Amino Acids (2005) 28: 183–191
DOI 10.1007/s00726-005-0166-0

Amino Acids
Printed in Austria

Nefopam inhibits calcium influx, cGMP formation, and NMDA receptor-dependent neurotoxicity following activation of voltage sensitive calcium channels

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Received July 1, 2004
Accepted August 1, 2004
Published online February 18, 2005;

Summary. Nefopam hydrochloride is a potent non sedative benzoxazocine analgesic that possesses a profile distinct from that of anti-inflammatory drugs. Previous evidence suggested a central action of nefopam but the detailed mechanism remains unclear. We have investigated the actions of nefopam on voltage sensitive calcium channels and calcium-mediated pathways. We found that nefopam prevented N-methyl-D-aspartate (NMDA)-mediated excitotoxicity following stimulation of L-type voltage sensitive calcium channels by the specific agonist BayK8644. Nefopam protection was concentration-dependent. 47 μM nefopam provided 50% protection while full neuroprotection was achieved at 100 μM nefopam. Neuroprotection was associated with a 73% reduction in the BayK8644-induced increase in intracellular calcium concentration. Nefopam also inhibited intracellular cGMP formation following BayK8644 in a concentration-dependent manner, 100 μM nefopam providing full inhibition of cGMP synthesis and 58 μM allowing 50% cGMP formation. Nefopam reduced NMDA receptor-mediated cGMP formation resulting from the release of glutamate following activation of channels by BayK8644. Finally, we also showed that nefopam effectively reduced cGMP formation following stimulation of cultures with domoic acid, while not providing neuroprotection against domoic acid. Thus, the novel action of nefopam we report here may be important both for its central analgesic effects and for its potential therapeutic use in neurological and neuropsychiatric disorders involving an excessive glutamate release.

Keywords: Nefopam – Voltage sensitive calcium channels – Excitotoxicity – cGMP – Cultured cerebellar neurons

Classification Items: THEME C: Excitable membranes and synaptic transmission. TOPIC: Calcium channels

1 Introduction

Nefopam hydrochloride is a potent analgesic compound commercialized in most of Western Europe for over 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; Villier and Mallaret, 2002). 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 postoperative 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). Furthermore, nefopam has recently been used to separate the modulation of vasoconstriction from shivering (Alfonsi et al., 2004). Unpleasant adverse effects consistent with a central mode of action of the drug have also been reported during therapeutic use and include dizziness, headache, nausea, vomiting and sweating. 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 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. Nefopam is a benzoxazocine and is considered as 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., 2003) which involves the activation of NMDA receptors. However, we have recently shown that in primary cultures of cerebellar neurons nefopam selectively prevents excitotoxicity that follows the activation of voltage sensitive sodium channels (VSSCs) but not the direct application of exogenous glutamate (Fernández-Sánchez et al., 2001). Consistently, we observed that nefopam does not possess a pharmacologically relevant affinity for NMDA receptors (Fernández-Sánchez et al., 2002). Furthermore, the finding that nefopam blocks VSSCs (Fernández-Sánchez et al., 2001) is consistent with the therapeutic properties of this drug, and suggest that other voltage sensitive ion channels may also be the target of nefopam action. Calcium influx plays a crucial role in transducing neuronal depolarization into activities controlled by excitation in various cellular processes, including glutamate release and excitotoxicity. In this study we specifically tested the action of nefopam on calcium influx, cGMP formation, and NMDA receptor-dependent neurotoxicity induced by the activation of voltage sensitive calcium channels (VSCC) in cultured cerebellar neurons. This experimental system has proved very useful in the study of the biochemical events coupled to excitatory aminoacid neurotransmission, and the conditions controlling excitotoxicity (McCaslin and Morgan, 1987; Nicoletti et al., 1986; Novelli and Henneberry, 1987; Novelli et al., 1987; Novelli et al., 1988; Novelli et al., 1992; Novelli et al., 1995; Lipsky et al., 2001). It express VSCC and ionotropic NMDA glutamate receptors coupled to the synthesis of cGMP (Novelli et al., 1987; Fernández-Sánchez and Novelli, 1993), allowing for the study of glutamate-mediated neurotransmission and excitotoxicity (Diáz-Trelles et al., 2002).

We show here that nefopam can fully prevent the occurrence of excitotoxicity by released endogenous excitatory aminoacids following activation of VSCC, but lacks any neuroprotective effect against excitotoxicity induced by exogenous excitatory amino acids.

2 Materials and methods

2.1 Methods

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 2.5×10^5 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. After 8 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 4 days and compensating for lost amounts of water, due to evaporation.

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).

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 (^{125}I). Protein content was determined on the membrane pellet from the same sample.

Confocal microscopy

For intracellular calcium determination neuronal cultures were loaded for 20–30 min. with 5 μM Fluo-3-AM ester in a 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. At the moment of the record the dye was removed and the indicated drugs were added. Fluo-3 emission (>515 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 analyzed by NIH Image (version 1.61). Concentrations of intracellular calcium ($[Ca^{2+}]_i$) could be estimated as a function of Fluo-3 intensity (F) using the calibration procedure described previously (Segal and Manor, 1992), and according to the following equation:

$$[Ca^{2+}]_i = K_d(F - F_{min})/(F_{max} - F)$$

where the dissociation constant K_d has been estimated at 400 nM for Fluo-3 at vertebrate ionic strength (Kao et al., 1989), and the mean values obtained for the camera signal F_{min} and for the maximum fluorescence F_{max} were 6 and 230 respectively.

Data presentation and analysis

For statistical analysis a one-way or a 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.

2.2 Materials

Fluo-3 AM (F1241) was from Molecular Probes. 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. (±)-methyl-1,4-dihydro-2,6-dimethyl-3-nitro-4-(2-trifluoromethylphenyl)-pyridine (BayK8644) was from Research Biochemicals International. Domoic acid was from Diagnostic Chemicals Ltd. (Charlottetown, PEI).

