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α Conotoxin ArIB[V11L,V16D] is a potent and selective antagonist at rat and human native $\alpha 7$ nicotinic acetylcholine receptors

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Abstract

A recently developed α -conotoxin, α -CtxArIB[V11L,V16D] is a potent and selective competitive antagonist at rat recombinant $\alpha 7$ nicotinic acetylcholine receptors (nAChRs), making it an attractive probe for this receptor subtype. $\alpha 7$ nAChRs are potential therapeutic targets that are widely expressed in both neuronal and non-neuronal tissues where they are implicated in a variety of functions. Here we evaluate this toxin at rat and human native nAChRs. Functional $\alpha 7$ nAChR responses were evoked by choline plus the allosteric potentiator PNU-120596 in rat PC12 cells and human SHSY5Y cells loaded with calcium indicators. α -CtxArIB[V11L,V16D] specifically inhibited $\alpha 7$ nAChR-mediated increases in Ca^{2+} in PC12 cells. Responses to other stimuli (5-iodo-A-85380, nicotine or KCl) that did not activate $\alpha 7$ nAChRs were unaffected. Human $\alpha 7$ nAChRs were also sensitive to α -CtxArIB[V11L,V16D]: ACh-evoked currents in *X. laevis* oocytes expressing human $\alpha 7$ nAChRs were inhibited by α -CtxArIB[V11L,V16D] (IC_{50} 3.4 nM) in a slowly reversible manner, with full recovery taking 15 min. This is consistent with the timecourse of recovery from blockade of rat $\alpha 7$ nAChRs in PC12 cells. α -CtxArIB[V11L,V16D] inhibited human native $\alpha 7$ nAChRs in SHSY5Y cells, activated by either choline or AR-R17779 plus PNU-120596. Rat brain $\alpha 7$ nAChRs contribute to dopamine release from striatal minces: α -CtxArIB[V11L,V16D] (300 nM) selectively inhibited choline-evoked dopamine release without affecting responses evoked by nicotine that activates heteromeric nAChRs. This study establishes that α -CtxArIB[V11L,V16D] selectively inhibits human and rat native $\alpha 7$ nAChRs with comparable potency, making this a potentially useful antagonist for investigating $\alpha 7$ nAChR functions.

Introduction

The $\alpha 7$ subtype of nicotinic acetylcholine receptor (nAChR) is currently attracting considerable interest for its roles in brain function, including synaptic plasticity (McKay *et al.*, 2007) and attentional aspects of cognitive performance (Levin *et al.*, 2006). Consequently, $\alpha 7$ nAChRs are therapeutic targets for cognitive impairments in Alzheimer's disease, schizophrenia and attention deficit hyperactivity disorder (Mazurov *et al.*, 2006; Chiamulera and Fumagalli, 2007; Wilens and Decker, 2007). $\alpha 7$ nAChRs also have therapeutic potential in several other processes, including pain, inflammation and wound healing (Decker and Meyer, 1999; de Jonge and Ulloa, 2007). Selective antagonists are essential research tools for elucidating and clarifying the molecular and cellular mechanisms that underpin these effects.

Research into $\alpha 7$ nAChRs has profited from the availability of two useful antagonists: α bungarotoxin (α Bgt) and methyllycaconitine (MLA). α Bgt, from the venom of the Elapidae snake *Bungarus multicinctus*, is a large polypeptide with slow binding kinetics. This confers high affinity binding with very slow dissociation, an advantage for some studies. It can also be a limitation in functional assays as increased incubation times and/or concentrations are required to achieve blockade. Moreover the slowly reversible block limits recovery in experiments where repeated agonist application is desirable, and the large size of the toxin can compromise its access to $\alpha 7$ nAChRs in intact preparations.

The norditerpenoid MLA from *Delphinium* and *Consolida sp.* is also a competitive antagonist of $\alpha 7$ nAChRs. It binds to $\alpha 7$ nAChRs with nanomolar affinity ($K_i \sim 1$ nM; Davies *et al.*, 1999). In addition, it exhibits moderately high affinity for $\alpha 6\beta 2$ -containing nAChRs (K_i 33 nM; Mogg *et al.*, 2002), which compromises its utility for identifying $\alpha 7$ nAChRs in areas where the $\alpha 6$ subunit is expressed, notably catecholaminergic and visual pathways. Furthermore, both α Bgt and MLA interact with high affinity with nAChRs containing $\alpha 9$ and $\alpha 10$ subunits (Elgoyhen *et al.*, 2001; Baker *et al.*, 2004). The potential co-localisation of $\alpha 7$, $\alpha 9$ and $\alpha 10$ subunits in sympathetic neurones (Lips *et al.*, 2006) and non-neuronal cells (Bschleipfer *et al.*, 2007) demands more discriminating antagonists.

The α -conotoxins, small peptides from the venom of *Conus sp.*, can exhibit exquisite specificity for nAChR subtypes (McIntosh *et al.*, 1999; Nicke *et al.*, 2004). Recently, a naturally occurring α -conotoxin from *C. arenatus* was modified by changing two amino acid residues to generate a novel toxin α -CtxArIB[V11L,V16D] (Fig. 1A; Whiteaker *et al.*, 2007). This toxin displayed high potency and specificity for discriminating $\alpha 7$ nAChRs (K_i 7 nM) from all other nAChR subtypes tested in binding assays. Functionally, α -CtxArIB[V11L,V16D] specifically blocked currents evoked by stimulation of rat homomeric $\alpha 7$ nAChRs expressed in *Xenopus* oocytes, whereas it was without effect on heteromeric nAChRs (including $\alpha 9\alpha 10$ nAChR) in the same expression system (Whiteaker *et al.*, 2007). However, the subunit composition of native nAChRs can be more diverse and more complex than that of recombinant receptors. Here we have assessed the ability of α -CtxArIB[V11L,V16D] to antagonise responses to native nAChRs.

