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PKC delta activation increases neonatal rat retinal cells survival *in vitro*: Involvement of neurotrophins and M1 muscarinic receptors

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ABSTRACT

Protein kinase C (PKC) is a family of serine/threonine kinases related to several phenomena as cell proliferation, differentiation and survival. Our previous data demonstrated that treatment of axotomized neonatal rat retinal cell cultures for 48 h with phorbol 12-myristate 13-acetate (PMA), a PKC activator, increases retinal ganglion cells (RGCs) survival. Moreover, this treatment decreases M1 receptors (M1R) and modulates BDNF levels. The aim of this work was to assess the possible involvement of neurotrophins BDNF and NGF in the modulation of M1R levels induced by PKC activation, and its involvement on RGCs survival. Our results show that PMA (50 ng/mL) treatment, via PKC delta activation, modulates NGF, BDNF and M1R levels. BDNF and NGF mediate the decrease of M1R levels induced by PMA treatment. M1R activation is essential to PMA neuroprotective effect on RGCs as telenzepine (M1R selective antagonist) abolished it. Based on our results we suggest that PKC delta activation modulates neurotrophins levels by a signaling pathway that involves M1R activation and ultimately leading to an increase in RGCs survival *in vitro*.

1. Introduction

Phorbol 12-Myristate 13-Acetate (PMA) is a phorbol ester originally isolated from seed oil of *Croton tiglium* and other plants of Euphorbiaceae family [1] that activates protein kinase C (PKC) isozymes, mimicking physiological activation of diacylglicerol [2].

PKC is a family of phospholipid-dependent serine/threonine kinases divided in three subfamilies: conventional (α, βI, βII, γ), novel (δ, ε, η, ζ) and atypical (θ, λ) [3]. Many hormone and neurotrophin signal transduction pathways involves PKC activation [4], being related to several phenomena as cell proliferation, differentiation and survival in different tissues including the nervous system [5]. Previous work of our group demonstrated that PKC activation by 50 ng/mL PMA treatment increases rat retinal ganglion cells (RGCs) survival *in vitro* after 48 h [6]. Damage of RGCs occurs in retinal neurodegenerative diseases, such as glaucoma and diabetic retinopathy, and the understanding of signaling pathways involved in neuronal survival is thus of clinical importance.

Natural development of nervous system depends on the action of different mediators as cytokines and neurotransmitters [7]. According to developmental stage of tissue maturation, a change in the expression of these mediators can be observed, as well as that of their specific receptors subtypes [8].

Cytokines are low molecular weight polypeptides or glycoproteins grouped into families: neurotrophins, interleukins, tumor necrosis factors, chemokines, interferons and growth factors [9,10]. Neurotrophins regulate different aspects of neuronal development, such as differentiation and survival, as well as axonal regeneration in peripheral nervous system [11,12]. Components of the neurotrophin family are Nerve Growth Factor (NGF), Brain-derived Neurotrophic Factor (BDNF) [13], NT-3 [14], NT-4/5 [15], NT-6 [16] and NT-7 [17]. BDNF regulates neuronal survival and differentiation during central nervous system development, and has a well known role in cognitive phenomena (memory consolidation and learning process) in adult life [18]. NGF regulates several important

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functions, including neurotransmitter and neuropeptides release [19].

Another molecule with an important role during neuronal development is acetylcholine, exerting its effects via nicotinic and muscarinic receptors [20]. Muscarinic receptors are divided in five subtypes (M1–M5). Members of the "M1-like" family (M1, M3, and M5 receptors) are associated with Gq subtype of G-protein and phosphatidylinositol turnover. Members of the "M2-like" family (M2 and M4 receptors) are generally linked to inhibition of adenylyl cyclase activity [21].

Our previous work showed that PKC activation by PMA modulates muscarinic receptors levels, leading to a decrease in M1 receptors (M1R) levels [22], and also modulates BDNF levels in culture [23]. Our group also demonstrated that M1R activation is involved in the increase in RGCs survival [24]. Since cytokines, including NGF and BDNF, are involved in cholinergic system development [25], our aim was to investigate the possible involvement of these neurotrophins in the modulation of M1R levels induced by PMA treatment in rat retinal cell cultures. An also, if NGF, BDNF and M1R are involved in the increase in RGCs survival mediated by PKC activation.

2. Materials and methods

2.1. Chemicals

PMA and telenzepine were obtained from Sigma (St. Louis, USA). Trypsin was purchased from Worthington (Freehold, USA). BDNF, NGF, rabbit anti-BDNF and rabbit anti-NGF antibody were obtained from PeproTech (New Jersey, USA). Secondary rabbit anti-goat HRP-linked, goat anti-rabbit HRP-linked, anti-actin (C-11), anti-M1 antibody, anti-pPKCδ and carbamylcholine were obtained from Santa Cruz Biotechnology (Santa Cruz, USA). K252a and rottlerin were obtained from Calbiochem (San Diego, USA). Luminata and PDVF membrane were purchased from Millipore (Darmstadt, GER).

