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Allosteric Modulator Desformylflustrabromine Relieves the Inhibition of $\alpha 2\beta 2$ and $\alpha 4\beta 2$ Nicotinic Acetylcholine Receptors by β -Amyloid_{1–42} Peptide

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Abstract

Nicotinic acetylcholine receptors (nAChRs) are pentameric transmembrane proteins that belong to the cys-loop ligand-gated ion channel family. These receptors are widely expressed in the brain and implicated in the pathophysiology of many neurological conditions, including Alzheimer's disease (AD), where typical symptoms include the loss of cognitive function and dementia. The presence of extracellular neuritic plaques composed of β amyloid ($A\beta_{1-42}$) peptide is a characteristic feature of AD. Desformylflustrabromine (dFBr) is a positive allosteric modulator (PAM) for $\alpha 4\beta 2$ nAChRs since it increases peak ACh responses without inducing a response on its own. Previously, the effect of dFBr on the $\alpha 2\beta 2$ nAChR subtype was not known. The action of dFBr was tested on $\alpha 2\beta 2$ receptors expressed in *Xenopus* oocytes. It was found that dFBr is also a PAM for the $\alpha 2\beta 2$ receptor. Next we tested whether dFBr had any effect on the previously known block of both the $\alpha 4\beta 2$ and $\alpha 2\beta 2$ receptors by $A\beta_{1-42}$. We found that the functional blockade of ACh-induced currents in oocytes expressing $\alpha 4\beta 2$ and $\alpha 2\beta 2$ receptors by $A\beta_{1-42}$ was prevented by dFBr. We conclude that dFBr is a positive allosteric modulator for both $\alpha 4\beta 2$ and $\alpha 2\beta 2$ subtypes of nAChRs and that it also relieves the blockade of these receptors by $A\beta_{1-42}$. This study demonstrates that PAMs for the non- $\alpha 7$ nAChRs have the potential to develop into clinically applicable drugs for AD and other disorders.

Keywords

Alzheimer's disease (AD); Beta amyloid ($A\beta_{1-42}$); Desformylflustrabromine (dFBr); Electrophysiology; Nicotinic acetylcholine receptors (nAChRs); Positive allosteric modulators (PAMs)

Introduction

Nicotinic acetylcholine receptors (nAChRs) are cys-loop ligand-gated ion channels activated by the neurotransmitter acetylcholine (ACh) and are distributed throughout the peripheral and central nervous system (Gay and Yakel 2007). The main neuronal subtypes of nAChRs which are widely expressed in the brain are the $\alpha 7$ and the $\alpha 4\beta 2$ receptors (Whiting et al. 1987; Flores et al. 1992; Gopalakrishnan et al. 1996; Lindstrom 1996; Lindstrom et al. 1996; Liu et al. 2009). Dysfunction of nAChRs is thought to be involved in various neurodegenerative disorders, including but not limited to Alzheimer's disease (AD; Jones et

al. 1999). A clear pathological feature of AD is the presence of amyloid plaques comprised of the β -amyloid peptide ($A\beta_{1-42}$) and neurofibrillary tangles formed by aggregates of hyperphosphorylated tau protein (Schmechel et al. 1993; Nathan et al. 1994; Huang et al. 2001). Severe functional loss of the cholinergic system in the basal forebrains of patients suffering from AD has been reported (Perry et al. 1986). In advanced cases of AD, cholinergic neurons are lost and the number of nAChRs is reduced in the hippocampus and cortex (Coyle et al. 1983).

$A\beta_{1-42}$ has been shown to directly affect nAChR function; however, there are conflicting reports of either activation or inhibition of various subtypes of nAChRs by $A\beta_{1-42}$. For example, it was first reported that $A\beta_{1-42}$ blocked whole cell and single channel native non- $\alpha 7$ nAChR-mediated currents in CA1 stratum radiatum inter-neurons in rat hippocampal slices, while the $\alpha 7$ receptors were less affected by $A\beta_{1-42}$ (Pettit et al. 2001). Consistent with this, heteromeric $\alpha 4\beta 2$ or $\alpha 2\beta 2$ subtype of nAChRs expressed in either SH-EP1 cells or *Xenopus* oocytes were also blocked by $A\beta_{1-42}$ (Wu et al. 2004; Lamb et al. 2005). Other studies have shown that $A\beta_{1-42}$ blocked $\alpha 7$ receptors in cultured rat hippocampal neurons (Liu et al. 2001), or in *Xenopus* oocytes expressing human $\alpha 7$ receptors (Grassi et al. 2003). Recently, it was shown that in cultured rat basal forebrain cholinergic neurons, the $\alpha 7$ -containing receptors, which appeared to be heteromeric $\alpha 7\beta 2$ receptors, had high sensitivity to blockade by low concentrations of oligomeric $A\beta_{1-42}$ (Liu et al. 2009). In complete contrast to these reports of blockade of various nAChR subtypes by $A\beta_{1-42}$, native presynaptic (Dougherty et al. 2003) or $\alpha 7$ receptors expressed in *Xenopus* oocytes (Dineley et al. 2002) and native non- $\alpha 7$ nAChRs from acutely dissociated rat basal forebrain neurons were directly activated by $A\beta_{1-42}$ peptide (Fu and Jhamandas 2003).

