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The 5-HT$_{1A}$ receptor agonist BAY x 3702 prevents staurosporine-induced apoptosis

Bettina Suchanek *, Heidrun Struppeck, Thomas Fahrig

Bayer, CNS Research, Pharma-Forschungszentrum, Aprather Weg, D-42096 Wuppertal, Germany

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Abstract

The 5-HT$_{1A}$ receptor agonist (−)-(*R*)-2-[4-[[(3,4-dihydro-2*H*-1-benzopyran-2-yl)methyl]amino]butyl]-1,2-benzisothiazol-3(2*H*)-one1,1-dioxide monohydrochloride (BAY x 3702) was recently shown to have pronounced neuroprotective effects in rat models of cerebral ischemia and traumatic brain injury. In the present study we investigated the neuroprotective effects of BAY x 3702 in primary cultures of hippocampal and cortical neurons. Cell death was induced by 25 nM of the apoptosis inducing agent staurosporine and analyzed 24 h later by release of lactate dehydrogenase, formation of apoptotic bodies and DNA fragmentation. A significant neuroprotection was seen after pretreatment of the affected neurons with 50 pM to 1 μM BAY x 3702. The effects of BAY x 3702 were completely blocked by the selective 5-HT$_{1A}$ receptor antagonist *N*-(2-(4-(2-methoxyphenyl)-1-piperazinyl)ethyl)-*N*-(2-pyridinyl) cyclohexanecarboxamide trihydrochloride) (WAY-100635). These results indicate that low concentrations of BAY x 3702 protect cortical as well as hippocampal neurons from apoptotic cell death via a 5-HT$_{1A}$ receptor mediated pathway.

Keywords: BAY x 3702; Staurosporine; 5-HT$_{1A}$ receptor; Apoptosis

1. Introduction

5-HT$_{1A}$ receptor agonists have been shown to mediate neuroprotective effects in various animal models of focal and global cerebral ischemia (Bielenberg and Burkhardt, 1990; Bode-Greuel et al., 1990; Prehn et al., 1991, 1993; Contreras et al., 1992). For example this has been demonstrated for the highly selective and potent 5-HT$_{1A}$ receptor full agonist (−)-(*R*)-2-[4-[[(3,4-dihydro-2*H*-1-benzopyran-2-yl)methyl]amino]butyl]-1,2-benzisothiazol-3(2*H*)-one1,1-dioxide monohydrochloride (BAY x 3702), in rat models of focal cerebral ischemia (permanent middle cerebral artery occlusion), of reperfusion injury (transient middle cerebral artery occlusion) and of traumatic brain injury (acute subdural hematoma) (De Vry et al., 1997; Horváth et al., 1997; Horváth and Augstein, 1997).

The mechanisms which lead to cellular protection after BAY x 3702 treatment are thought to be due to the suppression of increased electrical activity in certain brain areas, possibly by membrane hyperpolarization and reduction of glutamate release (Colino and Halliwell, 1987; Davies et al., 1987; Andrade, 1992; De Vry et al., 1998). Recently, an induction of the 5-HT$_{1A}$ receptor in neuronal cell lines and in primary cultures of hippocampal neurons during nutrient deprivation was shown and interpreted as a final attempt of the cells to survive (Singh et al., 1996). Apoptosis is a fundamental process required for neuronal development but also occurs under pathological conditions, for instance after acute insults to the CNS, such as ischemia or trauma, as well as in chronic degenerative conditions (Linnik et al., 1993; MacManus et al., 1993; Nixon et al., 1994; Rink et al., 1995; Vito et al., 1996). Nevertheless, there is increasing evidence that apoptosis and necrosis may just represent the two extremes of a continuous spectrum of possibilities for a cell population to die (Nicotera and Leist, 1997; PorteraCailliau et al., 1997).

