0026-895X/89/04525-07$02.00/0

MOLECULAR PHARMACOLOGY, 36:525-531

Ca^{2+}-Mediated Neuronal Death in Rat Brain Neuronal Cultures by Veratridine: Protection by Flunarizine

PETRUS J. PAUWELS, HARRIE P. VAN ASSOUW, JOSÉE E. LEYSEN, and PAUL A. J. JANSSEN

Department of Biochemical Pharmacology, Janssen Research Foundation, B-2340 Beerse, Belgium

Received May 2, 1989; Accepted July 10, 1989

SUMMARY

Neuronal cell degeneration was studied *in vitro* in primary rat brain neuronal cultures grown in serum-free, chemically defined, CDM R12 medium, by measuring lactate dehydrogenase (LDH) released in the culture medium. A Ca^{2+}-dependent neuronal cell degeneration was observed after prolonged and transient exposure to 30 μM veratridine. The release of LDH occurred gradually and could be completely prevented by 2 mM ethylene glycol bis (β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid, 0.1 μM tetrodotoxin, and 1 μM flunarizine. Flunarizine was without effect on neuronal cell loss induced by 1 mM glutamate, 1 mM kainic acid, and 5 mM KCN. The lack of effect on neurotoxicity induced by 1 mM glutamate differentiates flunarizine from *N*-methyl-D-aspartate antagonists such as MK-801. The latter protected at nanomolar concentrations against glutamate-induced neuronal cell death but had a maximal effect only at 0.1 mM on the veratridine-induced released LDH. It is suggested that, besides the excitatory amino acid receptor pathway, prolonged opening of the veratridine-sensitive Na^+ channel can be neurotoxic. The latter can be prevented by flunarizine. The role of Na^+ channel blockers as therapeutic agents in cerebral ischemia is discussed.

The biochemical mechanisms underlying neuronal death following cerebral ischemia and/or hypoxia, such as in stroke or cardiac failure, are not yet fully understood. The cytosolic concentration of Ca^{2+} may play a critical role in neuronal death, as has been hypothesized for cellular disintegration mechanisms in general (1, 2). Recently, hypoxic-ischemic neuronal injury has been linked to excessive activation of postsynaptic glutamate receptors, and glutamate neurotoxicity itself has been attributed to a lethal influx of extracellular Ca^{2+} through cell membrane channels (3). Using dissociated cultures of fetal neurons, Choi (4, 5) and Rothman *et al.* (6) showed that such neurons died within 24 h following the administration of glutamate. Whereas the neuronal cells are protected from excitatory amino acid neurotoxicity by the presence of 2 mM levels of the Ca^{2+} chelate EGTA in the medium, Ca^{2+} blockers applied to these cultures show only slight protective effects at the high dose of 10 μM (7). This indicates that Ca^{2+} entry, triggered by excitatory amino acid receptor activation, occurs via routes that are insensitive to the mechanisms of Ca^{2+} entry blockade of these drugs. Nevertheless, the class IV Ca^{2+} entry blocker flunarizine (8) is an effective cerebral protecting agent in models of brain hypoxia, ischemia, or metabolic intoxication, and in experimental models of epilepsy (9). However, it is not clear as to what extent brain protection may be attributed to direct effects on neurons or to indirect effects through an action on cerebral blood vessels. Previous studies showed that flunarizine competes with BTX-B binding to Na^+ channels and veratridine-induced ^{22}Na uptake (IC_{50} values are 0.3 and 0.2 μM, respectively) in rat brain synaptosomal preparations (10). Therefore, the opening of Na^+ channels in serum-free rat brain neuronal cultures by veratridine was investigated as a pathway for neuron-specific degeneration. Selection of variant neuroblastoma clones with missing or altered Na^+ channels has been reported (11-13). An acute, Na^+-dependent, veratridine-induced neuronal cell swelling in hippocampal cultures has been previously reported by Rothman (14).

We now report that prolonged opening of a Na^+ channel by veratridine is neurotoxic for rat brain neuronal cultures. This type of neuronal damage was compared with the damage induced by the excitatory amino acid glutamate. The observed Ca^{2+}-dependent, veratridine-induced, neuronal cell loss could be completely prevented by 1 μM flunarizine. The protective effect of flunarizine was compared with its effect against other types of neurotoxicity and with the effect of the noncompetitive NMDA antagonist MK-801.

Materials and Methods

Serum-free culture of neurons. Cultures of neurons were prepared from the hippocampal formation of 17-day-old rat embryos, as

Some of these results were presented at the Belgian Society for Fundamental and Clinical Physiology and Pharmacology (Antwerp, December 17, 1988).

[1] Unpublished observations.

ABBREVIATIONS: EGTA, ethylene glycol bis(β-amino ethyl ether)-*N,N,N′,N′*-tetraacetic acid; BTX-B, [^3H]batrachotoxin A 20-α-benzoate; LDH, lactate dehydrogenase; NMDA, *N*-methyl-D-aspartate; DMEM, Dulbecco's modified Eagle's medium; HEPES, 4-(2-hydroxyethyl)]-1-piperazineethanesulfonic acid.

described previously (15). Cells were plated in poly-L-lysine-coated (0.001%) 24-well tissue culture plates (Nunc) with 1 ml of DMEM/Ham's F12 (3:1, v/v) plus 10% heat-inactivated horse serum per well, at a density of 7×10^5 cells/cm^2 (well size, 1.76 cm^2). After 24 hr, cultures were switched from serum-containing medium to serum-free, chemically defined, CDM R12 medium (16) based on 25 mM HEPES-buffered DMEM/Ham's F12 (3:1, v/v). Once a week, the culture medium was partly replaced with fresh medium. Viability tests, using the trypan blue exclusion method, yielded viability values >95%. Cultures were maintained at 37° in an air/CO_2 (95/5) water-saturated atmosphere.

