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Effects of bilobalide on amino acid release and electrophysiology of cortical slices

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Summary. This study investigated the effects of bilobalide, a constituent of *Ginkgo biloba*, on potassium and veratridine-induced release of glutamate and aspartate from mouse cortical slices. We also studied its effects on spontaneous and N-methyl-D-aspartate (NMDA)-induced depolarizations elicited in magnesium-free artificial cerebrospinal fluid (aCSF) as well as its effect on NO-711 (a γ-aminobutyric acid (GABA) uptake inhibitor)-induced depolarizations. Bilobalide, 100 μM significantly reduced both glutamate and aspartate release elicited by potassium or veratridine. Bilobalide (5–100 μM) also significantly reduced the frequency of NO-711 induced depolarizations, however, it had no effect on spontaneous or on NMDA-induced depolarizations at 5–200 μM. These results suggest that the neuroactive properties of bilobalide may be mediated by a reduction in excitatory amino acid neurotransmitter release.

Keywords: Amino acids – Bilobalide – Mouse cortical slices – Glutamate – Depolarizations

Introduction

Although diverse therapeutic uses of extracts prepared from various parts of the *Ginkgo biloba* tree have been used in Chinese medicine for many centuries, it was only relatively recently that the medicinal uses of the leaf extracts of this ancient tree became known to the western world and to modern medicine (DeFeudis, 1998). Currently, various extracts of *Ginkgo biloba* leaves are being investigated as treatments for a variety of ailments from

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glaucoma (Ritch, 2000) to sexual dysfunction (Ashton et al., 2000), from tinnitus (Ernst and Stevinson, 2000) to a treatment for Alzheimer's disease (Dinsmore, 2000). The active constituents are considered to be the ginkgoflavone glycosides and the polycyclic lactone ginkgolides A, B, C, J and bilobalide (DeFeudis, 1998). Amongst these, the standardised extract referred to a EGb-761 is pharmacologically and clinically the most studied and its recommended therapeutic use is for the treatment of conditions related to disturbances in central and peripheral circulation (DeFeudis, 1998). Two recently reported controlled clinical trials indicate that it could also be used in the treatment of mild to moderate dementia associated with Alzheimer's disease (Le Bars et al., 1997; Maurer et al., 1997). These latter observations have renewed interest in the therapeutic uses of ginkgo extracts and have reopened the questions concerning the active constituents and their mechanisms of action.

Bilobalide is quantitatively the major single chemical constituent of EGb-761 and the first pharmacological action detected was a beneficial effect on cytotoxic brain oedema caused by triethyltin (Otani et al., 1986). Since then, several reports have not only revealed its diverse pharmacological properties but have also raised some speculative proposals concerning its mechanism of action. Bilobalide has been demonstrated to possess protective effects on infarct volume after focal cerebral ischaemia in rodents and against neuronal damage in cell culture (Krieglstein et al., 1995), it also accelerates the regeneration of rat motor neurones (Bruno et al., 1993).

Sasaki and colleagues (1990a) recently reported on the effects of bilobalide on population spikes in rat hippocampal slices. This group demonstrated that bilobalide significantly increased the amplitude of population spikes evoked by electrical stimulation of Schaffer collateral/commissural fibres. These results suggested that bilobalide enhanced the excitability of neurones. This group also reported that bilobalide was effective in significantly reducing muscimol (a $GABA_A$ receptor agonist)-induced inhibition of the population spikes. These observations led them to suggest that the actions of bilobalide may have in part involved a reduction in GABAergic inhibition. However, a second paper by Sasaki and colleagues (1999b) reported the effects of bilobalide on GABA levels and glutamic acid decarboxylase (GAD) activity, the enzyme responsible for production of GABA from glutamate. This paper reported that administration of bilobalide for four days prior to sacrifice caused a significant rise in levels of GABA in the cerebral cortex. It was also found that pre-treatment with bilobalide caused a significant enhancement of the total GAD activity in the cortex. The question whether the acute and chronic effects observed in these two studies were due to different mechanisms of action remain, however, unanswered. Bilobalide, has also been shown to inhibit hypoxia- or NMDA-induced phospholipase A_2 activation and phospholipid breakdown in rat hippocampus (Klein et al., 1997; Weichel et al., 1999). These observations suggest that the mode of action of bilobalide could be either due to inhibition of membrane depolarization caused by NMDA (or hypoxia) or via stimulation of the inhibitory GABAergic system.