3 Results

Activation of VSCC by exposure of cultures to the specific L-type VSCC agonist BayK8644 (2 μM) for 30 min. resulted in neuronal swelling and darkening compared to the control (Fig. 1A and B), and after 24 h in a significant reduction in surviving neurons (Fig. 1C). These signs of toxicity were similar to those elicited by exposure of cultures to toxic concentrations of exogenous glutamate (Novelli et al., 1988), and toxicity to BayK8644 both at 30 min. and 24 h, was completely abolished by the specific NMDA receptor antagonist MK-801 (2 μM) (Fig. 1D and Table 1). Exposure of neurons to 100 μM nefopam 15 min. before VSCC stimulation with BayK8644 completely prevented both neuronal swelling and darkening, and neuronal death (Table 1). Nefopam protection from toxicity by BayK8644 was concentration dependent (Fig. 2). Thus, nefopam at 47 μM resulted in a 50% protection, while full neuroprotection was achieved at 100 μM nefopam.

In order to test the possibility that nefopam may block calcium influx via L-type VSCCs, we performed calcium imaging experiments in which cells loaded with the fluorescent dye Fluo-3 were stimulated with BayK8644, and the cellular fluorescence intensity was visualized by confocal laser microscopy (see Fig. 3A, B). In these experiments the specific NMDA antagonist MK-801 was always present to avoid calcium influx via NMDA receptor channels. Neurons were maintained in a physiologic solution containing 5 mM KCl (see Methods), and 50 mM KCl was added together with BayK8644 to allow the action of this agonist on VSCCs. Application of BayK8644 resulted in a rapid and strong rise in fluorescence intensity in most cell bodies and neurites (Fig. 3A, B). In our experimental system, the increase in fluorescence intensity produced by BayK8644 has previously been shown to be long lasting

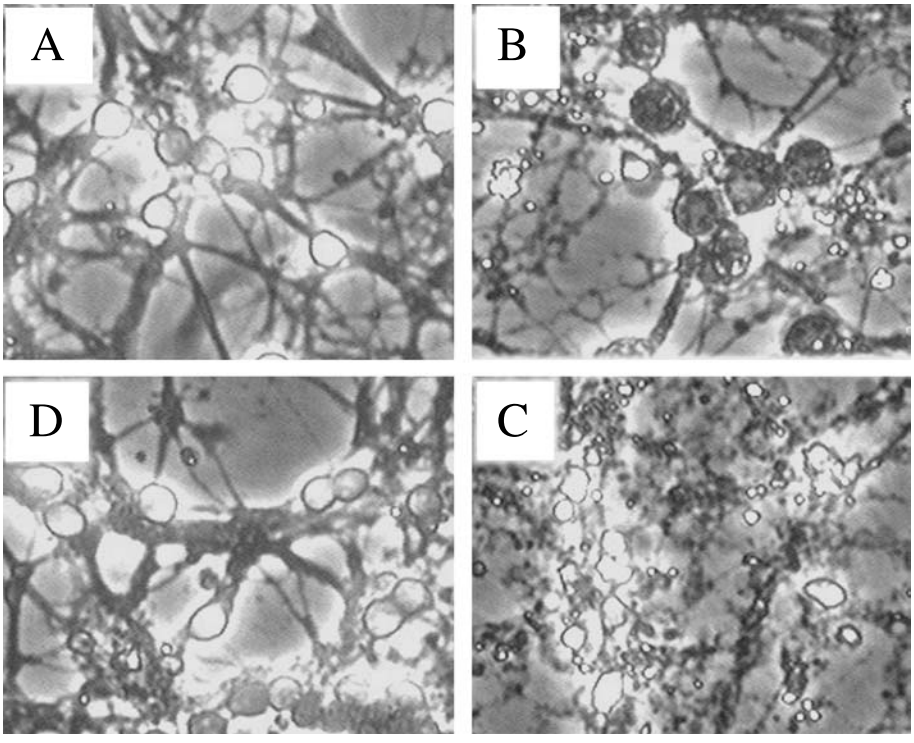

Fig. 1. Phase-contrast microscopy of cerebellar cultures exposed to BayK8644. Neuronal cultures were untreated (**A**) or exposed to 2 μM BayK8644 either in the absence (**B**, **C**) or in the presence of 2 μM MK-801 (**D**). Cultures were observed for neurotoxicity both at 30 min and 24 h and representative images are shown in (**B**) and (**C**) respectively. Cultures that were untreated (**A**) or received BayK8644 in the presence of MK801 (**D**) did not change their morphology throughout the experiment. Images are from one experiment that has been repeated more than 3 times with similar results

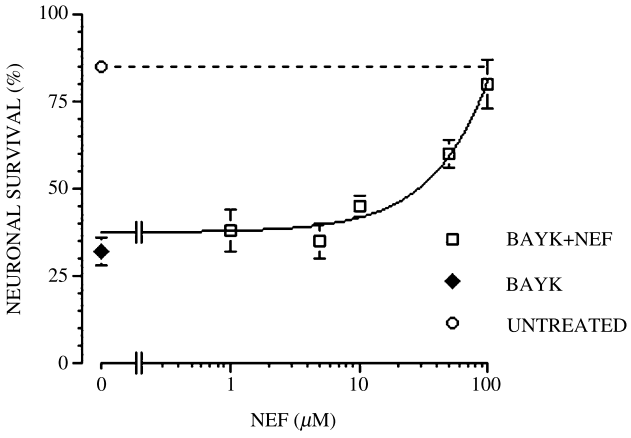

Fig. 2. Concentration-dependent protection by nefopam against neurodegeneration induced by BayK8644. Neuronal cultures were pretreated with the indicated concentrations of nefopam (*NEF*) for 15 min. before the addition of 2 μM BayK8644 (*BAYK*). All drugs were added to the culture growth medium. Neuronal survival was determined 24 h later as indicated in Methods. Data represent the mean ± SD (n = 5–8)

Table 1. Nefopam prevents both early signs of neurotoxicity and later neurodegeneration following exposure to BayK8644