Exposure of cultures to veratridine, excitatory amino acids, and KCN. Seven-day-old cultures were washed with serum-free medium and incubated with 0.2 ml of serum-free, chemically defined, DMEM medium in the presence of veratridine, KCN, kainic acid, and glutamate. Concentrations and exposure times are mentioned in the legends of figures and tables. Nutrient mixture Ham's F12 was excluded from CDM R12, because it contains L-aspartate and L-glutamate. Unless otherwise indicated, exposure was terminated after 16 hr by removal of the incubation medium. Media and cells were assayed for LDH activity.

LDH assay. LDH activity was measured by following NADH oxidation at 340 nm, as described previously (15, 17), using an automatic EPOS-5060 (Eppendorf, Hamburg) linked to a Macintosh SE personal computer. Extracellular LDH activity was defined as the LDH activity measured in the incubation medium. Intracellular LDH activity was measured after cell lysis by the addition of 1.0 ml of water and mechanical rupturing by repetitive suction through a Pasteur pipette. Total LDH activity was defined as the sum of intracellular and extracellular LDH activity. Released LDH was defined as the percentage of extracellular to total LDH activity. Specifically released LDH was obtained by subtraction of released LDH measured in cultures not exposed to a trigger. Concentration-response curves for released LDH by veratridine, glutamate, kainic acid, and KCN were presented as the percentage of specifically released LDH.

Treatment of cultures with flunarizine, MK-801, and tetrodotoxin. Cultures were pretreated for 30 min with tetrodotoxin or drug. Tetrodotoxin was dissolved in DMEM, whereas flunarizine and MK-801 were added as 10 μl of a 100-fold concentrated drug solution, in 10% hydroxypropyl-β-cyclodextrin, to the 1.0 ml of serum-free, chemically defined, CDM R12 medium. After 30 min, the cultures were washed with serum-free medium and incubated with 0.2 ml of serum-free, chemically defined DMEM in the absence or presence of veratridine, KCN, kainic acid, and glutamate, as described above, in the absence or presence of drug. The final concentration of hydroxypropyl-β-cyclodextrin in the incubation medium was 0.1%. In some experiments, flunarizine was added as 10 μl of a 100-fold concentrated drug solution in 10% ethanol. The released LDH in the presence of drug was calculated as the percentage of specifically released LDH in the absence of drug. IC_{50} values (concentration inhibiting 50% of specifically released LDH) were derived graphically.

Materials. The serum-free, chemically defined, CDM R12 medium was prepared every 6 weeks. DMEM, nutrient mixture Ham's F12, serum, and 24-well tissue culture plates were obtained from Gibco/Biocult Laboratories, Scotland. Tetrodotoxin was obtained from Janssen Biochimica (Beerse, Belgium). Veratridine and poly-L-lysine (molecular weight, >70,000) were purchased from Sigma Chemical Co. (St. Louis, MO). Serial dilutions of flunarizine (Janssen Pharmaceutica, Beerse, Belgium) and MK-801 (Merck Sharp & Dohme, Rahway, NJ) were made in 10% hydroxypropyl-β-cyclodextrin or 10% ethanol. Hydroxypropyl-β-cyclodextrin was obtained from Janssen Biotech (Olen, Belgium).

Results

Neuronal degeneration induced by veratridine. Exposure of 7-day-old neuronal cultures to 30 μM veratridine was followed by progressive neuronal degeneration. Fig. 1 shows that the neuronal network and cell bodies were hard to distinguish after 16-hr exposure to 30 μM veratridine. For quantitative assessment of the neuronal damage, the efflux into the culture medium of the cytosolic LDH was measured. Fig. 2a shows that LDH released in the medium over 16 hr in control cultures constituted only 6% of the total LDH activity; it reached 20% in cultures exposed for 16 hr to 30 μM veratridine.