In this paper we report the effects of bilobalide on veratridine- and potassium-stimulated amino acid release from mouse cortical slices and on magnesium-free artificial cerebrospinal fluid (aCSF)-, N-methyl-D-aspartate (NMDA)- and GABA uptake inhibitor (NO-711)-induced depolarizations in DBA/2 mouse cortical wedges.

Materials and methods

Amino acid release from BALB/c mouse cortical slices

Coronal cortical slices (400 μm) were prepared as previously described (Jones and Davies, 1999). Briefly, three of these slices were placed on a gauze disc and transferred to a heated (37°C) tissue bath and perfused with gassed (95% oxygen/5% carbon dioxide) aCSF (1 ml/minute). The slices were left for 60 minutes to equilibrate prior to commencing experiments as amino acid release following slicing has been shown to plateau after approximately 40 minutes (Barnes et al., 1988).

Eighteen consecutive 2-minute samples of perfusate were collected and neurotransmitter release was elicited using two-pulses of either veratridine hydrochloride (20 μM) or high potassium aCSF (60 mM). Veratridine or potassium was perfused for 1 or 2 minutes respectively during collection of the fourth and fifteenth samples. Bilobalide (10–100 μM) was dissolved in diethyl sulphoxide (DMSO, final concentration $<0.05\%$) and diluted to the required concentration with aCSF. The solution was gassed (95% oxygen/5% carbon dioxide) before being perfused onto the slices. Bilobalide (10–100 μM) was perfused for 14 minutes prior to and during the second pulse of stimulated amino acid release.

Samples were assayed for amino acid content using HPLC following pre-column derivatization with o-pthaldialdehyde (Turnell and Cooper, 1982). The following amino acids were measured, glutamate, aspartate, GABA, taurine, glycine, serine, and glutamine.

Electrical recording from cortical wedge preparation

Cortical wedges were prepared as previously described by Hu and Davies, (1995). Briefly, brains from male or female DBA/2 mice aged between 21 and 42 days were used. Coronal slices (500 μm) were cut and from these slices wedges of cerebral cortex, corpus callosum and striatum were prepared. Cuts of approximately 4 mm length were made close to the midline 2 mm apart at the cortex and tapering to 1 mm wide at the striatum.

One wedge was immediately transferred to a two-compartment tissue bath, where the grey cortical matter was separated from the callosum and striatum by a thin wall of silicon grease. The compartment containing the callosal side of the wedge was filled with aCSF and the second compartment, the cortical side, was continuously perfused with gassed aCSF at 2 ml/min. One hour was allowed for the tissue to equilibrate at room temperature (20–22°C). Drugs were perfused across the cortical side of the wedge. The direct current potential between the two compartments was continuously monitored using silver/silver chloride electrodes, amplified (Flyde 2601A) and displayed on a chart recorder (BBC Goertz-Metrawatt) and also recorded on a MacLab computer system (AD Instruments, Hastings, UK).

