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Short communication

The 5-HT_{1A} receptor agonist Bay × 3702 inhibits apoptosis induced by serum deprivation in cultured neurons

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Abstract

We examined whether the highly selective 5-HT_{1A} receptor agonist (−)-(*R*)-2-[4-[[(3,4-dihydro-2*H*-1-benzopyran-2-yl)methyl]-amino]butyl]-11,2-benz-isothiazol-3(2*H*)-one 1,1-dioxide monohydrochloride (Bay × 3702) could inhibit neuronal apoptosis induced by serum deprivation. In primary cultures of chick embryonic neurons and in mixed neuronal/glial cultures from neonatal rat hippocampus, Bay × 3702 (1 μM) rescued serum-deprived neurons from apoptosis. The antiapoptotic effect of Bay × 3702 (1 μM) was blocked in chick neurons by the selective 5-HT_{1A} receptor antagonists 4-iodo-*N*-[2-[4-(methoxyphenyl)-1-piperazin]ethyl]-*N*-2-pyridinyl-benzamide hydrochloride (*p*-MPPI, 10 μM) and 4-[3-benzotriazol-1-propyl]-1-(2-methoxyphenyl)-piperazine (BPMP, 10 μM) as well as by anti-nerve growth factor (anti-NGF) antibodies and in rat neurons by *N*-[2-[4-(2-methoxy)-1-piperazinyl]ethyl]-*N*-(2-pyridinyl)cyclohexane-carboxamide trihydrochloride (WAY 100635, 10 μM). We found only under control conditions (medium with serum), but not in serum-deprived cultures, that NGF secretion was 6-fold increased by Bay × 3702 (1 μM) compared to untreated cultures. Additionally, Bay × 3702 (4 μg/kg i.v.), infused within a period of 4 h, significantly increased the NGF content of the rat hippocampus, but not of the striatum. In summary, our data suggest that Bay × 3702 inhibited growth factor withdrawal-induced apoptosis by the stimulation of 5-HT_{1A} receptors and that the NGF signalling pathway is involved in the mechanism of action.

Keywords: Apoptosis; Chick neuron; NGF (nerve growth factor); Neuroprotection; Serum deprivation; 5-HT_{1A} receptor agonist

1. Introduction

5-HT_{1A} receptor agonists have already been shown to protect neurons from cell death after focal cerebral ischemia in rats and after glutamate toxicity in mixed cultures of hippocampal neurons and astrocytes (Prehn et al., 1993; Semkova et al., 1998). In addition, serotonin has a mitogenic and trophic effect on neurons and neuroblasts (Hanley, 1989) which was mediated by stimulation of 5-HT_{1A} receptors (Yan et al., 1997). Previously, we have shown that the 5-HT_{1A} receptor agonist 8-hydroxy-2-(di-*N*-propylamino)tetralin hydrobromide (8-OH-DPAT) inhibited apoptosis induced by serum deprivation in chick embryonic neurons and that induction of nerve growth factor (NGF) seemed to be partially involved in its mechanism of action (Ahlemeyer and Krieglstein, 1997). In the present study, we attempted to find out whether (−)-(*R*)-2-[4-[[(3,4-dihydro-2*H*-1-benzopyran-2-yl)methyl]-amino]-butyl]-11,2-benz-isothiazol-3(2*H*)-one 1,1-dioxide monohydrochloride (Bay × 3702), a newly synthesized, highly selective 5-HT_{1A} receptor agonist which is currently in clinical trials for acute ischemic stroke and traumatic brain injury (De Vry et al., 1998) will inhibit serum deprivation-induced apoptosis in different central nervous system cells and whether NGF contributes to its neuroprotective effect.

2. Materials and methods

2.1. Animals

Neonatal (PD1) Fischer 344 rats and male Long–Evans rats weighing 160–260 g (Møllegaard, Shenveds, Denmark) were used. The animals had free access to food (Altromin, Lage, Germany) and water and were kept under standardized environmental conditions (12-h light/dark

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cycle, 23 ± 1°C and 55 ± 1% relative humidity). The animal experiments were conducted with approval of the Government Commission of Animal Care, Germany.

