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Neuroprotective effect of 5-HT_{1A} receptor agonist, Bay × 3702, demonstrated in vitro and in vivo

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Abstract

It has been shown recently that Bay × 3702 ((−)-(*R*)-2-[4-[[(3,4-dihydro-2H-1-benzopyran-2-yl)methyl]amino]butyl]-1,2,-benzisothiazol-3(2H)-one 1,1-dioxide monohydrochloride), a highly potent and selective 5-HT_{1A} receptor agonist, has a neuroprotective potency associated with its ability to inhibit ischemia-induced excessive release of glutamate. 5-HT_{1A} receptors are highly expressed in brain areas, such as the hippocampus and the cerebral cortex, sensitive to neuronal damage induced by ischemic stroke or brain trauma. Therefore, we investigated whether Bay × 3702 can rescue cultured hippocampal neurons subjected to excitotoxic damage. The hippocampal neurons exposed to 0.5 mM L-glutamate for 1 h had pronounced damage characteristic of neuronal necrosis as evaluated 18 h later by trypan blue staining and morphological criteria. However, treatment with Bay × 3702 (0.001 to 1 μM) reduced the number of damaged neurons, and preserved cell morphology and integrity of the neuronal network. Bay × 3702 was added immediately after the end of exposure to glutamate and was present until the evaluation of neuronal damage. Furthermore, the neuroprotective activity of Bay × 3702 (0.1 μM) was abolished by WAY 100635 (*N*-[2-[4-(methoxyphenyl)-1-piperazinyl]ethyl]-*N*-2-pyridinyl cyclo-hexanecarboxamide) (1 μM), a selective 5-HT_{1A} receptor antagonist, indicating that the neurorescuing activity of Bay × 3702 was mediated via stimulation of 5-HT_{1A} receptors. Additionally, we attempted to find whether the drug could protect rat brain tissue from ischemic insult due to permanent occlusion of the middle cerebral artery in rats. Bay × 3702 (12 and 40 μg/kg), infused within a period of 4 h, immediately after induction of ischemia greatly reduced cortical infarct volume (57 and 55% of controls, respectively) suggesting that this drug might be useful for the treatment of acute cerebral infarction.

Keywords: Bay × 3702; Excitotoxicity; Hippocampal cell; Glutamate; Permanent focal ischemia; (Rat)

1. Introduction

After cerebral ischemia there is an excessive release of excitatory amino acids such as L-glutamate and L-aspartate into the extracellular space, followed by overactivation of glutamate receptors on neurons (Benveniste et al., 1984; Choi, 1988). As a result, elevation of intracellular free Ca^{2+} concentration and accumulation of free radicals have been shown to trigger a cascade of events leading to neuronal degeneration that occurs after ischemic insults and brain trauma (Desphande et al., 1987; Siesjö and Bengtsson, 1989). Furthermore, glutamate receptor antagonists and direct blockers of neuronal Ca^{2+} channels have been demonstrated to attenuate excitotoxic injury (Simon et al., 1984; Ferger and Krieglstein, 1996). However, the clinical benefit of these drugs is debatable, since they lack convincing effectiveness or have serious side-effects. The drugs that increase the resistance of the neuronal membrane to excitotoxicity by induction of hyperpolarization represent another approach to achieve neuroprotection. Electrophysiological studies have shown that activation of neuronal 5-HT_{1A} receptors leads to hyperpolarization due to an increase in the inwardly rectifying potassium (K^+) current (Araneda and Andrade, 1991; Rupalla et al., 1994). Thus, stimulation of neuronal 5-HT_{1A} receptors can inhibit glutamate-induced depolarization during cerebral ischemia and therefore could reduce neuronal degeneration. Additionally, 5-HT_{1A} receptor agonists have been reported to inhibit the ischemia-induced excessive release of gluta-

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mate, most likely through activation of 5-HT_{1A} receptors located on glutamatergic terminals (Maura et al., 1988). Furthermore, 5-HT_{1A} receptors are located in brain areas which are highly sensitive to neuronal damage induced by ischemic stroke or brain trauma, such as the hippocampus and cerebral cortex (Pazos and Palacios, 1985; Chalmers and Watson, 1991). In fact, it has been reported that 5-HT_{1A} receptor agonists exert neuroprotective properties in vitro as well as in vivo, in rodent models of cerebral ischemia (Bode-Greuel et al., 1990; Nuglisch et al., 1990; Prehn et al., 1993; Piera et al., 1995). However, these compounds have only limited 5-HT_{1A} receptor selectivity/activity. Therefore, the development of more selective full 5-HT_{1A} receptor agonists with strong neuroprotective properties is a promising approach (de Vry et al., 1997).

The aim of this study was to find whether Bay × 3702, a highly potent and selective 5-HT_{1A} receptor agonist, could rescue cultured hippocampal neurons subjected to excitotoxic damage and could protect rat brain tissue from ischemic insult due to permanent occlusion of the middle cerebral artery in rats.