Cortical wedges were perfused with magnesium-free aCSF to produce spontaneous depolarizations following the removal of the natural magnesium ion block that occurs at NMDA-receptors. Preparations were perfused for one hour in order to obtain a stabilized frequency of depolarizations. Bilobalide was then perfused for 30 minutes at varying concentrations. Perfusion of NMDA (2.5–8.0 μM) onto cortical wedges produced slow depolarizations that could be recorded as peaks on a MacLab computer system. At the beginning of each experiment 10 μM NMDA was perfused as 2-minute pulses, with 15

minutes between pulses, until three peaks of equivalent heights had been achieved. Once a standardised peak height for 10μM NMDA had been found, concentration-response experiments for NMDA-induced depolarizations, at concentrations of 2.5–80μM, were carried out. Concentration-response curves were then constructed to NMDA in the presence of bilobalide. NO-711 (25μM) was perfused onto the wedges for 15 minutes to induce depolarizations. The tissue was left for approximately 40 minutes to allow a suitable number of depolarizations to occur to ascertain a control, stable, frequency of peaks for comparison with subsequent drug treatments. Bilobalide was dissolved in DMSO (final concentration $<0.05\%$) and then diluted in magnesium-free aCSF.

The composition of aCSF in mM: NaCl 124, KCl 5, NaH_2PO_4 1.25, $CaCl_2$ 2, $MgSO_4$ 2, $NaHCO_3$ 26, D-glucose 10 and the pH was 7.4. The composition of the 60 mM potassium aCSF was adjusted to maintain osmolarity with a corresponding decrease in NaCl, similarly there was an increase in NaCl in magnesium-free aCSF.

Drugs

Bilobalide was a gift from Dr Willmar Schwabe Arzneimittel, Karlsruhe, Germany. Veratridine was purchased from Sigma and NMDA from Tocris, all other chemicals were of analytical grade.

Statistical analysis

Amino acid release data were calculated as pulse 1/pluse 2 percentages and expressed as mean ± S.E.M. Student's *t*-test (unpaired) was used to calculate significance levels. One-way ANOVA followed by the Student-Newman-Keuls test was used to calculate drug effects.

Magnesium-free aCSF-induced depolarizations in the wedge preparation were counted over three five-minute periods: five minutes immediately prior to drug perfusion (1st-period), for the final five minutes of 15 minute drug perfusion (2nd-period) and then for five minutes fifteen minutes after the end of drug perfusion (3rd period). Values from the second and third periods were then compared to the value from the first period using Student's *t*-test (paired) to calculate significance levels. The time intervals between depolarizations induced by NO 711 were compared to the intervals following treatment with bilobalide and significance levels were calculated using Student's t-test for unpaired data.

Results

Bilobalide on amino acid release

Veratridine (20μM) and potassium (60 mM) both produced significant increases in levels of glutamate and aspartate of between 6–8 fold over basal during the first and second pulses of stimulated release. The second pulse of potassium elicited release of both glutamate and aspartate which was approximately 90% of the first pulse whereas the release of both amino acids to the second pulse of veratridine was approximately 70% of the first pulse.

Basal (unstimulated) levels of the amino acids were unaffected by any concentration of bilobalide. The lowest concentration of bilobalide (10μM) had no significant effect on the release of either aspartate or glutamate however, increasing concentrations (50 & 100μM) significantly (both $P < 0.01$) reduced potassium-stimulated release of both aspartate and glutamate (Fig. 1).

