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Calcium preconditioning triggers neuroprotection in retinal ganglion cells

Sean K. Brandt^a, Monique E. Weatherly^a, Lillian Ware^a, David M. Linn^b, and Cindy L. Linn^a

^a Western Michigan University, Department of Biological Sciences, Kalamazoo, MI 49008

^b Grand Valley State University, Biomedical and Health Science Department, Seymour and Esther Padnos Hall of Science, Allendale, MI 49401

Abstract

In the mammalian retina, excitotoxicity has been shown to be involved in apoptotic retinal ganglion cell (RGC) death and is associated with certain retinal disease states including glaucoma, diabetic retinopathy and retinal ischemia. Previous studies from this lab (Wehrwein et al., 2004) have demonstrated that acetylcholine (ACh) and nicotine protects against glutamate-induced excitotoxicity in isolated adult pig RGCs through nicotinic acetylcholine receptors (nAChRs). Activation of nAChRs in these RGCs triggers cell survival signaling pathways and inhibits apoptotic enzymes (Asomugha et al., 2010). However, the link between binding of nAChRs and activation of neuroprotective pathways is unknown. In this study, we examine the hypothesis that calcium permeation through nAChR channels is required for ACh-induced neuroprotection against glutamate-induced excitotoxicity in isolated pig RGCs. RGCs were isolated from other retinal tissue using a two step panning technique and cultured for 3 days under different conditions. In some studies, calcium imaging experiments were performed using the fluorescent calcium indicator, fluo-4, and demonstrated that calcium permeates the nAChR channels located on pig RGCs. In other studies, the extracellular calcium concentration was altered to determine the effect on nicotine-induced neuroprotection. Results support the hypothesis that calcium is required for nicotine-induced neuroprotection in isolated pig RGCs. Lastly, studies were performed to analyze the effects of preconditioning on glutamate-induced excitotoxicity and neuroprotection. In these studies, a preconditioning dose of calcium was introduced to cells using a variety of mechanisms before a large glutamate insult was applied to cells. Results from these studies support the hypothesis that preconditioning cells with a relatively low level of calcium before an excitotoxic insult leads to neuroprotection. In the future, these results could provide important information concerning therapeutic agents developed to combat various diseases involved with glutamate-induced excitotoxicity.

Keywords

excitotoxicity; neuroprotection; retinal ganglion cells; preconditioning; calcium; nicotinic AChR

Excitotoxicity is defined as neuronal cell death caused by excessive excitatory neurotransmitter and has been linked to various diseases of the eye including ischemia,

Corresponding author: Cindy Linn, Ph.D., Professor, Department of Biological Sciences, Western Michigan University, Kalamazoo, MI 49008 USA, Cindy.linn@wmich.edu, 269–387–5615 (phone), 269–387–5609 (fax).

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diabetic retinopathy, and glaucoma (Lieth et al., 1998; Quigley, 1998; Romano et al., 1998; Lafuente et al., 2001). Each of these diseases can eventually lead to blindness. Glaucoma is one of the leading causes of blindness in the world, affecting an estimated 66 million people worldwide and is characterized by optic neuropathy, cupping of the optic disk, degeneration of retinal ganglion cells (RGCs) and eventual visual field loss. Although the fundamental cause of glaucoma is unknown, the primary risk factor associated with glaucoma is an increase in intraocular pressure. However, reduction in intraocular pressure is frequently insufficient to prevent progression of the disease and visual field loss. Rather, glutamate-induced excitotoxicity likely plays an important role in glaucoma (Vickers et al., 1995; Brooks et al., 1997; Dkhissi et al., 1999; Dreyer and Lipton, 1999). Using *in vivo* and *ex vivo* preparations (Romano et al., 1998; Saitoh et al., 1998; Luo et al., 2001; Kawasaki et al., 2002), relatively high concentrations of glutamate in the eye has been shown to lead to a prolonged influx of nonspecific cations into retinal ganglion cells, leading to apoptosis and cell death (Quigley et al., 1995; Lam et al., 1999). As the axons of RGCs form the optic nerve and convey visual information from the retina to the brain, the loss of RGCs through excitotoxicity-induced apoptosis leads to loss of the visual field.

One hypothesis on how to prevent excitotoxicity and cell death is through the process of preconditioning. Preconditioning occurs when small amounts of stressors are introduced to a group of cells before application of an insult. These preconditioning stressors trigger neuroprotection and prevent the insult from initiating cell death. There are many different types of preconditioning. For example, some types of preconditioning occur under hypoxic and ischemic conditions. The preconditioning effects of these conditions have been studied and shown to be effective in preventing cell death under various insults (Cescon et al., 2006; Webster et al., 1995). Other studies have analyzed the effects of drug-induced preconditioning. Youssef et al. (2006) studied the effects of drug-induced preconditioning in hippocampal slices in rats. Incubating slices in relatively low doses of NMDA or glutamate acted to precondition slices against subsequent NMDA insults and induced neuroprotection.

In the retina, acetylcholine (ACh) and nicotine may have a neuroprotective role against glutamate-induced excitotoxicity (Wehrwein et al., 2004; Thompson et al., 2006) as the result of preconditioning. ACh is an important endogenous neurotransmitter. In previous studies, ACh and nicotine have been shown to act as a neuroprotective agent in several regions of the central nervous system including the retina (Marin et al., 1994; Shimohama et al., 1996; Kaneko et al., 1997; Dajas-Bailador et al., 2000; Wehrwein et al., 2004; Thompson et al., 2006). For ACh-induced neuroprotection to occur in the retina, RGCs are incubated in relatively low concentrations of ACh or nicotine (1–10 μ M) before a large glutamate insult (500 μ M) (Wehrwein et al., 2004), suggesting that the cells are preconditioned against a subsequent glutamate insult. Pharmacological and immunocytochemical studies have provided evidence that ACh's and nicotine's neuroprotection against glutamate-induced excitotoxicity in adult pig RGCs is mediated through $\alpha 7$ nicotinic acetylcholine receptor (nAChR) subunits on the large RGCs and through $\alpha 4\beta 2$ nAChR subunits on small RGCs (Thompson et al., 2006).

