ELSEVIER

European Journal of Pharmacology 331 (1997) 93–96

ejp

Short communication

GYKI 53655, a 2,3-benzodiazepine, non-competitively protects cultured neurones against AMPA toxicity

Attila D. Kovács *, Géza Szabó

Department of Biochemistry, EGIS Biological Laboratories, EGIS Pharmaceuticals Ltd., P.O. Box 100, H-1475 Budapest 10, Hungary

Received 28 April 1997; revised 27 May 1997; accepted 30 May 1997

Abstract

The nature of the neuroprotection by the competitive α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor antagonist, 6-nitro-7-sulfamoyl-benzo[f]quinoxaline-2,3-dione (NBQX), and the non-competitive AMPA receptor antagonist, 1-(4-aminophenyl)-3-methylcarbamoyl-4-methyl-3,4-dihydro-7,8-methylenedioxy-5H-2,3-benzodiazepine (GYKI 53655), was investigated in mature telencephalic neurone cultures of the rat. NBQX protected cultured neurones against AMPA-induced delayed toxicity in a competitive manner: the AMPA concentration–response curve was shifted to the right in parallel and concentration dependently. In contrast, GYKI 53655 decreased the maximal neurotoxic effect of AMPA considerably but without affecting the EC_{50} for AMPA toxicity, which indicated the non-competitive mode of its action. Thus we found a clear relationship between the nature of in vitro neuroprotection and the mode of AMPA channel block. © 1997 Elsevier Science B.V.

Keywords: AMPA receptor; AMPA receptor antagonism; Neuroprotection; GYKI 53655; NBQX (6-nitro-7-sulfamoyl-benzo[f]quinoxaline-2,3-dione)

1. Introduction

Glutamate is the main mediator of excitatory synaptic transmission in the mammalian central nervous system. Glutamate-gated ion channels (ionotropic glutamate receptors) are classified pharmacologically and on the basis of gene homology into three distinct groups: *N*-methyl-D-aspartate (NMDA), α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) and kainate receptors (Hollmann and Heinemann, 1994). Excessive or persistent activation of glutamate-gated ion channels may cause neuronal degeneration and this process is involved in the neuropathology of several age-related neurodegenerative disorders (e.g., Parkinson's disease, Huntington's disease and amyotrophic lateral sclerosis), as well as in stroke and seizures (Danysz et al., 1995; Dawson et al., 1995).

Recently, interest has turned to research on AMPA receptor antagonists as potential therapeutic agents for the treatment of the above-mentioned diseases and brain insults. 6-nitro-7-sulfamoyl-benzo[f]quinoxaline-2,3-dione (NBQX) is one of the most potent competitive AMPA receptor antagonists and has been shown to be neuroprotective in vitro as well as in several animal models of global and focal ischaemia (for review, see Gill, 1994). The competitive nature of AMPA receptor antagonism by NBQX has been proved by receptor binding and patch clamp experiments as well (Sheardown et al., 1990; Donevan and Rogawski, 1993; Parsons et al., 1994).

The 2,3-benzodiazepine, GYKI 52466 (1-(4-aminophenyl)-4-methyl-7,8-methylenedioxy-5H-2,3-benzodiazepine hydrochloride), was originally described as a centrally active muscle relaxant and potent AMPA/kainate receptor antagonist (Tarnawa et al., 1989, 1990). Further examination of GYKI 52466 proved its neuroprotective effect in vitro against AMPA receptor mediated toxicity (May and Robison, 1993; Kovács and Egyed, 1996) and in vivo in models of focal and global ischemia (Gill, 1994; Vizi et al., 1996). Electrophysiological experiments revealed that GYKI 52466 and its more effective analogues, GYKI 53405 [1-(4-aminophenyl)-3-acetyl-4-methyl-3,4-dihydro-7,8-methylenedioxy-5H-2,3-benzodiazepine] and GYKI 53655 [1-(4-aminophenyl)-3-methylcarbamoyl-4-methyl-3,4-dihydro-7,8-methylenedioxy-5H-2,3-benzodiazepine], are selective, non-competitive AMPA receptor antagonists (Vizi et al., 1996; Bleakman et al., 1996).