2. Materials and methods

2.1. Animals

Neonatal (PD 1) Fischer 344 rats and male Long–Evans rats weighing 160-260 g (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 cycle, 23 ± 1°C and 55 ± 1% relative humidity). There were no significant differences between the mean body weight of treated and untreated animals.

2.2. Cell culture agents and other substances

Drug tested: Bay × 3702 ((−)-(*R*)-2-[4-[[(3,4-Dihydro-2H-1-benzopyran-2-yl)methyl]amino]butyl]-1,2,-benzisothiazol-3(2H)-one 1,1-dioxide monohydrochloride) was obtained from Bayer, CNS Research (Cologne, Germany); WAY 100635 (*N*-[2-[4-(methoxyphenyl)-1-piperazinyl]ethyl]-*N*-2-pyridinyl cyclo-hexanecarboxamide), a selective postsynaptic 5-HT_{1A} receptor antagonist, and dizocilpine (MK 801) were obtained from Research Biochemical International (RBI, Cologne, Germany). Minimum essential medium (MEM), B27 supplement, penicillin–streptomycin solution and NU^R-serum were purchased from Gibco (Eggenstein, Germany). Sodium L-glutamate, cytosine β-D-arabinofuranoside, papain, trypsin inhibitor and poly-L-lysine hydrobromide were purchased from Sigma (Deisenhofen, Germany). Antibody against glial fibrillary acidic protein was obtained from Boehringer-Mannheim (Mannheim, Germany) and antibody against neuron-specific enolase from Polysciences (USA).

2.3. Primary rat hippocampal cultures

Primary hippocampal cultures containing neurons and glial cells were prepared from postnatal (PD 1) Fischer 344 rats. Briefly, the hippocampi were isolated and incubated for 20 min at 37°C in medium supplemented with 1 mg/kg papain. The supernatant was removed and the tissue pieces were triturated in medium. Trypsin inhibitor solution (1% in medium) was added. The mixture was centrifuged at 600 rpm and 20°C for 10 min. The cells were resuspended in medium containing 10% NU^R serum and seeded on poly-L-lysine-coated 35-mm culture dishes (3×10^5 cells/dish). The cells were cultured in minimum essential medium (supplemented with Earle's salts, 2 mM L-glutamine, 5 g/l glucose and 2.2 g/l sodium bicarbonate) containing 10% NU^R serum, B27 supplement, 20 U/ml penicillin and 20 μg/ml streptomycin, in a humidified atmosphere of 95% air and 5% CO_2 at 37°C. After 2 days in vitro, cytosin-β-D-arabinofuranoside (1 μM) was added to the medium for another 2 days to minimize glial cell proliferation. All experiments were performed with 9–10-day-old hippocampal cultures. Immunostaining performed in cultured hippocampal cells at this stage showed that under our experimental conditions these cultures contained both neurons (neuron-specific enolase staining) and astroglia positive for glial fibrillary acidic protein.

2.4. Excitotoxic neuronal damage

Experiments were performed with 9–10-day-old cultures, a time period during which neurons express glutamate receptors and are vulnerable to excitotoxicity. The cells were damaged with L-glutamate as described previously (Koh and Choi, 1988). Briefly, the cultures were washed with serum-free medium and exposed to serum-free medium containing 0.5 mM L-glutamate for 1 h. Afterwards, glutamate was washed out and fresh medium with dissolved drugs or with vehicle was added to the cultures for 18 h (the point of measurement of glutamate toxicity). The damaged neurons were visualized with trypan blue. The identification of neuronal phenotype was based on standard morphological criteria, such as phase-bright fusiform and pyramidal cell bodies, which extend one or more processes. Neurons stained with trypan blue and possessing fragmented neurites were regarded as damaged. The nonstained neurons with intact neurites and soma were considered viable. Bay × 3702 (0.001 to 1 μM) was added immediately after the end of exposure to glutamate and was present in the culture medium until evaluation of neuronal damage (18 h later). In separate experiments, the effect of WAY 100635 on the neuroprotective activity of Bay × 3702 was tested. WAY 100635 (1 μM) was added

to the culture medium 30 min before exposure to Bay × 3702 (0.1 μM) and was present during the time of Bay × 3702 treatment. Control cultures received the vehicle only.

2.5. Surgical procedure and induction of ischemia

Focal cerebral ischemia was produced according to Tamura et al. (1981) with minor modifications. The animals were anesthetized with a mixture of 68.5% nitrous oxide and 30% oxygen that contained 1.5% halothane. A vertical incision was performed between the left orbit and the ear and the skull was exposed. The skull was opened with the aid of a drill (Proxxon, Niersbach, Germany). After removal of the Dura mater, the left middle cerebral artery was irreversibly occluded by microbipolar electrocoagulation. Body temperature was maintained at 37 ± 1°C with a heating lamp during the operation and the period of the i.v. infusion. Afterwards, to prevent a decrease of body temperature, the animals were kept at an environmental temperature of 30°C up to 2 h after middle cerebral artery occlusion. Mean arterial blood pressure and plasma glucose concentrations as well as arterial pH, pCO_2 and pO_2 were monitored (Corning 178, Corning, Germany) during the administration of Bay × 3702.