ACh and nicotine-induced neuroprotection studies in the retina also demonstrated that activation of these nAChR subunits initiates multiple neuroprotective pathways to induce overall neuroprotection. Specifically, ELISA studies provided evidence that activation of nAChRs on pig RGCs activates the PI3 AKT- Bcl2 and NF κ B cell survival pathway, while inhibiting the MAP KKK - p38 MAP kinase pathway associated with apoptosis to enhance neuroprotection (Asomugha et al., 2010).

What's the link between activation of nAChRs and modulation of enzymes in cell survival and apoptotic pathways? One possibility is that PI3 kinase physically associates with

nAChR subunits. When ACh or nicotine binds to the nAChRs, PI3 kinase is activated. The other scenario involves calcium. Activation of the nAChR's allows influx of sodium and calcium into cells (Decker and Dani, 1990; Mulle et al., 1992; Burnashev, 1998). Calcium has been shown to trigger many different secondary messenger pathways, including the PI3 - AKT- Bcl₂ pathway that is involved in neuroprotection in other systems (Kihara et al., 2001). It is likely that activation of the PI3 - AKT pathway leads to enhancement of Bcl₂ and NF κ B as well as inhibition of MAP kinases (Asomugha et al., 2009). However it has yet to be demonstrated whether calcium is required for neuroprotection to occur in isolated pig RGCs, whether activation of nAChRs is required for neuroprotection to occur, or whether preconditioning cells with calcium is required for neuroprotection to occur. Experiments performed in this study will address each of these issues.

EXPERIMENTAL PROCEDURES

Dissociation and Panning Procedure

Pure retinal ganglion cells were isolated from pig eyes using an immunoselective panning technique (Wehrwein et al., 2004; Thompson et al., 2006). Briefly, adult pig eyes were obtained immediately after sacrifice from a local slaughterhouse (Pease Slaughterhouse, Scotts, MI). The eyes were then transported on ice to the laboratory, dissociated and cultured. Upon arrival, excess muscle was trimmed off each eye then dipped in alcohol to sterilize the surface. The cornea, lens and vitreous humor was subsequently removed, leaving behind an eyecup preparation. Each eyecup was then moistened with a modified CO₂-independent media (Gibco) containing 4mM glutamine (Sigma), 5% fetal bovine serum (FBS, Sigma), 5% antibiotic/antimycotic (Gibco), and 4mM HEPES (Sigma) and retinas were gently scraped out of the sclera. Once removed, retinas were cut into 8 pieces and transferred into the modified CO₂-independent culture media. Each retina was enzymatically treated with a papain solution (Worthington Biochemicals) (27 units/ mg) for 20 minutes in a 37° C water bath, inverted every 2–3 minutes to ensure proper reaction. To stop the enzymatic reaction after the 20 minutes, fresh culture media was added to each tube along with DNase solution (Sigma). The tissue was then dissociated by gentle titration with a sterile Pasteur pipette and the dissociated cells were transferred to a 15ml conical tube.

Retinal tissue was then processed using a modified two-step panning technique to isolate the RGCs from other retinal cells (Wehrwein et al., 2004). In the first step of this procedure, dissociated retinal cells were placed onto 150 mm petri dishes containing goat anti-rabbit IgG antibody (Jackson ImmunoResearch; 0.5 mg in 48 ml of 20 mM Tris buffer) for one hour in a 37°C incubator to eliminate nonspecific binding. Afterwards, retinal tissue was transferred to petri dishes containing mouse anti-rat Thy 1.1 antibody (BD Biosciences; 10 µg of .5 mg/ml in 10 ml PBS containing zero calcium and zero magnesium) bound to goat anti-mouse IgM (Jackson ImmunoResearch; 0.3 mg in 48 ml of 20 mM Tris buffer). In the retina, the Thy 1.1 antibody selectively binds to glycoproteins found exclusively on RGCs (Barnstable and Drager, 1984). Cells were incubated for one hour in a 37°C incubator. At the end of the hour, the supernatant in each of the large petri dishes was discarded. The isolated RGCs that remained bound to Thy 1.1 in the petri dishes (Wehrwein et al., 2004) were released using 0.25% trypsin (Sigma) for 10 minutes at 37°C. Trypsin activity was stopped using 1 mg/ml soybean trypsin inhibitor (Sigma) and cells were strained. The cell density of the dissociated RGCs was calculated using a hemocytometer and cells were subsequently: 1) plated evenly at a density of 1×10^5 cells/ml in modified CO₂-independent medium into 50 mm petri dishes for pharmacology studies, 2) processed for ELISA studies, or 3) plated on round coverslips positioned on the bottom of petri dish wells for calcium imaging studies.

Pharmacology studies

In pharmacological studies, cells were allowed to settle for two hours, after which time media was replaced with fresh modified CO₂-independent media containing additional supplements that enhanced cell survival and growth of processes. The supplements included: 15 µg/ml NGF (nerve growth factor), 10 mg/ml insulin and 500 µg/ml transferrin (Wehrwein et al., 2004).

