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# Novel effect of nefopam preventing cGMP increase, oxygen radical formation and neuronal death induced by veratridine

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**Abstract**

Nefopam hydrochloride is a potent analgesic compound that possesses a profile distinct from that of opiods or anti-inflammatory drugs. Previous evidence suggested a central action of nefopam but the detailed mechanisms remain unclear. Here we have used cultured cerebellar neurons to test the hypothesis that nefopam may modulate voltage sensitive sodium channel (VSSC) activity. Nefopam (100 μM) effectively prevented NMDA receptor-mediated early appearance (30 min) of toxicity signs induced by the VSSC activator veratridine. Delayed neurotoxicity by veratridine occurring independently from NMDA receptor activation, was also prevented by nefopam. In contrast, excitotoxicity following direct exposure of neurons to glutamate was not affected. Neuroprotection by nefopam was dose-dependent. 50% protection was obtained at 57 μM while full neuroprotection was achieved at 75 μM nefopam. Veratridine-induced sodium influx was completely abolished in nefopam-treated neurons. Intracellular cGMP and oxygen radical formation following VSSC stimulation by veratridine were also effectively prevented by nefopam. Our data are consistent with an inhibitory action of nefopam on VSSC and suggest that nefopam may modulate the release of endogenous glutamate following activation of these channels. This novel action of nefopam may be of great interest for the treatment of neurodegenerative disorders involving excessive glutamate release and neurotransmission. 

*Keywords:* Nefopam; Glutamate release; Sodium channels; Excitotoxicity; Reactive oxygen species; Cultured cerebellar neurons

## 1. Introduction

Nefopam hydrochloride is a potent analgesic compound commercialized in most Western European countries for 20 years. Nefopam possesses a profile distinct from that of opioids or anti-inflammatory drugs. It does not cause tolerance, withdrawal reactions or physical dependence, and the potential for its abuse is very low (Heel et al., 1980). Furthermore, nefopam does not produce respiratory depression even in the post-operative period (Gasser and Bellville, 1975; Gerbershagen and Schaffner, 1979). Clinical studies have demonstrated nefopam to be very effective in the prevention of post-operative shivering in patients after general anesthesia (Rosa et al., 1995) without affecting the recovery time between the end of anesthesia and extubation (Piper et al., 1999). Unpleasant adverse effects during therapeutic use have been also reported including dizziness, headache, nausea, vomiting and sweating, consistent with a central mode of action of the drug. Although these side effects are usually minor and not very long lasting, they can probably explain the limited development of nefopam for post-operative use in the last years.

The analgesic properties of nefopam are now being reinvestigated. This drug has been recently demonstrated

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to induce a rapid and strong depression of the nociceptive flexion ($R_{III}$) reflex in humans (Guirimand et al., 1999), probably through a central mechanism of action (Hunskaar et al., 1987; Fasmer et al., 1987). However, the detailed mechanisms underlying the pharmacological actions of nefopam remain unclear. Evidence exists suggesting a possible action of nefopam on the neurotransmission mediated by glutamate. Thus, nefopam is a cyclic analogue of orphenadrine and diphenhydramine, drugs originally synthesized as central myorelaxants which exert unspecific antagonistic activity at the phencyclidine binding site of NMDA receptors (Kornhuber et al., 1995). Furthermore, nefopam shows pre-emptive analgesic effects in a model of neuropathy (chronic constriction injury of the sciatic nerve) (Biella et al., unpublished results) which involves the activation of NMDA receptors. In this study, we have used primary cultures of cerebellar neurons to test the hypothesis that nefopam modulated glutamate neurotransmission via NMDA receptors following depolarization of neurons by the voltage sensitive sodium channel activator veratridine. Cultured cerebellar neurons represent a neuronal model that has proved very useful in the study of the biochemical events coupled to glutamate receptors and the conditions controlling excitotoxicity (McCaslin and Morgan, 1987; Nicoletti et al., 1986; Novelli et al. 1987, 1988, 1992). In these cultures, the capability of depolarizing stimuli such as veratridine to release glutamate has been demonstrated (Gallo et al., 1982), and the amount of glutamate released is capable of activating NMDA receptors and promoting cGMP synthesis (Fernández-Sánchez and Novelli, 1993), as well as excitotoxicity (Díaz-Trelles et al., 1999). We report that nefopam effectively inhibits the influx of sodium following VSSC activation by veratridine. We also show that nefopam effectively prevents NMDA receptor-dependent and independent neuronal death as well as cGMP and oxygen radical formation following activation of voltage sensitive sodium channels by veratridine. The novel action of nefopam we report here may be of great interest for the treatment of neurodegenerative disorders involving excessive glutamate release and neurotransmission.

