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Acetylcholine and antibodies against the acetylcholine receptor protect neurons and astrocytes against beta-amyloid toxicity

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ABSTRACT

Aggregated amyloid-β causes pathological changes in mixed cultures of neurons and astrocytes such as sporadic cytoplasmic intracellular Ca^{2+}-signalling, increase in reactive oxygen species production and cell death. Some of the toxic effects of amyloid-β are mediated through the interaction of the peptide with α7-type nicotinic acetylcholine receptors at the cell surface. Here we demonstrated that affinity purified antibodies to synthetic fragment 173–193 of the α7-subunit of the nAChR are able to protect cells from amyloid-β induced cell death. The antibodies had no effect on the amyloid-β induced calcium signal in astrocytes. However, they significantly reduced amyloid-β induced and NADPH oxidase mediated ROS production. Modulation of the NADPH oxidase activity by either the antibodies, the receptor agonist acetylcholine or the antagonist of the α7-type nicotinic acetylcholine receptors α-bungarotoxin was vital in inhibiting both amyloid-β induced ROS production, caspase 3 cleavage as well as cell death. The uncovered details of the mechanism underlying the action of antibodies to α7-type nicotinic acetylcholine receptors gives additional insight into the involvement of this receptor in Alzheimer's disease pathology and provides a new approach to anti-Alzheimer's disease vaccine design.

1. Introduction

One of the most intensively studied hypotheses of the pathophysiology of Alzheimer's disease (AD) is that of the mechanism underlying the toxicity of the amyloid-β (Aβ) peptides, which are known to impair neuronal activities, leading to a decline in memory and cognitive function (Hardy and Selkoe, 2002). On a molecular level, the pathology of AD is associated with increased oxidative stress (Hensley et al., 1994; Abramov and Duchen, 2005; Ma et al., 2011), which is regarded as an important factor contributing to the impaired brain metabolism and mitochondrial dysfunction in AD (Abramov et al., 2004; Abramov and Duchen, 2005; Abeti et al., 2011). Aberrant Aβ accumulation along with altered expression and function of nicotinic acetylcholine receptors (nAChRs) feature prominently in the etiology of AD (Court et al., 2001). Since the discovery that Aβ is bound to α7 nAChRs in many experimental settings, including post-mortem AD brain, much effort has been exerted to understand the implications of this interaction in the disease milieu (Wang et al., 2000; Lilja et al., 2011).

Previous studies have shown that Aβ binds to nAChRs and activates signaling cascades that result in the disruption of synaptic functions. It has also been suggested that activation of pathological calcium signaling can be due to binding of Aβ to the nAChRs (Fayuk and Yakel, 2005). However, Aβ is known to induce a calcium signal by other mechanisms, including pore formation in the plasma membrane (Arispe et al., 1993; Abramov et al., 2003; Demuro et al., 2011).

Importantly, stimulation of nAChRs is protective against Aβ-induced neurotoxicity (Moon et al., 2008), as is application of antibodies against nAChRs (Kamynina et al., 2010). Furthermore, vaccination with only the α7-subunit fragment 173–193 was shown to rescue spatial memory, restore the level of α7 nAChR in the cortex, and prevent an increase in the Aβ level in brain tissue in mice with experimentally induced AD.

The cellular mechanism of protection of neurons against Aβ-induced cell toxicity by the activation of nAChRs or using antibodies against the α7-subunit of nAChRs remains unclear. In the present work we have therefore investigated the connection between activation of nAChRs, or application of antibodies to α7 nAChRs, and changes in $[Ca^{2+}]_c$ and reactive oxygen species production in the neurotoxicity of Aβ.

Abbreviations: Aβ, amyloid β peptide; AD, Alzheimer's disease; HBSS, HEPES-buffered salt solution; ROS, reactive oxygen species; α7 nAChRs, α7-type nicotinic acetylcholine receptors; AchRabs, affinity purified antibodies to synthetic fragment 173–193 of the α7-subunit of the nAChR.

