Chloride channel protein 2 prevents glutamate-induced apoptosis in retinal ganglion cells

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ARTICLE INFO

Article type: Original article

Article history:
Received: Apr 21, 2015
Accepted: Feb 22, 2016

Keywords:
Apoptosis
Chloride channels
Chloride channel protein 2
Neuroprotection
Retinal ganglion cells

ABSTRACT

Objective(s): The purpose of this study was to investigate the role of chloride channel protein 2 (ClC-2) in glutamate-induced apoptosis in the retinal ganglion cell line (RGC-5). Materials and Methods: RGC-5 cells were treated with 1 mM glutamate for 24 hr. The expression of ClC-2, Bax, and Bcl-2 was detected by western blot analysis. Cell survival and apoptosis were measured by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and flow cytometry assays, respectively. Caspase-3 and -9 activities were determined by a colorimetric assay. The roles of ClC-2 in glutamate-induced apoptosis were examined by using ClC-2 complementary deoxyribonucleic acid (cDNA) and small interference ribonucleic acid (RNA) transfection technology. Results: Overexpression of ClC-2 in RGC-5 cells significantly decreased glutamate-induced apoptosis and increased cell viability, whereas silencing of ClC-2 with short hairpin (sh) RNA produced opposite effects. ClC-2 overexpression increased the expression of Bcl-2, decreased the expression of Bax, and decreased caspase-3 and -9 activation in RGC-5 cells treated with glutamate, but silencing of ClC-2 produced opposite effects. Conclusion: Our data suggest that ClC-2 chloride channels might play a protective role in glutamate-induced apoptosis in retinal ganglion cells via the mitochondria-dependent apoptosis pathway.

Introduction

Retinal ganglion cells (RGCs), the projection neurons of the retina, transmit light information to the visual center in the brain via their axons (1). Apoptosis of RGCs is a hallmark of many retinopathies, such as glaucoma, retinal ischemia, and anterior ischemic optic neuropathy (2). Glutamate-mediated excitotoxicity has been shown to play an important role in RGC apoptosis in glaucoma and many other retinal diseases (3, 4). In glaucoma, excessive activation of glutamate receptors due to the release of glutamate from damaged RGCs has been implicated to contribute to RGC apoptosis (5).

Several lines of evidence have shown that mitochondria-dependent apoptosis contributes to RGC loss in glaucoma, and glutamate induces apoptosis of RGCs through the mitochondria-dependent pathway (6). However, the exact mechanism of RGC apoptosis induction by glutamate remains to be elucidated.

Emerging evidence has shown that chloride channels (CIC) are involved in apoptotic events in the death program of many cells including cardiac atrial and ventricular myocytes, cortical neurons, and rat hepatoma cells (7, 8). The CIC family is voltage-regulated chloride channels that are widely expressed in various cells (9). Recent studies have shown that CIC proteins, especially CIC-3 channels, prevent apoptosis in RGC, PC12, and basilar artery smooth muscle cells (10, 11). Some properties of CIC-2 channels such as ubiquitous expression and sensitivity to cell volume are similar to that of CIC-3 channels (12). However, it remains unknown whether CIC-2 is involved in cell apoptosis. In addition, the roles of CIC-2 in RGCs remain unexplored.

The RGC-5 cell line, derived from transforming postnatal day 1 rat retinal cells with psi2 EA1 virus, exhibits the characteristics of RGCs, including the
expression of Thy-1 and Brn-3C as well as sensitivity to glutamate excitotoxicity (4, 13). RGC-5 cells are similar to RGCs in terms of their molecular and genetic changes during apoptosis as well as their responses to neuroprotective agents (4, 6). A glutamate-induced RGC-5 apoptosis model has been widely used to investigate RGC apoptosis (14, 15). It has been reported that RGC-5 cells express various chloride channels (16). The purpose of this study was to investigate the roles of ClC-2 chloride channels in glutamate-induced apoptosis in RGC-5 cells.

