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NMDA receptor dependent and independent components of veratridine toxicity in cultured cerebellar neurons are prevented by nanomolar concentrations of terfenadine

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Summary. Exposure of cultured neurons to nanomolar concentrations of terfenadine prevented the NMDA receptor-mediated early appearance (30 min.) of toxicity signs induced by the voltage sensitive sodium channel activator veratridine. Terfenadine also provided an histamine-insensitive protection against delayed neurotoxicity by veratridine (24 h), occurring independently of NMDA receptor activation, while not protecting from excitotoxicity following direct exposure of neurons to glutamate. Terfenadine reduced tetrodotoxin-sensitive inward currents, and reduced intracellular cGMP formation following veratridine exposure.

Our data suggest that nanomolar concentrations of TEF may reduce excitatory aminoacid release following neuronal depolarization via a presynaptic mechanism involving voltage sensitive sodium channels, and therefore may be considered as a prototype for therapeutic drugs in the treatment of diseases that involve excitatory aminoacid neurotransmission.

Keywords: Amino acids – Glutamate release – Excitotoxicity – Terfenadine – Sodium channels

Introduction

The neurotransmitter glutamate is considered to be the cause of neuronal death in several acute and chronic disorders, including stroke, Parkinson's disease, and some forms of epilepsy (For review see Lee et al., 1999; Dunnett and Björklund, 1999; McNamara, 1999). Glutamate release and reuptake may play an important role in excitotoxicity (Nichols and Attwell, 1990), and some attention has been directed toward the possibility to modulate glutamate release following neuronal depolarization via voltage sensitive sodium channels (VSSC) (Taylor and Meldrum, 1995; Urenjak and Obrenovitch,

1996; Stys, 1998). Although this approach has not been successful in clinical trials yet (Lee et al., 1999), it remains of considerable interest given the well documented role of Na^+ influx in neuronal cell damage, both alone and in combination with excitatory amino acids (Taylor and Meldrum, 1995; Urenjak and Obrenovitch, 1996; Stys, 1998; Takahashi et al., 1999).

Terfenadine is widely used as a non sedating antihistamine. It is thought to penetrate poorly into the brain, and it is generally devoid of central nervous system (CNS) depressant activity. However, reported adverse effects include sedation, drowsiness, fatigue, and weakness (McTavish et al., 1990), suggesting that therapeutic concentrations of this drug may also act at the CNS. Besides acting as an histamine H1 receptor antagonist, terfenadine has been shown to possess a complex pharmacological profile. Thus, studies on the effects of terfenadine in non-neuronal cells have indicated that it may block voltage dependent ion channels (Woosley, 1996; Suessbrich et al., 1996; Valenzuela et al., 1997; Liu et al., 1997) and terfenadine appears to reverse drug resistance in a variety of cell types through its interaction with the P glycoprotein (Yang et al., 1994). However, the action of terfenadine on neurons has received little attention. Interestingly, terfenadine has been shown to bind on neuronal calcium channels (Zhang et al., 1993), and recently we have shown that nanomolar concentrations of terfenadine may reduce excitotoxicity by acting on presynaptic depolarization (Díaz-Trelles et al., 1999). In this study we have further characterized the mechanism of action of terfenadine.

Materials and methods

Cell cultures

Primary cultures of rat cerebellar neurons were prepared as described (Novelli et al., 1988), and used after 15 days in culture for neurotoxicity studies. Briefly, cerebella from 8-day-old pups were dissected, cells were dissociated and suspended in basal Eagle's medium with 25 mM KCl, 2 mM glutamine, 100 μg/ml gentamycin and 10% fetal calf serum. Cells were seeded in poly-l-lysine coated (5 μg/ml) 35 mm dishes at $2{,}5 \times 10^5$ cells/cm^2 and incubated at 37°C in a 5% CO_2, 95% humidity, atmosphere. Cytosine arabinoside (10 μM) was added after 20–24 h of culture, to inhibit the replication of non-neuronal cells. Cerebellar neurons were kept alive for more than 30 days in culture by replenishing the growth medium with glucose every 4 days and compensating for lost amounts of water, due to evaporation (Fernández et al., 1991).