2.2. Chemicals

Dulbecco's modified Eagle medium (DMEM), minimal essential medium (MEM), Leibovitz L-15 medium, antibiotics, NUSERUM™ (25% fetal bovine serum, 75% supplements), fetal bovine serum, papain and supplement B27 were purchased from Life Technologies, Karlsruhe, Germany. Propranolol, bisbenzimide (Hoechst 33258), poly-L-lysine (MW 30–70 kDa, MW 70–150 kDa), NGF, cytosine-β-arabinofuranoside and dimethylsulfoxide were purchased from Sigma, Deisenhofen, Germany. 4-Iodo-*N*-[2-[4-(methoxyphenyl)-1-piperazin]ethyl]-*N*-2-pyridinyl-benzamide hydrochloride (*p*-MPPI) and chlorpromazine hydroch loride were from RBI, Köln, Germany; 4-[3-benzotriazol-1-propyl]-1-(2-methoxyphenyl)-piperazine maleate (BPMP) was from Tocris, Köln, Germany. Apotag Kit (Oncor, USA) was used for terminal transferase-mediated d-UTP nick end labeling (TUNEL) assay. The trypsin inhibitor and the two-site enzyme immunoassay for NGF including anti-NGF antibodies were purchased from Boehringer Mannheim, Mannheim, Germany. *N*-[2-[4-(2methoxy)-1-piperazinyl]ethyl]-*N*-(2-pyridinyl)cyclohexanecarboxamide trihydrochloride (WAY 100653) and (−)(*R*)-2-[4-[[(3,4-dihydro-2*H*-1-benzopyran-2-yl) methyl]amino]butyl]-11,2-benz-isothiazol-3(2*H*)-one 1,1-dioxide monohydrochloride (Bay × 3702) were gifts from the companies Wyeth and Bayer, respectively.

2.3. Cell culture

Primary neuronal cultures from 7-day-old chick embryo telencephalons were prepared as previously described by Pettmann et al. (1979). Cerebral hemispheres were mechanically dissociated and the resulting cell suspension was seeded into poly-L-lysine (MW 70–150 kDa)-coated culture flasks with a density of 4×10^4 cells/cm^2. The cell cultures were maintained in DMEM supplemented with 20% fetal bovine serum and were used for experiments on day 5 after seeding. Mixed cultures of neurons and astrocytes were prepared from the hippocampi of neonatal Fischer 344 rats (PD1). Briefly, the dissected tissue was digested with papain (0.1% in Leibovitz L-15 medium) for 15 min at 37°C and then mechanically triturated. A trypsin inhibitor solution (1% in MEM containing 1% bovine serum albumin) was layered under the cell suspension which was then centrifuged at 200 × *g* at 20°C for 10 min. Finally, cells were resuspended and seeded into poly-L-lysine (MW 30–70 kDa)-coated Petri dishes. After 2 days, cells were treated with cytosine-β-arabinofuranoside (1 μM) for 1–2 days to inhibit glial cell proliferation. Cultures were incubated for 12 days in MEM containing 10% NUSERUM™ and supplement B27 before experiments.

2.4. Drug treatment

Apoptosis was induced by serum deprivation for 24 h in chick neurons and by serum plus supplement B27-deprivation for 24 h in hippocampal cells from neonatal rats. The 5-HT_{1A} receptor agonist Bay × 3702 was added 30 min before the induction of apoptosis. The neurotransmitter receptor antagonists and the anti-NGF antibody were added 30 min before the administration of Bay × 3702 (60 min before the induction of apoptosis).

2.5. Cell viability

The cell viability was determined by the Trypan blue exclusion method.

2.6. Nuclear staining with Hoechst 33258

Chick embryonic neurons were incubated for 15 min at 37°C with the DNA fluorochrome Hoechst 33258 (10 μg/ml) and then washed with phosphate-buffered saline. Nuclear morphology was observed under a fluorescence microscope. Viable cells (with a normal nuclear size and normal chromatin) and apoptotic cells (with reduced nuclear size, chromatin condensation and nuclear fragmentation) were counted and the results were expressed as the percentage of apoptotic neurons.