RGCs were cultured in petri dishes for three days under a variety of pharmacological treatments (Wehrwein et al., 2004; Thompson et al., 2006). In each experiment, plates contained untreated RGCs to use as an internal control, plates that contained RGCs treated with 500 µM glutamate to induce excitotoxicity (Wehrwein et al., 2004), and plates that contained cultured RGCs pretreated with 5 µM ACh for 1 hour before addition of 500 µM glutamate to induce neuroprotection (Wehrwein et al., 2004). The remaining petri dishes contained various agents to determine if calcium was required for neuroprotection to occur. For example, in some experiments, the extracellular calcium concentration was reduced to 0.125 mM from normal levels with EGTA (Sigma) to determine if extracellular calcium was required for ACh-induced neuroprotection to occur. In other experiments, agents were added to increase intracellular calcium levels in the RGCs before glutamate insult to determine if preconditioning cells with calcium triggered neuroprotection against glutamate-induced excitotoxicity. Agents were applied directly to each culture plates and allowed to incubate with the cells for three days. Dose-response experiments were performed to determine what concentrations of the various agents elicited maximal neuroprotection of RGCs against glutamate-induced excitotoxicity.

After three days in culture, cell viability was determined by incubating cells with 2µM calcein-AM (Molecular Probes, Carlsbad, CA) for one hour. Calcein labels the cell bodies of living viable cells through their esterase activity (Bozyczko-Coyne et al., 1993). Cells were photographed under a Nikon Diaphot epifluorescent research microscope illuminated by a 100-W mercury arc lamp with an excitation filter (EX 510–590), dichroic mirror (DM 580) and barrier filter (BA590). Fluorescent images were recorded by a Hamamatsu XC-77 CCD camera, captured and counted using a Metamorph Imaging system and software (Universal Imaging). Images of labeled cells were obtained from five different regions in each culture dish. The number of living cells obtained from the five sections in each eye was summed and averaged. The average number of cells from the treated eyes was compared to the average number of surviving RGCs from untreated dishes. Data was normalized to untreated values for each experiment to minimize variation. Each experiment was performed a minimum of 5 times from different animals.

Calcium Imaging Studies

Isolated dissociated RGCs were loaded with membrane permeable Fluo-4 (2µM) in normal pig Ringers for 30 minutes before imaging. After loading, RGCs cultured on round coverslips were transferred to a perfusion chamber on the stage of the Nikon Diaphot inverted microscope and allowed to settle for 10 minutes before perfusion with normal pig Ringers. Normal pig saline as well as nicotine (1µM, 10µM, and 100µM), KA/NMDA (100µM), and KCl (20mM) were perfused over the RGCs using a gravity fed solenoid controlled perfusion system (Warner Instruments) at the rate of 1 ml/min. Each agent was perfused for a duration of 3 seconds, which elicited a maximal response. In some experiments, cells were incubated for 5 minutes in 25 µM dantrolene +/- 10 µM nifedine before perfusion begun. At the end of each experiment, a maximal increase of intracellular calcium response was recorded by perfusing the cell with 20 mM KCl. After application of KCl, cells in the chamber were removed and replaced with a coverslip containing freshly loaded cells. Fluorescent images were obtained using the Nikon Diaphot epifluorescent

research microscope illuminated by a 100-W mercury arc lamp (excitation 488 nm) at a rate of 3 images/sec using MetaMorph software. MetaMorph software was also used for the analysis of any relative fluorescence intensity changes that occurred in response to perfusing different agents over the RGCs. Enhancement of fluorescence intensity has been demonstrated to indicate an increase in $[Ca^{+2}]_i$ (Pearson et al., 2004). For analysis, a consistent defined region in each RGC was used. From this region, the average relative fluorescence intensity was measured for each loaded RGC immediately before, during and after application of added pharmacological agents at the rate of 3 images/sec. To evaluate the effect of various pharmacological agents on $[Ca^{+2}]_i$, baselines were normalized to 1 (F_o) and the mean maximal change of fluorescence intensity upon addition of reagents (F_{max}) was measured and recorded.

ELISA procedure

ELISA techniques were used in this study to quantitatively measure the degree of up- or down-regulation of phosphorylated Akt and Bcl2 that is involved with calcium preconditioning. ELISAs were chosen to quantify protein content in this study as previous studies from this lab have used ELISAs to demonstrate changes of these proteins during ACh-induced neuroprotection (Asomugha et al., 2010).

After dissociation and cell plating, RGCs were cultured under a variety of pharmacological conditions to determine if relatively low concentrations of glutamate change levels of phosphorylated Akt or Bcl2. There were 5 different pharmacological conditions that cells were cultured in. They included: 1) untreated cells, 2) cells treated with 500 μ M glutamate, 3) cells treated with 50 μ M glutamate, 4) cells treated with 50 μ M glutamate one hour prior to adding 500 μ M glutamate, 5) cells treated with 10 nM wortmannin for 30 minutes prior to 50 μ M glutamate application and 1 1/2 hours before 500 μ M glutamate. Previous time studies conducted by Asomugha et al., (2010) calculated the optimal incubation times that correlated to peak phosphorylation of the various enzymes analyzed. After incubation, isolated pig RGCs were removed from petri dishes, washed with PBS and spun gently into a pellet. The cell pellet was lysed using a cell extraction buffer containing: 10 mM Tris, 100 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM NaF, 20 mM sodium pyrophosphate tetrabasic anhydrous, 2 mM sodium orthovanadate, 1% Triton X-100, 10% glycerol, 0.1% sodium dodecyl sulfate, 0.5% deoxycholate, 1 mM phenylmethanesulfonyl fluoride. Lysed cells were vortexed at 10-minute intervals and the cell extracts were transferred to microcentrifuge tubes and centrifuged at 13,000 rpm for 10 minutes at 4°C. The resulting lysate was kept at -800 C. until the next day.

Each ELISA kit was purchased from Biosource International (Invitrogen) and came with a precoated 96-well plate containing a monoclonal antibody raised against the specific protein to be assayed. ELISA kits were designed to detect and quantify the level of phosphorylated proteins at specific residue sites. The specific residue sites detected by antibodies in each ELISA kits include: Akt[pS473], p38[pTpY180/182] MAP kinase and Bcl-2[pS70]. For normalizing the protein contents of the samples, a total ELISA kit for each protein (Invitrogen) was purchased and used to calculate the total protein present in each sample as the total ELISA kits are independent of the enzyme's phosphorylation state. The percent phosphorylation of each protein was calculated for each experimental condition. All ELISA experiments were repeated a minimum of 3 times with similar results.