Seven days after occlusion of the middle cerebral artery the rats were anesthetized with chloral hydrate (400 mg/kg i.p.) and decapitated. The brains were removed from the skull and frozen in isopentane (Fluka, Buchs, Switzerland) on dry ice. The brains were stored at an environmental temperature of 25°C. With the aid of a cryomicrotome (Reichert-Jung, Nussloch, Germany) brain slices of 20 μm thickness were taken every 500 μm. The sections were stained for histology with 0.5% cresyl violet to differentiate between intact and damaged brain tissue. The infarct volume (mm^3) was calculated from the infarct area of each section and the distance between succeeding sections. The infarct volume was calculated, using an image analyzing system (IBAS 2, Kontron, Eching, Germany). Since the lenticulostriatal branches of the middle cerebral artery were not occluded, brain tissue damage was restricted to the cerebral cortex. Bay × 3702 (12 and 40 μg/kg) was infused i.v. with a perfusor (Secura, Braun, Melsungen, Germany) for a period of 4 h immediately after occlusion of the middle cerebral artery. The drug was infused into the tail vein with a constant flow rate of 3 μg/kg per h or 10 μg/kg per h. MK 801 (positive control) was injected i.v. as a bolus immediately after induction of ischemia. The experiments were conducted with approval of the Government Commission of Animal Care, Germany.

2.6. Statistics

All in vitro data are means ± S.D. from n experiments. The values for the infarct volume are presented as means ± S.E.M. of n experiments. Multiple comparisons were made by one-way analysis of variance (ANOVA) with subsequent Duncan or Scheffé tests. Homogeneity of errors was determined using Bartlet's test. P values of < 0.05 were considered to be significant.

3. Results

3.1. Cell culture morphology

After 10 days in vitro, control (untreated) cultures exhibited two morphological cell types: rounded neuron-like cells and flattened glial cells (Fig. 2B). Anti-neuron-specific enolase antibody labeled cells with a small rounded soma, with a highly developed neuritic network. Astrocytes were labeled with anti-glial fubrillary protein antibody (data not shown). The neurons grew on the feeder monolayer of flat-type astrocytes and were easily distinguishable from astrocytes by their specific cell morphology, pyramidal cell body and one or more extended major processes.

3.2. Characterization of glutamate-induced toxicity in rat hippocampal cultures

Hippocampal cultures were treated with 0.5 mM L-glutamate for 1 h and subsequently reincubated in culture medium lacking glutamate for 18 h. During the exposure to glutamate the neurons exhibited granulated soma which were moderately swollen. These somatic changes were accompanied by swelling of neurites. Thus, glutamate at a concentration of 0.5 mM induced changes characteristic of neuronal necrosis during the early stages of degeneration. Additionally, glutamate induced rapid cell death in a neuronal subpopulation since approximately 40% of the neurons die within 3 h after exposure to this excitotoxin as

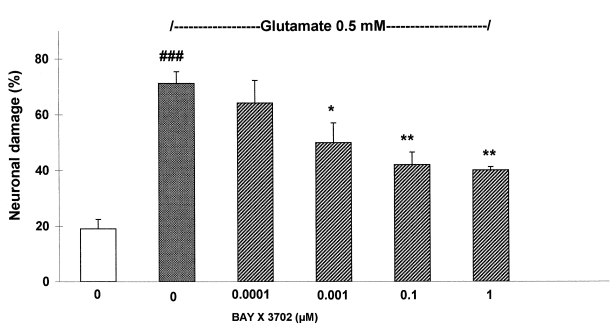
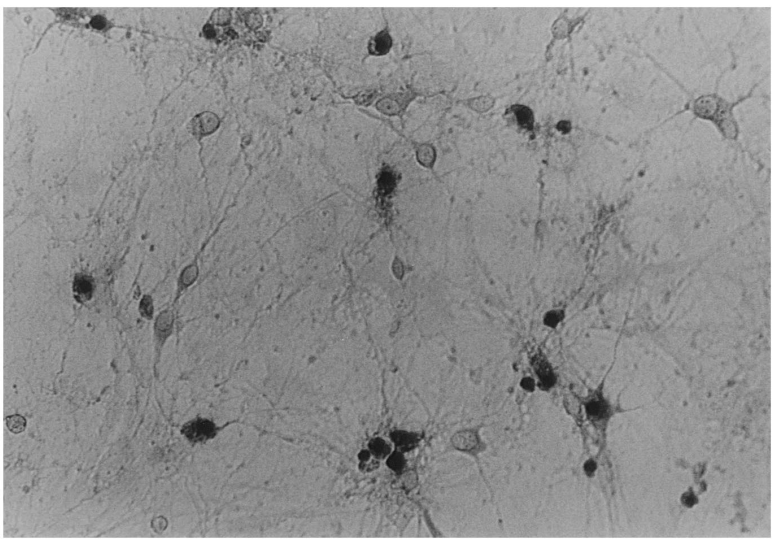