Clinical studies demand investigation of human tissues. The exquisite specificity of α -conotoxins for particular nAChR subtypes (Nicke *et al.*, 2004), coupled with subtle species differences that can affect pharmacological selectivity (Young *et al.*, 2007), requires caution when extrapolating from rodent to human tissues. Therefore we have exploited both PC12 and SH-SY5Y cell lines that are representative of rat and human peripheral autonomic neurones, respectively. Both cell lines express $\alpha 3$, $\alpha 5$, $\alpha 7$, $\beta 2$ and $\beta 4$ nAChR subunits (Henderson *et al.* 1994; Blumenthal *et al.* 1997; Lukas *et al.*, 1993; Peng *et al.*, 1994; Wang *et al.*, 1996) and have functional $\alpha 7$ and non- $\alpha 7$ nAChRs that can be discriminated using subtype-selective ligands (Dickinson *et al.*, 2007). Additional nAChR subtypes are expressed in the brain, and their heterogeneity has been particularly well defined with respect to nAChR-evoked [3 H] dopamine release from rodent striatal preparations. Several $\beta 2$ -containing heteromeric nAChRs ($\alpha 4\beta 2$, $\alpha 4\alpha 5\beta 2$, $\alpha 4\alpha 6\beta 2\beta 3$, $\alpha 6\beta 2\beta 3$, $\alpha 6\beta 2$) reside on dopamine terminals (Grady *et al.*, 2007). In addition, $\alpha 7$ nAChRs assigned to neighbouring glutamate afferents can also modulate striatal [3 H]dopamine release (Kaiser and Wonnacott, 2000; Barik and Wonnacott, 2006). We show that in rat striatal minces, as well as in the rat and human cell lines, α -CtxArIB[V11L,V16D] selectively inhibited responses attributable to $\alpha 7$ nAChRs while having no effect on non- $\alpha 7$ nAChR responses. These results establish the utility of this new antagonist for interrogating the actions of rat and human $\alpha 7$ nAChRs *in vitro*.

Materials and Methods

Materials

[7,8-³H]Dopamine (43 Ci/mmol) and [¹²⁵I]- α -bungarotoxin (263 Ci/mmol) were purchased from GE Healthcare (Little Chalfont, Buckinghamshire, UK). The PC12 cell line was a gift from D. Berg (University of California, San Diego, USA). SH-SY5Y cells were obtained from ECACC, Salisbury, UK. 5-Iodo-A85380 dihydrochloride (5-iodo-3-(2(S)-azetidinylmethoxy)pyridine dihydrochloride. 5-I-A85380) and PNU-120596 (1-(5-chloro-2,4-dimethoxy-phenyl)-3-(5-methyl-isoxazol-3-yl)-urea) were purchased from Tocris Cookson (Bristol, Avon, UK). α -Bungarotoxin (α Bgt), fluo-3 AM and pluronic F127 were purchased from Invitrogen (Poortgebouw, Netherlands). (-)-Nicotine hydrogen tartrate, pargyline hydrochloride, choline tartrate, ascorbic acid, nomifensine maleate, mecamlamine, RPMI 1640 medium, horse serum, foetal bovine serum, L-glutamine, penicillin and streptomycin were obtained from Sigma-Aldrich Co. (Poole, Dorset, UK). AR-R17779 ((2)-spiro[1-azabicyclo[2.2.2]octane-3,59-oxazolidin]-29-one) was provided by GlaxoSmithKline, (Harlow, UK). Optiplates and Optiphase Supermix were obtained from Perkin Elmer (N.V./S.A., Zaventem, Belgium). Other standard chemicals of analytical grade were attained from Sigma-Aldrich Co. Male Sprague-Dawley rats (approximately 250g) were from Charles River, UK.

Nicotine tartrate, choline and KCl solutions were prepared freshly for each experiment by dissolving in assay buffer and correcting the pH to 7.4 if necessary. Other drugs were diluted from stocks made up in water, with the exception of PNU-120596 (100 mM stock in dimethylsulfoxide).

Radioligand binding

Competition binding experiments for ¹²⁵I- α Bgt and [³H]-epibatidine binding sites were carried out on rat brain P2 membranes as previously described (Davies *et al.*, 1999; Whiteaker *et al.*, 2000). For ¹²⁵I- α Bgt, membranes (250 μ g) were incubated in a total volume of 1ml in 10 mM phosphate buffer, pH 7.4, containing 0.1 % bovine serum albumin (BSA) and 1 nM ¹²⁵I- α Bgt in the presence and absence of increasing concentrations of α -Ct_xArIB[V11L,V16D] or (-)-nicotine for 4 h at 37°C, followed by chilling to 4°C. Samples were filtered through a double thickness of Gelman A/E filters, pre-soaked overnight in 0.3 % polyethyleneimine (bottom filter) or 4 % milk powder (top filter), using a Brandel Cell Harvester. For [³H]-epibatidine, membranes (250 μ g) were incubated in a total volume of 2ml in 10 mM phosphate buffer, containing 0.2 nM [³H]-epibatidine in the presence and absence of increasing concentrations of α -Ct_xArIB[V11L,V16D] or (-)-nicotine for 2 h at room temperature. Samples were filtered through a double thickness of Gelman A/E filters, pre-soaked overnight in 0.3 % polyethyleneimine, using a Brandel Cell Harvester. Nonspecific binding was determined in the presence of 1 mM nicotine.

Filters were subsequently washed five times using cold PBS and counted for radioactivity in a Packard 1600 Tricarb scintillation counter (Packard Instrument Company, Meriden, CT, USA).

