The G protein-coupled 5-HT₁A receptor causes suppression of caspase-3 through MAPK and protein kinase Ca

Tatyana Adayev, Indrani Ray, Rachna Sondhi, Tomasz Sobocki, Probal Banerjee*

Department of Chemistry and the CSI/IBR Center for Developmental Neuroscience, City University of New York at the College of Staten Island, 2800 Victory Boulevard, Staten Island, NY 10314, USA

Received 29 July 2002; received in revised form 21 January 2003; accepted 5 February 2003

Abstract

The 5-HT₁A agonist 8-hydroxy-2-(di-n-propylamino) tetralin (8-OH-DPAT) causes inhibition of caspase-3 and apoptosis via the extracellular signal-regulated kinases (ERK1/2) in hippocampal HN2–5 cells. Two 5-HT₁A agonists, Repinotan hydrochloride (BAY x 3702) and 8-OH-DPAT, block caspase-3 activation and apoptosis caused by anoxia/reoxygenation and H₂O₂ treatment. This is reversed upon transient expression of dominant negative Ras (N17Ras) and Raf-1 (Raf301), confirming the involvement of Ras and Raf-1 in this 5-HT₁A-R → ERK1/2 → caspase-3 pathway. A selective inhibitor of phospholipase C₁₁ (PLC₁₁) (U73122) but not a general protein kinase C (PKC) inhibitor (GFX) reversed the 5-HT₁A-R-mediated ERK1/2 stimulation. However, both GFX and the PKCa and PKCβ₁ inhibitor Gö6976 reversed the ERK1/2-mediated inhibition of caspase-3. ERK-dependent activation of only PKCa was observed in immunoprecipitates obtained from 5-HT₁A agonist-treated HN2–5 cells. Finally, transient expression of kinase-negative PKCa eliminated the 8-OH-DPAT-evoked block on the H₂O₂-triggered caspase-3 stimulation, establishing PKCa as a link between ERK and caspase-3 (5-HT₁A-R → PLC → ERK1/2 → PKCa → caspase-3). Our results elucidate a novel yet general, neuroprotective pathway through which G protein-coupled receptors could cause inhibition of effector caspases, such as caspase-3.

© 2003 Elsevier Science B.V. All rights reserved.

Keywords: 5-HT₁A receptor; BAY x 3702; ERK1/2; PKC; Caspase-3

1. Introduction

The heptahelical, serotonin 1A receptor couples mainly to pertussis toxin (PTX)-sensitive G proteins, such as G₁i and G₁o [1,2]. Among these interactions, the most extensively studied signaling is in the inhibition of adenylate cyclase through G₁i [3,4]. In addition, agonist binding to this receptor causes inhibition of N-type Ca-channels and activation of phospholipase C through distinct pools of G proteins [5,6].

Abbreviations: MAPK, mitogen activated protein kinase; PI-3K, phosphoinositol-3-kinase; PTX, pertussis toxin; AcDEVD-AMC, N-acetyl-Asp-Glu-Val-Asp-(amino-4-methylcoumarin); AcDEVD-CHO, acetyl-Asp-Glu-Val-Asp-aldehyde; AcYVAD-CHO, acetyl-Tyr-Val-Ala-Asp-aldehyde; Ca/CaM, CaM; PBS, phosphate buffered saline; Krebs–Ringer buffer; MEK, MAPK kinase; 5-HT₁A-R, serotonin 1A receptor; PD, PD98059; WAY, WAY100635; ERK, extracellular signal-regulated kinase; DMSO, dimethyl sulfoxide; GFX, GF109203X (bis-indolylmaleimide I)

* Corresponding author. Tel.: +1-718-982-3938; fax: +1-718-982-3944.
E-mail address: banerjee@postbox.csi.cuny.edu. (P. Banerjee).

More recently, the 5-HT₁A receptor has been shown to couple to G₂ in Sβ cells [7]. Serotonin 1A-receptor coupling to such variety of G proteins, however, has a common denominator—in all these receptor–G protein interactions, the G protein βγ complex is always released, which can then activate multiple effector molecules or pathways, such as the phospholipase Cβ → ERK1/2 pathway or the PI-3 kinase (PI-3K) pathway [8–11]. By contrast, the G protein α subunits faithfully couple to one effector molecule—e.g. Gα₁i couples negatively to adenylate cyclase and Gα₁o couples positively to adenylyl cyclase and Gα₁q couples positively to PLCβ.

As discussed earlier, in various cell types including neurons, agonist-stimulated release of the G protein βγ complex results in the activation of ERK1/2 enzymes, which are members of the mitogen-activated protein kinase (MAPK) family [10–12]. In this pathway, termed MAP kinase pathway, activation of multiple signaling proteins could precede the stimulation of the key enzymes ERK1/2, which finally regulates many important cellular events, such as mitosis or survival. Our earlier studies have demonstrated
that agonist stimulation of the 5-HT\textsubscript{1A} receptor causes up-regulation of the MAP kinase pathway, which results in an inhibition of the proapoptotic, cysteinyI aspartate-specific protease, caspase-3 [13].

