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Research Report

Retinal ganglion cell death and neuroprotection: Involvement of the CaMKIIα gene

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Abstract

The purpose of this study is to determine if calcium/calmodulin-dependent protein kinase-II (CaMKII) plays a role in neuronal cell death and if inhibition of this kinase affords some neuroprotection in the RGC-5 retinal ganglion cell line. The RGC-5 cells were treated with glutamate at various concentrations for increasing increments of time. Cytotoxicity was assayed by measuring the lactate dehydrogenase (LDH) leakage from non-viable cells and TUNEL assays. The involvement of caspase-3, Bcl-2 and caspase-8 in glutamate-induced cytotoxicity was determined by immunoblots and/or real time RT-PCR. In addition, the autocamtide-2-related inhibitory peptide (AIP), a specific inhibitor of CaMKII, was used to determine the involvement of CaMKII in glutamate-induced RGC-5 cell death. Application of increasing concentrations of glutamate to RGC-5 cells caused a dose-dependent increase in the level of cell death after 24 h. There was a glutamate-stimulated increase in the expression of caspase-8 and caspase-3 and a corresponding decrease in Bcl-2. The active fragment of caspase-3 increased in glutamate-treated cells. An early transient increase in the expression of CaMKIIα_B gene and a corresponding CaMKIIα nuclear translocation was found in glutamate-treated cells. Treatment with AIP blocked the activation of caspase-3 and protected RGC from glutamate-mediated cell death but did not alter the glutamate-enhanced expression levels of caspase-8 or caspase-3. This report shows the likely involvement of a transcript of the CaMKIIα gene in the cytotoxicity response of RGC-5 cells similar to previous reports in the neural retina. AIP is shown to be a neuroprotectant for RGC-5 cells as was reported for the neural retina.

Theme: Development and regeneration
Topic: Neuronal death

Keywords: RGC-5; Excitotoxicity; Apoptosis; CaMKIIα; AIP; Neuroprotection

1. Introduction

The death of retinal ganglion cells (RGCs) is a hallmark of many retinopathies, such as glaucoma, retinal ischemia and anterior ischemic optic neuropathy [44,31]. There are several animal models of such retinal diseases that have their focus on, reproducing as best as possible, the correct etiological aspects of the disease [9,28,30] or reproducing certain aspects of ganglion cell death in vivo [20,48,54]. These animal models are difficult at best when it comes to handling large scale studies with multiple variables that are needed to examine the cell death pathways and to determine neurotherapeutic strategies.

Recently, a retinal ganglion cell line (RGC-5) has become available, and its phenotype has some of the hallmarks of retinal ganglion cells [16], although clearly, these cells do not mirror all of the characteristics of primary cells in culture or of the in situ retinal ganglion cells [29]. Nevertheless, they represent an opportunity to explore the cell death pathways in culture. This report demonstrates that

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some aspects of the excitotoxic cell death cascade known to be evident in the retina are indeed present in the RGC-5 cell line.

It is well documented that exposure to excitotoxins such as glutamate or its analogues can kill ganglion cells [2,22,26,32,35,39]. Excessive levels of glutamate have been implicated in the pathophysiology of RGC death in glaucoma [44], and its use in studies of receptor-mediated cell death is justified not only in the retina but also in other neuronal sites [7,49]. Glutamate in excess can cause the death of RGCs by hyperstimulating the glutamate receptors, especially the *N*-methyl-D-aspartate (NMDA) receptor, causing a cascade of events that involves an increase in intracellular calcium [21] and the activation of downstream enzymes and cellular processes [44]. One of these enzymes, calcium/calmodulin-dependent protein kinase-II (CaMKII), is a multifunctional Ser/Thr protein kinase, which plays important roles in controlling a variety of cellular functions in response to an increase in intracellular calcium [5]. It is known that CaMKII is located in the cells of the inner nuclear and ganglion cell layers [46], and recently, CaMKII has been shown to be involved in signaling pathways leading to apoptosis [8,17,52] in these cell layers. Neuroprotection in the CaMKII containing cell layers is afforded by treatment with the autocamtide-2-related inhibitory peptide (AIP), a specific inhibitor for CaMKII [17,19]. The CaMKIIα_B transcript produced by alternative splicing of the CaMKIIα gene [6] is upregulated in retinal neurons exposed to NMDA [18] and may be an important early indicator of neuronal cell death. This transcript has an 11 amino acid insert in the regulatory domain, which functions as a nuclear localization signal (NLS) [18].

