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A novel fluorescent α -conotoxin for the study of $\alpha 7$ nicotinic acetylcholine receptors

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Abstract

Homomeric $\alpha 7$ nicotinic acetylcholine receptors are a well-established, pharmacologically distinct subtype. The more recently identified $\alpha 9$ subunit can also form functional homopentamers as well as $\alpha 9\alpha 10$ heteropentamers. Current fluorescent probes for $\alpha 7$ nicotinic acetylcholine receptors are derived from α -bungarotoxin. However, α -bungarotoxin also binds to $\alpha 9^*$ and $\alpha 1^*$ receptors which are coexpressed with $\alpha 7$ in multiple tissues. We used an analog of α -conotoxin ArIB to develop a highly-selective fluorescent probe for $\alpha 7$ receptors. This fluorescent α -conotoxin, Cy3-ArIB[V11L;V16A], blocked acetylcholine-evoked $\alpha 7$ currents in *Xenopus laevis* oocytes with an IC₅₀ value of 2.0 nM. Observed rates of blockade were minute-scale with recovery from blockade even slower. Unlike fluorescein isothiocyanate-conjugated α -bungarotoxin, Cy3-ArIB[V11L;V16A] did not block $\alpha 9\alpha 10$ or $\alpha 1\beta 1\delta \epsilon$ receptors. In competition binding assays, Cy3-ArIB[V11L;V16A] potently displaced [¹²⁵I]- α -bungarotoxin binding to mouse hippocampal membranes with a K_i value of 21 nM. Application of Cy3-ArIB[V11L;V16A] resulted in specific punctate labelling of KX $\alpha 7$ R1 cells but not KX $\alpha 3\beta 2$ R4, KX $\alpha 3\beta 4$ R2, or KX $\alpha 4\beta 2$ R2 cells. This labelling could be abolished by pre-treatment with α -cobratoxin. Thus, Cy3-ArIB[V11L;V16A] is a novel and selective fluorescent probe for $\alpha 7$ receptors.

Keywords

$\alpha 7$ nAChR; α -conotoxin; fluorescent; HEK293 cells

Introduction

Nicotinic acetylcholine receptors (nAChRs) are ligand gated ion channels that are ubiquitously expressed throughout the nervous system but are also found on non-neuronal cells in the brain and periphery. nAChRs are pentameric assemblies of homologous subunits that combine to form ligand-gated ion channels. When activated by agonists, nAChRs open to allow the passage of ions across the cell membrane. To date, 17 nAChR subunits have been cloned encoding the $\alpha 1$ - $\alpha 10$, $\beta 1$ - $\beta 4$, δ , ϵ , and γ subunits; $\alpha 8$ is apparently only expressed in

* denotes the possible presence of additional nicotinic acetylcholine receptor subunits

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aves. These subunits assemble in various combinations with each other to form heteromeric receptors or, in the case of $\alpha 7$, $\alpha 8$, and $\alpha 9$, homomeric receptors (for review see (Dani & Bertrand 2007)).

For the last several decades α -bungarotoxin (α -BgTx), isolated from the venom of the Taiwanese banded krait *Bungarus multicinctus*, and α -cobratoxin (α -CbTx), from *Naja naja*, have been used to pharmacologically identify $\alpha 7$ nAChRs. However, it has recently become apparent that α -BgTx will also block $\alpha 9$ homomers and $\alpha 9\alpha 10$ heteromers with nanomolar potency $IC_{50} = 2.1$ and 14.0 nM, respectively, (Verbitsky *et al.* 2000, Sgard *et al.* 2002) in addition to its well characterized antagonist activity on the $\alpha 1\beta 1\delta \epsilon$ and to the avian $\alpha 8$ (Gotti *et al.* 1997) subtypes. α -CbTx will also block $\alpha 9\alpha 10$ ($IC_{50} = 3.8$ nM; our unpublished result) and the $\alpha 1\beta 1\delta \epsilon$ subtype. Methyllycaconitine (MLA), another widely used $\alpha 7$ antagonist, $IC_{50} = 0.03$ nM (Palma *et al.* 1996), is a plant alkaloid isolated from *Delphinium* and *Consolida* species. Unfortunately, MLA also blocks $\alpha 9$ homomers $IC_{50} = 1.1$ nM (Verbitsky *et al.* 2000), and $\alpha 6^*$ (Klink *et al.* 2001, Mogg *et al.* 2002). Thus, none of these ligands can be used to definitively identify $\alpha 7$ nAChRs if $\alpha 1^*$, $\alpha 6^*$, or $\alpha 9^*$ nAChR subtypes are also present.

