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T-477, a novel Ca^{2+}- and Na^{+} channel blocker, prevents veratridine-induced neuronal injury

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Abstract

To evaluate the effect of (*R*)-(+)-2-(4-chlorophenyl)-2,3-dihydro-4-diethyl aminoacetyl-4H-1,4-benzothiazine hydrochloride (T-477), a novel Na^{+}- and Ca^{2+} channel blocker, on neuronal injury in vitro, we studied veratridine-induced injury in cultured rat hippocampal neurons. Neurons swelled extensively 10 min after the addition of veratridine, and returned to their initial size within 2 h. Intracellular Na^{+} and Ca^{2+} concentrations and amino acid release from the cells, in particular, that of glutamate, increased after the treatment with veratridine. Approximately 70% of neurons died within 24 h. T-477 inhibited both veratridine-induced swelling and death in a concentration-dependent manner. Moreover, T-477 concentration dependently reduced the increases in Na^{+} and Ca^{2+} influx and amino acid release. These results suggest that T-477 prevented the veratridine-induced influx of Na^{+} and, thereby, reduced neuronal swelling. This, combined with the effects of T-477 on the inhibition of Ca^{2+} influx and glutamate release, possibly by the blockade of Na^{+} channels, may be the mechanism by which T-477 protects neurons from death induced by veratridine.

Keywords: Hippocampal neuron; Veratridine; Swelling; Death; Na^{+} channel; Ca^{2+} channel

1. Introduction

Veratridine, a Na^{+} channel site 2 agonist, induces an increase in intracellular Na^{+} concentration ($[Na^{+}]_i$), cellular depolarization, elevation of intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$), glutamate release, swelling and death in neuronal cells (Ohta et al., 1973; Janka and Jones, 1982; Haupman et al., 1984; Ramnath et al., 1992; Hubert et al., 1994). Na^{+} is reported to participate directly to cellular swelling (Goldberg and Choi, 1993), but does not appear to participate directly in cell death, as is the case with Ca^{2+} (Cheung et al., 1986). However, Na^{+} influx into cells results in glutamate release which does play an important role in neuronal death (Rothman and Olney, 1986; Choi, 1988; Neal et al., 1994). This release of glutamate is mediated by the reversal of glutamate transporters due to a decrease in the Na^{+} gradient (Nicholls and Attwell, 1990; Attwell et al., 1993; Levi and Raiteri, 1993). The glutamate transporters are Ca^{2+}-independent, and one of their physiological roles is to protect neurons from glutamate toxicity by transporting this amino acid into cells using a Na^{+} gradient (Kanai and Hediger, 1992). In addition, a $[Ca^{2+}]_i$ increase is also induced by veratridine, which also plays an important role in cellular death. Therefore, veratridine not only induces cellular swelling directly, but also, cell death indirectly. All the changes induced by veratridine described above are observed in neurons during cerebral ischemia (Goldberg et al., 1986; Szatkowski and Attwell, 1993; Berg-Johnsen et al., 1995). Although it is not the only event, the activation of Na^{+} channels occurs in brain ischemia. Therefore, it is of interest to assess the consequences of antagonizing Na^{+} channel hyperactivity for cerebral ischemia.

(*R*)-(+)-2-(4-chlorophenyl)-2,3-dihydro-4-diethyl aminoacetyl-4H-1,4-benzothiazine hydrochloride (T-477; Fig. 1) is a novel neuronal Na^{+}- and Ca^{2+} channel blocker. The IC_{50} value of T-477 on the fast Na^{+} current of isolated rat brain neurons, as determined by the nystatin-performed patch recording mode under voltage-clamp conditions, is 7.8 μM (personal communication with Dr. N. Akaike). In contrast, the IC_{50} values of T-477 on cardiac L-type (also exists in brain), neuronal Q-, R-, and N-type Ca^{2+} channels are 52, 45, 74, and 59 μM, respectively

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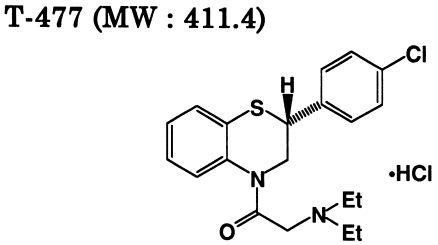

Fig. 1. Chemical structure and molecular weight (MW) of T-477.