Additions (μM)			
	None	BAYK (2) × (30 min)	BAYK (2) × (24 h)
None	–	+++	++
MK-801 (2)	–	–	–
NEF (100)	–	–	–

Cerebellar neurons in primary culture were exposed to the indicated drugs. Nefopam and MK-801 were added 5 min. before BayK8644. The presence (+) or the absence (–) of both, signs of early neurotoxicity such as darkening and swelling of cell bodies at 30 min, and neurodegeneration after 24 h as shown in Fig. 1B and C respectively, is indicated

(≥300 sec) (Diáz-Trelles et al., 2002) due to the drug preferential binding and stabilization of channel open states (Hockerman et al., 1997). This allows for the quantification of the effects of potential blockers of VSCCs on the same neurons where BayK8644 increases the intracellular

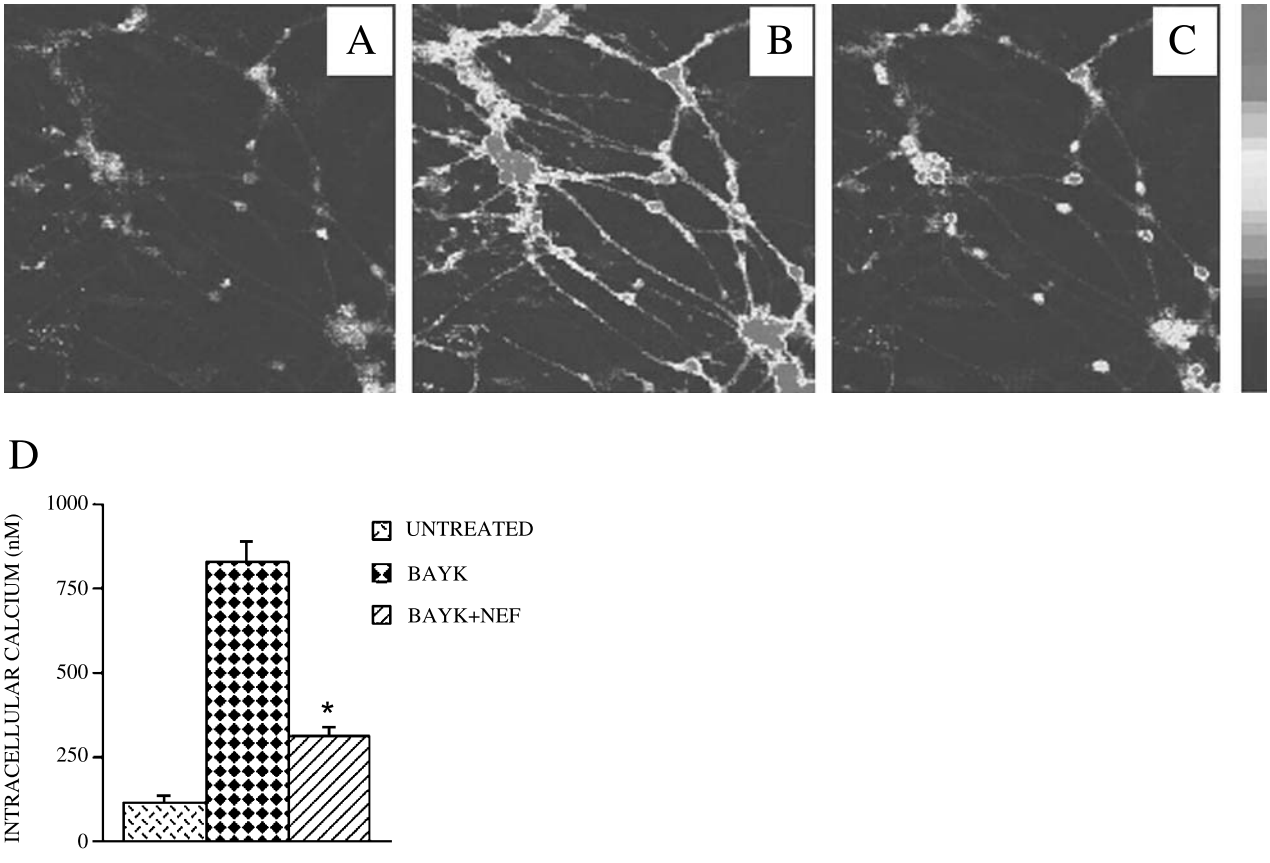

Fig. 3. Effects of nefopam on intracellular calcium following specific stimulation of voltage sensitive calcium channels. Neuronal cultures were loaded for 20–30 min. with 5 μM Fluo-3-AM ester and then examined under a laser confocal microscope. MK801 (2 μM) was always present to avoid both calcium influx via NMDA receptor channels and excitotoxicity. **A–C** Cultures were exposed to 2 μM BayK8644 (BAYK) +50 mM KCl. Nefopam (*NEF*, 100 μM) was added 1 min. later. Representative images were taken before (**A**) or 30 s after (**B**) the addition of BAYK and KCl, and 2 min. after the application of NEF (**C**). Fluorescence intensity scale bar goes from a minimum on the bottom, to a maximum on the top. Experiments were repeated 3 times with similar results. **D** Quantification of intracellular calcium concentration of the neurons shown in the upper panel (see text for details). $^{*}p < 0.0001$ vs. BAYK

calcium concentration. Thus, the application of 100 μM nefopam during BayK8644-induced fluorescence steady-state increase, significantly reduced fluorescence intensity (Fig. 3C). Quantification of the fluorescence intensity in about 12 neurons per field revealed a fluorescence increase (mean $\pm$ s.e.m.) from 57 ± 8 fluorescent units (f.u.) in unstimulated cultures to 156 ± 11 f.u. following stimulation with BayK8644, and this stimulated level was significantly decreased to 104 ± 8 f.u. by nefopam ($p < 0.0001$). By using the calibration procedure described by Segal and Manor, 1992, the intracellular concentration of calcium after stimulation with BayK8644 was estimated to increase from 118 ± 17 nM to 830 ± 62 nM, while after the addition of nefopam intracellular calcium was estimated to be approximately 312 ± 25 nM (Fig. 3D).