This difficulty in unequivocally identifying $\alpha 7$ nAChRs is particularly problematic in peripheral tissues. For example, multiple nAChR subtypes have been implicated in the modulation of pain and inflammation. The $\alpha 4\beta 2$, $\alpha 7$, and $\alpha 9\alpha 10$ subtypes have recently received considerable attention in this regard (Vincler & McIntosh 2007, Damaj *et al.* 2007, Damaj *et al.* 1998). The $\alpha 7$ subtype has been determined to be an essential component of the cholinergic anti-inflammatory pathway (Wang *et al.* 2003), and block of the $\alpha 9\alpha 10$ subtype has been demonstrated to be analgesic in animal models of neuropathic pain (Satkunanathan *et al.* 2005, Vincler *et al.* 2006). The detection of these two subtypes and the elucidation of their contributions to cellular processes have been complicated by the relative lack of subtype-specific ligands that can discriminate between $\alpha 7$ and $\alpha 9\alpha 10$ nAChRs (McIntosh *et al.* 2005). The ability of a ligand to discriminate between these two subtypes is essential given the likelihood that both may be co-expressed in a variety of cell types including T-lymphocytes (Kawashima & Fujii 2004, Peng *et al.* 2004) and macrophages (Biallas *et al.* 2007, Grau *et al.* 2007), cells especially relevant in pain and inflammatory conditions. In addition, $\alpha 7$, $\alpha 9$, and $\alpha 10$ subunits have been reported to be co-expressed in the dorsal horn of the spinal cord (Genzen & McGehee 2003) and dorsal root ganglia neurons (Genzen *et al.* 2001, Papadopolou *et al.* 2004, Rau *et al.* 2005, Haberberger *et al.* 2004, Lips *et al.* 2006, Lips *et al.* 2002).

We recently described a set of novel α -conotoxins (α -CTx) isolated from the venomous marine snail *Conus arenatus* (Whiteaker *et al.* 2007). Directed substitutions in the amino acid sequence of the cloned peptides were made which resulted in ligands that are highly selective for the $\alpha 7$ subtype. One such ligand, ArIB[V11L;V16A], was demonstrated to be >10,000-fold more selective for $\alpha 7$ over $\alpha 9\alpha 10$ and $\alpha 1\beta 1\delta \epsilon$. We have also shown that a radioligand version of ArIB[V11L;V16A] can be used in autoradiography and ligand binding assays (Whiteaker *et al.* 2008). With its selectivity for $\alpha 7$, potency ($IC_{50} = 0.52$ nM), and slow off-rate kinetics, we reasoned that ArIB[V11L;V16A] would be an attractive candidate for the development of a fluorescent probe that could be used to definitively identify $\alpha 7$ nAChRs in tissues where there are other receptors that are sensitive to α -BgTx and MLA.

In this study, we describe the development of a fluorescent conjugate of ArIB[V11L;V16A] namely, Cy3-ArIB[V11L;V16A]. We used oocyte electrophysiology, binding, and

* denotes the possible presence of additional nicotinic acetylcholine receptor subunits

fluorescence imaging techniques to assess its potency at and selectivity for $\alpha 7$ nAChRs. Cy3-ArIB[V11L;V16A] potentially competed with [125 I]- α -BgTx for binding sites in mouse hippocampal membranes. In HEK293 cells stably transfected with rat $\alpha 7$ nAChRs (KX $\alpha 7$ R1), punctuate labelling of $\alpha 7$ nAChRs was observed. No labelling was observed in HEK293 cells expressing $\alpha 3\beta 2$ (KX $\alpha 3\beta 2$ R4), $\alpha 3\beta 4$ (KX $\alpha 3\beta 4$ R2), or $\alpha 4\beta 2$ (KX $\alpha 4\beta 2$). Together, these results suggest that Cy3-ArIB[V11L;V16A] is a useful probe for the study of $\alpha 7$ nAChR.

Materials and Methods

Cy3 NHS ester dye and [125 I]- α -BgTx (specific activity 2000 Ci/mmol $^{-1}$) were purchased from General Electric Healthcare (Piscataway, NJ). Penicillin/streptomycin, minimum essential medium, G418 sulfate [Geneticin], 0.5% Trypsin/EDTA, Hoechst 33342, and calcium and magnesium free phosphate buffered saline (PBS) were from Invitrogen (Carlsbad, CA). Heat inactivated fetal bovine serum (FBS) was from Hyclone (Logan, UT). α -Conotoxin ArIB[V11L;V16A] was synthesized as previously described (Cartier *et al.* 1996). Fluorescein isothiocyanate- α -bungarotoxin (FITC- α -BgTx), α -CbTx, acetylcholine chloride (ACh), carbachol, poly-L-lysine hydrobromide, acetonitrile (ACN), trifluoroacetic acid (TFA), atropine sulphate, bovine serum albumin (BSA), phenylmethylsulfonyl fluoride, EGTA, EDTA, leupeptin trifluoroacetate, aprotinin, and pepstatin A were from Sigma Aldrich (St. Louis, MO). HEPES was from Research Organics (Cleveland, OH). Amikacin sulphate, sulfamethoxazole, and trimethoprim were purchased from the University of Utah Health Science Center (Salt Lake City, UT).

Conjugation of ArIB[V11L;V16A] with Cy3 NHS ester

To generate the fluorescent conjugate of ArIB[V11L;V16A], the peptide was suspended in a NaHCO $_3$ buffer (final concentration 100 mM), pH 8.5, and the dye in anhydrous DMSO. Two hundred μ g of Cy3 NHS ester dye suspended in 14 μ l of DMSO was reacted with 50 nanomoles of peptide suspended in 11 μ l of NaHCO $_3$ buffer. The reaction was kept protected from light at RT for 1 h with brief vortexing and centrifugation performed at least once during this time period. The reaction was terminated by dilution with 50 μ l of DMSO then 150 μ l of buffer A (0.1% (vol/vol) TFA in distilled water). The reactants were separated by reverse phase- HPLC (RP-HPLC) using a Vydac C $_{18}$ column. The buffers used were buffers A and B60 (0.092% (vol/vol) TFA, 60% (vol/vol) ACN). The peptides were eluted using a linear gradient of 20% to 90% B60 over 40 minutes. Quantification of the fluorescent conjugate was calculated using the Abs $_{\text{max}}$ for Cy3 at 550 nm (determined using a spectrophotometer), the published molar extinction coefficient (150,000 M $^{-1}$ cm $^{-1}$), and the Lambert-Beer equation. The masses were verified by matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS) which was performed at the Salk Institute, La Jolla, CA, under the direction of Dr. Jean Rivier.

