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Role of Phosphatidylinositol-3 and Mitogen-Activated Protein Kinases on Rat Retinal Cells Size and Neurite Outgrowth in the Absence and in the Presence of Taurine

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Abstract: ***This study examined the effect of phosphatidylinositol-3 proteinkinase (PI3K), LY 294002 and wortmannin, and of mitogen activated protein kinases (MAPK) inhibitors, PD 98059 and SB 203580, on rat retinal cells size and neurite outgrowth in the absence and in the presence of taurine. Isolated cells were cultured for 5 days in minimal essential medium containing taurine (4 mM)and the inhibitors at different concentrations (2.5, 10, 20, 25 µM). Size of ganglion cells was measured and outgrowth was evaluated by the length of the largest neurite (µm), in cells < 12 µm and ≥ 12 µm. The inhibition of IP3K and MAPK modifies the cell size and neuritic growth of retinal cells and the trophic effect of taurine on these parameters. The effect of the inhibitors, probably acting directly or at other cellular levels, indicates that the regulation of outgrowth by phosphorylation is a complex and dual process. These results contribute to the understanding of mechanisms involved in maintenance of retinal cells, and could be useful for possible therapeutic targets in retinopathies of various origins such as glaucoma, macular degeneration, among others.***

Keywords: retina; taurine; kinases; neurite; outgrowth

1. Abbreviations

DMSO dimethylsulfoxide; PI3K phosphatidylinositol-3kinase; MAPK mitogen activated protein kinases; LY 294002 2-morpholin-4-yl-8-phenylchromen-4-one; PD 98059 2'-amino-3'-methoxyflavone; SB 203580 4-(4-fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)-1H-imidazole

2. Introduction

Taurine (2-amino-ethanesulfonic acid)is the most abundant amino acid in retina [1,2], with concentrations greater than those in other ocular structures or in the brain of adult animals of all species examined [3,4], reaching up to 50 µmol/g of wet weight retina in rats [5,6]. Taurine exerts multiple functions, such as cell volume regulation, cytoprotective, substrate for the formation of bile salts, neuromodulation, and modification of protein phosphorylation, development, stimulator of axonal outgrowth, nutrition, and survival, and acts as a trophic agent in retina, among others [7-13]. Taurine increases the outgrowth of the goldfish retina in a bell-shaped concentration-manner, and at certain critical periods of time and concentration shown to be determinant in the regeneration of the retina [7,14]. The mechanisms by which taurine exerts its trophic role in the retina have been related to calcium fluxes [8], and to protein phosphorylation [10].

A variety of signals are involved incellular processes, in which certain kinases, such as phosphatidylinositol-3kinase (PI3K) and mitogen activated protein kinases (MAPK) function in the signaling pathway [15-17]. These kinases involved in a variety of fundamental cellular processes such as differentiation, proliferation, apoptosis, stress response, and survival.After binding at growth factors, their respective receptor tyrosine kinase, receptor dimerization triggers the intrinsic tyrosine-kinase activity [18-21].MAPK and PI3K pathways have been well studied in various types of neurons [22-24]. The roles of these pathways, however, are distinct in each cell subtypes. In PC12 cells, prolonged activation of AMPK promotes cell survival after damage of nerve growth factor [25]. The inactivation of proapoptotic protein and the increased transcription of prosurvival genes, such as cAMP-responsive element-binding protein, in MAPK signaling pathway, promotes cell survival through a dual mechanism [26]. On the other hand, recent evidence indicates that the PI3K / Akt pathway is more relevant to cell survival than the MAPK pathway in granule neurons of the cerebellum and spinal motor neurons [27].

The PI3K/AKT pathway is a signalingmodule that is also implicated in the proliferation of severaltypes of cells, including mouse embryonic stem cells [28], developing cells from the rat cerebral cortex [29], adult hippocampal neural progenitors [30], and Muller glial cells of the rat retina [31]. PI3K is now considered as one of the most important regulatory proteins, being involved in a number of diverse signaling pathways and in controlling the main functions of the cell [21, 32]. PI3K has been reported to stimulate neurite outgrowth in PC12 cells (a cell line derived from a pheochromocytoma of the rat adrenal medulla) [33], and to be required for the neural cell adhesion molecule-mediated neurite outgrowth of primary neurons [34]. The effects of PI3K and MAPK inhibitors could be relevant in studying their influence on neurite growth from rat retinal cells.

Knowing the signaling pathways involved in the regulation of cell size and neurite outgrowth in the retina is of great significance, regarding the possible therapeutic potential in several diseases, many of them associated with retinopathy of various origins. According to the modulation of the phosphorylation of specific proteins by taurine [9] together

with the influences of protein phosphorylation on retinal outgrowth, the aims of this study were: (1) to explorethe effects of PI3K and MAPK inhibitorson the basal neurite outgrowth from rat retinal cells; (2) to determine the possible effect of these inhibitors on the trophic effect of taurine and (3) to understand the signaling pathways involved in the trophic effect of taurine on retina.

3. Methods

3.1 Animals

Male Sprague–Dawley rats five days old were obtained from the hatchery of InstitutoVenezolano de Investigaciones Científicas. Commercial rat food and water were provided *ad libitum*. The animals were decapitated at 9:00 am, and the eyes were extracted from the orbit. All manipulations followed international ethical guide [35]. The total number of animals per experiment was 15 for each condition. The animal protocols were approved by Institute Ethical Committee following international rules.

