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TRPC1 protects human SH-SY5Y cells against salsolinol-induced cytotoxicity by inhibiting apoptosis

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Abstract

Salsolinol, an endogenous neurotoxin, may be involved in the pathogenesis of Parkinson's disease. In this study, we sought to determine whether salsolinol-induced cytotoxicity in SH-SY5Y human neuroblastoma cells, a cloned cell line which expresses dopaminergic activity, could be prevented by overexpressing a Ca²⁺ channel, transient receptor potential (TRPC1) protein. Exposure of SH-SY5Y cells to 500 μM salsolinol for 12 h resulted in a significant decrease in thapsigargin or carbachol-mediated Ca²⁺ influx. Consistent with these results, SH-SY5Y cells treated with salsolinol showed approximately 60% reduction in TRPC1 protein levels. Confocal microscopy also showed that SH-SY5Y cells treated with salsolinol had a significant decrease in the plasma membrane staining of the TRPC1 protein. Interestingly, overexpression of TRPC1 increases TRPC1 protein levels and also protected SH-SY5Y neuroblastoma cells against salsolinol-mediated cytotoxicity as determined by 3, [4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay. The protective effect of TRPC1 was blocked by the addition of TRPC1 blockers lanthanum, or 2APB. Activation of TRPC1 protein by either thapsigargin or carbachol further protected SH-SY5Y cells from salsolinol treatments. Staining of SH-SY5Y cells with an apoptotic marker (YO-PRO-1) showed that TRPC1 protein protects against apoptosis. Furthermore, TRPC1 overexpression also inhibited cytochrome *c* release and decreased BAX protein levels required for apoptosis. Taken together, these findings suggest that the reduction in cell surface TRPC1 protein expression in response to salsolinol may be a contributory factor in cellular toxicity of the dopaminergic neurons. Furthermore, overexpression of TRPC1 could inhibit apoptotic complex thereby increasing neuronal cell survivability in Parkinson's disease.

Keywords

Salsolinol; Carbachol; Thapsigargin; Apoptosis; Neuroprotection

1. Introduction

The cause and underlying mechanism of chronic cell death of the neural tissues in Parkinson's disease (PD) remains elusive (Ebadi and Pfeiffer, 2005). Both exogenous and endogenous neurotoxic substances are known to provide partial explanation of these processes. 1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) is an exogenous neurotoxin producing parkinsonism in humans, monkeys and various animals as the result of MAOB-catalyzed conversion of it to the 1-methyl-4-phenyl-pyridinium ion (MPP⁺), which selectively kills the

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nigrostriatal dopaminergic neurons. On the other hand, various isoquinoline derivatives were found in the brain, and they are considered to be the endogenous neurotoxins with neurochemical properties similar to those of MPTP, which cause PD. Among them, 1-methyl-5,6-dihydroxy-TIQ (salsolinol) is believed to have the most potent neurotoxic action.

Salsolinol derivatives are endogenously formed from dopamine and aldehydes (Nagatsu, 1997). Elevated levels of salsolinol have been found in the brain, the cerebrospinal fluid and the urine (Moser et al., 1995; Maruyama et al., 1996, 1997) of patients with idiopathic Parkinson's disease (PD), which have also been proposed as biological markers of PD as they may result from an altered metabolism of dopamine in these patients. It has been shown that salsolinol can be synthesized *in vivo* by three different mechanisms, namely, non-enzymatic Pictet–Spengler condensation of dopamine with aldehydes leading to the formation of racemic salsolinol isomers; non-enzymatic condensation of dopamine and pyruvate to form 1-*carboxyl*-tetrahydroisoquinoline, followed by decarboxylation and reduction to form (*R*)-salsolinol; and enantioselective synthesis of (*R*)-salsolinol from dopamine and acetaldehyde by (*R*)-salsolinol synthase.

