Activation of caspase-3 pathway by expression of sGαi2 protein in BHK cells

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Abstract

Treatment with dopamine and other dopamine D2 receptor agonists has been shown to induce cell death through activation of caspase-3 pathway. However, initial step that leads to the activation of caspase-3 in D2 receptor-mediated apoptotic pathway remains unclear. Recently, it was shown that a spliced variant of Gαi2 protein (sGαi2) forms intracellular complex with D2 receptors by protein-protein interaction and that D2 drugs treatment causes the liberation of sGαi2 protein from complex. Now, we show that the unbound form of sGαi2 protein is able to activate caspase-3 pathway in baby hamster kidney (BHK) cells. Expression of sGαi2 protein in BHK cells led to the production of active form of caspase-3 and activation of p38 mitogen-activated protein kinase (p38 MAPK) and extracellular regulated kinase 1/2 (ERK1/2). Co-expression of sGαi2 with either D2 short (D2S) or D2 long (D2L) isoforms of dopamine D2 receptors blocked the activation of caspase-3 pathway. Thus, our results demonstrate that high level of unbound sGαi2 protein can affect the cell survival and engagement of this protein with D2 receptors can block this process. It is suggested that this process may be a crucial step in the initiation of D2 receptor-mediated cellular apoptosis through this pathway.

Besides its classic role in signalling within the nervous system, dopamine and its agonists have been shown to be apoptosis inducer to cell cultures of striatal neurons [5,13], pituitary GH3 cells [1,5], and olfactory neuronal cell lines [3]. Dopamine induces apoptosis in neuroblastoma cells primarily through generation of reactive oxygen species (ROS) and activation of p38 kinase, which in turn mediate the release of cytochrome c triggering in turn caspase-3 and -9 activation [4]. Further studies have shown that this effect of dopamine was mediated through dopamine D2 receptors [3,13]. Agonist activation of dopamine D2 receptors activates caspase-3 dependent cell death through p38 MAPK and ERK1/2 pathways [1,5]. However in contrary to this D2 receptor function in cell death, Bozzi et al. [2] have demonstrated a neuroprotective nature of dopamine D2 receptor by using knockout mice. Systemic administration of the kainic acid is a well-characterized model of epilepsy-induced brain damage. D2 deficient mice showed extensive hippocampal cell death after exposure to kainic acid, whereas, mice expressing D2 receptor did not. This neuronal death was indicative of apoptosis because fragmented DNA and activation of pro-apoptotic protein BAX were observed in these neurons [2]. Therefore, these results suggest that the expression of dopamineD2 receptors may prevent apoptosis, whereas activation of the same receptor can lead to cell death.

Recently, we have shown that D2 receptor interacts with a spliced variant of Gαi2 protein called sGαi2 protein [7,12]. This protein–protein interaction led to the formation of intracellular D2-sGαi2 complex. Dopamine D2 drugs treatment resulted in the activation of the complex and separation of both proteins. Dissociated D2 receptors were then localized to the cell surface. Based on these evidences, it was concluded that sGαi2 protein participates in the formation of intracellular reservoir of D2 receptors and in the regulation of the receptor density of cell surface [12]. However, in contrary to D2 receptors, fate of the dissociated or unbound sGαi2 protein is not known. Apart from this, we also observed that high level of sGαi2 protein significantly reduced the number of viable cells in cell culture. Therefore, considering the intimate relation of this protein with D2 receptors and further D2 receptor association with caspase-dependent apoptosis, we hypothesize that unbound sGαi2 protein may activate the caspase-3 pathway. Therefore, here, we have tested the activation of caspase-3 pathway with the goal to understand the function of unbound sGαi2 protein in D2-mediated cell death. Our results using cDNA transfection in cell lines show that sGαi2 protein could activate the caspase-3 pathway and that this activation was blocked by the over-expression of D2 receptors.

