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Monoclonal antibodies to mouse butyrylcholinesterase

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ABSTRACT

Our immunization strategy introduced recombinant mouse butyrylcholinesterase (BChE) to naïve BChE knockout mice. An extraordinarily strong immune reaction gave rise to a whole spectrum of antibodies with different properties. Two selective and highly efficient monoclonal anti-mouse BChE antibodies 4H1 (IgG1) and 4C9 (IgG2a), with Kd values in the nanomolar range were generated. ELISA detected BChE in as little as 20–50 nl of mouse plasma using 2 μg (4H1) or 4 μg (4C9). Both antibodies cross-reacted with BChE in dog plasma but only 4H1 reacted with rat BChE, suggesting that the antibodies are targeted towards different epitopes. Surprisingly, neither recognized human BChE. The anti-mouse BChE antibodies were used in immunohistochemistry analysis of mouse muscle where they specifically stained the neuromuscular junction. The antibodies enable visualization of the BChE protein in the mouse tissue, thus complementing activity assays. They can be used to study a long-lasting question about the existence of mixed acetylcholinesterase/BChE oligomers in mouse tissues. Moreover, monoclonal anti-mouse BChE antibodies can provide a simple, fast and efficient way to purify mouse BChE from small amounts of starting material by using a single-step immunomagnetic bead-based protocol.

1. Introduction

Work with the recombinant and purified butyrylcholinesterase (BChE) of different origin over past 20 years widened our knowledge about structure and enzymatic behavior of this enzyme *in vitro*. Additionally, generation of BChE mutant mice with complete loss of BChE (BChE knockout, BChE −/− mouse [1]; or removal of particular cholinesterase (ChE) molecular forms (transmembrane ChE anchor knockout, PRiMA −/− mouse [2]; and collagen tail knock-out, ColQ −/− mouse [3]; provided tools to study BChE *in vivo* and *in situ*. Despite the existence of such animals, properties of "natural" BChE, precise BChE/BChE molecular forms localization and its/their specific physiological functions remain unknown. To follow-up with BChE studies in mouse models, selective and specific antibodies against mouse BChE were needed but unavailable. BChE had been considered to be a "difficult" antigen for antibody production for many years, mainly due to the glycosylation and the high interspecies protein sequence similarity. Only few efficient antibodies were generated in past, recognizing human and monkey protein [4] but to our knowledge, all effort to raise antibody against mouse BChE by standard immunization protocols failed. Recently we have re-introduced a successful immunization strategy for "difficult" antigens that showed up to be very efficient for BChE as well [5]. Presenting BChE of different origin to the naïve BChE −/− mouse led to an extraordinarily strong immune reaction and gave rise to the whole spectrum of antibodies with different properties. We have successfully generated monoclonal antibodies against mouse BChE, which we further characterize in this paper.

2. Methods

2.1. Monoclonal anti-mouse BChE antibodies

Two monoclonal antibodies raised against mouse BChE were used in the study, 4H1 and 4C9. Antibodies were generated as described before [5]. Briefly, BChE deficient mice were immunized with recombinant mouse BChE in the presence of Freund's adjuvants. Antigen injection was repeated until the anti-mouse BChE antibody reached plateau in plasma. Monoclonal antibody were generated commercially (P.A.R.I.S Production d'Anticorps & Services, Compiegne, France) by hybridoma fusion. All hybridomas were tested for antibody production in our lab by common immunological assays. One batch of purified 4H1 was biotinylated by P.A.R.I.S. company.

Abbreviations: AChE, acetylcholinesterase; BChE, butyrylcholinesterase; BChE −/− mouse, butyrylcholinesterase knock-out mouse; ChE, cholinesterases; DTNB, 5,5-dithiobis(2-nitrobenzoic acid); isoOMPA, tetraisopropylpyrophosphoramide.

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2.2. Plasma preparation

Plasma from human, mouse, rat and dog were used in the study. Venous blood was collected into the EDTA- or heparin- treated collection tubes (S-Monovette® EDTA K2 Gel, Sarstedt, #04.1931 or Microvette® 200 LH, Sarstedt, #20.1292) and centrifuged at 14,000*g* for 10 min at 4 °C. Supernatant containing plasma was flash frozen in liquid nitrogen and stored at −80 °C until used.

2.3. Tissue extraction

Mice were deeply anesthetized with sodium chloral hydrate, perfused transcardially with ice-cold physiological saline and euthanized. Liver, lungs, brain, heart and tibialis muscle were dissected and flash frozen in liquid nitrogen. Frozen tissue was then ground in a mortar with pestle in liquid nitrogen and homogenized in a glass Dounce homogenizer for 3 min in 10 mM Hepes, pH 7.4, 10 mM EDTA, 0.8 M NaCl, 1% CHAPS. Extraction was performed at constant mixing at 4 °C for 15 min followed by a centrifugation at 14,000*g* for 10 min.