Several studies indicated that salsolinol is toxic to dopaminergic neurons *in vitro* as well as *in vivo*. Salsolinol is known to inhibit tyrosine hydroxylase and monoamine oxidase (Bembenek et al., 1983) as well as mitochondrial complex-I and complex-II enzyme activities (Morikawa et al., 1998). However, the precise biochemical and molecular mechanisms underlying the oxidative stress-mediated neurotoxicity of salsolinol is still poorly understood. Among several causative factors, oxidative stress is known to be a major contributing factor to the biochemical cascade leading to degeneration of dopaminergic neurons in PD. Recently, neuroprotection to halt progressive death of neurons has been proposed as a future therapy for neurodegenerative disorders. In disorders such as PD and AD, apoptosis contributes to neuronal death in most cases (Tatton, 2000) and the slow apoptotic processes have been proposed as a target of neuroprotection (Thompson, 1995; Naoi and Maruyama, 2001). Apoptosis is induced in neurons by various insults including oxidative stress, metabolic compromise, excitotoxicity and neurotoxins. Apoptotic signaling is a multistep pathway induced by opening a mitochondrial mega-channel called permeability transition (PT) pore, followed by decline in membrane potential, $\Delta\psi_m$, release of apoptosis-inducing factors, activation of caspases and fragmentation of nuclear DNA.

We have previously shown that TRPC1 expression is decreased upon treatment of MPP⁺ an exogenous neurotoxin which causes PD (Bollimuntha et al., 2005). However, because PD could also occur due to the presence of endogenous neurotoxins, this study was undertaken to define if similar decrease in TRPC1 levels is observed upon salsolinol treatment and whether overexpression of TRPC1 could protect against endogenous neurotoxins. Our results indicate that TRPC1 protects SH-SY5Y cells from salsolinol-mediated cytotoxicity by suppressing apoptosis induced by salsolinol in human dopaminergic neuroblastoma SH-SY5Y cells. Because PD is a slowly progressing neurodegenerative disease, associated with excitotoxicity and apoptosis, therapeutic strategies exhibiting anti-apoptotic potential could be developed as a possible target to treat PD.

2. Results

2.1. Salsolinol treatment decreases Ca²⁺ influx in SH-SY5Y cells

To determine the effect of salsolinol on Ca²⁺ influx, we treated SH-SY5Y cells with either SERCA pump blocking drug thapsigargin (Tg) or with muscarinic agonist carbachol. Fig. 1A shows Tg-stimulated [Ca²⁺]_i increase on control SH-SY5Y cells. Increase in [Ca²⁺]_i upon Tg stimulation in a Ca²⁺ containing media is a combination of intracellular release as well as influx from the TRPC1 channel representing the store-operated Ca²⁺ entry (SOCE) component. As

shown in Fig. 1A, control cells stimulated with Tg in a Ca^{2+} containing media (1 mM) showed an increase in $[\text{Ca}^{2+}]_i$, whereas SH-SY5Y cells pretreated with 500 μM of salsolinol (12 h) showed a significant decrease (~60% reduction) in Tg-stimulated $[\text{Ca}^{2+}]_i$ influx (Fig. 1A, for average data, see also Fig. 1D). To study whether salsolinol have an effect on internal stores, we performed Ca^{2+} imaging experiments in the absence of external Ca^{2+} . Importantly, Tg-stimulated internal Ca^{2+} release was not altered in SH-SY5Y cells treated with salsolinol (Fig. 1B). Thus, only the increase in SOCE was disrupted upon salsolinol treatment. To study if salsolinol treatment has any effect on agonist stimulation, we performed similar Ca^{2+} imaging studies. Control SH-SY5Y cells were stimulated with 1 mM carbachol (CCh) in a Ca^{2+} containing media. As indicated in Fig. 1C, addition of CCh to control cells lead to an increase in $[\text{Ca}^{2+}]_i$, which was significantly decreased in salsolinol-treated cells (Fig. 1C, for average data, see also Fig. 1D). Salsolinol-treated cells showed a 50–60% decrease in $[\text{Ca}^{2+}]_i$ as compared with the control-untreated cells. Also similar to Tg-stimulated internal release, release of Ca^{2+} from internal stores (measured in the absence of Ca^{2+}) was not altered (data not shown). Similar results were also obtained when SH-SY5Y cells were treated with MPP^+ (Fig. 1D). These results are also consistent with our previous finding, which indicated that MPP^+ treatment decreases TRPC1 protein levels (Bollimuntha et al., 2005). To have more evidence that salsolinol decreases Ca^{2+} influx and not due to altered efflux activity via PMCA, we measured Ba^{2+} influx. As indicated in Fig. 1E, addition of salsolinol significantly decreased Ba^{2+} influx. This decrease was comparable to that with Ca^{2+} influx. Overall, these results suggest that MPP^+ and salsolinol drugs both decrease agonist and Tg-stimulated Ca^{2+} influx, whereas no change was observed in the release of Ca^{2+} from the internal stores.