The in vitro and in vivo neuroprotective effects of NBQX and GYKI 52466 are considered to be the consequence of their AMPA channel-blocking activity, although the relation between AMPA channel block and the neuroprotection has not been investigated.

* Corresponding author. Tel.: (36-1) 256-6387; Fax: (36-1) 257-7932.

PII S0014-2999(97)01046-7

The exact mechanism of AMPA-induced delayed neurotoxicity in primary neuronal cultures has not been explored (Coyle and Puttfarcken, 1993; Courtney et al., 1995; Patel et al., 1996). The proved in vitro neuroprotective effect of AMPA receptor antagonists, therefore, may be related — instead of to AMPA channel block — to their influence on other cellular process(es) involved in AMPA-induced toxicity.

Based on experimental data, it was hypothesised that in vivo neuroprotection by NBQX is due to hypothermia or to a decrease in glucose utilisation (Gill, 1994, Nurse and Corbett, 1996). Other contradictory results also exist. NBQX given 12 h after global ischemia still reduced markedly the loss of hippocampal CA1 neurones in rats, while GYKI 52466 was ineffective in the same test (Li and Buchan, 1993). NBQX showed a neuroprotective effect in global ischemia even when administered 24 h following ischemia (Sheardown et al., 1993). In recent studies, only pretreatment or 30 min posttreatment with GYKI 52466 reduced neuronal damage after global ischemia (Block et al., 1996; Lodge et al., 1996).

In addition, characterisation of GYKI 52466, 53405 and 53655 by receptor binding or autoradiographic studies has not yet been achieved. These GYKI compounds do not affect the membrane binding of any known receptor ligand, including [^{3}H]AMPA or [^{3}H]kainate (Vizi et al., 1996). The only specific binding site of these 2,3-benzodiazepines was found on *Xenopus* brain membranes (Szabó and Henley, 1993), but the connection of this specific binding site with AMPA/kainate receptors has not been proved.

On the basis of the above, the relation between AMPA channel block and the neuroprotection is not clear. Recently, we have found that the rank order of in vitro neuroprotective efficacy of NBQX, GYKI 52466, 53405 and 53655 against 20 μM AMPA-induced toxicity correlated well with their potency to block AMPA channels (Kovács and Szabó, 1997). The present study aimed to investigate whether the in vitro neuroprotection by NBQX or a 2,3-benzodiazepine (GYKI 53655) against the delayed toxicity induced by long AMPA exposure is related to their mode of AMPA channel-blocking activity. We examined the nature of the neuroprotection afforded by NBQX or GYKI 53655 against delayed AMPA toxicity in primary rat telencephalic cultures. The mode of neuroprotection by NBQX or GYKI 53655 was consistent with their mode of AMPA channel block.

2. Materials and methods

2.1. Cell culture

Primary cultures of rat embryonic telencephalic cells were prepared from Sprague-Dawley rat embryos on the 17th to 19th day of gestation as described by Kovács and Szabó (1997), with minor modifications. Briefly, telencephalons were dissected and after removal of meninges, mechanically dissociated in culture medium by trituration with a 1-ml automatic pipette above a nylon mesh with a pore diameter of 48 μm. The cells filtered through the mesh were plated onto 24-well plates: $(7–8) \times 10^5$ cells/well. The plates were previously coated with 2 μg/ml poly-L-lysine (MW > 300 000; Sigma) for 1 h. For dissociation, plating and maintenance, Eagle's Minimal Essential Medium (EMEM) supplemented with 10% heat-inactivated fetal calf serum, 4 mM glutamine, 20 mM KCl, gentamicin sulphate (50 mg/l) and amphotericin B (2.5 mg/l) was used. Cultures were incubated at 37°C in a humidified atmosphere containing 5% CO_2. Medium changes were carried out 24 h after plating. On the 5th–6th day in vitro the proliferation of non-neuronal cells was arrested by addition of 10 μM cytosine arabinofuranoside for 24 h. The cells were than transferred into EMEM, containing 10% heat-inactivated horse serum, 4 mM glutamine, 12 mM glucose, gentamicin sulphate and amphotericin B (EMEM-10% horse serum). Starting on the 11th day in vitro, to prevent the formation of a second layer of glia, the cultures were treated with 10 μM cytosine arabinofuranoside for 24 h. Following this the medium was exchanged for EMEM-10% horse serum containing penicillin (10^5 U/l) as antibiotic instead of gentamicin and amphotericin B. On the 15th day in vitro, half of the medium was exchanged for EMEM containing 5% horse serum, 4 mM glutamine, 12 mM glucose and penicillin as antibiotic. No further medium change was made prior to the AMPA toxicity experiments.

