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Research paper

Counter-regulation of cholinesterases: Differential activation of PKC and ERK signaling in retinal cells through BChE knockdown

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ABSTRACT

The ubiquitous cholinesterase (ChE) enzymes, functioning in the termination of acetylcholine mediated neural transmission, are also reported to have additional functions. Through application of siRNAs against butyrylcholinesterase (BChE) in R28 cells, a retinal cell line with pluripotent properties, a counter-regulation between ChEs was revealed. BChE knock down resulted in an up-regulation of not only acetylcholinesterase (AChE), but also altered the signaling status of PKC and ERK. Knockdown of BChE modified ERK signaling most notably through ERK1/2 proteins, together with the transcription activator P90RSK1 and c-fos. Stimulation of the R28 cell line by forskolin revealed that ChEs are involved in an intricate cross talk between different signaling pathways. Forskolin-stimulated R28 cells displayed a robust cholinergic response, as detected by both electrophysiology and ChE expression, and changed the activation status of PKC/ERK signaling pathways. The findings in R28 cells show that ChE expressions are inversely co-regulated and act through the transcription factors c-fos and P90RSK1. Since R28 cells have the capacity to differentiate into different cell types through stimulation of signaling pathways, ChEs are likely to be associated with cell fate determination, rather than just terminating cholinergic responses.

1. Introduction

Cholinesterases (ChE) are ubiquitous enzymes mostly associated with the termination of acetylcholine mediated neural transmission. Of the two enzymes belonging to this family, acetylcholinesterase (AChE; E.C. 3.1.1.7) has been termed “true” ChE whereas the other one is renamed butyrylcholinesterase (BChE; E.C.3.1.1.8), after being labeled as “pseudo” ChE for decades. Although similar in both structural and catalytic properties, the existence of a surplus enzyme with apparently the same function as AChE is intriguing. Apart from this clearly defined function, the presence of both ChEs in tissues with no apparent link to neural transmission like erythrocytes and plasma, have initiated the search for a novel function for both ChEs [1–3].

In fact, ChEs now can be considered pleiotropic or co-opting proteins. The AChE protein contains a calcium-binding motif, called an EF hand, and BChE can be activated through Ca^{2+} in a concentration-dependent manner [4]. Furthermore, AChE and BChE can regulate voltage-dependent Ca^{2+} currents and neurite growth [5,6]. The use of inhibitors and antisense technology had effects on neurite growth and AChE expression [7,8]. Knockout AChE mice studies have shown that BChE can partially compensate the lack of AChE [9]. Moreover, a family of cellular adhesion molecules including neurotactin and neuroligin has a high sequence homology to ChEs and, focused the search for a novel function of AChE in cell adhesion [10]. Not to the least, AChE has an ability to bind a variety of other proteins, mostly but not all that are involved with extracellular matrix like perlecan, heparin and laminin-1 [11–13]. Pathologically, in tissues like amyloid plaques ChEs are found to increase as in embryonic development. Moreover, there are numerous reports about abnormal BChE/AChE ratios during many proliferative processes like cancer [14–17]. All of these data correspond to a mutually interdependent co-regulation between ChEs and their involvement in differentiation, proliferation and pathologic processes. Long before synaptogenesis in the vertebrate embryo, BChE expression precedes AChE [1,18]. Often, BChE was

Abbreviations: AChE, acetylcholinesterase; AChR, acetylcholine receptor; ATCh, acetylthiocholine; BChE, butyrylcholinesterase; BTCh, butyrylthiocholine; ChAT, choline acetyltransferase; ChE, cholinesterase; ERK, extracellular signal-regulated kinases; PKC, protein kinase C.

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shown to be involved with proliferation, while AChE was found in differentiating and/or mature tissues. These processes were accompanied by an increase in AChE content and the activation of several cell growth and differentiation-related pathways [2,7,15]. The molecular mechanisms, however, of how ChEs could signal into target cells and interact with well-known cell-internal pathways need much more detailed investigations. It was the aim of this study to get some more insights into ChE-dependent cell-internal mechanisms.

Therefore, using siRNAs here we have knocked-down BChE, and the effects of BChE knockdown on the cell growth- and differentiation-related signaling pathways ERK and PKC was investigated in the retinal cell line R28. This cell line was developed by 12S E1A-induced immortalization of postnatal day 6 rat retina [19]. It is a highly proliferative cell line, shows no tumorigenicity and expresses abundant retinal and neuronal markers [20], like muscarinic acetylcholine receptors (AChR) and ChEs, making it a useful tool for developmental studies. Here we employ siRNAs to knockdown BChE according to the principles of Elbashir et al. [21] and show that BChE knockdown changes the signaling of ERK and PKC pathways displaying a further link in elucidating non-classical roles of ChEs. Through stimulation of the cell line by forskolin, an adenylate cyclase activator, the R28 cells displayed cholinergic properties and change the activation status of ERK/PKC-related signaling pathways.