2.4. ELISA

Assay was performed in 96-well Nunc-Immuno-Immuno F96 Maxi-Sorp plates (Nunc GmbH & Co). Each well was coated (48 h incubation at 4 °C) with 1 μg of affinity pure goat anti-mouse IgG (Jackson Immunoresearch laboratories, #115-005-003) in final volume 100 μl adjusted with 0.05 M phosphate buffer pH 7.4, 0.15 M NaCl. Plates were blocked with 0.1% BSA for minimum 48 h. Plates were then sequentially incubated with affinity pure monoclonal antibodies and plasma. Incubations were done overnight at 4 °C. Monoclonal antibody dilutions and plasma dilution were determined experimentally (see Sections 3.1 and 3.2). Plates were washed after each incubation with 0.01 M phosphate buffer pH 7.4, 0.04% Tween in ELx50™ Microplate Strip Washer (BioTek). Signal was revealed as BChE activity. In saturation experiments (Sections 3.1 and 3.2), the Kd values were calculated using GraphPad Prism® software for PC from a non-linear fit of the curve (choosing built-in model of specific saturation binding). As only monoclonal antibodies were used in the experiments, full saturation was assumed at the value of 2Kd.

2.5. Ellman's assay

BChE activity in plasma was followed using 1 mM butyrylthiocholine and 0.5 mM 5,5-dithiobis(2-nitrobenzoic acid) (DTNB) in 5 mM Hepes buffer, pH 7.5 for the time period up to 20 min [6]. Total cholinesterase activity was followed with 1 mM acetylthiocholine under same conditions as described above. BChE was inhibited by 10 mM tetraisopropylpyrophosphoramide (iso-OMPA) (Sigma–Aldrich). Inhibitor of acetylcholinesterase (AChE), BW284C51 (Sigma–Aldrich), was used in the final concentration 5 μM. Inhibitors were pre-incubated with enzyme for 30 min before substrate was added.

2.6. Immunohistochemistry

Muscles from Thy1-YFP mice (B6.Cg-Tg(Thy1-YFP)16Jrs/J, The Jackson Laboratory) were dissected and fixed for 30 min with 4% paraformaldehyde in 10 mM phosphate-buffered saline pH 7.4 (PBS). They were incubated in 0.1 M glycine/PBS and then incubated in 5% goat serum/0.2% saponine in PBS for 3 h. Muscle was incubated with biotinylated 4H1 (1 mg/ml, 1/500) overnight in 5% goat serum/0.2% saponine/PBS, washed with PBS several times, followed by incubation (90 min) with streptavidin, Alexa Fluor 594 conjugate (Molecular probes). Preparations were mounted on glass slides with Vectashield medium (Vector Laboratories) before examination by confocal microscopy.

2.7. Immunoblotting

Recombinant mouse BChE and plasmas from wild-type mouse, BChE −/− mouse, human, dog and rat were used for Western blot. The recombinant enzyme was a gift from A. Saxena (WRAIR, Silver Spring, MD, USA). Denatured SDS/PAGE gel (12%) was used for recombinant BChE. Transfer to the PVDF membrane was achieved overnight at 300 mA at 4 °C. Plasmas were analyzed under non-reduced non-denatured conditions, 0.5, 2 and 4 μl were loaded on a gel. Two native precast 4–20% gels (Mini-PROTEAN, TGX, BIO-RAD, #4561095) were run simultaneously under same conditions. One gel was stained for BChE activity by method of Karnovsky and Roots in presence of 2 mM butyrylthiocholine as described before [7]. The second gel was transferred to the PVDF membrane using Transfer Pack (BIO-RAD, #1704156) in TRANSBLOT TURBO (BIO-RAD, #1704150). Membranes were blocked at room temperature for 4 h and incubated with primary antibodies diluted in TBS (20 mM Tris/HCl, 140 mM NaCl) for 4 h at room temperature. Incubation with secondary anti-mouse antibodies (Jackson ImmunoResearch laboratories, #315-036-003) was performed overnight at 4 °C. Signal was revealed after washing, using ECL Western Blotting Substrate (Pierce).

3. Results

Two monoclonal anti-mouse BChE antibodies were selected for further characterization, 4H1 and 4C9. Isotype analysis performed by the production company proved 4H1 to be IgG1 and 4C9 to be IgG2a.

3.1. Avidity

Avidity of the antibodies for tetrameric mouse BChE was analyzed by ELISA. Different antibody dilutions were used to capture the enzyme from 10 μl of mouse plasma (Fig. 1). The Kd values calculated by computer from the non-linear fit of the dilution curves were 0.3 nM for 4H1 and 0.1 μM for 4C9 (assuming IgG molecular weight 150 kDa). Based on the obtained data, 2 μg of 4H1 and 4 μg of 4C9 were chosen for further experiments in order to ensure saturation of the anti-mouse secondary antibody – coated plates.

3.2. Sensitivity

Sensitivity of the antibodies towards mouse plasma BChE was determined in ELISA based on the saturation curve (Fig. 2) at 20 min of the recording. The plateau was reached at the plasma volume 3.2 μl in case of 4H1 and 5.9 μl for 4C9. Detection limit was set to the plasma volume that gave 40% higher signal comparing to the background, which was in case of 4H1-facilitated capturing 20 nl and in case of 4C9 capturing 50 nl of plasma.

3.3. Efficiency with BChE hetero-oligomers

Capability of 4H1 antibody to recognize BChE assembled with anchoring peptides was tested in ELISA assay with tissue extracts from lungs, brain, heart and tibialis muscle (Fig. 3). BChE was captured from all tested extracts, while captured activity was 80–100% of the original activity in extracts as measured by Ellman's method.