The preparation of pseudivirions was done as described previously [12]. In brief, full length cDNAs of sGαi2 (GenBank accession number AY677118), D2L (accession number NM000795) or D2S receptors (accession number NM016574) were cloned into pSin-Rep5 plasmid vector (Sindbis Expression System from Invitrogen) at Mlu I and Apa I restriction sites. After confirming the DNA sequence of inserts by sequencing, DNA was prepared using Wizard Maxi-Plasmid Preparation System (Promega). These recombinant DNA samples were further processed for in vitro transcription to produce RNA. The RNA product was quantified and stored at −80 ˚C.

For transfection of RNA, BHK cells were used. These cells were obtained from the American Type Culture Collection and were cultured at 37 ˚C in CO2 incubator. Cells were grown in αMEM medium supplemented with 2mMl-glutamine and 5% fetal bovine serum. 2×105 cells were seeded into six-well culture plates and incubated for 12–18 h until 80% confluency. For transfection, RNA lipid complexes were prepared by adding 9μl of liposome reagent (DMRIE-C from Gibco), 9μg of recombinant RNA (from above) and 9μg of helper RNA (Invitrogen) to 1ml of OPTI-MEM I. The lipid-RNA complexes were added to BHK cells in culture plate and incubated for 4 h. Following the incubation, transfection medium was replaced with complete growth medium and the cells were incubated for an additional 36 h. During this incubation period, packaged pseudovirion particles are released into the medium. The culture medium containing pseudovirions was used for the infection into BHK cells and protein expression.

For infection, BHK cells were grown to 70–80% confluency in 60mm tissue culture plates and sGαi2 pseudovirions (50μl containing 175,000 particles) diluted to 450μl were added in each well. After incubation at room temperature for 1 h, 4 ml of medium was included and cells were left in CO2 incubator for 60 h. Coinfection of sGαi2 and dopamine D2 receptors was performed by adding equal amount of both pseudoparticles. As control, infection with pseudovirions obtained from pSinRep5 plasmid (without any insert) was also performed.

Viable cells were counted after trypan blue staining according to the method of Invitrogen. 1×10^5 cells in 0.5 ml complete medium were mixed with 50_l of 0.4% Trypan blue stain (Invitrogen) and incubated at room temperature for 5 min. Both stained and non stained cells were counted. Non-stained cells were considered as viable cells and presented as percent of total number of cells.

The effect of varying amounts (0–90μl) of pseudovirions of sGαi2 and Gαi2 (as control) on the viability of cells was tested (see Supplement Fig. 1). In light of these results, we decided to use 50μl of pseudovirions of sGαi2 where viable cells counts were approximately 50% in the experiments of this manuscript. In addition, we also tested psudovirions corresponding to D2L, D2S and plasmid control. In cells infected with Gαi2, D2L, D2S or plasmid control, there was a reduction of 6–10% in the viable cells.

Polyclonal antibodies to sGαi2[7,11,12] and to both D2 receptors [9,10,12] were prepared in our laboratory. The specificity of these antibodies were determined [7,9,10,12]. Antibodies to Gαi2were from Santa Cruz Biotechnology. These antibodies recognize single polypeptide band of expected size on the Western blot analysis and the immunoreactive bands were completely abolished after the pre-absorption of the antibodies with their respective cognate peptides. Antibodies to cleaved (active form) caspase-3, phospho-p38 MAPK and phospho-ERK1/2 were obtained from the Cell Signaling Technology. In addition to the manufacturer data showing that these antibodies recognize a single protein band on blot assays in various cell lines after activation, we have also tested in BHK cells in our laboratory. We found that cleaved caspase-3, phospho-p38 MAPK and phospho-ERK1/2 antibodies react with 17 KDa, 43 KDa and 44/42 KDa proteins, respectively.

