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PKC stimulation increases expression of cholinesterases in R28 cell line

[PKC uyarımı R28 hücrelerinde artan kolinesteraz ifadesine neden olur]

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ABSTRACT

Objective: Previously we have shown a counter-regulation between cholinesterases (ChEs) through both anti-sense Butyrylcholinesterase (BChE) transfection and knock-down by siRNAs in R28 cells. In course of this counter-regulation, the status of the cell growth- and differentiation-related signaling pathways PKC and ERK were also changed. Down-regulation of BChE led to an increased PKC-α expression. Here, we demonstrate that this interaction between ChEs and PKC is reciprocal.

Methods: R28 cells were treated with final 10 μM Di-octanoyl glycerol (DOG) and 10 nM siRNAs against BChE. Expression analysis was done by RT-PCR, Western Blot, IHC and activity assays.

Results: DOG treatment along with BChE knockdown resulted in increased PKC-α expression, as compared with DOG treatment alone. Change in ERK1 expression was not as profound. In R28 cells, DOG stimulation led to marked rapid increase in both AChE and BChE expression.

Conclusion: PKC stimulation during BChE knockdown caused functional, saturated AChE expression. Increased PKC-α expression suggests that PKC-α is not only regulated by its stimulators but also by the absence of BChE. While ChEs are counter-regulated, activation of PKC has both reciprocal and additive outcome through cholinergic pathway. Thus, effects of DOG stimulation expose yet another interaction between calcium dependent PKC-α and ChEs showing that any measure of disclosure on neurodegenerative diseases must not only consider cholinergic control but also monitor the PKC pathway.

Key Words: Acetylcholinesterase, Butyrylcholinesterase, siRNA, Signaling, R28, PKC, ERK, di-octanoyl glycerol

Conflict of Interest: The authors have nothing to declare.

ÖZET

Amaç: Kolinesteraz (ChE) enzimleri anti-sens ve siRNA uygulamaları ile aralarında bir karşıt düzenlenme gösterir. Önceki çalışmamızda bu karşıt düzenlenim sırasında hücre çoğalması ve farklılaşması ile ilişkili PKC ve ERK yolaklarının aktivasyon durumunu değiştiğini gözlemlemiştik. BChE ifadesinde gerçekleşen azalma PKC-α yolağında artışa yol açar. Bu çalışmada ChE enzimleri ile PKC yolağı arasında var olan bu etkileşimin karşılıklı olduğunu gösteriyoruz.

Gereç ve Yöntemler: Nihai 10 μM Di-octanoyl glycerol (DOG) ve BCHE'a yönelik 10 nM siRNA uygulanan R28 hücrelerinde gen/protein ifadesindeki değişiklikler RT-PCR, Western Blot, IHC ve aktivite ölçümleri ile incelenmiştir

Bulgular: BChE nakavtı ile beraber DOG uygulaması, PKC-α ifadesinde tek başına DOG uygulamasından daha fazla artışa yol açtı. ERK1 ifadesindeki artış aynı derecede çarpıcı değildi. R28 hücrelerinin DOG ile uyarılmaları ise hem AChE hem de BChE ifadelerinde belirgin ve hızlı artışa neden oldu.

Sonuçlar: BChE nakavtı sırasında PKC uyarımı işlevsel ve doygun AChE ifadesine neden olmuştur. Gözlenen PKC ifadesi, PKC-α'nın, uyarıcıları dışında BChE varlığı / yokluğu ile de düzenlendiğini düşündürür. PKC ve ERK uyarımı kolinerjik yolak üzerinde karşılıklı ve tamamlayıcı sonuca yol açar. PKC-α ve ChE'lar arasındaki bu etkileşim, nörodejeneratif hastalıkların değerlendiriminde ChE'lar ile beraber PKC yolağını da dikkate alınması gerektiğini gösterir.

Anahtar Kelimeler: Asetilkolinesteraz, Butirilkolinesteraz, siRNA, Sinyal iletimi, R28, PKC, ERK, di-octanoyl glycerol

Çıkar Çatışması: Yazarların makale konusu dahilinde herhangi bir çıkar çatışması bulunmamaktadır.