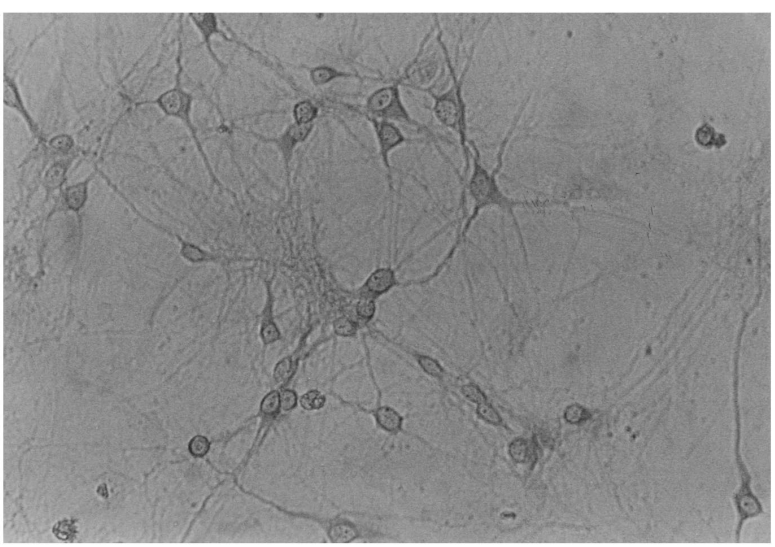
Fig. 1. Bay × 3702 protects hippocampal neurons against excitotoxic damage. The cells were grown for 9 days and then exposed to 0.5 mM L-glutamate for 1 h in serum-free medium. Neuronal damage was determined 18 h later by trypan blue exclusion and based on morphological criteria. The cells were treated with different concentrations of Bay × 3702 (0.0001 to 1 μM) or vehicle immediately after the end of exposure to glutamate (posttreatment). The drug was present in the culture medium until the evaluation of neuronal damage. The values are means ± S.D. from five different experiments. Different from glutamate-exposed cells: $^{*}P < 0.05$; $^{**}P < 0.01$ and from nondamaged cells: $^{###}P < 0.001$ by Scheffé test.

A.



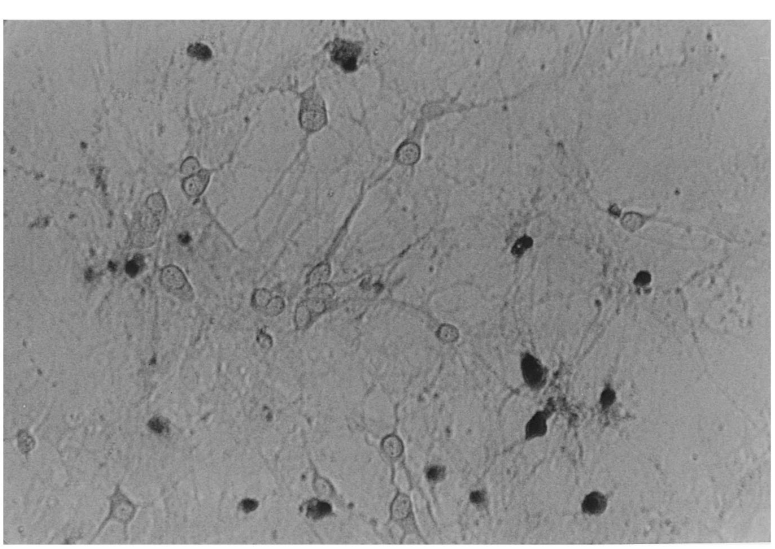
Glutamate
(0.5 mM)

B.



Control

C.



Bay x 3702
(1 μM)
+
Glutamate
(0.5 mM)

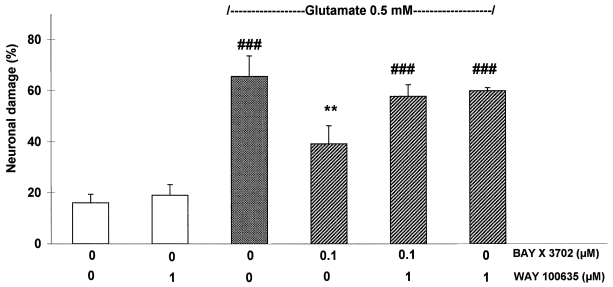


Fig. 3. Bay×3702 protects hippocampal neurons from excitotoxicity. After 9 days in vitro, hippocampal cells were exposed to 0.5 mM L-glutamate for 1 h (A, C) or to saline (B). The cells were treated with 1 μM Bay×3702 (C) immediately after the end of exposure to glutamate. The drug was present in the culture medium until evaluation of neuronal damage (18 h later). Bay×3702 (1 μM) reduced the number of trypan-blue positive cells and preserved cell morphology as shown in photomicrograph C.