## 2. Methods

### 2.1. Cell cultures

Primary cultures of rat cerebellar neurons were prepared as previously described (Novelli et al. 1988, 1992). 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 $2.5\times10^5$ 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. After eight days in vitro, morphologically identifiable granule cells accounted for more than 95% of the neuronal population, the remaining 5% being essentially GABAergic neurons. Astrocytes did not exceed 3% of the overall number of cells in culture. Cerebellar neurons were kept alive for more than 40 days in culture by replenishing the growth medium with glucose every four days and compensating for lost amounts of water, due to evaporation.

### 2.2. Neurotoxicology

Neurons were used between 14–20 days in culture. Drugs were added into the growth 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. To quantify neuronal survival, cultures were stained with fluorescein diacetate and ethidium bromide (Novelli et al., 1988; Fernández et al., 1991), photographs of three randomly selected culture fields were taken and live and dead neurons were counted. Results were expressed as percentage of live neurons. Total number of neurons per dish was calculated considering the ratio between the area of the dish and the area of the picture (~3000).

### 2.3. cGMP determination

Intracellular cGMP concentration was determined as previously reported (Novelli and Henneberry, 1987). Briefly, cultures were washed 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. Dishes were incubated at 37°C for 10 min with 1 ml new incubation buffer and for an additional 20 min with a second 1 ml new incubation buffer in which $MgCl_2$ was omitted. Drugs were added at the end of the 20 min incubation period for the indicated times. Incubation was stopped by aspiration of the solution and addition of 1 ml $HClO_4$ (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.

### 2.4. Confocal microscopy

Oxygen radical formation was detected with carboxy-2'7'-dichlorodihydrofluoresceine diacetate (carboxy-$H_2$DCFDA). Following uptake, the carboxy-$H_2$DCFDA is converted by endogenous esterases to carboxy-$H_2$DCF, which upon exposure to oxidative species is oxidized to the fluorescent probe carboxy-DCF. Neuronal cultures were treated with the indicated drugs and

loaded with 20 μM carboxy-$H_2$DCFDA in the culture medium for 1 h. Then, the dye was removed and cultures were washed twice with a 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. Carboxy-DCF fluorescence was recorded in a Bio–Rad confocal microscope with a krypton–argon laser excitation source (488 nm). Signals were digitized using Bio–Rad interface and fluorescence intensity was quantified in 10–15 cell bodies per field using the software NIH Image (1.61).

Intracellular sodium was determined using Sodium Green. A stock solution of Sodium Green tetraacetate (1 mM) and the nonionic detergent Pluronic F127 (20% w/v) were prepared in dimethyl sulfoxide and mixed in equal volumes just prior to cell loading. Neuronal cultures were loaded for 20–30 min at 20°C with 3 μM Sodium Green tetraacetate in an 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. The dye was removed and cells were washed twice with the same buffer. Sodium Green emission (532 nm) was recorded in a Bio–Rad confocal microscope with a krypton–argon laser excitation source (488 nm). Signals were digitized using Bio–Rad interface and analysed by the software NIH Image (1.61). As the increase in fluorescence intensity affected mostly the neurites, for each treatment we determined the average signal obtained from 8–10 fields of the same size (41×62 pixels). Concentrations of intracellular sodium ($[Na^+]_I$) could be estimated using the following equation:

$$[Na^+]_I = K_d(F - F_{min})/(F_{max} - F)$$

where $F$ is the fluorescence of the indicator at experimental sodium levels, $F_{min}$ is the fluorescence in the absence of sodium and $F_{max}$ is the fluorescence of the sodium-saturated probe. Mean values for $F_{min}$ and $F_{max}$ were ascertained using cells that have been loaded with Sodium Green tetraacetate and then treated with the pore-forming antibiotic gramicidin (40 μM). To determine $F_{min}$ a $Na^+$-free incubation buffer was used in which NaCl was substituted by coline chloride. The dissociation constant $K_d$ of Sodium Green for $Na^+$ is about 21 mM at 22°C. Although calculation of $[Na^+]_I$ using single-wavelength dyes is inherently less accurate than using dual-wavelength ratioable indicators, published comparisons show good quantitative agreement between Sodium Green and the dual-wavelength dye SBFI (Amorino and Fox, 1995).