To investigate how neuroprotective concentrations of nefopam would affect second messenger formation following the specific activation of VSCC, we determined the effect of nefopam on the intracellular formation of cGMP upon stimulation of VSCC. In order to properly compare the effects of nefopam on both neuroprotection and intracellular signaling, we determined the effect of nefopam on cGMP formation in cultures kept in their growth medium where the presence of 25 mM KCl guaranteed a trophic influx of calcium (Gallo et al., 1987; Novelli et al., 1995; Fernández-Sánchez et al., 1996). As shown in Fig. 4, stimulation of cultures with BayK8644 significantly increased the intracellular concentration of cGMP, and such elevation could be prevented by nefopam in a dose-dependent manner. Thus, 58 μM nefopam reduced cGMP formation elicited by BayK8644 by 50%, while 100 μM nefopam fully prevented it. Since VSCC activation by BayK8644 may induce the release of toxicologically relevant amounts of endogenous NMDA receptor agonists as shown in Fig. 1, we asked whether nefopam was reducing the glutamate receptor-mediated contribution to cGMP formation upon stimulation of VSCC.

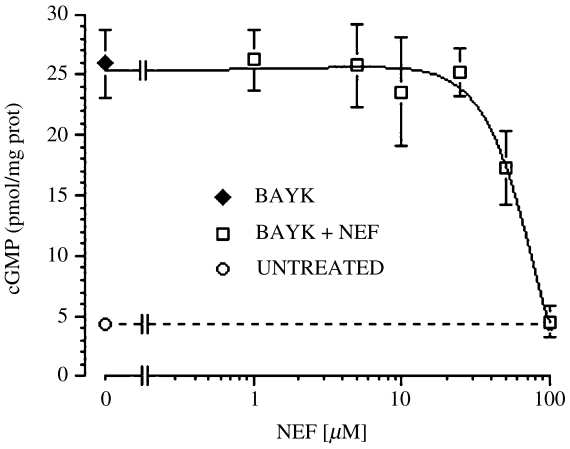

Fig. 4. Nefopam inhibited in a concentration-dependent manner intracellular cGMP formation following specific activation of voltage sensitive calcium channels. Intracellular concentration of cGMP was measured in cerebellar culture at 12–14 DIC following 1 min. stimulation with 2 μM BayK8644 (*BAYK*) in the growth medium, in the absence (filled square symbol) or in the presence of the indicated concentrations of nefopam (*NEF*). NEF was added 10 min. prior to BayK8644. cGMP content was determined by radioimmunoassay. Dashed line represents intracellular cGMP formation in unstimulated cultures. Represented values are the mean $\pm$ SD of duplicate values from three independent experiments

As shown in Fig. 5, the increase in cGMP synthesis induced by BayK8644 was reduced by MK-801 by approximately 27%. However, nefopam (100 μM) did not reduce cGMP synthesis elicited by NMDA, while significantly reduced cGMP synthesis elicited by the ionotropic Non-NMDA receptor agonist domoic acid (Dingledine

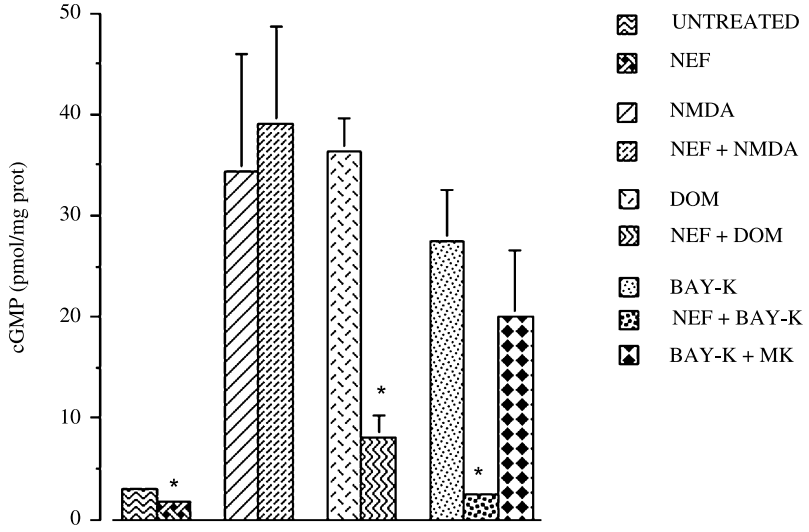

Fig. 5. Pharmacological characterization of cGMP formation following stimulation of voltage sensitive calcium channels. Intracellular cGMP formation was measured after exposure of cultures to the indicated drugs. Nefopam (NEF) and MK801 were added 10 min. before BayK8644 (*BAYK*), and cultures were exposed to BAYK for 1 min. Concentrations were: BAYK, 2 μM; MK801, 2 μM; NEF, 100 μM, NMDA 100 μM, Domoic acid (DOM) 10 μM. Represented data are the mean $\pm$ SD of duplicate values from three independent experiments. $^{\star}p < 0.01$ vs. Same treatment in the absence of NEF

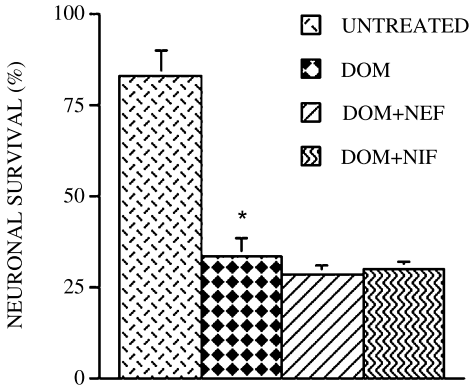

Fig. 6. Nefopam effect on excitatory amino acid receptor-mediated neurodegeneration. Cultures were exposed to 10 μM domoic acid (*DOM*) either in the absence or in the presence of 100 μM nefopam (*NEF*) or 10 μM nifedipine (*NIF*). Both NEF and NIF were added 5 min. before DOM. All drugs were added to the growth medium. Neuronal survival was measured after 24 h. $^*p < 0.001$ vs. untreated

et al., 1999). Culture exposure to AMPA did not result in any stimulation of cGMP synthesis (data not shown). Culture exposure to nefopam alone resulted in a small but significant reduction of cGMP basal levels.