2.2. Effect of salsolinol on the expression of the TRPC1 protein

SH-SY5Y cells were incubated with salsolinol (500 μM) and expression of the TRPC1 protein was studied. As indicated in Fig. 2A, prolonged incubation with salsolinol (12 or 24 h) showed a decrease in the level of the TRPC1 protein. Densitometry on these bands revealed that 50–60% reduction of the TRPC1 protein was observed upon 12 h of treatment with salsolinol (Fig. 2D). In contrast the blots probed with anti-actin antibodies showed no significant decrease in the level of actin proteins upon treatment with salsolinol. Moreover, SERCA2 antibodies were also used, which did not show a significant decrease in SERCA2 protein levels. Thus, overall these results suggest that the decrease in the level of the TRPC1 protein is specific to salsolinol treatments. Localization of the endogenous TRPC1 protein displayed a punctate plasma membrane staining in SH-SY5Y cells (Fig. 2B). Incubation of SH-SY5Y cells without the TRPC1 antibody showed no staining (data not shown). These results are consistent with our previous findings that TRPC1 protein is expressed in the plasma membrane of epithelial cells (Liu et al., 2000; Brazer et al., 2003). Treatment of cells with salsolinol for 12 h significantly decreased plasma membrane staining of the TRPC1 protein (Fig. 2B). TRPC1 protein was predominantly localized in the cytosol upon salsolinol treatment. Similar results were also obtained upon 24 h of incubation with salsolinol where most TRPC1 protein was localized in the cytosol (data not shown).

2.3. Overexpression of TRPC1 in SH-SY5Y cells significantly increases TRPC1 protein and protects SH-SY5Y neuroblastoma cells from cell death

To evaluate the role of TRPC1 in SH-SY5Y cells, we transiently overexpressed TRPC1 protein using adenoviral method. Ad-TRPC1 (5 MOI) was used to infect SH-SY5Y cells. We have previously used this virus to overexpress TRPC1 protein (Singh et al., 2002). As indicated in Fig. 2C, western blots performed on crude membranes isolated from control-untreated cells detected TRPC1 protein. Whereas, cells treated for 12 h with salsolinol had a significant decrease in the TRPC1 protein level. This decrease in TRPC1 protein level was similar as observed in Fig. 2A. In contrast, salsolinol treatment on SH-SY5Y cells overexpressing TRPC1 (using Ad-TRPC1, 5 MOI) showed a significant increase in the levels of the TRPC1 protein

(Fig. 2C). SH-SY5Y cells infected with 5 MOI of control virus (Ad-Luciferase) showed no increase in the TRPC1 protein level upon 12 h of salsolinol treatment (data not shown). These blots were stripped and re-probed with actin antibodies, which showed no decrease in actin protein in all three sets of cells, indicating that overexpression of TRPC1 protein attenuates the effect of salsolinol. Quantification of these bands is shown in Fig. 2D.

