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BRAIN RESEARCH

Research Report

Role of glycine receptors and glycine release for the neuroprotective activity of bilobalide

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ABSTRACT

Bilobalide, a constituent of *Ginkgo biloba*, has neuroprotective properties. Its mechanism of action is unknown but it was recently found to interact with neuronal transmission mediated by glutamate, γ-aminobutyric acid (GABA) and glycine. The goal of this study was to test the interaction of bilobalide with glycine in assays of neuroprotection. In rat hippocampal slices exposed to *N*-methyl-D-aspartate (NMDA), release of choline indicates breakdown of membrane phospholipids. NMDA-induced choline release was almost completely blocked in the presence of bilobalide (10 μM). Glycine (10–100 μM) antagonized the inhibitory action of bilobalide in this assay. In a second assay of excitotoxicity, we measured tissue water content as an indicator of cytotoxic edema formation in hippocampal slices which were exposed to NMDA. In this assay, edema formation was suppressed by bilobalide but bilobalide's action was attenuated in the presence of glycine and of D-serine (100 μM each). To investigate bilobalide's interaction with glycine receptors directly, we determined ^{36}chloride flux in rat cortico-hippocampal synaptoneurosomes. Glycine (100 μM) was inactive in this assay indicating an absence of functional glycine-A receptors in this preparation. [^{3}H]Glycine was used to assess binding at the glycine binding site of the NMDA receptor but bilobalide was found to be inactive in this assay. Finally, [^{3}H]glycine release was monitored in hippocampal slices exposed to oxygen-glucose deprivation. In this model, glycine release was induced by ischemia, an effect that was strongly reduced by bilobalide. We conclude that bilobalide does not interact with glycine receptors in neurochemical assays but it significantly reduces the release of glycine under ischemic conditions. This effect likely contributes to bilobalide's neuroprotective effects in assays of excitotoxicity and ischemia.

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Abbreviations: AU, arbitrary units; AUC, area under the curve; DCKA, 5,7-dichlorokynurenic acid; DFP, di-isopropyl fluorophosphates; DIDS, 4,4′-di-isothiocyanostilbene-2,2′-disulfonic acid; GABA, γ-aminobutyric acid; HEPES, (4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid; NMDA, *N*-methyl-D-aspartate; OGD, oxygen-glucose deprivation; TLC, thin-layer chromatography

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1. Introduction

EGb761, a defined extract of the leaves of the Ginkgo tree (*Ginkgo biloba*), is a clinically useful medication for chronic neurodegenerative diseases such as Alzheimer's dementia (Oken et al., 1998; DeFeudis and Drieu, 2000). In experimental work, Ginkgo extracts were found to interfere with amyloid formation, aggregation and toxicity (Bastianetto et al., 2000; Luo et al., 2002; Colciaghi et al., 2004), and this activity may underlie their antidementia actions. Recent publications have also demonstrated anti-ischemic and anti-edema properties of Ginkgo extract and its constitutent, bilobalide, which represents 3% of EGb761 (DeFeudis, 2002; Ahlemeyer and Krieglstein, 2003). Neuroprotection by bilobalide was observed in models of ischemia and excitotoxicity, both *in vitro* and *in vivo* (Krieglstein et al., 1995; Chandrasekaran et al., 2001). In our hands, bilobalide blocked cellular toxicity and phospholipid breakdown in hippocampal slices exposed to hypoxia or to N-methyl-D-aspartate (NMDA) acting in the low micromolar range (Klein et al., 1997; Weichel et al., 1999). Recently, we reported potent anti-edema effects of bilobalide after middle cerebral artery occlusion in mice (Mdzinarishvili et al., 2006). The mechanism of action of bilobalide in these models, however, remains elusive.