(Kobayashi et al., 1997). We have already reported the protective effect of T-477 in a brain ischemia and edema models in rats (Ishii et al., 1996; Okuyama et al., 1999). The blocking action of T-477 on both Ca^{2+} and Na^{+} channels is hypothesized to inhibit the increases in $[Ca^{2+}]_i$ and $[Na^{+}]_i$, resulting from the excessive transmission with prolonged cellular excitation, as well as to reverse the uptake of glutamate. However, a precise mechanism of neuroprotection by T-477 has not been established yet. Moreover, there are no reports about the effect of T-477 on cellular injury in vitro.

Anoxic depolarization of neurons occurs during cerebral ischemia, which is mediated by the voltage-dependent Na^{+} channels (Prenen et al., 1988; Wahl et al., 1994). In addition, T-477 blocks Na^{+} channels more potently than Ca^{2+} channels. For these reasons, we focused on the Na^{+} channel effects to evaluate the effect of T-477 in vitro. In this study, the effect of T-477 on the damage to cultured rat hippocampal neurons induced by veratridine was investigated.

2. Materials and methods

2.1. Preparation of cells

Primary cultures of hippocampal neurons were prepared by a modification of Ogura's method (Ogura et al., 1988). Hippocampi were isolated from 17-day-old rat embryos and treated with 0.25% trypsin and 0.01% DNase I at 37°C for 15 min. Cells were dissociated by pipetting and then filtering the suspension through lens-cleaning paper (Eastman Kodak, NY, USA). The cells were pelleted by centrifugation and resuspended in fresh high glucose (25 mM) Dulbecco's modified Eagles medium with 10% fetal bovine serum containing 50 U/ml penicillin and 50 μM streptomycin. Then, cells were plated onto either poly-L-lysine-coated 24 or 48-well multiplates (Sumitomo Bakelite, Tokyo, Japan) or polyethylenimine-coated 8-well LAB-TEK cover glass chambers (Nunc, IL, USA). Cells were cultured with 0.2, 0.3 and 0.6 ml medium at a density of 2.5, 3.8 and 7.0×10^5 cells/well, respectively. After 2–4 days in culture, cytosine β-D-arabino-furanoside (3 μM) was added to the medium. To prevent the proliferation of glial cells, the fetal bovine serum concentration was decreased from 10% to 5% after 4 days in culture, and finally, to 2.5% after 7 days in culture. Medium was changed every 2 or 3 days.

Neurons of 9–11 days in culture were used in this study.

2.2. Assessment of cellular injury

Cellular injury was induced by adding 30 μM veratridine to the culture medium in 48-well multiplates. Photomicrographs of identical fields from each well were taken before and 10, 30, 60, 120 min, and 24 h after the addition of veratridine. Cellular swelling was assessed by a modification of Choi's method (Choi et al., 1988). Outlines of 25 identical neurons in a series of photomicrographs, except those 24 h after the addition of veratridine, were traced, and their areas were analyzed by using an imaging analyzer (MCID imaging analyzer; Imaging Research, Ontario, Canada). Means of 25 neuronal areas were calculated and expressed as Δ% of baseline. Percentages of surviving neurons were assessed by counting the neurons in each photomicrograph before and after the addition of veratridine. The number of living neurons was counted according to the following criteria (Ogura et al., 1988): (1) phase-brightness; (2) possession of one or more cell processes longer than the diameter of the cell; (3) absence of vesiculations or granulations in those processes. When the effects of drugs were studied, T-477 (0.1, 0.3, 1, 3, 10 μM), diltiazem (3, 10, 30 μM), or tetrodotoxin (1 μM) was added together with veratridine. Three or five sister cultures were examined, and the data are expressed as means ± S.E.M.