To investigate if TRPC1 has a role in the protection of dopaminergic SH-SY5Y cells, we performed cell survival assay (MTT). SH-SY5Y cells were grown on a 96-well plate and treated with salsolinol both in control and transiently overexpressing TRPC1 cells. SH-SY5Y cells treated with salsolinol showed a decrease in the cell viability (~60% reduction in cells as compared with control-untreated cells) (Fig. 3A). Interestingly, SH-SY5Y cells overexpressing TRPC1 protein showed a significant increase in the cell viability (~90% cell survived, $P < 0.05$; Fig. 3A). To further examine the role of the TRPC1 in the protection of SH-SY5Y cells, we overexpress TRPC1 in SH-SY5Y cells and certain TRPC1 agonists as well as antagonists were studied. Activation of the TRPC1 protein using muscarinic agonist CCh or thapsigargin significantly increases protection of SH-SY5Y cells against salsolinol (Fig. 3B). However, pretreatment of SH-SY5Y cells with La^{3+} (a non-specific TRPC1 channel blocker) or an ER antagonist 2APB (which indirectly effect TRPC1 activity; Ma et al., 2000) significantly decreased TRPC1-mediated protection of SH-SY5Y cells (Fig. 3B).

2.4. Expression of the TRPC1 protein prevents SH-SY5Y cell death via inhibition of the apoptotic pathway

Salsolinol-induced cell death could occur via two pathways either via necrosis or by apoptosis. Thus, to understand the role of TRPC1 in the protection of SH-SY5Y cells, we examined the effect of TRPC1 overexpression in both these processes. Necrotic-mediated cell death was identified using propidium iodide staining and to differentiate cell death from apoptosis an apoptotic marker YO-PRO-1 was used. As indicated in Fig. 3C, control SH-SY5Y cells without salsolinol treatment showed very little cell death (2 cells/100 cells) (Fig. 3C, average data are shown in panel D). Whereas, cells treated with salsolinol showed both necrosis-mediated (15 cells/100 cells) and apoptosis-mediated (14 cells/100 cells) cell death. TRPC1 overexpressing SH-SY5Y cells showed a ~60% reduction in the apoptotic-mediated death of SH-SY5Y cells occurred in response to salsolinol (Fig. 3C, average data are shown in panel D). However, only ~20% reductions were observed in necrotic-mediated cell death in TRPC1 overexpressing cells treated with salsolinol. In aggregate, the results presented here strongly suggest that TRPC1 protects SH-SY5Y cells against salsolinol via inhibiting the apoptotic-mediated cell death.

To more directly demonstrate that TRPC1 has anti-apoptotic and neuroprotective activities; we investigated proteins necessary for the apoptotic-mediated cell death process. Consistent with our above results, TRPC1 has a profound role in regulating the proteins required for apoptotic pathway. As indicated in Fig. 4A, cytochrome *c* protein was present in the mitochondrial membrane fractions of control SH-SY5Y cells. Whereas, treatment with salsolinol decreases cytochrome *c* protein level in the mitochondrial membrane of SH-SY5Y cells (Fig. 4A, upper blot). In contrast, SH-SY5Y cells overexpressing TRPC1 showed a significant increase in the cytochrome *c* levels (in the mitochondria), treated with salsolinol (Fig. 4A, upper blot). Western blots using Bax antibody showed that the Bax protein levels were substantially increased in SH-SY5Y cells treated with salsolinol. This increase in Bax levels was again reduced in cells overexpressing TRPC1 protein. During apoptotic-mediated cell death, cytochrome *c* binds to the apoptotic protease-activating factor-1 (Apaf-1). This complex activates procaspase-9, resulting in caspase-mediated execution of apoptotic neuron cell death. Thus, we investigated Apaf-1 proteins level in all sets of cells. TRPC1 overexpression significantly decreased the amount of Apaf-1 protein levels in salsolinol-treated cells, suggesting that TRPC1 protects SH-SY5Y neurons by inhibiting the pro-apoptotic

complex. Taken together, the data in Figs. 3 and 4 demonstrate that overexpression of TRPC1 protects SH-SY5Y cells against salsolinol-mediated cytotoxicity by inhibiting proteins important for apoptotic process.