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2. Material and Methods

2.1. Cell culture

Mixed cultures of hippocampal or cortical neurons and glial cells were prepared as described previously (Abramov et al., 2003) with modifications, from Sprague-Dawley rat pups 2–4 days post-partum (UCL breeding colony). Experimental procedures were performed in full compliance with the United Kingdom Animal (Scientific Procedures) Act of 1986. Hippocampi and cortex were removed into ice-cold PBS (Ca^{2+}, Mg^{2+}-free, Invitrogen, Paisley, UK). The tissue was minced and trypsinised (0.25% for 5 min at 37 °C), triturated and plated on poly-D-lysine-coated coverslips and cultured in Neurobasal A medium (Invitrogen, Paisley, UK) supplemented with B-27 (Invitrogen, Paisley, UK) and 2 mM L-glutamine. Cultures were maintained at 37 °C in a humidified atmosphere of 5% CO_2 and 95% air, fed twice a week and maintained for a minimum of 12 days before experimental use to ensure expression of glutamate and other receptors. Neurons were easily distinguishable from glia: they appeared phase bright, had smooth rounded somata and distinct processes, and lay just above the focal plane of the glial layer. Cells were used at 12–15 days in vivo (DIV) unless otherwise stated.

2.2. Peptides and treatments

Aβ 25–35 and Aβ 1–40 (Bachem, St. Helens, UK) were dissolved at 1–5 mM in sterile ultrapure water (Milli-Q standard, Millipore, Watford, UK) and kept frozen until use. The peptides were added to cells during experimental recordings, except for the neurotoxicity measurements, where they were added 24 h before the assays of cell death (see below). Aβ 25–35 was used at concentrations of up to 50 μM and Aβ 1–40 was used at concentration of 10 μM. Acetylcholine (Sigma, Aldrich) and α-bungarotoxin (Tocris, Bioscience, UK) were dissolved in sterile ultrapure water (Milli-Q standard, Millipore, Watford, UK) at concentrations 10 mM and 100 μM, respectively, and kept frozen until use.

For collecting affinity purified antibodies specific for the α7 nAChR, peptide 173–193 ^{173}EWDLVGIPGKRSERFYECCKE^{193} corresponding to the sequence of the human AChR α7-subunit (Swiss-Prot Q5W554) was synthesized manually by solid-phase Fmoc-chemistry (Udenfriend et al., 1987). The homogeneity of the synthesized peptide was estimated by analytical reverse-phase HPLC chromatography on Jupiter columns 5 μ C18 300 A, 250 mm × 4.6 mm (Phenomenex, USA), amino acid analysis on Biotronik LC-3000 (Germany) and MALDI mass spectrometry on a VISION 2000 instrument (Bioanalysis, UK). The synthetic peptide was >98% homogeneous when analyzed by these methods.

For ELISA assays the N-terminal extracellular domain of the human α7-subunit nAChR (Sigma, Aldrich) and Aβ 1–42 (Sigma, Aldrich) were dissolved at concentration 5 mg/ml in sterile ultrapure water and kept frozen until use.

2.3. Rabbit blood sera collection for affinity purification of antibodies

To obtain sera with antibodies specific to peptide 173–193 rabbits were double immunized subcutaneously with 1 mg of the peptide in saline solution mixed with equal volume of an adjuvant to obtain emulsion. The first immunization was in Freund's complete adjuvant, the second immunization was on the 45th day in Freund's incomplete adjuvant. Blood sera samples were taken from the rabbit ear vessels on the 55th day of the experiment. Sera were prepared from each blood sample and stored at −20 °C until use.