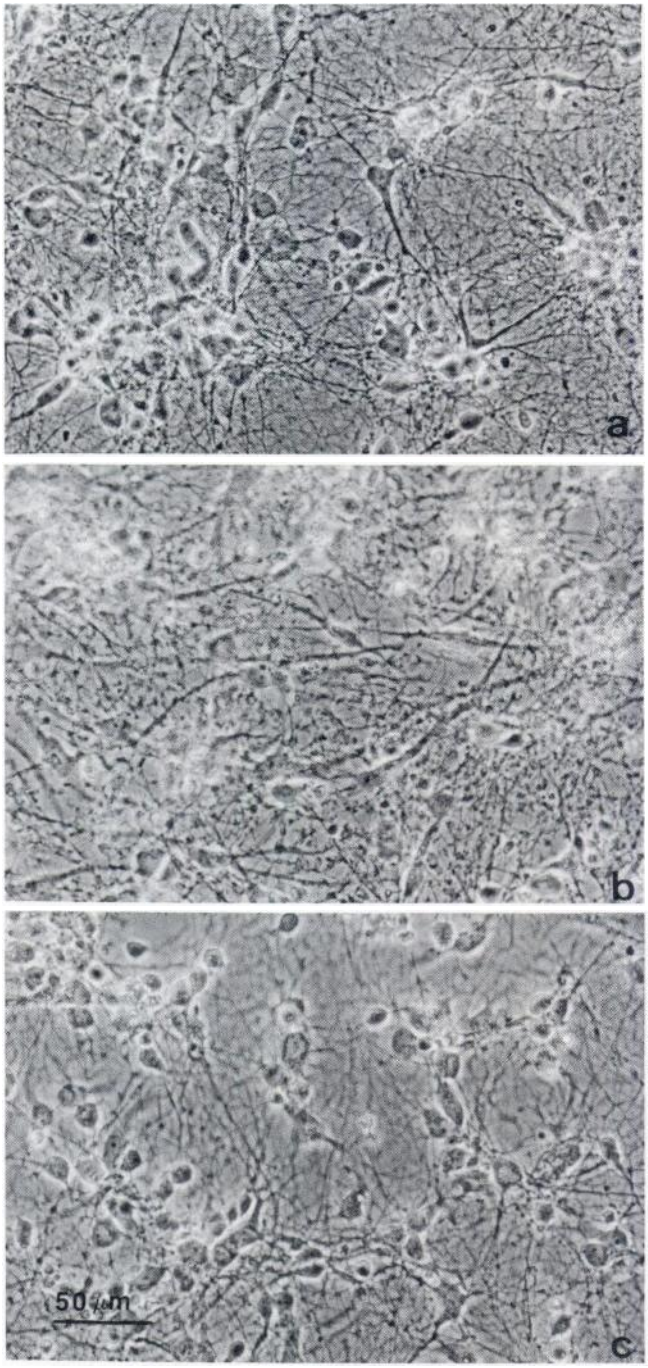

Fig. 1. Representative phase-contrast micrographs of 7-day-old neuronal cultures exposed for 16 hr to 0.1% cyclodextrine, 30 μM veratridine in 0.1% cyclodextrine, and 30 μM veratridine in the presence of 1 μM flunarizine in 0.1% cyclodextrine. After 16 hr in the presence of 30 μM veratridine, neuronal cell bodies were replaced by debris and the neuronal network was hard to distinguish (b), whereas in the presence of 1 μM flunarizine (c) cultures were not different from control (a). *Bar* = 50 μm.

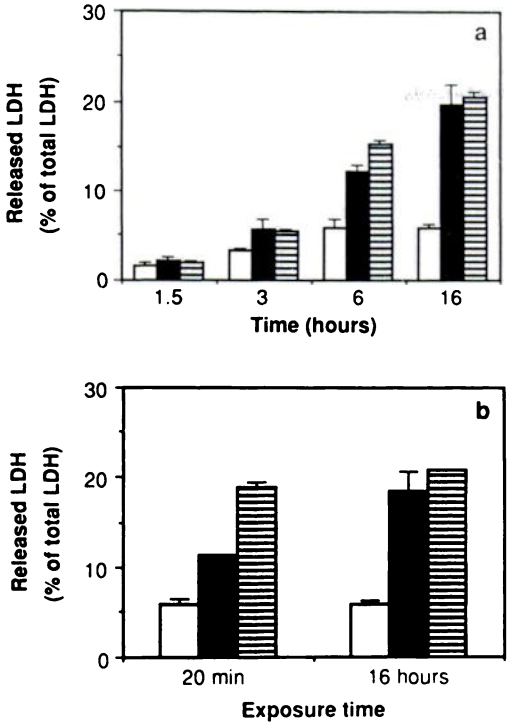

Fig. 2. Effect of veratridine and glutamate exposure time on released LDH in neuronal cultures. a, Seven-day-old cultures were incubated with 0.2 ml of serum-free, chemically defined, DMEM in the absence or presence of 30 μM veratridine or 1 mM glutamate. At the times indicated, intracellular and extracellular LDH activity was measured as described in Materials and Methods. Results are expressed as the ratio of extracellular to total (extracellular plus intracellular) LDH activity. A representative experiment is shown; all values are the mean ± standard deviation of three cultures. b, Seven-day-old cultures were exposed for 20 min and 16 h, respectively, to 30 μM veratridine or 1 mM glutamate. In both cases, intracellular and extracellular LDH activity was measured 16 hr after the beginning of the trigger exposure. Results are expressed as the ratio of extracellular to total LDH activity. *Bars* were constructed using mean values ± standard deviations of two separate experiments in triplicate. □, control; ■, 30 μM veratridine; ■, 1 mM glutamate.

Fig. 2a also indicates that the first signs of veratridine-induced damage were already apparent after 3 hr. Thereafter, LDH release increased gradually with time. A similar time course and total amount of LDH release were obtained with prolonged exposure (up to 16 hr) to 1 mM glutamate (see Fig. 2a). Released LDH was greater after 20-min exposure to 1 mM glutamate than to 30 μM veratridine, although 16-hr exposure to both agents caused the same effect. Fig. 2b shows that, after 20-min exposure to 30 μM veratridine or 1 mM glutamate, released LDH reached, within an interval of 16 hr, 11 and 19%, respectively.

Prevention of neuronal degeneration induced by veratridine. In the presence of 2 mM EGTA, 0.1 μM tetrodotoxin, or 1 μM flunarizine, 16-hr exposure to 30 μM veratridine failed to produce neuronal degeneration. The neuronal network and neuronal cell bodies were preserved; the protective effect of 1 μM flunarizine on neurons exposed for 16 hr to 30 μM veratridine is shown in Fig. 1c. In accordance with the morphological integrity, no specific release of LDH occurred in response to veratridine. During the incubation with 30 μM veratridine EGTA (2 mM) lowered released LDH from 19% to the control value of 6% (see Table 1). Concentration-response curves for LDH released by veratridine in control cultures and in the presence of various concentrations of tetrodotoxin and flunarizine are shown in Fig. 3a. Half-maximal neurotoxicity with veratridine in control cultures was obtained at 12 ± 2 μM (Table 2). Tetrodotoxin and flunarizine shifted the dose-response curve of released LDH to the right; the concentrations of veratridine producing half-maximal neurotoxicity in the presence of 0.1 μM tetrodotoxin and 1 μM flunarizine increased to more than 100 μM. The protective effect of flunarizine was greatly affected by the solvent, as shown in Fig. 3a. One micromolar flunarizine that was prepared from a 100-fold concentrated solution in 10% cyclodextrin was fully protective. In contrast, 1 μM flunarizine that was prepared from a 100-fold concentrated solution in 10% ethanol was only partially protective. Therefore, further experiments with flunarizine were performed with cyclodextrin at a final concentration of 0.1%. At this concentration, cyclodextrin did not influence the released LDH values under the different test conditions (Table 1). The dose-response curves of tetrodotoxin and flunarizine for inhibition of released LDH induced by 30 μM veratridine are shown in Fig. 3b. Released LDH induced by 30 μM veratridine was inhibited by 50% at 22 ± 14 nM tetrodotoxin. IC_{50} values of flunarizine at a final concentration of 0.1% cyclodextrin and 0.1% ethanol were 0.12 ± 0.04 and 0.40 ± 0.10 μM, respectively (Table 3).