cRNA preparation and injection

Rat nAChR subunit cDNA clones $\alpha 2$, $\alpha 3$, $\alpha 4$, $\alpha 7$ and $\beta 2$ - $\beta 4$ were from S. Heinemann (Salk Institute, La Jolla, CA), $\alpha 9$ and $\alpha 10$ were from A.B. Elgoyhen (Universidad de Buenos Aires, Buenos Aires, Argentina), $\beta 2$ and $\beta 3$ in the high expressing vector pGEMHE were from C. Luetje (University of Miami, Miami, FL). Construction of the $\alpha 6/\alpha 3$ chimera has been previously described (McIntosh *et al.* 2004) and provided by J. Garret (Cognetix Inc., Salt Lake City, UT). Capped cRNA was made using the mMACHINE T7 *in vitro* transcription kit (Ambion, Austin, TX) and purified using the Qiagen RNeasy kit (Qiagen, Valencia, CA). The concentration of cRNA was determined by the absorbance at 260 nm. The mouse muscle subunit cDNA clones in the CMV-based pRBG4 vector were provided by S.M. Sine (Mayo Clinic College of Medicine, Rochester, MN). Fifty nl (at least

5-10 ng) of cRNA or 18.3 nl of cDNA (25 ng/ul of each subunit) were injected into each *Xenopus* oocyte with a Drummond microdispenser (Drummond Scientific, Broomall, PA) and incubated at 17° C in ND96 (96.0 mM NaCl, 2.0 mM KCl, 1.8 mM CaCl₂, 1.0 mM MgCl₂, 5 mM HEPES, pH 7.5) containing antibiotics (100 U/ml penicillin, 100 µg/ml streptomycin, 100 µg/ml amikacin sulfate, 160 µg/ml sulfamethoxazole, and 32 µg/ml trimethoprim). Oocytes were injected within 1-2 day of harvesting and recordings were made 3-6 days post-injection.

Electrophysiology

Oocytes were voltage-clamped and exposed to ACh and toxins as previously described (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 ND96 containing 0.01% (wt/vol) BSA and 1 µM atropine to block endogenous muscarinic receptors. For experiments involving α7 and α9α10, atropine was excluded from the perfusion solution because it has been shown to block these receptor subtypes (Gerzanich *et al.*, 1994). Oocytes were exposed once a minute to a 1 sec pulse of ACh. The ACh concentrations used were 200 µM for α7, 50 µM for α1β1δε, and 100 µM for all other subtypes. Once a stable baseline was achieved, either ND96 alone or ND96 containing varying concentrations of toxins was perfusion-applied during which 1-sec pulses of ACh were applied every minute until a constant level of block by the toxins was achieved. For the baseline response, at least 3 ACh-induced responses were averaged. After a steady-state block had been achieved, the % block by the toxins was determined by taking the final value and dividing by the pre-toxin baseline value to yield a % response. For experiments involving concentrations >1 µM, toxins were bath applied to conserve material. In this case, toxins were applied at the beginning of a 5 minute static bath to ensure equilibrium. For bath applied toxins, the % block was calculated as a % of control response (ND96 + ACh, see above for ACh concentrations used for each subtype) where no toxin was applied. The concentration-response data were fit to the equation $Y = 100 / (1 + 10^{((\text{LogIC}_{50} - \text{Log}[\text{Toxin}]) \cdot \text{Hill Slope}))}$ by nonlinear regression analysis and the observed rate of block data by a one phase exponential decay equation using GraphPad Prism (GraphPad Software, San Diego, CA). Each data point represents the mean ± SEM from at least 3 oocytes. Statistical differences for observed rates of block and recovery were determined by a two-tailed Student's t-test and considered significant if $p < 0.05$. K_d values were calculated from functional IC_{50} using the equation $\text{IC}_{50}/K_d = 2^{1/N} - 1$, where N is equal to the number of binding sites and in the case of α7 homomers N=5 (Whiteaker *et al.* 2007).

Radioligand binding assay

To prepare brain membranes, adult male (60-90 days) C57B6/J mice were sacrificed by cervical dislocation and the brains removed and placed on an ice-cold platform. Tissue was collected from the hippocampus. Hippocampal tissue was chosen because it expresses high concentrations of α7 nAChRs, is relatively large, and is simple to dissect. Individual hippocampal samples were homogenized in ice-cold hypotonic buffer (14.4 mM NaCl, 0.2 mM KCl, 0.2 mM CaCl₂, 0.1 mM MgSO₄, 2 mM HEPES, pH 7.5) using a glass-Teflon tissue grinder. Particulate fractions were collected by centrifugation at 25,000 x g for 15 min at 4°C (Eppendorf 5417 C centrifuge; Eppendorf North America, New York, NY). The pellets were resuspended in fresh homogenization buffer, incubated on ice for 10 min, and then harvested by centrifugation as described above. Each pellet was washed twice more by resuspension/centrifugation before storage (in pellet form under homogenization buffer) at -70°C until use.

