracting the response to the same voltage ramp obtained prior to the application of acetylcholine.

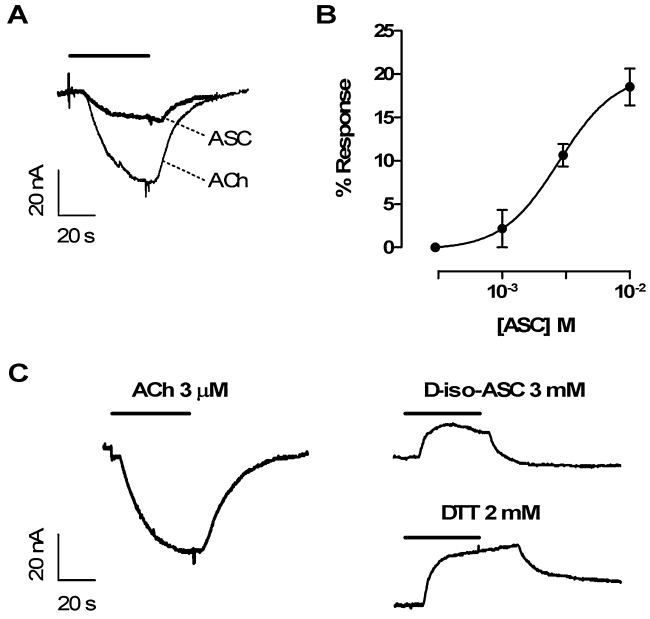

Figure 8

ASC is a partial agonist of α9α10 V13′T mutant receptors. (A) Representative traces of responses evoked by 10 mM ASC and by a saturating concentration of ACh (3 μM), in oocytes expressing V13′T mutant α9α10 receptors (n = 5, two frogs). (B) Concentration–response curve to ASC in oocytes expressing V13′T mutant α9α10 receptors. Peak current values were normalized and referred to the peak response to a saturating 3 μM ACh concentration. The mean and SEM of five experiments per concentration, using oocytes from two frogs, are shown. (C) Representative traces of the effect of 3 mM D-iso-ASC and 2 mM DTT in V13′T mutant α9α10 receptors (n = 6 in all cases, using oocytes from at least two frogs).

activity elicited by agonists by increasing the gating process and mainly affecting the peak current response (type I) and/or by decreasing desensitization (type II). The observation that ASC increased responses to ACh without modifying the macroscopic current decay kinetics would possibly classify it as a type I PAMs. However, type I PAMs in general also increase the apparent affinity of the agonist (Arias, 2010), an effect that was not observed in the case of ASC potentiation of the α9α10 nAChR. Thus, the classification of PAMs could be oversimplified, and future analyses might incorporate new mechanisms mediating allosteric modulation. It has been reported that the α7 PAM TQS behaves as an agonist of the L9′T mutant receptor (Gill *et al.*, 2011), a receptor that has increased gating properties and spontaneous openings in the absence of the agonist (Labarca *et al.*, 1995). In accordance to described models for allosteric proteins (Monod *et al.*, 1965), the agonist effect of TQS has been explained in terms of its influence on rate constants for transitions between resting and open states of the receptor, favoring the open state (Gill *et al.*, 2011). A similar effect of ASC can be hypothesized, since it also becomes a partial agonist of the V13′T α9α10 nAChR. In addition, the fact that D-iso-ASC does not behave as an agonist of the V13′T mutant receptor but produces a deflection of the baseline current in the positive direction (that most likely results from the closure of spontaneously opened mutant receptors) (Plazas *et al.*, 2005a) could suggest that it binds to the ASC binding site and acts as a negative allosteric modulator, a mechanism of action reported for some compounds acting on nAChRs (Arias, 2010). Moreover, the observation that the reducing agent DTT produces a positive deflection of the leak current and not an inward current as observed with ASC indicates that an antioxidant effect is probably not the underlying mechanism of action of ASC on the α9α10 nAChR. Although arginine residues have been described to be at the interaction site with ASC in proteins that have been crystalized with this compound (Pande and Myer, 1980; Burmeister *et al.*, 2000; Sharp *et al.*, 2003; Mishra *et al.*, 2009), they seem not to play a role in the case of the α9α10 receptor. Several amino acid residues present at both the extracellular or transmembrane portions of the nAChR have been reported to be involved in the site of action of PAMs (Arias, 2010). Further experiments that will involve molecular docking and the generation of chimeric and mutant receptors will aid to delineate the site of action of ASC on the α9α10 nAChR.

Does the potentiating effect of ASC on α9α10 nAChRs have a potential physiological or pathophysiological role? Antioxidants prevent cochlear damage from toxic reactive