2.4. Purification of monospecific polyclonal antibodies against peptide 173–193 of the α7 nAChR using affinity chromatography

2.4.1. Preparation of affinity adsorbent

One gram of CNBr-activated Sepharose 4B (GE health care, Sweden) was suspended in 3 ml 1 mM NaCl. The adsorbent was washed for 15 min with 1 mM NaCl on a sintered glass filter. Then 1 mg peptide 173–193 was dissolved in 1 ml coupling buffer, 0.1 M $NaHCO_3$ pH 8.3 containing 0.5 M NaCl. The coupling solution was mixed with the prepared adsorbent. The mixture was gently rotated for 1 h at room temperature. Not bound ligand was washed away with 5 medium volumes of coupling buffer. Remaining active groups were blocked in blocking buffer, 0.2 M Gly, 0.1 M NaCl, pH 8.0 for 2 h at room temperature. The adsorbent was then washed with three cycles of 5 medium volumes of buffers with alternating pH: 0.1 M acetic acid/sodium acetate, pH 4.0 containing 0.5 M NaCl followed by a wash with borate buffer, pH 8.0: 0.1 M $Na_2B_4O_7$ containing 0.1 M NaCl. 0.05% NaN_3 was added to the adsorbent and it was stored at 4 °C until use. 1 ml Sepharose conjugated with peptide 173–193 contains 0.4 mg of peptide 173–193 according to amino acid analysis data.

2.4.2. Affinity chromatography

An adsorbent column was prepared by pouring 5 ml of the Sepharose conjugated with peptide 173–193 into a column and settled. The column was equilibrated with 50 ml of PBS, pH 7.4, 10 mM Na_2HPO4, 150 mM NaCl. 2 ml of rabbit sera against peptide 173–193 was applied into the column for 1 h. The column was washed with 20 mL of PBS. Elution of affinity antibody against peptide 173–193 was done with 100 mM glycine–HCl, pH 2.2, and the eluate was collected in 1.8 ml fractions in 2 ml Eppendorf tubes containing 50–100 μl (one-tenth volume of glycine) of 1.5 M Tris–HCl, pH 8.7. The affinity purified rabbit antibodies against fragment 173–193 of the α7-subunit nAChRs (AChRabs) were dialyzed against PBS, pH 7.4 for 24 hrs at +4 °C and stored at −20 °C until use. The protein concentration of the samples was determined by UV absorbance (280 nm) and calculated in accordance with c (mg/ml) = D/1.4, D – optical density. Final concentration of AChRabs was 0.279 mg/ml. AChRabs were used in the experiments at the final concentration 13 μg/ml (50 μl/ml).

2.5. Enzyme-linked immunosorbent assay (ELISA)

Rabbit blood sera or affinity purified antibodies were pooled for analysis by ELISA as described in (Udenfriend et al., 1987). Shortly, wells of a 96-well plate Maxisorp (Nunc, Denmark) were coated with 20 μg/ml of either peptide 173–193 or N-terminal extracellular domain of the human α7-subunit nAChR or and Aβ 1–42, incubated with 100 μl prediluted sera or affinity purified antibodies starting from dilution 1:40 or 1:1000, followed by addition of peroxidase-conjugated goat antibody to rabbit IgG (Sigma, USA). Antibody titers of sera were quantified by an end-point dilution with OD > 0.1 which three times exceeded the binding with ChromPure rabbit IgG (Johnson ImmunoResearch laboratories, USA).

2.6. Measurements of $[Ca^{2+}]_c$ and ROS

For measurements of $[Ca^{2+}]_c$ cells were loaded for 30 min at room temperature with 5 μM fura-2 AM and 0.005% pluronic acid in a HEPES-buffered salt solution (HBSS) composed of 156 mM NaCl, 3 mM KCl, 2 mM $MgSO_4$, 1.25 mM KH_2PO_4, 2 mM $CaCl_2$, 10 mM glucose and 10 mM HEPES; pH adjusted to 7.35 with NaOH.

For measurement of ROS production dihydroethidium (HEt – 2 μM) was present in the solution during the experiment. No