Introduction

ChEs are classified into two subtypes through their genetic location, substrate-inhibitor specificity and response to carbamates, organophosphates, and xenobiotics [1-7]. The fact that ChEs follow a sequential expression has long led their association with processes of proliferation and differentiation. Preceding synapse formation, BChE expression leads AChE expression [1]. Hence, BChE is involved with proliferation, while AChE is found in differentiating tissues. These processes are accompanied by an increase in AChE content and activation of several cell growth and differentiation-related pathways [1, 7-8]. Furthermore, there seems to be a counter-regulation between ChEs which is reflected in proliferation and differentiation related signaling [7, 8].

As major proliferation and differentiation related signaling pathway the PKC/ ERK status is profoundly important in determining cell fate. Classical PKC pathway is activated by calcium, the universal differentiation signal [9]. Whereas association of ChEs with proliferation and differentiation is established, the association with Ca^{2+} and PKC pathway is relatively novel. AChE contains a Ca^{2+} binding motif, called an EF hand, and BChE can be activated through Ca^{2+} in a concentration-dependent manner [10]. ChEs also regulate voltage-dependent Ca^{2+} currents and neurite growth [11, 12]. In addition, Ca^{2+} reactivates inhibited BChE, signifying an important link between Ca^{2+} and functionally active ChE proteins [13].

Previously down-regulation of BChE leading to increased AChE and PKC-α expression along with the change in activation status of ERK pathway was displayed [8, 14]. The effectors of change were found as transcription factor and activator associated with ERK/ PKC pathway, c-fos and P90RSK1 [8]. Also in embryonic stem cells, stimulation with nicotine and acetylcholine, lead to transient increase in intracellular Ca^{2+} levels [15].

In both proliferative neural tissues under physiological and pathological conditions, e.g. embryonic development and Alzheimer's disease, AChE is over-expressed. In such conditions, alternatively spliced AChE variants are associated with alterations in PKC signaling and tau phosphorylation [16]. In this study, we have tried to clarify this interaction through the application of both siRNAs against BChE and stimulation of the classical Ca^{2+}-activated PKC-α pathway by diacylglycerol analog, dioctanoyl glycerol (DOG).

Materials and Methods

Cell culture and transient transfections with siRNAs

The rat retinal cell line R28 (passages 47-50), 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. Transient transfections with siRNAs against BChE were performed as described elsewhere [7, 8]. In DOG stimulation, cells incubated 12 hr with reduced medium and DOG was applied in final 10 µM concentrations up to 4 days. In experiments where stimulation was applied during transfection, DOG concentration was maintained.

RNA isolation and RT-PCR studies

Total RNA from cells was isolated with the Triagent (Sigma, USA). 1 µg RNA per sample was used to generate cDNA with reverse reaction using AMV-RT from Promega. cDNAs coding for AChE, BChE, PKC-α and ERK1 were amplified by PCR. GAPDH was used as internal control. Primers used 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'; PKC-α, 5'-CCC ATT CCA GAA GGA GAT GA-3', 5'-CAT GTG TTC CTT GCA CAT CA-3'; ERK1, 5'> AAT AAG GTG CCG TGG AAC AG <3', 5'> ACG GCT CAA AGG AGT CAA GA <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-α, ERK1) - 35 (ChEs) cycles (1 min denaturation at 94°C, followed by 1 min primer annealing at 55°C 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.

Protein quantitation and Immunoblotting

Cultured R28 cells were processed in ice-cold lysis buffer containing 50 mM Tris pH 7.5, 0.5 % Triton X-100, 5 mM EDTA, 250 mM sucrose, 1 mM Na_3VO_4/phosphate-buffered saline and protease inhibitor cocktail followed by centrifugation at 12,000 x g for 5 min at 4 °C. Protein concentration was measured by BCA method, with bovine serum albumin as standard. Samples were denatured at 100 °C for 5 min in loading buffer containing 1% SDS, 1% DTT and, separated by %7.5 SDS-PAGE. Ten µg of protein loaded per lane were blotted onto nitrocellulose for 2 hrs and blocked with % 5 BSA/TBST. Detection was performed with ECL. Anti-AChE antibody (BD Transduction Laboratories, San Diego) was used at 1:1000 dilution. Anti-BChE antibody (1:5000) was a generous gift from K. Tsim.