Postsynaptic 5-HT$_{1A}$ receptors are highly expressed in the hippocampus and neocortex (for reviews see De Vry, 1995; De Vry et al., 1998). These brain regions are especially sensitive to neuronal damage induced by ischemic stroke or brain trauma (Chalmers and Watson, 1991). Therefore, we established a model using primary rat

* Corresponding author. Tel.: +49-202-36-4628; E-mail: bettina.suchanek.bs@bayer-ag.de

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cortical and hippocampal neurons to investigate the neuroprotective effect of BAY x 3702 in vitro. In the present study we exposed the primary neurons to 25 nM staurosporine, which was shown in preliminary experiments to result in an optimal cell death rate for the evaluation of the neuroprotective efficacy of BAY x 3702. Staurosporine, a alkaloid produced by Streptomyces staurospores, is a potent inhibitor of protein kinases (Fujita-Yamaguchi and Kathuria, 1988; Tamaoki, 1991; Yanagihara et al., 1991) and an established agent known to induce apoptosis (Bertrand et al., 1994; Jarvis et al., 1994; Boix et al., 1997). We observed reduced lactate dehydrogenase release, reduced DNA fragmentation and less apoptotic body formation after BAY x 3702 treatment in hippocampal and cortical neurons. These effects were blocked by the high affinity 5-HT_{1A} receptor antagonist *N*-(2-(4-(2-methoxyphenyl)-1- piperazinyl)ethyl)-*N*-(2-pyridinyl)cyclohexanecarboxamide trihydrochloride) (WAY-100635), which has no, or only very low affinity to other putative central receptors (Forster et al., 1995; Khawaja et al., 1995). We conclude that activation of 5-HT_{1A} receptors by BAY x 3702 exerts an anti-apoptotic neuroprotective action that is distinct from activities such as hyperpolarization and modulation of glutamate release. This mechanism of neuroprotection may contribute significantly to the neuroprotective efficacy demonstrated for BAY x 3702 in several models of neurodegeneration.

2. Materials and methods

2.1. Primary cultures

Rat primary cortical and hippocampal cultures were prepared using modifications of established techniques (Choi et al., 1987). In brief, five to eight fetuses were removed on embryonic day 18 ± 1 from maternal rats. Cortical hemispheres and hippocampi were dissected under sterile conditions and placed into Dulbecco's modified Eagle's medium (DMEM, Gibco BRL, Gaithersburg, MD, USA). The tissue was dissociated according to the Papain Dissociation System protocol from Worthington Biochemical. Dissociated cells were suspended in Neurobasal Medium (Gibco BRL), supplemented with B-27 (Gibco BRL) and 2 mM L-glutamine (Sigma, St. Louis, MO, USA) and plated onto 96-well culture dishes (Costar, Cambridge, MA, USA) which had been coated with poly-D-lysine (0.1 mg/ml). The approximate plating density was 3.1×10^5 cortical cells per cm^2 and 1.2×10^5 hippocampal cells per cm^2. For the Hoechst stain cells were plated onto poly-D-lysine coated chamber slides (Nunc, Wiesbaden-Biebrich, Germany) at a cell density of 6.2×10^5 cells/cm^2. The cortical cultures were maintained under standard conditions at 37°C in an environment of 95% humidity and 10% CO_2. The hippocampal neurons were kept under the same conditions except that CO_2 was 5%. Every four to six days half of the medium was replaced by fresh medium. A 90% pure population of neurons was maintained as estimated from immunostaining for glial fibrillary acidic protein (GFAP, Sigma) and microtubule-associated protein-2 (MAP-2, Boehringer Mannheim, Mannheim, Germany).

2.2. Treatment of cultures with 5-HT_{1A} receptor ligands

To asses the cellular effects of BAY x 3702 (Bayer, Leverkusen, Germany) primary neurons which have been in culture for 9 to 13 days were preincubated with various concentrations of the agonist for 30 min at 37°C and 10%