Specificity of the protective effect of flunarizine. The protective effect of flunarizine on neurons exposed to veratridine was compared with its effect against neuronal damage induced by 1 mM glutamate, 1 mM kainic acid, and 5 mM KCN. Data on released LDH are shown in Figs. 4 and 5. The corresponding concentrations producing half-maximal neurotoxicity and IC_{50} values are summarized in Tables 2 and 3, respectively. Flunarizine did not prevent the neuronal damage induced by various concentrations of glutamate, kainic acid, or KCN. The IC_{50} values with flunarizine for released LDH obtained with 1 mM glutamate, 1 mM kainic acid, and 5 mM KCN were >10 μM. Tetrodotoxin, which was tested between 0.01 and 1 μM, was also without any effect on released LDH induced by these triggers. By contrast, the released LDH values in the presence of the Ca^{2+} chelate EGTA were not different from the released LDH values of control cultures (Table 1).

MK-801 was fully protective against neuronal damage induced by 1 mM glutamate and 5 mM KCN. Fig. 4 shows that released LDH induced by various concentrations of these triggers was completely absent at 1 μM MK-801. Fig. 5 shows the dose-response curves of MK-801. Half-maximal protection against damage induced by 1 mM glutamate and 5 mM KCN was obtained at 13 ± 11 and 53 ± 4 nM, respectively (Table 3). A shift to the right in the concentration-response curve for LDH released by kainic acid was noted with 1 μM MK-801 (Fig. 4). However, the dose-response of MK-801 in Fig. 5 indicates that the IC_{50} value at 1 mM kainic acid was >10 μM. The concentration of veratridine producing half-maximal neurotoxicity shifted in the presence of 1 and 10 μM MK-801 from 12 to 50 and 80 μM, respectively (Fig. 6a). The dose-response curve of MK-801 was biphasic (Fig. 6b). Fifty percent of released LDH was inhibited between 3 and 30 nM. Subsequently, a plateau was noted up to 3 μM, whereas at 100 μM released LDH was inhibited 92%.

Discussion

Neuronal degeneration induced by veratridine. Neuronal cultures degenerated following exposure to veratridine.

TABLE 1

Released LDH values in neuronal cultures following 16-hr exposure to veratridine, glutamate, kainic acid, or KCN in the absence or presence of 0.1% cyclodextrine, 0.1% ethanol, and 2 mM EGTA

Seven-day-old cultures were incubated as described in the legend to Fig. 2. After 16-hr exposure, intracellular and extracellular LDH activity was measured as described in Materials and Methods. Results are expressed as the ratio of extracellular to total LDH activity. All values are the mean ± standard deviation of three separate experiments performed in triplicate.

Condition/trigger	Released LDH				
	Nontreated	30 μM Veratridine	1 mM Glutamate	1 mM Kainic acid	5 mM KCN
	% of total LDH				
Control	6.0 ± 1.8	19.4 ± 2.5	24.9 ± 5.2	24.8 ± 2.2	25.2 ± 5.4
0.1% Ethanol	5.8 ± 2.7	18.8 ± 0.8	24.3 ± 5.4	23.7 ± 3.8	24.6 ± 8.0
0.1% Cyclodextrine	5.4 ± 1.1	17.2 ± 4.3	23.5 ± 5.6	23.5 ± 2.4	27.3 ± 1.3
2 mM EGTA	6.1 ± 0.1	6.8 ± 0.4	7.9 ± 3.2	5.3 ± 1.2	5.8 ± 1.0

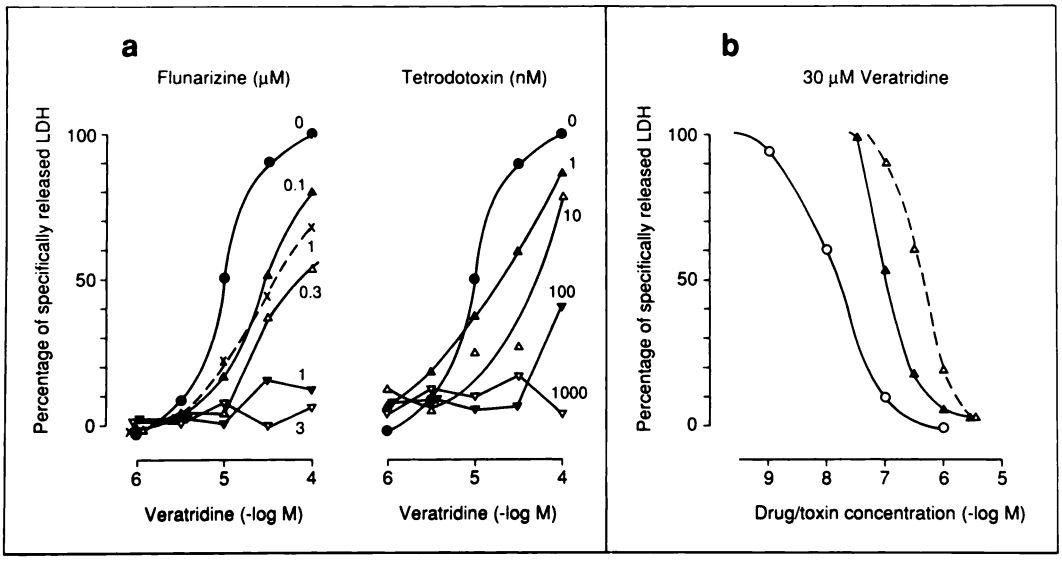

Fig. 3. The effect of flunarizine and tetrodotoxin on neurotoxicity induced by veratridine. a, Concentration-response curves for released LDH by veratridine in control neuronal cultures and in the presence of flunarizine (0.1% cyclodextrine) and tetrodotoxin. Released LDH was measured after 16-hr exposure. All LDH values were corrected by subtraction of released LDH in control cultures (cultures not exposed to veratridine) and expressed as the percentage of specifically released LDH obtained with 100 μM veratridine. *Curves* were constructed using mean values of three separate experiments performed in triplicate. The response curve by veratridine in the presence of 1 μM flunarizine prepared from a 100-fold concentrated solution in 10% ethanol is shown by the *dotted line*. b, Dose-response curves of flunarizine and tetrodotoxin on inhibition of released LDH induced by 30 μM veratridine. Released LDH was measured after 16-hr exposure. LDH values were expressed as the percentage of specifically released LDH in the absence of flunarizine or tetrodotoxin. *Curves* were constructed using mean values of three to six separate experiments in triplicate. ○, Tetrodotoxin; ▲, flunarizine (0.1% cyclodextrine); △, flunarizine (0.1% ethanol).