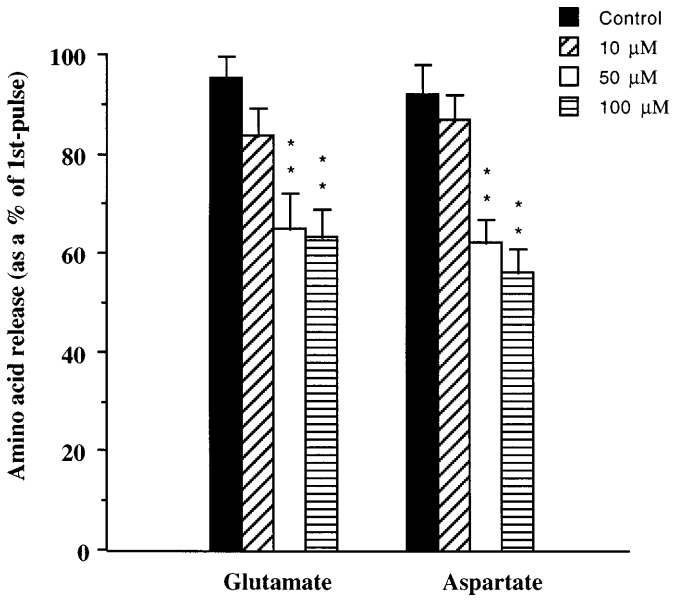

Fig. 1. The effect of bilobalide (10–100μM) on potassium-induced (60mM) release of glutamate and aspartate. The results are expressed as the mean ± s.e.mean stimulated release in the second pulse as percentage of the first pulse. n = 6–8 slice preparations. $^{**}P < 0.01$. Student's t-test was used to calculate significance levels

Bilobalide (10μM) did not significantly block veratridine-stimulated release of either aspartate or glutamate whilst 50μM reduced veratridine-stimulated release of aspartate ($P < 0.01$). 100μM bilobalide significantly blocked release of both aspartate and glutamate (Fig. 2; $P < 0.01$). Bilobalide, at all concentrations, had no effect on the release of the other amino acids assayed.

Effects of bilobalide on Mg^{2+}-induced depolarizations

Approximately 90% of wedges prepared from DBA/2 mice (aged 21–30 days) have been shown to exhibit fast spontaneous depolarizations when perfused with magnesium-free aCSF and the frequency of these depolarizations was between 5–15/min and were relatively fast in character with rise times of 70–120ms (Hu and Davies, 1997a). The results obtained in this present study were in agreement with those described previously from our laboratory; however, bilobalide (10–200μM) was ineffective in blocking these magnesium-free aCSF-induced depolarizations (Fig. 3a).

Effects of bilobalide on NMDA-induced depolarizations

Perfusion of increasing concentrations of NMDA (2.5–80μM) resulted in concentration-dependent depolarisations, bilobalide (200μM) had no significant effect on the NMDA-induced depolarizations (Fig. 3b).

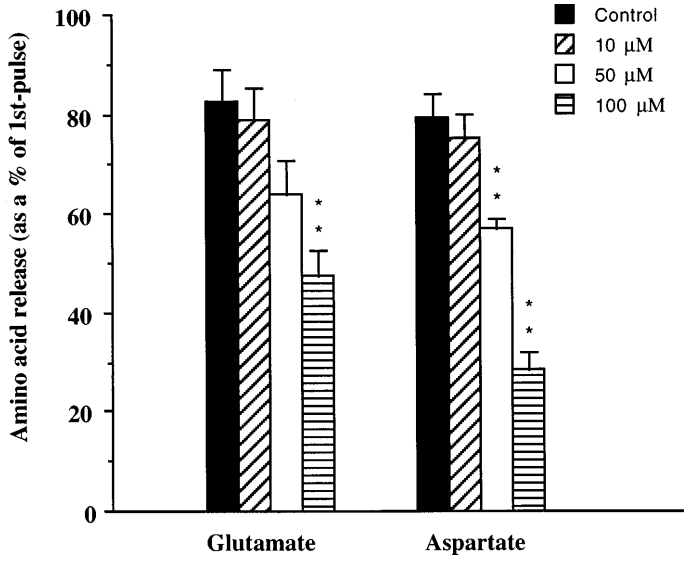

Fig. 2. The effect of bilobalide (10–100μM) on veratridine-induced (20μM) release of glutamate and aspartate. The results are expressed as the mean ± s.e.mean stimulated release in the second pulse as a percentage of the first pulse. n = 6–8 slice preparations. $^{**}P < 0.01$. Student's t-test was used to calculate significance levels

Effects of bilobalide on NO-711-induced depolarizations

Perfusion of NO-711 (25μM) for 15 minutes onto the cortical side of the wedges resulted in regular depolarizations (Fig. 4). The depolarizations observed following the perfusion of NO-711 continue at a steady frequency for up to 6 hours if left untreated.