ELISA's were performed according to the manufacturer's instructions. Absorbance was measured on a PowerWave 200 microplate scanning spectrophotometer. For each assay, a standard curve was calculated from known protein standard concentrations. The standard curve was used to calculate unknown protein concentrations.

Statistical analysis

Statistical analysis was performed on all normalized data using Kruskal-Wallis non-parametric analysis of variance (ANOVA) with post hoc multiple comparisons (Dunn's test). For data that was not normalized, statistical analysis was performed using ANOVA followed by a Tukey post hoc multiple comparison test. $P < 0.05$ was considered statistically significant for all tests.

RESULTS

Previous studies from this lab have provided evidence that ACh-induced neuroprotection in cultured adult pig RGCs is mediated through multiple pathways through activation of the PI3 kinase – Akt cell survival pathway and inhibition of p38 MAP kinase involved in apoptosis (Asomugha et al., 2009). However, the link that ties activation of nAChRs to these neuroprotective signaling pathways is unknown. To address this, we have tested the hypothesis that extracellular calcium is required for ACh-induced neuroprotection against glutamate in isolated adult pig RGCs. Calcium has been shown to permeate nAChR channels in a number of different tissues (Decker and Dani, 1990, Mülle et al., 1992; Burnashev, 1998). Fig. 1 demonstrates that intracellular calcium levels also increase when nAChRs are activated in cultured isolated pig RGCs. In fig. 1A, an isolated pig RGC has been loaded with 2 μM of the membrane permeable fluorescence calcium indicator, fluo-4, for 30 minutes before (left panel), during (middle panel) and after (right panel) perfusion of 5 μM nicotine. Images were captured at a frequency of 3 images/sec and relative fluorescent intensities were measured using Metamorph software. In this typical example, the relative fluorescent intensity increases from a baseline reading of 3.9 (Fig 1Ba) obtained immediately before the response to 7.9 (Fig 1Bb) in response to nicotine. After recovery of the nicotine response, 20 mM KCl was perfused over the same RGC to obtain a maximal fluorescent response from the same cell (fig 1Bc and d). In the presence of KCl, the baseline changed in this typical case from 0.25 to 11.4. These experiments were repeated 10 times with similar results. The mean F_{max}/F_0 values obtained under these conditions are listed in Table 1.

The relative fluorescence intensity increase due to 5 μM nicotine occurred if the voltage-gated calcium channel blocker, 10 μM nifedipine, was present or absent from the perfusate as shown in the example shown in fig. 2A and B respectively. In addition, the fluorescence intensity increase due to 5 μM nicotine occurred even if cells were incubated in dantrolene (25 μM) before addition of nicotine as shown in the typical response obtained and illustrated in fig. 2C. Dantrolene acts to inhibit calcium release from intracellular stores (Zhao et al., 2001). Experiments using nifedipine and dantrolene were each repeated 10 times and the mean F_{max}/F_0 values are listed in Table 1. These results suggest that neither voltage-gated calcium channels nor calcium-induced calcium release are involved in the ACh-induced increase of intracellular calcium.

Fig. 3 demonstrates that the increase of relative fluorescence occurs in a dose dependent manner. To generate the dose response results illustrated in fig. 3, RGCs were loaded with 2 μM fluo-4 and saline or various concentrations of nicotine were perfused over loaded RGCs using the gravity fed perfusion system. The relative fluorescence intensity changes obtained under these conditions were normalized and plotted and placed on top of each other for direct comparison (fig. 3, Table 1). When normal saline was perfused over loaded pig RGCs, the F_{max}/F_0 equaled 1.0 ± 0.05 (Table 1). However, when various concentrations of nicotine were perfused, the F_{max}/F_0 values obtained from loaded RGCs shown in fig. 3 ranged from 1.4 to 3.5 and correlated with increasing amounts of nicotine (Table 1). The maximal fluorescence change occurred when 100–500 μM nicotine was applied to fluo-4 loaded RGCs. However, the mean F_{max}/F_0 values measured in the presence of nicotine

were significantly lower than mean values obtained if 100 to 500 μM KA/NMDA or 500 μM glutamate was perfused over loaded RGCs (Table 1). This supports the hypothesis that more calcium can permeate glutamate channels than nAChR channels on pig RGCs.

The results from these calcium imaging experiments demonstrate that calcium permeation occurs through nAChR channels in isolated pig RGCs. To determine if permeation of calcium through the nAChR channels in pig RGCs is involved in ACh-induced neuroprotection, experiments were performed in culture medium containing reduced calcium. For these experiments, adult pig RGCs were cultured at 1×10^5 cells/ml under different conditions for 3 days and then labeled with calcein-AM for visualization. In fig. 4 (panel A), cultured RGCs were left untreated for 3 days. In panel B, isolated RGCs were incubated with 500 μM glutamate for 3 days to induce excitotoxicity (Wehrwein et al., 2004). In panel C, RGCs were pretreated with 5 μM nicotine for 2 hours before replacing the culture medium with medium containing 500 μM glutamate to induce neuroprotection (Wehrwein et al., 2004). The culture medium used for panels A, B and C contained 0.75 mM calcium and is the normal concentration of extracellular calcium in CO_2 -independent culture medium. In panel D, RGCs were pretreated with 5 μM nicotine for 1 hour in CO_2 -independent culture medium containing EGTA that decreased the calcium concentration from 0.75 mM to 0.25 mM. After the hour, the culture medium was replaced with normal culture medium containing 0.75 mM calcium and 500 μM glutamate to induce excitotoxicity. Under reduced calcium conditions, neuroprotection against glutamate significantly decreased (Fig. 4D). In control studies, experiments were performed to determine if a decrease of extracellular calcium alone would cause the decrease in the number of RGCs recorded in fig. 4D or if the change of culture medium alone would cause the decrease in cell numbers. Results from these control studies demonstrated that a decrease of extracellular calcium alone did not cause a significant loss of RGCs. In addition, cell loss also did not occur solely due to a change of culture medium (N=5; data not shown).