Enzyme Assays

Cholinesterase activities of R28 cells were determined from whole cell extracts with Ellman assay [17]. Total ChE activities were measured using ATCh as substrate at 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 triplicate. One unit of ChE is the amount of enzyme that catalyzes the formation of 1 µmol product per min under the conditions mentioned above.

2.5. Immunohistochemistry

R28 cells transiently transfected with siRNAs (as above), were grown on coverslips in 12-well dishes. The coverslips were rinsed 3 times for 5 min with phosphate-buffered saline (PBS), fixed with 4% formaldehyde in PBS for 30 min, and washed as above. The cells were incubated for 30 min in 500 µl of blocking solution (PBS containing 5% bovine serum albumin, and 0.1% Triton X-100) followed by overnight incubation at 4 °C with anti-PKC-α (1:100) in blocking solution. Control coverslips were incubated with blocking solution alone. After three washes for 5 min each in PBS, the cells were incubated with 500 µl of secondary antibody conjugated to cyanine 3 (1:50, Dianova) in blocking solution for 1 h at 22 °C. After three washes for 5 min each in PBS, coverslips were removed from the wells, dipped in distilled water, and dried before being mounted onto slides. The fluorescence was visualized with a ZEISS Axiophot microscope and documented with an INTAS image analysis system (INTAS Göttingen, Germany).

Results and Discussion

The PKC family of isozymes is sub-typed as either calcium dependent (PKC-α, β) or independent (PKC-ε). DOG is a diacyl glycerol analog of the classical stimulator of calcium-dependent PKC-α signaling. Previously, the interaction between ChE and PKC-α is shown [7, 8-14]. In this study, we stimulated embryonic cell line R28 with DOG under normal cell conditions as well as knocking BChE down.

Stimulation of R28 cells with DOG led to an increased PKC-α expression compared to control cells (Fig 1). The surprising finding is that application of DOG along with BChE knockdown leads to more pronounced increase in PKC-α expression as compared with controls (Fig 1). BChE knockdown leads to an increase in PKC-α expression (Fig 2), [7, 8, 14]. Here, we show that DOG stimulation along with BChE knockdown leads to an over-stimulation of PKC-α, adding an effect. This finding suggests that in R28 cells, while PKC stimulation is important, the ratio of AChE / BChE is as significant for the calcium-dependent PKC-α expression. Examination of ERK1 protein, representative for the ERK pathway,

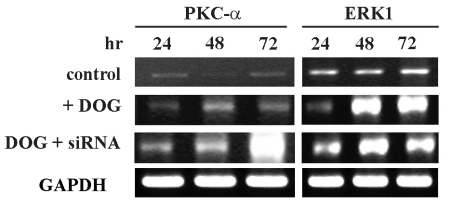

Figure 1. DOG stimulation along with BChE knockdown changes PKC and ERK signaling in R28 cells.
RNAs from cells transiently transfected with BChE siRNAs and stimulated by DOG were isolated and subjected to RT-PCR as described in Materials and Methods. Note the increase in ERK1 and especially PKC expression during BChE knockdown.