In order to determine whether nefopam reduction of cGMP formation induced by domoic acid was toxicologically relevant due to either, a direct antagonistic action of nefopam at the ionotropic Non-NMDA receptors, or a reduced formation of nitric oxide (NO) (Novelli et al., 2004), we exposed neuronal cultures to domoic acid (10 μM), both in the presence and in the absence of either nefopam (100 μM) or nifedipine (10 μM), a blocker of L-type VSCCs (Janis et al., 1987). In order to avoid any contribution of NMDA receptors to domoic acid toxicity, MK-801 (2 μM) was also present. As shown in Fig. 6, domoic acid produced a significant reduction in neuronal survival after 24 h, that was not ameliorated by the presence of either nefopam or nifedipine.

4 Discussion

We have previously demonstrated that nefopam reduced sodium influx and protected cultured cerebellar neurons from NMDA-receptor dependent excitotoxicity following sodium channel activation (Fernández-Sánchez et al., 2001). In this study we show for the first time that nefopam may also reduce the neuronal response to the specific activation of L-type VSCC, as determined by measuring intracellular calcium concentration, cGMP synthesis and neuronal survival. The permanent activation of VSCC by BayK8644 has recently been shown to be capable of reducing neuronal survival via an NMDA receptor-dependent excitotoxic process involving the release of endogenous NMDA receptor agonists (Diáz-Trelles et al., 2002). Thus, neurotoxicity by BayK8644 is completely prevented by the NMDA receptor antagonist MK-801, which does not prevent the elevation of intracellular calcium concentration via VSCC (Diáz-Trelles et al., 2002, Figs. 1D and 3). On the other hand, nefopam did not reduce NMDA receptor-mediated excitotoxicity due to exogenous glutamate exposure (Fernández-Sánchez et al., 2001), further suggesting a role for BayK8644 activated VSCC in the release of endogenous NMDA receptor agonists. Several investigations using dihydropyridines have led to the idea that L-type calcium channels make only a minor contribution to neurotransmitter release in the brain (Middlemiss and Spedding, 1985; Miller, 1987; Jensen et al., 1999). However, our results presented herein confirm previous evidence (Fernández-Sánchez and Novelli, 1993) and show that the amount of released glutamate due to specific activation of L-VSCC by BayK8644 is sufficient to activate NMDA receptors and promote NMDA receptor-dependent excitotoxicity (Fig. 1). A possible explanation for this discrepancy may be that the significance of each VSCC type in controlling transmitter release differs among types of neurons and synapses. Indeed, a differential contribution of presynaptic P- and N-type channels in neurotransmitter release has been demonstrated in hyppocampal, cerebellar, and spinal cord slices, respectively (Takahashi and Momiyama, 1993). As for cerebellar granule neurons, pharmacological studies have demonstrated that glutamate release in response to depolarization with KCl involves calcium currents that are sensitive to nifedipine, omega-agatoxin-IVA, and also omega-conotoxin-MVIIC, suggesting that L, N, and P channels act in concert in the neurotransmitter release process (Graham and Burgoyne, 1995; Varming et al., 1997). Although it is possible that N-and P/Q-type VSCC could also be affected by nefopam, several observations argue against a major contribution of VSCC other than L-channels: 1) nefopam reduced by 73% the sustained increase in intracellular calcium concentration due to the specific activation of L-type VSCC by BayK864 (Fig. 3). Because BayK8644 is applied to neurons under depolarizing conditions (50 mM KCl) in order to promote the opening of VSCCs, it is likely that the 27% increase in intracellular calcium concentration unaffected by nefopam may be due to the contribution of all but L-type VSCCs together with the release of calcium from intracellular stores (Simpson et al., 1993; Berridge, 1998); 2) neither N nor P/Q VSCC appear to be abundant in cerebellar neurons maintained in a culture medium containing 25 mM KCl

(Forti et al., 1994); 3) nefopam fully prevented excitotoxicity (Fig. 2), suggesting that the amount of calcium influx that can be blocked by nefopam is necessary for the release of endogenous excitotoxins.

Our results also demonstrate that cGMP formation following the specific activation of VSCCs could be effectively reduced by nefopam (Figs. 3 and 4). Nefopam reduction of the intracellular calcium increase induced by BayK8644 was associated with a reduction of the synthesis of cGMP, although it is worth noting that the decrease in intracellular calcium (73%) was smaller than the inhibition of cGMP formation (100%). The reason for this difference is unclear. Nefopam had no inhibitory effects on either the synthesis of cGMP in response to NMDA (Fig. 5), or by ionomycin (data not shown). Consequently, there is not a direct inhibitory effect of nefopam on any component of the cGMP synthetic pathway. On the other hand, it should be considered that the addition of 50 mM KCl together with BayK8644 for the confocal microscopy experiments, lead to the activation of VSCC other than L-types that may possibly not be either physically coupled or closely associated to the PSD-95/nitric oxide synthase complex (Brenman et al., 1996; Choi et al., 2002; Moss et al., 2003). Such channels would therefore contribute to increased intracellular calcium but not cGMP concentrations. Calcium released from intracellular stores following depolarization may also be of little importance for the synthesis of cGMP, since metabotropic glutamate receptor stimulation has been shown not to be associated to it (Nicoletti et al., 1986; Novelli et al., 1987).