In our mature telencephalic cultures, neurones connected by an intensive neurite network are on a glia (astrocyte) monolayer as verified by immunocytochemistry with monoclonal antibodies against neurofilament 200 and glial fibrillary acidic protein (GFAP) (Kovács and Egyed, 1996). The ratio of neurones and glial cells was 1:2 as assessed on the basis of lactate dehydrogenase (LDH) activity in neurones and glial cells, respectively. LDH activity of neurones was determined after 24-h exposure to 500 μM glutamate. The pure glial culture remaining in the same culture well was lysed by freezing and thawing and LDH activity of the glia lysatum was measured.

2.2. Assessment of neuronal cell death

Experiments were performed on 17 to 18-day-old cultures. Cultures were treated for 22–24 h with various concentrations of AMPA (Tocris Neuramin) alone or with NBQX or GYKI 53655. (NBQX and GYKI 53655 were synthesised in EGIS Pharmaceuticals, Budapest). NBQX and GYKI 53655 were dissolved in dimethyl sulfoxide (DMSO). At its final concentration (0.1%), DMSO failed to evoke any detectable response in our cultures. Treating medium (EMEM containing 5% horse serum, 12.5 mM HEPES, 4 mM glutamine, 12 mM glucose and 10^5 U/l

penicillin) was used for dilution of NBQX and GYKI 53655 and for the additions. Cultures were preincubated with NBQX or GYKI 53655 for 30 min at 37°C before addition of AMPA (8 μl from the appropriate dilution to a culture well). In the case of addition of 500 μM or 1 mM AMPA, 40 or 80 μl from a stock solution of 6.25 mM was added to a culture well. The antagonists were present during the entire AMPA exposure period. NBQX or GYKI 53655 alone has no effect on the viability of cultured telencephalic neurones.

Neuronal cell death was assessed visually by phase-contrast microscopy and quantified by measuring the activity of LDH released into the culture medium. After 22 to 24 h exposure to AMPA or AMPA and one of its antagonists, the culture supernatants (supernatants A) were removed for LDH determination and replaced by treating medium lacking horse serum and supplemented with 500 μM glutamate to destroy the surviving neurones. After 22 to 24 h incubation the culture supernatants (supernatants B) were removed. The LDH activity of supernatants A and B (LDH_A and LDH_B) was determined. Neurotoxicity was expressed as LDH activity in the culture supernatant after AMPA treatment as percent of the total neuronal LDH activity in the same culture well: $[LDH_A/(LDH_A + LDH_B)] \times 100$. The sum of LDH_A and LDH_B corresponds to LDH activity of all the neurones in a culture well.

2.3. Measurement of LDH activity

LDH activity was determined at 37°C, in 96-well microtiter plates in duplicate from 60 μl aliquots of culture supernatants. The decrease of NADH absorbance at 340 nm was measured with a kinetic ELISA reader. The LDH activity of 60 μl treating medium was subtracted as background (LDH activity of 5% horse serum) in the case of culture supernatants A.