2.2. Retinal cultures

Primary retinal cell cultures from Lister Hooded rats at postnatal day 1 (P1) were prepared using techniques previously described [6].

Cells were placed on Petri dishes previously treated with 25 μg/mL poly-L-ornithine at a plating density of 10^5 cells/cm^2. For western blot technique, cultures were maintained with medium (Control cultures) or medium containing the specific treatment, in a final volume of 2 mL, in a humidified atmosphere of 5% CO_2/95% air at 37 °C for different periods of time. For RGCs survival evaluation, cultures were incubated in 1 mL of culture medium for 2 h to allow cells to attach to substrate. Then, 1 mL of culture medium (control cultures) or 1 mL of medium containing the drugs to be tested was added to each Petri dish, and cultures were maintained in a humidified atmosphere of 5% CO_2/95% air at 37 °C for 48 h. All procedures using animals were performed according to the Society for Neuroscience guidelines and were approved by the local committee for animal care and experimentation (CEPA-UFFprojects #642-15). All efforts were made to minimize the number of experimental animals and their suffering.

2.3. Western blot

Retinal tissue from P0 to P30 rats was dissected in CMF solution. Cells from cultures or retinal tissue were lysed by sonication in lysis buffer (SDS-2% and 0.5 M Tris-pH6.8). Protein concentration determination was made by Bradford method [26].

M1R, NGF, BDNF and actin levels were determined and for each antibody used, the control of antibody specificity was performed, using a full membrane to compare with the information present in data sheet.

Samples (60μg/lane) were subjected to SDS-polyacrylamide gel electrophoresis (9 or 15%) and transferred to polyvinulidenedifluoride (PVDF) membranes. Membranes were pre-incubated with BSA-3% for 2 h, followed by overnight incubation with anti-NGF (0.2 μg/mL), anti-pPKCδ (1:1000), anti-M1 (1:750), anti-BDNF (0.2 μg/mL) or anti-actin (C-11) (1:500) antibody. They were washed in TBS and exposed to horseradish peroxidase (HRP)-conjugated secondary anti-rabbit (1:15000) or anti-goat IgG antibody (1:15000) at room temperature for 60min. Detection was performed at L-PIXChemi Molecular Imaging (Loccus Biotechnology) using chemiluminescence system. Protein bands were analyzed by densitometry with Image J. Mean value for the control was set at 100%.

2.4. Retrograde labeling of RGCs

Rats at P0 were anaesthetized by hypothermia. As in newborn rats the cerebral cortex still not covered mesencephalic region, 1 μL of HRP-30% in DMSO-2% was injected directly into each superior colliculus. Animals were returned to their mothers and survived for ~16 h before the procedures used for cell culture.

2.5. Identification and quantification of RGCs in culture

RGCs were identified by the presence of HRP in its cytoplasm revealed according to the protocol of Mesulam [27]. Cultures were fixed (paraformaldehyde-1% and glutaraldehydein-2% 0.1 M in sodium phosphate buffer) for 5min, washed in phosphate buffer and reacted with tetramethylbenzidine and H_2O_2. RGCs were quantified by counting using an Olympus BX41 microscope at a magnification of 400×, under bright field. As an internal control for the variable percentage of RGCs labeled with HRP in distinct experiments, the number of labeled cells at 4 h in culture was taken as 100% and results were reported as percentage of this control. Approximately 800 RGCs were labeled in 4 h control coverslips. Independently from the number of labeled cells, the 48 h survival was always in the same range (40–60%).

2.6. Statistical analysis

All data were expressed as mean ± S.E.M. from at least three independent experiments, each performed in duplicate. The overall statistical analysis was first obtained by one-way analysis of variance (ANOVA). Statistical significance of all pairs of multiple groups of data was assessed by Newman-Keuls comparison test. Student's t-test was used to analyze two experimental groups. We considered statistically significant $P < 0.05$.

3. Results

3.1. NGF levels in post-natal retinal development and in retinal cell cultures treated with PMA

NGF levels were analyzed during post-natal development of rat retinas. Retinal tissues were evaluated from the first until the thirty post-natal days (P0-P30). Results demonstrate a gradual increase in NGF levels up to P14 (122.3% increase compared to P0). In P30, NGF levels decrease returning to P0 levels (Fig.1A). Subsequently, it was investigated if PMA treatment could modulate NGF levels in retinal cultures. NGF levels were measured in cultures treated with 50 ng/mL PMA in different periods of time. Figure 1B shows a 112% increase in NGF levels in PMA treated cultures for 45min. However,

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