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Phenytoin protected mouse cortical cell cultures against neurotoxicity induced by kainate but not by NMDA

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Summary. Phenytoin (PHT) protected cultured mouse cortex neurons against kainate-induced excitotoxicity, but failed to protect against the N-methyl-D-aspartate (NMDA)-induced excitotoxicity. The voltage-clamp experiments showed that PHT significantly blocked kainate-induced currents but did not block NMDA-induced currents in the cultured neurons. These results indicate that PHT protects the cultures by blocking non-NMDA receptors and suggest that PHT has clinical efficacy against neuronal cell death through the excessive stimulation of non-NMDA receptors.

Keywords: Amino acids – Excitotoxicity – Voltage clamp – Diphenylhydantoin – DPH – PHT – LDH

Introduction

Glutamate-induced excitotoxicity has been proposed to participate in the neuronal cell death associated with various neurological diseases, typically ischaemia (Benveniste et al., 1984; Kirino et al., 1990; Monaghan et al., 1983; Wieloch et al., 1985). Although the mechanism of the neuronal cell death is still unclear, it has been found that blockers against the glutamate receptor subtypes protected against the excitotoxicity (Choi et al., 1988; Gill et al., 1987; Hao et al., 1991; Sheardown et al., 1990). To date, therefore, the search of the clinical blockers has been a center of attention.

PHT is a clinical antiepileptic drug exerting a variety of anticonvulsant actions on neurons with relative lack of neurological side effects at therapeutic doses (Rogawski and Porter, 1990). That is, PHT blocks voltage-gated Na channels, a principal anticonvulsant action (McLean and Macdonald, 1983; Willow et al., 1985) and voltage-gated Ca channels (Twombly et al., 1988), and potentiates GABA-mediated inhibition (McLean and Macdonald, 1983). Also, PHT is indicated to block glutamate receptors (Matthews and Connor, 1977; Sastry and Phillis, 1976) though there have been conflicting reports (McLean and Macdonald, 1983; Nicoll and Wojtowicz, 1980).

Several investigators reported that PHT protected the neurons of dogs (Suzuki et al., 1987) and rabbits (Cullen et al., 1979) against cerebral ischemia. However, they did not consider PHT as a glutamate blocker probably because the PHT blockage of glutamate receptors had been doubtful.

Recently we found that PHT, at clinical concentrations, competitively blocked the non-NMDA receptors, a glutamate receptor subtype, produced by Xenopus oocytes injected with ddY-strain mice whole brain mRNA (Kawano et al., 1994). These results imply that PHT is clinically effective against the excitotoxicity.

In the present study, we investigated the protective effect of PHT by measuring the lactate dehydrogenase (LDH) efflux in cultured mouse cerebral cortex neurons. Also, the blocking effect of PHT on voltage-clamp currents elicited by glutamate analogs was investigated to discuss the protective mechanisms.

Materials and methods

Cerebral cortex neurons of ddY-strain mouse were dissociated and cultured with established techniques (Matsuoka et al., 1987). In brief, cerebral cortex removed from mouse fetus (16-day gestation) was minced and incubated in trypsin for 15 min at 37°C. The cortex was then dissociated by trituration and plated as a single cell suspension on cover slips in 60 cm dishes (1.8×10^4 cell/cm^2) for the voltage-clamp experiments in the presence of Eagle's MEM supplemented with glucose, bicarbonate, 5% heat-inactivated fetal bovine and 5% house serum. For LDH efflux measurement, the single cell suspension was plated on 24 well plates (1.6×10^5 cell/cm^2). One day after plating, the medium was replaced with B18 (Brewer and Cotman, 1989). The cultures were fed with fresh B18 every 3 days for 10 days before exposure to kainate or NMDA in the presence or absence of PHT.

The blocking effect of PHT on the glutamate receptor subtypes was investigated under voltage-clamp conditions. The cultured neuron was whole cell clamped with a single glass electrode filled with 150 mM CsCl, 5 mM HEPES, 5 mM EGTA (pH 7.4, ~5 MΩ). The neurons eliciting transient inward currents on depolarization were irrigated with a saline solutions (145 mM NaCl, 5 mM KCl, 2.4 mM $CaCl_2$, 10 mM glucose, 10 mM HEPES, pH 7.4) and kainate or NMDA supplemented with 10 μM glycine dissolved in the saline solution with/without 100 μM PHT. The neurons responded to both kainate and NMDA were used.