3. Discussion

Parkinson's disease is the only neurodegenerative disorder in which pharmacological interventions have resulted in marked decrease in morbidity and a significant delay in mortality. Nevertheless, the unrelenting progression of disease underlying PD and the degeneration of neuromelanin containing dopaminergic neurons in the substantia nigra have led to large numbers of proposed pathogenic factors, which include excessive generation of free radicals, impairment of mitochondrial function, production of inflammatory mediators and loss of trophic support causing apoptosis of dopaminergic neurons. Of all theories concerning PD pathogenesis, the oxidative stress hypothesis is the most firmly rooted in scientific literature and offers an attractive target for neuroprotective drug development (Ebadi and Pfeiffer, 2005). In the present study, we have investigated the role of TRPC1 in protecting against salsolinol-induced toxicity in human dopaminergic SH-SY5Y cells. TRPC channels have been found in many cell types, including both neuronal and non-neuronal tissues (Montell, 2003; Putney, 2004). Functionally, the most prominent cellular signaling pathways in which TRPCs play a role are mediated via phosphoinositide-mediated Ca^{2+} influx (Minke and Cook, 2002; Singh et al., 2004).

A number of studies suggested that alterations in cytoplasmic free Ca^{2+} are important contributory factors for apoptosis. Both increased (McConkey et al., 1989, 1991; Bellomo et al., 1992) and decreased cytoplasmic-free Ca^{2+} (Baffy et al., 1993; Magnelli et al., 1993, 1994) are known to play a major role in the initiation of apoptosis. Free Ca^{2+} in the cytoplasm is maintained at approximately 100 nM, whereas specific compartments such as the endoplasmic reticulum (ER) exhibit a several fold higher concentration (Berridge et al., 2000). Changes in cytoplasmic Ca^{2+} levels occur because of opening and closing of Ca^{2+} channels located at the ER and in the plasma membrane. The subsequent depletion of ER Ca^{2+} stores is considered to be main signaling mechanism in the activation of plasma membrane Ca^{2+} channels and the capacitative Ca^{2+} entry (CCE) channel.

In the present study, we showed that salsolinol treatment for 12 or 24 h significantly decreased TRPC1 protein levels. Confocal microscopic studies confirmed that 12 h of salsolinol treatment decreased TRPC1 localization to the plasma membrane. Interestingly, the remaining TRPC1 staining was found mainly in the cytosol upon salsolinol treatment, suggesting that this toxin disrupt TRPC1 localization to the plasma membrane. In order to see any change in Ca^{2+} levels after salsolinol treatment, we performed Ca^{2+} imaging studies. Addition of salsolinol significantly reduced thapsigargin (Tg) and carbachol-stimulated Ca^{2+} influx; however, no effect on the release of Ca^{2+} from internal stores was observed. These results support our above data indicating that salsolinol affect the Ca^{2+} influx by disrupting the TRPC1 channels in SH-SY5Y cells. However, the possibility of other Ca^{2+} channels being disrupted by salsolinol cannot be ruled out.

Because salsolinol is known to decrease cell viability, we hypothesized that the decrease in TRPC1 protein levels and Ca^{2+} influx could be associated with the decrease in cell viability, indicating that TRPC1 may be an important Ca^{2+} influx channel in dopaminergic neurons. Any decrease in this protein may severely impair cell viability. To further confirm the specific role of TRPC1 in preventing the loss of cell viability against salsolinol toxicity, we overexpressed TRPC1 in SH-SY5Y cells. The TRPC1 levels were significantly increased upon overexpression and salsolinol treatments did not decrease TRPC1 levels as observed in control SH-SY5Y cells. Moreover, a significant increase in cell survival was observed in TRPC1

overexpressing cells treated with salsolinol. These data suggest the specific role of TRPC1 in protecting the dopaminergic SH-SY5Y cells against salsolinol-mediated cytotoxicity. These results also suggest that activation of TRPC1 increases protection against salsolinol-induced cell death. Inhibition of the TRPC1 channel by either addition of La^{3+} or addition of an ER antagonist (2APB, which indirectly regulates TRPC1 activity) decreases TRPC1-mediated protection of SH-SY5Y cells. These results imply that Ca^{2+} release from internal cellular stores such as the endoplasmic reticulum, followed by the activation of the TRPC1 protein may be more important in salsolinol-induced cell death.