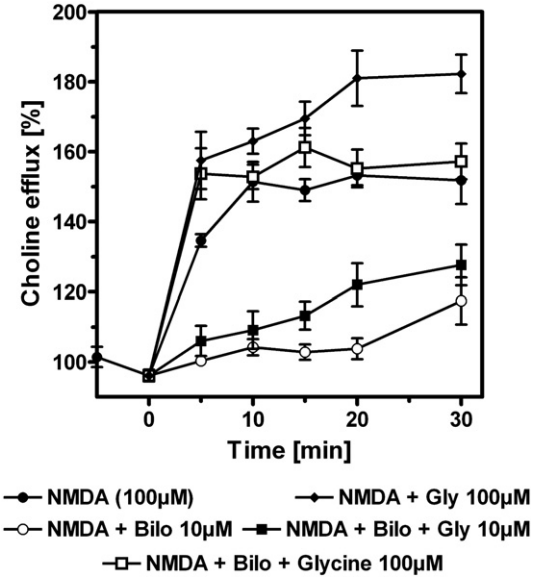

Fig. 1 – Choline release from hippocampal slices induced by NMDA receptor activation: effects of bilobalide and glycine. — Hippocampal slices were superfused with magnesium-free solution. N-methyl-D-aspartate (NMDA) was added at time zero. Choline efflux was measured using a chemoluminescence assay. Data are as follows: "NMDA (100 μM)" indicates the effect of NMDA alone. "NMDA + Bilo", "NMDA + Gly" and "NMDA + Bilo + Gly" show the responses of NMDA in the presence of bilobalide (Bilo, 10 μM) or glycine (10 or 100 μM), or both. Bilobalide and glycine, when present, were added together with NMDA. Bilobalide or glycine, when given alone or together, did not affect basal choline efflux (data not shown). Data are given as relative changes of the basal choline efflux (determined from three consecutive samples before addition of NMDA) and are means ± S.E.M. of 4–6 experiments.

Table 1 – Area under the curve (AUC) values from choline efflux experiments

Condition	AUC Value*	N
NMDA	267 ± 15.8	5
NMDA + Glycine (100 μM)	369 ± 12.8[a]	4
NMDA + Bilobalide	26.5 ± 10.3[b]	5
NMDA + Bilo + Glycine (10 μM)	88.9 ± 26.5[b]	5
NMDA + Bilo + Glycine (100 μM)	308 ± 22.3[c]	6

*AUC values were calculated from the data shown in Fig. 1, for 30 min of choline release. They are given as arbitrary units (AU, formally representing [%] min). Statistical evaluation (ANOVA with Tukey's Multiple Comparison Test): [a], $p<0.05$ vs. NMDA. [b], $p<0.01$ vs. NMDA. [c], $p<0.01$ vs. NMDA + Bilobalide.

A variety of cellular mechanisms were proposed for the neuroprotective effects of bilobalide, among them effects on nitric oxide and platelet-activating factor, on mitochondrial function and apoptosis as well as genomic and proteomic effects (DeFeudis, 2002; Ahlemeyer and Krieglstein, 2003). Much recent evidence from our and other laboratories points to an interference of bilobalide with amino acidergic neurotransmission which is mediated by glutamate, γ-aminobutyric acid (GABA) and/or glycine. For instance, tissue levels of these transmitter amino acids in the brain were increased following chronic bilobalide administration (Sasaki et al., 2002). In hippocampal slices, bilobalide blocked glutamatergic, NMDA receptor-mediated actions both in electrophysiological and in neurochemical assays (Chatterjee et al., 2003; Klein et al., 2003). Bilobalide was also found to interfere with the release of glutamate under hypoxic/hypoglycemic conditions (Davies et al., 2003). Bilobalide was reported as a potent noncompetitive antagonist at $GABA_A$ receptors (Huang et al., 2003; Ivic et al., 2003) and was also found to block glycine-A receptors, albeit at lower potency (Ivic et al., 2003; Hawthorne and Lynch, 2005). Moreover, it was reported that bilobalide structurally resembles picrotoxin as a ligand of GABA and glycine channels (Hawthorne and Lynch, 2005). However, we have previously reported that interactions with GABAergic mechanisms cannot explain bilobalide's neuroprotective activities (Kiewert et al., 2007).

In the present communication, we tested if bilobalide's neuroprotective activity can be explained by interactions with glycine. For this purpose, we tested bilobalide-glycine interactions in two models of excitotoxicity. We also determined if bilobalide directly interferes with glycine receptors or with glycine release.

2. Results

2.1. *Choline release from hippocampal slices*

Membrane breakdown is a typical consequence of excitotoxicity and can be monitored using the choline release assay which was established in our laboratory (Weichel et al., 1999; Klein, 2000). In the present study, we superfused rat hippocampal slices with NMDA (100 μM) in magnesium-free Tyrode solution (Fig. 1). Basal choline efflux from the slices (1.5 ± 0.1 pmol/10 μl, $N=24$)