The protective effect of PHT was estimated by monitoring the LDH efflux from damaged neurons during the 24 h exposure (Koh and Choi, 1987b). The cultured neurons released LDH through the exposure protocol even in the absence of the glutamate agonists. This background LDH efflux determined on sister cultures within each experiment was used as a unit to normalize values obtained from treated cultures. The mean ± standard deviation of the background LDH activity (IU/l) was 90.8 ± 18.4 (n = 21).

Results

Voltage clamp experiments showed that PHT preferably blocked non-NMDA receptors. Figure 1 shows typical voltage-clamp currents as responses of non-NMDA and NMDA receptors. 100 μM PHT substantially decreases the voltage-clamp current responses to 100 μM kainate whereas PHT slightly decreases those to 100 μM NMDA. Table 1 summarizes the blocking effect.

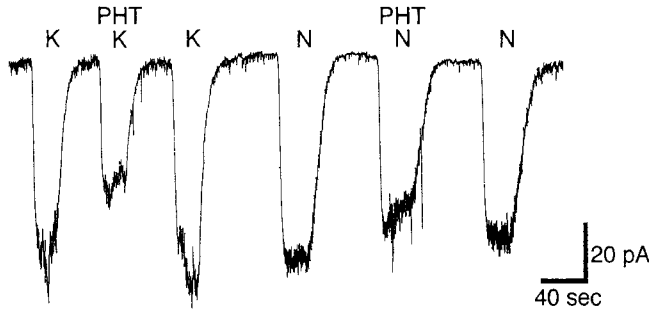

Fig. 1. Voltage-clamp currents elicited by kainate and NMDA in the absence and presence of PHT. The responses are recorded from the same cultured neuron voltage-clamped at − 50mV. *K* 100μM kainate; *N* 100μM NMDA + 10μM Gly; *PHT* 100μM phenytoin

Table 1. Relative magnitude of inward currentes elicited by kainate and NMDA in the presence of PHT

	mean ± S.D.
100μM kainate + 100μM PHT	0.60 ± 0.04*
100μM NMDA + 100μM PHT	0.92 ± 0.04

The inward currents were recorded from 5 cultured neurons voltage clamped at −50mV. The values are means and SD of the magnitude of inward currents calculated relative to the responses in the absence of PHT. *significant decrease in magninitude of relative inward currents by the addition of PHT (two-tailed t-test; $p < 0.001$).

The addition of 100μM PHT significantly blocked non-NMDA receptors whereas PHT hardly blocked NMDA receptors.

Although PHT blocked the kainate receptors as mentioned above, the protective efficacy had been unknown. Hence we examined the protective effect of PHT on cultured mouse cerebral cortex cells. As Fig. 2A shows, 100μM PHT hardly decreases NMDA-induced LDH efflux. No significant differences are observed (two-way ANOVA). In contrast, 100μM PHT lowers LDH efflux induced by kainate (Fig. 2B). There is a significant interaction between kainate concentrations and PHT on LDH efflux (two-way ANOVA, $p < 0.001$). PHT appears to shift the LDH efflux curve to right. This shift agrees with our previous results that PHT competitively blocked electrical responses of non-NMDA receptors produced by brain mRNA-injected Xenopus oocytes (Kawano et al., 1994). Further experiments will clarify the relations between the extent of the block and the protection.

Figure 3 shows the protective effect of different concentrations of PHT against the neurotoxicity induced with 100μM kainate, the Kd value of kainate on the non-NMDA receptors (Kawano et al., 1994). The protective