Cell culture

PC12 cells were cultured as described by Dickinson *et al.* (2007). Cells were grown in a humidified incubator under 7% CO₂ at 37°C in RPMI medium supplemented with 5% foetal bovine serum, 10% heat inactivated donor horse serum, 2 mM L-glutamine and 50 U/ml penicillin/streptomycin. Cells were plated at a density of 2.5×10^4 cells/cm² onto 25 mm glass coverslips in 6 well plates or into 96 well plates, both coated with poly-D-lysine (5 μ g/ml). Assays were conducted 24 h later with confluent cultures.

Human neuroblastoma SH-SY5Y cells were cultured as described previously (Ridley *et al.*, 2001). Cultures were maintained in 175 cm² tissue culture flasks in DMEM:F12, supplemented with 15 % foetal bovine serum, 2 mM L-glutamine, 1 % non-essential amino acids, 190 U/ml penicillin and 0.2 mg/ml streptomycin, until confluent. Cells were plated (1:5 dilution) into 96 well plates and experiments performed 72 h later with confluent cultures.

Calcium fluorimetry (1) cell populations

Increases in intracellular Ca²⁺ in PC12 and SH-SY5Y cells were monitored by measuring changes in fluorescence in cells loaded with fluo-3, as previously described (Dickinson *et al.*, 2007). In brief, cells cultured in 96 well plates were washed twice with Tyrode's salt solution (TSS, in mM: NaCl, 137.0; KCl, 2.7; MgCl₂, 1.0; CaCl₂, 2.5; NaH₂PO₄, 0.2; NaHCO₃, 12.0; glucose, 5.5; pH 7.4; supplemented with 0.1% bovine serum albumin (BSA) to prevent α -CtxArIB[V11L,V16D] binding to plastic or glass) and incubated with fluo-3 AM (10 μ M) and 0.02% pluronic F127 for 1 h at room temperature (22°C) in the dark. After washing the cells twice with TSS, cells were preincubated with 80 μ l TSS, with or without antagonist (10 min for α -CtxArIB[V11L,V16D], mecamylamine or DH β E; 20 min for α Bgt). Where used, PNU-120596 was added 1 min prior to the end of preincubation. Basal fluorescence (excitation 485 nm, emission 538 nm) was recorded for 5 s in a Fluoroskan Ascent fluorescent plate reader (Labsystems, Helsinki, Finland). Then nAChR agonist, KCl or vehicle (20 μ l) was added and the change in fluorescence was recorded for a further 20 s. Under all conditions examined, increases in fluorescence reached a maximum level within 5 s and this response was sustained over the remainder of the time course of the experiment. In order to normalise fluo-3 signals, the maximum and minimum fluorescence from each well was determined by addition of 0.3% Triton-X100 (F_{max}) followed by 100 mM MnCl₂ (F_{min}). Drug-evoked responses were expressed as a percentage of the corresponding ($F_{max}-F_{min}$) value. None of the drugs or vehicles used significantly altered the basal level of fluorescence.

Calcium fluorimetry (2) timecourse experiments

Changes in intracellular Ca²⁺ in individual PC12 cells loaded with fura-2 were monitored using dynamic video imaging (Concord System, Perkin Elmer, UK). PC12 cells cultured on coverslips were washed twice with Ca²⁺ buffer (in mM NaCl, 140.0; KCl, 5.0; MgCl₂, 1.0; CaCl₂, 1.8; glucose, 10.0; HEPES, 5.0; pH 7.4; supplemented with 0.1% BSA) and incubated with fura-2 AM (5 μ M) and 0.02% pluronic F127 for 1 h at room temperature (22°C) in the dark. Coverslips were placed in a temperature-controlled microscope chamber maintained at 37 °C (Harvard Apparatus, MA) and continuously perfused with buffer (5 ml/min). Agonist with or without α -CtxArIB[V11L,V16D] was applied via the perfusion system for 1 min, followed by washout before re-application of agonist alone. Fura-2 was excited at 340 and 380 nm using a SpectroMaster I (Perkin Elmer, UK) and emissions at 510 nm were detected with an intensified UltrapiX PDCI low light level CCD camera (Perkin Elmer, UK). Data were analysed using Ultraview software (Perkin Elmer, UK) and expressed as a ratio of F₃₄₀:F₃₈₀. Each cell that responded to agonist was analysed separately and these data were pooled for each experiment.

cRNA preparation and injection

Human α 7 nAChR subunit cDNA was sub-cloned into the RNA expression vector pBluescript II SK⁻ (Stratagene, La Jolla, CA) and the resulting construct (from J. Garrett, Cognetix Inc., Salt Lake City, UT) was used to make cRNA. Capped cRNA was made using the mMessage mMachine *in vitro* transcription kit (Ambion, Austin, TX) following linearization of the plasmid with NotI and transcription under the control of a T7 promotor. The cRNA was purified using the Qiagen RNeasy kit (Qiagen, Valencia, CA). The concentration of cRNA was determined by the absorbance at 260 nm. Fifty nl (608 ng/ μ l) of cRNA was injected into each

Xenopus oocyte with a Drummond microdispenser (Drummond Scientific, Broomall, PA), as described previously (Cartier *et al.*, 1996), and incubated at 17 °C. Oocytes were injected within one day of harvesting and recordings were made 3-6 days post-injection.

Voltage-clamp Recording

Oocytes were voltage-clamped and exposed to ACh and peptide as described previously (Cartier *et al.*, 1996). Briefly, the oocyte chamber consisting of a cylindrical well (~30 µl in volume) was gravity perfused at a rate of ~2 ml/min with ND-96 buffer (96.0 mM NaCl, 2.0 mM KCl, 1.8 mM CaCl₂, 1.0 mM MgCl₂, 5 mM HEPES, pH 7.5) containing 0.1 mg/ml BSA. Oocytes were subjected once a minute to a 1 sec pulse of 200 µM ACh. Once a stable baseline was achieved, either ND-96 alone or ND-96 containing varying concentrations of α -CtxArIB [V11L;V16D] was perfusion-applied during which 1-sec pulses of 200 µM ACh were applied every minute until a constant level of block was achieved. For experiments involving PNU-120596, the ACh concentration was reduced to 20 µM and PNU-120596 was added to the ND-96 at a final concentration of 10 µM.