Fig. 5 demonstrates that nicotine-induced neuroprotection in RGCs is dependent on the concentration of extracellular calcium in a dose-dependent manner. Each bar graph show in fig. 5 represents the mean percent survival of RGCs. To obtain each bar graph, isolated RGCs were cultured under the various pharmacological conditions illustrated for 3 days, loaded with calcein, counted and normalized to the number of cells cultured under control untreated conditions. In normal CO_2 -independent culture medium containing 0.75 mM calcium, 5 μM nicotine induced neuroprotection against glutamate-induced excitotoxicity. However, if 5 μM nicotine was applied to cultured pig RGCs an hour before the glutamate insult in reduced extracellular calcium containing 0.25 or 0.125 mM calcium, the nicotine-induced neuroprotection was lost. These results support the hypothesis that extracellular calcium is required for ACh-induced neuroprotection in pig RGCs.

If extracellular calcium is the link between AChR binding and activation of neuroprotective signaling cascades, it raises an interesting question. Can anything that increases intracellular calcium concentration lead to neuroprotection against glutamate-induced excitotoxicity? There are many preconditioning stimuli that can lead to increases in intracellular calcium in RGCs, including NMDA receptor activation, opening of voltage-gated calcium channels, release of calcium from intracellular stores, hormones, cytokines and neuromodulators. To address this issue, intracellular calcium levels was increased through several different mechanisms and the effect on excitotoxicity and neuroprotection was assessed.

Glutamate Treatment

Previous studies have demonstrated that RGCs contain both NMDA and non-NMDA ionotropic glutamate receptor channels that are permeable to non-specific cations, including calcium and sodium (Hamassaki-Britto et al., 1993; Watanabe et al., 1994; Goebel et al.,

1998; Lin et al., 2002). Influx of excessive calcium through these glutamate channels trigger activation of apoptotic intracellular signaling cascades and ultimately leads to calcium-induced cell death (Quigley et al. 1995; Lam 1999; Asomugha et al. 2010). To determine if lower influx of calcium through glutamate channels can lead to neuroprotection of RGCs, experiments were performed using several low concentrations of glutamate before application of 500 μ M glutamate (Fig 6). This procedure preconditioned cells with intracellular calcium before introducing an excitotoxic insult. The bar graphs shown in fig. 6 summarize the results obtained from these experiments. Each bar graph represents the mean percent of RGCs that survive under each of the treated conditions compared to the percent of cells that survived under untreated control conditions. In the presence of 500 μ M glutamate, an average of 42% ($\pm$ 6) of RGCs die. However, if cells are preconditioned with lower concentrations of glutamate for an hour before an excitotoxic glutamate concentration is applied (500 μ M), RGC survival significantly increases. As seen in fig. 6, if cells are pretreated with 50 μ M glutamate before 500 μ M glutamate, the average percent of RGC death decreased from 42% when 500 μ M glutamate is applied alone, to 18% ($\pm$ 8). These results suggest that low concentrations of glutamate can have a neuroprotective effect against excitotoxicity in pig RGCs.

Potassium Chloride Treatment

If cells are treated with KCl, neurons depolarize due to a shift in membrane potential. As cells depolarize, voltage-gated calcium channels open, allowing calcium influx and an increase of intracellular calcium. This procedure was used as another way to precondition cells with intracellular calcium before introducing the 500 μ M glutamate insult to induce excitotoxicity. To generate the bar graphs in fig. 7, isolated RGCs were preincubated in various concentration of KCl before applying 500 μ M glutamate. In fig. 7A, the summarized bar graphs represent that pretreatment of cells with 5 and 10 mM KCl eliminated glutamate's excitotoxic effect.

If KCl-induced neuroprotection is due to depolarization of the cells and opening of voltage-gated calcium channels to increase calcium influx into the cells, voltage-gated calcium channel blockers should eliminate this effect. In fig. 7B, RGCs were pretreated with 10 μ M nifedipine before application of KCl or 500 μ M glutamate. As shown from the bar graph results, 10 μ M nifedipine eliminated the neuroprotective effect associated with 5 or 10 mM KCl. This result supports the hypothesis that KCl-induced neuroprotection was due to calcium permeation through voltage-gated calcium channels in pig RGCs.

Can nAChR activation induce cell death?

If relatively low levels of glutamate receptor activation can protect against a higher glutamate insult, can high levels of ACh or nicotine applied to cultured RGCs lead to calcium-induced apoptotic cell death? To address this issue, various concentrations of nicotine were applied to isolated cultured pig RGCs. As shown by the summarized bar graphs shown in fig. 8, even high concentrations of nicotine failed to induce RGC death. This is likely due to the desensitization characteristic of nAChRs (Giniatullin et al., 2005; Papke et al., 2009; Yamodo et al., 2010), which limits the amount of calcium permeation through ACh channels.

Does calcium preconditioning lead to an increase in phosphorylated Akt?