### 2.5. Data presentation and analysis

For statistical analysis the one-way or the two-way analysis of variance (ANOVA) was used to identify overall treatment effects, followed by the unpaired two-tailed Student's *t*-test for selective comparison of individual data groups. Only significances relevant for the discussion of the data are indicated in each figure.

### 2.6. Materials

Carboxy 2',7'-dichlorodihydrofluoresceine diacetate (carboxy-$H_2$DCFDA, C-400) and Sodium Green tetraacetate (S-6901) were from Molecular Probes. Saxitoxin was a generous gift of Dr. V. Zitko of St. Andrews Biological Station, N.B. (Canada). Nefopam, veratridine, L-glutamate, nifedipine and (+)-10,11-dihydro-5-methyl-5H-dibenzo-[a,d]-cyclohepten-5,10-imine hydrogen maleate (MK-801) were from Sigma.

## 3. Results

Activation of voltage sensitive sodium channels (VSSC) by veratridine (10 μM) resulted within 30 min in swelling and darkening of cell bodies and appearance of varicosities in neurites (Fig. 1B). These signs were similar to those elicited by exposure of cultures to toxic concentrations of exogenous glutamate (Novelli et al., 1988), and they could be completely prevented by the N-methyl-D-aspartate (NMDA) receptor antagonist MK-801 (1 μM) (Fig. 1D). Exposure of cultures to nefopam (100 μM) for 5 min before veratridine stimulation completely prevented the appearance of veratridine-induced NMDA receptor-dependent excitotoxicity (Fig. 1F). It has to be noted that nefopam did not reduce neurotoxicity by exogenous glutamate (40 μM) (see Table 1), although both veratridine and glutamate-induced signs of excitotoxicity could be effectively blocked by MK-801 (Fig. 1D and data not shown). Longer exposures to veratridine (24 h) led to a significant reduction in neuronal survival that occurred independently of the presence of MK-801 (Table 1 and Fig. 1C and E). A progressive shrinking of cell bodies could be observed in neurons treated with veratridine in the presence of MK-801, until a complete degeneration occurred (Fig. 1E). In contrast, neurons pretreated with nefopam showed no signs of toxicity after 24 h exposure to veratridine, and appeared similar to controls (see Fig. 1G and A). Nefopam effectively protected neurons from toxicity by veratridine both in the absence and in the presence of MK-801 (Table 1). To confirm the involvement of VSSC in veratridine toxicity we used the VSSC blocker saxitoxin (Terlau et al., 1991) and nifedipine, a blocker of L-type voltage sensitive calcium channels (Janis et al., 1987) which effectively reduced neurotoxicity following depolarization by KCl (Fernández et al., 1991). Veratridine toxicity was completely prevented by 50 nM saxitoxin while nifedipine (5 μM) was ineffective (Table 1).

Nefopam protection from veratridine-induced neurotoxicity was concentration dependent (Fig. 2). A slight but statistically significant protective effect was observed as low as 20 μM nefopam, 50% protection was achieved at approximately 57 μM, and full neuroprotection required at least at 75 μM nefopam.