TABLE 2

Concentrations of veratridine, glutamate, kainic acid, and KCN producing half-maximal neurotoxicity in the absence and presence of 1 μM flunarizine, 1 μM tetrodotoxin, or 1 μM MK-801

Concentration-response curves for released LDH by veratridine, glutamate, kainic acid, and KCN were obtained as described in the legend to Figs. 3a and 4. Concentrations producing half-maximal neurotoxicity were derived graphically. All values are the mean ± standard deviation. The number of independent experiments performed in triplicate is given in parentheses.

Agent/trigger	Trigger concentrations producing half-maximal neurotoxicity			
	Veratridine	Glutamate	Kainic acid	KCN
	μM			
None	12 ± 2 (11)	61 ± 18 (11)	67 ± 21 (6)	610 ± 220 (6)
0.1% Cyclodextrine				
1 μM Flunarizine				
0.1% Cyclodextrine	>100 (12)	140 ± 46 (5)	150 ± 42 (5)	711 ± 112 (2)
0.1% Ethanol	48 ± 36 (3)			
1 μM Tetrodotoxin				
0.1% Cyclodextrine	>100 (5)	75 ± 17 (3)	110 ± 27 (4)	825 ± 163 (2)
1 μM MK-801				
0.1% Cyclodextrine	40 ± 20 (3)	>10,000 (4)	290 ± 67 (5)	>10,000 (3)

TABLE 3

Potencies of flunarizine, tetrodotoxin, and MK-801 to prevent neurotoxicity induced by veratridine, glutamate, kainic acid, and KCN

Inhibition of released LDH induced by 30 μM veratridine, 1 mM glutamate, 1 mM kainic acid, and 5 mM KCN was measured as described in the legend to Figs. 3b and 5. IC_{50} values were derived graphically. All values are the mean ± standard deviation. The number of independent experiments performed in triplicate is given in parentheses.

Agent/trigger	IC_{50}			
	30 μM Veratridine	1 mM Glutamate	1 mM Kainic acid	5 mM KCN
	nM			
Flunarizine				
0.1% Cyclodextrine	122 ± 35 (5)	>10,000 (3)	>10,000 (2)	>10,000 (3)
0.1% Ethanol	400 ± 100 (3)			
Tetrodotoxin				
0.1% Cyclodextrine	22 ± 14 (6)	>1000 (3)	>10,000 (2)	>1000 (3)
MK-801				
0.1% Cyclodextrine	Biphasic	13 ± 11 (5)	>10,000 (2)	53 ± 4 (2)

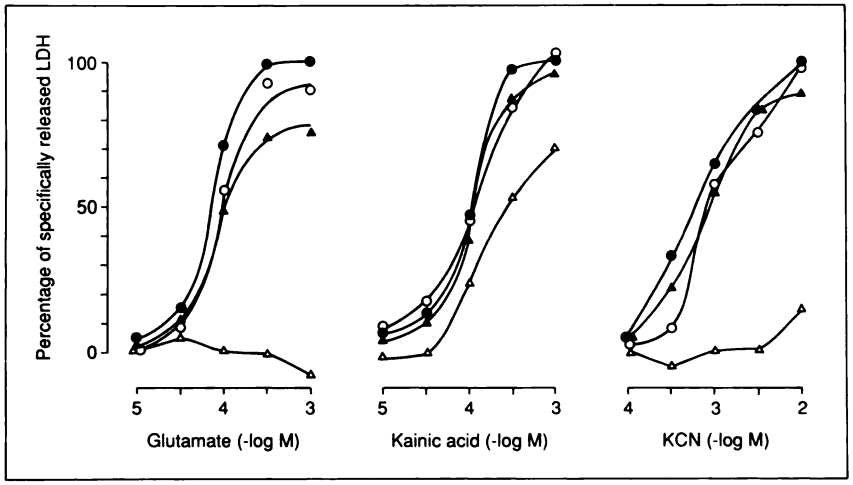

Fig. 4. Concentration-response curves for released LDH by glutamate, kainic acid, and KCN in control neuronal cultures and in the presence of 1 μM flunarizine, 1 μM tetrodotoxin, and 1 μM MK-801. Released LDH was measured after 16 hr. LDH values for glutamate, kainic acid, and KCN were expressed as the percentage of specifically released LDH obtained with 1 mM glutamate, 1 mM kainic acid, and 10 mM KCN, respectively. Representative curves are shown. ●, Control; ○, 1 μM tetrodotoxin; ▲, 1 μM flunarizine; △, 1 μM MK-801.