3.2 Isolation of rat retinal cells

Retina was dissected in a laminar flow hood and cells were isolated with 0.25 % trypsin in MinimumEssential Medium (MEM) with 0.1 mg/ml of gentamicin and 20 mM of (N-2-hydroethyl]piperazine-N_-2-ethanesulfonicacid) (HEPES), at 37 ºC for 15 min, followed by mechanical separation with Pasteur pipette. The cells were washed twice with MEM at 1500 rpm for 10 min and counted in a Neubauer chamber. Integrity of membrane was determined by 50 % Trypan blue exclusion (>96 %) [36].

3.3 Retinal cell cultures and treatments

Retinal cells suspended in MEM were placed (400,000 cells/ml) in poly-L-lysine (0,025 %) (Sigma) pre-coated culture flasks. The nutrient medium was Leibovitz, L-15 (free of taurine), and 5 ml per dish (Sigma). Taurine, 4 mM, was added to some cultures of retinal cells [37], [38]. Inhibitors LY 294002, wortmannin, PD 98059 and SB 203580 diluted in dimethyl sulfoxide (DMSO) were added in concentration of 2.5, 10, 20 and 25 µM. Control group for each test corresponds to cells with DMSO (0.25, 1 and 2 %) in the absence and presence of taurine and inhibitors. The cells size and neurite length (µm) was measured at 5 days after plating, using the program SigmaScanPro [17].

3.4 Statistical analysis

Results are expressed as mean ± standard error of the mean, analysis of variance was performed followed by Tukey–Kramer Multiple Comparisons Test for evaluating results (GraphPadInStat 3). Values of $p<0.05$ were considered significant.

4. Results & Discussion

Effect of phosphatidylinositol-3kinase inhibitors on neuritic outgrowth

In this study, we examined the role of PI3K signaling cascades on cell size and neurite length in rat retinal cells. The results with the inhibitor of IP3K LY 294002 on cell size and length of neuritis are shown in Fig. 1. Five days after plating, Ly 294002 did not change the size of the cells in the range of concentrations used (Figure 1a, 1c). The length of neurites was significantly reduced in a dose-dependent manner in all cells by Ly 294002 in the range of concentrations used (Figure 1b, 1d). The irreversible inhibitor of PI3K, Wortmannin, significantly decreases cell size and length of neuritis of cells (Figure 2), except the cells size in cells ≥ 12 µm (Figure 2c). These results suggest that neurite growth and cellsize are processes that occur through different signaling pathways.The inhibition of PI3K by Ly 294002 and Wortmannin inhibitor indicates the relevance of phosphorylation for outgrowth of neuritesin rat retina. The mechanism by which PI-3K affects neurite outgrowth is presently unclear, but prominent targets downstream of PI-3K include Akt (PKB), regulators of the Rho-GTPase family, and the RAS/RAF/ERK pathway [21, 39-43]. The effects of LY 294002 depend on the experimental conditions, such as the type of cell, the degree of injury and the time of cell culture. In the retina, LY 294002 was shown to exert a protective effect on retinal ganglion cells in the absence of ciliary neurotrophic factor (CNTF) after peripheral surgery of the optic nerve [44]. In contrast, other studies showed that LY 294002 decreased the number of surviving retinal ganglion cells after optic nerve clamping compared to injury alone [42]. Thus, our results with LY 294002 and wortmannin were different. The mechanisms exerted by LY 294002 and wortmannin on growth are not clear, however, it is well known that these 2 compounds are structurally distinct inhibitors of PI3K that act to suppress the activity of this enzyme through different mechanisms [45-50]. Further studies are required to elucidate the mechanisms of the effects of LY 294002 and wortmannin on retinal cells.

Effect of phosphatidylinositol-3kinase inhibitors on neuritic outgrowth in the presence of taurine

Our previous studies on the mechanisms involved in taurine effect on neuritic outgrowth of retinal cells examined substrates, calcium fluxes, but never focused on the influence of protein kinases. Diseases of the retina such as diabetic retinopathy and retinopathy of prematurity are characterized by the formation of abnormal new blood vessels. Studies indicate that inhibition of PI3K signaling interferes with angiogenesis [39]. In presence of taurine, Ly 294002 significantly decreases in a dose-dependent manner cell size and length of neuritis of all cells as compared with controls (Figure 3). In the range of concentrations used, Wortmann insignificantly decreased the trophic effect of taurine on cell size (Figure 4a, 4c), but not altered the length of neurites (Figure 4b, 4d). These results suggest that protein phosphorylation modulates the trophic effect of taurine in retinal cells, mainly ganglion cells. On the other hand, IP3K inhibitors reduce the efflux of taurine in chicken retina [51] indicating the posible protein phosphorylation relationship with the transport of taurine and therefore with the trophic effect of this amino acid.