2.7. TUNEL-staining

In mixed neuronal/glial cultures from neonatal rat hippocampus, it was difficult to distinguish nuclei of neurons from those of the underlying astrocytes because both were stained with Hoechst 33258. Therefore, we evaluated apoptosis in this cell culture model by the TUNEL reaction using the Apotag Kit. The staining was performed according to the manufacturer's instructions and the results were expressed as the percentage of TUNEL-positive neurons.

2.8. Two-site enzyme immunoassay for NGF

The two-site NGF enzyme-linked immunoassay (ELISA) was used for the measurement of NGF protein in the hippocampus and striatum of rats as well as in the culture medium of chick embryonic neurons. One series of rats ($n = 6$ per group) was treated with Bay × 3702 (0.004 mg/kg i.v. for 4 h). The brains were removed 6, 12 and 24 h after the beginning of the infusion. Hippocampus and striatum were taken separately and frozen immediately on dry ice. The extraction buffer (0.1 M Tris–HCl, 0.4 M NaCl, 2% bovine serum albumin, 0.05% NaN_3, 1 mM phenylmethylsulfonylfluoride, 4 mM EDTA, 0.007 mg/ml trypsin inhibitor) was added to the tissue sample. The tissue was homogenized, centrifuged at 20 000 rpm at 4°C for 30 min and the supernatant was used for NGF-ELISA.

Culture medium was stored at −20°C in the presence of trypsin inhibitor (0.02 mg/ml) to avoid NGF degradation. The two-site NGF-ELISA was performed according to the manufacturer's instructions with minor modifications as described in our previous study (Ahlemeyer and Krieglstein, 1997).

3. Results

Serum deprivation for 24 h reduced the viability of cultured chick neurons to 70% compared to 90% in controls and increased the percentage of apoptotic neurons from 9% in controls to 30% (Table 1). In a previous report, we distinguished apoptotic and necrotic neurons by nuclear staining. Apoptotic neurons showed reduction in nuclear size, chromatin condensation and DNA fragmentation, whereas necrotic cells contained nuclei of diffuse and irregular size together with chromatin condensation. Under serum-free conditions, we found 28% apoptotic and 17% necrotic neurons (Ahlemeyer and Krieglstein, 1997) showing that the 30% Trypan blue-stained neurons represented in part, necrotic and in part, apoptotic cells with secondary necrosis. The 5-HT_{1A} receptor agonist Bay × 3702 was able to block neuronal damage induced by serum deprivation in primary cultures of chick embryonic neurons as well as in mixed cultures of neurons and astrocytes from neonatal rat hippocampus (Table 1). To find out whether the antiapoptotic effect of Bay × 3702 was mediated through the selective stimulation of 5-HT_{1A} receptors, we evaluated whether the 5-HT_{1A} receptor antagonists *p*-MPPI (Kung et al., 1996), BPMP (Mokrosz et al., 1994) and WAY 100635 (Fletcher et al., 1996), the dopamine receptor antagonist chlorpromazine, or the β-adrenoceptor antagonist propranolol could block neuroprotection. In rat hippocampal neurons, but not in chick neurons, WAY 100635 (10 μM) was able to block the antiapoptotic effect of 1 μM Bay × 3702 (Table 1). In cultured chick neurons, we were able to block the protective effect of 1 μM Bay × 3702 with two other selective 5-HT_{1A} receptor antagonists, e.g., with *p*-MPPI (10 μM, Table 1) and with the pre- and postsynaptic 5-HT_{1A} receptor antagonist BPMP. Bay × 3702 (1 μM) increased the percentage of viable neurons after 24 h of serum deprivation from 72.5 ± 5.5% to 85.9 ± 5.8% ($n = 8$) and decreased the percentage of apoptotic neurons from 28.1 ± 5.2% to and 12.9 ± 2.2% ($n = 8$), but we determined 69.0 ± 6.0% viable and 27.5 ± 4.9% apoptotic neurons ($n = 8$) after serum deprivation in the presence of 1 μM Bay × 3702 plus 10 μM BPMP. BPMP (10 μM), WAY 100635 (10 μM) and *p*-MPPI (10 μM) did not change cell viability or the percentage of apoptotic chick and rat neurons under control conditions as well as after 24 h of serum deprivation (data not shown).