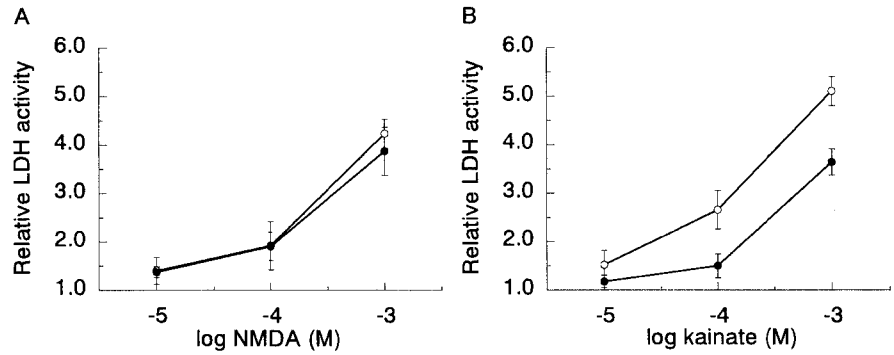

Fig. 2. LDH efflux induced by NMDA (**A**) or kainate (**B**) in the absence (open circles) or presence (closed circles) of 100μM PHT. Plotted data are obtained from at least 3 separate experiments each of which has three replicate culture plates and calculated relative to the LDH efflux from buffer-treated sister cultures. The interaction was significant between the kainate concentration and PHT on LDH efflux (two-way ANOVA; $p < 0.001$)

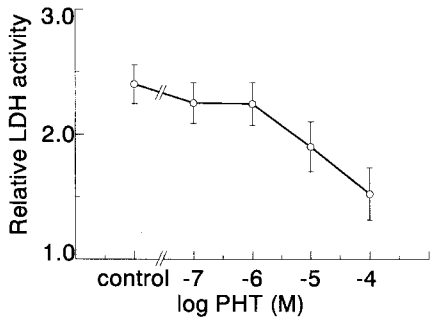

Fig. 3. PHT concentration-dependent protection against 100μM kainate-induced excitotoxicity. Plotted data are obtained and calculated in the same manner as those shown in Fig. 2. IC50 value was estimated by an intersecting point between horizontal line crossing 50% of the mean of control LDH efflux and a solid line connecting data points. The difference was significant between the LDH efflux in the absence and presence of PHT (one-way ANOVA; $p < 0.001$). Also, the LDH efflux in the presence of 10 and 100μM PHT was significantly different from that in the absence or presence of lower concentrations of PHT (Tukey's multiple comparison, $p < 0.01$)

effect is significant (one-way ANOVA, $p < 0.001$). Also, the LDH efflux in the presence of 10 and 100μM PHT is significantly different from that in the absence or presence of lower concentrations of PHT (Tukey's multiple comparison, $p < 0.01$). IC50 estimated from the figure is ~30μM.

Discussion

The present experiments showed that PHT protected only against kainate-induced neurotoxicity. The protective effect of PHT against ischemic brain damage (Cullen et al., 1979; Fukuda et al., 1983; Suzuki et al., 1987) and

kainate-induced neurotoxicity (Zaczek et al., 1978) in vivo agreed with our present results. No protective effect of PHT was found against glutamate-induced neurotoxicity in cortical cell culture (Koh and Choi, 1987a). Since PHT hardly protected against NMDA induced neurotoxicity, no protective effect reported is consistent with the present results.

Although IC50 against 100μM kainate-induced neurotoxicity was ~30μM, 10μM PHT significantly lowered the neurotoxicity (Fig. 3). That is, 10μM PHT significantly protects neurons when a half of non-NMDA receptors there are activated, since the Kd value of kainate on non-NMDA receptors were close to 100μM (Kawano et al., 1994). As an anticonvulsant, PHT is clinically used at ~10μM in CSF without serious neurological side effects (Rogawski and Porter, 1990). Therefore, it is likely that PHT clinically protects neurons against kainate-induced excitotoxicity without serious neurological side effects.

References

Benveniste H, Drejer J, Schousboe A, Diemer NH (1984) Elevation of the extracellular concentrations of glutamate and aspartate in rat hippocampus during transient cerebral ischemia monitored by intracerebral microdialysis. J Neurochem 43: 1369–1374

Brewer GJ, Cotman CW (1989) Survival and growth of hippocampal neurons in defined medium at low density: advantages of a sandwich culture technique or low oxygen. Brain Res 494: 65–74

Choi DW, Koh J, Peters S (1988) Pharmacology of glutamate neurotoxicity in cortical cell culture: attenuation by NMDA antagonists. J Neurosci 8: 185–196

Cullen JP, Aldrete JA, Jankovsky L, Romoo-Salas F (1979) Protective action of phenytoin in cerebral ischemia. Anesth Analg 58: 165–169