Effect of mitogen activated protein kinases inhibitors on neuritic outgrowth

MAPK pathways have been well characterized in different neuronal types [52], but the roles of these pathways are distinct in each neuronal subtype. The effect of the inhibitor

of MAPK, PD 98059, is shown in Figures 5. The cell size significantly decreased in the presence of PD 98059 inhibitor in the range of concentrations used (Figure 5a, 5c), and did not modified the length of neuritis (Figure 5b, 5d). The inhibitor of MAPK, SB 203580, significantly reduced cell size and length of neuritis of all cells (Figure 6).In the retina, the MAPK pathway was demonstrated to play pivotal roles in the extension of RGC axons [53], and in postnatal rats promote of survival of RGCs [25, 53]. In the adult rat retina, inhibitions of caspase-3 also avoid RGCs from secondary cell death in vivo [54-56].The MAPK family comprises at least three distinct signaling cascades: the p42/p44 MAPK (extracellular signal-regulated kinase,-ERK-1/2) cascade, which preferentially regulates cell growth and differentiation, and the p38 and c-Jun N-terminal kinase/stress-activated protein kinase (JNK/ SAPK) cascades, both of which function mainly in stress responses such as apoptosis and inflammation [57]. PD98059 has been shown to act in vivoas a highly selective inhibitor of MEK1 activation and the MAP kinase cascade [58,59]. PD98059 binds to the inactive forms of MEK1 and prevents activation by upstream activators such as c-Raf [60]. On the other hand, SB 203580 is a selective inhibitor of p38 MAPK, this compound inhibits the activation of MAPKAPK-2 by p38 [61] and inhibits the catalytic activity of p38 by binding to the pocket of ATP binding, but does not inhibit phosphorylation from p38 by kinases upstream. As in our study, neurite growth is apparently regulated by p42 / p44 [62]. It has also been shown that the p42 / p44 pathway mediates neurite growth by netrin-1 [63].

Effect of mitogen activated protein kinases inhibitors on neuritic outgrowth in presence of taurine

The role of taurine in protein phosphorylation has been extensively studied [46-47]. Our results demonstrate that the MAPK signaling pathway is involved in cell size and cells neurite outgrowth. Figure 7 shows theeffect of PD 98059 on the trophic effect of taurineoncellsize and neuritic length. The cell size of all the cells was not modified by the addition of the inhibitor (Figure 7a, 7c). In contrast, it has a dual effect on the neuritic length of the cells, increases significantly at 10 µM and decreases significantly at 25 µM (Figure 7b, 7d). SB 203580 significantly increases cell size and neuriticlength in cells<12 µm (Figure 8a, 8b) and significantly decreases both parameters in cells> 12 µm (Figure 8c, 8d). The modifications of the trophic effect of taurine suggest that taurine could be acting as a growth stimulator by phosphorylating specific proteins.

5. Conclusion

IP3K and MAPK participate in the signaling cascade for the regulation of cellsize and neuritic growth in rat retina cells and in the trophic effect exerted by taurine on these parameters. Further studies are needed to clarify the precise mechanisms, but the present results allow starting to make connections between protein phosphorylation and taurine trophic effect in the retina.

6. Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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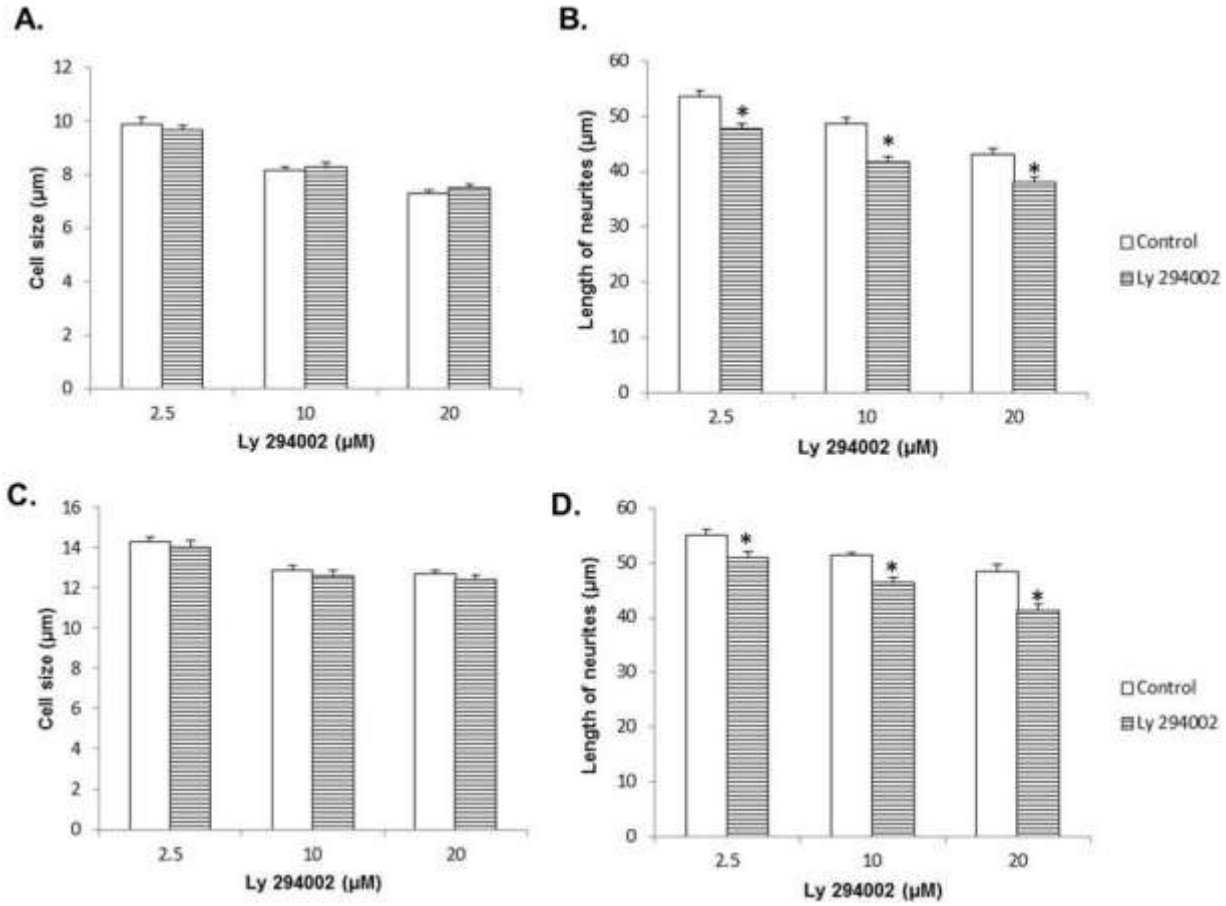