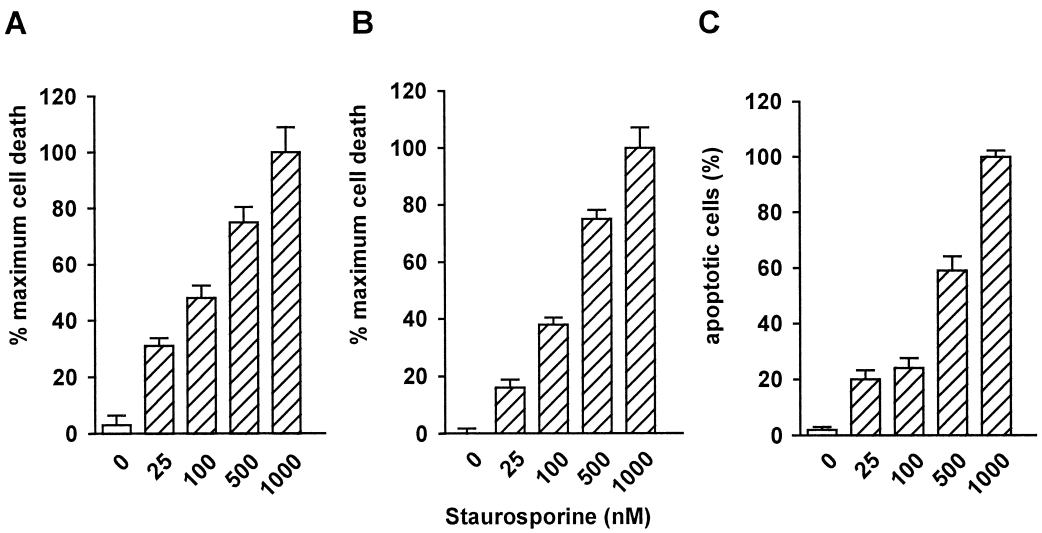

Fig. 1. Quantitative analysis of staurosporine-induced cell death by LDH release (A) DNA fragmentation ELISA (B) and Hoechst 33258 staining (C) after 24 h incubation of cortical neurons. Results of the LDH release assay and ELISA assay are expressed as percentage of the maximum cell death caused by 1 μM staurosporine treatment. The data from Hoechst 33258 staining represent the percentage of apoptotic cells of the total number of stained cells. (Each column is the mean + S.E.M. of eight measurements).

CO_2. BAY x 3702 was dissolved in water at a concentration of 10 mM. The dilutions were performed in Locke buffer (NaCl 154 mM, KCl 5.6 mM, $CaCl_2$ 2.3 mM, $MgCl_2$ 0.98 mM, glucose 5.5 mM, Hepes 10 mM). Subsequently staurosporine dissolved in DMSO was added and the cultures were incubated for 24 h. After diluting the staurosporine the DMSO concentration in the assay was less than 0.01% and did not affect neuronal viability. Cell death was analyzed by staining with Hoechst 33258, the release of lactate dehydrogenase (LDH) and by the Cell Death Detection ELISA Plus. To examine the effects of the 5-HT_{1A} receptor antagonist WAY-100635 (Bayer, Leverkusen, Germany), cells were preincubated with the antagonist for 10 min prior to addition of BAY x 3702. WAY-100635 was prepared in water at a concentration of 10 mM and diluted to the final concentration in Locke buffer.

2.3. Cell death assays

For staining with Hoechst 33258 (Sigma) cultures were fixed with 4% formaldehyde in phosphate buffered saline (PBS) for 10 min at room temperature, permeabilized for 2 min in 0.1% Trition X-100, 0.1% sodium citrate at 4°C, washed three times in PBS, and then incubated with 1 μg/ml Hoechst 33258 for 10 min at room temperature. After two rinses with distilled water the chamberslides were mounted with Fluor Save (Calbiochem, Cambridge, USA) and analyzed by fluorescent microscopy. For each sample three fields (40 to 60 cells) per well were counted. Observers were unaware of the treatment conditions.

The release of lactate dehydrogenase (LDH) into the culture medium, due to the loss of membrane integrity, is an index of cell death. The assay was performed using the Cytotoxicity Detection Kit from Boehringer Mannheim according to the manufacturer's recommendations. Measurements of six wells per treatment of two to four independent cultures were analyzed.