**Materials and Methods**

**Cell culture**

RGC-5 cells (ATCC, USA) were cultured in 75-cm² flasks in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin. Cultures were maintained at 37 °C in a humidified incubator containing a 95% O₂ plus 5% CO₂ atmosphere. All cells within the same passage were passaged every 2 to 3 days when they reached 80% confluence. Glutamate-induced apoptosis was performed as previously described (11, 14). Briefly, after passage, cells were incubated overnight, and then they were treated with serum-free DMEM supplemented with 1 mM glutamate for 24 hr (14).

**Reverse transcription-polymerase chain reaction**

Total RNA was extracted from RGC-5 cells using Trizol reagents (Invitrogen, USA) according to the manufacturer’s protocol. RNA was reverse transcribed into complementary DNA using a reverse transcription system (Takara, Japan).

PCRs were performed as follow: 94 °C for 5 min; 30 cycles of 94 °C for 1 min, 54°C for 1 min, 72 °C for 1 min, and 72 °C for 2 min. Primers were synthesized by Sangon Biotech (Sangon, China). The PCR primers used were as follows (15): 5’-CAGTCTCC-TCTCTCTTGG-3’ (sense) and 5’-GAACTGTCCAAA-GCCAGGG-3’ (antisense) for ClC-2 channels (product size, 499 bp), and 5’-CAGGCTGTGATGGTGGGTATG (sense) and 5’-TCCCTCAGCTGTGGTGG-3’ (antisense) for β-actin (product size, 500 bp) (17). The PCR products were analyzed by electrophoresis on 1% agarose gel containing ethidium bromide.

**Measurement of cell viability**

Cell viability was examined using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (15). Briefly, cells were seeded at a density of 5×10⁵/ml in 96-well plates. MTT dye (Sigma, USA) was added at a final concentration of 0.5 mg/ml into each well, and the plate was incubated for 4 hr at 37 °C. Cell plates were shaken for 15 min after the MTT was discarded. The precipitate was then solubilized in dimethyl sulfoxide (150 μl/well; Amresco, USA) for another 15 min. The absorbance of the samples was measured on a microplate reader at a wavelength of 570 nm. The cell survival rate was calculated according to the following equation: cell survival rate (%) = absorbance value of treatment group/absorbance value of control group×100%. Experiments were repeated three times.

**Annexin-V FITC/propidium iodide (PI) assay**

Apoptosis was induced by 1 mM glutamate treatment for 24 hr (14). The apoptosis rate was measured by flow cytometry using an annexin-V FITC/PI detection kit (KeyGEN Biotech, China). Briefly, cells in 6-well plates were harvested with 0.125% trypsin, washed twice with ice-cold phosphate-buffered saline, and resuspended in binding buffer. After centrifugation at 600×g for 5 min, the cells were suspended in 500 μl of binding buffer and mixed with 5 μl of annexin-VFITC and 5 μl of PI. After incubating for 15 min at room temperature in the dark, the cells were analyzed by flow cytometry within 1 hr. Independent experiments were performed in triplicate.

**ClC-2 cDNA transfection**

The ClC-2 cDNA/pcDNA3.1 plasmid was transfected with lipofectamine 2000 reagents into cells according to the manufacturer’s instructions (Life Technologies, USA). The pcDNA3.1 empty vector was used as a negative control. Briefly, cells were plated in 6-well plates for 24 hr until they reached 70–80% confluence. The plasmid and lipofectamine 2000 were diluted in DMEM-F12. Then, the diluted plasmid and lipofectamine 2000 were mixed together and incubated at room temperature for 20 min. The transfection mixture was added to the cells, and the cells were cultured in a humidified incubator containing 95% O₂ plus 5% CO₂ atmosphere for 24 hr. The vector contains a genetin-resistant marker. Stably transfected cells were selected using 500 μg/l G418 in DMEM. After 2 weeks, the surviving G418-resistant cells were further plated and passaged in the presence of 200 μg/l G418 in DMEM. The expression of ClC-2 was detected by western blot analysis (11).