Figure 1: Effect of 2-morpholin-4-yl-8-phenylchromen-4-one (LY 294002) on retinal cell size and length of neurites.
Isolated cells were cultured for 5 days in minimal essential medium containing Ly 294002 at different concentrations (2.5, 10, 20 µM) on cells < 12 µm (a,b) and cells ≥ 12 µm (c,d). Control: DMSO. $^{*}P < 0.05$ with respect to control.

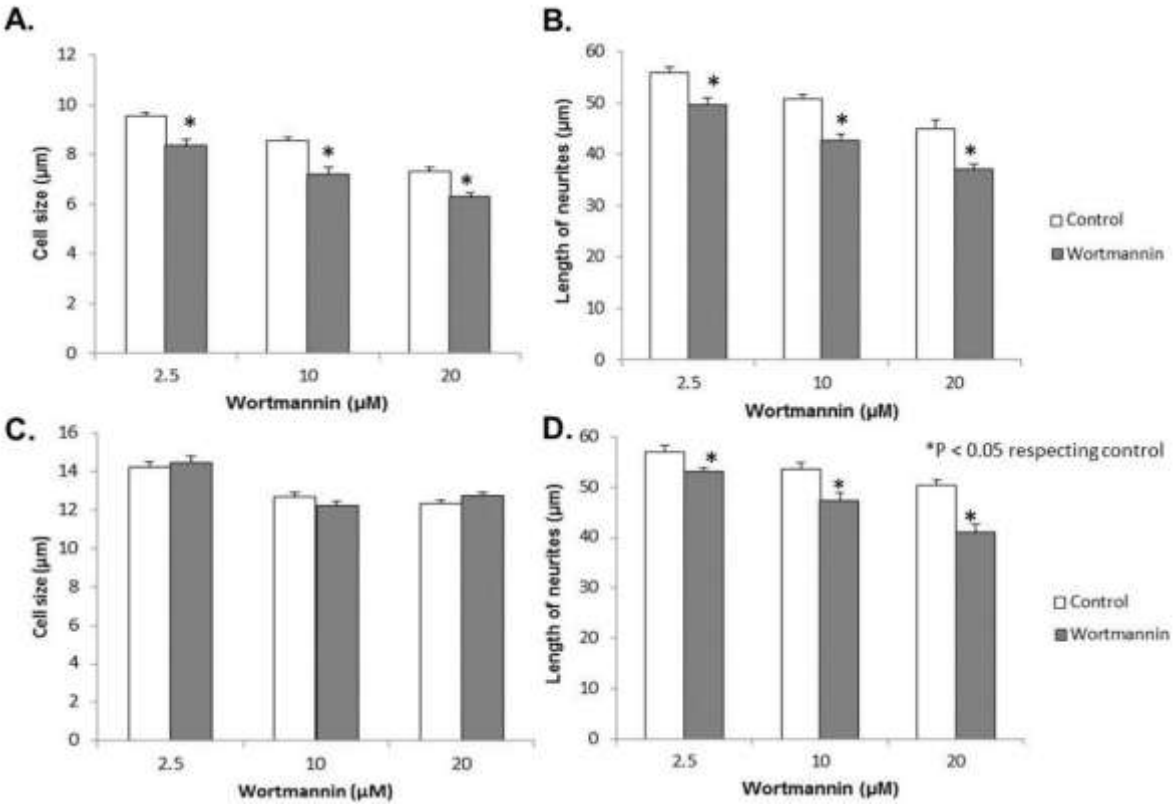

Figure 2: Effect of Wortmannin on retinal cell size and length of neurites. Isolated cells were cultured for 5 days in minimal essential medium containing Wortmannin at different concentrations (2.5, 10, 20 µM) on cells < 12 µm (a,b) and cells ≥ 12 µm (c,d). Control: DMSO. *P < 0.05 with respect to control.