Furthermore, in cultured chick neurons, the addition of anti-NGF antibodies (10 ng/ml)—which were able to block the antiapoptotic effect of externally added NGF under serum-free conditions (data not shown)—blocked the protective effect of 1 μM Bay × 3702 in serum-free medium (Fig. 1) suggesting that the induction of NGF

Table 1
The antiapoptotic effect of Bay × 3702. Cells were treated for 24 h with Bay × 3702, chlorpromazine, propranolol, WAY 100635 and *p*-MPPI in serum-containing (controls) and serum-free medium (SD)

Treatment	Chick neurons		Hippocampal neurons
	Trypan blue exclusion (% viable cells)	Hoechst staining (% apoptotic cells)	TUNEL-staining (% apoptotic cells)
Controls	76.0 ± 6.2[a]	9.9 ± 4.2[a]	14.6 ± 5.5[a]
Controls + 10 μM Bay × 3702	72.0 ± 6.3[a]	6.3 ± 1.7[a]	10.1 ± 7.6[a]
SD	45.2 ± 10.8	26.7 ± 2.6	
SD + 0.01 μM Bay × 3702	61.9 ± 7.9	16.2 ± 3.6[a]	
SD + 0.1 μM Bay × 3702	74.0 ± 6.3[a]	18.5 ± 6.0[b]	
SD + 1 μM Bay × 3702	76.8 ± 4.9[a]	11.1 ± 5.8[a]	
SD + 10 μM Bay × 3702	76.5 ± 5.0[a]	15.7 ± 3.9[a]	
Controls	90.7 ± 3.8[a]	8.5 ± 4.5[a]	
SD	70.0 ± 5.5	29.8 ± 4.8	38.0 ± 6.7
SD + 1 μM Bay × 3702	85.8 ± 2.8[a]	13.4 ± 4.8[a]	21.3 ± 7.7[a]
SD + 1 μM Bay × 3702 + 1 μM chlorpromazine	82.7 ± 3.4[a]	15.4 ± 7.7[a]	
SD + 1 μM Bay × 3702 + 10 μM propranolol	80.6 ± 8.3[a]	12.1 ± 4.7[a]	
SD + 1 μM Bay × 3702 + 10 μM WAY 100635	83.8 ± 4.3[a]	11.3 ± 7.1[a]	37.4 ± 4.0
SD + 1 μM Bay × 3702 + 10 μM *p*-MPPI	74.4 ± 3.7	28.7 ± 8.2	

Values are given as means ± S.D. of eight experiments; different from S.D.
[a] $P < 0.001$ (ANOVA-1 and post-hoc Scheffé-test).
[b] $P < 0.01$ (ANOVA-1 and post-hoc Scheffé-test).

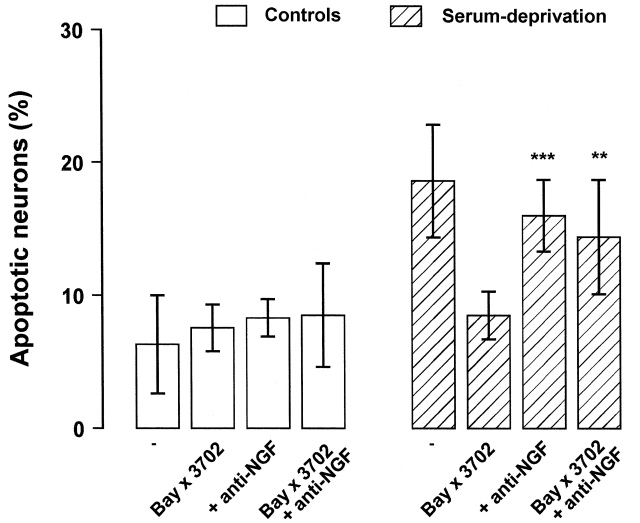

Fig. 1. The inhibition of the antiapoptotic effect of Bay×3702 with anti-NGF antibodies. Chick neurons were incubated for 24 h in medium with serum (controls) and serum-free medium (serum-deprivation) in the absence or presence of Bay×3702 (1 μM) and 10 ng/ml anti-NGF antibodies (anti-NGF). Values are given as means±S.D. of six experiments. $^{**}P < 0.01$; $^{***}P < 0.001$ by ANOVA-1 and post-hoc Scheffé-test.