3. Results

We examined the concentration dependence of AMPA-induced delayed neurotoxicity in mature telencephalic cultures of the rat. The dose–response curve is shown in Fig. 1. The EC_{50} value for AMPA was 8 μM (95% confidence intervals (CI) 6–11 μM) as determined with the GraphPad Prism program.

NBQX protected cultured neurones against AMPA toxicity in a competitive manner. The AMPA concentration–response curve was shifted to the right in parallel and concentration dependently (Fig. 1A). The EC_{50} for AMPA toxicity was increased by 2 and 50 μM NBQX to 54 μM (CI 47–62 μM) and 672 μM (CI 609–741 μM), respectively (Table 1). In contrast, GYKI 53655 decreased the maximal neurotoxic effect of AMPA considerably but without significantly affecting the EC_{50} for AMPA toxicity, which indicated the non-competitive mode of its action

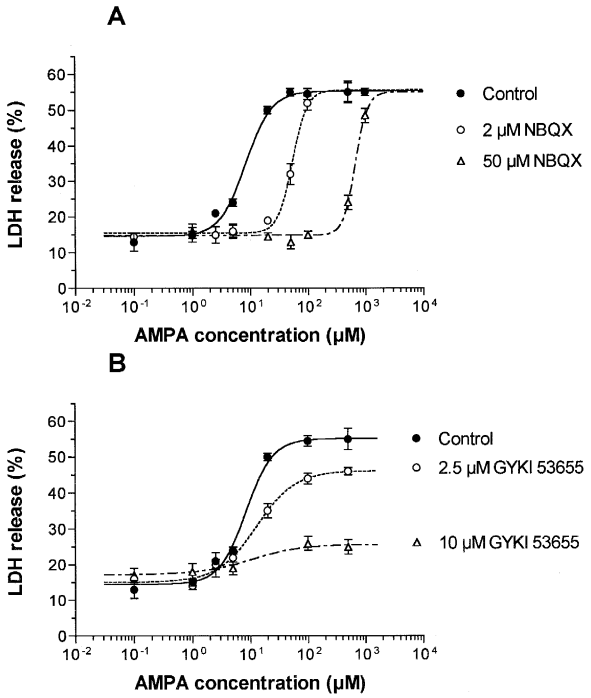

Fig. 1. (A) Competitive nature of the neuroprotection by NBQX against AMPA-induced toxicity in rat telencephalic cultures. (B) Non-competitive nature of the neuroprotection by GYKI 53655 against AMPA-induced toxicity in rat telencephalic cultures. Cultures of 17–18 days in vitro were treated for 22–24 h with various concentrations of AMPA alone or with one of its antagonists. NBQX (2 or 50 μM) or GYKI 53655 (2.5 or 10 μM) was added to the cultures 30 min prior to the AMPA exposure and was present during the whole exposure. Neuronal cell death was quantified by measuring the activity of LDH released into the culture medium. LDH release (%) was expressed as LDH activity in the culture supernatant after AMPA treatment as percent of the total neuronal LDH activity in the same culture well (see Section 2 for details). Points are means ± S.E.M. of two or three independent experiments ($n = 5–9$; in the case of 20 μM AMPA alone, $n = 12$). Non-linear curve fitting was performed with the GraphPad Prism program.

(Fig. 1B). The EC_{50} values for AMPA toxicity were 13 μM (CI 7–23 μM) and 10 μM (CI 1–95 μM) in the presence of 2.5 and 10 μM GYKI 53655, respectively (Table 1).

GYKI 53655, due to its non-competitive mode of action, was a much more effective neuroprotective com-

Table 1
EC_{50} values of AMPA neurotoxicity in the presence of NBQX or GYKI 53655 in rat telencephalic cultures

Treatment	EC_{50} values for AMPA toxicity (μM)
AMPA (control)	8 (6–11)
+ 2 μM NBQX	54 (47–62)
+ 50 μM NBQX	672 (609–741)
+ 2.5 μM GYKI 53655	13 (7–23)
+ 10 μM GYKI 53655	10 (1–95)

EC_{50} values were determined from the concentration–response curves presented in Fig. 1, with the GraphPad Prism program. The 95% confidence intervals are given in parentheses.

pound at higher AMPA concentrations than NBQX. For example, 10 μM GYKI 53655 protected cultured neurones almost completely against 500 μM AMPA (see Fig. 1B). In contrast, 50 μM NBQX was needed to obtain the same neuroprotection (see Fig. 1A), and 10 μM NBQX gave no neuroprotection (data not shown).