2. Materials and methods

2.1. Generation of siRNAs targeting to BChE

Short interfering RNAs against BChE were designed according to the methods described by Elbashir et al. [21]. Twenty-one nucleotide long siRNAs were chemically synthesized by Dharmacon USA; designed with 3′ overhangs of UU. The siRNA pair corresponded to the coding regions 1127–1145 relative to the start codon. The sequences of the siRNA pair were as follows: 5′-GGG ACA GCU UUC CUA GUG UUU-3′ and 5′-ACA CUA GGA AAG CUG UCC CUU-3′. As control, a scrambled siRNA pair was designed with a single nucleotide difference in the siRNA target sequence. The sequence of the control siRNA pair was as follows: 5′-GGG AUA GCU UUC CUA GUG UUU-3′ and 5′-ACA CUA GGA AAG CUA UCC CUU-3′. To generate duplexes of siRNAs, 20 μM sense and antisense single strand RNAs were annealed by incubating the mixed siRNAs in 100 mM acetic acid-TEMED, pH 3.8, for 45 min at 60 °C, followed by desalting the complex with 10 M ammonium acetate at −20 °C for 16 h. The desalted and annealed siRNAs were dissolved in 6 mM HEPES–KOH, pH 7.5, 20 mM KCl, and 0.2 mM $MgCl_2$ at a final concentration of 20 μM.

2.2. Cell culture and transient transfections with siRNAs

The rat retinal cell line R28 (passages 47–49), a generous gift from Dr. G.M. Seigel, was grown in Dulbecco's modified Eagle's medium (Gibco) supplemented with 10% fetal calf serum (Gibco), 2 mM glutamine, 20 units/ml penicillin, 20 μg/ml streptomycin, 40 μg/ml gentamycin and were passaged after reaching 70% confluence. In expression studies, cell lysates were collected after definite time intervals and stored at −80 °C. Transient transfections with siRNAs were performed with the siPORT lipid transfection agent from Ambion, USA following the manufacturer's instructions. For the stimulation of R28 cells with forskolin, R28 cells incubated 12 h with reduced medium and forskolin was applied in final 10 μM concentrations and incubated up to 4 days. The cell lysates were then collected at definite time points and stored at −80 °C. In the experiments where stimulation was applied during transfection, the forskolin concentration was maintained.

2.3. RNA isolation and RT-PCR studies

Total RNA from cells was isolated with the Triagent (Sigma, USA) and used in RT-PCR studies. 1 μg RNA per sample was used to generate cDNA with the reverse reaction using AMV-RT from Promega. cDNAs coding for AChE, BChE, and α-PKC were amplified by PCR reaction. GAPDH was used as internal control. Primers used to amplify were for AChE, 5′-GGA TGC TAC CAC CTT CCA AA-3′, 5′-ACG AAG GAA AAC CGG AAG AT-3′; BChE, 5′-CCA GAG GAA GCC AGA AAC AG -3′, 5′-AGC CAT GCA TTA CTC CCA TC-3′; α-PKC, 5′-CCC ATT CCA GAA GGA GAT GA-3′, 5′-CAT GTG TTC CTT GCA CAT CA-3′; GAPDH, 5′> GGT GAT GCT GGT GCT GAG TA <3′, 5′> GGA TGC AGG GAT GAT GTT CT <3′. The PCR reactions were carried out for 30 (PKC)-35 (ChEs) cycles (denaturation for 1 min at 94 °C, followed by primer annealing at 55 °C for 1 min and extension at 72 °C for 1 min). For PKC-α, primer annealing was carried out for 1 min at 60 °C and extension for 1 min at 72 °C, respectively. In each experiment, the last cycle was followed by a 10-min elongation step at 72 °C.

2.4. Immunoblotting and phosphorylation assays

R28 cells cultured in 50 ml culture dishes were washed with 1 mM Na_3VO_4/phosphate-buffered saline and homogenized in ice-cold lysis buffer containing 50 mM Tris pH 7.5, 0.5% Triton X-100, 5 mM EDTA, 250 mM sucrose, and protease inhibitor cocktail (0.52 mM AEBSF, 0.4 μM Aprotinin, 0.01 mM Leupeptin, 0.02 mM Bestatin, 7.5 μM Pepstatin A, 7 μM E-64, and 1 mM Na_3VO_4) followed by centrifugation at 12,000×*g* for 5 min at 4 °C. The protein concentration was measured by the Bradford method. Protein samples were denatured at 100 °C for 5 min in loading buffer containing 1% SDS, 1% dithiothreitol and, separated by %7.5 SDS-PAGE. Ten μg of supernatant was used in SDS-PAGE. Proteins from gels were electroblotted (Bio-Rad Mini-Protean II transblot system) onto nitrocellulose paper for 2 h. The blots were blocked with either 3% skimmed milk or 5% BSA in Tris-buffered saline-containing 0.1% Tween-20. The detection was performed according to the ECL protocol provided by the supplier (Amersham Biosciences). The anti-AChE and anti-α-PKC antibodies (BD Transduction Laboratories, San Diego) were used at 1:1000 dilutions. The anti-BChE antibody (1:5000) was a generous gift from K. Tsim. The anti-phospho-p44/42 MAP Kinase, anti-phospho-p90RSK1, and anti-phospho JNK1/2 antibodies (1:1000) were from Cell Signaling Technology, USA. The peroxidase-conjugated secondary antibodies for the phospho-antibodies (1:2000) were from Cell Signaling Technology, USA. The other peroxidase-conjugated secondary antibodies (1:40,000) were from Sigma.

2.5. Enzyme assays

Cholinesterase activities of R28 cells were determined from whole cell extracts with the Ellman assay [22]. Total ChE activities were measured using ATCh as substrate at a final concentration of 1 mM ATCh, plus 50 mM MOPS pH 7.4, 0.25 mM DTNB at 412 nm and 25 °C and AChE and BChE activities in the presence of 10^{-5} M ethopropazine and 10 μM BW284C51, respectively. All assays were carried out in triplicates. Possible interference of the lysis buffer was eliminated using different combinations of blank measurements. One unit of ChE is the amount of enzyme that catalyzes the formation of 1 μmol product per min under the conditions mentioned above.