Mitochondrial impairment caused by diverse types of tissue insults leads to apoptosis through a generally accepted pathway, which involves the release of cytochrome c from the impaired mitochondria, its association with the apoptosis-activating factor Apaf-1 and caspase-9, and subsequent partial activation of caspase-9 [14–17]. The partially activated caspase-9 undergoes autocatalytic cleavage to yield fully active caspase-9, which proteolytically activates the downstream caspase, caspase-3. Therefore, inhibition of this post-mitochondrial pathway is expected to cause attenuation of apoptosis caused by diverse conditions, such as anoxia/reoxygenation, neurodegenerative diseases, or excitotoxicity triggered by various conditions of neuronal injury, all of which are known to work through mitochondrial impairment. Earlier studies have shown that signaling from growth factor receptors can cause inhibition of apoptosis through PI-3K-mediated activation of Akt, which exerts its kinase activity to inhibit either the proapoptotic mitochondrial protein Bad [17] or the proapoptotic protease caspase-9 [16]. By contrast, our previous studies have shown that a G protein-coupled receptor, the 5-HT\textsubscript{1A} receptor, causes inhibition of caspase-3 and apoptosis by stimulating a novel, PI-3K-independent pathway in the hippocampal neuron-derived HN2–5 cells [11].

As mentioned earlier, in the circuitry of such complex mechanisms, caspase-3 is clearly the common downstream protease that has to be somehow inhibited to cause suppression of apoptosis. Since many conditions of neural insult and degeneration culminate in the activation of caspase-3, the 5-HT\textsubscript{1A}-R-mediated inhibition of caspase-3 could be used as a general tool to prevent neuronal apoptosis. This study establishes PKCa as a novel link between the MAP kinase pathway and caspase-3 and also introduces a measurable effect (readout), such as the 5-HT\textsubscript{1A} receptor-evoked caspase-3 inhibition. Such a readout could also be used to study the connectivity and hierarchy of the upstream members of the G protein-coupled receptor-activated MAPK cascade, such as the kinases like Src or Pyk2, which often connect G protein-coupled receptors to the MAPK pathway.

2. Materials and methods

The anti-active MAPK antibody was obtained from New England Biolabs (MA), and the ERK2 and other antibodies were obtained from Santa Cruz Biotechnology (CA). The vectors pZipneo, pZipneoN17Ras and pNMC, pNMCRaf301 were obtained as kind gifts from D. Foster (Hunter College, CUNY) and S. Gutkind (NIH), respectively. In immunoblotting analysis, the protein bands were detected by enhanced chemiluminescence using the Super Signal kit from Pierce (IL). The caspase substrate, acetyl-DEVD-(amino-4-methylcoumarin) (AcDEVD-AMC), the caspase inhibitor, acetyl-DEVD-aldehyde (AcDEVD-CHO) as well as PTX, WAY100635, and 8-OH-DPAT were obtained from Sigma/RBI. bis-indolylmaleimide I (GFX), Go6976, U73122 (a PLC\textbeta inhibitor) and PD98059 (an MEK inhibitor) were obtained from Calbiochem (CA). Repinotan hydrochloride (BAY x 3702) was obtained from Bayer Corporation (CT). PBS denotes phosphate-buffered saline.

2.1. Cell culture and anoxia

The mouse hippocampal neuron-derived HN2–5 cells were cultured to about 70–80% confluence in DMEM containing 10% fetal bovine serum and penicillin–streptomycin in poly-l-lysine-coated plates and then differentiated by 16 h (overnight) treatment with either phosphate-free Krebs–Ringer buffer (KRB) (25 mM HEPES, 20 mM glucose, 5 mM KCl, 1.28 mM CaCl\textsubscript{2}, 1.2 mM MgCl\textsubscript{2}, 145 mM NaCl, pH 7.4) or 5 mM retinoic acid in DMEM containing 1% fetal bovine serum for 24 h. Both processes confer to the cells neuronal morphology and strong immunoreactivity to the neurofilament protein-antibody SMI33 [18]. For both MAPK as well as caspase assays, cells in 10 cm plates were used. Following differentiation, the medium was changed to serum-free DMEM, supplemented with drugs (8-OH-DPAT, BAY x 3702, antagonists, inhibitors, etc.), and then, for caspase assays, the cells were subjected to anoxia as described in our previous report [11]. Prior to caspase assays, differentiated HN2–5 cells were subjected to 6 h anoxia followed by 16 h of reoxygenation.

2.2. MAPK assays

For MAP kinase (ERK2) assays, confluent, 10 cm plates of HN2–5 cells were differentiated over night in KRB, the medium changed to serum-free DMEM followed by drug treatment as described in the figure legends, medium aspirated and cells lysed in RIPA buffer (PBS containing 1% NP40, 0.5% sodium deoxycholate, 0.1% SDS, 0.5 mM Na\textsubscript{3}VO\textsubscript{4} plus freshly added protease inhibitor cocktail; Boehringer), immunoprecipitated using 1 μg/ml of MAPK (ERK2) antibody (polyclonal, Santa Cruz) and protein A-sepharose, immunoprecipitate resolved on a 7–16% gradient acrylamide gel, proteins transferred to nitrocellulose membrane, blocked in 5% solution of dry milk and then probed using a monoclonal, phospho-MAPK (P-MAPK) specific (anti-active) antibody at 1:1000 dilution and then with horseradish peroxidase-labeled anti-mouse IgG (1:1000). Following probing with P-MAPK antibody, the blot was stripped by incubating for 1 h at room temperature in 0.2 M glycine (pH 2.5), and then blocked in 5% dry milk and reprobed with ERK2 antibody (1:500). The ERK2 bands were used for normalization to confirm that the increase in P-MAPK bands was not due to an increase in the amount of ERK2 protein.
2.3. HOECHST33342 staining to assess apoptosis