2.3. Determination of $[Na^{+}]_i$

$[Na^{+}]_i$ was determined by a modification of Harootunian's method (Harootunian et al., 1989), and expressed as the F340/F380 excitation ratio (Ratio) of the sodium-binding dye agent benzofuran isophthalate (SBFI). Cells cultured in an 8-well LAB-TEK chamber were washed twice with control salt solution (consisting of NaCl 120 mM, KCl 5.4 mM, $MgCl_2$ 0.8 mM, $CaCl_2$ 1.8 mM, Tris 25 mM, and glucose 15 mM; Choi, 1988). Then, the cells were loaded with 15 μM SBFI acetoxymethyl ester in control salt solution with 0.025% cremophor at 37°C for 60 min, and again, washed twice with control salt solution. Basal $[Na^{+}]_i$ level of SBFI-loaded cells was measured first for 1 min, the cells were treated with 100 μM veratridine with or without T-477 or tetrodotoxin, and then $[Na^{+}]_i$ was measured again for 3 or 10 min with a microscopic dual-wavelength fluorometer (CAM-230; Japan Spectroscopic, Tokyo, Japan) at 10-s intervals. During the measurement, 100 μM veratridine with or without the drug in control salt solution was perfused at a rate of 2 ml/min; for a control experiment, only control salt solution was

used instead of the veratridine solution. Data were analyzed by using an image processor (ARGUS 50; Hamamatsu Photonics K.K., Hamamatsu, Japan). The concentration of T-477 used was 0.3, 1, 3, 10 and 30 μM. Five sister cultures were used for this experiment and one well was used for one measurement. Three sister cultures were used for the experiment with 100 μM veratridine and 1 μM tetrodotoxin.

2.4. Determination of $[Ca^{2+}]_i$

$[Ca^{2+}]_i$ was determined by a modification of Kudo's method (Kudo and Ogura, 1986), and $[Ca^{2+}]_i$ is expressed as the F340/F380 excitation ratio (Ratio) of the dye agent fura-2. Cells cultured in an 8-well LAB-TEK chamber were washed twice with control salt solution, loaded with 5 μM fura-2 acetoxymethyl ester in control salt solution containing 0.05% cremophor at 37°C for 30 min, and then washed twice with control salt solution. After stabilization of basal $[Ca^{2+}]_i$ level, the high K^+ solution (80 K; consisting of NaCl 45.4 mM, KCl 80 mM, $MgCl_2$ 0.8 mM, $CaCl_2$ 1.8 mM, Tris 25 mM and glucose 15 mM) was added to cells to confirm the ability of cells to depolarize. Cells were washed twice with control salt solution after the $[Ca^{2+}]_i$ reached a peak level. The same procedure was repeated, and 1 min after the last wash, veratridine (100 μM) with or without T-477 was added to cells for 3 min. Cells were then washed twice with control salt solution and the 80 K treatment was repeated again. The excitation ratio was continuously recorded with a microscopic dual-wavelength fluorometer (CAM-200; Japan Spectroscopic). Data were analyzed by the following method. The line connecting the Ratio before and after the second 80 K treatment was designated as the baseline (Fig. 5). The area surrounded by the baseline and the Ratio during veratridine treatment was analyzed with the MCID imaging analyzer as the increase in $[Ca^{2+}]_i$. The T-477 concentration and other experimental conditions used were the same as those used for the $[Na^+]_i$ determination.