Bilobalide (2.5–100μM) was perfused onto the cortical wedges for 30 minutes and concentration-dependently, significantly (5–25μM: $P < 0.05$, 50–100μM: $P < 0.01$) reduced the frequency of these recurrent NO-711-induced depolarizations (Fig. 5). Baclofen (20μM) perfused for 30 minutes had no effect on NO-711-induced depolarizations, nor did it have any effect, at the same concentration, on the action of bilobalide (results not shown).

Discussion

The results presented show that bilobalide is effective in reducing both potassium- and veratridine-stimulated release of the excitatory amino acids. Bilobalide was also effective in blocking NO-711-induced depolarizations, but had no significant effects on either NMDA-induced or Mg^{2+}-free aCSF-induced depolarizations.

The mechanism by which elevated levels of potassium ions stimulate release is secondary to changes in the transmembrane potential. This dramatic increase in potassium ion concentration results in the opening of voltage-sensitive calcium channels with a subsequent influx of calcium ions that results in exocytosis. The release of neurotransmitters in response to potassium does

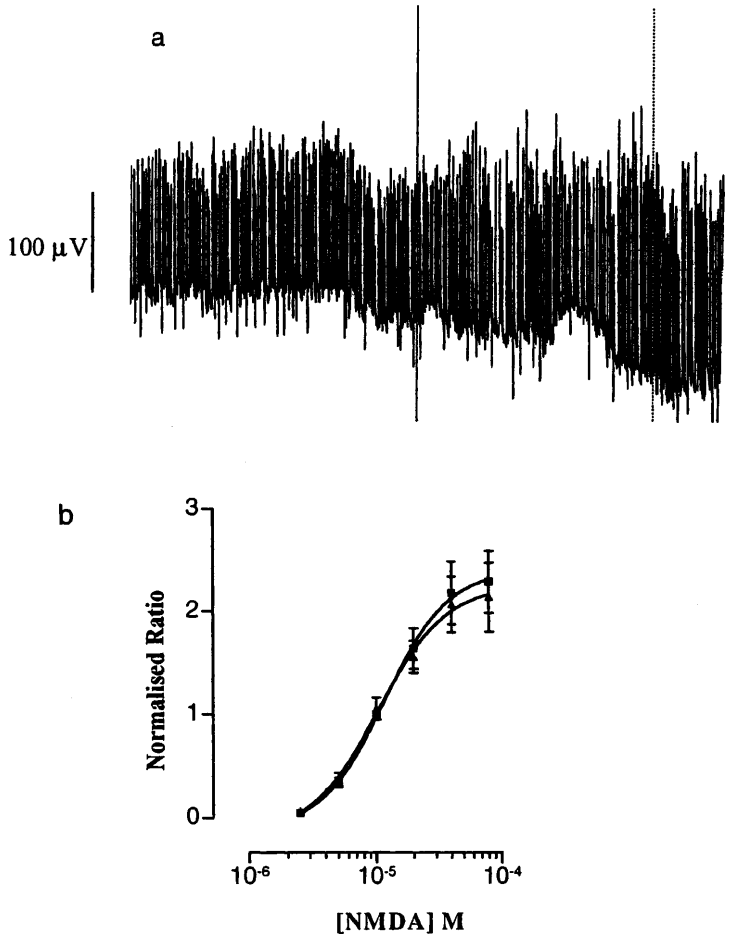

Fig. 3. a The effect of bilobalide (200μM) on magnesium-free aCSF-induced depolarizations. Bilobalide was perfused for 30 minutes (between lines) onto a cortical wedge prepared from a DBA/2 mouse. **b** The lack of effect of bilobalide (200μM) on NMDA-induced depolarizations. Control (■); bilobalide (▲) n = 4–6. Peak height was calculated as a ratio normalised to the height obtained with 10μM NMDA

not involve sodium channels as it has been shown that tetrodotoxin (TTX) is ineffective in reducing this release (Dickie and Davies, 1992). A possible explanation for bilobalide blocking potassium-stimulated excitatory amino acid release would be that perfusion of bilobalide, prior to the second-pulse of stimulated release, is causing a significant increase in levels of inhibitory amino acid neurotransmitters, an action of bilobalide that has been previously reported in rat cortex following chronic treatment with bilobalide (Sasaki et al., 1999b). We did not, however, detect a significant rise in GABA levels in the perfusate during basal or stimulated release in the presence of bilobalide.