Controlled treatment of cells with the membrane-permeable dye HOECHST33342 has been shown to effectively stain apoptotic nuclei and detect chromatin condensation [19,20]. HN2–5 cells were transfected with control vectors (pZipneo and pNMC) and those harboring the dominant negative mutants of Ras (N17Ras) and Raf-1 (Raf301), differentiated, treated with the agonist, and then subjected to hypoxia and reoxygenation. Following this the cells were fixed and permeabilized and then assessed for chromatin condensation and disintegration of the nucleus by Hoechst staining [19]. A 1 μM concentration of HOECHST33342 was used to label the cells for 15 min, following which the dye was washed out with 10 mM PBS and then pictures shot using a 360 nm excitation/420 nm emission filter. The apoptotic cells were identified by their disintegrated nuclear structure and counted to assess the proportion of healthy and apoptotic cells in multiple fields.

2.4. Caspase-3 assay using a fluorescent substrate

Caspase assays were performed using monolayers of HN2–5 cells at 80% confluence. Cell lysates were prepared from hydrogen peroxide-treated or normoxic and anoxic cell pellets obtained after two washes with ice-cold PBS. Cells were next lysed in 0.5 ml Yama buffer (10 mM HEPES pH 7.5, 142 mM KCl, 1 mM EGTA, 1 mM DTT, 0.2% NP-40, 0.1 mM PMSF) [21]. Each sample was homogenized with a Potter Elvehjem homogenizer and then ultracentrifuged at 100,000 × g for 5 min. The supernatant obtained was assayed for protein concentration, which was adjusted to 2 μg/μl and then an aliquot containing 300 μg protein was added to each well of a 96-well plate. This was followed by the addition of 1.5 μl of 10 mM stock AcDEVD-AMC in oxygen-free water (final concentration, 100 μM). The released fluorescence (due to the fluorescent group, AMC, which is cleaved by the caspases) was monitored after 30-min incubation using a fluorescence plate reader set at excitation wavelength 360 nm and emission wavelength 460 nm. Results from multiple caspase assays, each performed with triplicate samples, were combined after conversion into percentage of control caspase activity. Statistical analysis and determination of P values were carried out using Student’s t-test.

2.5. Protein kinase C (PKC) enzyme assays

Differentiated HN2–5 cells were treated for 30 min with 1 μM phorbol-myristate (PMA—a positive control), carrier or 1 μM 8-OH-DPAT in the absence and presence of 25 μM PD98059, following which the cells were lysed in RIPA buffer as described in MAPK assays. PKCα and PKCβ were immunoprecipitated from the cell lysates using the respective antibodies (Santa Cruz). The immunoprecipitates were washed thoroughly, and then resuspended in 10 μl PKC dilution buffer as provided in the Pierce PKC Assay Kit. This suspension was supplemented on ice with 15 μl of a pre-mixed reaction mixture containing equal volumes of 5 × reaction buffer, 5 × activator solution (saponicated in a bath sonicator prior to addition), PKC substrate which also contained 5 μCi/tube of AT[γ-32P]. The final volume with the protein A-Sepharose beads was 30 μl. The tubes were vortexed and then incubated at 30 °C for 30 min, followed by microcentrifugation and transfer of 25 μl of the supernatant to SpinZyme columns. The columns were microcentrifuged to elute the aqueous phase. The non-phosphorylated peptide was next washed off by addition of 250 μl of Phosphopeptide Binding Buffer (provided in the kit), incubation for 3 min, followed by centrifugation for 1 min at 6000 rpm. This procedure was repeated once for a total wash of 500 μl, and then the column was placed in a fresh tube before elution of the phosphopeptide adhering to the column. For the elution, 250 μl of Phosphopeptide Elution Buffer (provided in the kit) was applied to the column, followed by incubation for 3 min and centrifugal elution. This process was repeated once to obtain a total elution volume of 500 μl. Next, 20 μl of each eluate was mixed with 3 ml of scintillation fluid and subjected to scintillation counting using a Beckman scintillation counter.

2.6. Transfection of PKCα vectors and pEGFPC1 into HN2–5 cells

pEGFPC1 (2 μg) alone or 1 μg each of pEGFPC1 and SRDpkcαKN or pEGFPC1 and SRDpkcαWT were cotransfected into HN2–5 cells in 24-well plates [22,23]. Transfection was carried out using the SuperFect Transfection kit from Qiagen Inc., optimized for transfection of HN2–5 cells in 24-well plate without pre-plating. The cells (1 × 105 per well) were allowed to settle and attach in the wells for 5 h with the transfection complex containing 2 μg of total DNA and 5 μl of SuperFect reagent. Medium was then changed to the normal growth medium (10% fetal bovine serum and 1% penicillin–streptomycin in DMEM) and cells were allowed to recover for 19–24 h before differentiation. Differentiation was carried out in 5 μM retinoic acid in 1% fetal bovine serum containing DMEM for 24 h, following which the medium was changed to serum-free DMEM and appropriate wells were treated with carrier or 8-OH-DPAT in the absence or presence of 25 μM PD98059. H2O2 (200 μM) was added and the cells were incubated for 16 h in a tissue culture incubator as described in the following section.