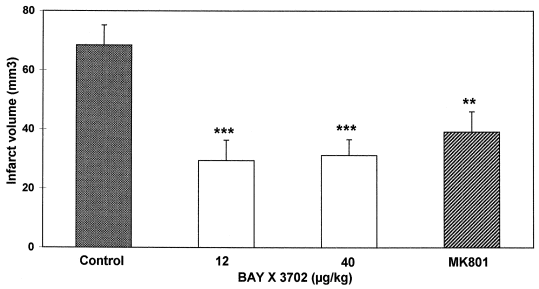


Fig. 4. Bay×3702 reduces cortical infarct volume in a rat model of permanent focal ischemia. Bay×3702 was administered i.v. immediately after occlusion of the MCA for a period of 4 h. The drug was infused into the tail vein with a constant flow rate of 3 μg/kg per h or 10 μg/kg per h. MK 801 (positive control) was injected i.v. as a bolus immediately after occlusion of the MCA. Values are given as means ± S.E.M. of $n = 10–12$ animals. Different from control: $^{**}P < 0.01$; $^{***}P < 0.001$.

indicated by trypan blue staining (data not shown). However, there was a progressive increase in the number of neurons displaying chromatin condensation and formation of typical apoptotic nuclei 8–12 h after treatment with glutamate. These neurons excluded trypan blue, i.e., they had intact cell membranes. Eighteen hours after exposure to glutamate (the time of evaluation of the drug effect on glutamate toxicity), approximately 70% of the neurons lost their membrane integrity and stained with trypan blue. Photomicrographs show pronounced neuronal degeneration, a widely destroyed neuronal network and many neurons stained with trypan blue displaying characteristics typical of necrotic damage (Fig. 2A).

3.3. Bay × 3702 protected hippocampal neurons from glutamate-induced excitotoxicity

Treatment of hippocampal cultures with Bay × 3702 (0.0001 to 1 μM) increased concentration—dependently the number of viable neurons (Fig. 1). The drug was added immediately after the end of the exposure to glutamate and was present in the culture medium until the evaluation of neuronal damage. The most pronounced protection was observed at a concentration of 1 μM Bay × 3702 ($P < 0.01$ vs. glutamate-exposed controls, Scheffé test; Fig. 1). Additionally, Bay × 3702 preserved cell morphology and the integrity of the neuronal network (Fig. 2C). The neuroprotective activity of Bay × 3702 (0.1 μM) was abolished by WAY 100635 (1 μM) added to the medium 30 min before and remaining during the exposure to Bay × 3702 (Fig. 3). WAY 100635, described as a selective postsynaptic 5-HT_{1A} receptor antagonist, itself, or in combination with glutamate, had no effect on neuron viability (Fig. 3).

3.4. Bay × 3702 reduced cortical infarct volume in a rat model of focal cerebral ischemia

Permanent occlusion of the middle cerebral artery in rats caused ischemic cortical injury as evaluated 7 days later as infarcted brain volume (mm^3). Intravenous infusion of Bay × 3702 (3 μg/kg per h; 10 μg/kg per h) for 4 h immediately after occlusion of the middle cerebral artery greatly reduced the infarct volume compared with that in vehicle-treated control animals (Fig. 4). The infarct volume of untreated animals measured seven days after induction of cerebral ischemia was 68.4 ± 3.6 mm^3 ($n = 12$ animals). An infarct volume of 29.4 ± 6.9 mm^3 ($n = 11$ animals) was measured after treatment with 12 μg/kg Bay × 3702, implying a 57% reduction of damaged brain tissue ($P < 0.001$, Duncan test). A similar reduction of ischemic damage was observed with 40 μg/kg Bay × 3702 (55% reduction of the infarct volume compared with untreated animals, $P < 0.001$, Duncan test). Intravenous injection of 1 mg/kg MK 801 (positive control) immediately after middle cerebral artery occlusion also significantly reduced the damage of brain tissue by approximately 40% ($P < 0.01$ vs. control animals; Duncan test) (Fig. 4). Mean arterial blood pressure and blood glucose levels were not changed during the period of infusion of Bay × 3702 (Fig. 5D, E). The values for arterial pH,

Fig. 2. WAY 100635, a specific 5-HT_{1A} receptor antagonist, abolished the neuroprotective activity of Bay × 3702. After 9 days in vitro, neuronal injury was induced by adding 0.5 mM L-glutamate for 1 h. Neuronal degeneration was evaluated 18 h later by trypan blue staining and morphological criteria. WAY 100635 (1 μM) was added to the medium 30 min before exposure to Bay × 3702 (0.1 μM). Both compounds were present in the culture medium after exposure to glutamate until evaluation of neuron degeneration. The values are means ± S.D. from four different experiments. Different from glutamate-exposed cells: $^{**}P < 0.01$ and from nondamaged controls: $^{###}P < 0.001$ by Scheffé test.