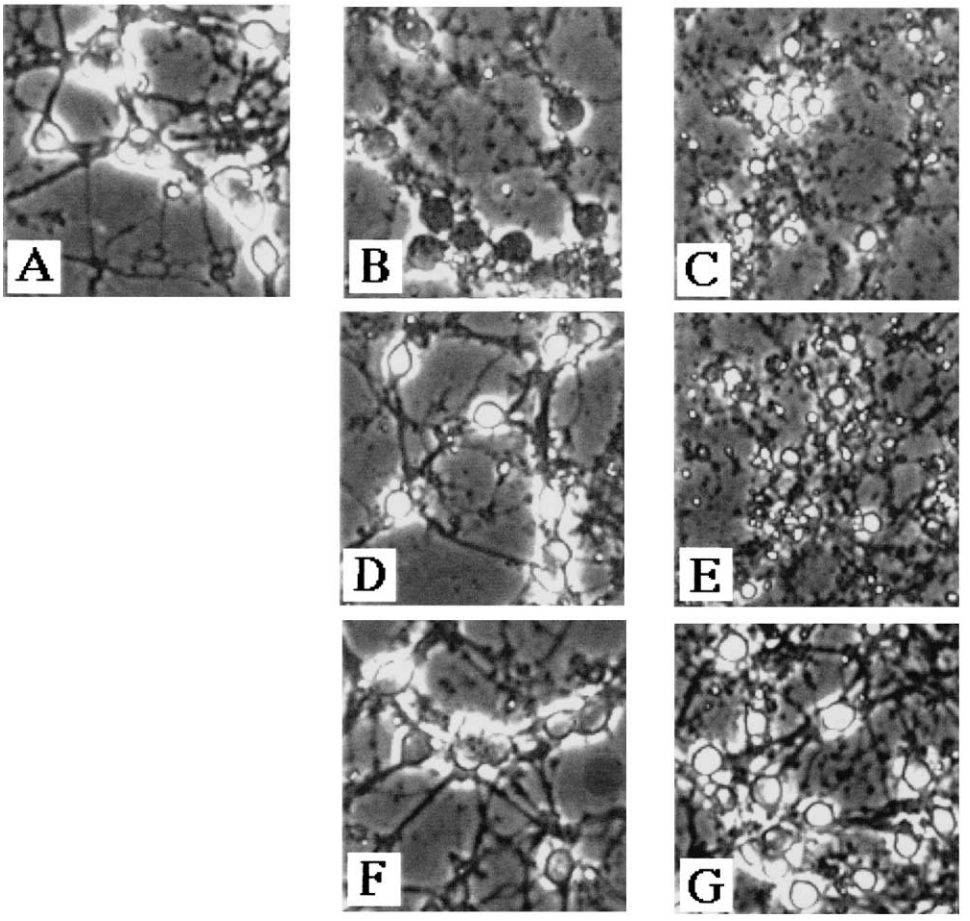


Fig. 1. Nefopam protects neurons from NMDA receptor dependent and independent components of veratridine toxicity. Phase contrast images represent neuronal cultures at 14 DIC untreated (A) or exposed to veratridine (VTD 10 μM) for 30 min (B,D,F) or 24 h (C,E,G), in the absence (B,C) or in the presence of either 1 μM MK-801 (D,E) or 100 μM nefopam (F,G). MK-801 and nefopam were added 5 min prior to VTD. Similar data was obtained at least five times using different neuronal cultures.

Table 1
Nefopam protects neurons from veratridine-induced neurotoxicity[a]

| | Neuronal survival (%) | |
|---|---|---|
| | None | Nefopam |
| Control | 94±4[a] | 96±5 |
| VTD | 10±5 | 92±8[d] |
| VTD+MK-801 | 7±4 | 89±8[d] |
| VTD+STX | 93±3[b] | n.d. |
| VTD+NIF | 8±7 | n.d. |
| GLU | 5±5 | 7±6 |
| GLU+MK-801 | 90±9[c] | n.d. |

[a] Cerebellar neurons in primary culture were exposed to the indicated drugs and neuronal survival was determined 24 h later. MK-801, saxitoxin (STX) and nifedipine (NIF) were added 5 min before other drugs. Concentrations were: Nefopam 100 μM; VTD 10 μM; STX 50 nM; MK-801 1 μM; NIF 10 μM; glutamate (GLU) 40 μM. Values represent the mean±SD from three independent experiments ($n$=6).

[b] $P<0.01$ vs. VTD in the same column.

[c] $P<0.01$ vs. GLU in the same column.

[d] $P<0.01$ vs. None in the same line.