2.7. Hydrogen peroxide treatment and caspase assays of cells

In all experiments, differentiated HN2–5 cells were used. Hydrogen peroxide was added from a freshly diluted and sterile filtered stock solution in serum-free DMEM. Typically, hydrogen peroxide treatment was carried out for 16 h before analyzing caspase-3 activity using either a fluores-
cent substrate (as discussed above) or immunocytochemistry. For the fluorescent substrate-based assays, HN2–5 cells grown in 10 cm plates to 70–80% confluence were differentiated and then subjected to H₂O₂ treatment in serum-free DMEM. Subsequently, the cells were pelleted down and subjected to caspase assays as described above.

### 2.8. Immunostaining to assess caspase-3 activation

In experiments requiring immunostaining, the cells were grown in 24-well plates, transfected as described above, differentiated, and then subjected to 200 μM hydrogen peroxide treatment for 16 h. Subsequently, the cells were fixed in freshly prepared 3% paraformaldehyde for 30 min at room temperature, washed two times with PBS, and then blocked in PBS containing 2% bovine serum albumin (Fraction V), 2% normal goat serum and 0.4% Triton X-100 for 1 h at room temperature. Next, the blocking solution was replaced with 1° antibody (CM1) diluted 1:3000 in blocking solution. After overnight treatment at 4 °C, the cells were washed three times with wash buffer (0.2% Tween-20 in PBS) at room temperature and then incubated with the 2° antibody (goat anti-rabbit IgG-Alexa Fluor 568) (1:2500) in blocking solution for 1 h at room temperature. After three washes with the wash buffer and then once with PBS, the cells were visualized by fluorescence microscopy using filters with emission wavelengths of 580 nm (for Alexa Fluor 568) and 510 nm (for green fluorescent protein).

### 2.9. Statistical analysis

After comparing multiple data sets, results were obtained as average (± S.D.), and “P” values were obtained using Student’s t-test.

### 3. Results

#### 3.1. The peak of 5-HT₁A-R-mediated activation of ERK1/2 occurs after 30 min of treatment with 100 nm BAY x 3702

Our earlier studies had established the time of optimal activation of ERK1/2 in 8-OH-DPAT-treated HN2–5 cells. Similar analysis of BAY x 3702-elicted ERK1/2 activation revealed a prominent increase in 5 min of agonist treatment, however, as observed for 8-OH-DPAT, the BAY x 3702-elicited ERK1/2 stimulation also reached a maximum in 30 min. However, following this, it decreased dramatically to virtually basal levels in 3 h (Fig. 1a and b). This is different from the prolonged activation of ERK observed in 8-OH-DPAT-treated HN2–5 cells [11] and could be attributed to the lesser chemical stability of BAY x 3702 in aqueous solutions. Dose–response analysis revealed that BAY x 3702-triggered ERK1/2 activation was quite significant even at 1 nM agonist concentration (Fig. 1c). Similar to the dose–response curve of 8-OH-DPAT, the ERK1/2 stimulation reached a maximum at 100 nM BAY x 3702 and then remained unchanged up to at least 1 μM.

The 5-HT₁A-R-mediated ERK1/2 stimulation was not affected by the PI-3K inhibitor wortmannin (Fig. 2a, lane 5) but was eliminated in the presence of PD98059 (Fig. 2a, lane 6), which inhibits MEK, the factor immediately upstream of ERK1/2 in the MAP kinase pathway [24,25]. This is an important difference from the protective pathways stimulated by other G protein-coupled receptors, which have been shown to involve PI-3K [25]. The non-involvement of PI-3K and the involvement of ERK1/2 in the 5-HT₁A-R-mediated inhibition of apoptosis had also been observed in our earlier studies [11].

Agonist stimulation of the 5-HT₁A receptor also causes a simultaneous increase in intracellular inositol phosphates, which is a direct measure of PLC activity [11]. Thus, there was a possibility that this increased PLC activity was essential for the observed activation of ERK1/2, and this is supported by earlier studies confirming that ERK stimulation by other G protein-coupled receptors requires activation of PLCβ upstream of Ras [12]. Elimination of the 5-HT₁A-R mediated activation of both ERK1/2 (Fig. 2a) and inhibition of caspase-3 (Fig. 2b and c) by the PLCβ inhibitor U73122 confirmed the involvement of PLCβ upstream of ERK1/2 [12]. Activation of PLC results in an increase in intracellular calcium concentration, which causes activation of PKC. PKC is known to phosphorylate and activate the signaling protein Raf-1, which stimulates ERK1/2 via the signaling protein MEK [24]. Therefore, a PKC isozyme could relay the signal from the activated PLCβ to activate ERK1/2.