DNA fragmentation was measured spectrophotometrically using the Cell Death Detection ELISA Plus from Boehringer Mannheim according to the manufacturer's recommendations. The ELISA assay provides a quantitative in vitro determination of histone-associated DNA fragments (mono- and oligonucleosomes) in the cytoplasmic fraction of cell lysates. Measurements of four wells per treatment of at least two independent cultures were analyzed.

2.4. Statistical analysis

The main effects of treatment were assessed by analysis of variance and, if significant ($P < 0.0001$), the means of treatment groups were compared by post hoc analysis with the least-significant difference test (Fisher's LSD-test) (SyStat 6.1, SPSS).

3. Results

3.1. Staurosporine induces cell death in primary cultures of cortical neurons

Cell death induced by staurosporine in cortical neurons was measured by LDH release, DNA fragmentation ELISA

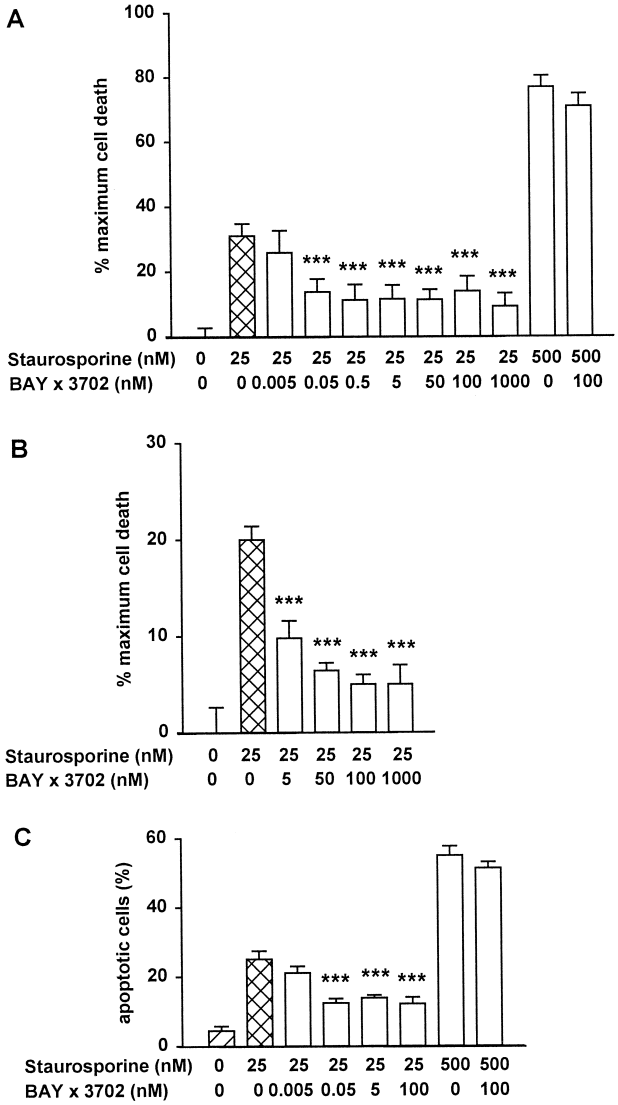

Fig. 2. BAY x 3702 inhibits the staurosporine-induced neurotoxicity in cortical neurons. Cortical neurons which have been in culture for 9 to 13 days were preincubated for 30 min with various concentrations of BAY x 3702 before treatment with staurosporine (25 nM) for 24 h. A significant neuroprotection was observed at BAY x 3702 concentrations of 50 pM to 1 μM as judged by LDH release assay (A, each column is the mean + S.E.M. of six measurements from two to four independent cultures), DNA fragmentation ELISA (B, each column is the mean + S.E.M. of at least three measurements) and Hoechst 33258 stain (C, each column is the mean + S.E.M. of six measurements from two to four independent cultures). No neuroprotection could be seen using 5 pM BAY x 3702. Cell death induced by 500 nM staurosporine was not prevented by 100 nM BAY x 3702. ANOVA: $P < 0.0001$, Fisher's LSD: $* P < 0.05$, $** P < 0.001$, $*** P < 0.0001$.