4. Discussion

This is the first report demonstrating a clear relationship between the mode of in vitro neuroprotection and AMPA channel block for NBQX and a 2,3-benzodiazepine (GYKI 53655). Our results suggest that neuroprotection by NBQX or GYKI 53655 is the consequence of their permanent AMPA channel-blocking activity. However, the possibility that NBQX or GYKI 53655 inhibits other cellular process(es) involved in AMPA-induced delayed neurotoxicity, cannot be ruled out but seems unlikely. Recently, we have found that the rank order of in vitro neuroprotective efficacy of NBQX, GYKI 52466, 53405 and 53655 against 20 μM AMPA-induced toxicity correlated well with their potency to block AMPA channels (Kovács and Szabó, 1997). This finding also supports the close relation between the neuroprotective effect of, and the AMPA channel block by the above AMPA receptor antagonists.

Recently, Bleakman et al. (1996) reported that the channel-blocking activity of GYKI 53405 and GYKI 53655 at recombinant human and native rat AMPA receptors is stereoselective, with only the (−) isomer being active. The same stereoselectivity was demonstrated for the neuroprotection in the gerbil global ischemia model (Lodge et al., 1996), suggesting a direct role of AMPA channel block in the in vivo neuroprotective effect of 2,3-benzodiazepines.

In our previous study, NBQX was found to be a ten times more effective neuroprotectant than GYKI 53655 against 20 μM AMPA toxicity in rat telencephalic cultures (EC_{50} of NBQX was 0.5 μM; Kovács and Szabó, 1997). Here we showed for the first time that GYKI 53655, due to its non-competitive mode of action, is a much more effective neuroprotective compound than NBQX when AMPA concentrations are high.

Acknowledgements

The authors thank Katalin Hadarics for her excellent technical assistance and Dr. András Egyed and Dr. Károly Tihanyi for the helpful discussions.

References

Bleakman, D., Ballyk, B.A., Schoepp, D.D., Palmer, A.J., Bath, C.P., Sharpe, E.F., Woolley, M.L., Bufton, H.R., Kamboj, R.K., Tarnawa, I., Lodge, D., 1996. Activity of 2,3-benzodiazepines at native rat and recombinant human glutamate receptors in vitro: Stereospecificity and selectivity profiles. Neuropharmacology 35, 1689–1702.

Block, F., Schmitt, W., Schwarz, M., 1996. Pretreatment but not post-treatment with GYKI 52466 reduces functional deficits and neuronal damage after global ischemia in rats. J. Neurol. Sci. 139, 167–172.

Coyle, J.T., Puttfarcken, P., 1993. Oxidative stress, glutamate, and neurodegenerative disorders. Science 262, 689–695.

Courtney, M.J., Enkvist, M.O.K., Åkerman, K.E.O., 1995. The calcium response to the excitotoxin kainate is amplified by subsequent reduction of extracellular sodium. Neuroscience 68, 1051–1057.

Danysz, W., Parsons, C.G., Bresink, I., Quack, G., 1995. Glutamate in CNS disorders. Drug News Perspect. 8, 261–276.

Dawson, R., Beal, M.F., Bondy, S.C., DiMonte, D.A., Isom, G.E., 1995. Excitotoxins, aging, and environmental neurotoxins: Implications for understanding human neurodegenerative diseases. Toxicol. Appl. Pharmacol. 134, 1–17.

Donevan, S.D., Rogawski, M.A., 1993. GYKI 52466, a 2,3-benzodiazepine, is a highly selective, noncompetitive antagonist of AMPA/kainate receptor responses. Neuron 10, 51–59.