2.5. Cellular amino acid release

Cells cultured on 24-well multiplates were washed four times with control salt solution or Ca^{2+}-free control salt solution, and treated for 5 min with 100 μM veratridine with or without the agent. The supernatant was collected. Cells were washed twice with control salt solution or Ca^{2+}-free control salt solution and solubilized with a solution containing 50 mM NaOH, 0.05% sodium dodecyl sulfate, 1 mM phenylmethylsulfonyl fluoride and 1 mM ethylenediaminetetraacetic acid. Total protein content of the cells was measured by a modified Lowry method (DC Protein Assay; BIO-RAD, CA, USA). The contents of glutamate, aspartate, glutamine, glycine, taurine, alanine and γ-animobutyric acid (GABA) in the supernatant were measured using high-performance liquid chromatography (HPLC) and electrode voltammetry after precolumn derivatization (Lindroth and Mopper, 1979). Each sample was diluted if necessary, and was automatically mixed with *o*-phthaldialdehyde and 2-mercaptoethanol, incubated for 10 min at 10°C, and then injected into the HPLC system. The HPLC system consisted of a pump (DB-100; Eicom, Kyoto, Japan, at a flow rate of 1.0 ml/min), a reverse-phase column (Eicompac MA-5 ODS; 4.6 × 150 mm, Eicom) and an electrode voltammetry detector (VMD-501; Yanaco, Kyoto, Japan). The mobile phase was 0.1 M sodium phosphate (pH 6.0) containing 30% methanol. Amino acid content was determined by comparison of the peak area of each sample with the peak area of an authentic sample. Amino acid release is expressed as nmol/mg protein. Concentrations of T-477 and tetrodotoxin were the same as those used for the determination of $[Na^+]_i$. Three sister cultures were used for this experiment.

2.6. Statistical analysis

All data are expressed as means ± S.E.M. and statistical analysis on veratridine-induced cellular injury was performed by one or two-way ANOVA with randomized block design followed by Dunnett's multiple comparison. IC_{50} values were calculated from the concentration–inhibition relationships by the method of least squares. Basal release of amino acids under the two conditions was analyzed by Student's *t*-test.

2.7. Reagents

Trypsin, high glucose concentration Dulbecco's modified Eagles medium, fetal bovine serum, penicillin and streptomycin were obtained from Gibco BRL (Life Technologies, ND, USA). Veratridine, tetrodotoxin, DNase I, poly-L-lysine, polyethylenimine, cytosine β-D-arabino-furanoside, fura-2 acetoxymethyl ester, cremophor, sodium dodecyl sulfate, phenylmethylsulfonyl fluoride, and ethylenediaminetetraacetic acid were obtained from Sigma Chemical (St. Louis, MO, USA). SBFI acetoxymethyl ester was obtained from Molecular Probes (Eugene, OR, USA). *o*-Phthaldialdehyde and methanol were obtained from Wako Pure Chemical Industries (Osaka, Japan) and 2-mercaptoethanol was purchased from Katayama Chemical (Osaka, Japan). T-477 and diltiazem were synthesized in our research laboratory.

3. Results

3.1. Cellular injury

Cultured hippocampal neurons swelled extensively 10 min after the addition of 30 μM veratridine, and gradually returned to their original size by 120 min. At this point,