For electrophysiological studies, both primary cultures of rat cerebellar neurons and primary cultures of rat hippocampal neurons were used. The latter was prepared according to the procedure described by Galdzicki and co-workers (Galdzicki et al., 1993), with the following modifications: a) hippocampal tissue was from P0 rat pups, b) dissociated cells were resuspended in the same medium as for cerebellar neurons, and seeded in poly-l-lysine coated dishes.

Neuronal treatment and survival

Neurotoxicity studies were performed in cultured cerebellar neurons by adding the drugs to the growth medium. Drugs were added to the culture medium at the indicated concentrations, and neuronal cultures were observed for signs of early neurotoxicity at

30 min., as well as for neuronal survival 24 h thereafter, by phase contrast microscopy and by staining of cultures with fluorescein diacetate and ethidium bromide, respectively (Novelli et al., 1988; Fernández et al., 1991). In order to quantify neuronal survival, photographs of randomly selected culture fields were taken, and live and dead neurons were counted. Results were expressed as percentage of live neurons.

Electrophysiological studies

Electrophysiological recordings were performed as previously indicated (Galdzicki et al., 1991; Galdzicki et al., 1993).

Biochemistry

Intracellular cGMP concentration was determined as previously reported (Novelli et al., 1987). Briefly, cultures were washed twice with 1 ml prewarmed (37°C) incubation buffer containing (in mM): 154 NaCl, 5.6 KCl, 5.6 glucose, 8.6 HEPES, 1 $MgCl_2$, 2.3 $CaCl_2$, pH 7.4, and incubated at 37°C for 10 min. with 1 ml fresh incubation buffer and for an additional 20 min. with a second 1 ml fresh incubation buffer. Veratridine was added at the end of the 20 min. incubation period for 1 min. Terfenadine was added 5 min. before veratridine. Incubation was stopped by aspiration of the solution and addition of 1 ml $HCl0_4$ (0.4 N). After neutralizing the perchlorate extract, cGMP content was determined by radioimmunoassay. Protein content was determined on the membrane pellet from the same sample.

Materials

Veratridine, glutamate, mepyramine and tetrodotoxin were purchased from SIGMA. Terfenadine, saxitoxin and (5R,10S)-(+)-5-methyl-10,11-dihydro-5H-dibenzo-(a,d)-cyclohepten 5, 10-imine hydrogen maleate (MK-801), were a generous gift of Dr. J. R. Fernández González of Asturpharma, Dr. V. Zitko of St. Andrews Biological Station, N.B. (Canada) and Dr. G. J. Kaczarowski (Merck Sharp & Dohme Lab., NJ, USA) respectively.

Data presentation and analysis

The mean ± SD of the data is reported unless otherwise indicated. Statistical significance was determined by two-way analysis of variance (ANOVA) and two-tailed unpaired Student's t test.

Results

Neuronal depolarization was obtained by activating voltage sensitive sodium channels (VSSC) with veratridine (20 μM), a stimulus capable of eliciting the release of endogenous glutamate in these cultures (Gallo et al., 1982). Following exposure to veratridine for 30 min., swelling and darkening of cell bodies and varicosities in neurites were observed (Fig. 1A and 1B). These signs appeared to be similar to those elicited by exposure of cultures to toxic concentrations of exogenous glutamate (Novelli et al., 1988). Furthermore, they could be completely prevented by the N-methyl-D-aspartate (NMDA)