Data Analysis

For the baseline response, at least 3 ACh-induced responses were averaged. To determine the percent block induced by toxin, after a steady-state block had been achieved, ACh-induced responses were averaged and the value divided by the pre-toxin baseline value to yield a % response. The concentration-response data were fit to the equation, $Y = 100/(1 + 10^{((\text{LogEC}_{50} - \text{Log}[\text{Toxin}]) \times n_H))}$, where n_H is the Hill coefficient, by non-linear regression analysis using GraphPad Prism (GraphPad Software, San Diego, CA). Each data point represents the mean $\pm$ SEM from at least 4 oocytes.

[³H]Dopamine Release

A 96 well filter plate assay was used to monitor [³H]dopamine release from striatal minces (Puttfarcken *et al.*, 2000; Barik & Wonnacott, 2006). For each experiment, 2 rats were killed by cervical dislocation, brains were rapidly removed and striata dissected and transferred to ice cold Krebs buffer (KB, in mM: NaCl 118.0, KCl 2.4, CaCl₂ 2.4, KH₂PO₄ 1.2, MgSO₄·7H₂O 1.2, NaHCO₃ 25, D-glucose 10, ascorbic acid 1, pargyline 0.01, gassed with 95 % O₂ and 5 % CO₂ for at least 1 h at 37 °C; pH 7.4). Tissue was chopped using a McIlwain tissue chopper to give 150 µm prisms. Following 2 washes with warm KB, striatal minces were incubated for 30 min with 50 nM [³H]dopamine in 5 ml KB at 37°C. After 5 washes in KB containing 0.5 µM nomifensine, minces (100 µl / well) were loaded into a 96 well filter plate (model MABVN1250, Millipore, Hertfordshire, UK) and preincubated for 10 min with buffer (70 µl) in the presence or absence of antagonist or modulator. Buffer was removed by filtration and replaced with fresh buffer (70 µl) containing agonist and/or antagonist. After a further 5 min at 37°C, buffer containing released [³H]dopamine was collected by filtration into a 96-well Packard Optiplate™ (Perkin Elmer, Belgium). Filters were removed to a separate Optiplate containing 70 µl water/well. Radioactivity was determined using a Microbeta liquid scintillation counter (Wallac 1450 Microbeta Trilux, Perkin Elmer, Finland), counting efficiency 30%. The amount of [³H]dopamine released was expressed as a percent of total radioactivity present in the slices prior to stimulation (i.e. tritium remaining in the tissue + amount of tritium released). Each condition was examined in 6 replicates and repeated in at least 3 independent experiments.

Statistics

Statistical significance ($P < 0.05$) was determined using student's *t*-test, carried out using Sigma Plot V8.0.

Results

α -CtxArIB[V11L,V16D] (Fig. 1A) potently displaced ^{125}I - α -bungarotoxin binding to rat P2 membranes with a K_i value of 4.0 ± 0.6 nM (Fig 1B), confirming that α -CtxArIB[V11L,V16D] interacts competitively at $\alpha 7$ nAChRs, as previously described (Whiteaker *et al.*, 2007). α -CtxArIB[V11L,V16D] did not compete for [^3H]-epibatidine (0.2 nM) binding to rat brain membranes (Fig 1C). At this concentration, [^3H]-epibatidine will not label $\alpha 7$ nAChRs ($K_i \sim 0.2$ μM , Davies *et al.*, 1999, see Marks *et al.*, 2006). Therefore this result indicates that over the concentration range 0.1 nM - 1 μM α -CtxArIB[V11L,V16D] does not interact with non- $\alpha 7$ nAChRs. α -CtxArIB[V11L,V16D] was then tested for functional potency at native nAChRs.

Effects of α -CtxArIB[V11L,V16D] on nAChR-mediated Ca^{2+} responses in PC12 cells

PC12 cells express rat $\alpha 7$ and non- $\alpha 7$ nAChRs (Blumenthal *et al.* 1997) and we have recently characterised the differential coupling of these nAChR subtypes to increases in intracellular Ca^{2+} (Dickinson *et al.* 2007). Here, we first used choline to increase Ca^{2+} by selectively activating $\alpha 7$ nAChRs. Choline (1, 3 mM) elicited very small increases in fluorescence in PC12 cells loaded with fluo-3 AM but these responses were substantially amplified in the presence of 10 μM PNU-120596, an $\alpha 7$ -selective positive allosteric modulator (Fig 2A,B; Hurst *et al.*, 2005). PNU-120596 alone produced no response (data not shown). αBgt (100 nM; 20 min pre-incubation) reduced responses to 1 mM and 3 mM choline plus PNU-120596 by 90.9 ± 0.9 % and 80.6 ± 1.8 %, respectively (Fig 2B).

In initial experiments, cells were pre-incubated with 100 nM α -CtxArIB[V11L,V16D] for 10 min prior to stimulation with choline (1 and 3 mM) and 10 μM PNU-120596. This concentration of α -CtxArIB[V11L,V16D] significantly inhibited responses, by 62.0 ± 5.4 % and 49.9 ± 6.2 %, respectively (Fig. 2B). α -CtxArIB[V11L,V16D] was then tested over the concentration range 10 - 300 nM against 1 mM choline plus PNU-120596. α -CtxArIB[V11L,V16D] blocked $\alpha 7$ nAChR-mediated responses in a concentration-dependent manner with an IC_{50} of 88.0 ± 17.4 nM (Fig. 2C). Maximum inhibition of 83.6 % was observed with 300 nM α -CtxArIB[V11L,V16D]. Higher concentrations were not tested in order to conserve the toxin.