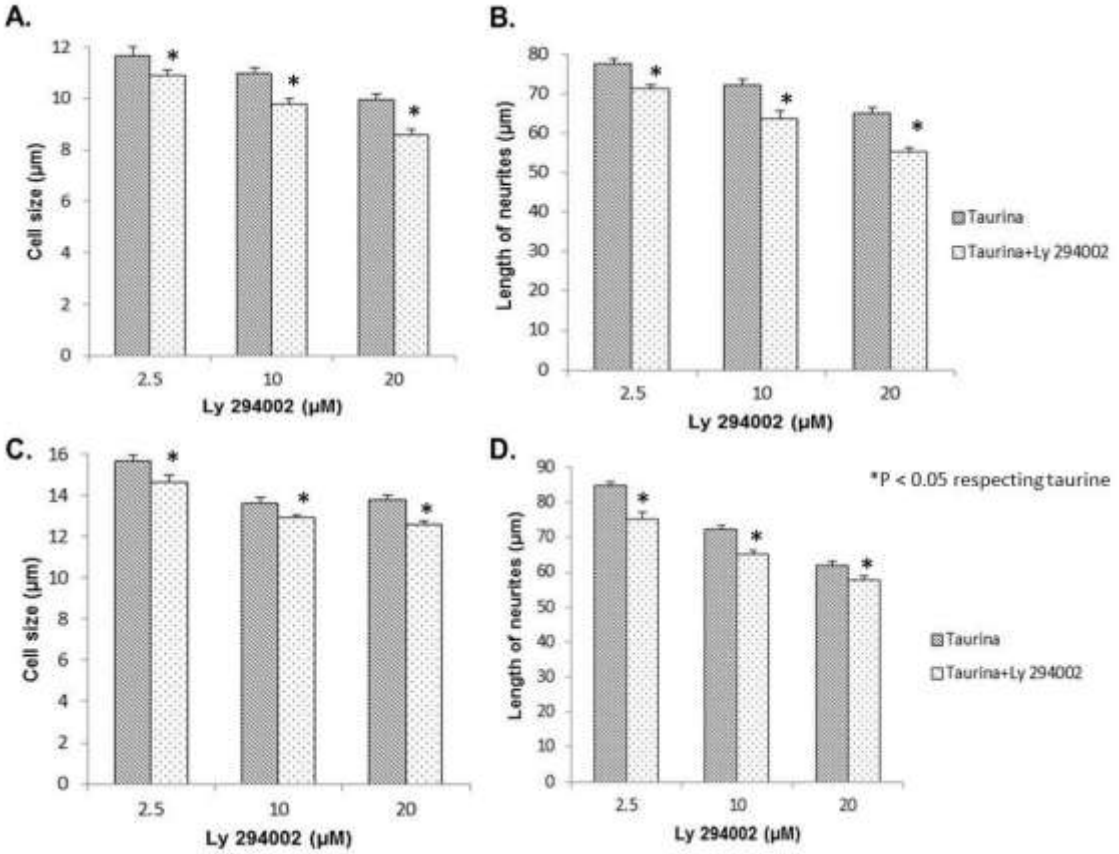

Figure 3: Effect of 2-morpholin-4-yl-8-phenylchromen-4-one (LY 294002) on retinal cell size and length of neurites in presence of taurine. Isolated cells were cultured for 5 days in minimal essential medium containing Ly 294002 at different concentrations (2.5, 10, 20 µM) in presence of taurine (4 mM) on cells < 12 µm (a,b) and cells ≥ 12 µm (c,d). *P < 0.05 with respect to taurine.