Immunoblots were done as described previously [6,8–12]. Solubilization of proteins from harvested intact cells was done with solubilization buffer provided in Seize X Mammalian Immunoprecipitation kit (Pierce). Solubilized proteins (40μg/lane) were separated by 10% SDS-PAGE and transblotted to PVDF membranes. Membranes containing proteins were incubated with 5μg/ml of sGαi2, D2S or D2L antibodies and 1:1000 dilution of cleaved caspase-3, phospho-p38 MAPK or phospho-ERK1/2 antibodies, followed by incubation with anti-rabbit IgG-HRP (1:2500; Amersham-GE). Bands were visualized by using an ECL Western Blotting Detection Reagents (Amersham-GE) according to the manufacturer's protocol.

This assay was performed according This assay was performed according to manufacturer manual with the use of ApoAlert Caspase-3 assay kit from Clontech. Briefly, BHK cells (2×106) were treated with 50μl of cell lysis buffer and after centrifugation, supernatant was collected. In cell lysate, 50μl of 2X Reaction Buffer/DTT Mixwas added followed by 5μl of 1mM caspase-3 Substrate (DEVD-pNA). After incubation at 37 °C for 1h in a water bath, samples were read in a spectrophotometer at 405nmto detect the level of chromophore pNA after its cleavage by caspase-3. The caspase activity was calculated using the standard calibration curve.

Data are presented as mean±S.D. Data were analyzed using two-way ANOVA. *p* Values less than 0.05 were considered statistically significant.

It has been shown that dopamine D2 receptors mediate cell death through caspase-3 activation and downstreamp38MAPKand ERK1/2 pathways [1,5]. Now, we show that

sGαi2 protein may be an important component of this D2 receptors-mediated caspase-3 activation pathway. We found that over-expression of sGαi2 protein in BHK cells leads to nuclear condensation (Fig. 1B) and significant loss in viable cell counts (Fig. 1C), as compared to cells transfected with plasmid control which showed normal appearance (Fig. 1A). Cells transfected with plasmid control displayed the same basal appearance as in the absence of plasmid. To further confirm the expression of sGαi2 protein, we tested the cell lysates of these cells by immunoblots analysis using antibodies to sGαi2 (Fig. 1D). sGαi2 was absent in cells infected with plasmid control but was labeled to an expected size polypeptide band of 41 KDa in cells infected with sGαi2 (Fig. 1D). Based on the finding that sGαi2 protein expression in BHK cells can provoke nuclear condensation and that sGαi2 protein interacts and forms intracellular complex, we next tested the activation of caspase-3 by immunoblots assay using antibodies against cleaved (active form) caspase-3 (Fig. 2A) and by caspase-3 activity assay using ApoAlert Kit (Fig. 2B). Cell lysates from both non-transfected cells and plasmid control infected cells showed no reactivity, however, cells infected with sGαi2 displayed immunoreactivity at 17 KDa polypeptide band corresponding to the size of active caspase-3 (Fig. 1A). Presence of a similar level of _-actin in all the samples suggests that reactivity of a band in sGαi2 sample is specific to sGαi2 protein expression. In addition to this, we also found 13-fold increase in the caspase-3 enzyme activity in cells expressing sGαi2 protein. These findings suggest that sGαi2 protein might be activating caspase-3 pathway in BHK cells. Therefore, we explored further into the activation of p38 MAPK and ERK1/2, known downstream components of dopamine D2 receptor-mediated apoptosis pathway [1,5]. To identify the active (phosphorylated) form of p38 MAPK and ERK1/2, we performed immunoblots assay. Antibodies to phospho-p38 MAPK (Fig. 2C) and phospho-ERK1/2 (Fig. 2D) showed reactivity to the correct protein size bands in cell lysates obtained from the cells infected with sGαi2. There was no reaction in the samples obtained from noninfected control and plasmid vector infected BHK cells but these cells revealed presence of _-actin at the same level as samples from sGαi2 infected cells. Previously, we have shown that intracellular interaction of sGαi2 with dopamine D2 receptor plays a crucial role in maintaining the intracellular D2 receptor pool and in controlling the D2 receptor density on plasma membrane [12]. However, apart from this, results shown here suggest that sGαi2 protein may also catalyze the cellular apoptosis pathway. Thus, it is argued that the interaction of sGαi2 with dopamine D2 receptors, may simultaneously serve two functions, one in regulation of receptor density at cell surface and other in prevention of cellular apoptosis. This prevention could be due to the formation of sGαi2 +D2 complex.