Desformylflustrabromine (dFBr) is a metabolite of the marine bryozoan *Flustra foliacea*, commonly found in the North Sea (Lysek et al. 2002). When the *Flustra* derivatives (including dFBr) were tested on rat $\alpha 4\beta 2$ and $\alpha 7$ subtypes of nAChRs using radioligand binding assays, it was shown that dFBr had low micromolar affinities for these nAChRs (Peters et al. 2004). When dFBr was extracted from its natural source and functionally tested on $\alpha 4\beta 4$, $\alpha 3\beta 2$, $\alpha 3\beta 4$, $\alpha 4\beta 2$, and $\alpha 7$ nAChRs expressed in *Xenopus* oocytes, it potentiated $\alpha 4\beta 2$ receptors but had no effect on the other subtype of nAChRs tested (Sala et al. 2005). Synthetic dFBr reversibly potentiated ACh-induced $\alpha 4\beta 2$ responses at nanomolar concentrations ($pEC_{50}=120$ nM) and inhibited these responses at micromolar concentrations ($IC_{50}=150$ μ M; Kim et al. 2007). Synthetic dFBr did not potentiate homomeric $\alpha 7$ receptors, and only inhibition was seen (Kim et al. 2007). It has been proposed that dFBr potentiates $\alpha 4\beta 2$ receptors by an allosteric mechanism, while its inhibition is via an open-channel block (Weltzin and Schulte 2010). Furthermore, the effect of dFBr on $\alpha 2\beta 2$ receptors is not known.

In this study, we initially tested whether dFBr potentiates $\alpha 2\beta 2$ receptors and then whether the sensitivity of both $\alpha 4\beta 2$ and $\alpha 2\beta 2$ receptors to $A\beta_{1-42}$ is affected by dFBr. We found that dFBr is a positive allosteric modulator (PAM) for both the $\alpha 2\beta 2$ and $\alpha 4\beta 2$ receptors and that dFBr prevents the inhibition of both the $\alpha 4\beta 2$ and $\alpha 2\beta 2$ receptors by $A\beta_{1-42}$.

Methods and Material

RNA Preparation and Expression in *Xenopus* Oocyte

The mRNAs for rat $\alpha 2$, $\alpha 4$, and $\beta 2$ nAChR subunits were transcribed in vivo from linearized cDNA using the mMessage Machine kit (Ambion) according to the conditions suggested by the manufacturer. All procedures with *Xenopus laevis* frogs were approved by the Animal Care and Use Committee of the National Institute of Environmental Health Sciences. Female frogs housed under a 12-h day/night cycle were placed in an ice bath for 15 min and

anesthetized by immersion in cold water containing 0.1% ethylmetaaminobenzoate (MS-222; Sigma-Aldrich, St. Louis, MO, USA). Oocytes were dissected and defolliculated by treatment with collagenase B (2 mg/ml; Roche Diagnostics, Indianapolis, IN, USA) and trypsin inhibitor (1 mg/ml; Invitrogen, Carlsbad, CA, USA) for 2 h. The total volume of RNA injected was approximately 50 nl (1 µg/µl). The subunit mRNAs were mixed at a ratio of 1:1 (25 nl of each was injected).