Previous work from this lab has demonstrated that ACh and nicotine-induced neuroprotection involves up-regulation of phosphorylated Akt and Bcl₂ (Asomugha et al., 2010). To determine if a relatively small increase of intracellular calcium through other mechanisms will also lead to up-regulation of these enzymes, the protein content of phosphorylated Akt and Bcl₂ was analyzed after cells were preconditioned with 50 μ M

glutamate before applying 500 μ M glutamate. The bar graphs shown in figure 9 represent the mean percent phosphorylation of Akt (fig. 9A) or Bcl₂ (fig. 9B) that resulted after incubating RGCs under a variety of conditions. As shown in figure 9A, there was no significant change in Akt phosphorylation levels compared to control untreated conditions when cells were incubated in 500 μ M glutamate. However, there was a significant change in Akt phosphorylation from control levels if RGCs were incubated in 50 μ M glutamate or if cells were incubated in 50 μ M glutamate for an hour before a larger 500 μ M glutamate insult. The increases of Akt phosphorylation measured with 50 μ M glutamate were similar to results obtained when cells were incubated in 5 μ M ACh or 1 μ M nicotine (Asomugha et al., 2010) and suggests that the PI3 kinase – Akt pathway is activated by 50 μ M glutamate. This hypothesis is supported by the results obtained when the PI3 kinase inhibitor, wortmannin was applied before application of the two glutamate concentrations (right bar). If wortmannin is applied to cells before the two glutamate concentrations, the significant increase of Akt phosphorylation was eliminated.

Bcl₂ governs mitochondrial outer membrane permeabilization (Dejean et al., 2006) and was found to be a downstream target for ACh and nicotine resulting in up-regulation of phosphorylated Bcl₂ (Asomugha et al., 2010). As shown in figure 9B, 500 μ M glutamate reduced phosphorylated Bcl₂ levels to below detection capabilities of the ELISA. However, if cells were incubated in 50 μ M glutamate instead of 500 μ M glutamate, there was a significant increase in Bcl₂ phosphorylation. This increase remained if 50 μ M glutamate was applied before a 500 μ M glutamate insult. The increase of Bcl₂ phosphorylation due to 50 μ M glutamate was eliminated if wortmannin was applied to cells before the two glutamate concentrations (right bar). These results support the hypothesis that 50 μ M glutamate activates the PI3 kinase – Akt – Bcl2 pathway, similar to results obtained when ACh or nicotine is applied (Asomugha et al., 2010).

DISCUSSION

Previous studies using cultured isolated pig RGCs have demonstrated that activation of nAChRs is linked to neuroprotection against glutamate-induced excitotoxicity in the retina (Wehrwein et al., 2004; Thompson et al., 2006; Asomugha et al., 2010). In this study, we hypothesize that calcium permeation through nAChR channels is the trigger linking receptor activation to enhanced cell survival. In the calcium imaging experiments, we demonstrated that calcium permeates nAChR channels on isolated pig RGCs. The rise of $[Ca^{+2}]_i$ in fluo-4 loaded RGCs occurred in a dose dependent manner between 1 and 100 μ M nicotine and did not involve activation of voltage-gated calcium channels or release of calcium from intracellular stores. Calcium, however, also permeates glutamate receptor channels and is responsible for initiating apoptosis and cell death in these same cells (Asomugha et al., 2010). Therefore, calcium appears to be the ion that initiates both events leading to two opposite physiological effects.

To explore this dichotomy, a number of experiments were conducted to test the hypothesis that preconditioning cells with low concentrations of calcium initiates neuroprotection against glutamate-induced excitotoxicity. If this hypothesis is correct, neuroprotection of RGCs occurs whenever relatively low concentrations of calcium are introduced into RGCs before a larger excitotoxic insult. On the other hand, large amounts of calcium introduced to cells without a preconditioning dose should lead to activation of apoptosis and cell death. In this study, we tested these issues by preconditioning cells with relatively low levels of calcium before trying to induce excitotoxicity. In the first experiment, various concentrations of glutamate were applied to isolated RGCs before application of 500 μ M glutamate. In previous experiments, 500 μ M glutamate induced excitotoxicity and cell death in isolated pig RGCs (Wehrwein et al., 2004; Thompson et al., 2006; Asomugha et al.,

2010). However, if cells were preconditioned with 50 μM glutamate for an hour before 500 μM glutamate application, excitotoxicity was significantly reduced. At 50 μM , a lower concentration of calcium would permeate glutamate channels. We propose that these results support the idea that a lower concentration of calcium initiates neuroprotection against a later and larger glutamate insult. The exact concentrations of calcium required for neuroprotection to occur or for triggering apoptosis needs to be explored in future studies.

This concept of preconditioning suggests that any method used to slightly increase $[\text{Ca}^{+2}]_i$ before a larger insult will lead to neuroprotection against glutamate-induced excitotoxicity. To test this, we performed another experiment that depolarized RGCs to open voltage-gated calcium channels. KCl is used routinely to depolarize neurons. If cells depolarize enough, voltage-gated calcium channels open in a voltage-dependent manner. When RGCs were incubated in 5 or 10 mM KCl, RGC death due to 500 μM glutamate was eliminated. Experiments were performed to confirm that the effect was due to calcium permeation through voltage-gated calcium channels using the calcium channel blocker, nifedipine. When cells were incubated in 10 μM nifedipine before KCl and glutamate, KCl's neuroprotective effect was eliminated. These results also support the hypothesis that a preconditioning calcium pulse initiates neuroprotection against glutamate-induced excitotoxicity.

As previously mentioned, incubation of RGCs in 500 μM glutamate for 3 days leads to significant cell death (Wehrwein et al., 2004). Excitotoxic cell death is likely due to excessive calcium permeation through channels that initiates apoptosis (Asomugha et al., 2010). Therefore, any mechanism that allows large concentrations of calcium into cells may trigger apoptosis. To address this issue we asked the following question: Would high concentrations of nicotine allow enough calcium into isolated pig RGCs to trigger apoptosis? This was tested by culturing isolated pig RGCs in relatively large concentrations of nicotine. The results of these studies demonstrated that relatively high concentrations did not lead to cell death. In fact, neuroprotection against glutamate-induced excitotoxicity occurred even when 500 μM nicotine was applied to cells. This is likely due to the rapid desensitization property of nAChRs, which would limit the amount of calcium entry into the cells (Giniatullin et al., 2005; Papke et al., 2009; Yamodo et al., 2010). Even at high concentrations of nicotine, intracellular calcium levels only increased to the point of inducing neuroprotection.