The two reports by Sasaki and colleagues (1999a,b) would appear to contradict one another. They first suggest that bilobalide is enhancing excitatory responses by blocking inhibitory input, then, in subsequent experiments, they demonstrated that bilobalide increased levels of GABA and

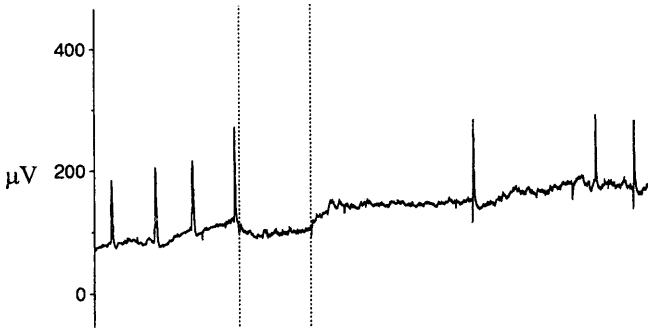

Fig. 4. The effect of bilobalide (50μM) on NO-711-induced depolarizations. Bilobalide was perfused for 30 minutes (between lines) onto a cortical wedges from a DBA/2 mouse following perfusion of NO-711 (25μM)

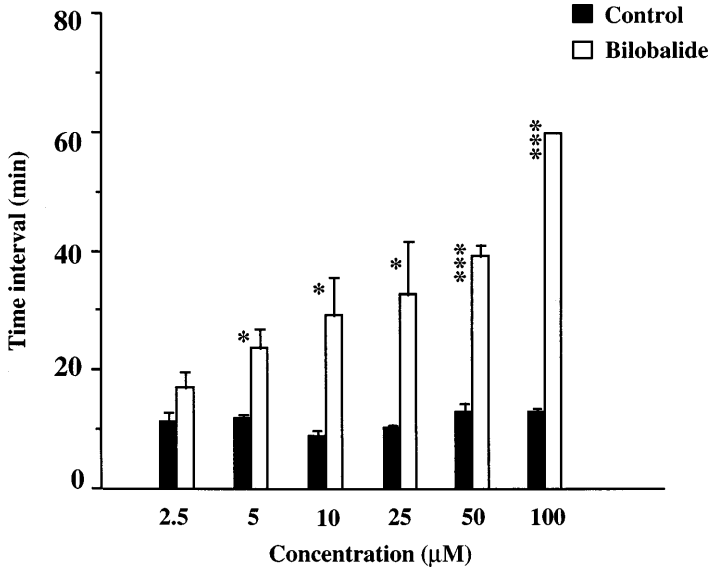

Fig. 5. The effects of bilobalide (2.5–100μM) on NO-711 (25μM)-induced depolarizations on DBA/2 mouse cortical wedges. The columns represent time-intervals between depolarizations. $^{*}P < 0.05$, $^{***}P < 0.01$. n = 6

GAD activity in rat cortex. If bilobalide, as they state, was blocking inhibitory transmission, then the fact that it is also increasing levels of GABA would seem to suggest that the two effects cancel each other out, or at least reduce the overall effect of either action.

Veratridine elicits the release of neurotransmitters by preventing the inactivation of sodium channels and TTX is effective in preventing this release (Levi et al., 1980; Dickie and Davies, 1992). The fact that bilobalide reduced potassium- as well as veratridine-stimulated release would suggest that it is not acting solely through blockade of voltage-operated sodium channels, however, such an action could contribute to its action on veratridine-stimulated release. Another possibility, in view of the report by Weichel et al.