Oocyte Electrophysiology

Current responses were obtained by two-electrode voltage clamp recording at a holding potential of -60 mV using a Geneclamp 500 amplifier and pClamp 8 software (Molecular Devices, Sunnyvale CA, USA). All electrophysiological experiments were done at room temp (~24°C). Electrodes contained 3 M KCl and had a resistance of <1 MΩ. The oocytes were continuously perfused with bath solution (96 mM NaCl, 2 mM KCl, 1.8 mM CaCl₂, 1 mM MgCl₂, 5 mM HEPES). Drugs were applied using a synthetic quartz perfusion tube (0.7 mm i.d.) operated by a computer-controlled valve for 3 to 4 s. Drugs were applied at an interval of 4 to 5 min. The peak amplitude with 100 µM ACh was obtained for each oocyte, and all responses were normalized to responses obtained at this concentration.

Chemicals and Solutions

Aβ₁₋₄₂ peptide was purchased from Anaspec (Fremont, CA, USA) and reconstituted in basic buffer (1% NH₄OH). The stock solution of Aβ₁₋₄₂ peptide was diluted fresh in bath solution daily. Acetylcholine was purchased from Sigma-Aldrich (St. Louis, MO, USA). Desformylflustrabromine was purchased from Tocris, Inc. (Ellisville, MO, USA), and stock solutions were diluted directly in the bath solution.

Data Analysis and Statistical Procedures

Concentration–response curves were fit by non-linear curve fitting in GraphPad Prism Software (San Diego, CA, USA) using standard built-in algorithms. For obtaining the potentiation/inhibition curve for α2β2 receptor modulation by dFBr, the data were fit to a bell-shaped dose–response equation (in GraphPad) as was done previously for the potentiation of α4β2 receptor by dFBr (Kim et al. 2007). This equation simultaneously fits both the potentiation and inhibition phases to give an EC₅₀ for the potentiation component and an IC₅₀ for the inhibitory component. For determining the EC₅₀ value for ACh, the data were fit to the Hill equation:

$$I = I_{\max} / (1 + EC_{50}/[A]^n)$$

where *I* is the current at a given agonist concentration, *I*_{max} is the maximal current obtained at a saturating agonist (ACh) concentration, EC₅₀ is the agonist concentration that elicits a half-maximal current, *A* is the agonist, and *n* is the Hill coefficient. The percent response and inhibition was calculated from the averaged data expressed as mean±SE. GraphPad Prism Software (San Diego, CA, USA) was also used to measure associations between the variables and assess statistical significance using the two-tailed paired *t* test. All statistical differences were deemed significant at the level of *p*<0.05.

Results

dFBr Potentiates α2β2 Receptors

Initially we tested whether dFBr has any effect on α2β2 nAChRs, since dFBr has previously been tested only on the α3β2, α3β4, α4β2, α4β4, and the α7 subtypes. In *Xenopus* oocytes expressing α2β2 receptors, dFBr on its own failed to elicit any currents (data not shown) in

oocytes clamped at holding potential of -60 mV. When dFBr was co-applied with 100 μ M ACh, it potentiated the ACh-induced currents by $127 \pm 18\%$ ($n=4$; Fig. 1a, left traces). At higher concentrations, dFBr inhibited the ACh-induced responses (Fig. 1a, right traces). The dose–response curve of dFBr at varying concentrations is shown in Fig. 1b. The concentration of dFBr inducing half-maximal potentiation was 446 ± 124 nM, and the concentration causing half-maximal inhibition was 11.3 ± 2.3 μ M. The extent of the potentiation is similar to that observed previously for $\alpha 4\beta 2$ receptors (Sala et al. 2005).

A comparison of ACh dose–response curves in the presence and absence of 1 μ M dFBr (Fig. 1c) indicates that dFBr causes a decrease in the apparent EC_{50} value of ACh from 50.3 ± 3.8 to 18.2 ± 2.3 μ M. Therefore, the presence of dFBr (1 μ M) leads to an increase in the apparent affinity of ACh for the $\alpha 2\beta 2$ receptors, along with an increase in the amplitude of the maximal response.