To test this concept, we co-expressed both sGαi2 protein and D2 receptor in BHK cells with the assumption that D2 receptor will occupy the sGαi2 protein and that this process will block the activation of caspase-3 pathway. Indeed, we found that co-expression of either D2L or D2S isoforms of D2 receptor prevented the activation of caspase-3 provoked by sGαi2 protein (Fig. 3A and B). This was confirmed by two methods: one by immunoblots (Fig. 3A) and other by caspase-3 enzyme activity measurement (Fig. 3B). Expression of D2 receptors alone did not show caspase-3 activation (Fig. 3A). In contrary to the observation made in cells expressing sGαi2 alone where there were nuclear

condensation and significant reduction in the number of viable cells (Fig. 1B and C), these co-infected cells showed no difference in appearance (Fig. 3C) and in the number of viable cells (Fig. 3D) when compared to normal non-infected cells or plasmid control infected cells. Thus our results clearly demonstrate that expression of sGαi2 protein provoked activation of caspase-3 pathway and the co-expression of dopamine D2 receptor blocked this activation. Earlier, we have reported that co-infection of sGαi2 protein and D2 receptor leads to intracellular sGαi2 +D2 complex formation and further treatment with D2 receptor agonists including dopamine breaks-up this complex and releases both proteins [12]. We found that released D2 receptors go on to localize at cell surface. And now with the consideration of current evidences, we suggest that liberated sGαi2 protein might activate caspase-3 pathway. It has been shown that dopamine and other D2 receptor agonists treatment produce D2 receptor-mediated apoptosis in various cell types [1,3,5,13]. Therefore, it is proposed that dopamine D2 receptor agonist treatment may generate unbound sGαi2 protein by breaking-up sGαi2+D2 complex and that this unbound sGαi2 protein can provoke activation of caspase-3 pathway of cell death. Though this mechanism may be of great importance in the survival of cells containing dopamine D2 receptors such as in case of Parkinson's disease where cell death of D2-cells in midbrain has been shown as a prime cause to this disease, increased expression of sGαi2 protein may be damaging to all cells. Conversely, the unavailability of free sGi2 can be a preventive step inD2-medatedapoptotic process. In literature, there are description of both apoptotic as well as neuroprotective functions of dopamineD2receptor [1,2,5]. These opposite functions could be explained by this mechanism where the balance between bound and unbound sGαi2 protein might play a critical role in the outcome of both of these D2-mediated actions.

Acknowledgements
We thank O. Civelli for cDNAs of dopamine D2L and D2S receptors and R. Reed for cDNA of Gαi2. This work was supported by grants BFU2006-0306 from MEC and CTS 586 from Junta de Andalucía.