Our results strongly suggest nefopam to inhibit sodium influx via voltage sensitive sodium channels. To test this possibility we did sodium imaging experiments using the fluorescent dye Sodium Green, previously shown to provide resolution of $Na^+$ concentrations with sufficient selectivity in the presence of physiological concentrations of other monovalent cations (Minta and Tsien, 1989; Amorino and Fox, 1995). In the absence of nefopam, 20 μM veratridine significantly enhanced the fluorescence intensity of Sodium Green (see Fig. 3), consistent with an increase in the concentration of intracellular sodium following the application of veratridine. Although a rise in fluorescence intensity could be observed in both cell bodies and neurites, the most prominent increase occurred in the neuritic network (Fig. 3B). In contrast, no increase in the fluorescence intensity could be observed in cultures stimulated with 20 μM veratridine in the presence of 100 μM nefopam (Fig. 3C and D). Quantification of the fluorescence intensity signals (see Methods) showed that veratridine elicited a significant mean increase from 9±2 to 17±3 fluorescence units (f.u.) in the absence of nefopam, while no increase

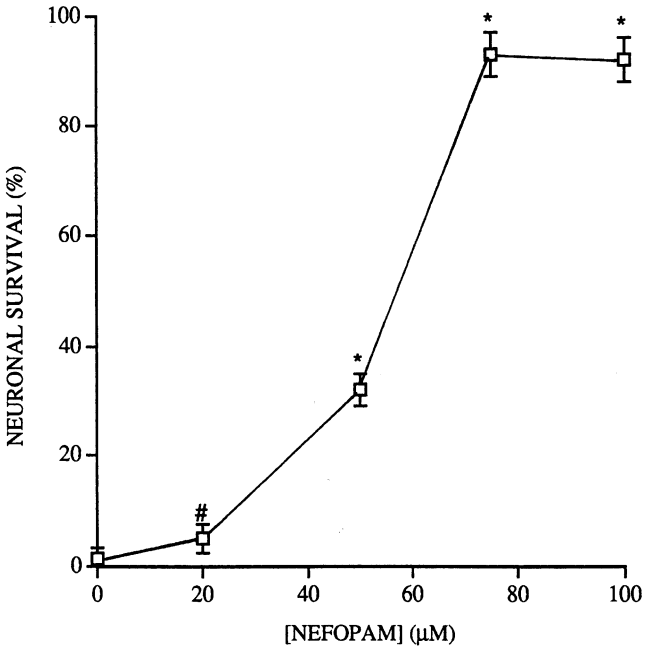


Fig. 2. Nefopam protection from veratridine-induced neurotoxicity is concentration-dependent. Neuronal cultures at 14–18 DIC were treated with increasing concentrations of nefopam added 5 min before veratridine (10 μM). Neuronal survival was determined 24 h later. Data represent the mean±SD of two independent experiments ($n$=6). $^{\#}P<0.01$ and $^{*}P<0.001$ vs. veratridine in the absence of nefopam.

in fluorescence intensity could be observed in nefopam-treated neurons (Fig. 3E). Following the addition of veratridine, the concentration of sodium was estimated (see Methods) to rise from approximately 12±4 to 45±10 mM (mean±SD, $n$=12), values similar to those reported by other authors in cultured hippocampal neurons (Rose and Ransom, 1997). In nefopam-treated neurons the concentrations of sodium were estimated to be 6±3 before and 2±2 mM after the application of veratridine respectively.

To investigate how neuroprotective concentrations of nefopam would affect second messenger formation, we determined the effect of nefopam on the intracellular formation of cGMP following the activation of VSSC by veratridine. As shown in Fig. 4, veratridine (10 μM) resulted in an approximately 10 fold increase in intracellular concentrations of cGMP, and such elevation could be effectively prevented by the application of 100 μM nefopam 5 min before veratridine. cGMP formation by the direct guanylate cyclase activator sodium nitroprusside (1 mM) was not affected by nefopam.

We also assessed for the participation of oxidative stress in veratridine-induced excitotoxicity. We used the fluorescent probe carboxy-dichlorodihydrofluorescein diacetate (carboxy-$H_2$DCFDA), as dichlorodihydrofluorescein diacetate and derivatives have proved extremely useful for assessing the overall oxidative stress in toxicological phenomena in a variety of experimental systems including cultured neurons (Behl et al., 1994; Cubells et al., 1994). As shown in Fig. 5, application of veratridine