Action of dFBr on Inhibition of $\alpha 2\beta 2$ Receptors by $A\beta_{1-42}$

Previously, we have shown that 1 μ M $A\beta_{1-42}$ inhibits carbachol-induced currents in *Xenopus* oocytes expressing $\alpha 2\beta 2$ receptors (Lamb et al. 2005). We studied the effect of dFBr on inhibition of $\alpha 2\beta 2$ receptors by $A\beta_{1-42}$. When 1 μ M $A\beta_{1-42}$ was co-applied with 100 μ M ACh, the average response amplitude obtained was $77.5 \pm 6.9\%$ ($n=8$) of that obtained by 100 μ M ACh alone (Fig. 2); the peak amplitudes of ACh responses were significantly decreased with 1 μ M $A\beta_{1-42}$ by $22.4 \pm 6.8\%$ ($n=8$; Fig. 2; $p=0.0166$). In oocytes where the $\alpha 2\beta 2$ receptor-mediated responses were potentiated by 1 μ M dFBr, co-application with 1 μ M $A\beta_{1-42}$ now had no significant effect on the response amplitude (Fig. 2). The response amplitude increased slightly by $3.43 \pm 2.3\%$ ($n=8$) in the presence of $A\beta_{1-42}$, which was not significantly different from the response amplitude obtained with ACh and dFBr alone (Fig. 2; $p=0.1120$). Therefore, the presence of dFBr appeared to block the inhibition of the $\alpha 2\beta 2$ receptors by $A\beta_{1-42}$.

Action of dFBr on Inhibition of $\alpha 4\beta 2$ Receptors by $A\beta_{1-42}$

For $\alpha 4\beta 2$ receptors expressed in *Xenopus* oocytes, co-application of 1 μ M $A\beta_{1-42}$ with 100 μ M ACh resulted in average response amplitudes that were $85.2 \pm 3.3\%$ of that obtained by 100 μ M ACh alone (Fig. 3). The peak amplitudes were significantly decreased with 1 μ M $A\beta_{1-42}$ by $16.9 \pm 3.8\%$ ($n=5$; Fig. 3; $p=0.0118$). In oocytes where the $\alpha 4\beta 2$ receptor-mediated responses were potentiated by 1 μ M dFBr, co-application with 1 μ M $A\beta_{1-42}$ now had no significant effect on the response amplitude (Fig. 3). The response amplitude decreased slightly by $2.02 \pm 2.4\%$ ($n=5$), which was not significantly different from the response amplitude obtained with ACh and dFBr alone (Fig. 3; $p=0.5718$). Therefore, as with the $\alpha 2\beta 2$ receptors, the presence of dFBr appeared to block the inhibition of the $\alpha 4\beta 2$ receptors by $A\beta_{1-42}$.

Discussion

Previously, dFBr had been identified as a positive allosteric modulator for the $\alpha 4\beta 2$ receptors; however, it had no effect on other nAChR subtypes such as the $\alpha 3\beta 2$, $\alpha 3\beta 4$, $\alpha 4\beta 2$, $\alpha 4\beta 4$, and the $\alpha 7$ subtypes (Sala et al. 2005). Therefore, we initially tested the action of dFBr on rat $\alpha 2\beta 2$ nAChRs as this was not previously examined. For $\alpha 2\beta 2$ nAChRs expressed in *Xenopus* oocytes, dFBr, at lower concentrations, potentiated ACh-induced responses to a similar extent as for the $\alpha 4\beta 2$ receptors. In addition, we observed that higher doses of dFBr decreased the amplitude of the $\alpha 2\beta 2$, similar to what was previously observed for the $\alpha 4\beta 2$ receptor (Kim et al. 2007). The decline in amplitude of the ACh-induced responses with dFBr on $\alpha 2\beta 2$ receptor is consistent with an open-channel block mechanism suggested for the $\alpha 4\beta 2$ receptors (Weltzin and Schulte 2010).

Furthermore, the ACh dose–response curves in the presence and absence of dFBr shows that dFBr increases the apparent affinity and efficacy of ACh for $\alpha 2\beta 2$ receptors. This increase in apparent efficacy and affinity suggests that the potentiating effect of dFBr is due to its binding to a distinct site separate from the agonist binding site as has been proposed for PAMs for the $\alpha 7$ nAChRs (Young et al. 2008).

Previously, we have reported that $A\beta_{1-42}$ inhibited various subtypes of the non- $\alpha 7$ nAChRs expressed in oocytes, including the $\alpha 2\beta 2$ and $\alpha 4\beta 2$ receptors, but not the $\alpha 7$ nAChRs (Lamb et al. 2005). These receptors are known to be expressed in rat CA1 hippocampal interneurons (McQuiston and Madison 1999; Sudweeks and Yakel 2000; Yakel and Shao 2004), and it is well-known that the non- $\alpha 7$ nAChRs have a positive role in regulating cognitive function (Picciotto et al. 1995; Levin and Simon 1998). Therefore, inhibition of these receptors (e.g., with $A\beta_{1-42}$) could lead to impairment in cognitive function. Furthermore, there is considerable decline in the number of nAChRs (especially the $\alpha 4$ subunit-containing receptors) in patients suffering from AD (Paterson and Nordberg 2000; Court et al. 2001). Here we have found that the inhibition of the $\alpha 4\beta 2$ and $\alpha 2\beta 2$ receptors by $A\beta_{1-42}$ is blocked by the presence of dFBr. While the mechanism of inhibition of these receptors by $A\beta_{1-42}$ is unknown, as is the mechanism whereby dFBr blocks this action, it is possible that dFBr and similar compounds might have positive effects on cognitive function in patients with AD in part through these actions. Further studies will directly address these issues.