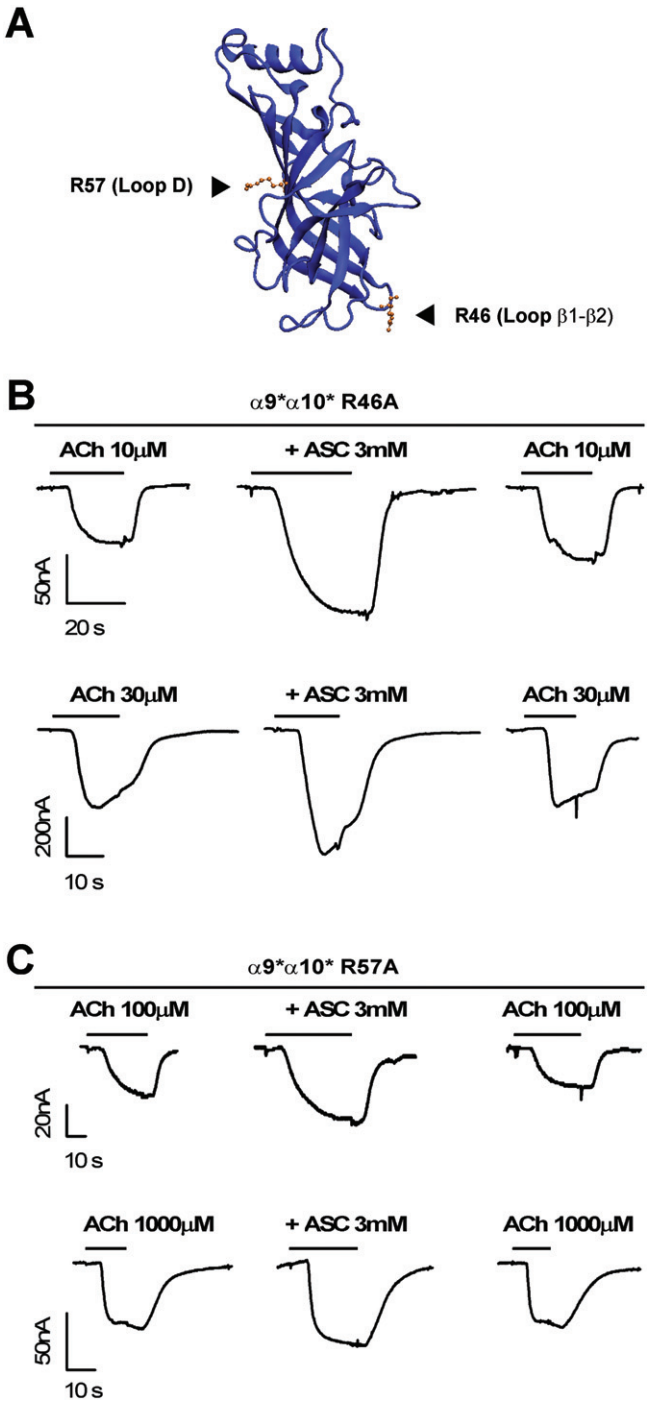

Figure 9

Mutation of arginine residues in the extracellular domain of α9 and α10 subunits does not abolish ASC potentiation. (A) Ribbon diagram of the extracellular domain of the α9 subunit derived from a molecular homology model, displaying candidate arginine residues residues in loop D (R57, torpedo α1 numbering) and loop β1-β2 (R46). These residues are also present in the same domains in an α10 homology model. (B) Representative traces (n = 6–7, three frogs) of responses evoked by the co-application of different concentrations of ACh and 3 mM ASC in oocytes expressing R46A α9α10 receptors. (C) Representative traces (n = 4, two frogs) of responses evoked by the co-application of different concentrations of ACh and 3 mM ASC, in oocytes expressing R57A α9α10 receptors.

oxygen species that are produced at high levels during and after noise exposure (Lynch and Kil, 2005; Le Prell *et al.*, 2007). In particular, it has been described in rodents that ASC is beneficial in reducing the sensitivity to noise-induced hearing loss (McFadden *et al.*, 2005). Thus, in addition to its antioxidant effects and based on the described protective effect of the medial olivocochlear system (Maison *et al.*, 2002; Taranda *et al.*, 2009), potentiation of responses mediated by α9α10 nAChRs at the efferent-hair cell synapse might also play a role.

In summary, we report that α9α10 nAChR responses are potentiated by ASC. This is an important contribution to the pharmacology of this receptor, for which few positive modulators have been described and paves the way to the design of clinically useful compounds for inner ear pathologies.

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Conflicts of interest

None.

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Figure S1 Effect of ASC injection on α9α10 responses to ACh. (A) Responses evoked by 10 μM ACh prior to injection of 4.6 nL (mean oocyte volume ~300 nL) of 300 mM ASC, pH balanced with equimolar KOH, 5 s and 5 min after injection (representative of n = 4, one frog). Arrowheads show time of injection. (B) Responses evoked by 10 μM ACh prior to control injection of 4.6 nL of 300 mM KCl, 5 s and 5 min after injection (representative of n = 4, one frog). (C) Scatter plot displaying the experimental variability of the injection of ASC on the responses to 10 μM ACh (n = 4, 1 frog) or the effect of the control injection with KCl (n = 4, one frog). Peak current values are shown only for the 5 s responses, expressed as the percentage of the peak control current elicited by 10 μM ACh prior to injection. The mean and SEM for each group is shown in black; P = 0.07, Mann–Whitney test.

Figure S2 Effect of 10 mM ASC on responses to ACh of recombinant α4β2 or α7 nAChRs. Responses evoked by ACh either alone, plus 10 mM ASC or after a 3 min wash, in oocytes expressing α4β2 or α7 nAChRs (representative of $n = 6$, two frogs).

Figure S3 Role of extracellular Cys residues in the potentiation by ASC. Responses evoked by ACh either alone, plus 3 mM ASC or after a 3 min wash, in oocytes expressing mutant α9 or α10 subunits with CC 192/193 SS substitutions (*Torpedo* α1 numbering) ($n = 3$, one frog).