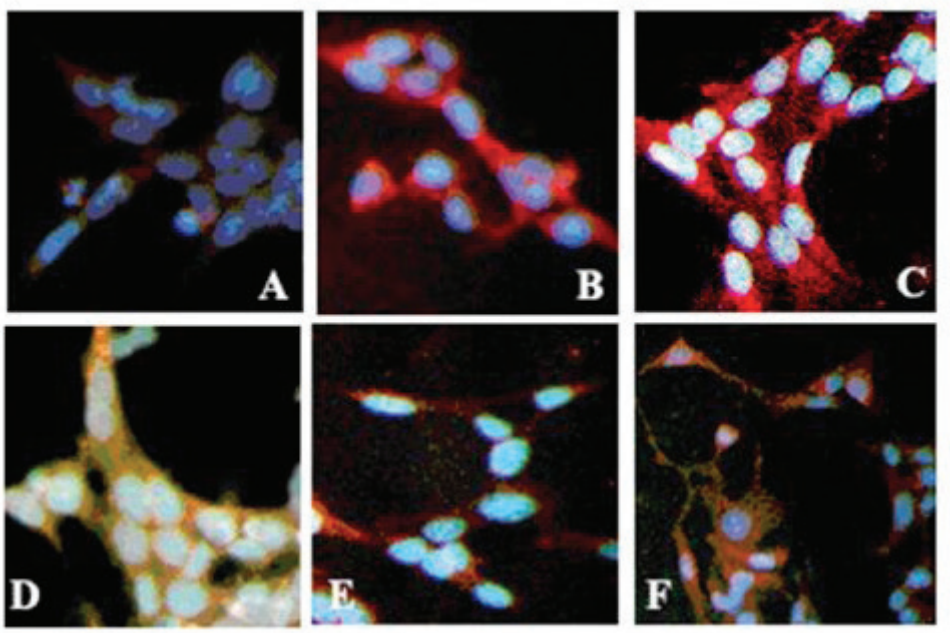

Figure 2. PKC-α expression during BChE knockdown
R28 cells were plated on coverslips and transiently transfected with BChE siRNAs. 24-72 hrs after transfection cells were washed with PBS and fixed in 4 % formaldehyde. PKC-α during knockdown **(A-C,** after 24, 48 and 72 hrs PT, respectively**)** and in control cells (**D-F**, accordingly) was visualized by anti-PKC-α antibody with a fluorescent second antibody, as described in Materials and Methods. The cell nuclei are labeled by DAPI staining. The view was under a 50 X objective on ZEISS Axiophot microscope. Bar, 50 µM.

did not display such drastic results (Fig 1). DOG stimulation of R28 cells resulted in increased ERK1. The only observed difference during BChE knockdown along with DOG application was on the first day of knockdown, which signifies early involvement of ERK pathway. Since active ERK proteins are translocated to the nucleus binding a number of transcription factors and regulators like c-fos and P90RSK1 this seems a plausible explanation for the observed expression.

Stimulation of PKC-α pathway by DOG resulted in R28 cells in a marked immediate increase of ChE expression, observed by mRNA (Fig 3A), protein (Fig 3B) and functional enzyme levels (Fig 3C). Most striking were increased enzyme activity (Fig 3C); displaying that at this modified signaling state of alert reactivity, ChEs not only retain but display a hyperactive function. The increase in ChE expression and function was much more profound than at the normal status of ChEs in R28 cells. Stimulation with DOG alongside that of BChE knockdown also caused an increase AChE expression but this was not as strong as the increase observed in control cells (Fig 3). During knockdown, it seems that AChE expression reached quickly a saturation level and stayed at this state. BChE expression and activity on the other hand, was highest on the first day of knockdown, which afterwards was quickly abolished.

ChEs are known to interact with Ca^{2+}. AChE contains a Ca^{2+} binding motif, called an EF hand, and BChE can be activated through Ca^{2+} in a concentration-dependent manner [10]. ChEs also regulate voltage-dependent Ca^{2+} currents and neurite growth [11, 12]. Recently, it was shown that ACh and nicotine could induce the mobilization of the intracellular Ca^{2+}, whereas muscarine could not [15]. Our finding that stimulation of Ca^{2+}-dependent PKC pathway leads to increased ChE expression sheds a new light on these reports. It seems that not only ChEs are affected by Ca^{2+} but that they can also be influenced by PKC pathway activation, and in return, influence this signaling pathway by their presence. The R28 cell line expresses the M2 receptor and, although in a previous