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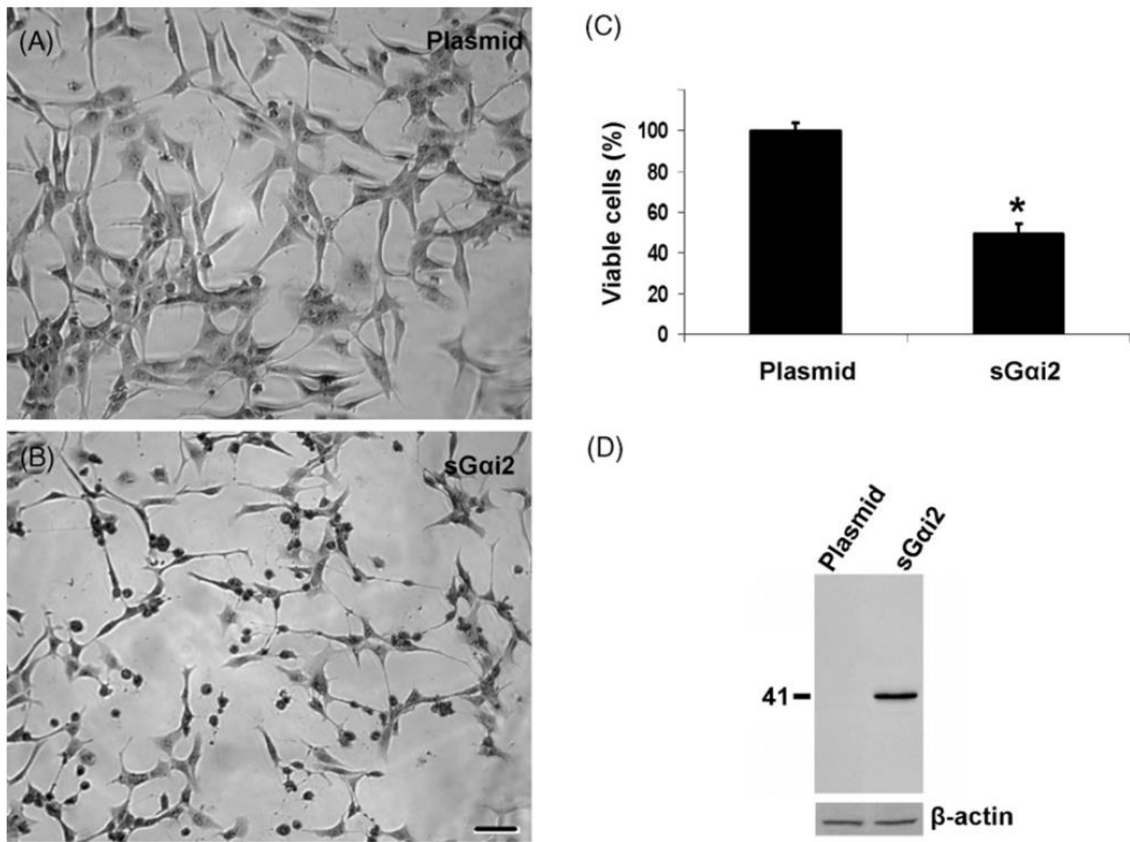

Fig. 1. BHK cells expressing sGαi2 protein show reduced number of viable cells. (A) Shows image obtained from cells infected with plasmid and (B) is from cells expressing sGαi2 protein. After trypan blue staining of both (A) and (B), stained and non-stained cells were counted. (C) Represents the percent viable cells (non-stained) in both cases. Expression of sGαi2 significantly reduced the number of viable cells. Data of concentration-dependent effect of sGαi2 and Gαi2 (as control) is shown in Supplement Fig. 1. (D) Shows the immunoblots of cell lysates from (A) and (B) with antibodies to sGαi2. Immunoblots result confirm the expression of sGαi2 protein in cells obtained from (B) and not from (A). Bottom image of (D) shows the presence of _-actin in both lanes. Values in plot (C) are shown as mean ± S.D. of five experiments. (*) values significantly different from control ($p < 0.05$). Scale bars in (A) and (B) are 12_m (shown in (B)).