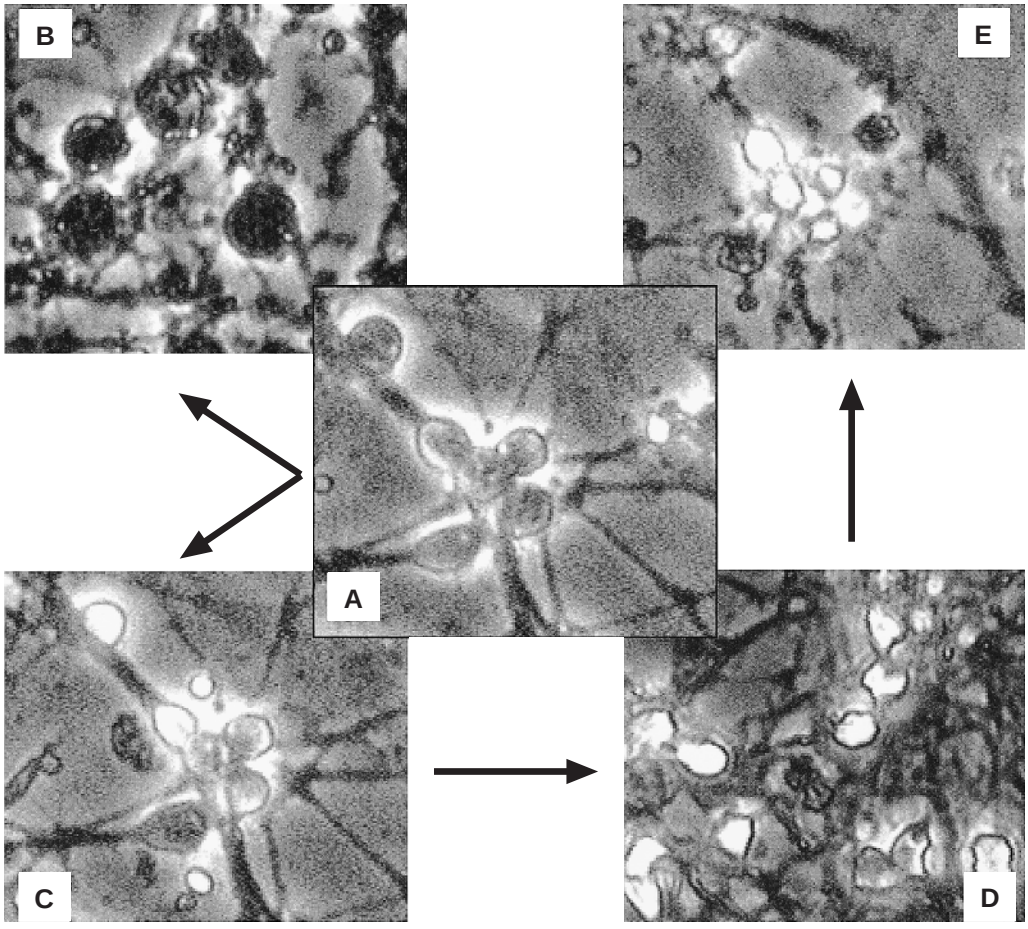

Fig. 1. Characterization of veratridine neurotoxicity in cultured cerebellar neurons. **A** Untreated neuronal culture; **B** exposure to veratridine (VTD, 20μM) for 30 min.; **C** exposure to VTD for 30 min. in the presence of 2μM MK-801; **D** exposure to VTD+MK-801 for 12 h; **E** exposure to VTD+MK-801 for 24 h. Similar data were obtained at least 10 times using different neuronal cultures

receptor antagonist MK-801 (2μM), thus confirming the excitotoxic nature of the process (Fig. 1C).

Exposure of cultures to terfenadine at concentrations as low as 250 nM for 5 min. before veratridine stimulation, completely prevented the appearance of veratridine-induced excitotoxicity (Table 1). Interestingly, terfenadine treatment did not reduce the appearance of excitotoxicity signs following exposure to exogenous glutamate (40μM), although both veratridine and glutamate-induced signs of excitotoxicity could be effectively blocked by MK-801 (2μM) (Table 1).