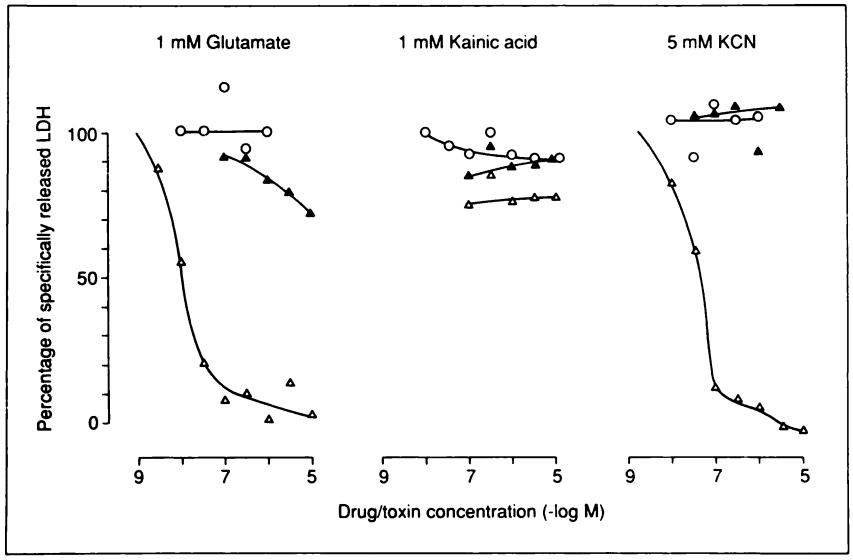

Fig. 5. Dose-response curves of flunarizine, tetrodotoxin, and MK-801 on inhibition of released LDH induced by 1 mM glutamate, 1 mM kainic acid, and 5 mM KCN. Released LDH was measured after 16 hr. LDH values were expressed as the percentage of specifically released LDH in the absence of drug. *Curves* were constructed using mean values of two to five separate experiments in triplicate. ○, Tetrodotoxin; ▲, flunarizine; △, MK-801.

The measurements of the efflux into the culture medium of LDH indicated that the release of LDH occurred gradually. Twenty-minute exposure to 30 μM veratridine yielded, within an interval of 16 hr, half-maximal neurotoxicity. The toxicity of veratridine was Ca^{2+} dependent, because it could be antagonized by 2 mM levels of the Ca^{2+} chelate EGTA. An acute, Na^+-dependent, veratridine-induced, neuronal cell swelling has been described previously by Rothman (14), who employed the cellular swelling as an index for acute neuronal death immediately following a 30-min exposure to veratridine. It should be noted that acute and nonacute neuronal death are clearly separable in *in vivo* models of global cerebral ischemia (18). The present neuronal cell loss *in vitro* showed no clear dissociation between an acute and a nonacute phase. This difference can be attributed to differences in the maturational stage of the cells (fully matured neurons in adult animals *in vivo* versus cells undergoing the process of maturation from an embryonic stage *in vitro*), differences in supporting cells, and/or the presence or absence of blood flow and nutritional supplies (19).

It is suggested that voltage-dependent Na^+ channels are suggested to be opened by veratridine, because tetrodotoxin blocks veratridine-induced neuronal degeneration in the na-

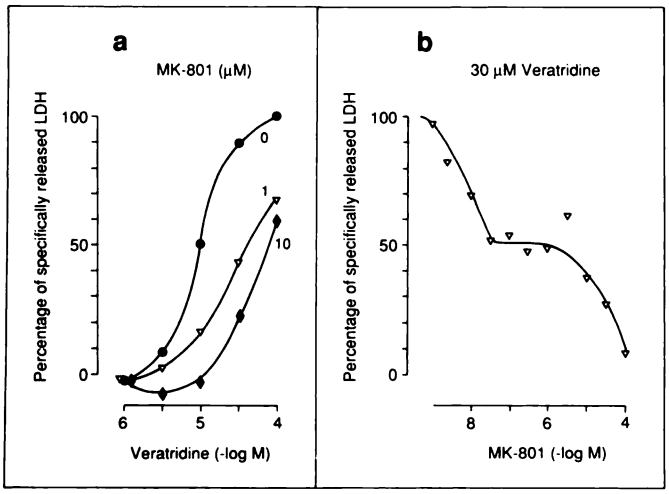

Fig. 6. The effect of MK-801 on neurotoxicity induced by veratridine. a, Concentration-response curves for released LDH by veratridine in control neuronal cultures and in the presence of 1 and 10 μM MK-801. Released LDH was measured after 16-hr exposure. All LDH values were expressed as the percentage of specifically released LDH obtained with 100 μM veratridine. *Curves* were constructed using mean values of two separate experiments in triplicate. b, Dose-response curves of MK-801 on inhibition of released LDH induced by 30 μM veratridine. Cultures were pretreated for 30 min with MK-801. LDH values were expressed as the percentage of specifically released LDH in the absence of MK-801. *Curves* were constructed using mean values of three separate experiments in triplicate.

nomolar range. At least five hypotheses can be put forward as to the mechanism involved in the toxic Ca^{2+} influx induced by veratridine. First, veratridine could trigger a Ca^{2+} influx directly through voltage-dependent Na^+ channels. This has been shown in neuroblastoma cells (20). Second, Ca^{2+} could enter the cells through voltage-gated Ca^{2+} channels opened by the membrane depolarization induced by veratridine. However, voltage-gated L-type Ca^{2+} channels are probably not involved, because slow Ca^{2+} channel blockers, such as dihydropyridines, do not yield protection.[1] The third possible route of Ca^{2+} entry is via the membrane Na^+/Ca^{2+} exchanger, a mechanism that normally acts to extrude Ca^{2+} but that might operate in reverse under conditions of elevated cytosolic Na^+ (21). Fourth, Ca^{2+} could enter the cell via a nonspecific membrane leak. Finally, cytosolic Ca^{2+} concentrations can be elevated not only by an influx of extracellular Ca^{2+} but also by release from intracellular Ca^{2+} stores, as has recently been reported for quisqualate receptors (22). According to Gusovsky and Daly (23), intracellular Na^+ may have a regulatory role in the turnover of phosphatidylinositol in synaptosomes. The mechanism responsible for the effects of Na^+ channel agents is unclear but might involve activation, by elevation of intracellular Na^+, of a Na^+/Ca^{2+} exchange mechanism, thereby increasing Ca^{2+} at the sites of hydrolysis of phosphatidylinositol.