Although it has been suggested that the nigral dopaminergic neurons in PD die by apoptosis (Anglade et al., 1997; Mochizuki et al., 1996), our results indicate that salsolinol induced both apoptotic and necrotic-mediated cell death. The proteins encoded by the Bcl-2 gene family are known to play a major role in the regulation of apoptosis. Bax is a pro-cell death driving force within the central decision point at the onset of apoptosis, and the ratio of Bax to cell death repressors including Bcl- x_L modulates the activation of downstream effectors of cell death (Pettmann and Henderson, 1998). In the present study, we observed that exposure to salsolinol lead to an increased Bax expression, and overexpression of TRPC1 significantly decreased the level of the Bax protein; however, the increase in Bax levels was not greater as observed with MPP^+ treatment (Bollimuntha et al., 2005). Furthermore, salsolinol decreased mitochondrial cytochrome *c* levels and TRPC1 overexpression prevented the decrease of mitochondrial cytochrome *c*. Release of cytochrome *c* into the cytosol activates the caspase cascade of protease, which mediate the biochemical and morphological alterations characteristic of apoptosis. Interestingly, mitochondrial dysfunction has been reported in PD (Schapira et al., 1990). Increase in mitochondrial Bax protein expression and decrease in mitochondrial cytochrome *c* release by salsolinol suggest that mitochondrial dysfunction may play an important role in the cascade of cell death.

Ca^{2+} concentration is tightly regulated in neuronal cells. Disturbances in neuronal Ca^{2+} homeostasis have been implicated in a variety of neuropathological conditions. Several lines of evidence suggest that neuronal toxicity is not simply a function of increased $[\text{Ca}^{2+}]_\text{i}$. Treatments with AMPA or KCl can cause increases of up to 1–2 μM in $[\text{Ca}^{2+}]_\text{i}$ in neurons, without causing toxicity. However, equally high Ca^{2+} loads are toxic when entering via the NMDA channels, but not when entering via the voltage-dependent Ca^{2+} channels, suggesting that the source of increased $[\text{Ca}^{2+}]_\text{i}$ can be critical. Our results indicate that addition of salsolinol not only decreases TRPC1 protein levels but also affects both agonist and thapsigargin-stimulated Ca^{2+} entry. Moreover, a modest increase in $[\text{Ca}^{2+}]_\text{i}$ can be neuroprotective, whereas too much Ca^{2+} could be toxic, suggesting a ‘set-point’ mechanism for $[\text{Ca}^{2+}]_\text{i}$ effects. It has also been proposed that a critical component in neuronal damage is depletion of Ca^{2+} from the endoplasmic reticulum. Because depletion of the intracellular Ca^{2+} stores activates plasma membrane TRPC1 Ca^{2+} channels, it could be postulated that TRPC1 should have a significant role in PD.

In summary, this study provides the first evidence that treatments that cause Parkinsonism (salsolinol or MPP^+) have an altered Ca^{2+} influx and TRPC1 protein levels. Inhibition of TRPC1 could contribute in the activation of the pro-apoptotic pathways. Also reduction in the physiological $[\text{Ca}^{2+}]_\text{i}$ may trigger apoptotic process by activating caspase-3 (Moran et al., 1999). Our results further indicate that activation of TRPC1 is more important in protecting dopaminergic cells against salsolinol-mediated toxicity, indicating that this could be mediated by Ca^{2+} entry via the TRPC1 channel, which could regulate translocation of the key proteins necessary for apoptotic-mediated cell death. However, it remains to be seen whether TRPC1 activation further inhibits the translocation of the key proteins required for apoptotic cell death. Thus, future studies will elucidate the intracellular signaling mechanisms that are responsible

for these effects, identifying the causal relationship between loss of TRPC1 protein and mitochondrial dysfunction in PD.