To assess the specificity of α -CtxArIB[V11L,V16D] for $\alpha 7$ nAChR, its effects on responses induced by a non-selective nAChR agonist (nicotine), an agonist selective for $\beta 2$ -containing nAChRs (5-I-A85380) and a general depolarising agent (KCl) were examined (Dickinson *et al.*, 2007). Nicotine (100 μM), 5-I-A85380 (30 μM) and KCl (60 mM) elicited increases in fluorescence of 26.7 ± 2.1 %, 14.8 ± 1.7 % and 44.2 ± 3.7 %, respectively (Fig 2D, mean $\pm$ SEM, $n = 4$). α -CtxArIB[V11L,V16D], tested at the highest concentration used previously (300 nM; Fig. 2C), failed to reduce the responses to any of these stimuli. To determine if 100 μM nicotine activated $\alpha 7$ nAChRs in addition to non- $\alpha 7$ nAChRs, it was applied in the presence of PNU-120596 (10 μM). Under this condition responses were modestly enhanced by 53.6% (from 27 % to 41 % of the maximum response, in the absence and presence of PNU-120596 respectively). This is consistent with the response to 100 μM nicotine including a small $\alpha 7$ nAChR-mediated component. In the presence of α -CtxArIB[V11L,V16D], the PNU-120596-enhanced response was not observed (Fig. 2D).

The timecourse of recovery from blockade by 300 nM α -CtxArIB[V11L,V16D] was assessed in PC12 cells loaded with fura2 AM and monitored using dynamic video imaging. The response to 3 mM choline plus 10 μM PNU-120596 (60 s) was blocked by 300 nM α -CtxArIB[V11L,V16D] by 87.1 ± 1.6 % (mean $\pm$ S.E.M. from 34 agonist-responsive cells [40.5 % of total number of cells monitored] in 3 separate experiments). Following removal of the toxin from the perfusing buffer, responses to choline plus PNU-120596 slowly recovered, with complete recovery after 15 min washout (Fig. 2E).

Blockade of human $\alpha 7$ nAChR-mediated responses by α -CtxArIB[V11L,V16D]

To determine if α -CtxArIB[V11L,V16D] is similarly effective at human $\alpha 7$ nAChRs, the toxin was examined for blockade of human $\alpha 7$ nAChRs heterologously expressed in *X. laevis* oocytes. Responses to 200 μ M ACh by oocytes transfected with human $\alpha 7$ nAChR were blocked by α -CtxArIB[V11L,V16D] with an IC_{50} value of 3.4 ± 0.037 (2.9-4.11) nM and a Hill slope of 1.20 ± 0.12 (1.5-1.0) (Fig. 3A). A maximum block of 100 % was obtained with 100 nM toxin. Repeating this experiment with 20 μ M ACh plus 10 μ M PNU-120596 resulted in a slight rightward shift of the concentration response curve for inhibition by α -CtxArIB[V11L,V16D]; the IC_{50} value was increased 2-fold (6.7 nM; Fig. 3A). This indicates that amplification of $\alpha 7$ nAChR responses by PNU-120596 does not significantly perturb the sensitivity to toxin. Kinetic analysis shows that α -CtxArIB[V11L,V16D] blocks $\alpha 7$ nAChR currents evoked by 200 μ M ACh within 60 s of exposure, with responses beginning to recover 180 s after removal of the toxin (Fig. 3B).

SH-SY5Y cells resemble human foetal sympathetic neurones grown in primary cultures and express a similar range of nAChR subunits to the PC12 cell line (Lukas *et al.*, 1993; Peng *et al.*, 1994). Changes in intracellular Ca^{2+} in SH-SY5Y cells loaded with fluo-3 were determined to assess the effects of α -CtxArIB[V11L,V16D] on native human $\alpha 7$ nAChRs. In agreement with the data from PC12 cells (Fig. 2), 3 mM choline alone was without effect but in the presence of 10 μ M PNU-120596 it evoked an increase in intracellular Ca^{2+} in SH-SY5Y cells that was inhibited by α Bgt by 93.4 ± 1.0 %, whereas DH β E had no significant effect (Fig. 4A). α -CtxArIB[V11L,V16D] produced a concentration-dependent inhibition of responses to choline plus PNU-120596 with an IC_{50} value of 74.0 ± 1.8 nM. Another $\alpha 7$ nAChR-selective agonist, AR-R17779 (Jensen *et al.*, 2005; Fig. 4B) was examined for comparison with choline. This agonist was also ineffective in the absence of PNU-120596; responses in the presence of the allosteric modulator were blocked by α Bgt by 95.4 ± 1.8 % but not by DH β E. The concentration-dependence of inhibition by α -CtxArIB[V11L,V16D] resulted in an IC_{50} value of 51 ± 1.0 nM; maximum inhibition by 1 μ M α -CtxArIB[V11L,V16D] was 95.9 %.

Effects of α -CtxArIB[V11L,V16D] on nicotine- and choline-evoked [3 H]dopamine release from rat striatal minces

The ability α -CtxArIB[V11L,V16D] to inhibit nAChR-evoked release of radiolabeled dopamine *in vitro* was investigated using a high throughput 96 well filter plate assay. BSA (0.01 %) was included in all conditions to prevent non-specific adsorption of α -CtxArIB[V11L,V16D]: BSA had no effect on basal release (Fig 5). Nicotine (10 μ M) significantly increased [3 H]dopamine release (10.5 ± 2.1 %) compared with buffer alone, and this increase was abolished in the presence of 10 μ M mecamylamine. However, α -CtxArIB[V11L,V16D] (300 nM) had no effect on either basal (not shown) or nicotine-evoked [3 H]dopamine release (Fig 5A).