Gill, R., 1994. The pharmacology of α-amino-3-hydroxy-5-methyl-4-isoxazole propionate (AMPA)/kainate antagonists and their role in cerebral ischemia. Cerebrovasc. Brain Metab. Rev. 6, 225–256.

Hollmann, M., Heinemann, S., 1994. Cloned glutamate receptors. Annu. Rev. Neurosci. 17, 31–108.

Kovács, A.D., Egyed, A., 1996. Protection against non-NMDA receptor-mediated excitotoxicity by GYKI 52466 in mature telencephalic cultures of the rat. Neurobiology 4, 59–72.

Kovács, A.D., Szabó, G., 1997. Comparison of neuroprotective efficacy of competitive and non-competitive AMPA antagonists in vitro. Environ. Toxicol. Pharmacol. 3, 69–72.

Li, H., Buchan, A.M., 1993. Treatment with an AMPA antagonist 12 h following severe normothermic forebrain ischemia prevents CA1 neuronal injury. J. Cereb. Blood Flow Metab. 13, 933–939.

Lodge, D., Bond, A., O'Neill, M.J., Hicks, C.A., Jones, M.G., 1996. Stereoselective effects of 2,3-benzodiazepines in vivo: Electrophysiology and neuroprotection studies. Neuropharmacology 35, 1681–1688.

May, P.C., Robison, P.M., 1993. GYKI 52466 protects against non-NMDA receptor-mediated excitotoxicity in primary rat hippocampal cultures. Neurosci. Lett. 152, 169–172.

Nurse, S., Corbett, D., 1996. Neuroprotection after several days of mild, drug-induced hypothermia. J. Cereb. Blood Flow Metab. 16, 474–480.

Parsons, C.G., Gruner, R., Rozental, J., 1994. Comparative patch clamp studies on the kinetics and selectivity of glutamate receptor antagonism by 2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo(F)quinoxaline (NBQX) and 1-(4-amino-phenyl)-4-methyl-7,8-methylenedioxy-5H-2,3-benzodiazepine (GYKI 52466). Neuropharmacology 33, 589–604.

Patel, M., Day, B.J., Crapo, J.D., Fridovich, I., McNamara, J.O., 1996. Requirement for superoxide in excitotoxic cell death. Neuron 16, 345–355.

Sheardown, M.J., Nielsen, E.O., Hansen, A.J., Jacobsen, P., Honoré, T., 1990. 2,3-Dihydroxy-6-nitro-7-sulfamoyl-benzo(F) quinoxaline: A neuroprotectant for cerebral ischemia. Science 247, 571–574.

Sheardown, M.J., Suzdak, P.D., Nordholm, L., 1993. AMPA, but not NMDA, receptor antagonism is neuroprotective in gerbil global ischemia, even when delayed 24 h. Eur. J. Pharmacol. 236, 347–353.

Szabó, G., Henley, J.M., 1993. Binding of the AMPA receptor antagonist [^{3}H]GYKI 53405 to Xenopus brain membranes. Neuroreport 5, 93–94.

Tarnawa, I., Farkas, S., Berzsenyi, P., Pataki, A., Andrási, F., 1989. Electrophysiological studies with a 2,3-benzodiazepine muscle relaxant: GYKI 52466. Eur. J. Pharmacol. 167, 193–199.

Tarnawa, I., Engberg, I., Flatman, J.A., 1990. GYKI 52466, an inhibitor of spinal reflexes is a potent quisqualate antagonist. In: Lubec, G., Rosenthal, G.A. (Eds.), Amino Acids: Chemistry, Biology and Medicine. Escom Science Publishers B.V., Leiden, pp. 538–546.

Vizi, E.S., Mike, Á., Tarnawa, I., 1996. 2,3-benzodiazepines (GYKI 52466 and analogs): Negative allosteric modulators of AMPA receptors. CNS Drug Rev. 2, 91–126.