---

Fig. 1. (a) Time course of BAY x 3702-evoked ERK2 stimulation. Differentiated HN2–5 cells were treated with BAY x 3702 (100 nM) for the indicated time periods. (b) Extended time course. The control was carrier-treated for 3 h, whereas the other samples were treated for the indicated time periods with BAY x 3702 (1 μM). (c) Dose dependence for BAY x 3702-evoked ERK2 stimulation. Lanes: 1, control; 2–5, treatment for 30 min with the following concentrations of BAY x 3702: 5 pM (lane 2), 1 nM (lane 3), 100 nM (lane 4), and 1 μM (lane 5). Data shown represent two independent experiments.
phospholipase molecule to ERK1/2. As shown in Fig. 2a, GFX (2 μM) did not affect the BAY x 3702-evoked ERK1/2 activation (Fig. 2a, lane 7). However, as shown in Fig. 2b and c, GFX did indeed block signaling downstream of ERK1/2 where PKC could be involved (explained below). Results presented in Fig. 2a elucidate that the cytoprotective, 5-HT1A receptor-mediated activation of ERK1/2 occurs not through a PKC→Raf-1 pathway, but most probably through a Ras→Raf-1→MEK→ERK1/2 cascade as reported earlier for the same receptor in non-neural cells [10].

Although data presented in Fig. 1 demonstrate that BAY x 3702 causes ERK1/2 activation in HN2−5 cells, it was essential to test if BAY x 3702 affords ERK1/2-mediated caspase-3 inhibition as 8-OH-DPAT does in HN2−5 cells [11]. As shown in Fig. 2b and c, BAY x 3702 was even more effective than 8-OH-DPAT in causing an ERK1/2-dependent inhibition of caspase-3 in HN2−5 cells. Caspase-3 levels were assessed by enzyme assays (Fig. 2b), as well as immunoblotting using the CM1 antibody (IDUN, CA), which recognizes only the 17 kDa fragment of proteolyzed and activated caspase-3 (Fig. 2c). We also asked if PKC was

Fig. 2. The 5-HT1A agonist-activated MAPK pathway causes a PKC-dependent caspase-3 inhibition, but PKC is not involved in the activation of ERK1/2. Differentiated HN2−5 cells were treated for 30 min with carrier or 8-OH-DPAT (1 μM) (D) or BAY x 3702 (1 μM) (B) in the absence and presence of GFX (2 μM) (GFX), or PD98059 (25 μM) (PD), WAY100635 (1 μM) (WAY), or U73122 (1 μM) (U). (a) Differentiated HN2−5 cells were treated for 30 min with either carrier (lane 1), or 8-OH-DPAT (1 μM) (lane 2), or BAY x 3702 (1 μM) in the absence (lane 3) or presence of WAY100635 (1 μM) (lane 4), wortmannin (0.1 μM) (lane 5), PD98059 (25 μM) (lane 6), GFX (lane 7), or U73122 (1 μM) (lane 8). In a separate set, the cells were treated with 1 μM U73122 alone (lane 9). Next, the cells were lysed and subjected to immunoprecipitation using an ERK1/2 antibody followed by Western blotting using an anti-active ERK1/2 antibody. In a separate set of experiments, cells were subjected to 6 h hypoxia and 16 h reoxygenation, following which cell lysates were either (b) subjected to caspase assay or (c) analyzed by Western blotting using the anti-active caspase-3 antibody (CM1). Decreased presence of the 17 kDa fragment of active caspase-3 indicates inhibition of caspase-3 activity. Data presented (a) are the mean (± S.D.) of three independent experiments performed with triplicate samples. Results are representative of three independent experiments. Normalized intensities of bands shown in (a) and (b) are graphically shown.
involved downstream of ERK1/2 in this cytoprotective pathway. Although the broad-range PKC-inhibitor GFX eliminated the BAY x 3702-elicited suppression of caspase-3 (Fig. 2b and c), as shown Fig. 2a (lane 7), the same concentration of GFX (2 μM) did not affect the BAY x 3702-evoked ERK1/2 activation. Therefore, our results collectively show that, unlike PLCβ, which is involved upstream of ERK1/2, PKC is involved downstream of ERK1/2 in the 5-HT1A agonist-triggered inhibition of caspase-3 in HN2–5 cells.

3.2. Involvement of Ras and Raf-1 in serotonin 1A receptor-mediated protection of HN2–5 cells against anoxia-triggered apoptosis

Our previous studies had established that 5-HT1A receptor-mediated activation of ERK1/2 is an obligate step in the protection of HN2–5 cells from anoxia and reoxygenation [11]. However, the link between the G protein-coupled, 5-HT1A receptor and ERK1/2 was not clear. One possibility was that the 5-HT1A receptor was connected to MEK and ERK1/2 through the growth factor pathway, i.e. through Ras and Raf-1. However, since direct activation of Raf-1 through phosphorylation by receptor-activated PKC (and Ras-independent activation of ERK1/2, thereafter) has been reported [20], we sought to test the involvement of Ras in the 5-HT1A receptor-mediated protection of HN2–5 cells by selectively inhibiting Ras and Raf-1 activity through transient expression of the corresponding dominant inhibitory mutants, N17Ras and Raf301, respectively. By cotransfecting pEGFP, which encodes a photoenhanced mutant of the green fluorescent protein (GFP), approximately 60–80% transfection efficiency was recorded. Additionally, it was observed that transfection of a relatively large amount (20 μg/10⁷ cells) of plasmid encoding N17Ras (pZipneoN17Ras) or Raf301 (pNMCRaf301) killed all cells within 3–5 days. Both of these observations confirmed that the transfection efficiency was high enough for the recording of a downstream effect after partially inhibiting Ras and Raf-1 in the HN2–5 cells by using lower amounts (10 μg) of these plasmids.