It is worth noting that neuroprotection from BayK8644-induced excitotoxicity was concentration dependent and was coincident with a significant reduction in the intracellular formation of cGMP (see Figs. 2 and 4). Thus, approximately 50 μM nefopam significantly reduced by 50% both BayK8644-induced excitotoxicity and cGMP formation, and 100 μM nefopam provided both full neuroprotection and a 100% reduction of cGMP stimulation. While no correlation between BayK8644 neurotoxicity and cGMP increase can be easily established because full prevention of BayK8644 neurotoxicity by MK801 reduced cGMP formation only by 27% (Fig. 5), the similarities in the concentration-dependency of nefopam inhibition of both neurotoxicity and cGMP synthesis, suggest that this drug may act with the same affinity on all L-type VSCCs independently of their role in either neurotransmitter release or nitric oxide synthase stimulation.

The reduction of cGMP formation by nefopam following exposure to domoic acid further suggests that this drug is acting on L-type VSCCs. In fact, most of domoic acid-induced intracellular calcium and cGMP increase is due to the activation of dihydropiridine-sensitive VSCCs (Fernández-Sánchez and Novelli, 1996, and Fig. 5). Such activation of L-type VSCCs may be responsible for the early (30 min.) signs of excitotoxicity by domoic acid and other ionotropic Non-NMDA receptor agonists such as kainic acid (Dingledine et al., 1999). In our experimental system the effects of these drugs can be reduced by the presence of MK801 without altering the extent of neurodegeneration after 24 h (Novelli et al., 1992; Fernández-Sánchez and Novelli, 1996; Tasker et al., 2002). The failure of nefopam to protect against domoic acid neurotoxicity rules out both the possibility that nefopam may act as an antagonist of ionotropic Non-NMDA receptors, and that domoic acid toxicity may be due to the formation of NO (Novelli et al., 2004) (Fig. 6). On the other hand, the observation that nefopam effectively reduced NMDA receptor-mediated BayK8644 neurotoxicity, further supports its role as an L-type VSCC blocker.

The recent finding that nefopam blocks VSSCs (Fernández-Sánchez et al., 2001), together with the results we presents here on its block of VSCCs, provide a new perspective on the therapeutic use of nefopam. Nefopam may be useful in neuropathic pain (Biella et al., 2003), similarly to drugs such as carbamazepine and lamotrigine (Finnerup et al., 2002). The usefulness of these two drugs in neuropathic pain as well as in bipolar disorders and epilepsy, has been related to their capability to block voltage dependent sodium and calcium channels (Goldsmith et al., 2003; Okada et al., 2002), a feature that appears to be common to nefopam. Furthermore, the molecular structure of nefopam resembles that of lamotrigine and carbamazepine. Thus, nefopam may share functional and structural properties with other drugs that are used for pathologies other than neuropathic pain, and may be a potential new drug for mood disorders and epilepsy. In addition, although investigations of the application of calcium channel antagonists for the treatment of acute neurodegenerative disorders that are caused by an excessive release of glutamate, such as stroke, have not yet led to the discovery of compounds with efficient neuroprotective activity (Kobayashi and Mori, 1998), it is interesting to note that drugs exerting multiple actions appeared to block the cascades of neuronal death more efficiently than drugs with a single action site (Spedding et al., 1995). For example, an efficient neuroprotective action of flunarizine has been shown in animal models of ischemia (Deshpande and Wieloch, 1986). Flunarizine is a potent non-selective VSCC antagonist which also interacts with Na^+ channels. The same effect has also been reported for other VSCC

antagonists with little channel selectivity (Benham et al., 1993; Goldin et al., 1995; Varming et al., 1996). Furthermore, a diltiazem analog (T-477) with little selectivity among VSCC types, showed neuroprotective activity in a rat stroke model (Ishii et al., 1996) while the more selective mother compound diltiazem did not show neuroprotection (Wauquier et al., 1985).

In conclusion, this study has demonstrated that the analgesic nefopam hydrochloride can reduce calcium influx and effectively prevent intracellular formation of cGMP and neuronal death following the activation of voltage-sensitive calcium channels in cultured cerebellar neurons. Our data are consistent with a presynaptic action of nefopam that may be of interest in reducing the excessive release of endogenous glutamate involved in neurological and neuropsychiatric disorders.

Acknowledgements

We thank Dr. A. Sampedro and Dr. A. M. Nistal from the Image Process Service of the University of Oviedo for the use of the laser confocal microscope. This work was supported by CICYT, Grants SAF98-0171 and HI1998-0188, and by the University of Oviedo, Grant NP-00-530-27, and by the University of Milan, Grant AE 2002.

Note added in proof

After submission of this article, an independent group (Verleye et al., 2004) reported that nefopam blocks VSSCs and modulates glutamatergic transmission ''in vivo'' without binding to ionotropic glutamate receptors. Furthermore, it did show that nefopam can protect against electroshock-induced seizures in rodents. This new data confirm and expand our previous observations (Fernández-Sánchez et al., 2001; Fernández-Sánchez et al., 2002), and provide experimental evidence for the anticonvulsant properties of nefopam we speculated in the discussion.

References

Alfonsi P, Adam F, Passard A, Guignard B, Sessler DI, Chauvin M (2004) Nefopam, a nonsedative benzoxazocine analgesic, selectively reduces the shivering threshold in unanesthetized subjects. Anesthesiology 100: 37–43

Benham CD, Brown TH, Cooper DG, Evans ML, Harries MH, Herdon HJ, Meakin JE, Murkitt KL, Patel SR, Roberts JC, Rothaul AL, Smith SJ, Wood N, Hunter AJ (1993) SB 201823-A, a neuronal Ca^{2+}-antagonist is neuroprotective in two models of cerebral ischaemia. Neuropharmacology 32: 1249–1257

Berridge MJ (1998) Neuronal calcium signaling. Neuron 21: 13–26

Biella GE, Groppetti A, Novelli A, Fernández-Sánchez MT, Manfredi B, Sptgiu ML (2003) Neuronal sensitization and its behavioral correlates in a rat model of neuropathy are prevented by a cyclic analog of orphenadrine. J Neurotrauma 20: 593–601