The results performed in this study, support the hypothesis that calcium preconditioning is involved in neuroprotection. Although this is the first demonstration of calcium's preconditioning role in retinal ganglion cells to our knowledge, other literature have tested various forms of preconditioning and the underlying mechanisms associated with preconditioning. Ischemic preconditioning is one of the most common forms of preconditioning tested. The mechanism behind ischemic preconditioning involves activation of NMDA glutamate receptors with glutamate or NMDA to protect hippocampal cells from NMDA insults (Youssef et al. 2006). In other preconditioning studies conducted by Bickler et al. (2005), isoflurane was used to induce intracellular calcium concentrations within cells in the hippocampus before the cells were subjected to an ischemic-like injury of oxygen-glucose deprivation. The results from this study supported the hypothesis that increases in intracellular calcium were required for the preconditioning protective effect to occur. In addition, it has been demonstrated that low levels of calcium permeation through NMDA receptors in the hippocampus protect cells against later ischemic insult via activation of ERK (Wang et al., 2006). This was also found in a study by Yamamura et al. (2005), which demonstrated that a reduced uptake of calcium into the sarcoplasmic reticulum, and therefore an increase in intracellular concentration, results in increased protection for adult

rate cardiomyocytes. Other studies by Tauskela et al. (2003) using cortical neurons also showed the importance of calcium in preconditioning protection.

ELISA results obtained in this study demonstrated that the levels of calcium influx through glutamate channels was sufficient to activate the PI3 kinase – Akt – Bcl2 pathway, which is one of the survival pathways activated when 5 μ M ACh was applied to the same cells (Asomugha et al., 2010). However, this pathway activation only occurred when 50 μ M glutamate was applied to cells and did not occur when higher concentrations of glutamate was applied, supporting the hypothesis that relatively low levels of intracellular calcium are required for triggering neuroprotection pathways.

Physiological Significance

The results of this study have demonstrated that any stimuli that preconditions RGCs with a relatively low concentration of calcium before glutamate insult, produces neuroprotection against glutamate-induced excitotoxicity. This raises an important question concerning the role of nAChRs located on pig RGCs. Do the nAChRs on RGCs have a neuroprotective role under physiological conditions? In other words: *does ACh have a physiological neuroprotective role in the retina?* In the retina, RGCs receive cholinergic input from a well-described population of cholinergic input from a well-described population of amacrine cells, known as starburst amacrine cells. Physiologically, these starburst amacrine cells receive strong excitatory input from bipolar cells and synapse onto RGCs (Masland et al., 1984; Mariani and Hersh, 1988). They are the only source of ACh in the vertebrate retina. Release of ACh from these starburst amacrine cells should lead to an increase of $[Ca^{+2}]_i$ in RGCs and subsequent activation of neuroprotective pathways if the results obtained using cultured cells also occur under physiological conditions. To determine if ACh has a neuroprotective effect in the retina under physiological conditions, experiments using an *in vivo* model of glaucoma are currently underway.

In summary, this study has provided results consistent with our main hypothesis that calcium is the trigger linking activation of nAChRs to activation of neuroprotective signaling cascades in pig RGCs. In addition, any stimuli that preconditioned cells with a relatively low concentration of calcium before glutamate insult, produced a neuroprotective effect against glutamate-induced excitotoxicity through activation of the PI3 kinase – Akt – Bcl₂ pathway. A better understanding of calcium's role in preconditioning cells against excitotoxicity may bring us closer to discovering a medicinal or preventative treatment for many diseases within the CNS that involves excitotoxicity.

Research Highlights

Evidence of calcium permeation through activation of nACh receptors.
Calcium links activation of nAChRs to neuroprotection against excitotoxicity.
Low levels of calcium preconditions RGCs to trigger neuroprotection.
Preconditioning RGCs trigger the PI3-Akt-Bcl2 survival pathway.

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ABBREVIATIONS

ACh	acetylcholine
Akt	protein kinase B
AM	membrane permeable form
ANOVA	analysis-of-variance
Bcl₂	B-cell lymphoma protein 2
[Ca⁺²]_i	intracellular calcium concentration
DM	dichroic mirror
DNase	deoxyribonuclease
EGTA	Ethylene glycol-bis(beta-aminoethyl ether)-N,N,N',N'-tetra acetic acid
ELISA	enzyme-linked-immunosorbent serologic assay
EX	excitation filter
F_o	relative fluorescence intensity baselines normalized to 1
F_{max}	maximal change of fluorescence intensity upon addition of reagents
IgG	immunoglobulin G
IgM	immunoglobulin M
KA	kainic acid
KCl	potassium chloride
MAP	mitogen activated protein
MAPK	mitogen activated protein kinase
NF-κβ	nuclear factor kappa-light-chain-enhancer of activated beta cells
NGF	nerve growth factor
nAChR	nicotinic acetylcholine receptor
NMDA	N-methyl-d-aspartate
PI3	phosphatidylinositol 3-kinase
RGC	retinal ganglion cell
Thy	glycoprotein originally identified in thymus gland

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Fig 1.

Calcium permeation through nAChR channels in pig RGCs. A) An isolated pig RGC was loaded with fluo-4 for 30 minutes and fluorescent images were displayed from 4 different time points. The left image (image a) was obtained immediately before perfusion of 5 μ M nicotine, followed by an image obtained during nicotine perfusion (image b) and after recovery of the nicotine response (image c). The right image in fig. 1A (image d) was obtained during the response to 20 mM KCl and represents a maximal response. B) Represents the relative fluorescence intensities that correspond to the images shown in fig. 1A. The arrows indicate where each agent was applied. The letters a,b,c and d correspond to the images in fig 1A.

Fig 2.