A progressive shrinking of cell bodies could be observed in neurons treated with veratridine in the presence of MK-801 for 12 hours (Fig. 1D). After 24 hours, a significant reduction in neuronal survival was observed (Fig. 1E and Fig. 2). In the absence of MK-801, the neurodegenerative process was

Table 1. Effect of terfenadine on early veratridine-induced neurotoxicity

TREATMENT	Toxicity signs at 30 min.				
	NONE	VTD	VTD + MK-801	GLU	GLU + MK-801
NONE	−	+	−	+	−
TEF	−	−	−	+	−

Cerebellar neurons in primary culture were exposed to the indicated drugs. MK-801 was added 5 min. before other drugs. Drugs were used at the following concentrations: veratridine (*VTD*), 20 μM; glutamate (*GLU*), 40 μM; terfenadine (*TEF*), 250 nM; MK-801, 2 μM. The presence (+) or the absence (−) of signs of early neurotoxicity such as darkening and swelling of cell bodies were evaluated at 30 min. These observations are based on a TV camera recording as in Fig. 1, and were repeated at least 10 times using different cultures.

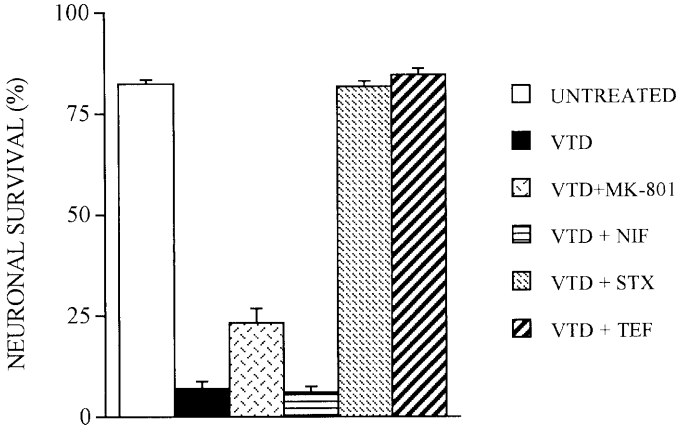

Fig. 2. Effect of terfenadine on veratridine-induced late neurotoxicity. Cerebellar neurons in primary culture were exposed to the indicated drugs: VTD 20 μM; saxitoxin (*STX*) 50 nM; terfenadine (*TEF*) 250 nM; MK-801, 2 μM; nifedipine (*NIF*) 5 μM. Neuronal survival was determined 24 h later as indicated in the text. Data are the mean ± SD of 4–10 experiments

faster (data not shown) and neuronal survival was lower (Fig. 2), but no higher neuronal survival to veratridine was observed in the presence of MK-801 after 48 h (data not shown). Terfenadine treatment significantly increased neuronal survival in veratridine-treated neurons both in the presence and in the absence of MK-801 (Fig. 2). No protection by terfenadine was observed against excitotoxicity following direct exposure to either NMDA (1 mM) or glutamate (40 μM) (Fig. 3). In order to prove that veratridine neurotoxicity was mediated by the specific activation of VSSC, neuronal cultures were exposed to veratridine in the presence of either the VSSC blocker saxitoxin (Terlau et al., 1991) or nifedipine, a blocker of L-type voltage-sensitive calcium channels (Janis et al., 1987), that has been shown to be effective in reducing neurotoxicity following depolarization by KCl (Fernández et al.,

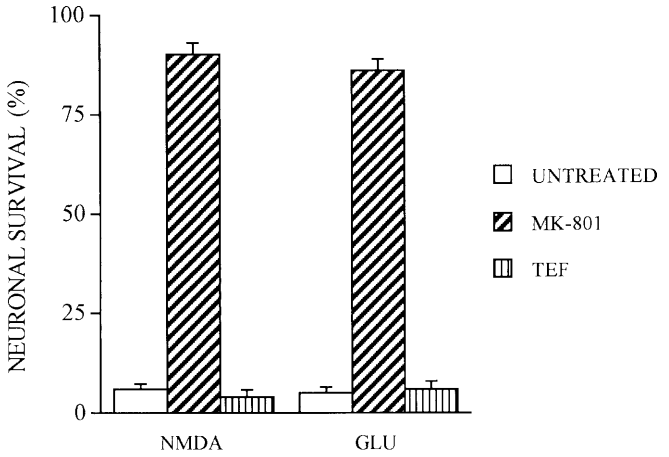

Fig. 3. Terfenadine does not reduce excitotoxicity following direct activation of excitatory amino acid receptors. Neuronal cultures were exposed to either Glutamate (*GLU*) 40 μM or N-methyl-D-aspartate (*NMDA*) 1 mM, alone or in the presence of either 2 μM MK-801 2 or 500 nM TEF. Mk-801 and TEF were added 5 min. before excitatory amino acids, and toxicity was measured 24 h later. Data are the mean ± SD of at least four experiments