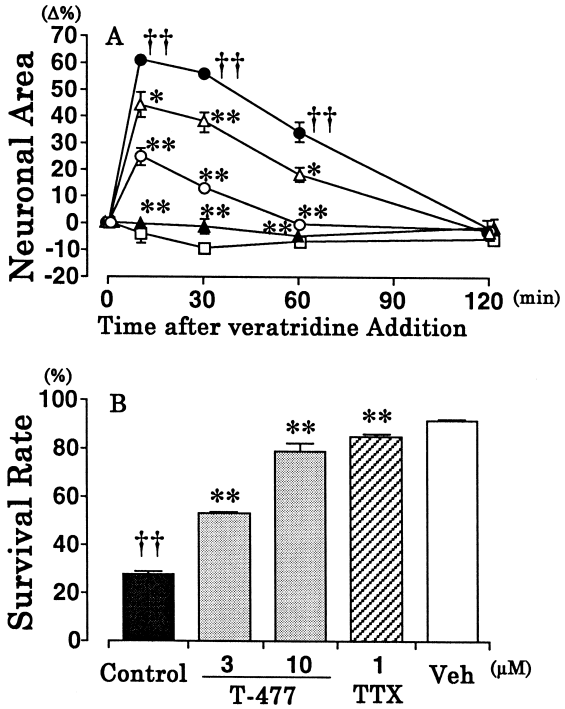

Fig. 2. Effects of T-477 and tetrodotoxin (TTX) on neuronal swelling and death induced by 30 μM veratridine in cultured rat hippocampal neurons. Each point or column and vertical bar represents mean ± S.E.M. of the three sister cultures. $^{\dagger\dagger}P < 0.01$ vs. vehicle-treated cells (Veh), $^{*}P < 0.05$, $^{**}P < 0.01$ vs. control by randomized block design followed by Dunnett's multiple comparison. (A) The time course of changes in neuronal area. ●: Control (veratridine); ▲: vehicle-treated cells; △: veratridine + 3 μM T-477; ○: veratridine + 10 μM T-477; and □: veratridine + 1 μM tetrodoxin. (B) Survival rate of hippocampal neurons 24 h after the treatment with veratridine.

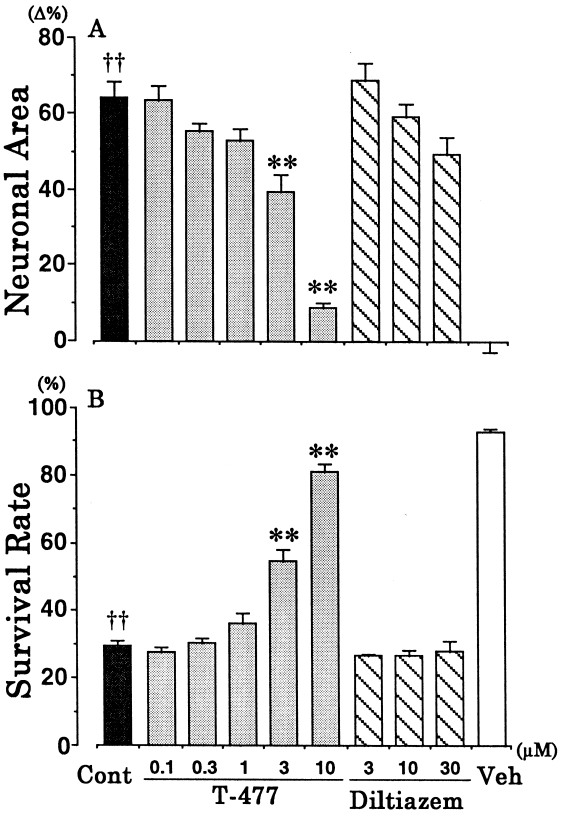

Fig. 3. Effects of T-477 and diltiazem on neuronal swelling and death induced by 30 μM veratridine in cultured rat hippocampal neurons. Each column and vertical bar represnts mean ± S.E.M. of the five sister cultures. $^{\dagger\dagger}P < 0.01$ vs. vehicle-treated cells (Veh), $^{**}P < 0.01$ vs. control (Cont) by randomized block design followed by Dunnett's multiple comparison. (A) Neuronal swelling 30 min after veratridine addition. (B) Survival rate of hippocampal neurons 24 h after the treatment with veratridine.

almost all neurons survived. However, 24 h after the treatment, only 28.0 ± 0.7% of the neurons were viable (Fig. 2). T-477 protected the cells from both swelling and death induced by veratridine in a concentration-dependent manner with IC_{50} values of 4.1 μM and 3.6 μM, respectively (Fig. 3 and Table 1). Diltiazem did not prevent veratridine-induced neuronal swelling and death (Fig. 3). Tetrodotoxin (1 μM) almost completely protected the cells from injury. Vehicle-treated cells did not swell and the survival rate at 24 h after vehicle treatment was 91.4 ± 0.9% (Figs. 2–4).