and Hoechst 33258 staining. All three assays reflected a clear dose-dependent toxic effect of staurosporine after an incubation period of 24 h (Fig. 1). At a concentration of 25 nM, staurosporine induced a LDH release which was about 30% of the maximal release induced by 1 μM staurosporine. Since staurosporine is known to induce mainly apoptosis, the LDH release is an index of the proportion of cells dying of secondary necrosis (Fig. 1A). In addition, significant DNA fragmentation was detected by the ELISA after staurosporine exposure (Fig. 1B). Almost all cells developed bright apoptotic nuclei after incubation with 1 μM staurosporine, in contrast to an incubation with 25 nM staurosporine after which apoptotic bodies were detected in 20% of the cells (Fig. 1C).

3.2. Neuroprotective properties of BAY x 3702

Cell death was determined by LDH release following exposure to staurosporine in the presence or absence of different concentrations of BAY x 3702. BAY x 3702 dose-dependently reduced cell death of 25 nM staurosporine-treated cortical neurons which have been in culture for 9 to 13 days.

Preliminary experiments revealed that BAY x 3702 prevented neuronal cell death in cultures treated with up to 100 nM staurosporine (data not shown) but failed to prevent cell death at higher staurosporine concentrations. Maximal neuroprotective efficacy was obtained after treatment of cells with 25 nM staurosporine.

Significant reductions of LDH release were observed after treatment with BAY x 3702 at concentrations as low as 50 pM and up to 1 μM (Fig. 2A). No neuroprotection was visible with 5 pM BAY x 3702 pretreatment as well as after cell death induction with 500 nM staurosporine and pretreatment with 100 nM BAY x 3702. Comparable results were obtained by quantitative analysis with photometric DNA fragmentation ELISA (Fig. 2B). This assay detected significant reductions of DNA fragmentation after treatment with 5 nM to 1 μM BAY x 3702. Further, a specific pattern of chromatin condensation was observed by means of fluorescence microscopy after Hoechst 33258 staining. Apoptotic cells have clustered chromatin that appears as bright round bodies. In contrast, normal growing cells display a nucleus with homogenous fluorescent chromatin. Upon induction of cell death with 25 nM staurosporine, about 20% of nuclei with the characteristic images of apoptosis were found (Fig. 2C). A reduction to 10% bright apoptotic nuclei was detected in cells preincubated with 50 pM to 100 nM BAY x 3702 but not with 5 pM BAY x 3702. As described for the LDH assay after cell death induction with 500 nM staurosporine and pretreatment with 100 nM BAY x 3702 no protective efficacy was detectable.

A similar series of experiments was conducted with hippocampal neurons which have been in culture for 9 to 13 days treated with BAY x 3702 and 25 nM staurosporine for 24 h. Results of the LDH release assay (Fig. 3A) and DNA fragmentation ELISA (Fig. 3B) demonstrated neuroprotection by the 5-HT_{1A} receptor agonist in a concentration range of 50 pM to 1 μM.

3.3. Inhibition of the neuroprotective effect of BAY x 3702 by the 5-HT_{1A} receptor antagonist WAY-100635

BAY x 3702 is a high affinity, full agonist of 5-HT_{1A} receptors. In order to determine if the anti-apoptotic effect