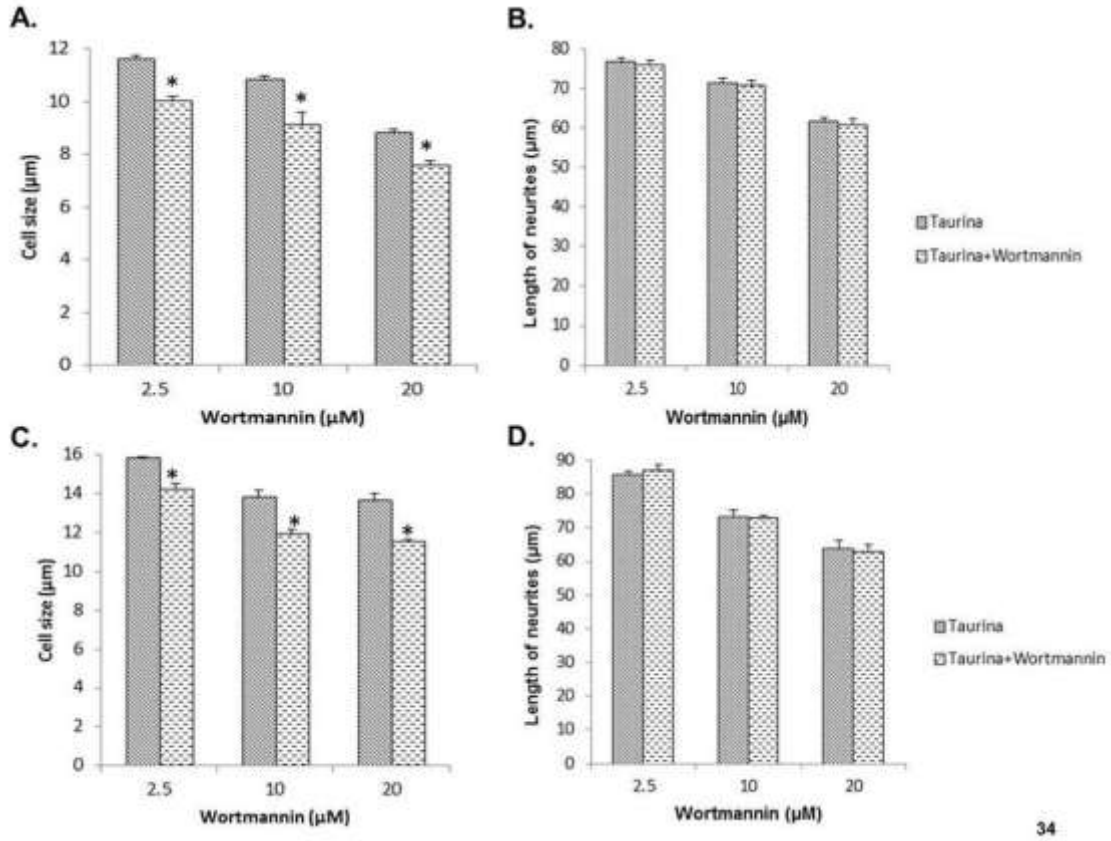

Figure 4: Effect of Wortmannin on retinal cell size and length of neurites in presence of taurine. Isolated cells were cultured for 5 days in minimal essential medium containing Wortmannin at different concentrations (2.5, 10, 20 µM) in presence of taurine (4 mM) on cells < 12 µm (a,b) and cells ≥ 12 µm (c,d). *P < 0.05 with respect to taurine.

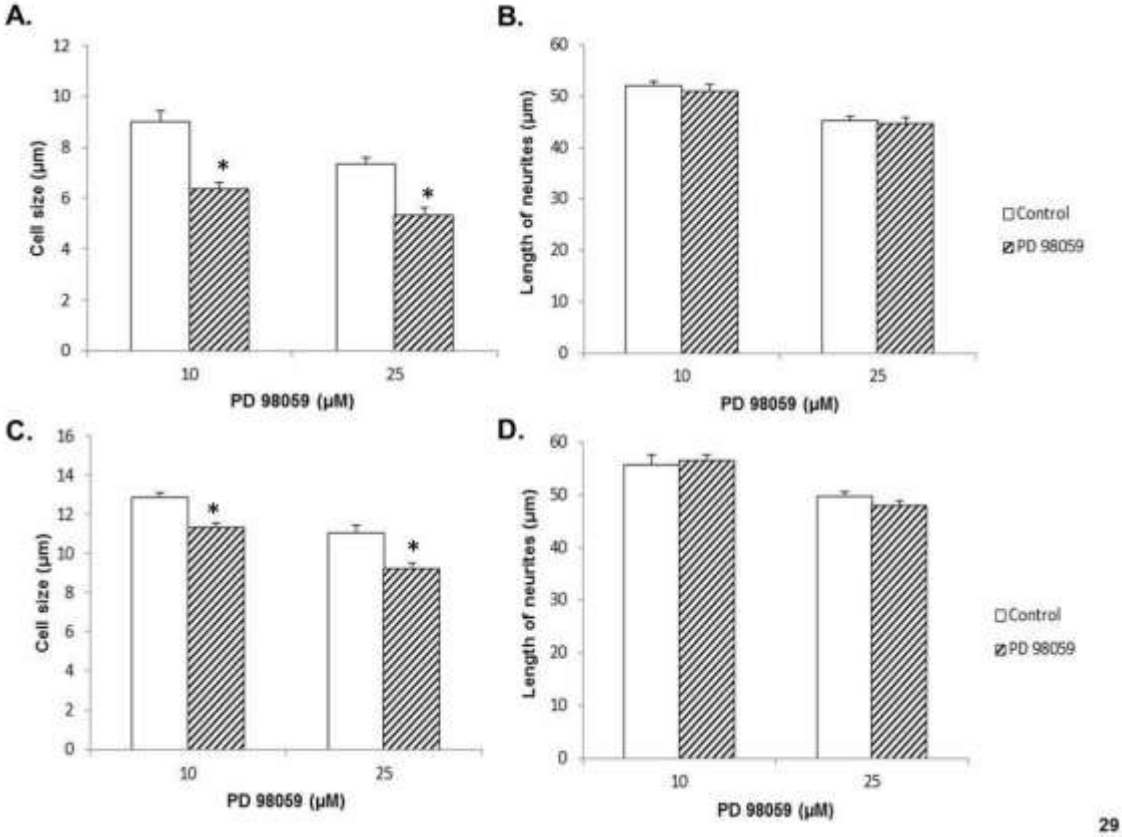

Figure 5: Effect of 2'-amino-3'-methoxyflavone (PD 98059) on retinal cell size and length of neurites. Isolated cells were cultured for 5 days in minimal essential medium containing PD 98059 at different concentrations (10, 25 µM) on cells < 12 µm (a,b) and cells ≥ 12 µm (c,d). Control: DMSO. *P < 0.05 with respect to control.