4. Experimental procedure

4.1. SH-SY5Y cell culture, transformation and reagents

SH-SY5Y cells were obtained from the American Type Culture Collection (Manassas, VA, USA). They were cultured in a medium containing minimum essential medium, F-12 medium, HBSS (2:1:1) with 10% fetal bovine serum (Biofluids), 1 U/ml penicillin and 1 µg/ml streptomycin and maintained at 37 °C with 95% humidified air–5% CO₂. Culture medium was changed twice weekly. SH-SY5Y cells were maintained in complete media, until reaching 90% confluence, then trypsinized, centrifuged and resuspended in complete DMEM without phenol red at a concentration 5×10^6 cells/ml. Salsolinol was added to culture wells and was present during the duration of the experiment (12–24 h) unless otherwise noted. Agents being tested for protective/inhibitory effects were added 10 min prior to introduction of the toxic drug. SH-SY5Y cells were cultured and transfected as described earlier (Bollimuntha et al., 2005). Salsolinol and LaCl₃ were obtained from Sigma Biochemical (St. Louis, MO, USA). Thapsigargin, carbachol and BAPTA-AM were obtained from Calbiochem; 2APB was obtained from Tocris-Cookson.

4.2. Calcium measurements

SH-SY5Y cells were cultured on glass bottom coverslips (MaTeck Corporation) for 24 h and were treated for another 12 h with MPP⁺ or salsolinol. After incubation cells were incubated with 2 µM fura-2 (Molecular Probes) for 45 min at 37 °C under an atmosphere of 5% CO₂–95% air. The cells were washed twice with Ca²⁺ containing buffer (10 mM HEPES, 120 mM NaCl, 5.4 mM KCl, 1 mM MgCl₂, 1 mM CaCl₂, 10 mM glucose, pH 7.4). For fluorescence measurements, the fluorescence intensity of Fura-2-loaded control cells was monitored with a CCD camera-based imaging system (Compix) mounted on an Olympus XL70 inverted microscope equipped with an Olympus 40× (1.3 NA) fluor objective. A monochromator dual wavelength enabled alternative excitation at 340 and 380 nm, whereas the emission fluorescence was monitored at 510 nm with an Okra Imaging camera (Hamamatsu, Japan). The images of multiple cells collected at each excitation wavelength were processed using the C imaging, PCI software (Compix Inc., Cranberry, PA), to provide ratios of Fura-2 fluorescence from excitation at 340 nm to that from excitation at 380 nm (F_{340}/F_{380}). Analog plots of the fluorescence ration (340/380) in single cells are shown.

4.3. Vybrant staining assay

Vybrant Apoptosis Assay Kit (Molecular Probes, Eugene, OR) was used to evaluate apoptosis as per manufacturer's instruction. This kit can distinguish apoptotic and necrotic cells by propidium iodide dye and lipid dye (YO-PRO-1) staining. The cells were visualized using a fluorescence microscope using 10× objective. The dead and necrotic cells exhibit red fluorescence whereas apoptotic cells fluoresce green. The total and apoptotic cells were counted and the percentage of cells exhibiting apoptosis was calculated.

4.4. Membrane preparation and western blotting

SH-SY5Y cells were cultured and transfected as described earlier (Shavali et al., 2004). Cells were harvested, lysed and stored at –80 °C. Crude membranes were prepared from cell lysates (Lockwich et al., 2000). Mitochondrial enriched fraction (P2) was isolated as described by Muralikrishnan and Ebadi (2001). Protein concentration was determined by using the Biorad protein assay kit. Proteins were resolved on 4–20% SDS–PAGE gels and western blotting was performed (Singh et al., 2002). Anti-TRPC1, anti-Apaf1, anti-Bax, anti-SERCA2 and anti

Actin were used at 1:1000 dilutions. Peroxidase-conjugated respective secondary antibodies were used to label the proteins. Proteins were detected using ECL reagent and proteins on the membrane were analyzed using Lumiimager (Roche).

4.5. Confocal microscopy

For immunofluorescence, SH-SY5Y cells were grown on coverslips for overnight. Cells were washed with PBS and fixed for 30 min using 3% paraformaldehyde. Cells were then permeabilized using cold methanol and blocked for 20 min using donkey serum. For staining, cells were treated with TRPC1 antibody at 1:100 dilution, washed and labeled with rhodamine-linked anti-rabbit secondary antibody (1:100 dilution). Confocal images were collected using an MRC 1024-krypton/argon laser scanning confocal equipped with a Zeiss apotome photomicroscope.