Control transfection was achieved using the vectors without the insert cDNAs (pZipneo and pNMC), and 10 μg of each plasmid DNA along with 10 μg of pEGFP were transfected into 10⁷ HN2–5 cells by electroporation. Our assays were performed 96 h after transfection by electroporation at 220 V and 1000 μF in serum-free DMEM, when the maximum recovery of cells was observed. Additionally, the transfected and post-differentiation HN2–5 cells were more fragile to anoxia and reoxygenation than the untransfected but post-differentiation HN2–5 cells. Thus, in contrast to only 20–30% of apoptosis in the untransfected HN2–5 cells, about 50–80% apoptosis was observed in the transfected HN2–5 cells. In the control cells (transfected with pZipneo and pNMC), apoptosis was inhibited in a dose-dependent manner by BAY x 3702 (Fig. 3a and b).

Since agonist-stimulated PLCβ activity results in IP3 synthesis, which eventually causes calcium release from the ER, we postulated that intracellular calcium might play an important role in the transmission of the ERK signal to caspase-3. To test this hypothesis, we investigated if the PKC isozyme involved was either of the calcium-sensitive molecules, PKCα or PKCβ, by using a selective inhibitor of PKCα and PKCβ, (Gö6976, Calbiochem). It was observed that even as little as 1–5 nM
concentration of Gö6976 completely reversed the 5-HT1A receptor-mediated inhibition of the anoxia-induced caspase-3 activity, and this effect was observed using both 8-OH-DPAT (Fig. 4a) as well as BAY x 3702 (Fig. 4b) as agonist. Therefore, the 5-HT1A-R-mediated caspase-3 inhibition involved a calcium-dependent PKC, probably PKCα or PKCβ.

3.4. PKCα but not PKCβ is stimulated downstream of ERK1/2

Although the above experiments demonstrate that activation of a calcium-sensitive PKC is essential for the ERK1/2-mediated inhibition of caspase-3, the suppression of caspase-3 could still be due to synergistic action of the activated forms of PKC and ERK1/2 (Fig. 5, mechanism 2). In fact classical G protein coupled receptor pathways show that stimulation of PLCβ by the receptor-activated G protein results in the production of diacylglycerol (DAG) by PLC-catalyzed hydrolysis of phosphoinositide-bis-phosphate. DAG is a potent activator of PKC. Thus, it was possible that PKC was activated in a parallel pathway by the ligand-bound 5-HT1A receptor. Following this, both PKC and MAPK could act together in a synergistic manner on a downstream target to finally cause inhibition of caspase-3 (Fig. 5, mechanism 2). Such synergism between activated PKC and ERK1/2 would preclude activation of PKC by ERK1/2.

The next experiment was designed to test the possibility described above and also to identify the PKC isozyme involved in this 5-HT1A-R-mediated caspase-3 inhibition. To achieve this, we immunoprecipitated two typical, calcium-dependent PKC isozymes, PKCα and PKCβ, from the lysates of 5-HT1A-agonist-stimulated and control HN2–5 cells. These immunoprecipitates were next tested for PKC activity using a PKC-assay kit (Pierce) and an MBP peptide as substrate. Agonist (8-OH-DPAT) stimulation of the cells caused a dramatic stimulation of PKCα but not PKCβ (Fig. 6). Moreover, this 5-HT1A agonist-evoked activation of PKCα was eliminated in the presence of the selective inhibitor of the MAPK pathway PD98059 (Fig. 6). Thus, PKCα is likely to be the isozyme involved in the 5-HT1A-R-mediated signaling to caspase-3. Also, PKC is not activated in a parallel pathway; rather, it is downstream of the 5-HT1A-R-ERK1/2 pathway, such that blocking ERK1/2 activation could eliminate the stimulation of PKCα (Fig. 5, mechanism 1).

**Mechanism 2**

**Mechanism 1**

Fig. 5. Possible mechanisms of 5-HT1A receptor-mediated inhibition of caspase-3.

PKCα was eliminated in the presence of the selective inhibitor of the MAPK pathway PD98059 (Fig. 6). Thus, PKCα is likely to be the isozyme involved in the 5-HT1A-R-mediated signaling to caspase-3. Also, PKC is not activated in a parallel pathway; rather, it is downstream of the 5-HT1A-R-ERK1/2 pathway, such that blocking ERK1/2 activation could eliminate the stimulation of PKCα (Fig. 5, mechanism 1).