Brenman JE, Chao DS, Gee SH, McGee AW, Craven SE, Santillano DR, Wu Z, Huang F, Xia H, Peters MF, Froehner SC, Bredt DS (1996) Interaction of nitric oxide synthase with the postsynaptic density protein PSD-95 and alpha1-syntrophin mediated by PDZ domains. Cell 84: 757–767

Choi J, Ko J, Park E, Yoon J, Lim S, Kim E (2002) Phosphorylation of stargazin by protein kinase A regulates its interaction with PSD-95. J Biol Chem 277: 12359–12363

Deshpande JK, Wieloch T (1986) Flunarizine, a Ca^{2+} entry blocker, ameliorates ischemic brain damage in the rat. Anesthesiology 64: 215–224

Diáz-Trelles R, Fernández-Sánchez MT, Marini A, Novelli A (2002) Antihistamine terfenadine inhibits calcium influx, cGMP formation, and NMDA receptor-dependent neurotoxicity folowing activation of voltage sensitive calcium channels. Neurotox Res 4: 15–24

Dingledine R, Borges K, Bowie D, Traynelis SF (1999) The glutamate receptor ion channels. Pharmacol Rev 51: 7–61

Fasmer OB, Berge OG, Jçrgensen HA, Hole K (1987) Antinoceptive effects of (±)-, (+)- and (−) nefopam in mice. J Pharm Pharmacol 39: 508–511

Fernández-Sánchez MT, Novelli A (1993) Basic fibroblast growth factor protects cerebellar neurons in primary culture from NMDA and non-NMDA receptor mediated neurotoxicity. FEBS Lett 335: 124–131

Fernández-Sánchez MT, Novelli A (1996) Characterization of neurotoxicity by the amnesic shellfish toxin domoic acid in cultured neurons. Neurologia 11 S3: 29–39

Fernández MT, Zitko V, Gascón S, Novelli A (1991) The marine toxin okadaic acid is a potent neurotoxin for cultured cerebellar neurons. Life Sci 49: PL157–PL162

Fernández-Sánchez MT, García-Rodríguez A, Díaz-Trelles R, Novelli A (1996) Inhibition of protein phosphatases induces IGF-1 blocked neurotrophin-insensitive neuronal apoptosis. FEBS Letters 398: 106–112

Fernández-Sánchez MT, Díaz-Trelles R, Groppetti A, Manfredi B, Brini AT, Biella G, Sotgiu ML, Novelli A (2001) Novel effect of nefopam preventing cGMP increase, oxygen radical formation and neuronal death induced by veratridine. Neuropharmacology 41: 935–942

Fernández-Sánchez MT, Díaz-Trelles R, Groppetti A, Manfredi B, Brini AT, Biella G, Sotgiu ML, Novelli A (2002) Nefopam, an analogue of orphenadrine, protects against both NMDA receptor-dependent and independent veratridine-induced neurotoxicity. Amino Acids 23: 31–36

Finnerup NB, Gottrup H, Jensen TS (2002) Anticonvulsants in central pain. Expert Opin Pharmacother 3: 1411–1420

Forti L, Tottene A, Moretti A, Pietrobon D (1994) Three novel types of voltage-dependent calcium channels in rat cerebellar neurons. J Neurosci 14: 5243–5256

Gallo V, Kingsbury A, Balázs R, Jørgensen OS (1987) The role of depolarization in the survival and differentiation of cerebellar granule cells in culture. J Neurosci 7: 2203–2213

Gasser JC, Bellville JW (1975) Respiratory effects of nefopam. Clin Pharmacol Ther 18: 175–179

Gerbershagen HU, Schaffner E (1979) Respiratory effect of nefopam. Clin Ther 2 [Suppl B]: 39–42

Goldin SM, Subbarao K, Sharma R, Knapp AG, Fischer JB, Daly D, Durant GJ, Reddy NL, Hu LY, Magar S, Perlman ME, Chen J, Graham SH, Holt WF, Berlove D, Margolin LD (1995) Neuroprotective use-dependent blockers of Na^{+} and Ca^{2+} channels controlling presynaptic release of glutamate. Ann N Y Acad Sci 765: 210–229

Goldsmith DR, Wagstaff AJ, Ibbotson T, Perry CM (2003) Lamotrigine: a review of its use in bipolar disorders. Drugs 63: 2029–2050

Graham ME, Burgoyne RD (1995) Effects of calcium channel antagonists on calcium entry and glutamate release from cultured rat cerebellar granule cells. J Neurochem 65: 2517–2524

Guirimand F, Dupont X, Bouhassira D, Brasseur L, Chauvin M (1999) Nefopam strongly depresses the nociceptive flexion (R(III)) reflex in humans. Pain 80: 399–404

Heel RC, Brogden RN, Pakes GE, Speight TM, Avery GS (1980) Nefopam: a review of its pharmacological properties and therapeutic efficacy. Drugs 19: 249–267

Hockerman GH, Peterson BZ, Johnson BD, Catterall WA (1997) Molecular determinants of drug binding and action on L-type calcium channels. Annual Rev Pharmacol Toxicol 37: 361–396

Hunskaar S, Fasmer OB, Broch OJ, Hole K (1987) Involvement of central serotonergic pathways in nefopam-induced antinoception. Eur J Pharmacol 138: 77–82

Ishii T, Okamoto T, Kume T, Narita H, Kudo Y, Matsuoka Y (1996) T-477, a novel neuronal Ca^{2+} blocker, reduces infarct volume following middle cerebral artery occlusion in rats. Japanese J Pharmacol 71: 175P

Janis DA, Silver P, Triggle DJ (1987) Drug action in cellular calcium regulation. Adv Drug Res 16: 309–591

Jensen K, Jensen MS, Lambert JD (1999) Role of presynaptic L-type Ca^{2+} channels in GABAergic synaptic transmission in cultured hippocampal neurons. J Neurophysiol 81: 1225–1230

Kao JPY, Harootunian AT, Tsien RY (1989) Photochemically generated cytosolic calcium pulses and their detection by Fluo-3. J Biol Chem 264: 8179–8184