Affinity for α7 nAChRs was measured by displacement of [¹²⁵I]-α-BgTx binding to hippocampal membranes, as described in Whiteaker *et al.* (2008). Incubations were performed at 22 °C, for 4 h, in 1.2-ml polypropylene tubes arranged in a 96-well format.

Hippocampal membranes were incubated with 2 nM [¹²⁵I]- α -BgTx in a total volume of 30 μ l of buffer (144 mM NaCl, 2 mM KCl, 2 mM CaCl₂, 1 mM MgSO₄, BSA 0.1% (wt/vol), 20 mM HEPES, pH 7.5) containing protease inhibitors (5 mM EDTA, 5 mM EGTA, 1 mM phenylmethylsulfonyl fluoride, and 10 μ g/ml each of aprotinin, leupeptin trifluoroacetate, and pepstatin A), to minimize proteolysis. Displacement of [¹²⁵I]- α -BgTx binding was assessed in duplicate. Total (no peptide added) and nonspecific binding (defined using 10 μ M α -CbTx) was also measured in duplicate. After the initial incubation, 1 ml of isotonic binding buffer (144 mM NaCl, 1.5 mM KCl, 2 mM CaCl₂, 1 mM MgSO₄, 20 mM HEPES, BSA 0.1% (wt/vol), pH 7.2) was added to each tube, and the incubation was continued for a further 5 min at 22°C. This dilution and further incubation step allow some of the nonspecific [¹²⁵I]- α -BgTx binding to dissociate, but it has no measurable effect on specific binding, increasing the signal-to-noise ratio of the binding assay. Binding reactions were terminated by filtration using a 96-place manifold (Inotech Biosystems, Lansing, MI). Particulate fractions were collected onto single layers of Inotech 0.75 μ m retention glass fiber filters that had been soaked in 5% (wt/vol) nonfat skim milk dissolved in isotonic binding buffer. Samples were washed 6 times, and all filtration and washing steps were conducted in a 4°C cold room, using ice-cold binding buffer. Bound radioligand was quantified by gamma counting at ~83 to 85% efficiency, using a Packard Cobra counter (PerkinElmer Life and Analytical Sciences, Waltham, MA). Results for inhibition of [¹²⁵I]- α -BgTx binding were calculated using a one-site fit: $B = B_0 / (1 + I / IC_{50})$, where B is ligand bound at inhibitor concentration I , B_0 is the binding in the absence of inhibitor, and IC_{50} is the concentration of inhibitor required to reduce binding to 50% of B_0 . Values for K_i (inhibition binding constant) were derived by the Cheng and Prusoff method (Cheng & Prusoff 1973): $K_i = IC_{50} / (1 + L / K_d)$.

Cell transfection and culture

The cell lines KX α 3 β 2R4, KX α 3 β 4R2, KX α 4 β 2R2, and KX α 7R1 were established previously by stably transfecting HEK293 cells with rat nAChR subunit genes (Xiao & Kellar 2004, Xiao *et al.* 1998, Xiao *et al.* 2009). The cells were routinely grown in a selective growth medium (SGM-7) which consists of minimum essential medium containing 2.2 mg/ml NaHCO₃, 10% FBS (vol/vol), 100 U/ml penicillin, 100 μ g/ml streptomycin, 0.7 mg/ml Geneticin, and 0.18 mg/ml carbachol, pH 7.4. Cells were grown in 75cm² culture flasks and passaged every 3-4 days upon reaching ~80-90% confluency.

Live cell fluorescence microscopy

Cells were plated on 15 mm glass coverslips (catalog # 64-0703, Warner Instruments, Hamden, CT) treated with 0.1 mg/ml poly-L-lysine and grown in 12 well tissue culture plates. Cells were suspended at RT in SGM-7 at a density of 8×10^4 /ml. One ml of the cell suspension was added to each well plus 500 μ l of SGM-7 and cultured at 37 °C in 95% air and 5% CO₂ for 2-3 days prior to use. For labelling experiments, cells were washed 3 times with RT PBS (pH 7.5) then exposed to a PBS solution containing 2 μ M Hoechst 33342 and 100 nM Cy3-ArIB[V11L;V16A] for 45 min at 4°C. To determine non-specific binding, cells were pre-incubated with 2 μ M α -CbTx for 30 min at 4°C then with 100 nM Cy3-ArIB[V11L;V16A] in the presence of 2 μ M α -CbTx for 45 min at 4°C. Cells were washed 3x with 4°C PBS and immediately examined using a Nikon FN1 microscope equipped with an epiilluminator, a 40x NIR Apo water immersion objective numerical aperture 0.80 and a filter set appropriate for FITC, Hoechst 33342 and Cy3 fluorophores. The images were captured at 640x480 resolution with 2x2 binning. The light source was a Lambda LS xenon arc lamp with a 300 watt bulb (Sutter Instruments, Novato, CA). For z-series images, a remote focus adaptor (Sutter Instruments, Novato, CA) was used to capture 1 μ m interval optical sections. All images were processed using NIH ImageJ software.