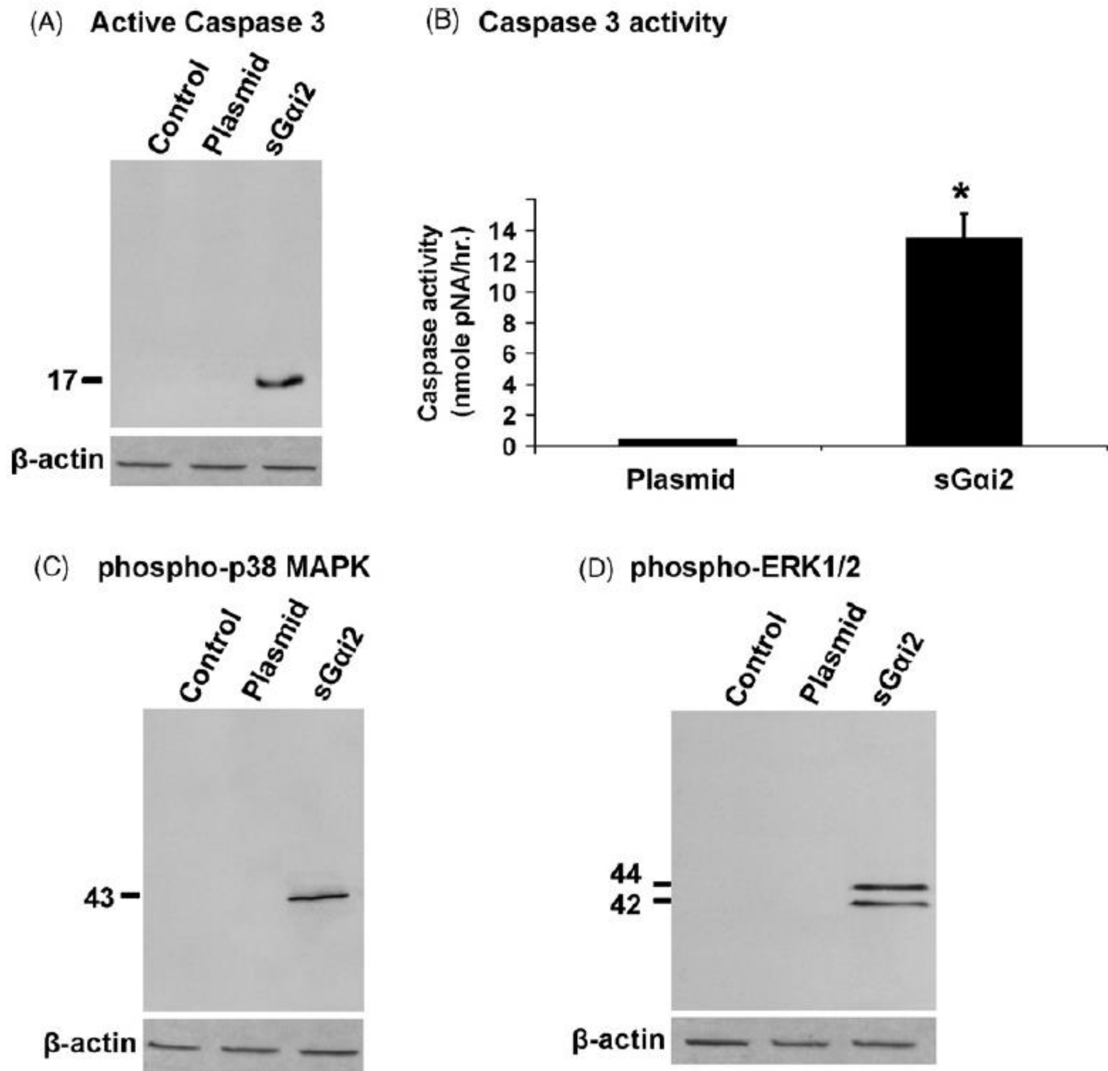

Fig. 2. Activation of caspase-3 pathway by expression of sGαi2 protein in BHK cells. Cleaved and active form of caspase-3was identified in immunoblots of cells infected with sGαi2 (A). Non-infected (control) and plasmid infected cells showed no reactivity, however, the presence of _-actin was detected in these cells. Activation of caspase-3 was also confirmed by the caspase-3 activity assay (B). The downstream activation of p38 MAPK and ERK1/2 was also observed. In immunoblot assays, cells with sGαi2 protein showed reactivity with antibodies to phospho-p38MAPK (C) and phospho-ERK1/2 (D). Values in plot (B) are shown as mean ± S.D. of five experiments. (*) values significantly different from control ($p < 0.05$).