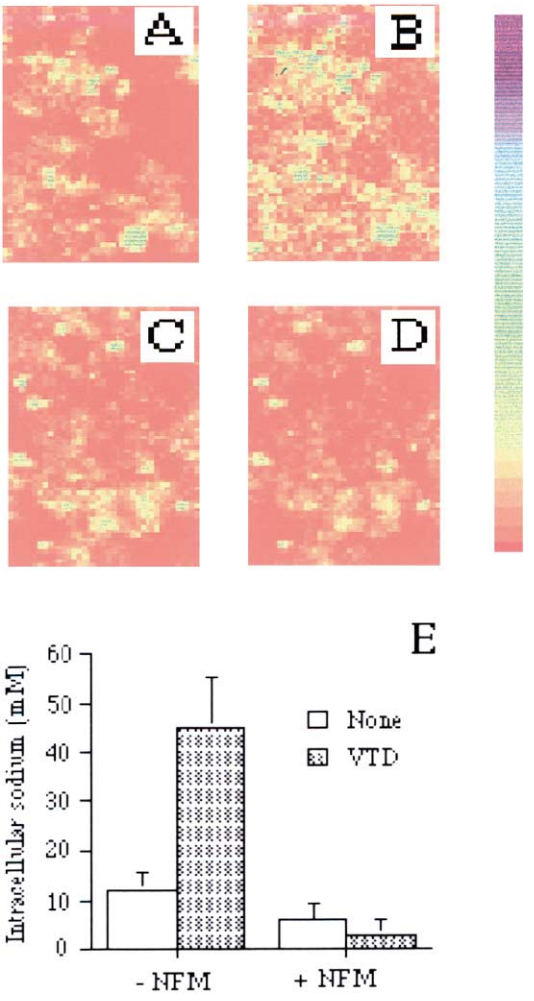


Fig. 3. Nefopam prevents the increase in intracellular sodium induced by veratridine. Neuronal cultures were loaded for 20–30 min at 20°C with 3 μM Sodium Green tetraacetate and then examined under a laser confocal microscope. (A–D) Representative images taken before (A,C) or 30 s after (B,D) the addition of 20 μM veratridine (VTD) in the absence (A,B) or in the presence (C,D) of 100 μM nefopam (NFM). Scale colour bar spans from orange (minimum) to purple (maximum) corresponding to fluorescence intensity values of 0 and 255 respectively. (E). Images as the ones represented in (A–D) were used to estimate intracellular sodium concentrations before (none) or after the application of 20 μM veratridine in the absence (−NFM) or in the presence of nefopam (+NFM). As the increase in fluorescence intensity affected mostly the neurites, for each treatment we report the concentration calculated from the fluorescence intensity obtained from 8–10 fields of the same size (41×62 pixels). Results represent the mean±SD of two independent experiments ($n$=6–10). $^{\circ}P<0.05$ vs. none in the absence of NFM.

(20 μM) resulted after 2 h in a strong rise in fluorescence intensity in most cell bodies and neurites (Fig. 5B) compared to unstimulated cultures (Fig. 5A), consistent with a significant increase in the generation of reactive oxygen species. Radical formation was completely abolished in neurons to which nefopam was added 5 min prior to the application of veratridine (Fig. 4C). Quantification of the fluorescence intensity revealed a veratridine-induced increase from 67±7 to 174±42 f.u. in the absence of

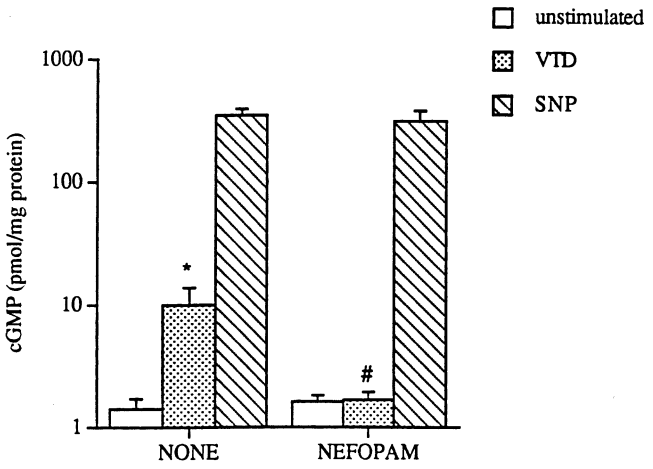


Fig. 4. Nefopam prevents depolarization-induced stimulation of cGMP formation. Cerebellar neurons at 12–14 DIC were either untreated (NONE) or exposed to nefopam (100 μM) for 5 min, before stimulation of cultures with veratridine (VTD; 10 μM×1 min) or sodium nitroprusside (SNP; 1 mM×2 min). cGMP content was determined by radioimmunoassay. Data are reported as the mean±SD of two independent experiments performed in triplicate ($n$=6). $^{*}P<0.001$ vs. unstimulated in the same group; $^{\#}P<0.001$ vs. VTD in the absence of nefopam.