Results

Conjugation of ArIB[V11L;V16A] with Cy3 NHS ester dye

α -CTx ArIB[V11L;V16A] (Fig. 1A) is a synthetic peptide composed of 20 amino acids and 2 disulfide bridges connecting cysteines residues 3 to 8 and 4 to 17. Since there are no lysine residues in the amino acid sequence, the only free amino group available for coupling with Cy3 NHS ester (Fig. 1B) is located at the N-terminus of the peptide. The reaction between the free amino group and the NHS ester proceeds via nucleophilic acyl substitution to form a carboxamide bond between the peptide and the dye (Fig. 1C). The desired product, Cy3-ArIB[V11L;V16A], was separated from the reactants by RP-HPLC (Fig. 2). RP-HPLC analysis revealed that conjugation of the peptide resulted in a shift in retention time for the conjugate compared to the unconjugated peptide indicating increased hydrophobicity. The desired product, Cy3-ArIB[V11L;V16A], eluted at 24.2% B60 and the unconjugated peptide at 17.7% B60. Cy3-ArIB[V11L;V16A] eluted as a single peak indicating a single product. MALDI-TOF MS confirmed the RPHPLC results. The calculated monoisotopic mass for Cy3-ArIB[V11L;V16A] is 2922.17 Da and the observed monoisotopic mass was 2922.6 Da.

Effect of conjugation of Cy3 NHS ester on ArIB[V11L;V16A] pharmacology

Because substitutions in the amino acid sequence of α -CTx's can dramatically affect the binding affinity and specificity of the peptide for its target receptor, we determined whether conjugation with Cy3 would also affect the pharmacology of the peptide. Conjugation with Cy3 resulted in a right-ward shift in the IC_{50} value for inhibition of ACh-induced $\alpha 7$ responses in *Xenopus laevis* oocytes (Fig. 3A) from 0.52 nM to 2.0 nM with corresponding Hill coefficients of 2.4 ± 0.2 (2.0-2.8) and 1.5 ± 0.1 (1.3-1.8), respectively. Assuming there are 5 binding sites per $\alpha 7$ homopentamer, that occupation of only 1 site is required to block function, and all sites are equal with respect to ligand binding affinity (Whiteaker *et al.* 2007, Palma *et al.* 1996), this yields a K_d value of 13.2 ± 1.45 nM. In contrast to FITC- α -BgTx (Fig. 3B), Cy3-ArIB[V11L;V16A] (10 μ M) did not have any significant activity on $\alpha 9\alpha 10$ or $\alpha 1\beta 1\delta \epsilon$ nAChRs. Next we assessed whether the observed rates of block and unblock were affected by conjugation with Cy3. At the concentrations of toxins used, 5 nM for ArIB[V11L;V16A] and 20 nM for Cy3-ArIB[V11L;V16A], the observed rates of block were similar (Fig. 4) indicating that the observed rate constant (k_{ob}) for Cy3-ArIB[V11L;V16A] is substantially smaller than the k_{ob} for ArIB[V11L;V16A]. Measurement of current recovery showed no difference ($p > 0.05$) in the rate of unblock between Cy3-ArIB[V11L;V16A] and the parent peptide indicating similar dissociation rate constants (k_{off}). The ACh response recovered from block by Cy3-ArIB[V11L;V16A] to $5.7 \pm 1.8\%$ compared to $5.9 \pm 0.7\%$ for ArIB[V11L;V16A] after 15 minutes of wash (Fig. 4A,B). Thus, conjugation of the parent peptide with Cy3 produces an analog with slower on-rate kinetics and with a small loss of potency compared to the parent peptide. However, Cy3-ArIB[V11L;V16A] retains the slow off-rate and remarkable selectivity of the parent peptide for the $\alpha 7$ nAChR subtype. At 100 nM Cy3-ArIB[V11L;V16A], the average block of $\alpha 3\beta 4$ was $4.6 \pm 1.8\%$ (Fig. 3A) and for $\alpha 3\beta 2$ no block was detected (average response was $104.8 \pm 3.4\%$; Fig. 3A). Even at a concentration 5000-fold (10 μ M) greater than the IC_{50} for $\alpha 7$, significant block was only detected for $\alpha 3\beta 4$ (37%) (Fig. 4C) and $\alpha 3\beta 2$ (74%) (Fig. 4D). At more realistic concentrations (5-10x the K_d for $\alpha 7$) little-to-no block of $\alpha 3^*$ would occur. Furthermore, the off-rate kinetics of Cy3-ArIB[V11L;V16A] on these 3 subtypes are substantially different. Full recovery from block of $\alpha 3\beta 2$ and $\alpha 3\beta 4$ by Cy3-ArIB[V11L;V16A] occurred within 60 seconds (Fig. 4C,D).

To assess binding affinity of the Cy3 derivative at native $\alpha 7$ nAChRs, we used Cy3-ArIB[V11L;V16A] to compete with [125 I]- α -BgTx for binding to $\alpha 7$ nAChRs from mouse hippocampal membranes. [125 I]- α -BgTx is a standard radioligand for binding assays

involving $\alpha 7$ nAChRs and is considered $\alpha 7$ -selective when used on preparations from mammalian brain because other nAChRs that are bound by α -BgTx are not expressed in this tissue. Cy3-ArIB[V11L;V16A] potently displaced [125 I]- α -BgTx from mouse hippocampal membranes with a K_i value of 20.8 ± 1.4 nM and Hill coefficient value of 0.83 ± 0.05 (Fig. 5). Using the equation $IC_{50}/K_d = 2^{1/N} - 1$ (see methods), this K_i value corresponds to a functional IC_{50} of 3.0 nM which is in good agreement with the IC_{50} value (2.0 nM) determined by the preceding *Xenopus* oocyte experiments. This confirms that addition of Cy3 allows retention of competitive binding at $\alpha 7$ nAChRs, similar to that of the parent compound ArIB[V11L;V16A]. However, conjugation with Cy3 does induce a modest loss of potency compared to ArIB[V11L;V16A]; binding $K_i = 10.5$ nM (Whiteaker *et al.* 2008).