contributed to its mechanism of action. Under control conditions (medium with serum), we found an increase in the NGF content of the culture medium from 2.2 ± 1.5 pg/ml in untreated to 14.7 ± 3.5 pg/ml in Bay × 3702 (1 μM)-treated chick neurons which corresponded to a secretion of 18.5 and 123 pg NGF/mg cell protein, respectively. Similar results were found in vivo where the infusion of 4 μg/kg Bay × 3702 (i.v.) for 4 h significantly increased the NGF content of the rat hippocampus after 6 h to 0.94 ± 0.09 ng/g tissue compared to 0.59 ± 0.18 ng/g tissue in untreated rats. The NGF content returned to basal levels 12 and 24 h after the infusion with Bay × 3702. Bay × 3702 had no effect on the NGF content of the rat striatum. However, when cultured chick neurons were deprived of serum, the NGF content of the culture medium was 6-fold increased compared to neurons incubated with serum-containing medium, but it was not further increased in the presence of Bay × 3702.

4. Discussion

We have previously reported that apoptotic damage in chick neurons due to serum deprivation was time-dependent and could be inhibited by the 5-HT_{1A} receptor agonist 8-OH-DPAT (Ahlemeyer and Krieglstein, 1997). In the present study, the highly selective 5-HT_{1A} receptor agonist Bay × 3702 was able to block apoptosis induced by serum deprivation in neurons from chick embryo telencephalons and from neonatal rat hippocampus. Similarly, Bay × 3702 has been shown to protect rat hippocampal neurons from staurosporine-induced apoptosis (Suchanek et al., 1998) and from glutamate excitotoxicity (Semkova et al., 1998). In chick neurons, we found a dose–response curve by measuring neuronal viability, but not by determining the percentage of apoptotic neurons (Table 1). Similarly, De Vry et al. (1998) did not report a clear dose–response relationship between Bay × 3702 and the firing rate of dorsal root ganglion neurons because of the small range between the no effect and full effect concentration.

The antiapoptotic effect of Bay × 3702 was mediated through selective stimulation of 5-HT_{1A} receptors, because the protective effect was completely blocked with WAY 100635 in rat hippocampal neurons, and with the 5-HT_{1A} receptor antagonists *p*-MPPI and BPMP in chick neurons. However, the absence of WAY 100635 sensitivity in chick neurons was puzzling. It may be suggested that WAY 100635 has a lower affinity to the avian than to the rat 5-HT_{1A} receptors (which could be concluded by comparing our data on chick and rat neurons) or that the three 5-HT_{1A} receptor antagonists used in our study differ in their potency to block the various effects due to pre- or postsynaptic 5-HT_{1A} receptor stimulation. For example, WAY 100635 did not inhibit 8-OH-DPAT-induced presynaptic receptor stimulation (Hajós-Korcsok and Sharp, 1996), but it has been shown to block the effect of Bay × 3702 on somatodendritic (presynaptic) 5-HT_{1A} receptors of 5-HT neurons in the dorsal raphe, although a dosage of WAY 100635 500-fold higher than that of Bay × 3702 was needed (Dong et al., 1998). Because we could block the antiapoptotic effect of Bay × 3702 with the presynaptic 5-HT_{1A} receptor antagonist MPPI (Bjorvatn et al., 1998) and the pre- and postsynaptic 5-HT_{1A} receptor antagonist BPMP, Bay × 3702 may act through specific stimulation of presynaptic 5-HT_{1A} receptors. However, the studies on the pre- and postsynaptic activity of different 5-HT_{1A} receptor antagonist were performed in vivo and the existence of pre- and postsynaptic 5-HT_{1A} receptors in neuronal cell cultures is poorly documented. In addition, the inhibitory potency of WAY 100635 also showed subcellular and regional differences, e.g., WAY 100635 did not block the activation of dendritic 5-HT_{1A} receptors (Chaput and De Montigny, 1988) and it has been shown by Larsson et al. (1998) that WAY 100635 inhibited the effects of 8-OH-DPAT more effectively in the hippocampus than in the striatum, hypothalamus and in the frontal cortex. Consistently, we found only in neurons from the hippocampus and not in neurons from the telencephalon (including the striatum, cortex, thalamus and hippocampus) an inhibition of the antiapoptotic effect of Bay × 3702 by WAY 100635. Thus, we suggest that species and/or tissue-specific differences in the affinity of WAY 100635 to the 5-HT_{1A} receptor caused the different sensitivities of chick and rat neurons to WAY 100635.