Veratridine compared with glutamate as a neurotoxic agent. Sixteen-hour exposure to 30 μM veratridine or 1 mM glutamate led to the same amount of released LDH. Under both conditions, LDH was released gradually with time, although transient exposure studies indicated that the release with glutamate (7, 19) (Fig. 2b) starts to occur faster than that with veratridine. Neurotoxicity could be prevented under both conditions by chelation of extracellular Ca^{2+}. In contrast to its effect on veratridine, 1 μM tetrodotoxin could not block the neurotoxicity induced by 1 mM glutamate. Recently, Ogura *et al.* (19) reported that tetrodotoxin was partially effective, at 1 μM, in reducing cell death induced by 1 mM glutamate. We have no explanation for this discrepancy. Nervous tissue contains voltage-dependent Na^+ channels with nanomolar affinity for tetrodotoxin (24). Therefore, the protective effect of tetrodotoxin should be in the nanomolar range, as observed with veratridine.

Protection from neurotoxicity induced by veratridine and glutamate. Flunarizine was able to prevent neuronal cell loss induced by veratridine. A maximal protective effect was observed at 1 μM, whereas the IC_{50} value of flunarizine was 0.1 μM. This activity correlates well with the observed affinity, 0.3 μM, for BTX-B binding to rat brain synaptosomes (10, 25). This observation suggests that flunarizine can interact with Na^+ channels. The action of flunarizine on Na^+ channels can be distinguished from that of local anaesthetics. The latter drugs reveal lower affinity for BTX-B binding (10, 25). It is not excluded that different Na^+ channel subtypes are involved. Noda *et al.* (26) have reported that there are two distinct, Na^+ channel, large polypeptides in rat brain.

A 4-fold difference in potency was observed between flunarizine prepared from a 100-fold concentrated solution in 10% hydroxypropyl-β-cyclodextrin and 10% ethanol, respectively (Fig. 3). Hydroxypropyl-β-cyclodextrin itself was without effect on released LDH at a final concentration of 0.1%. Therefore, we recommend 10% hydroxypropyl-β-cyclodextrin as a suitable solvent for solubilization and stabilization of flunarizine. According to Pitha *et al.* (27), hydrophobic interactions drive lipophilic drugs into the cyclodextrin cavity by displacing water molecules. After dilution in the medium, dissociation of the drug-cyclodextrin complex results in free drug, which is available for the cells.

Flunarizine can be considered as a specific blocker of neuronal cell loss induced by veratridine, because it was without effect on neuronal cell loss induced by glutamate, kainic acid, and KCN. The lack of effect on neurotoxicity induced by glutamate differentiates flunarizine from the noncompetitive NMDA antagonist MK-801 and other NMDA antagonists (D-2-amino-5-phosphonovalerate, 2-amino-7-phosphonoheptanoate, ketamine, and phencyclidine) (6, 28, 29). The latter produce almost complete blockade of glutamate-induced late neuronal cell loss on most cortical and hippocampal neurons. A partial effect of MK-801 on released LDH induced by veratridine was observed in the nanomolar range. This suggests that about half of the neuronal damage provoked by veratridine is mediated by secondary release of endogenous glutamate and consequent activation of NMDA receptors. However, this effect was not related to the micromolar affinity of MK-801 for Na^+ channels.[1] This implies that MK-801 acts at two different sites, 1) at low MK-801 concentrations, the NMDA receptor-activated channel, and 2) at high MK-801 concentrations, the veratridine-activated Na^+ channel. We suggest that, besides the excitatory amino acid receptor pathway, prolonged opening of the veratridine-sensitive Na^+ channel in rat brain neurons can be neurotoxic. The latter can be prevented by flunarizine.

Are Na^+ channel blockers beneficial for damaged neurons in the brain? Recent observations of Prenen *et al.* (30) showed that tetrodotoxin can, at least partially, prevent the detrimental effects of ischemic brain damage *in vivo*. Its protective action may be brought about by delaying cell depolarization and by shortening the actual duration of the depolarized

state. The authors concluded that Na^+ influx and, consequently, neurotransmission may play a crucial role in the development of cerebral damage. In addition, administration of flunarizine 30 min after unilateral photochemical infarction in the hindlimb sensorimotor cortex of the rat resulted in marked sparing of the neurological function (31). It cannot be excluded that several mechanisms probably exist to explain this beneficial effect. It has been reported that flunarizine blocks Ca^{2+} channels (32). This study shows that, in neurons, blockade of a Na^+ channel may be a possible mechanism. Therefore, the neuronal culture model offers, as a simplified system for quantification of neurotoxicity, an easy tool to explore new cerebroprotective drugs.

Acknowledgments

Dr. M. De Ryck is acknowledged for critical reading of the manuscript. We thank Luc Peeters for excellent technical assistance, Jef Peeters for measuring the LDH activities and Suzy De Cauwer for skillful secretarial assistance.