Choline was used to specifically target $\alpha 7$ nAChRs capable of modulating [3 H]dopamine release (Fig. 5B). Choline (3 mM) increased fractional release by 6.1 ± 0.9 % and this was further increased in the presence of 10 μ M PNU-120596 to 11.9 ± 1.2 %. α -CtxArIB[V11L,V16D] (300 nM) inhibited responses to choline and choline plus PNU-120596 by 81.4 ± 1.2 % and 77.9 ± 1.0 % respectively (Fig 5B).

Discussion

The α -conotoxin ArIB analogue α -CtxArIB[V11L,V16D] potently displaced [125 I] α -bungarotoxin binding to rat brain membranes and inhibited functional rat and human $\alpha 7$ nAChR responses in cell lines, transfected oocytes and rat striatal minces. In contrast, α -CtxArIB[V11L,V16D] did not inhibit [3 H]-epibatidine binding or functional responses evoked by non-

$\alpha 7$ nAChR agonists or KCl. These results confirm the specificity of α -CtxArIB[V11L,V16D] for $\alpha 7$ nAChR over other native nAChRs.

There is increasing evidence for a role for $\alpha 7$ nAChRs in both neural and non-neural signalling and plasticity (McKay *et al.*, 2007; de Jonge and Ulloa, 2007). $\alpha 7$ nAChRs are a therapeutic target for psychiatric and neurological disorders with cognitive or attentional impairments as well as other non-neurological conditions (Mazurov *et al.*, 2006; Chiamulera and Fumagalli, 2007). Investigation of the physiological and pathological roles of $\alpha 7$ nAChRs requires selective ligands. Synthetic efforts in this area have generated several novel $\alpha 7$ nAChR-selective agonists (Jensen *et al.*, 2005; Mazurov *et al.*, 2006; Biton *et al.*, 2007; Hajós *et al.*, 2004) and allosteric modulators (Hurst *et al.*, 2005; Grønlien *et al.*, 2007; Timmermann *et al.*, 2007). However, antagonism of $\alpha 7$ nAChR responses relies on α Bgt and MLA, whose utility is constrained by slow kinetics and lack of specificity, respectively. The development of α -CtxArIB[V11L,V16D] and demonstration that it displays high specificity for blockade of $\alpha 7$ nAChRs versus other recombinant nAChRs (including muscle and $\alpha 9$ nAChRs) represents a potentially important advance for $\alpha 7$ nAChR research (Whiteaker *et al.*, 2007). Here we have established that this specificity for $\alpha 7$ nAChRs is preserved with respect to functional native nAChRs and encompasses human as well as rat receptors.

Choline was employed to elicit responses from native $\alpha 7$ nAChRs. Choline is a weak $\alpha 7$ nAChR-selective agonist (Alkondon *et al.*, 1997) and a non-competitive inhibitor of heteromeric nAChRs, including the $\alpha 3\beta 4$ subtype (Alkondon and Albuquerque, 2006). In PC12 and SH-SY5Y cells, the small responses to choline were substantially enhanced by PNU-120596, an allosteric modulator that selectively prolongs agonist-evoked $\alpha 7$ nAChR currents by reducing desensitisation (Hurst *et al.*, 2005; Grønlien *et al.*, 2007). This is in agreement with our previous observations using another $\alpha 7$ nAChR-selective agonist, compound A, in PC12 cells (Dickinson *et al.*, 2007). Similarly, the structurally unrelated $\alpha 7$ nAChR agonist AR-R17779 (Jensen *et al.*, 2005) provoked negligible responses in SH-SY5Y cells when applied alone but in the presence of PNU-120596 significant increases in intracellular Ca^{2+} resulted. Blockade of the amplified responses by α Bgt confirms the specific activation of $\alpha 7$ nAChRs by choline and AR-R17779. α -CtxArIB[V11L,V16D] effectively inhibited choline- and AR-R17779-evoked increases in fluorescence, consistent with its antagonism of $\alpha 7$ nAChRs.

In addition to its potent and selective interaction with rat $\alpha 7$ nAChRs (this study; Whiteaker *et al.*, 2007), α -CtxArIB[V11L,V16D] inhibited recombinant human $\alpha 7$ nAChRs expressed in *X. laevis* oocytes or native human $\alpha 7$ nAChRs in SH-SY5Y cells. The timecourse of blockade and recovery indicates full inhibition within 2 mins, with a slower recovery taking 15-20 min washout to regain full agonist responsiveness. This rate of recovery is similar to that observed for rat $\alpha 7$ nAChRs in PC12 cells. Although the IC_{50} values for inhibition of whole cell currents recorded from oocytes expressing recombinant receptors (~ 3 nM) were in close agreement for the two species, the IC_{50} values for inhibition of $\alpha 7$ nAChR-evoked increases in intracellular Ca^{2+} in both rat and human cell lines were about 20-fold higher (50-80 nM). This is not attributable to differences between agonists used, as choline and AR-R17779 resulted in comparable IC_{50} values in SH-SY5Y cells. Although the IC_{50} value for a competitive antagonist is dependent on agonist concentration, all the agonists were applied at a concentration approximating to their EC_{50} value. Nor is it due to using PNU-120596 to elicit measurable responses from the cell lines, as the IC_{50} value derived from oocytes was not significantly increased in the presence of the allosteric modulator, in agreement with the lack of effect of PNU-120596 on the ACh inactivation curve (Grønlien *et al.*, 2007). Therefore the consistent discrepancy between recombinant and native preparations is likely to reflect methodological differences between the assays. For example, the apparent potency of competitive antagonists is increased when the agonist duration is very short (Wyllie and Chen,

2007): oocyte recordings used 1 s agonist applications, compared with 20 s for the calcium fluorimetry assays.