Fukuda A, Tabuse H, Ihara N, Tanabe J, Ikeda H, Kohama A (1983) Effects of phenytoin on regional cerebral blood flow, electroencephalogram, and electrolyte contents in cerebral blood and cerebral cortex following total cerebral ischemia in dogs. Circulatory Shock 10: 341–350

Gill R, Foster AC, Woodruff GN (1987) Systemic administration of MK-801 protects against ischemia-induced hippocampal neurodegeneration in the gerbil. J Neurosci 7: 3343–3349

Hao JX, Xu XJ, Aldskogius H, Seiger A, Wiesenfeldhallin Z (1991) The excitatory amino acid receptor antagonist MK-801 prevents the hypersensitivity induced by spinal cord ischemia in the rat. Exp Neurol 113: 182–191

Kawano H, Sashihara S, Ohno K, Mita T, Kawamura M, Yoshii K (1994) Phenytoin, an antiepileptic drug, competitively blocked non-NMDA receptors produced by Xenopus oocytes. Neurosci Lett 166: 183–186

Kirino T, Tamura A, Sano K (1990) Chronic maintenance of presynaptic terminals in gliotic hippocampus following ischemia. Brain Res 510: 17–25

Koh JY, Choi DW (1987a) Effect of anticonvulsant drugs on glutamate neurotoxicity in cortical cell culture. Neurology 37: 319–322

Koh JY, Choi DW (1987b) Quantitative determination of glutamate mediated cortical neuronal injury in cell culture by lactate dehydrogenase efflux assay. J Neurosci Methods 20: 83–90

Matsuoka I, Syoto B, Kurihara K, Kubo S (1987) ADP-ribosylation of specific membrane proteins in pheochromocytoma and primary-cultured brain cells by botulinum neurotoxins type C and D. FEBS Lett 216: 295–299

Matthews W, Connor JD (1977) Actions of iontophoretic phenytoin and medazepam on hippocampal neurons. J Pharmacol Exp Ther 201: 613–621

McLean MJ, Macdonald RL (1983) Multiple actions of phenytoin on mouse spinal cord neurons in cell culture. J Pharmacol Exp Ther 227: 779–789
Monaghan DT, Holets VR, Toy DW, Cotman CW (1983) Anatomical distributions of four pharmacologically distinct 3H-L-glutamate binding sites. Nature 306: 176–179
Nicoll RA, Wojtowicz JM (1980) The effects of pentobarbital and related compounds on frog motoneurons. Brain Res 191: 225–237
Rogawski MA, Porter RJ (1990) Antiepileptic drugs: Pharmacological mechanisms and clinical efficacy with consideration of promising developmental stage compounds. Pharmacol Rev 42: 223–286
Sastry BSR, Phillis JW (1976) Antagonism of glutamate and acetylcholine excitation of rat cerebral conrtical neurones by diphenylhydantoin. Gen Pharmacol 7: 411–413
Sheardown MJ, Nielsen EO, Hansen AJ, Jacobsen P, Honore T (1990) 2,3-Dihydroxy-6-nitro-7-sulfamoyl-benzo(F)quinoxaline: a neuroprotectant for cerebral ischemia. Science 247: 571–574
Suzuki J, Abiko H, Mizoi K, Oba M, Yoshimoto T (1987) Protective effect of phenytoin and its enhanced action by combined administration with mannitol and vitamin E in cerebral ischaemia. Acta Neurochir 88: 56–64
Twombly DA, Yoshii M, Narahashi T (1988) Mechanisms of calcium channel block by phenytoin. J Pharmacol Exp Ther 246: 189–195
Wieloch T, Lindvall O, Blomqvist P, Gage FH (1985) Evidence for amelioration of ischaemic neuronal damage in the hippocampal formation by lesions of the perforant path. Neurol Res 7: 24–26
Willow M, Gonoi T, Catterall WA (1985) Voltage clamp analysis of the inhibitory actions of diphenylhydantoin and carbamazepine on voltage-sensitive sodium channels in neuroblastoma cells. Mol Pharmacol 27: 549–558
Zaczek R, Nelson MF, Coyle JT (1978) Effects of anaesthetics and anticonvulsants on the action of kainic acid in the rat hippocampus. Eur J Pharmacol 52: 323–327

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