Cy3-ArIB[V11L;V16A] labels $\alpha 7$ nAChRs in KX α 7R1 cells

The stably transfected HEK293 cell line KX α 7R1 was used to assess the ability of Cy3-ArIB[V11L;V16A] to label $\alpha 7$ nAChRs. Application of 100 nM ($K_d = 13.2$ nM) Cy3-ArIB[V11L;V16A] to KX α 7R1 cells resulted in bright and punctate labelling of the cells. This labelling was prevented by preapplication of the competitive $\alpha 7$ antagonist α -CbTx (Fig. 6A) indicating that Cy3-ArIB[V11L;V16A] labelling of the cells represents specific binding to $\alpha 7$ nAChRs. No labelling was observed in KX α 3 β 2R4, KX α 3 β 4R2, or KX α 4 β 2R2 cells (Fig. 6B) indicating that Cy3-ArIB[V11L;V16A] labelling is specific to the $\alpha 7$ subtype. Using 100 nM ($K_d = 23$ nM) FITC- α -BgTx under these same conditions, we could not distinguish specific labelling from nonspecific labelling of KX α 7R1 cells due to a large amount of apparent nonspecific binding to cellular debris and the coverslip. To determine whether the nonspecific FITC- α -BgTx binding could be reduced, the cells were pre-incubated for 20 min in PBS containing 10% (vol/vol) FBS and 10% (vol/vol) FBS was included in all toxin and wash solutions. Under these conditions, we were still not able to detect specific labelling of KX α 7R1 cells although it appeared that nonspecific binding to the coverslip was reduced (see supplemental data).

Discussion

Multiple subtypes of nAChRs are expressed within the same tissue creating a need for highly specific probes with which to study these subtypes. The 500 species of carnivorous cone snails in aggregate produce thousands of distinct nAChR antagonists that they use to help immobilize their prey. The subtype specificity of these peptides enables their development as novel, specific tools (Olivera *et al.* 2008). In this study, we utilized the recently reported α -CTx ArIB as a platform to develop a fluorescent ligand for $\alpha 7$ nAChRs. The ArIB[V11L;V16A] analog was chosen due to both its high potency and slow off-rate kinetics. The addition of Cy3 to α -CTx ArIB[V11L;V16A] resulted in a single homogeneous product (Fig. 2) owing to the single conjugation site present at the N-terminus of the synthetic parent peptide. This contrasts to the situation with α -BgTx whose sequence is ~4-fold longer than α -CTx ArIB[V11L;V16A] and that has multiple free amino groups from multiple Lys residues and the N-terminus to which FITC may couple (Basus *et al.* 1993). HPLC analysis of FITC- α -BgTx confirms the presence of multiple compounds; this heterogeneity may be compounded by the heterogeneous nature of the parent venom-derived compound (unpublished observations).

Analysis of the functional effects of Cy3-ArIB[V11L;V16A] indicates that it retains the high potency and selectivity of ArIB[V11L;V16A] for $\alpha 7$ nAChRs. However, because of the very slow off-rate kinetics, we were unable to determine precise k_{off} values for Cy3-ArIB[V11L;V16A]. Functional studies often introduce variables that can make accurate measurements of peptide kinetics difficult. In this case, full recovery from block by both the parent peptide and the Cy3 conjugate takes hours. This exceeds the practical limitations of oocyte recording time. Furthermore, current run-up or current run-down in oocytes can also

hinder accurate measurements of recovery rates. For these reasons, we chose to provide values for percent recovery after 15 minutes of toxin wash-out rather than k_{off} values. Since we cannot accurately measure k_{off} , determining k_{on} is also difficult. The values provided allow only an estimate of peptide kinetics but nevertheless provides useful information for functional and labelling experiments. Despite the difficulties in accurately measuring kinetics, estimates of peptide affinities determined by concentration-response studies in oocytes are in good agreement with the affinities estimated by radioligand binding assays. Of note, the slow reversibility of Cy3-ArIB[V11L;V16A] allows long wash times which reduces non-specific binding in labelling experiments. The combination of properties possessed by Cy3-ArIB[V11L;V16A] make it well-suited for use in fluorescent labelling applications and it should be especially useful in native tissues where multiple nAChR subtypes are expressed.

Next we used stably transfected mammalian cell lines that express $\alpha 7$, $\alpha 3\beta 2$, $\alpha 3\beta 4$, and $\alpha 4\beta 2$ to assess the fluorescent labelling properties of Cy3-ArIB[V11L;V16A]. Cy3-ArIB[V11L;V16A] labelled $\alpha 7$ nAChRs in KX $\alpha 7$ R1 cells and the labelling could be prevented by preincubation with the competitive antagonist α -CbTx. The labelling had the appearance of puncta located on the membrane of the cells. The majority of the observed labelling is likely to be associated with receptors expressed on the cell surface as the binding was performed at 4°C to prevent active internalization of the ligand-bound receptor by endocytotic mechanisms (Wilkins *et al.* 2008). Furthermore, when 1 μm interval z-series images or 3D volume renditions of a z-series stack were examined, the labelling appeared to be associated only with the membrane (Fig 6C). In contrast to these results, we did not observe specific labelling in KX $\alpha 7$ R1 exposed to FITC- α -BgTx. This indicates that under the conditions used in this study, Cy3-ArIB[V11L;V16A] has superior specific labelling properties. We did not detect any significant Cy3-ArIB[V11L;V16A] labelling in HEK293 cells expressing $\alpha 3\beta 2$, $\alpha 3\beta 4$, or $\alpha 4\beta 2$.