Kobayashi T, Mori Y (1998) Ca^{2+} channel antagonists and neuroprotection from cerebral ischemia. Eur J Pharmacol 363: 1–15

Kornhuber J, Parsons CG, Hartmann S, Retz W, Kamolz S, Thome J, Riederer P (1995) Orphenadrine is an uncompetitive N-methyl-D-aspartate (NMDA) receptor antagonist: binding and patch clamp studies. J Neural Transm 102: 237–246

Lipsky RH, Xu K, Zhu D, Kelly C, Terhakopian A, Novelli A, Marini AM (2001) Nuclear factor k_B is a critical determinant in N-methyl-D-aspartate receptor-mediated neuroprotection. J Neurochem 78: 254–264

McCaslin PP, Morgan WW (1987) Cultured cerebellar cells as an *in vitro* model of excitatory amino acid receptor function. Brain Res 417: 380–384

Middlemiss DN, Spedding M (1985) A functional correlate for the dihydropyridine binding site in rat brain. Nature 314: 94–96

Miller RJ (1987) Multiple calcium channels and neuronal function. Science 235: 46–52

Moss FJ, Dolphin AC, Clare JJ (2003) Human neuronal stargazin-like proteins, gamma2, gamma3 and gamma4; an investigation of their specific localization in human brain and their influence on CaV2.1 voltage-dependent calcium channels expressed in Xenopus oocytes. BMC Neurosci 4: 23

Nicoletti F, Wroblewski JT, Novelli A, Alho H, Guidotti A, Costa E (1986) The activation of inositol phospholipid metabolism as a signal-transducing system for excitatory amino acids in primary cultures of cerebellar granule cells. J Neurosci 6: 1905–1911

Novelli A, Henneberry RC (1987) cGMP synthesis in cultured cerebellar neurons is stimulated by glutamate via a Ca^{2+}-mediated, differentiation-dependent mechanism. Dev Brain Res 34: 307–310

Novelli A, Nicoletti F, Wroblewski JT, Alho H, Costa E, Guidotti A (1987) Excitatory amino acid receptors coupled with guanylate cyclase in primary cultures of cerebellar granule cells. J Neurosci 7: 40–47

Novelli A, Reilly JA, Lypsko PG, Henneberry RC (1988) Glutamate becomes neurotoxic via the NMDA receptor when intracellular energy levels are reduced. Brain Res 451: 205–212

Novelli A, Kispert J, Fernández-Sánchez MT, Torreblanca A, Zitko V (1992) Domoic acid-containing toxic mussels produce neurotoxicity in neuronal cultures through a synergism between excitatory amino acids. Brain Res 577: 41–48

Novelli A, Torreblanca A, Losa-Uría P, Fernández-Sánchez MT (1995) Role of intracellular calcium in neuronal survival to excitatory aminoacid stimulation. Soc Neurosci Abstr 21: 530.13

Novelli A, Perez-Basterrechea M, Fernández-Sánchez MT (2005) Glutamate and neurodegeneration. In: Reith MEA, Schmidt WJ (eds) Dopamine and glutamate in psychiatric disorders. Humana Press Inc., Totowa, NJ, USA (in press)

Okada M, Zhu G, Yoshida S, Kanai K, Hirose S, Kaneko S (2002) Exocytosis mechanism as a new targeting site for mechanisms of action of antiepileptic drugs. Life Sci 72: 465–473

Piper SN, Suttner SW, Schmidt CC, Maleck WH, Kumle B, Boldt J (1999) Nefopam and clonidine in the prevention of postanaesthetic shivering. Anaesthesia 54: 695–699

Rosa G, Pinto G, Orsi P, de Blasi RA, Conti G, Sanita R, La Rosa I, Gasparetto A (1995) Control of post anaesthetic shivering with nefopam hydrochloride in mildly hypothermic patients after neurosurgery. Acta Anaesthesiol Scand 39: 90–95

Segal M, Manor D (1992) Confocal microscopic imaging of $[Ca^{2+}]i$ in cultured rat hippocampal neurons following exposure to N-methyl-D-aspartate. J Physiol 448: 655–676

Simpson PB, Challiss RAJ, Nahorski SR (1993) Involvement of intracellular stores in the Ca^{2+} response to N-methyl-D-aspartate and depolarization in cerebellar granule cells. J Neurochem 61: 760–763

Spedding M, Kenny B, Chatelain P (1995) New drug binding sites in Ca^{2+} channels. Trends Pharmacol Sci 16: 139–142

Takahashi T, Momiyama A (1993) Different types of calcium channels mediate central synaptic transmission. Nature 366: 156–158

Tasker RAR, Bernard PB, Doucette TA, Kerr DS, Zabidin Y, Alvarez-Fernández L, Fernández-Maroto B, Fernández-Sánchez MT, Novelli A (2002) Comparison of the *in vitro* and *in vivo* neurotoxicity of three new sources of kainic acid. Amino Acids 23: 45–54

Varming T, Christophersen P, Moller A, Axelsson A, Nielson EO (1996) Synthesis and biological activity of the neuronal Ca^{2+} blocker 2-amino-1-(2,5-dimethoxyphenyl)-5-trifluoromethyl benaimidazole (NS-649). Biorganic Med Chem Lett 6: 245–248

Varming T, Christopherson P, Schousboe A, Drejer J (1997) Pharmacological characterization of voltage-sensitive calcium channels and neurotransmitter release from mouse cerebellar granule cells in culture. J Neurosci Res 48: 43–52

Verleye M, André N, Heulard I, Gillardin J-M (2004) Nefopam blocks voltage-sensitive sodium channels and modulates glutamatergic transmission in rodents. Brain Res 1013: 249–255

Villier C, Mallaret MP (2002) Nefopam abuse. Ann Pharmacother 36: 1564–1566

Wauquier A, Ashton D, Clincke G, Fransen J (1985) Calcium entry blockers as cerebral protecting agents: comparative activity in tests of hypoxia and hyperexcitability. Japanese J Pharmacol 38: 1–7

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