Brain $\alpha 7$ nAChRs that modulate striatal [^3H]dopamine release from rat striatal minces were also substantially inhibited by 300 nM α -CtxArIB[V11L,V16D]. Although $\alpha 7$ nAChRs are generally accepted to be absent from dopaminergic terminals in the striatum, inhibition of the αBgt -sensitive portion of release by glutamate receptor antagonists supports the localisation of $\alpha 7$ nAChRs on glutamatergic afferents (Kaiser and Wonnacott, 2000). This indirect action is discernable in striatal slices or minces that preserve some local anatomical connections and can be elicited with choline (Barik and Wonnacott, 2006). In the present experiments, PNU-120596 potentiated responses to 1 mM choline and α -CtxArIB[V11L,V16D] was equally effective at blocking responses to choline alone and PNU-120596-potentiated responses.

The specificity of α -CtxArIB[V11L,V16D] for $\alpha 7$ nAChR over other native nAChR subtypes is demonstrated by its failure to inhibit Ca^{2+} increases elicited by nicotine, 5-I-A85380 or KCl in PC12 cells or nicotine-evoked dopamine release from striatal slices. The inability of α -CtxArIB[V11L,V16D] to displace [^3H]epibatidine binding to rat brain membranes confirms that it does not interact with the agonist binding site of non- $\alpha 7$ nAChRs, in agreement with previous binding assays on mouse brain preparations (Whiteaker *et al.*, 2007). In PC12 cells, non- $\alpha 7$ nAChRs comprise $\alpha 3\beta 2$ - and $\alpha 3\beta 4$ -containing subtypes (Henderson *et al.*, 1994): the lack of any significant blockade by 300 nM α -CtxArIB[V11L,V16D] is consistent with the evaluation of recombinant $\alpha 3\beta 2$ nAChRs in *X. laevis* oocytes (Whiteaker *et al.*, 2007). Nicotine-evoked [^3H]dopamine release is mediated by $\alpha 4\beta 2$ - and $\alpha 6\beta 2$ -containing nAChRs (Grady *et al.*, 2007). The lack of effect of α -CtxArIB[V11L,V16D] on [^3H]dopamine release evoked by 10 μM nicotine contrasts with the modest inhibition of this response by 30 - 100 nM MLA (Mogg *et al.*, 2002). Moreover, MLA selectively inhibits $\alpha\text{conotoxin MII}$ -sensitive nAChR-responses on dopaminergic cell bodies (Klink *et al.*, 2001), and this predilection for $\alpha 6\beta 2$ -containing nAChRs confounds the use of MLA as an $\alpha 7$ nAChR-selective antagonist in tissue preparations where $\alpha 6\beta 2^*$ nAChRs might be present. The present evidence for the insensitivity to α -CtxArIB[V11L,V16D] of $\alpha 4\beta 2$ - and $\alpha 6\beta 2$ -containing nAChRs that mediate striatal dopamine release is supported by its very weak effect on recombinant $\alpha 6/\alpha 3\beta 2\beta 3$ nAChRs ($\text{IC}_{50} > 20 \mu\text{M}$; Whiteaker *et al.*, 2007), giving α -CtxArIB[V11L,V16D] a clear advantage over MLA in this respect. Together with its lack of effect at $\alpha 9\alpha 10$ nAChRs and $\alpha 1$ muscle-type nAChRs (see Whiteaker *et al.*, 2007), α -CtxArIB[V11L,V16D] exhibits greater selectivity for $\alpha 7$ nAChRs than either MLA or αBgt .

These findings extend the previous characterisation of α -CtxArIB[V11L,V16D] (Whiteaker *et al.*, 2007) and establishes that it is a specific inhibitor of both rat and human native $\alpha 7$ nAChRs. It exhibits relatively fast blockade and is slowly reversible over several minutes, offering advantages over the very slow kinetics of αBgt inhibition. Its high degree of selectivity, with no effect on $\alpha 6\beta 2$ -containing nAChRs or $\alpha 9$ nAChRs, is superior to that of MLA. With effective blockade achieved by sub-micromolar concentrations, α -CtxArIB[V11L,V16D] will be a useful addition to the portfolio of nicotinic ligands.

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Abbreviations

αBgt , $\alpha\text{bungarotoxin}$
 α -Ctx, α -conotoxin

α -CtxArIB[V111,V16D], α -conotoxin *Arenatus* IB[V111,V16D]
 AR-R17779, (2)-spiro[1-azabicyclo[2.2.2]octane-3,59-oxazolidin]-29-one
 BSA, bovine serum albumin
 cDNA, complementary DNA
 cRNA, complementary RNA
 DH β E, dihydro- β -erythroidine
 5-I-A85380, 5-Iodo-A85380, 5-iodo-3-(2(S)-azetidinylmethoxy)pyridine dihydrochloride
 KB, Krebs's buffer
 MLA, methyllycaconitine
 nAChR, nicotinic acetylcholine receptor
 PNU-120596, 1-(5-chloro-2,4-dimethoxy-phenyl)-3-(5-methyl-isoxazol-3-yl)-urea
 TSS, Tyrode's salt solution

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Figure 1.

(A) Peptide sequence of α -CtxArIB[V11L,V16D]. Bars indicate the disulphide bond pattern and the mutated amino acids are underlined. (B) Inhibition of ^{125}I - α Bgt binding to rat brain membranes by α -CtxArIB[V11L,V16D] (solid line) or nicotine (dashed line). Data points were fitted to the Hill equation, giving K_i values of 4.0 ± 0.6 nM and 2.3 ± 0.6 μM , respectively. Data represent mean $\pm$ SEM, $n = 3$ (C) α -CtxArIB[V11L,V16D] has no effect on $[^3\text{H}]$ epibatidine binding to rat brain membranes (solid line), whereas nicotine displaced $[^3\text{H}]$ epibatidine binding, K_i value 22.5 ± 16.8 nM (dashed line). Data represent mean $\pm$ range, $n = 2$.