Recent recognition that $\alpha 7$ nAChRs are co-expressed with $\alpha 9\alpha 10$ and $\alpha 1^*$ nAChRs has made the creation of an $\alpha 7$ -selective fluorescent ligand a priority. The detection of $\alpha 7$ nAChRs using standard pharmacological techniques has been complicated due to the relative lack of highly selective ligands for the $\alpha 7$ nAChRs. This is particularly problematic in tissues that express multiple nAChR subtypes that are sensitive to α -BgTx and MLA. These receptor subtypes include $\alpha 1^*$, $\alpha 6^*$, $\alpha 7$, and $\alpha 9^*$. Although $\alpha 9^*$ is not expressed in mammalian brain, it is co-expressed with $\alpha 7$ and $\alpha 1^*$ in several peripheral tissues. It is well established that DRG neurons express $\alpha 7$ nAChRs (Genzen *et al.* 2001, Genzen & McGehee 2003, Rau *et al.* 2005, Haberberger *et al.* 2004) and there is increasing evidence to support DRG expression of $\alpha 9^*$ (Lips *et al.* 2006, Rau *et al.* 2005, Haberberger *et al.* 2004). In Rau *et al.* (2005), the authors suggest the presence of a nAChR subtype with a distinct pharmacological profile but were unable to definitively identify this receptor as $\alpha 9\alpha 10$ because of the lack of selective antagonists of $\alpha 9\alpha 10$ nAChRs.

The $\alpha 9\alpha 10$ nAChR has been most extensively characterized in the auditory system where it functions as a ligand-gated ion channel (Zorrilla de San Martin *et al.* 2007, Gomez-Casati *et al.* 2009, Taranda *et al.* 2009, Vetter *et al.* 2007, Ellison *et al.* 2006, Elgoyhen *et al.* 1994). The $\alpha 1\beta 1\delta\gamma$ (fetal) and $\alpha 1\beta 1\delta\epsilon$ (adult) nAChR subtypes are generally thought of as being expressed exclusively at the neuromuscular junction and are sensitive to α -BgTx. However, a recent report suggests the presence of the fetal subtype in the vestibular and cochlear systems of the mouse inner ear (Scheffer *et al.* 2007). Immune cells, including lymphocytes and macrophages, also express the $\alpha 7$ subtype which has been extensively implicated in the inflammatory roles of these cell types (for review see (de Jonge & Ulloa 2007)). Macrophages and lymphocytes also express $\alpha 9\alpha 10$ receptors (Peng *et al.* 2004, Grau *et al.* 2007, Biallas *et al.* 2007, Kawashima & Fujii 2004) but as is with the case of DRG, the

functional role of this subtype expressed by these cells is not known. In addition, $\alpha 7$ and $\alpha 9\alpha 10$ nAChRs have been reported in human sperm (Kumar & Meizel 2005) and keratinocytes (Chernyavsky *et al.* 2007, Arredondo *et al.* 2002). The $\alpha 10$ subunit has been suggested to require the $\alpha 9$ subunit for functional expression (Sgard *et al.* 2002). Interestingly, there are several sites where $\alpha 7$ and $\alpha 10$ have been detected but not $\alpha 9$. In the rat, these sites include the heart (Dvorakova *et al.* 2005), thoracic DRG neurons (Haberberger *et al.* 2004) and sympathetic ganglia (Lips *et al.* 2006) but the functional significance of $\alpha 10$ expression in the absence of $\alpha 9$ is not known.

The overlapping pharmacological profiles and expression patterns of $\alpha 1^*$, $\alpha 7$, $\alpha 9^*$, and $\alpha 10^*$ nAChRs presents a difficult problem when studying these receptor subtypes. Other techniques for identifying $\alpha 7$ receptors present their own unique caveats. For example, the specificity of commercially available $\alpha 7$ antibodies has been questioned following two recent reports of antibody labelling in $\alpha 7$ -knockout mice (Moser *et al.* 2007, Herber *et al.* 2004). In the present study we show that Cy3-ArIB[V11L;V16A] binds $\alpha 7$ nAChRs but fails to bind to cells that lack this subtype. Thus, Cy3-ArIB[V11L;V16A] represents a valuable new addition to the collection of available nAChR probes.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

ACh	acetylcholine
nAChR	nicotinic acetylcholine receptor
α-BgTx	α -bungarotoxin
α-CbTx	α -cobratoxin
FITC-α-BgTx	fluorescein isothiocyanate- α -bungarotoxin
[¹²⁵I]-α-BgTx	monoiodinated α -bungarotoxin
α-CTx	α -conotoxin
MLA	methyllycaconitine
FBS	fetal bovine serum
BSA	bovine serum albumin
DMSO	dimethylsulfoxide
RT	room temperature
PBS	phosphate buffered saline
RP-HPLC	reverse phase-HPLC
MALDI-TOF MS	matrix assisted laser desorption ionization-time of flight mass spectrometry
CCD	charged coupled device

DRG dorsal root ganglia
HEK293 human embryonic kidney 293

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Fig. 1.