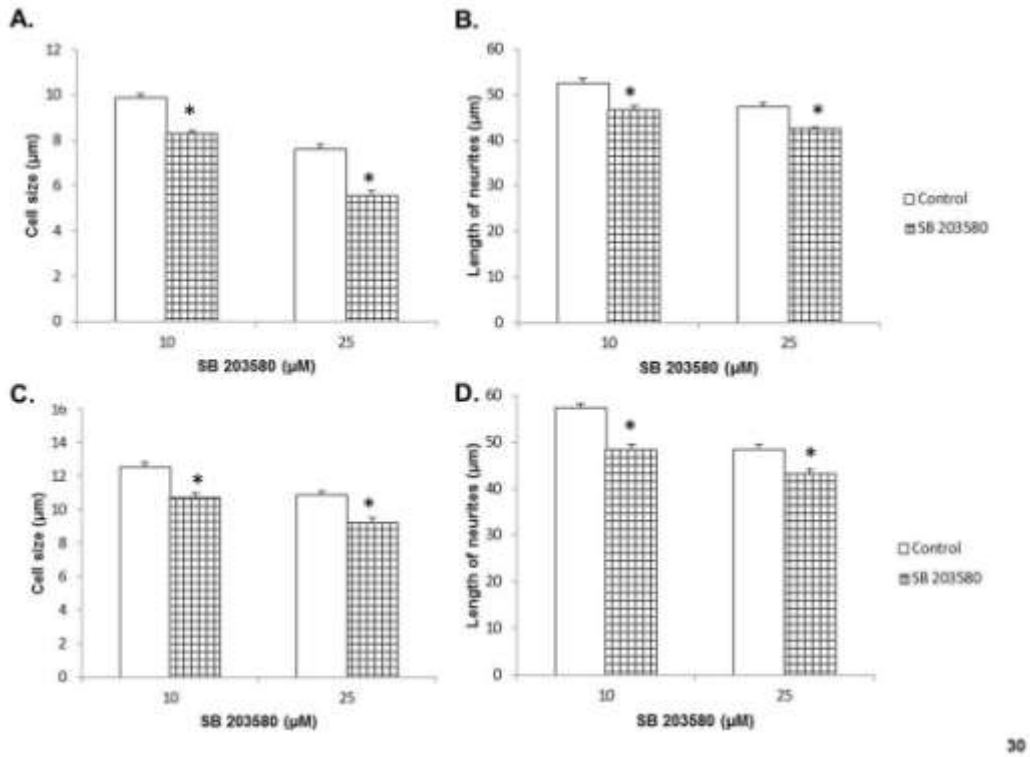

Figure 6: Effect of 4-(4-fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)-1H-imidazole (SB 203580) on retinal cell size and length of neurites. Isolated cells were cultured for 5 days in minimal essential medium containing SB 203580 at different concentrations (10, 25 µM) on cells < 12 µm (a,b) and cells ≥ 12 µm (c,d). Control: DMSO. *P < 0.05 with respect to control.