3.2. $[Na^+]_i$ and $[Ca^{2+}]_i$ increase

$[Na^+]_i$ increased slowly for 3 min after the addition of 100 μM veratridine (Fig. 5), and thereafter, it was stable for at least 7 min (data not shown). Under these conditions, the effect of T-477 on the increase in $[Na^+]_i$ was determined 3 min after the addition of veratridine. In contrast, veratridine induced a rapid increase in $[Ca^{2+}]_i$ after a short lag time, and $[Ca^{2+}]_i$ was stable thereafter even when veratridine was washed out with control salt solution (Fig. 6). The effect of T-477 on the increase in $[Ca^{2+}]_i$ was determined as described in Section 2. T-477

Table 1
IC_{50} values of T-477 on veratridine-induced cellular changes

	IC_{50} values (95% confidence limits), μM	
Swelling	4.1 (3.4–5.0)	
Death	3.6 (2.8–4.7)	
Na^+ influx	4.8 (3.6–6.2)	
Ca^{2+} influx	13.8 (10.4–19.3)	
Amino acid release	Normal conditions	Ca^{2+}-free conditions
Glutamate	4.5 (3.6–5.4)	3.4 (2.8–4.0)
Aspartate	[a]	[a]
Glutamine	2.1 (1.4–3.0)	0.7 (0.6–0.8)
Glycine	17.2 (14.8–20.1)	3.7 (3.1–4.3)
Taurine	[b]	3.5 (3.0–4.1)
Alamine	59.0 (31.6–265.1)	6.1 (4.8–7.7)
GABA	[a]	5.6 (4.1–7.2)

[a] IC_{50} value cannot be calculated because the basal release was undetectable.

[b] IC_{50} value was not calculated because veratridine did not increase the release.