4.6. Cell viability (MTT) assay

SH-SY5Y cells were seeded in 96-well plates at a density of 0.5×10^6 cells/well. The cultures were grown for 24 h followed by new medium containing salsolinol or MPP⁺. Cell viability was determined by MTT assay. Briefly, after incubation for 12 h with the desired drug, 30 μ l of MTT reagent (0.5 mg/ml MTT in PBS containing 10 μ M HEPES) was added to each well and incubated in a CO₂ incubator for 2 h. The medium was aspirated from each well and the culture plate was dried at 37°C for 1 h. The resulting formazan dye was extracted with 100 μ l of 0.04 N HCl in isopropanol and the absorbance was measured in a microplate reader (Molecular Device, Sunnyvale, CA) at 570 and 630 nm.

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Abbreviations

MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
PD	Parkinson's disease
MAOB	Monoamine oxidase B
AD	Alzheimer's disease
SOCE	Store-operated calcium entry
Cch	carbachol
Tg	thapsigargin

Fig. 1.

MPP⁺ and salsolinol decrease CCh- and Tg-stimulated TRPC1 activity. Ca^{2+} influx was measured in thapsigargin (Tg)- and carbachol (CCh)-stimulated SH-SY5Y cells with or without salsolinol treatment (12 h). (A, C) Fluorescence traces in either Tg (A)- or CCh (C)-treated cells in Ca^{2+} containing media. (B) Release of Ca^{2+} from intracellular stores in control and salsolinol-treated cells. (D) Average values. *Values that are significantly different from that of the respective control condition ($P < 0.05$, number of cells is indicated in each case). (E) Fluorescence traces of Ba^{2+} influx in control and salsolinol-treated cells.

Fig. 2.

Salsolinol treatment decreases TRPC1 levels whereas overexpression of TRPC1 protects SH-SY5Y cells. (A) Western blot on crude membranes prepared from SH-SY5Y cells treated with salsolinol (500 μ M) in a time-dependent manner (0, 12 and 24 h). Upper blot was probed using anti-TRPC1 antibody and the lower portion with anti-actin antibodies. (B) Anti-TRPC1 antibody and rhodamine-conjugated secondary antibody were used to detect endogenous TRPC1 protein in control- and salsolinol-treated cells. (C) Western blot on crude membranes prepared from control SH-SY5Y cells, or TRPC1 overexpressing cells treated with 500 μ M of salsolinol for 12 h. (D) Bar graph indicating quantitative analysis of TRPC1 and actin proteins.

Fig. 3.

Activation and overexpression of TRPC1 increases protection against salsolinol. (A) MTT assays were performed on control and TRPC1 overexpressing cells treated with 500 μ M of salsolinol. (B) Represents bar graph showing percent cell survival as detected via MTT assay. These data are an average of 3 independent experiments performed in triplicate. *Significant values ($P < 0.05$). Cells were transiently transfected (for 24 h) with Ad-TRPC1 encoding virus. Other details of the experiment are provided in the Experimental procedures section. CCh, Tg, La³⁺ and 2APB were added 10 min prior to the addition of salsolinol. Marker for necrosis (PI staining) and apoptosis (YO-PRO-1) were used for staining of control or cell overexpressing TRPC1 with or without salsolinol treatment (C). Rhodamine-conjugated propidium iodide and

FITC-conjugated YO-PRO-1 was added to control cells or cells treated with salsolinol for 12 h. Fluorescence images were taken immediately using either a 10× or 40× objective and the red and green cells were counted. (D) Represents mean bar graph from 700 to 900 cells in each group. *Values significantly different from its counterpart ($P < 0.05$).

Fig. 4.

Overexpression of TRPC1 decreases protein required for apoptotic pathway. (A) Western blot performed on mitochondrial membrane fractions. Membrane fractions (25 μ g) were resolved on a 4–20% SDS gel and probed with various antibodies as indicated. Bound cytochrome *c* in the mitochondria was assayed using anti-cytochrome *c* antibody (upper panel). Bax protein was identified using anti-Bax antibody. Similarly, Apaf1 and Actin antibodies were used to detect individual proteins, respectively. Details about the antibodies and method are described in the Experimental procedures section. (B) The proposed model for the neuroprotective role of TRPC1 in PD.