![Fig. 4. A calcium-dependent PKC isozyme is involved in the 5-HT1A receptor-mediated inhibition of caspase-3-like activity. (Go=Gö6976). Differentiated HN2–5 cells were treated with (a) 8-OH-DPAT (1 μM) or (b) BAY x 3702 (1 μM) in the absence and presence of GFX (2 μM) or increasing concentrations of Go. Results represent the mean (± S.D.) of two discrete experiments performed with triplicate samples.](image-url)
pretreatment of the differentiated HN2–5 cells attenuated this caspase-3 activity by 40% (Fig. 7b). Also, this caspase-3 inhibition was eliminated upon inhibition of the MAPK pathway with PD98059 (PD), or inhibition of PKC with GFX (Fig. 7b). Therefore, it is possible that 8-OH-DPAT-evoked caspase-3 inhibition in anoxia/reoxygenation as well as H2O2-treatment occurs through a ERK1/2 and PKC-dependent pathway as shown in Fig. 5 (mechanism 1).

3.4. A kinase negative mutant of PKCα reverses the 5-HT1A receptor-mediated blockage of H2O2-triggered caspase-3 activation

To confirm that the observed 8-OH-DPAT-evoked check on the H2O2-triggered caspase-3 activation specifically required PKCα, we performed transient expression of a kinase-negative mutant of PKCα in the HN2–5 cells (Fig. 8). The GFP was cotransfected to identify the transfected cells by fluorescence microscopy. After transfection, the cells were differentiated as in the previous experiments and then subjected to 200 μM H2O2 treatment for 16 h followed by fixing of cells, and immunostaining using the same anti-active caspase-3 (CM1) antibody as used for Western blotting in an earlier experiment (Fig. 2c). Unlike the enzyme assays for caspase-3 presented earlier, immunostaining does not give a quantitative representation of caspase-3 activity. So, the H2O2 concentration was adjusted to 200 μM (instead of 500 μM) to obtain a greater number of cells at the initial stages of caspase-3 activation. This procedure allowed us to carry out an effective analysis of the effect of 8-OH-DPAT on caspase-3 activation in the HN2–5 cells. Representative images show punctate anti-active caspase-3 staining (red) in the perinuclear regions, which indicates proteolytic cleavage of caspase-3 following secretion of cytochrome c and Apaf1 from the mitochondria (Fig. 8a). Flat cells with...
reduced caspase-3 activity, visible only as a faint, diffused, red staining were considered to be negative in terms of caspase-3 activation. It should be noted from our caspase-3 assays that caspase-3 activation is inhibited but not completely eliminated in the presence of 8-OH-DPAT. While cells transfected with GFP alone or GFP plus SRDpkcaWT showed the expected inhibition of CM1 antibody staining upon 8-OH-DPAT pretreatment (red), cells cotransfected with the kinase-negative mutant of PKCα (SRDpkcaKN) showed complete reversal of this 8-OH-DPAT-evoked attenuation of anti-active caspase-3 staining (Fig. 8b). Such elimination of 8-OH-DPAT-evoked suppression of caspase-3 was also observed in cells pretreated with the MEK inhibitor PD98059. Data obtained from this study and that shown in Fig. 7 confirmed that the general, 5-HT1A-R-mediated protective pathway observed in the HN2–5 cells indeed involved PKCα downstream of ERK1/2 as proposed earlier in Fig. 5 (mechanism 1).
4. Discussion

Apoptosis is an important event that is essential for embryonic development as well as efficient removal of irreversibly damaged cells. This signaling program has been studied extensively in blood cells, and as a result, major biochemical mechanisms for the apoptotic program have been established. Simultaneously, research on growth factors has revealed the existence of a receptor tyrosine kinase-activated PI-3K pathway, which eventually causes inhibition of this apoptotic programming, such that some of the partially injured cells can be revived [15,16]. This is particularly important in the central nervous system, since, typically, after a stroke or neuronal injury caused by other insults, delayed death of a large number of neurons is observed, which often results in the loss of cognitive and motor functions. Additionally, during embryonic development, a large number of pre-neuronal cells undergo apoptosis to promote survival of neurons that have already made synaptic connections. Beneficial effects of apoptosis are also observed when chemotherapeutic agents eliminate cancer cells.

Receptor-mediated regulation of apoptosis could serve as an effective way of protecting brain neurons after a stroke or brain injury. As is true for the PI-3 kinase pathway, the MAP kinase cascade, which is stimulated by the growth factor receptors, is also linked to the protection of various cell types against apoptosis. Intriguingly, the isoforms ERK1/2 and p38 of MAPK that are stimulated at the end of the MAPK pathway have been linked to the inhibition and promotion of apoptosis, respectively [27]. Although earlier studies have demonstrated the importance of the MAPK pathway in protection against apoptosis [27,28], molecular events connecting this pathway to apoptosis are not clear. Results presented here provide a direct demonstration of MAPK-dependent inhibition of an enzyme, caspase-3, which actually causes the signature cytoskeletal breakdown that defines apoptosis.

Our studies in the hippocampal neuron-derived cell line HN2–5 has revealed a novel link between the 5-HT1A receptor and the proapoptotic enzyme caspase-3 through the important enzyme PKCα. It had been reported by multiple research teams that inhibition of PKC with either staurosporine or PKCα-targeted ribozyme causes apoptosis [29,30]. If PKCα is indeed a downstream target of ERK1/2, then the activity of this protein should be regulated by ERK1/2. Our immunoprecipitation experiments confirm that 8-OH-DPAT treatment of HN2–5 cells indeed causes activation of PKCα via ERK1/2 (Fig. 6). If this occurs by direct ERK1/2-catalyzed phosphorylation of PKC, then the particular PKC isoform involved should have one or more consensus ERK1/2 phosphorylation sites. Earlier studies have revealed that the PKCα molecule is phosphorylated at Thr638, Thr638, and Ser657 residues. Following activation by membrane attachment, these phosphate groups are removed by a PKCα-activated phosphatase and the dephosphorylated PKCα is rapidly degraded [31]. Thus phosphorylation is important for proper activation and turnover of the PKCα molecule. Of these phosphorylation sites, the Thr638 residue is within a MAPK recognition sequence. Therefore, it is possible that the PKCα molecule is regulated by ERK1/2 through direct phosphorylation.