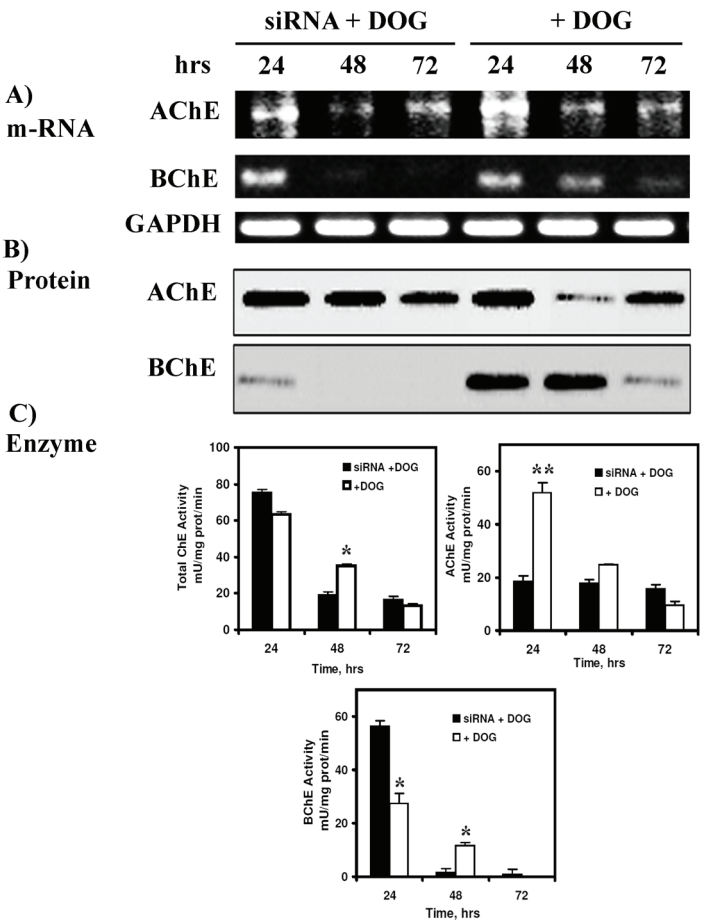

Figure 3. DOG stimulation of R28 cells causes cholinergic activation.
(A) Effects of DOG stimulation on BChE knockdown were analyzed by RT-PCR and Western Blot. (B) Ten µg of cell extract were loaded per lane. The hours on the lanes correspond to post transfection hours. (C) Total ChE, BChE and AChE activity from whole cell extracts were prepared from transiently siRNA transfected cells, stimulated by DOG application determined by the Ellman method, using BW284c51 as specific inhibitor for AChE and ethopropazine for BChE. Protein quantification was performed by Bradford assay. Each point represents data from three different transfections, and all assays were done in triplicates (* p=0.01, **p=0.001)

study no mobilization of Ca^{2+} could be observed with muscarine, here we observe direct activation of ChEs through Ca^{2+}-dependent stimulation; displaying that such a phenomenon may not only depend on the respective cholinergic receptors. Ca^{2+} acts as universal differentiation signal [9]. Such a signal would play an important role in tissues that have the ability to differentiate into new cell types as the R28 cells in this study.

In conclusion, calcium signal signifies a change in cell status as observed and, by increasing the more stable and active form of AChE, enhances the capacity of R28 cells to become more cholinergic. The counter-regulation between ChEs also seems to aid this transformation. By rapid activation of cholinolytic activity of both AChE and BChE, cells quickly to pass from a proliferative state to differentiating state. Having once become cholinergic, no new response to a differentiating signal is needed. The AChE expression and function remains stable. Such a phenomenon would play an important role in differentiating tissues most notable of which is regulating the available ACh concentration, a morphogenetic substance essential for neural differentiation. Hence, we conclude that responding to changes in active signaling pathways; cell responses to ChEs define cellular fate.

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Conflict of interest statement: Nothing to declare.

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