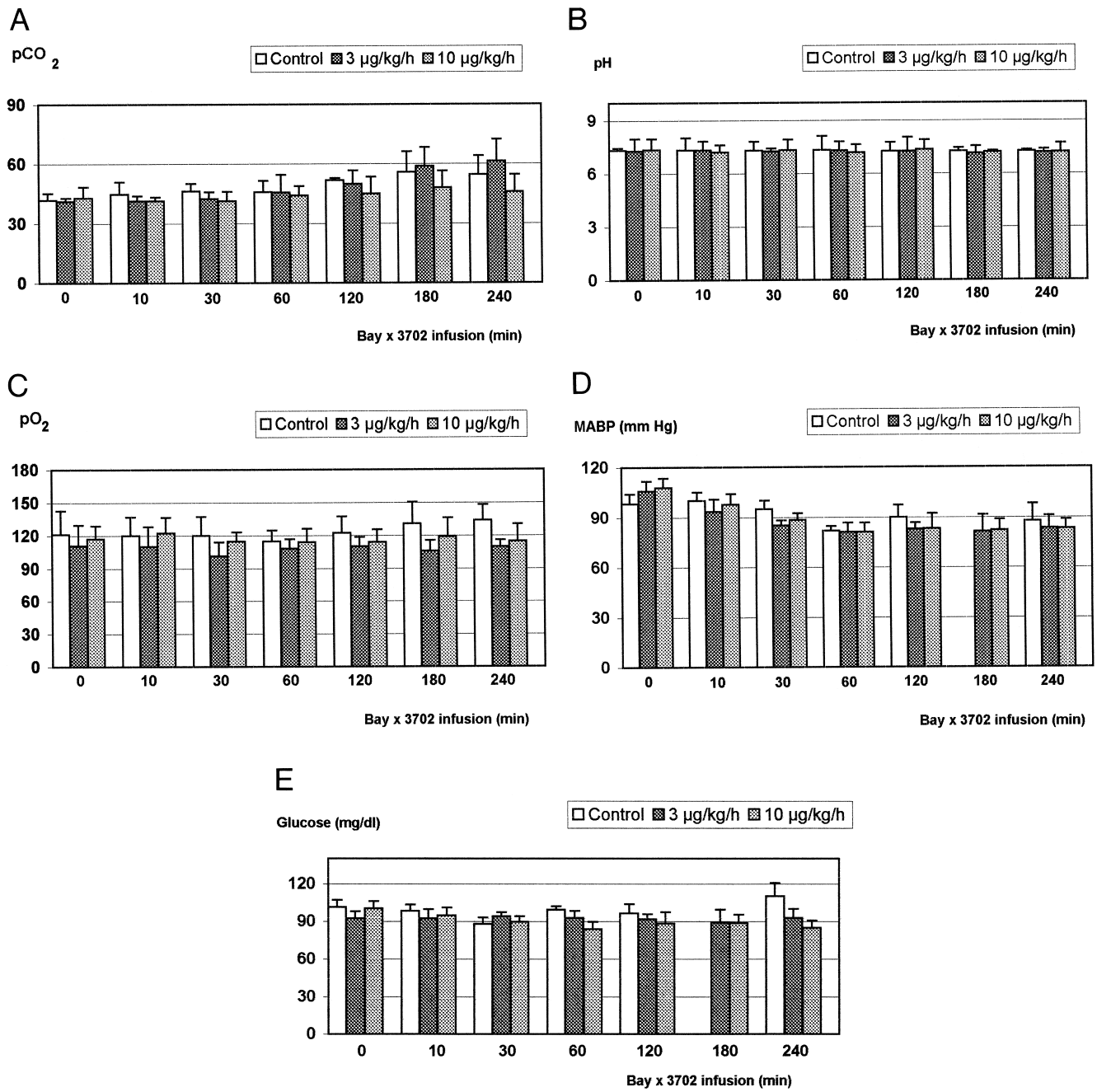


Fig. 5. Physiological variables. Bay × 3702 was infused to Long–Evans rats for a period of 4 h. 0: start of Bay × 3702 infusion; 240: end of Bay × 3702 infusion. MABP = mean arterial blood pressure. (A, C) Values for arterial pCO_2 and pO_2. (B, D) MABP and pH values. (E) blood glucose levels. Values are given as means ± S.D. from 10 to 12 animals.

pCO_2 and pO_2, monitored at the same time, were not influenced by treatment with this drug (Fig. 5A, B, C).

4. Discussion

The role of the serotonergic system in the pathophysiology of cerebral ischemia is not well understood. Kozuka and Iwata (1995) pointed out that the concentration of 5-HT in the cerebral cortex was decreased in incompletely ischemic rat brain. On the other hand, destruction of the 5-HT system by administration of a neurotoxin resulted in aggravation of the neuronal degeneration after cerebral ischemia (Nakata et al., 1997). It is well known that the neurotransmitter, 5-HT, produces its effects through activation of several subtypes of receptors (Humphrey et al., 1993) and has both excitatory and inhibitory effects on neuronal activity (Segal, 1980; Davies et al., 1987). Stimulation of the 5-HT_{1A} subtype has been shown to induce neuronal hyperpolarization, most likely mediated by acti-