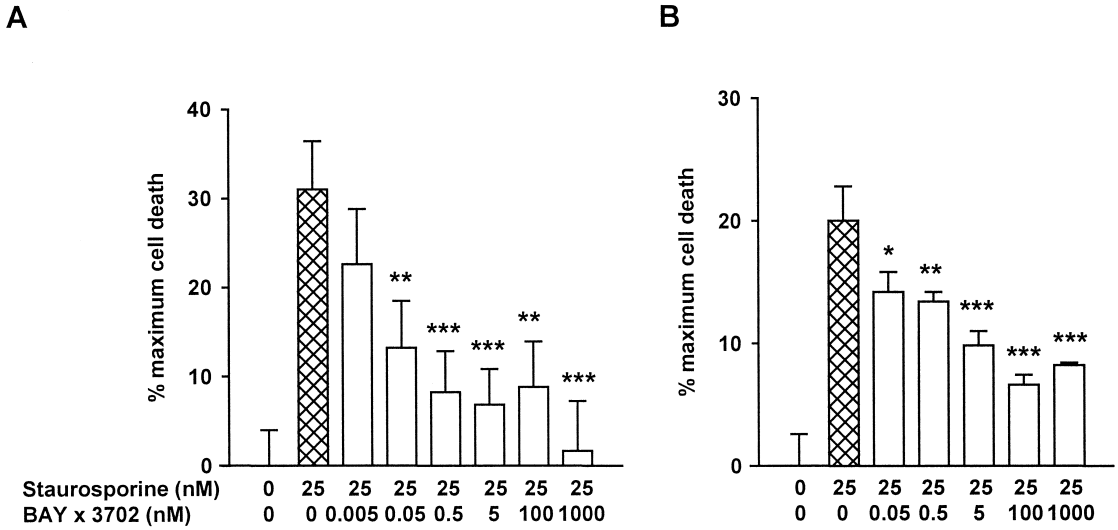

Fig. 3. Protection of hippocampal neurons by BAY x 3702 from staurosporine-induced apoptosis. Hippocampal neurons which have been in culture for 9 to 13 days were preincubated with different concentrations of BAY x 3702 followed by an addition of 25 nM staurosporine. LDH release assay (A) and DNA fragmentation ELISA (B) were performed 24 h later. BAY x 3702 at concentrations of 50 pM to 1 μM showed significant protective effects in both assays (A, each column is the mean + S.E.M. of six measurements from two to four independent cultures; B, each column is the mean + S.E.M. of two to four measurements). ANOVA: $P < 0.0001$, Fisher's LSD: $* P < 0.05$, $** P < 0.001$, $*** P < 0.0001$.