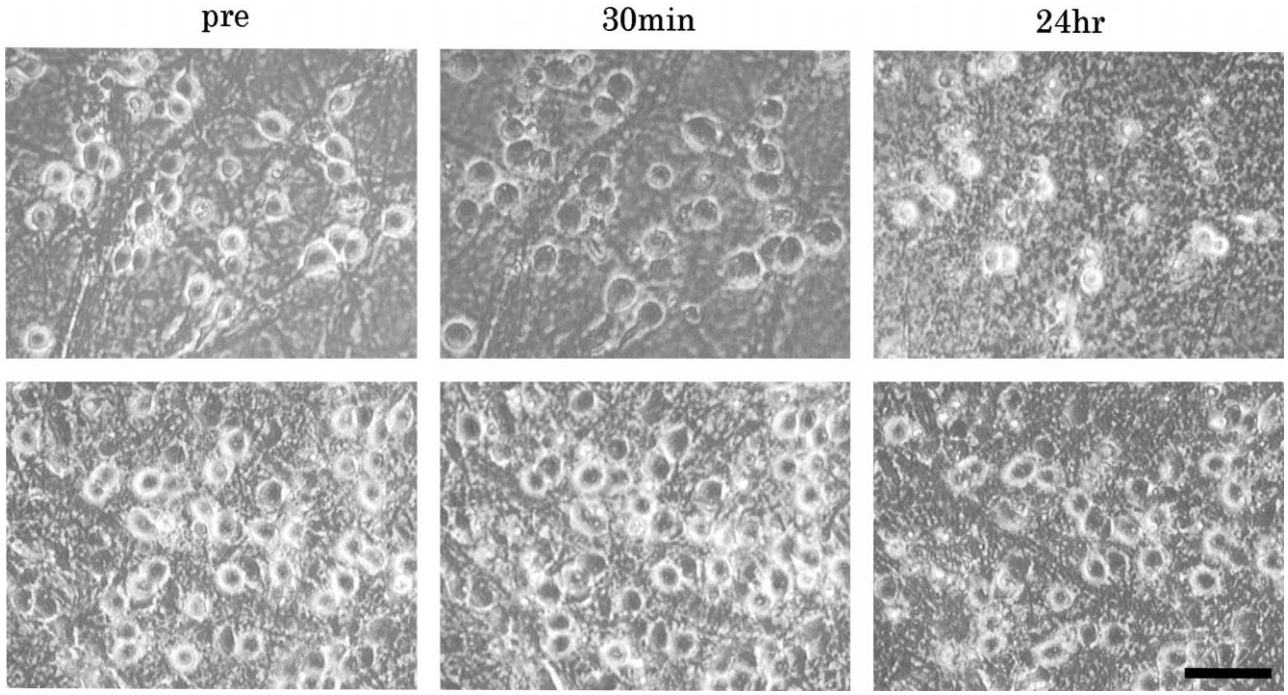

Fig. 4. Effects of T-477 on veratridine-induced neurotoxicity. Phase-contrast photomicrographs of an identical field of cultured rat hippocampal neurons. Pre: before, 30 min:30 min after and 24 h:24 h after the treatment with veratridine. Upper panels: 30 μM veratridine and lower panels:30 μM veratridine + 30 μM T-477. Bar indicates 50 μm.

concentration dependently inhibited the increases of both $[Na^+]_i$ and $[Ca^{2+}]_i$. The IC_{50} value of T-477 for Na^+ influx was very similar (4.8 μM) to that for cellular swelling and death, but the IC_{50} value for Ca^{2+} influx was 2.7-fold higher (13.8 μM) (Table 1). Tetrodotoxin (1 μM) almost completely inhibited the increases of both $[Na^+]_i$ and $[Ca^{2+}]_i$ (data not shown).

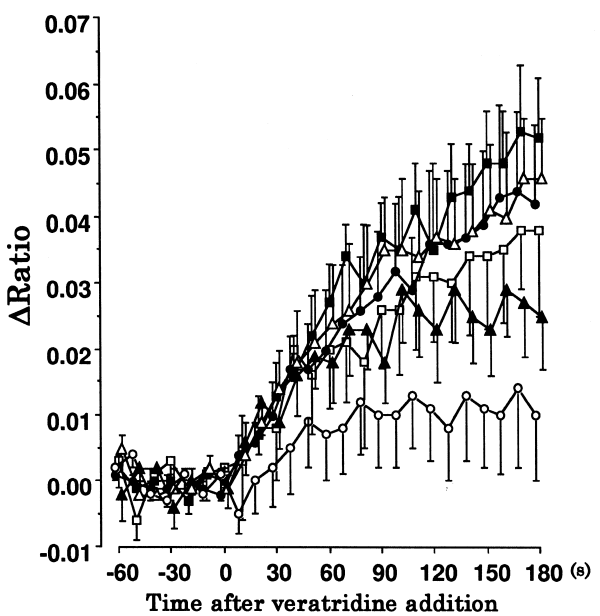

Fig. 5. Veratridine-induced change in Ratio of SBFI (ΔRatio) in cultured rat hippocampal neurons. Each point and vertical bar represents mean ± S.E.M. of the five sister cultures. Neurons were treated with 100 μM veratridine with or without T-477 for 180 s. ■: Control (veratridine); △: veratridine + 0.3 μM T-477; ●: veratridine + 1 μM T-477; □: veratridine + 3 μM T-477; ▲: veratridine + 10 μM T-477; and ○: veratridine + 30 μM T-477.

3.3. Ca^{2+}-dependent and -independent amino acid release

Amino acid release under normal and Ca^{2+}-free conditions is shown in Table 2. Under normal ionic conditions,