The present study provides evidence that CaMKIIα_B is involved in glutamate-mediated cell death in the RGC-5 cell line, and AIP, the specific inhibitor for CaMKII, is a neuroprotectant for glutamate-mediated cell death. AIP inhibits the activation of caspase-3, a principal executioner of apoptosis.

2. Materials and methods

2.1. Cell culture

A rat RGC cell line, RGC-5, transformed with E1A virus was used in this study. These cells have been shown to express RGC-specific markers thy-1, Brn-3C and NMDA receptors [16]. They do not express GFAP (marker for Muller cells), HPC-1 (marker for amacrine cells) or 8A1 (marker for horizontal cells), suggesting that they represent the RGC phenotype [4,16]. In addition, these cells are known to be sensitive to glutamate excitotoxicity [16]. Cultures of the cells were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum, 100 U/ml penicillin and 100 μg/ml streptomycin in a humidified atmosphere of 95% air and 5% CO_2 at 37 °C. The cells were subcultured at 1:20 split by trypsinization when they reached confluence.

2.2. Assays of glutamate-induced cytotoxicity and the effects of drugs

RGC-5 cells (passages 3–8) were seeded at a density of 5000 cells/well in black-wall 96-well plates. After an overnight incubation, the cells were treated with various concentrations of L-glutamate prepared in DMEM (serum-free) and maintained for 24 h. Glutamate-induced cytotoxicity was assayed by measuring the lactate dehydrogenase (LDH) leaked from the cells into the culture media with Cytotox-One Homogeneous Membrane Integrity Assay kit (Promega, Madison, WI) following the manufacturer's protocol. To test for neuroprotection by AIP (Calbiochem, La Jolla, CA), 10 μM myristoylated AIP (m-AIP) or 10 μM AIP-II (an AIP analog with greater potency) was added to the culture medium 1 h prior to the 24 h exposure to various concentrations of glutamate. The data were presented as the percent cytotoxicity when compared with respective controls. AIP and m-AIP did not affect the basal levels of LDH (data not shown).

2.3. TUNEL assay

RGC-5 cells were plated on poly-L-lysine-coated four-chamber slides (Nalge Nunc International, Naperville, IL) and treated with or without 1000 μM glutamate for 24 h. The TUNEL assay was performed using an *In Situ Cell Death Detection Kit, Fluorescein* (Roche Applied Science) following the manufacturer's guide. Briefly, the cells were fixed in 4% paraformaldehyde in PBS, pH 7.4 and permeabilized in 0.1% Triton X-100 in 0.1% sodium citrate. After washing in PBS, the cells were incubated with 50 μl/well TUNEL reaction buffer in a humidified atmosphere for 1 h at 37 °C. The cells incubated with label solution (without terminal transferase) or pre-incubated with DNase I and then TUNEL reaction buffer served as negative or positive controls, respectively. The slides were washed three times with PBS, mounted with mounting medium with DAPI (Vector Laboratories, Burlingame, CA) and analyzed with the aid of a fluorescence microscope. To quantify apoptotic cells, TUNEL positive cells were counted in at least six random fields of each chamber. Data are presented as the percentage of the total number of cells in the six fields. The experiments were performed in duplicate wells and repeated three times.

2.4. RT-PCR

Total RNA was extracted from RGC-5 cells and rat retina (served as positive control) using an RNeasy Mini Kit (QIAGEN, Valencia, CA) according to manufacturer's directions. The yield and purity of RNA were estimated