The mechanism of PKCα-mediated caspase-3 inhibition also requires further analysis. Earlier studies have shown that drugs which activate PKC promote cell survival [32], and inhibitors of PKC, such as staurosporin, induce apoptosis [29,33,34]. It has also been observed that activators of PKC stimulate phosphorylation of Bad at serine 112, indirectly, through a downstream target, p90Rsk [35]. Although each of Bad, caspase-9 and caspase-3 harbors one or more copies of the PKC phosphorylation sequence (S/TXR/K), recent studies have shown that a PKC substrate, p90Rsk, actually catalyzes PKC-dependent phosphorylation of Bad at Ser112, which ablates interactions of Bad with Bcl-2, thus blocking apoptosis [35,36]. Therefore, phosphorylation of Bad, caspase-3 or caspase-9 could be a reason for the inhibition of caspase-3.

Caspases are kept under check in some viruses that harbor genes encoding caspase inhibitory proteins. In this way, such viruses suppress host defense mechanisms that otherwise would eliminate virus-infected cells by apoptosis. The first inhibitor of apoptosis protein (IAP) was discovered in baculoviruses [37]. Subsequently, cellular IAP homologs were found in many animal species [38,39]. Each IAP molecule contains one to three copies of an 80-amino acid baculovirus IAP repeat (BIR), which are potentially involved in Zn2+ ion coordination via a DX2CXXC/HX6C consensus [40]. Additionally, BIR proteins also contain a C3HC4 RING finger [39]. Mammalian IAP proteins (XIAP, NAIP, cIAP1, cIAP2, and survivin) block apoptosis by inactivating the terminal effector caspases caspase-3 and -7 and by interfering with upstream activation of caspase-9 [39,41,42].

A likely mechanism of inhibition of caspase-3 involves NFκB mediated induction of IAP expression. Cowen et al. [43] have shown that agonist activation of the 5-HT1A-R causes activation of NFκB. Also, it has been reported that PKC activation could cause stimulation of the transcription factor NFκB, which in turn causes induced expression of the IAPs [44,45]. As discussed earlier, the IAPs are known to block caspase-3 activity and apoptosis. Therefore, it is possible that a 5-HT1A-R-mediated NFκB activation causes induced expression of IAPs, thereby resulting in an inhibition of caspase-3.

There has been a considerable debate over the molecular signals that relay the effect of any receptor-mediated increase in intracellular calcium ([Ca2+]) to the MAPK pathway in various cell types (Fig. 5). Our earlier studies had implicated [Ca2+]i in the 5-HT1A-R → MAPK signaling in HN2–5 cells [11]. Other reports in diverse cell types have shown the involvement of the calcium-sensitive signaling molecules like Src and Pyk2 in receptor-mediated MAPK
signaling [9,46]. Our investigations to complete this link between the 5-HT1A-R-mediated increase in [Ca2+]i and the MAPK pathway in the HN2–5 cells will be reported elsewhere.

We have demonstrated that the 5-HT1A receptor-mediated blockage of insult-triggered caspase-3 activation requires PKCa. Collectively, this study elucidates a novel, neuroprotective pathway, 5-HT1A-R → G protein → PLCβ → Ca2+ → → → MAPK → PKCa → caspase-3 (inhibition), placing PKCa in a novel role as a signaling molecule linking the MAPK pathway to caspase-3. Selective activation of PKCa over PKCβ in the HN2–5 cells indicates that specific PKC molecules are sequestered in the cytosol as a sequel to agonist activation of a receptor. Current literature is replete with examples of such channeled transmission of molecular signals in the neural and other cell types. Further investigation of this pathway would reveal new mechanisms and proteins linking G protein-coupled receptors to ERK1/2 activation and caspase-3 inhibition in neuronal cells.

Acknowledgements

We wish to thank Dr. D. Foster and Dr. S. Gutkind for DNA vectors. Technical assistance by Mr. Matthew Sclar and Mr. Oleg Rivkin and Ms. Karen Mungroo is appreciated. We are grateful to Dr. Anu Srivinavan and the IDUN Pharmaceuticals for the gift of CM1 antibody and to Dr. Christopher Unsworth and Dr. Reinhard Jork (Bayer Corporation, CT) for helpful discussions and the gift of Repinotan hydrochloride (BAY x 3702). We also wish to express our gratitude to Dr. Trevor Biden and Dr. Mary Reylan for their generous gift of PKCα mutant cDNA constructs. This project was supported by grants from Bayer Corporation, National Institute on Aging (AG015465), and PSC-CUNY (622170031).

References


[27] I. Laszkiewicz, R. Mozuzanner, R.S. Wiggins, G.W. Konat, Delayed