**RNA interference and cell transfection**

To knockdown ClC-2 gene expression, RGC-5 cells were transfected with lentiviral particles encoding ClC-2 short hairpin (sh) RNA or control shRNA (catalog no. sc-61868-V and sc-108080; Santa Cruz Biotechnology). The clones with stable expression of shRNA were selected using titrated concentrations of puromycin dihydrochloride (10 μg/ml for initial selection, and 6 μg/ml for maintenance). The transfection efficiency was detected by Western blot analysis.
Measurement of caspase-3 and caspase-9 activities

Caspase-3 and caspase-9 activities were measured using a colorimetric assay kit (KeyGen, China), according to the manufacturer’s instructions. Briefly, cells were harvested and resuspended in the cell lysis buffer. The proteins from each cell lysate were collected by centrifugation at 12,000×g for 1 min at 4 °C. The protein concentration in the supernatant was determined using a BCA protein assay kit (KeyGen, China). Protein samples were incubated with caspase-3- and caspase-9-specific substrates with reaction buffer at 37 °C for 4 hr. The absorbance density was measured using a spectrophotometer (Bio-Rad, Tokyo, Japan) at 400 nm. The experiments were performed in triplicate.

Western blot analysis

Cultured cells were harvested and lysed using RIPA buffer (50 mM Tris-HCl, 150 mM NaCl, 0.02% NaN3, 1% NP-40, 0.5% sodium deoxycholate, and 1% protease inhibitor cocktail) for 30 min on ice. Lysates were sonicated for 10 sec. After centrifugation at 12000×g for 10 min at 4 °C, the protein concentration was determined using a BCA protein assay kit (KeyGen, China). Proteins were incubated in 2×buffer (100 mM Tris, pH 6.8, 0.2% bromophenol blue, 4% SDS, 20% glycerol, and 200 mM dithithreitol) in boiling water for 5 min. Equal amounts of proteins were loaded into each lane on SDS-PAGE gels and transferred to polyvinylidene difluoride membranes (Millipore, USA). The membranes were blocked at room temperature for 1 hr in Tris-buffered saline solution and 0.1% Tween-20 (TBST) containing 5% nonfat dry milk and 5% bovine serum albumin and then incubated overnight in primary antibodies against Bax (Cell Signaling Technology Inc., USA), Bcl-2 (Cell Signaling Technology Inc., USA), or ClC-2 (Santa Cruz Biotechnology, USA) at 4 °C (18). β-actin was used as a loading control. Membranes were incubated with horseradish peroxidase-conjugated anti-mouse or anti-rabbit secondary antibodies (Santa Cruz Biotechnology, USA) for 90 min at room temperature. An enhanced chemiluminescence kit (Millipore, USA) was used to detect blotting signals, and the bands were visualized by exposure to Kodak X-ray film. Images were acquired by scanning, and gray band values were analyzed. For quantitative assays, the gray values of target protein bands were normalized to the β-actin protein and compared with the controls. Independent experiments were performed in triplicate.

Statistical analysis

Statistical analysis was performed using SPSS 13.0 statistical software (SPSS, Chicago, IL, USA). All quantitative data were presented as the mean± standard error of the mean. One-way analysis of variance was used to determine the difference between groups. Statistical difference determination between two groups was performed by the Student’s t-test. Values of P<0.05 were considered statistically significant.

Results

Effects of ClC-2 channels on glutamate-induced cell viability in retinal ganglion cells