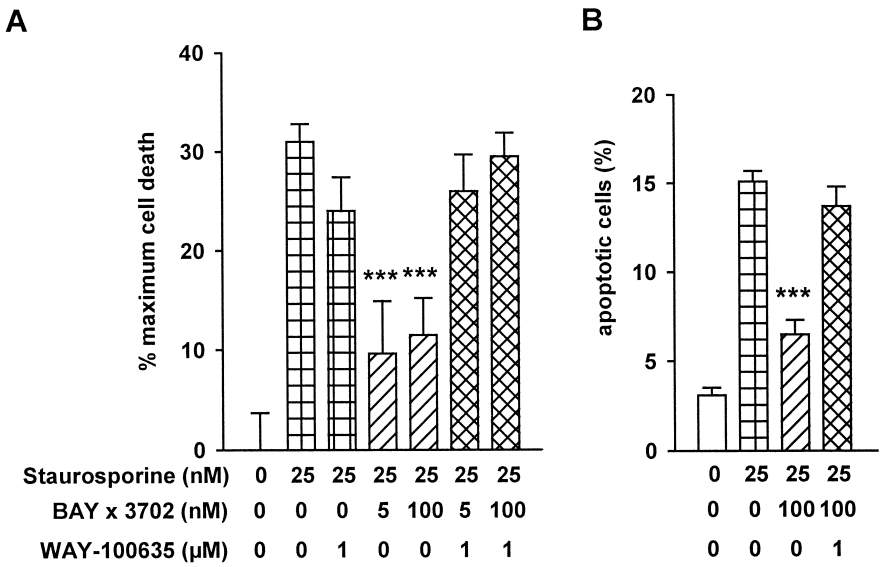

Fig. 4. WAY-100635, a selective 5-HT_{1A} receptor antagonist inhibits the neuroprotective effect of BAY x 3702. Cortical neurons were pretreated with 1 μM WAY-100635 for 10 min, followed by a 30 min treatment with BAY x 3702 and a 25 nM staurosporine challenge for 24 h (in the continued presence of BAY x 3702 and WAY-100635). A significant decrease in LDH release (A) was evident after 5 and 100 nM BAY x 3702 incubation. This effect was almost completely abolished by 1 μM WAY-100635 incubation. WAY-100635 itself had no significant influence on the cells or on the toxic effect of staurosporine. In the Hoechst 33258 stain (B) which was performed in parallel a reversal of the neuroprotective effect of 100 nM BAY x 3702 by 1 μM WAY-100635 was also evident (each column is the mean + S.E.M. of six measurements). ANOVA: $P < 0.0001$, Fisher's LSD: $* P < 0.05$, $** P < 0.001$, $*** P < 0.0001$.

of BAY x 3702 is mediated by activation of 5-HT_{1A} receptors, the effect of staurosporine on primary cultures of cortical neurons cotreated with BAY x 3702 and the selective 5-HT_{1A} receptor antagonist WAY-100635 was examined. The neuroprotective effect of BAY x 3702 was almost completely inhibited by 1 μM WAY-100635 as shown by LDH release (Fig. 4A) and Hoechst 33258 stain (Fig. 4B). At this concentration, WAY-100635 alone exerted no detectable effects on the cultured neurons.

4. Discussion

The present studies provide evidence that staurosporine-induced injury to rat cortical or hippocampal neurons can be prevented by the aminomethylchroman derivative BAY x 3702, a highly potent 5-HT_{1A} receptor full agonist. Previously, neuroprotective activity was demonstrated for the 5-HT_{1A} receptor agonists 8-OH-DPAT (8-hydroxy-2-(di-*n*-propylamino)tetralin) and BAY x 3702 (Bielenberg and Burkhardt, 1990; Bode-Greuel et al., 1990; Prehn et al., 1991, 1993; De Vry et al., 1997) in various animal models of cerebral ischemia. Whether the neuroprotection in these models is due to inhibition of excitotoxicity or ischemic apoptosis or a combination of both is not known. To find out what type of cell death is prevented by BAY x 3702 we chose an in vitro model in which apoptosis is the predominant mechanism of cell death. We demonstrated that the 5-HT_{1A} receptor agonist BAY x 3702 protects cortical and hippocampal neurons by inhibiting apoptosis induced by 25 nM staurosporine. However, no prevention of cell death was seen at staurosporine concentrations higher than 100 nM. This may be due to the fact that with increasing concentrations of staurosporine multiple pathways leading to apoptotic cell death may be activated, which are not influenced by BAY x 3702.

The neuroprotective effect of BAY x 3702 as indicated by decreased release of LDH as well as decreased DNA fragmentation and reduced apoptotic body formation was dose-dependent indicating a selective and specific mode of action. Even low concentrations of BAY x 3702, within the range of binding affinity for the 5-HT_{1A} receptor, were shown to inhibit cell death. The nearly complete reversal of the anti-apoptotic effect of BAY x 3702 by the 5-HT_{1A} receptor antagonist WAY-100635 strongly suggests that this process is mediated by activation of the 5-HT_{1A} receptor. The data reported in this paper support the previous findings demonstrating that 5-HT_{1A} receptor agonists can inhibit cell death in vitro (Singh et al., 1996; Banerjee et al., 1996; Ahlemeyer and Krieglstein, 1997).

Currently it is not known which second messenger pathways are involved in the neuroprotective effect of 5-HT_{1A} receptor agonists. Recent reports suggest that neuroprotective events initiated by 5-HT_{1A} receptor activation may involve Pertussis Toxin-sensitive G-proteins and the mitogen-activated kinase (MAPK) cascade (Banerjee et al., 1996; Ahlemeyer and Krieglstein, 1997).

Since staurosporine is known to be a protein kinase inhibitor capable of activating caspases (Jacobson et al.,

1996; Posmantur et al., 1997), increasing ceramide levels (Dowson et al., 1997), and increasing intracellular and mitochondrial calcium levels (Kruman et al., 1997) further experiments are warranted to characterize the downstream signalling cascade induced by BAY x 3702 which leads to inhibition of apoptotic cell death.

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