(1999) who showed that bilobalide inhibited NMDA-induced phospholipid breakdown in rat hippocampus, is that bilobalide is blocking NMDA-operated channels and thus reducing the release of stimulated transmitter. The NMDA channel blocker, dizocilpine (MK801), has been shown to reduce both veratridine- and potassium-stimulated release of glutamate (Srinivasan et al., 1995). However, Weichel et al. (1999) postulate that bilobalide is acting "downstream" from the NMDA receptor, which would not be contradictory to our electrophysiological results presented in this paper (see below).

Despite the observation that bilobalide inhibited NMDA-induced phospholipase A_2 activation and phospholipid breakdown in the rat hippocampus (Weichel et al., 1999) we did not find evidence that bilobalide is an NMDA antagonist in our electrophysiological experiments. Spontaneous depolarizations of cortical wedges occur with a much enhanced frequency when Mg^{2+} are omitted from the aCSF, which implies NMDA receptor involvement, and it has also been shown that compounds acting at different sites on the NMDA receptor complex inhibit these depolarizations (Hu and Davies, 1997a). This, together with the fact that bilobalide had no effect on NMDA-induced depolarizations strongly suggests that the compound has no effect on any of the modulatory sites on the NMDA receptor complex. This does not invalidate the fact that bilobalide has been shown to protect membranes against excitotoxic damage as the effect of the compound is probably acting at a site distant from the NMDA receptor.

The GABA uptake inhibitor, NO-711, when perfused onto cortical wedges for 15 minutes, at relatively low concentrations, elicits slow depolarizations which persist for 4–5 hours with little decrement in amplitude or frequency (Davies and Shakesby, 1999). These depolarizations are inhibited by the $GABA_A$ receptor antagonist, bicuculline, are calcium-dependent but are insensitive to NMDA receptor antagonists. However, they are blocked by AMPA receptor antagonists and by compounds which reduce excitatory amino acid neurotransmitter release (Hu and Davies, 1997b). Bilobalide significantly reduced the frequency of these depolarizations at low concentrations (5–50μM) and completely inhibited them at 100μM. Sasaki et al. (1999a) reported that bilobalide initiated population spikes in rat hippocampal slices and postulated that the this effect was possibly due to bilobalide reducing GABAergic inhibition in the hippocampus as bilobalide also inhibited the action of the GABA receptor agonist muscimol. In our experiments we did not see any evidence of increased electrical activity in normal or Mg^{2+}-free aCSF with bilobalide. However, a possible explanation for the inhibition of NO-711-induced depolarizations with bilobalide could be through an antagonistic effect at $GABA_A$ receptors in a similar manner to the action of bicuculline. Although such an effect would have been expected to increase transmitter release if inhibition was blocked rather than decreased as described above. It is probable, therefore, that the action of bilobalide on NO-711-induced depolarizations is related to its ability to decrease stimulated transmitter release. A similar action has been postulated for dextromethorphan acting through sigma receptors. It has been shown to decrease both veratridine- and K^+-stimulated release of glutamate from rabbit

hippocampal slices (Annels et al., 1991) and to decrease the frequency of depolarizations induced by the GABA uptake inhibitor, tiagabine (Hu and Davies, 1997b).

Although the observations reported here do not allow for any definitive conclusions to be made regarding the precise molecular mechanism(s) involved in the diverse effects of bilobalide, the work demonstrates that neither NMDA receptor-operated channels nor voltage-operated sodium channels are involved in its mode of action. As bilobalide reduced the release of the excitatory amino acids it would suggest that modulation of synaptic Ca^{2+} homeostasis (Swoboda and Mainen, 1999) or any of the multiple components of the exocytotic process may be involved in this action of bilobalide (Kasai and Takahashi, 1999). Since bilobalide has been reported to possess a protective action on mitochondrial coupling mechanisms (Janssens et al., 1995), and mitochondria are known to be an important regulator of glutamate release (Scotti et al., 1999) this could account for its neuroactive properties. Further efforts to experimentally verify this possibility could, therefore, not only be helpful for clarifying bilobalide's mode of action, but also could establish it as a specific pharmacological tool for studies related to excitatory amino acid release.

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