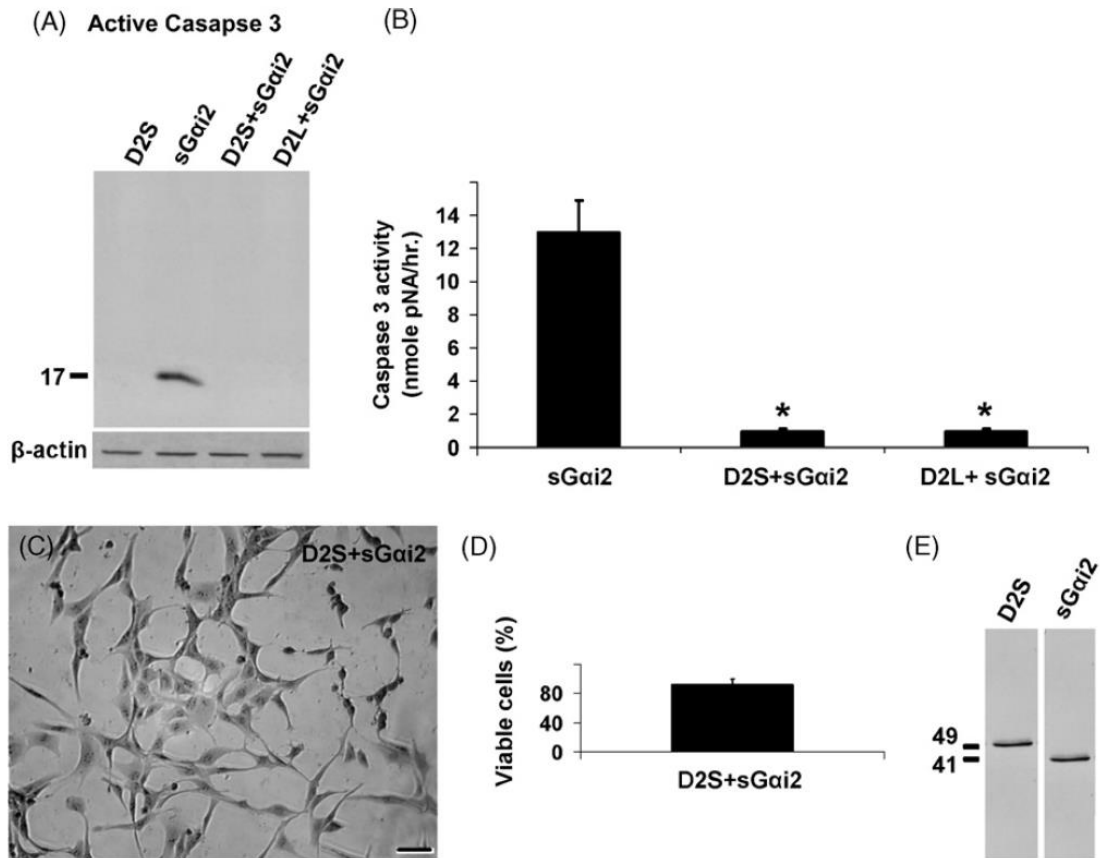

Fig. 3. Blockade of caspase-3 activation by co-expression of D2S or D2L in BHK cells. Cells infected with sGαi2 showed activation of caspase-3 (A and B), however, co-expression with either D2S or D2L blocked the caspase-3 activation. Presence of β-actin in all the samples was observed. (C) is the image of cells after co-transfection with D2S and (D) is the viable cell count of (C) after trypan blue staining. Both results (C and D) show that the blockage of caspase-3 activation normalized the cell morphology as well as the number of viable cells. As shown in Supplement Fig. 2A and B, co-expression of sGαi2 with D1 instead of D2 receptor posed no effect. E is immunoblots of the cell lysate from C to demonstrate the co-expression of D2S and sGαi2 protein. Co-expression of D2S with sGαi2 did not change the protein level in comparison to when sGαi2 was expressed alone (Supplement Fig. 2C and D). Values in plots (B and D) are shown as mean ± S.D. of 4-5 experiments. (*) values are significantly different from control ($p <$ 0.05). Scale bar in (C) is 12 μm.