First, the expression of ClC-2 channels in RGC-5 cells was examined using RT-PCR. The RT-PCR results showed that the ClC-2 mRNA was expressed in RGC-5 cells (Figure 1A). Afterward, the protein expression of ClC-2 in RGC-5 cells was investigated using Western blot analysis. Consistent with the mRNA expression, ClC-2 protein was detected in RGC-5 cells. Transfection of ClC-2 cDNA into RGC-5 cells resulted in an increase in the protein expression of ClC-2 by 25.3±4.3% (Figure 1B, P=0.031), and transfection of pcDNA3.1 empty vector failed to increase the protein expression of ClC-2. In addition, ClC-2 shRNA decreased the protein expression of ClC-2 by 54.8±3.5% (Figure 1C, P=0.0089). The effect of glutamate was then examined on the expression of ClC-2 in RGCs. Glutamate treatment (1 mM) significantly downregulated the expression of ClC-2 by 29.8±2.4%. Furthermore, overexpression of ClC-2 increased the protein expression by 27.1±3.6%, similar to that (25.3±4.3%) in RGC-5 cells in the absence of glutamate treatment, suggesting that overexpression of ClC-2 significantly prevented glutamate-induced downregulation of ClC-2. Moreover, transfection of control pcDNA3.1 cells failed to increase the protein expression of ClC-2 in RGC-5 cells treated with glutamate (Figure 1D, P=0.073). In RGC-5 cells treated with glutamate, down-regulation of ClC-2 significantly decreased ClC-2 expression by 52.1±3.5% (Figure 1E, P=0.0091), similar to that (54.8±3.5%) in RGC-5 cells in the absence of glutamate (Figure 1C, P=0.0089). In contrast, negative shRNA transfection did not significantly decrease the ClC-2 expression in RGC-5 cells treated with glutamate (Figure 1E, P=0.079).

The effect of ClC-2 on glutamate-treated cell viability was further measured by MTT assay. After treatment of RGCs with 1 mM glutamate for 24 hr, the cell viability rate was decreased to 76.71±6.13%, compared with untreated controls. ClC-2 overexpression significantly inhibited glutamate-induced cell death and increased the cell viability to 84.1±5.03% (Figure 1F, P=0.043), whereas transfection of control pcDNA3.1 did not prevent glutamate-induced cell death (Figure 1F, P=0.064). In contrast, transfection with ClC-2 shRNA promoted glutamate-induced cell death and further reduced the cell viability to 58.26±6.04% (Figure 1G, P=0.0071). Meanwhile, treatment with control shRNA failed to change the glutamate-induced cell viability.
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Figure 1A. Expression of chloride channels (ClC-2) mRNA in retinal ganglion cells (RGC-5). Reverse transcription-polymerase chain reaction was performed using RGC-5 cells. PCR products were electrophoresed on agarose gel. Figure 1B-G. Effects of ClC-2 cDNA and ClC-2 shRNA transfection on glutamate-induced RGC-5 cell viability measured by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. (B) Densitometric analysis shows a significant increase in ClC-2 protein expression in cells transfected with ClC-2 cDNA, n=3; *P<0.05 vs. control; (C) Densitometric analysis shows a significant decrease in ClC-2 protein expression induced by ClC-2 shRNA transfection, n=3; +P<0.01 vs. control; (D) Densitometric analysis shows a significant decrease in ClC-2 protein expression in cells treated with 1 mM glutamate for 24 hr, n=3; *P<0.05 vs. control; +P<0.01 vs. glutamate treatment. (E) Densitometric analysis showing a significant decrease in ClC-2 protein expression induced by ClC-2 shRNA transfection, n=3; *P<0.05 vs. control; +P<0.01 vs. glutamate treatment. (F) Effects of ClC-2 cDNA transfection on the cell viability of RGC-5 cells. n=3; *P<0.05 vs. control; +P<0.01 vs. glutamate treatment. (G) Effects of ClC-2 shRNA transfection on the cell viability of RGC-5 cells. n=3; *P<0.05 vs. control; +P<0.01 vs. glutamate treatment.

Effects of ClC-2 on glutamate-induced apoptosis

Anti-apoptotic effect of ClC-2 was also examined using flow cytometry with annexin V/PI double staining. Treatment with 1 mM glutamate for 24 hr produced an apoptotic population of 20.56±2.38% in RGC-5 cells. ClC-2 overexpression significantly decreased the glutamate-induced apoptotic population to 14.76±2.54% (Figure 2A, P=0.0087), whereas control pcDNA3.1 transfection did not show any effect on glutamate-induced cell death. In contrast, ClC-2 shRNA transfection increased the glutamate-induced apoptotic population to 32.67±1.41% (Figure 2B, P<0.0073), whereas control shRNA transfection did not produce any significant effects. These findings suggested that ClC-2 chloride channels had a protective role on glutamate-induced apoptosis in RGC-5 cells.