vation of G protein-coupled K^+-channels, and consequent inhibition of neuronal activity (Andrade et al., 1986; Brandao et al., 1991; Rupalla et al., 1994; de Vry, 1995). Moreover, an inhibitory effect of 5-HT on glutamate release, mediated through 5-HT_{1A} receptors has also been demonstrated (Maura et al., 1988). Furthermore, 5-HT_{1A} receptors are widely distributed within the brain and are highly expressed in some areas which are sensitive to ischemia-induced neuronal injury such as the hippocampus and neocortex, as well as on the serotonergic cell bodies and dendrites in the raphe nuclei (Pazos and Palacios, 1985; Chalmers and Watson, 1991; Azmitia et al., 1996). The highest density of 5-HT_{1A} receptors has been shown in the dentate gyrus, CA1, CA2 and CA3 hippocampal subfields (Miquel et al., 1994; Wright et al., 1995). Therefore, the brain distribution of 5-HT_{1A} receptors, as well as the hyperpolarizing effects resulting from their activation, suggest that 5-HT_{1A} receptor agonists offer an opportunity for effective neuroprotection. In fact, it has been already demonstrated that 5-HT_{1A} receptor agonists such as ipsapirone, urapidil and roxindole exert neuroprotective properties against excitatory amino acid-induced damage in vitro as well as in rodent models of cerebral ischemia (Bielenberg and Burkhardt, 1990; Nuglisch et al., 1990; Shibata et al., 1992; Prehn et al., 1993). However, these compounds possess only limited 5-HT_{1A} selectivity and therefore, limited intrinsic activity for 5-HT_{1A} receptors (Doods et al., 1985; Gross et al., 1987). Thus, the development of highly selective 5-HT_{1A} receptor agonists with a strong neuroprotective potency is of interest. Bay × 3702 is a newly synthesized compound which has been characterized as a selective and potent 5-HT_{1A} receptor agonist (de Vry et al., 1997). Therefore, we attempted to investigate the neuroprotective potency of this drug against excitotoxic damage in primary hippocampal cultures. Hippocampal neurons express 5-HT_{1A} receptors and are vulnerable to glutamate-induced degeneration. It has been indicated that glutamate-induced excitotoxicity in hippocampal neurons is due to massive Ca^{2+} influx through NMDA-receptors since NMDA receptor antagonists are able to attenuate the degree of neuronal injury (Simon et al., 1984). Under our experimental conditions, the exposure to 0.5 mM L-glutamate for 1 h induced pronounced neurotoxicity as evaluated 18 h later by trypan blue staining and morphological criteria. Glutamate-induced degeneration of cultured rat hippocampal neurons produced morphological changes characteristic of neuronal necrosis. These changes included swelling of the neuronal soma and neurites still observed during the exposure to this neurotoxin and early loss of membrane integrity. Therefore, a few hours after exposure to 0.5 mM glutamate, approximately 40% of the neurons stained with trypan blue (data not shown). On the other hand, certain morphological changes characteristic of neuronal apoptosis became evident subsequent to the glutamate exposure. These latter changes included cell shrinkage and nuclear pyknosis. Thus, glutamate-induced toxicity (0.5 mM for 1 h) appeared to involve both necrotic and apoptotic processes, which is in agreement with previously published results for cerebellar neuronal cultures (Ankarcrona et al., 1995). However, Bay × 3702 reduced the number of trypan blue-positive neurons in a concentration-dependent manner (maximal effect at 1 μM) as evaluated 18 h after exposure to glutamate. Additionally, the drug preserved cell morphology and the integrity of the neuronal network. Since the glutamate-damaged neurons showed, at various times within 18 h after damage, both necrotic and apoptotic features, we suggest that Bay × 3702 exerts both antiapoptotic and antinecrotic activities. This supports previously published data about an antiapoptotic potency of 8-hydroxydipropylaminotetralin (8-OH-DPAT), a selective ligand for 5-HT_{1A} receptors, in cultured chick embryonic neurons (Ahlemeyer and Krieglstein, 1997). The neuroprotective activity of Bay × 3702 was observed at very low concentrations (1 nM, for example) which is consistent with the reported high potency and selectivity of this compound for 5-HT_{1A} receptors (inhibition constant for rat 5-HT_{1A} receptors $K_i = 0.2$ nM). Concerning its intrinsic activity, the compound was characterized as a full agonist of 5-HT_{1A} receptors (de Vry et al., 1997). An agonistic profile with an IC_{50} value of 3.5 nM, for inhibiting forskolin-stimulated adenylate cyclase in rat hippocampal tissue was reported (de Vry, 1995). Furthermore, pretreatment with Bay × 3702 was not necessary to achieve neuroprotection. This drug greatly reduced neuronal degeneration when it was added immediately after induction of injury and was present in the culture medium until evaluation of neuronal damage. We demonstrated that the neuroprotective effect of Bay × 3702 was mediated through selective stimulation of 5-HT_{1A} receptors by the 5-HT_{1A} receptor antagonist, WAY 100635. The neuroprotective activity of Bay × 3702 was completely abolished by WAY 100635. The protective effect of Bay × 3702 could be associated with G-protein dependent opening of K^+ channels and hyperpolarization of the cell membrane (Penington et al., 1991; Rupalla et al., 1994). The mechanism underlying the neuroprotective effect of Bay × 3702 might not be restricted to the opening of a potassium conductance since Murase et al. (1990) have shown that 5-HT_{1A} receptor agonists reduce NMDA-evoked currents in isolated spinal dorsal horn neurons. Additionally, results of other studies indicated that stimulation of 5-HT_{1A} receptors inhibits the voltage-dependent Ca^{2+} influx (Rupalla et al., 1994; Chen and Penington, 1996) which also can contribute to the neuroprotective activity of 5-HT_{1A} receptor agonists. In this respect, recent studies on cortical neurons indicated that mainly N- and P-type Ca^{2+} currents were modulated by 5-HT_{1A} receptor agonists (Foehring, 1996). Furthermore, it has been shown that serotonin can induce differentiation and stimulates neurite extension and sprouting in serotonergic neurons during early development (Whitaker-Azmitia and Azmitia, 1986; Ueda et al.,