1991). Veratridine toxicity was completely prevented by 50 nM saxitoxin, while nifedipine (5 μM) was ineffective (Fig. 2), as well as the lowering of extracellular calcium with the calcium quelator EGTA (data not shown). Finally, we studied whether terfenadine neuroprotection involved histamine receptors (Hill et al., 1997). Thus, cultures were preincubated with both terfenadine (250 nM) and histamine (1 mM), before exposure to veratridine. Histamine failed to reverse the protective effect of terfenadine against veratridine toxicity (Fig. 4).

To further confirm that terfenadine may provide protection from veratridine neurotoxicity by reducing ion influx through VSSC, we performed patch-clamp studies. For these experiments, we used cultured hippocampal neurons which exhibit a sodium current density of approximately 160 pA/pF, that is up to 3 times larger than that of cerebellar granule cells, as well as a 5–6 times larger size as inferred from their capacitance, thus providing larger sodium currents (Galdzicki et al., 1991; Galdzicki et al., 1993). We found that neuronal exposure to 200 nM terfenadine significantly reduced tetrodotoxin-sensitive peak inward currents by 60 ± 5% (n = 10). We also observed that both transient and delayed rectifier potassium currents (I_A and I_K respectively) were reduced by terfenadine, the latter showing the larger inhibition, comparable to that of sodium currents (17% for I_A vs 58% for I_K).

Finally, we decided to study how a fully neuroprotective concentration of terfenadine would affect second messenger formation following activation of VSSC. For this purpose, we measured the intracellular formation of cGMP following depolarization by veratridine both alone and in the presence of 500 nM terfenadine. We observed that terfenadine inhibited cGMP formation by veratridine, and the extent of the inhibition depended upon the magnitude

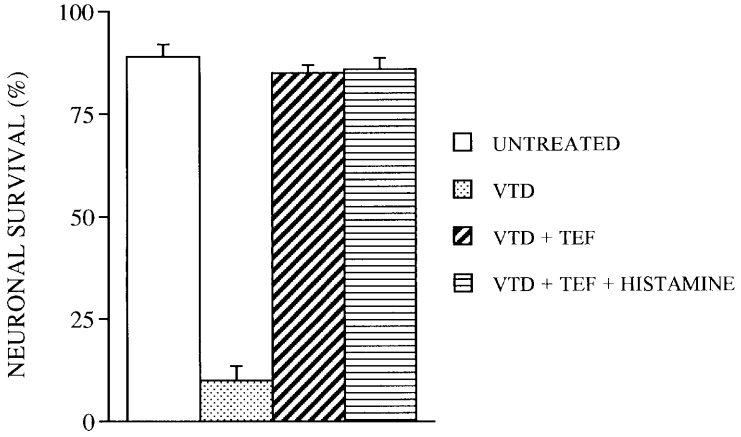

Fig. 4. Terfenadine protection from veratridine toxicity is histamine receptor independent. Neuronal cultures were exposed to 20μM VTD alone or in the presence of either 250 nM TEF or 250 nM TEF + 1 mM histamine. TEF was added 5 min. before VTD and histamine was added just before TEF. Neurotoxicity was determined 24 h later. Data are the mean ± SD of at least four experiments

of the response elicited by veratridine. The results could be fitted by linear regression with high correlation (Fig. 5).