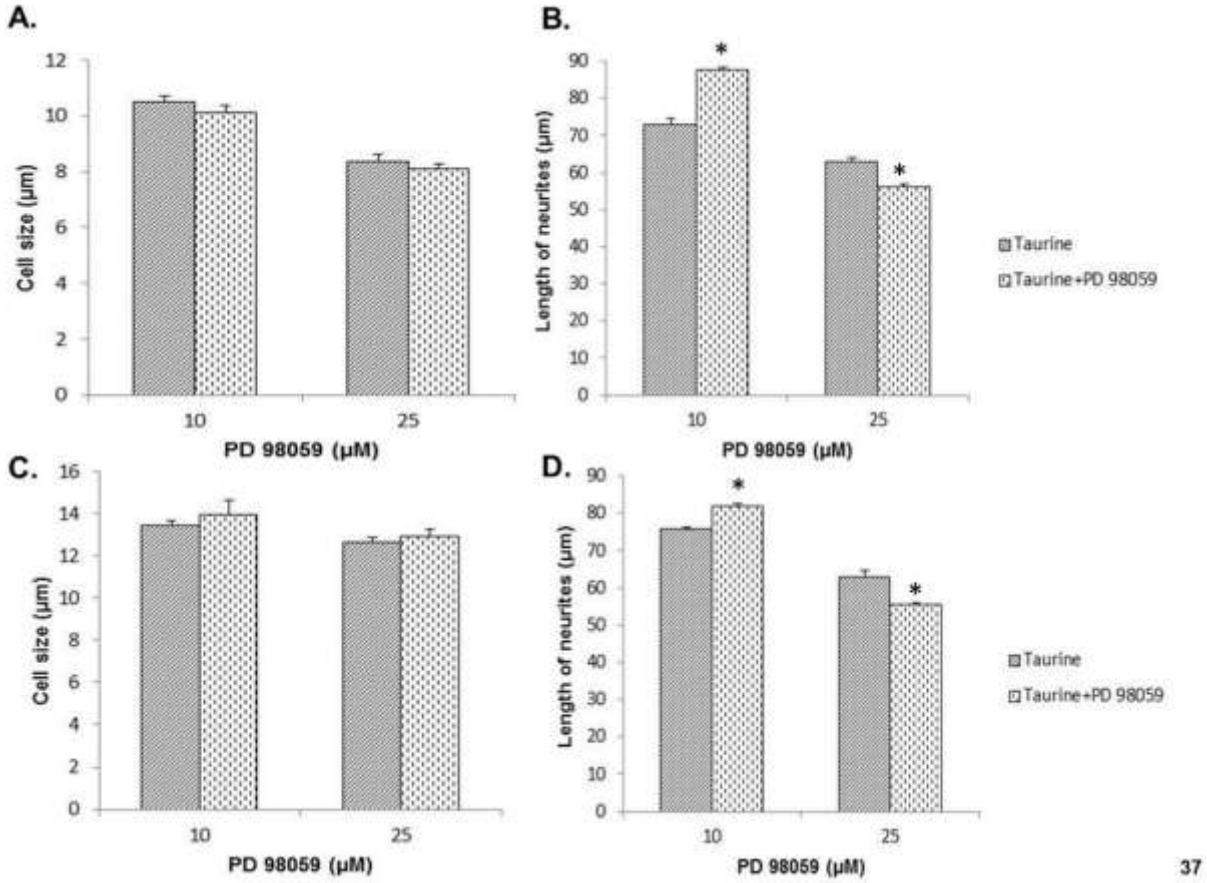

Figure 7: Effect of 2'-amino-3'-methoxyflavone (PD 98059) on retinal cell size and length of neurites in presence of taurine. Isolated cells were cultured for 5 days in minimal essential medium containing PD 98059 at different concentrations (10, 25 µM) in presence of taurine (4 mM) on cells < 12 µm (a,b) and cells ≥ 12 µm (c,d). Control: DMSO. *P < 0.05 with respect to taurine.

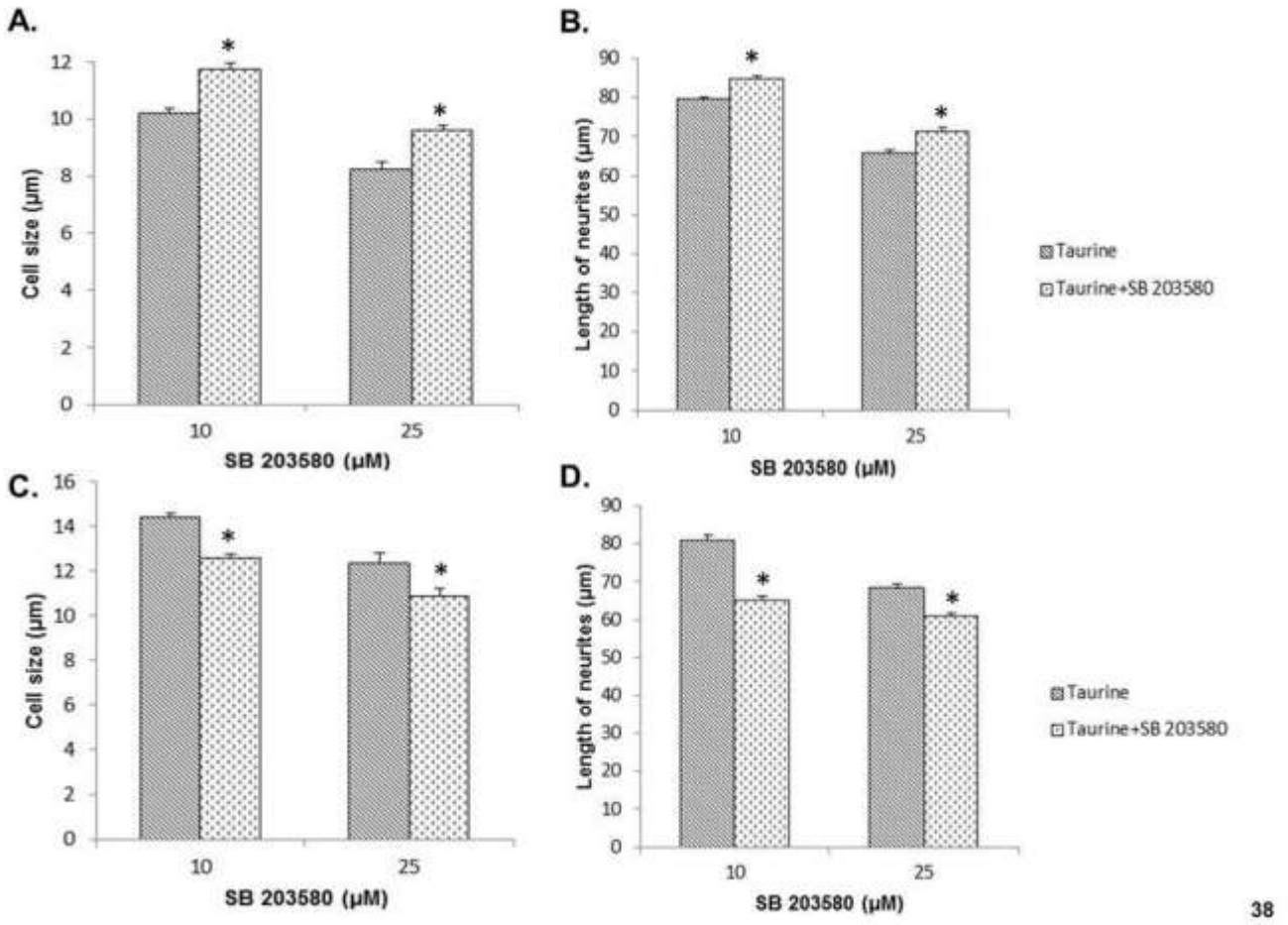

Figure 8: Effect of 4-(4-fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)-1H-imidazole (SB 203580) on retinal cell size and length of neurites in presence of taurine. Isolated cells were cultured for 5 days in minimal essential medium containing SB 203580 at different concentrations (10, 25 µM) in presence of taurine (4 mM) on cells < 12 µm (a,b) and cells ≥ 12 µm (c,d). Control: DMSO. $^{*}P < 0.05$ with respect to taurine.