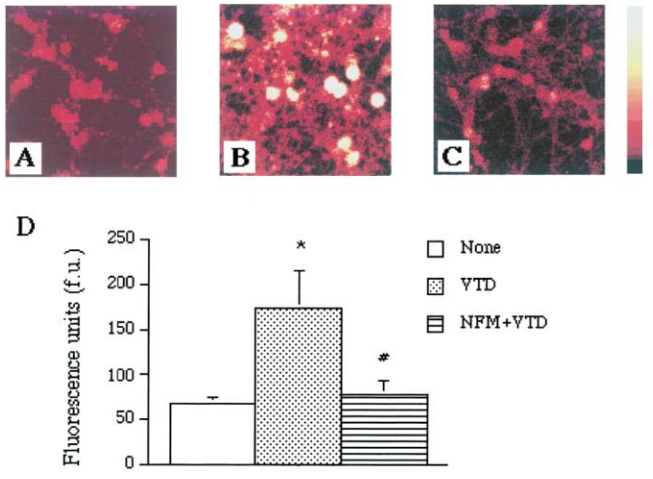


Fig. 5. Nefopam prevents the formation of oxygen radicals induced by veratridine. Neuronal cultures untreated (A) or exposed to veratridine (20 μM) either in the absence (B) or in the presence of 100 μM nefopam (C), were loaded with 20 μM carboxy-$H_2$DCFDA in the culture medium for 1 h. The carboxy-DCF fluorescence was recorded in a Bio–Rad confocal microscope with a krypton–argon laser excitation source (488 nm). Signals were digitized using Bio–Rad interface and analyzed by NIH Image (1.61). Scale colour spans from black (minimum) to white (maximum) corresponding to fluorescence intensity values of 0 and 255 respectively. Nefopam was added 5 min before veratridine and carboxy-$H_2$DCFDA was added 1 h after veratridine. The experiment was repeated three times with similar results. $^{*}P<0.05$ vs. none; $^{\#}P<0.05$ vs. VTD.

nefopam, while no significant increase was observed in the presence of 100 μM of this drug (78±16).

## 4. Discussion

Our results demonstrate the analgesic compound nefopam to inhibit sodium influx, second messenger formation, and neuronal death following the stimulation of VSSC with veratridine. The capability of depolarizing stimuli such as veratridine to release glutamate from cultured cerebellar neurons has been demonstrated previously (Gallo et al., 1982), and the amount of glutamate released is sufficient to activate NMDA receptors, to stimulate cGMP synthesis (Fernández-Sánchez and Novelli, 1993), and to induce excitotoxicity (see Fig. 1B). Thus, our data provide novel evidence suggesting that nefopam may modulate glutamatergic neurotransmission.

The early (30 min) MK-801-sensitive excitotoxic component of veratridine toxicity was important in determining the speed of the neurodegenerative process, but it had a very minor contribution to the overall toxicity by veratridine after 24 h (see Fig. 1C and E). This late, MK-801-independent toxicity, may be attributed to the large influx of sodium through the persistently activated VSSC. Thus, our observation is consistent with studies by other authors showing that NMDA receptor antagonists failed to protect against toxicity after 24 h exposure to veratridine (Ramnath et al., 1992; Dargent et al., 1996; Takahashi et al., 1999), and confirms previous results from our group (Díaz-Trelles et al., 1999). The failure of the NMDA receptor specific antagonist aminophosphonovalerate (APV) in reducing neuronal swelling shortly after exposure to veratridine reported by other authors (Ramnath et al., 1992) could be due to differences in the amount of glutamate released by different neuronal types, or to the competitive characteristics of APV.