We also tested propranolol (10 μM) and chlorpromazine (1 μM) to exclude an effect of Bay × 3702, especially at the high dosage of 1 μM on β-adrenoceptors and dopamine receptors, respectively. The stimulation of

dopamine receptors has also been described to protect neurons from apoptosis (Iwasaki et al., 1996) and it has been shown in vivo, that the administration of low doses of 8-OH-DPAT induces behavioural syndromes which can be blocked by the β-adrenoceptor antagonist propranolol (Lucki, 1992). As shown in Table 1, the stimulation of dopamine or β-adrenoceptors was not involved in the antiapoptotic effect of Bay × 3702.

Previous studies have shown that serotonin has neurotrophic effects (Hanley, 1989) which are mediated by stimulation of 5-HT_{1A} receptors (Yan et al., 1997). Therefore, we measured the neuronal NGF secretion of chick neurons incubated in medium with or without serum in the presence and absence of Bay × 3702. Under control conditions (medium with serum), Bay × 3702 increased significantly NGF secretion into the culture medium and similarly, we found in vivo an increase in the NGF content of the rat hippocampus, but not of the striatum 6 h after the infusion of 4 μg/ml Bay × 3702 (i.v.). Under conditions of serum withdrawal, the NGF content of the culture medium was already increased without drug, but was not further elevated by Bay × 3702.

However, anti-NGF antibodies inhibited the neuroprotective effect of Bay × 3702 in chick neurons suggesting that Bay × 3702 affected the NGF signalling pathway leading to a synergistic effect of NGF and Bay × 3702. Consistently, the stimulation of the 5-HT_{1A} receptor has been reported to activate ras-dependent mitogen activated kinases (MAPK), a pathway which is also shared by NGF, and it increased the activity of phosphoinositide-3-kinase (Garnovskaya et al., 1996), an enzyme which has been shown to be essential for cell survival (Yao and Cooper, 1995). Bay × 3702 induced NGF synthesis in chick neurons cultured in medium with serum. Other drugs which have been shown to induce NGF protein and NGF m-RNA probably by increasing the intracellular c-AMP concentrations are the β-adrenoceptor agonists (Mocchetti et al., 1989; Schwartz and Mishler, 1990; Semkova et al., 1996). Analogues of c-AMP were also able to increase NGF synthesis (Kew et al., 1996). In the coexistence of 5-HT_{1A}/5-HT_{1B}/5-HT_{1D} receptors, the 5-HT_{1A} receptor seemed to be negatively coupled to adenylate cyclase (Fanburg and Lee, 1997). However, in chick neurons, lacking the 5-HT_{1B} receptor (Heuring et al., 1986), the stimulation of 5-HT_{1A} receptor led to an activation of adenylate cyclase and to an increase in the intracellular c-AMP content (Fanburg and Lee, 1997).

In addition, other mechanisms of action of Bay × 3702 should be discussed such as the opening of potassium channels which leads to a hyperpolarization of the cell membrane and thereby to neuroprotection (Penington et al., 1993; Rupalla et al., 1994; Kew et al., 1996). On the other hand, elevated intracellular potassium concentration increased the concentration of NGF-m-RNA in cultured hippocampal neurons (Lu et al., 1991; Zafra et al., 1992). It has been shown that the stimulation of 5-HT_{1A} receptors inhibited the voltage-dependent Ca^{2+} influx (Rupalla et al., 1994; Chen and Penington, 1996) and others have found that the prevention of the increase in $[Ca^{2+}]_i$ was able to abolish the induction of apoptosis (Trump and Berezesky, 1995).

5. Conclusion

Our data suggest that neuronal apoptosis induced by serum deprivation can be inhibited by the highly selective 5-HT_{1A} receptor Bay × 3702 and that the NGF signalling pathway seems to be involved in the mechanism of action.

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