1994). It has been suggested that this neurotrophic effect of serotonin is mediated via activation of 5-HT_{1A} receptors on astrocytes and subsequent release of S-100β (Azmitia et al., 1990; Whitaker-Azmitia et al., 1990; Yan et al., 1995). S-100β is a Ca^{2+}-binding protein exclusively produced in astroglial cells (Donato, 1991) and characterized as a serotonergic growth factor (Haring et al., 1993) which can induce neurite extension in primary neurons (Azmitia et al., 1990; Liu and Lauder, 1992; Yan et al., 1995). Moreover, S-100β has been reported to protect hippocampal neurons from glucose deprivation-induced damage by modulation of intracellular calcium homeostasis and preservation of mitochondrial function (Barger et al., 1995). The synthesis of S-100β in astrocytes is up-regulated by 5-HT_{1A} receptor agonists. Since our cultures contain both neurons and astrocytes we cannot exclude the possibility that the stimulation of S-100β synthesis contributes to the protective activity of Bay × 3702 on hippocampal neurons.

Based on the results of the in vitro study and on recently published data (Horvath et al., 1997), we tested the protective potency of Bay × 3702 in a rat model of permanent focal ischemia. We could also demonstrate that this drug protects brain tissue against ischemic damage due to permanent occlusion of the middle cerebral artery when it was administered immediately after the onset of ischemia. The cortical infarct volume was greatly reduced by post-ischemic infusion of Bay × 3702 (12 and 40 μg/kg) to 57 and 55%, respectively. Therefore, the protective effect of Bay × 3702 in vitro was confirmed in a rat model of focal ischemia. The enhanced concentration of glutamate in the synaptic space, followed by neuronal depolarisation and accumulation of Ca^{2+} in the postsynaptic neuron underlies the neurodegeneration that occurs after ischemic insults. Various pathophysiological processes, i.e., activation of proteolytic enzymes, enhanced formation of oxygen free radicals and nitric oxide (NO) synthesis are triggered when the neuron is overloaded with Ca^{2+} (for review, see Rami and Krieglstein, 1993; Krieglstein, 1996). It has been reported that 5-HT_{1A} receptor agonists block the glutamate-induced increase in the intracellular Ca^{2+} concentration. The protective potency of Bay × 3702 in vivo could be explained by stimulation of 5-HT_{1A} receptors followed by hyperpolarization and increased resistance of the neuronal membrane to excitotoxicity. This, together with the reported inhibition of voltage-dependent Ca^{2+}-influx (Rupalla et al., 1994), leads to prevention of the increase in intracellular Ca^{2+} after ischemia. Additionally, stimulation of 5-HT_{1A} receptors has been shown to inhibit the ischemia-induced excessive release of glutamate (Maura et al., 1988; Murase et al., 1990; de Vry et al., 1997) which can also contribute to the protective effect of Bay × 3702. Except for the changes in the electrophysiological profile of the neurons, we cannot exclude the involvement of other factors in the protective activity of Bay × 3702 such as stimulated production and release of neurotrophic protein S-100β from astrocytes, an effect, also mediated through activation of 5-HT_{1A} receptors. Additional experiments will be required to clarify this point. Furthermore, our results are in agreement with the previously reported potency of 5-HT_{1A} receptor agonists to protect brain tissue from ischemic damage in different rodent models of ischemia (Prehn et al., 1993; Piera et al., 1995; Horvath et al., 1997). However, Bay × 3702 was effective at relatively lower doses than other 5-HT_{1A} receptor agonists, which is consistent with its higher selectivity for 5-HT_{1A} receptors and therefore, higher intrinsic activity and neuroprotective potency.

The data from this study clearly demonstrate that treatment with Bay × 3702 reduced neuronal death due to exposure of hippocampal cultures to a toxic concentration of glutamate. In vivo, post-ischemic infusion of Bay × 3702 greatly reduced cortical infarct volume due to permanent occlusion of the middle cerebral artery in rats, suggesting that the drug might be useful for the treatment of acute cerebral infarction. Furthermore, our results confirm the hypothesis that the synthesis of highly selective and potent 5-HT_{1A} agonists like Bay × 3702 is a promising approach to achieve neuroprotection.

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