(A), Amino acid sequence of α -CTx ArIB[V11L;V16A] showing the disulfide connectivity between cysteine residues. (B), chemical structure of Cy3 NHS ester dye. (C), the conjugation reaction between α -CTx ArIB[V11L;V16A] and Cy3 NHS dyes resulting in the formation of a carboxamide bond between the dye and the N-terminus of the peptide.

Fig. 2. Separation of Cy3-ArIB[V11L;V16A] from the reactants by RP-HPLC. The labelled peaks correspond to unconjugated α -CTx ArIB[V11L;V16A] (1), unconjugated Cy3 NHS ester dye (2), and the fluorescent conjugate Cy3-ArIB[V11L;V16A] (3).

Fig. 3.

Concentration-response analysis of ACh-induced current block by Cy3-ArIB[V11L;V16A] and FITC- α -BgTx. (A), Cy3-ArIB[V11L;V16A] blocks $\alpha 7$ with an IC_{50} of 2.0 (1.7-2.2) nM, $n_H = 1.5$ (1.3-1.8). Conjugation with Cy3 NHS ester shifts the concentration-response curve of ArIB[V11L;V16A] (dashed line) to the right. Note lack of activity for Cy3-ArIB[V11L;V16A] on $\alpha 9\alpha 10$ and $\alpha 1\beta 1\delta\epsilon$ whereas (B), FITC- α -BgTx blocks $\alpha 7$ with an IC_{50} of 3.4 (2.3-5.0) nM, $n_H = 1.1$ (0.7-1.4), $\alpha 9\alpha 10$ 92.4 (64.3-132.7) nM, $n_H = 1.1$ (0.7-1.5), and $\alpha 1\beta 1\delta\epsilon$ 2.2 (1.7-2.8) μ M, $n_H = 1.4$ (1.0-1.8). In graph A, dashed line indicates concentration-response data for ArIB[V11L;V16A] binding to $\alpha 7$ denoted by ■ $\alpha 7$, solid lines indicate concentration-response data for Cy3-ArIB[V11L;V16A] binding to ▼ $\alpha 7$, ● $\alpha 1\beta 1\delta\epsilon$, * $\alpha 2\beta 2$, + $\alpha 2\beta 4$, ○ $\alpha 3\beta 2$, ◆ $\alpha 3\beta 4$, △ $\alpha 4\beta 2$, ▲ $\alpha 4\beta 4$, ◆ $\alpha 6/\alpha 3\beta 4^*$, □ $\alpha 6/\alpha 3\beta 2\beta 3^*$, × $\alpha 9\alpha 10$; *, denotes $\alpha 6/\alpha 3$ chimera. In graph B, solid lines indicate concentration-response data for FITC- α -BgTx binding to ■ $\alpha 7$, × $\alpha 9\alpha 10$, and, ● $\alpha 1\beta 1\delta\epsilon$. r, rat; m, mouse; n_H , Hill slope; (), 95% confidence interval.

Fig. 4.

Kinetics of block and unblock of ArIB[V11L;V16A] and Cy3-ArIB[V11L;V16A]. (A,B), representative trace recordings of *Xenopus laevis* oocytes expressing $\alpha 7$ nAChRs. After a stable ACh baseline was achieved, the oocytes were exposed to a concentration of toxin equal to 10x the IC_{50} for each respective toxin until a steady state level of block was achieved. The toxin solution was then changed to ND96 without toxin and the responses were monitored for recovery. At the concentrations used, the observed rates of block were similar. The responses recovered from block by ArIB[V11L;V16A] and Cy3-ArIB[V11L;V16A] to $5.9 \pm 0.7\%$ and $5.6 \pm 1.8\%$ respectively, after 15 minutes of wash which were also similar ($p > 0.05$). (C), 10 μM Cy3-ArIB[V11L;V16A] blocked $\alpha 3\beta 4$ by $36.7 \pm 6.2\%$ and $\alpha 3\beta 2$ by $74.1 \pm 8.8\%$. C, ACh control response without toxin; W, washout; $\pm$, SEM from at least 4 individual determinations.

Fig. 5.

Cy3-ArIB[V11L;V16A] competes with [125 I]- α -BgTx for binding sites from mouse hippocampal membranes. A competition binding assay was performed as described in materials and methods and the data were fit using a single-site model. Cy3-ArIB[V11L;V16A] displaced [125 I]- α -BgTx with a K_i of 20.8 ± 1.4 , n_H 0.83 ± 0.05 . n_H , Hill slope; each point represents the mean $\pm$ SEM of 4 individual experimental determinations.

Fig. 6. Cy3-ArIB[V11L;V16A] selectively labels cells expressing $\alpha 7$ nAChRs. (A), KX $\alpha 7$ R1 cells were labelled with 100 nM Cy3-ArIB[V11L;V16A] (red). The labelling was abolished by pre-incubating the cells with 2 μ M α -CbTx. (B), No labelling was observed with 100 nM Cy3-ArIB[V11L;V16A] on KX $\alpha 3\beta 2$ R4, KX $\alpha 3\beta 4$ R2, or KX $\alpha 4\beta 2$ R2 cells. Cells in all images were counter stained with 2 μ M Hoechst 33342 (blue) to label the nuclei. Scale bar is 20 μ m. (C), 1 μ m interval Z-series projection of KX $\alpha 7$ R1 cells labelled with 100 nM Cy3-ArIB[V11L;V16A]. All images were taken with a cooled CCD camera at 40x magnification and processed with NIH ImageJ.