In conclusion, we have demonstrated that dFBr is a PAM for both $\alpha 4\beta 2$ and $\alpha 2\beta 2$ nAChRs and that it interferes with the inhibitory actions of $A\beta_{1-42}$ on these receptors. Therefore, the nAChR-specific PAMs may be a clinically useful tool in combating the inhibition of these receptors by $A\beta_{1-42}$ in patients suffering with AD by the restoring the cholinergic tone.

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Fig. 1.

Effects of dFBr on $\alpha 2\beta 2$ receptors. **a** In *Xenopus* oocytes expressing rat $\alpha 2\beta 2$ receptors voltage-clamped to -60 mV, $100 \mu\text{M}$ ACh induces inward current responses that are potentiated by the co-application (with ACh) of $1 \mu\text{M}$ dFBr (*left* two traces). Co-applying a higher dose of dFBr ($100 \mu\text{M}$) with ACh inhibited the amplitude of ACh responses (*right* two traces). The time of drug exposure is indicated by the *horizontal bar* above the traces. **b** Dose-response curve for varying concentrations of dFBr applied in the presence of $100 \mu\text{M}$ ACh. The peak amplitudes were normalized to peak currents obtained from $100 \mu\text{M}$ ACh in the absence of dFBr. **c** ACh dose-response curves in the absence (*circle*) and presence (*square*) of dFBr ($1 \mu\text{M}$). The *vertical dotted lines* from the two curves correspond to the EC_{50} value of ACh with and without dFBr. Responses were obtained using two-electrode voltage clamp on *Xenopus* oocytes expressing rat $\alpha 2\beta 2$ receptors. Peak amplitudes were normalized to peak currents obtained with $100 \mu\text{M}$ ACh in the absence of dFBr. Amplitudes are expressed as $\text{mean} \pm \text{SE}$; $n=4$ for each data point

Fig. 2.

Effect of dFBr on inhibition of $\alpha 2\beta 2$ receptors by $A\beta_{1-42}$. **a** In oocytes expressing $\alpha 2\beta 2$ receptors, the peak amplitude of ACh (100 μ M)-induced responses is reduced by the co-application with 1 μ M $A\beta_{1-42}$ (*left* two traces). When the ACh-induced responses have been potentiated with dFBr (1 μ M), co-application with $A\beta_{1-42}$ now has no effect on the amplitude of the responses (*right* two traces). **b** Normalized amplitudes of ACh-induced responses; 1 μ M $A\beta_{1-42}$ significantly inhibited ACh responses in the absence of dFBr ($p=0.0166$; $n=8$), but not in its presence ($p=0.1120$; $n=8$). **c** Percent inhibition of ACh responses by $A\beta_{1-42}$ in absence (*left*) and presence of dFBr (*right*); the difference was significant ($p=0.0066$; $n=8$). Asterisk indicates significant difference

Fig. 3.

Effect of dFBr on inhibition of $\alpha 4\beta 2$ receptors by $A\beta_{1-42}$. **a** In oocytes expressing $\alpha 4\beta 2$ receptors, the peak amplitude of ACh (100 μ M)-induced responses is reduced by the co-application with 1 μ M $A\beta_{1-42}$ (*left* two traces). When the ACh-induced responses have been potentiated with dFBr (1 μ M), co-application with $A\beta_{1-42}$ now has no effect on the amplitude of the responses (*right* two traces). **b** Normalized amplitudes of ACh-induced responses; 1 μ M $A\beta_{1-42}$ significantly inhibited ACh responses in the absence of dFBr ($p=0.0118$; $n=5$), but not in its presence ($p=0.5718$; $n=5$). **c** Percent inhibition of ACh responses by $A\beta_{1-42}$ in absence (*left*) and presence of dFBr (*right*); the difference was significant ($p=0.0201$; $n=5$). Asterisk indicates significant difference