Available online at www.sciencedirect.com
ScienceDirect
Biochemical and Biophysical Research Communications 355 (2007) 1069–1074
BBRC
www.elsevier.com/locate/ybbrc

Calcium-activated butyrylcholinesterase in human skin protects acetylcholinesterase against suicide inhibition by neurotoxic organophosphates

Karin U. Schallreuter [a,c,*], Nicholas C.J. Gibbons [a,c], Souna M. Elwary [a], Susan M. Parkin [b], John M. Wood [a]

[a] *Clinical and Experimental Dermatology, Department of Biomedical Sciences, University of Bradford, Bradford BD7 1DP, West Yorkshire, UK*
[b] *Department of Biomedical Sciences, University of Bradford, Bradford, UK*
[c] *Institute for Pigmentary Disorders in Association with EM Arndt University of Greifswald, Germany and University of Bradford, UK*

Received 17 January 2007
Available online 23 February 2007

Abstract

The human epidermis holds an autocrine acetylcholine production and degradation including functioning membrane integrated and cytosolic butyrylcholinesterase (BuchE). Here we show that BuchE activities increase 9-fold in the presence of calcium (0.5×10^{-3} M) via a specific EF-hand calcium binding site, whereas acetylcholinesterase (AchE) is not affected. ^{45}Calcium labelling and computer simulation confirmed the presence of one EF-hand binding site per subunit which is disrupted by H_2O_2-mediated oxidation. Moreover, we confirmed the faster hydrolysis by calcium-activated BuchE using the neurotoxic organophosphate *O*-ethyl-*O*-(4-nitrophenyl)-phenylphosphonothioate (EPN). Considering the large size of the human skin with 1.8 m^2 surface area with its calcium gradient in the 10^{-3} M range, our results implicate calcium-activated BuchE as a major protective mechanism against suicide inhibition of AchE by organophosphates in this non-neuronal tissue.

Keywords: Butyrylcholinesterase; Acetylcholinesterase; Calcium; EF-hand; Human epidermis; Organophosphates; Hydrogen peroxide

The role of butyrylcholinesterase (BuchE, EC 3.1.1.18) in human cells and tissues is still obscure because no specific endogenous substrate has been identified for this enzyme. Moreover, although its expression in human serum is high, peripheral tissues have overall lower levels compared to acetylcholinesterase (AchE) [1]. Earlier it has been proposed that BuchE functions as a protective mechanism for AchE [2]. Interestingly BuchE has 53% sequence homology with AchE and both enzymes share the same active site catalytic triad of Glu, His and Ser. Moreover, both enzymes contain aromatic gorges for substrate binding where AchE has 14 aromatic residues lining the gorge, while BuchE has only eight such residues [3]. Based on these structures, AchE owns a very specific substrate binding site for the neurotransmitter acetylcholine, whereas BuchE is non-specific, hydrolysing many ester substrates including potent neurotoxins e.g., organophosphates and cocaine [4–6].

In this context it is interesting that the human epidermis holds the full capacity for a non-neuronal cholinergic signal transduction [7]. Only very recently it was recognised that this tissue also holds the capacity of a functioning BuchE in the cytosol and on cell membranes [8]. Enzyme activities are significantly higher compared to AchE, implicating an important role for BuchE in this compartment. Moreover, it was also recognised that both

* Corresponding author. Address: Clinical and Experimental Dermatology, Department of Biomedical Sciences, University of Bradford, Bradford BD7 1DP, West Yorkshire, UK. Fax: +44 1274 236489.
E-mail address: K.Schallreuter@bradford.ac.uk (K.U. Schallreuter).

0006-291X/$ - see front matter © 2007 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2007.02.078

BuchE and AchE are regulated by hydrogen peroxide (H_2O_2) in a concentration dependent manner. Both enzymes are inhibited/deactivated by H_2O_2 concentrations $>0.5\times10^{-3}$ M, whereas concentrations $<0.5\times10^{-3}$ M activate transcription and enzyme activities [8,9]. BuchE is more sensitive to this reactive oxygen species (ROS) than AchE. This was explained due to H_2O_2-mediated oxidation of methionine (Met) and tryptophan (Trp) residues in the active site as well as in the tetramerisation domain yielding a more severe structural alteration in the case of BuchE compared to AchE, where only the enzyme active site is affected [8,9]. Considering the strong expression of both enzymes in the outermost layer of human skin which in turn presents a first line target for many exogenous insults including UV-generated H_2O_2, it was tempting to invoke a major function for BuchE as an important protection/defence mechanism in this tissue. Preliminary in situ results on protein expression of membrane integrated and cytosolic BuchE suggested that this enzyme followed the well-established calcium gradient in the human epidermis [8,10]. This result is in agreement with previously reported data on membrane integrated BuchE in other tissues [1].