Perfusion of 5 μ M nicotine produced relatively similar responses whether nicotine was applied alone (A), whether RGCs were incubated in 10 μ M nifedipine before nicotine (B) or whether cells were incubated in 25 μ M dantrolene before nicotine (C). F_{max} represents the mean maximal change of fluorescence intensity upon addition of reagents, while F_0 represents the normalized baseline fluorescence obtained before addition of any pharmacological agent. Standard error (S.E.) values for each mean value illustrated is listed in Table 1.

Fig 3.

Fmax/Fo values obtained from RGCs loaded for 30 minutes with 2 μ M fluo-4. Various concentrations of nicotine were perfused over loaded RGCs: 1 μ M (a), 5 μ M (b), 10 μ M (c), 50 μ M (d), 100 μ M (e), 500 (f). The traces a-f represent the mean Fmax/Fo values obtained from each concentration of nicotine. Standard error (S.E.) for each mean value illustrated is listed in Table 1.

Fig 4.

Effect of reduced extracellular calcium on neuroprotection. Isolated RGCs were cultured at a density of 1×10^5 cells/ml for 3 days under various pharmacological conditions: (A) untreated, (B) in 500 μ M glutamate (C) pretreated with 5 μ M nicotine for 1 hour before 500 μ M glutamate. The cells in panels A,B and C were cultured in normal calcium CO_2 -independent medium. In (D), isolated RGCs were pretreated with 5 μ M nicotine in reduced calcium for 1 hour. After an hour, the culture medium was replaced with 500 μ M glutamate in culture medium containing normal calcium. After 3 days, cells were labeled with 2 μ M calcein-AM and photographed.

Fig 5.

Nicotine-induced neuroprotection against glutamate-induced excitotoxicity is dependent on extracellular calcium. All bar graphs represent the mean percent RGC survival compared to control untreated conditions. To obtain each bar, isolated RGCs were cultured for 3 days under the various pharmacological conditions, labeled with calcein-AM, counted and normalized to the untreated controls. 5 μM nicotine was applied 1 hour before the 500 μM glutamate insult in various concentrations of extracellular calcium. Error bars represent S.E. * represents significance from glutamate treated conditions, # represents significance from control untreated conditions. $P < 0.05$.

Fig 6.

Preconditioning cells with relatively low concentrations of calcium provides neuroprotection against a larger glutamate insult. All bar graphs in this figure represent the mean percent RGC survival compared to control untreated conditions. To generate these bar graphs, isolated RGCs were preconditioned with various low concentrations of glutamate for an hour before applying 500 μ M glutamate. After 3 days in culture, cells were labeled, counted and normalized for comparison. Error bars represent S.E. * represents significance from glutamate treated conditions, # represents significance from control untreated conditions. $P < 0.05$.

Fig 7.

Opening of voltage-gated calcium channels can provide neuroprotection against glutamate-induced excitotoxicity. In (A), isolated RGCs were cultured in various concentrations of KCl to depolarize the cells and open voltage-gated calcium channels for an hour before application of 500 μ M glutamate. In (B), 10 μ M nifedipine was applied to cultured cells before KCl and subsequent 500 μ M glutamate application. After 3 days in culture, cells were labeled, counted and normalized for comparison. Error bars represent S.E. * represents significance from glutamate treated conditions, # represents significance from control untreated conditions. $P < 0.05$.

Fig 8.

Activation of nAChRs does not lead to excitotoxicity. To obtain the bar graphs in this figure, various concentrations of nicotine were applied to isolated RGCs for 3 days. After 3 days in culture, cells were labeled, counted and normalized for comparison. Error bars represent S.E. * represents significance from glutamate treated conditions, # represents significance from control untreated conditions. $P < 0.05$.

Fig 9.

Preconditioning cells with calcium activates the PI3-Akt-Bcl2 survival pathway. The bar graphs represent the mean percent phosphorylation of Akt (Fig. 9A) or Bcl2 (Fig. 9B) that occurred when isolated adult pig RGCs were cultured under various pharmacological conditions. RGCs were processed using ELISA techniques. To obtain the results in Fig. 9A, isolated RGCs were collected 12 hours after plating and represents Akt's peak phosphorylation. To obtain the results in Fig. 9B, RGCs were collected 20 hours after plating, representing Bcl2's peak phosphorylation time (Asomugha et al., 2010). Stars represent significance from the untreated control condition, from the 500 μ M glutamate condition and from the condition when 10 nM wortmannin was applied. The filled circle represents that the percent of Bcl2 phosphorylation was below detection levels of the ELISA assay. The assay standard curve detected concentrations as low as 2.0 pg/ml of protein. $P < 0.05$.

Table 1Effect of various pharmacological agents on F_{max}/F_o.

Pharmacological Treatment	F _{max} /F _o	N
nicotine (1 μM)	1.4 ± 0.12 ^a	12
nicotine (5 μM)	1.8 ± 0.23 ^b	20
nicotine (10 μM)	2.4 ± 0.24 ^c	20
nicotine (50 μM)	2.9 ± 0.11 ^d	10
nicotine (100 μM)	3.0 ± 0.23 ^d	10
nicotine (500 μM)	3.1 ± 0.32 ^d	10
KA/NMDA (100 μM)	8.2 ± 0.55 ^e	12
KA/NMDA (500 μM)	9.2 ± 0.46 ^f	10
glutamate (500 μM)	9.5 ± 0.44 ^f	15
nifedipine (10 μM)/nicotine (5 μM)	1.9 ± 0.21 ^b	10
dantrolene (25 μM)/nicotine (5 μM)	1.7 ± 0.33 ^b	10
KCl (20 mM)	10.5 ± 0.83 ^f	35

N represents the number of experiments performed with each agent. ± values represent S.E.

Letters a-f following each S.E. correspond to significant difference from all F_{max}/F_o values that do not have the same letter. P<0.05.