References

1. Schanne, F. A. X., A. B. Kane, E. E. Young, and J. L. Farber. Calcium dependence of toxic cell death: a final common pathway. *Science (Wash. D. C.)* **206**:700–702 (1979).
2. Lemasters, J. J., J. DiGuiseppi, A. L. Nieminen, and B. Herman. Blebbing, free Ca^{++} and mitochondrial membrane potential preceding death in hepatocytes. *Nature (Lond.)* **325**:78–80 (1987).
3. Choi, D. W. Calcium-mediated neurotoxicity: relationship to specific channel types and role in ischemic damage. *Trends Neurosci.* **11**:465–469 (1988).
4. Choi, D. W. Glutamate neurotoxicity in cortical cell culture is calcium dependent. *Neurosci. Lett.* **58**:293–297 (1985).
5. Choi, D. W. Ionic dependence of glutamate neurotoxicity in cortical cell culture. *J. Neurosci.* **7**:369–379 (1987).
6. Rothman, S. M., J. H. Thurnston, and R. E. Hauchart. Delayed neurotoxicity of excitatory amino acids *in vitro*. *Neuroscience* **22**:471–480 (1987).
7. Pauwels, P. J., H. P. Van Assouw, and J. E. Leysen. Degeneration of cultured rat brain neurons by excitotoxic agents: role of Ca^{++} — biochemical approaches to cellular calcium. *Proc. R. Soc. Lond.*, in press.
8. Vanhoutte, P. M. The expert committee of the World Health Organization on classification of calcium antagonists: the view-point of the raporteur. *Am. J. Cardiol.* **59**:3A–8A (1987).
9. Wauquier, A., D. Ashton, and G. Clincke. Brain ischemia as a target for Ca^{++}-entry blockers. *Ann. N. Y. Acad. Sci.* **522**:478–490 (1988).
10. Pauwels, P. J., J. E. Leysen, and P. M. Laduron. [^{3}H]Batrachotoxinin A 20-α-benzoate binding to sodium channels in rat brain: characterization and pharmacological significance. *Eur. J. Pharmacol.* **124**:291–298 (1986).
11. West, G. J., and W. A. Catterall. Selection of variant neuroblastoma clones with missing or altered sodium channels. *Proc. Natl. Acad. Sci. USA* **76**:4136–4140 (1979).
12. Costa, M. R., and W. A. Catterall. Characterization of variant neuroblastoma clones with missing or altered sodium channels. *Mol. Pharmacol.* **22**:196–202 (1982).
13. Gonoi, T., B. Hilles, and W. A. Catterall. Voltage clamp analysis of sodium channels in normal and scorpion toxin-resistant neuroblastoma cells. *J. Neurosci.* **4**:2836–2842 (1984).
14. Rothman, S. M. The neurotoxicity of excitatory amino acids is produced by passive chloride influx. *J. Neurosci.* **5**:1483–1489 (1985).
15. Pauwels, P. J., H. P. Van Assouw, and J. E. Leysen. Attenuation of neurotoxicity following anoxia or glutamate receptor activation in EGF- and hippocampal extract-treated neuronal cultures. *Cell. Signalling* **1**:45–54 (1989).
16. Romijn, H. J., F. van Huizen, and P. S. Wolters. Towards an improved serum-free, chemically defined medium for long-term culturing of cerebral cortex tissue. *Neurosci. Biobehav. Rev.* **8**:301–334 (1984).
17. Bergmeyer, H. U. Biochemical reagents, in *Methods of Enzymatic Analysis* (H. U. Bergmeyer, ed.), 2nd Ed. Academic Press, New York, 480 (1974).
18. Pulsinelli, W. A., J. B. Brierly, and F. Plum. Temporal profile of neuronal damage in a model of transient forebrain ischemia. *Ann. Neurol.* **11**:499–509 (1982).
19. Ogura, A., M. Miyamoto, and Y. Kudo. Neuronal death *in vitro*: parallelism between survivability of hippocampal neurones and sustained elevation of cytosolic Ca^{++} after exposure to glutamate receptor agonist. *Exp. Brain Res.* **73**:447–458 (1988).
20. Jacques, Y., C. Frelin, P. Vigne, G. Romey, M. Parjari, and M. Lazdunski. Neurotoxins specific for the sodium channel stimulate calcium entry into neuroblastoma cells. *Biochemistry* **20**:6219–6225 (1981).
21. Nachsen, D. A., S. Sanchez-Armass, and A. M. Weinstein. The regulation of cytosolic calcium in rat brain synaptosomes by sodium-dependent calcium efflux. *J. Physiol. (Lond.)* **381**:17–28 (1986).
22. Murphy, S. N., and R. J. Miller. A glutamate receptor regulates Ca^{++} mobilization in hippocampal neurons. *Proc. Natl. Acad. Sci. USA* **85**:8737–8741 (1988).
23. Gusovsky, F., and J. W. Daly. Formation of inositol phosphates in synaptoneurosomes of guinea-pig brain: stimulatory effect of receptor agonists, sodium channel agents and sodium and calcium ionophores. *Neuropharmacology* **27**:95–105 (1988).
24. Lombet, A., T. Kazazoglou, E. Delpont, J. F. Renaud, and M. Lazdunski. Ontogenic appearance of Na^+ channels characterized as high affinity binding sites for tetrodotoxin during development of the rat nervous system and skeletal muscle cells. *Biochem. Biophys. Res. Commun.* **10**:894–901 (1983).
25. McNeal, E. T., G. A. Lewandowski, J. W. Daly, and C. R. Creveling. [^{3}H] Batrachotoxinin A 20α-benzoate binding to voltage-sensitive sodium channels: a rapid and quantitative assay for local anesthetic activity in a variety of drugs. *J. Med. Chem.* **28**:381–388 (1985).
26. Noda, M., T. Ikeda, H. Suzuki, H. Takeshima, T. Takahashi, M. Kuno, and S. Numa. Expression of functional sodium channels from cloned cDNA. *Nature (Lond.)* **322**:826–828 (1986).
27. Pitha, J., T. Irie, P. B. Sklar, and J. S. Nye. Drug solubilizers to aid pharmacologists: amorphous cyclodextrin derivatives. *Life Sci.* 43:493–502 (1988).
28. Goldberg, M. P., J. H. Weiss, P.-C. Pham, and D. W. Choi. *N*-Methyl-D-aspartate receptors mediate hypoxic neuronal cell injury in cortical culture. *J. Pharmacol. Exp. Ther.* **243**:784–791 (1987).
29. Choi, D. W., J. Koh, and S. Peeters. Pharmacology of glutamate neurotoxicity in cortical cell culture: attenuation by NMDA antagonists. *J. Neurosci.* **8**:185–196 (1988).
30. Prenen, G. H. M., K. Gwan Go, F. Postema, F. Zuiderveen, and J. Korf. Cerebral cation shifts in hypoxic-ischemic brain damage are prevented by the sodium channel blocker tetrodotoxin. *Exp. Neurol.* **99**:118–132 (1988).
31. De Ryck, M., J. Van Reempts, M. Borgers, A. Wauquier, and P. A. J. Janssen. Photochemical stroke model: flunarizine prevents sensorimotor deficits after neocortical infarcts in rats. *Stroke*, in press.
32. Godfraind, T., R. Miller, and M. Wibo. Calcium antagonism and calcium entry blockade. *Pharmacol. Rev.* **38**:321–416 (1986).

Send reprint requests to: Dr. P. J. Pauwels, Dept. of Biochemical Pharmacology, Janssen Research Foundation, B-2340 Beerse, Belgium.