To study the direct effect of calcium on enzyme activities, we used pure human serum BuchE and rh AchE following the kinetics in the presence and absence of calcium in a concentration dependent manner. The results reveal that calcium increases BuchE activities 9-fold over the lanthanum inhibited calcium free enzyme, while AchE activities were not affected. In order to substantiate the nature of the calcium effect on BuchE, we utilised radiolabelled ^{45}calcium identifying stoichiometric binding of one calcium atom per enzyme subunit. Since H_2O_2-mediated oxidation severely altered the active site and the tetramerisation domain of the enzyme, we asked the question whether calcium binding would be also affected by this ROS [8]. These results show that calcium binding is severely altered after oxidation by H_2O_2. Evaluation of the primary sequence of BuchE suggested the presence of a single EF-hand binding domain per subunit which was confirmed by computer simulation. After H_2O_2-mediated oxidation of Met and Trp residues of the protein a significant shift in the backbone structure results in disruption of the calcium binding site. Calcium-activated BuchE revealed a 9-fold faster turnover of the organophosphate EPN.

In summary, our results show for the first time that BuchE is regulated by calcium via a single EF-hand binding domain per subunit while AchE is not influenced by this ion. Moreover, we show that this calcium binding is regulated by H_2O_2. Since the human epidermis with its size of 1.8 m^2 presents a major source for BuchE, we conclude that the function of this enzyme in this outermost tissue of the human body provides a major protection mechanism for AchE to prevent suicide inhibition of this enzyme by neurotoxins such as organophosphates.

Materials and methods

Materials. Human serum BuchE, human recombinant AchE, butyrylthiocholine iodide, acetylthiocholine iodide, *O*-ethyl-*O*-(4-nitrophenyl) phenylphosphonothioate (EPN), 5′,5′-dithiobis-2-nitrobenzoic acid (DTNB), lanthanum nitrate and all other chemicals were obtained from Sigma (Poole Dorset, UK). Radiolabelled ^{45}calcium chloride (12.2 mCi/mg) was obtained from ICN (Basingstoke, Hants, UK).

BuchE enzyme assay. For enzyme kinetics pure human serum BuchE was used. In order to ensure a calcium free enzyme, lanthanum (10^{-3} M) was utilised to exchange any bound calcium from the native enzyme prior to the kinetic analysis. One unit of enzyme hydrolysed 1.0 μmol of butyrylthiocholine iodide per minute at room temperature (pH 7.4). Enzyme activities were determined spectrophotometrically at 405 nm by following the reduction of DTNB according to Ellman et al. [11]. BuchE and AchE activities were measured in the presence and absence of $CaCl_2$ (0–2×10^{-3} M) under saturating substrate conditions (10^{-3} M). In order to test the effect of calcium on the hydrolysis of the organophosphate EPN (10^{-4} M), reaction rates for its turnover were determined in the presence and absence of 10^{-3} M $CaCl_2$.

AchE enzyme assay. AchE enzyme activities were measured at 405 nm using acetylthiocholine as substrate under saturating conditions (10^{-3} M) coupled to the reduction of DTNB [11].

Enzyme activities were determined in the presence of $CaCl_2$ (0–1×10^{-3} M).

Quantitation of calcium binding to BuchE using the isotope ^{45}calcium. To follow the specific binding of calcium to BuchE, 2.2 mg of pure enzyme in 0.5 ml distilled water was incubated with 2×10^{-3} M $^{45}CaCl_2$ for 30 min at room temperature. The labelled enzyme was applied to a G25 Sephadex column (1.5 × 6 cm) and eluted with distilled water in 0.5 ml fractions. BuchE (340 kDa) eluted in the void volume. In order to detect the enzyme, the protein content was determined in each fraction at 280 nm [12]. ^{45}calcium binding was followed in 100 μl aliquots from each fraction and counted in 3 ml scintillation fluid (Ready Safe, Beckman Coulter, Fullerton, CA, USA) at the ^{14}C channel of a Tricarb 2001TR scintillation counter (Packard Instruments, Meriden, CT, USA). To prove the specificity of ^{45}calcium binding, we utilised lanthanum (10^{-3} M) which has a stronger irreversible affinity for calcium binding sites. For this purpose the radiolabelled enzyme was preincubated with lanthanum for 15 min at room temperature and separated by Sephadex chromatography. The exchange of the isotope was followed by counting the ^{45}calcium in each fraction.

Hyperchem™ molecular modelling of the single EF-hand calcium binding site per subunit of BuchE. The molecular 3D structure of BuchE based on the X-ray crystallographic analysis was obtained from the Protein Data Bank [3]. This structure was compared with the primary sequence of the enzyme and it was realised that the crystal structure from the Data Bank missed two Asp residues in its sequence (Asp378 and Asp379). Since analysis of the sequence implicated these two acidic residues in the putative EF-hand site, both residues were added to generate the complete structure of the proposed EF-hand site prior to the Deep View analysis (Swiss Institute of Bioinformatics, Lausanne, Switzerland) by sealing the loop and minimising the structure in water using Hyperchem™ software (Hypercube Gainsville, FL, USA). In order to follow the effect of H_2O_2-mediated oxidation of Met and Trp residues on the protein structure in the vicinity of the EF-hand binding domain, the enzyme was oxidised and compared to the native enzyme structure.

Results

BuchE activities are increased 9-fold by calcium but AchE activities are not affected

In order to follow the effect of calcium on serum BuchE, we pre-incubated the enzyme with lanthanum (10^{-3} M) for 15 min to ensure that any residual calcium is displaced