Figure 2. Effects of chloride channels (ClC-2) on glutamate-induced apoptosis (A) Flow cytometry analysis showing the effects of ClC-2 overexpression and ClC-2 shRNA on glutamate-induced apoptosis. (B) The percentage of apoptotic cells by quantitative analysis. n=3, *P<0.01 vs. control; +P<0.05 vs. glutamate treatment.
**Effects of ClC-2 on glutamate-induced Bcl-2 and Bax expression in Retinal ganglion cells**

The effects of ClC-2 was then examined on the expression of Bax, a pro-apoptotic protein, and Bcl-2, an anti-apoptotic protein in RGC-5 cells treated with glutamate (19, 20). Western blot analysis showed that glutamate treatment increased the expression of Bax but decreased the expression of Bcl-2 (Figure 3A-B, P<0.01). The effects of glutamate were further aggravated by transfection with ClC-2 shRNA. In contrast, ClC-2 cDNA transfection increased the expression of Bcl-2 but decreased the expression of Bax. Meanwhile, the expression levels of Bcl-2 and Bax were not significantly changed by pcDNA3.1 and negative siRNA transfection.

**Effects of ClC-2 on glutamate-induced caspase-3 and -9 activation**

The activities of caspases-3 and -9 were examined using a colorimetric assay kit. Compared with controls, glutamate treatment significantly increased cleaved caspase-3 and -9 activities (Figure 3C, P<0.01 vs. control). P<0.01 vs. glutamate treatment). The glutamate-induced increase in cleaved caspase-3 and caspase-9 activities was inhibited by ClC-2 cDNA transfection but further increased by ClC-2 shRNA transfection. The glutamate-induced activation of caspases-3 and -9 was not significantly changed by transfection with pcDNA3.1 and negative shRNA.

**Discussion**

RGC apoptosis is an important pathological feature of many eye diseases such as glaucoma and retinal ischemia (21, 22). In glaucoma, RGC apoptosis results in cupping of the optic disk, and overexpression of glutamate plays an important role in RGC apoptosis (22). RGC-5 apoptosis induced by glutamate has been used as an in vitro model to investigate RGC apoptosis in glaucoma (14). Chloride channels have been reported to be involved in apoptotic events in the death program of many cells (7, 8, 23). In particular, RGC-5 cells have been found to express various chloride channels (16). However, it has remained unknown whether these chloride channels are involved in glutamate-induced apoptosis in RGC-5 cells. In the present study, we investigated the roles of ClC-2 chloride channels in glutamate-induced apoptosis in RGC-5 cells by using ClC-2 cDNA and shRNA transfection techniques. For this purpose, expression of ClC-2 channels in RGC-5 cells was first confirmed by RT-PCR and western blot analyses. Overexpression of ClC-2 resulted in improved survival and decreased apoptosis in cells treated with glutamate, accompanied by increased expression of Bcl-2, decreased expression of Bax, and decreased caspase-3 and -9 activation. But, silencing of ClC-2 produced the opposite effects. Our findings suggest that ClC-2 chloride channels play a protective role in glutamate-induced apoptosis via mitochondria-dependent pathways involving Bcl-2, Bax, and caspases-3 and -9. Targeting ClC-2 may be effective in protecting RGC-5 cells against glutamate cytotoxicity, and thus ClC-2 activators may be used as potential neuroprotectants in neurodegenerative diseases such as glaucoma.