Discussion

Our results confirm and extend previous evidence that terfenadine may modulate glutamatergic neurotransmission via a presynaptic mechanism (Díaz-Trelles et al., 1999). Depolarizing stimuli such as veratridine, can release glutamate from cultured cerebellar neurons (Gallo et al., 1982), and the amount of glutamate released is sufficient to activate NMDA receptors and to promote cGMP synthesis (Fernández-Sánchez and Novelli, 1993). The excitotoxic role of the amounts of glutamate released following veratridine depolarization, has been object of study by several authors (Ramnath et al., 1992; Dargent et al., 1996; Takahashi et al., 1999) who did not observed protection by NMDA receptor antagonists against veratridine toxicity after 24 h. Thus, it should be noted that the early excitotoxic component in veratridine toxicity we observed was important in determining the speed of the neurodegenerative process, but contributed only minimally to the overall toxicity of veratridine after 24 h (Fig. 2), and had no contribution at all after 48 h. On the other hand, the reported failure of amino-phosphonovalerate in reducing neuronal swelling shortly after veratridine exposure (Ramnath et al., 1992), may depend on the amount of glutamate released and the use of a competitive receptor antagonist.

Terfenadine neuroprotection from the excitotoxic early component of veratridine toxicity appears to occur independently of excitatory amino acid receptors, as demonstrated by terfenadine failure to protect from direct

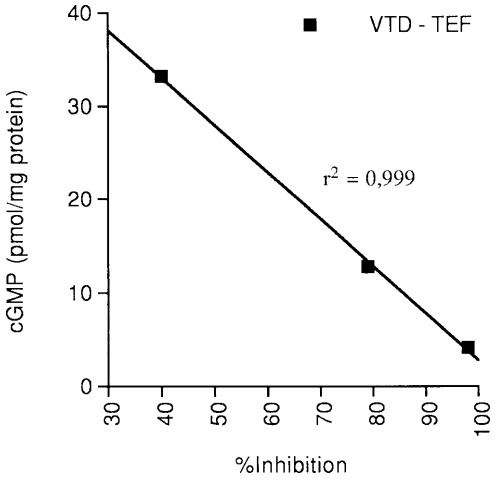

Fig. 5. Correlation between cGMP formation by veratridine and its inhibition by terfenadine. Neuronal cultures were exposed to either 10μM VTD alone or 10μM VTD in the presence of 500nM TEF. TEF was added 5min. before VTD. cGMP intracellular concentration was measured after 1min. of exposure to the drug. Data are from three different neuronal cultures and three culture dishes were used for each experiment. SD was $\leq$20%. cGMP was assayed in duplicate

activation of excitatory aminoacid receptors, and may involve the release of glutamate from the neurons in culture. This presynaptic mechanism receives further support by the observation that terfenadine is also able to prevent the late NMDA-receptor independent effects of veratridine, that can be attributed to the large Na^+ influx through the persistently activated VSSCs. In fact, Na^+ influx appears to be the cause of neuronal death following veratridine but not other depolarizing agents (Takahashi et al., 1999), while the influx of Ca^{++} subsequent to depolarization, does not appear to be the cause of neurodegeneration following either veratridine stimulation (Dargent et al., 1996; Takahashi, 1999), or elevation of extracellular KCl (Fernández et al., 1991; Novelli et al., 1995). Our present results confirm this view, as both, early and late veratridine toxicity were blocked by saxitoxin, while neither the L-type voltage sensitive calcium channel antagonist nifedipine, nor removal of extracellular calcium, did reduce its toxicity at 24h. The protective effect of terfenadine we describe against veratridine toxicity at 24h is then in agreement with the electrophysiological data indicating that this drug may act blocking VSSC. Furthermore, considering the capability of this drug to also reduce K^+ currents, the block of VSSC appears to be absolutely necessary for protection against neurodegeneration due to persistent activation of VSSC, and may deserve further studies.

The effects of terfenadine on veratridine-mediated cGMP formation also deserve further studies, as the correlation we observed between the magnitude of intracellular cGMP formation and its inhibition by terfenadine, may be due to the binding of the drug to a particular population of VSSC, whose block is sufficient to produce neuroprotection while calcium-

dependent formation of second messengers, such as cGMP, may be maintained to a certain extent.

Finally, it is worth noting that the neuroprotective effect of terfenadine we describe here is independent of histamine receptors, indicating that this drug may have unexpected effects at therapeutically useful concentrations, and may be used as a prototype for the development of new drugs with a therapeutic potential in the treatment of neurodegenerative diseases involving glutamatergic neurotransmission.

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