Figure 2.

Effect of α -CtxArIB[V11L,V16D] (α -Ctx) on Ca^{2+} responses in PC12 cells. PC12 cells loaded with fluo-3 AM were preincubated with α -CtxArIB[V11L,V16D] for 10 min, with or without PNU-120596 (PNU) for the last 60 s, and stimulated with agonist or KCl as described in Materials and Methods. The change in fluorescence was measured over 20 s (**A**) Representative traces of change in fluorescence in response to 1 mM choline, in the absence (dotted line) or presence of 10 μ M PNU-120596 (PNU) (solid line) or with 10 μ M PNU-120596 and 100 nM α -CtxArIB[V11L,V16D] (α -Ctx) (dashed line) (**B**) Increases in fluorescence evoked by 1 and 3 mM choline, with or without 10 μ M PNU-120596, were measured in the presence and absence of 100 nM α Bgt (mean $\pm$ range, $n = 2$) or 100 nM α -CtxArIB[V11L,V16D] (mean $\pm$ SEM, $n = 4$; ** $p < 0.01$, student's t -test) (**C**) The concentration-dependence of inhibition by α -CtxArIB[V11L,V16D] of responses to 1 mM choline plus 10 μ M PNU-120596. Data points were fitted

to the Hill equation, giving an IC_{50} value of 88.0 ± 17.4 nM (mean $\pm$ SEM, $n = 4$) **(D)** Effect of 300 nM α -CtxArIB[V11L,V16D] (hatched bars) on increases in fluorescence in response to 100 μ M nicotine (with or without 10 M PNU-120596), 30 μ M 5-I-A85380 (5-I-A) or 60 mM KCl. PNU-120596 significantly increased responses to nicotine in the absence (but not in the presence) of 300 nM α -CtxArIB[V11L,V16D] (mean $\pm$ SEM, $n = 4$; * $p < 0.05$, student's t -test). **(E)** Timecourse of recovery from inhibition by α -CtxArIB[V11L;V16D]. PC12 cells were loaded with fura-2 AM and perfused with buffer as described in Materials and Methods. Fluorescence changes in response to one minute applications of 3 mM choline and 10 μ M PNU-120596 (PNU), in the presence and absence of 300 nM α -CtxArIB[V11L,V16D] (α -Ctx), were monitored. A representative trace from one experiment in which responses from 13 cells that responded to agonist stimulation have been combined.

Figure 3.

Effect of α -CtxArIB[V11L;V16D] on human $\alpha 7$ nAChRs heterologously expressed in *X. laevis* oocytes. **(A)** Concentration-response analysis of peptide block of ACh-induced currents was performed as described in the Materials and Methods. α -CtxArIB[V11L;V16D] blocked human $\alpha 7$ nAChRs activated by 200 μ M ACh (squares) with an IC_{50} value of 3.4 ± 0.04 (2.9-4.1) nM. The Hill slope was 1.2 ± 0.12 (1.5-1.0). Values are the mean $\pm$ SEM from an n of 4-5 for each concentration. Activation of currents by 20 μ M ACh plus 10 μ M PNU-120596 yielded an IC_{50} value of $6.7 \pm .05$ (5.3-8.6) nM (diamonds). Values are the mean $\pm$ SEM from an n of 3-4 for each concentration. The Hill slope was 1.1 ± 0.13 (1.4-0.8). 95% confidence interval is given in parentheses. **(B)** Kinetics of block and unblock. A representative trace recording of current amplitudes induced by 200 μ M ACh applied to human $\alpha 7$ nAChRs heterologously expressed in *X. laevis* oocytes before, during, and after perfusion with 100 nM α -CtxArIB[V11L;V16D]. c; control, prior to toxin.

Figure 4.

Effect of α -CtxArIB[V11L,V16D] on native human $\alpha 7$ nAChRs in SH-SY5Y cells. SH-SY5Y cells were loaded with fluo-3 AM and preincubated with inhibitors for 10 min and / or PNU-120596 for 1 min before stimulation with choline (A) or AR-R17779 (B), as described in the Materials and Methods. Increases in fluorescence evoked by agonist were measured in the presence or absence of 100 nM α Bgt, 1 μ M DH β E or increasing concentrations of α -CtxArIB[V11L,V16D]. Insets: Concentration response curve for inhibition of responses to agonist plus 10 μ M PNU-120596 by α -CtxArIB[V11L,V16D]. Data points were fitted to the Hill equation, giving IC₅₀ values of 74 ± 1.8 nM and 51.3 ± 1.4 nM for choline and AR-R1779, respectively (mean $\pm$ SEM, $n = 4$).

Figure 5.

Effect of α -CtxArIB[V11L,V16D] on [³H]dopamine release from rat striatal minces evoked by (A) nicotine (Nic) and (B) choline (with or without PNU-120596). Striata were chopped, loaded with [³H]dopamine and release was measured by filtration as described in Materials and Methods. Minces were preincubated for 5 min with or without 300 nM α -CtxArIB [V11L,V16D] (α -Ctx) or 10 μ M mecamylamine (Mec). They were then exposed to buffer, 10 μ M nicotine (A) or 3 mM choline (B), in the presence or absence of 0.01 % BSA, 10 μ M PNU-120596 and/or toxin for a further 5 min before recovery of released [³H]dopamine by filtration. Fractional release of [³H]dopamine was expressed as a percentage of the total in the tissue at the beginning of the stimulation period. Values are mean $\pm$ SEM, $n = 3$ independent experiments, * $p < 0.05$, significantly different from response to buffer; + $p < 0.05$, significantly different from evoked release in the absence of α -CtxArIB[V11L,V16D], student's t -test.