In the present study, we found that glutamate treatment induced apoptosis in RGC-5 cells, accompanied by an upregulation of ClC-2. Overexpression of ClC-2 significantly inhibited glutamate-induced apoptosis in RGC-5 cells, whereas downregulation of ClC-2 by transfection with ClC-2 shRNA accelerated glutamate-induced apoptosis. These results strongly suggest that in RGC-5 cells, ClC-2 channels act to inhibit glutamate-induced apoptotic processes, and the activation of ClC-2 protein is beneficial for cell resistance against apoptotic inducers. These findings confirm the reports that chloride channels play protective roles in the process of apoptosis (10, 11). However, it remains unclear how chloride channels exert their anti-apoptotic activity. It is well known that ClC-2 and ClC-3 channels, which are activated by cell swelling, participate in cell volume regulation,
and cell volume regulation is involved in the process of apoptosis (24). It is likely that CIC-2 exerts its anti-apoptotic activity in RGC-5 cells by functioning as a swelling-activated chloride channel. This theory is consistent with the previous report that inhibition of swelling-activated chloride channels increases apoptosis in the rabbit myocardium (7). In addition, it has been reported that in rat basilar arterial smooth muscle cells, CIC-3 is upregulated in the HO2-induced apoptotic process, accompanied by increased expression of Bcl-2 (10). It has been reported that overexpression of Bcl-2 results in upregulation of CIC-3 in human prostate cancer epithelial cells (18), suggesting that the Bcl-2 signaling pathway may be involved in the regulation of CIC-3 expression. Similarly, we found up-regulation of CIC-2 and increased expression of Bcl-2 in the glutamate-induced apoptotic process, suggesting that CIC-2 may be regulated in a similar way.

Apoptosis is a type of programmed cell death that can be activated via mitochondria-dependent pathway. This pathway involves mitochondrial translocation of pro-apoptotic members of the Bcl-2 family, such as Bax, which open the mitochondrial permeability transition pore and subsequently release cytochrome c to initiate apoptosis (25). Bcl-2 is an anti-apoptotic protein that plays an important role in controlling the release of cytochrome c from mitochondria and regulating the activation of effector caspases-3, -6, and -7 (26). The Bcl-2 family members are the key regulators in RGCs, and overexpression of Bcl-2 is known to protect RGCs from glutamate toxicity (18, 19). In the present study, treatment of RGC-5 cells with glutamate resulted in increased expression of Bax, decreased expression of Bcl-2, and activation of caspases-3 and -9, suggesting that glutamate activates apoptosis via the classical mitochondria-dependent pathway. Overexpression of CIC-2 via transfection with CIC-2 cDNA inhibited glutamate-induced apoptosis, accompanied by decreased Bax expression and increased Bcl-2 expression; whereas, CIC-2 silencing via transfection with shRNA produced the opposite effects, indicating that CIC-2 protects RGC-5 cells against mitochondrial apoptosis.

Caspase-3 is an intracellular cysteine protease that exists as an activated proenzyme during the cascade of apoptotic events. The activated caspases kill cells by degrading structural elements and DNA repair enzymes and by indirectly activating chromosomal endonucleases. One important pathway for caspase-3 activation is via caspase-8, which can activate caspase-3 by directly cleaving it (25) or indirectly causing cytochrome c release from mitochondria that triggers caspase activation through Apaf1 (27). Caspase-9 is the active enzyme upstream of the mitochondrial caspase-dependent apoptosis pathway (28). Activated caspases kill cells by degrading structural elements and DNA repair enzymes and by indirectly activating chromosomal endonuclease (29). In the present study, we found that compared with the control, glutamate treatment significantly increased cleaved caspase-3 and -9 activities, which were apparently counteracted by CIC-2 cDNA transfection, but further increased by CIC-2 shRNA transfection. Our results suggest that CIC-2 channels prevent glutamate-induced apoptosis via the caspase-3 and -9 pathway.

Although RGC-5 is a well-described cell model to study apoptosis of RGCs in vitro, but it remains to be determined whether the in vitro findings can be extrapolated to the native RGCs in vivo. Future studies are required to confirm our finding that CIC-2 prevented glutamate-induced apoptosis in the animal models.

Conclusion

In summary, we found that CIC-2 has protective effects on glutamate-induced apoptosis of RGC-5 cells by influencing the mitochondrial signaling pathway, involving Bcl-2, Bax, and caspases-3 and -9. Our results suggest that increasing the function of CIC-2 channels may result in an improvement of cell survival and a reduction of cell apoptosis. Thus, the neuroprotective effects of CIC-2 channels may lead to a novel approach for the treatment of retinopathies, such as glaucoma.

Conflicts of interest

None declared

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