It is tempting to speculate that the actions on VSSC we report may play a significant role in the analgesic effects of nefopam. Several lines of evidence suggest sodium channel inhibition to be a mechanism for analgesia (Besson, 1999). The sodium channel blocker carbamazepine has been shown to be effective in the treatment of neuropathic pain (Rizzo, 1997; Harke et al., 2001). Intrathecal administration of the sodium channel blocker lamotrigine produced a spinal long-lasting antihyperalgesic effect in short- and long-term neuropathic models of hyperalgesia (Klamt, 1998). Also, an inhibition of veratridine-induced sodium influx has been found for antidepressants and neuroleptics used in chronic pain (Deffois et al., 1996)

Evidence exists for a potential antagonistic action of nefopam on NMDA receptors. The nefopam analogue orphenadrine compete with MK-801 at the phencyclidine binding site of the NMDA receptor (Kornhuber et al., 1995). Nefopam strongly reduces behavioral and electrophysiological signs of the neuropathy induced by ligature of the sciatic nerve (Biella et al., unpublished

results), and NMDA receptor activation appears to be critical for the development and maintenance of the centrally mediated events in the course of this neuropathy (Kim et al., 1997; Kawata and Omote, 1996). Also, here we show nefopam to effectively prevent the veratridine-induced formation of oxygen radicals (Fig. 5), a key event in the occurrence of NMDA receptor mediated neurotoxicity (Coyle and Puttfarcken, 1993; Díaz-Trelles et al., 2000). However, our data suggest an inhibitory action of nefopam on sodium channels rather than a direct action of nefopam on NMDA receptors. Indeed, nefopam failed to prevent neuronal death following the direct activation of receptors by exogenous glutamate (Table 1). Receptor binding studies using rat hyppocampus homogenates showed that nefopam significantly displaced MK-801 only at concentrations of at least 1 mM (data not shown), also excluding a possible block of NMDA receptors by nefopam. Instead, nefopam neuroprotection from the NMDA receptor-dependent excitotoxic early component of veratridine toxicity, may be associated to a reduction of the release of glutamate from neurons in culture due to the inhibition of VSSC. The inhibitory action of nefopam on VSSC is supported by the observation that it significantly prevented the rise in intracellular sodium induced by veratridine (Fig. 3), as well as the late (24 h) NMDA-receptor independent effects of veratridine (Fig. 1G and Table 1). Moreover, veratridine was not toxic for neurons maintained in a $Na^+$-free incubation buffer in which NaCl was substituted by coline chloride (data not shown). Such an inhibitory effect of nefopam would be also consistent with the observation that it completely abolished the increase in cGMP synthesis following depolarization of neurons with veratridine (Fig. 4), as approximately 65% of total veratridine-induced intracellular increase in cGMP is NMDA receptor stimulation-independent (Fernández-Sánchez and Novelli, 1993).

Several lines of evidence indicate sodium influx to be the cause of neuronal death following activation of VSSC with veratridine (Takahashi et al., 1999; Dargent et al., 1996). Our results here further confirm this view as both early and late veratridine toxicity could be totally prevented by the specific VSSC blocker saxitoxin, while the L-type voltage sensitive calcium channel antagonist nifedipine was not effective (see Table 1). Other depolarizing stimuli capable of increasing intracellular calcium concentrations failed to induce neurodegeneration in the presence of MK-801 (Fernández et al., 1991; Novelli et al., 1995). Therefore, veratridine neurotoxicity in the presence of MK-801 cannot be due to the release of endogenous amino acids activating other types of excitatory amino acid receptors, or to an excessive influx of calcium via voltage sensitive calcium channels. Cultured cerebellar neurons express GABA receptors (Gallo et al., 1985), and GABAergic neurons account for approximately 3% of total neuronal population in these cultures (Nicoletti et al., 1986). Therefore, it is possible to speculate that nefopam could mimic the action of GABA receptor agonists and protect neurons by increasing GABAergic inhibitory neurotransmission. However, the GABA receptor agonist muscimol did not prevent veratridine-induced toxicity (data not shown), rendering such possibility unlikely.

In conclusion, this study demonstrates the analgesic compound nefopam hydrochloride to reduce sodium influx and to effectively prevent intracellular formation of cGMP and oxygen radicals as well as neuronal death following the activation of voltage-operated sodium channels in cultured cerebellar neurons. This novel action of nefopam may be of interest in reducing the excessive release of endogenous glutamate involved in some neurodegenerative disorders.

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