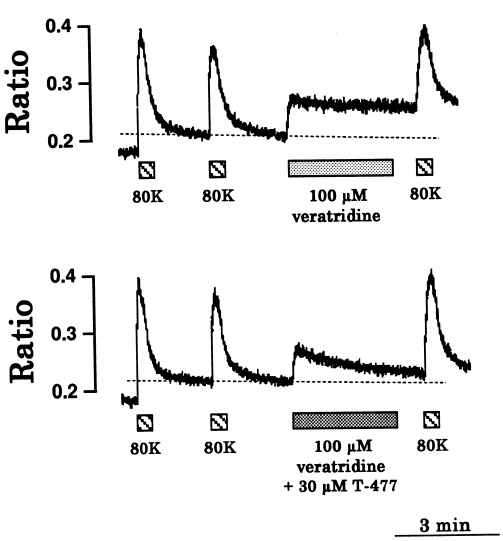

Fig. 6. Tracing of the Ratio of fura-2 in cultured rat hippocampal neurons. Dotted line: baseline of the Ratio.

Table 2
Veratridine-induced amino acid release
Data are expressed as means ± S.E.M. (nmol/mg protein) of the three sister cultures. ND: not detectable.

	Normal conditions		Ca^{2+}-free conditions	
	Basal	Veratridine-induced	Basal	Veratridine-induced
Glutamate	0.10 ± 0.00	0.18 ± 0.01**	0.42 ± 0.08***	9.38 ± 1.44**
Aspartate	ND	ND	ND	0.40 ± 0.11
Glutamine	2.80 ± 0.24	3.91 ± 0.24*	3.86 ± 0.60	6.03 ± 0.44*
Glycine	0.93 ± 0.02	2.69 ± 0.41**	1.72 ± 0.25***	11.53 ± 1.18**
Taurine	0.70 ± 0.08	0.73 ± 0.17	1.49 ± 0.38	11.9 ± 0.50**
Alamine	0.52 ± 0.07	1.16 ± 0.03**	0.79 ± 0.08	1.92 ± 0.12**
GABA	ND	0.55 ± 0.03	0.10 ± 0.03	1.39 ± 0.09**

* $P < 0.05$ vs. basal release of the same conditions.
** $P < 0.01$ vs. basal release of the same conditions.
*** $P < 0.05$ vs. basal release of normal conditions.

veratridine (100 μM) induced the release of glutamate, glutamine, glycine, alanine and GABA. Taurine release was not changed by veratridine treatment, and aspartate release was undetectable even after veratridine addition. Removal of Ca^{2+} significantly enhanced the basal release of glutamate and glycine, and the release of the other five amino acids tended to increase. The release of all the amino acids increased upon veratridine addition under Ca^{2+}-free conditions. T-477 inhibited these veratridine-induced increases in amino acid release in a concentration-dependent manner under both conditions. The IC_{50} values of T-477 for the increase in amino acid release induced by veratridine are shown in Table 1. The IC_{50} values for GABA release under normal conditions and aspartate release under both conditions could not be calculated because the basal release of these amino acids was undetectable. The IC_{50} values of T-477 under Ca^{2+}-free conditions were smaller than those under normal conditions. Tetrodotoxin almost completely inhibited all changes in amino acid release induced by veratridine under both conditions (data not shown).

4. Discussion

Veratridine is a Na^+ channel site 2 agonist and is known to induce both cellular swelling and death (Janka and Jones, 1982; Ramnath et al., 1992). In the previous reports, veratridine-induced swelling and death were shown independently, so that the progression of these changes was not clear. Thus, we investigated the time course of cellular swelling and death induced by veratridine. We found that swelling of neurons reached a peak level within 10 min after veratridine addition. Thereafter, neurons almost completely recovered from swelling 2 h after the treatment with veratridine even though veratridine was still present in the medium, and at that time, almost all neurons were viable. However, 70% of neurons died within 24 h after the veratridine treatment, even though they had exhibited a complete recovery from swelling.

Veratridine opens Na^+ channels and induces an increase in $[Na^+]_i$ which is accompanied by water influx, which leads to cellular swelling. We have shown here a rapid increase in $[Na^+]_i$, indicating that this phenomenon is responsible for the cellular swelling seen after the treatment with veratridine. The mechanism of the reversal of cellular swelling cannot be determined from our study; however, an efflux of cation and/or anion must have occurred by some mechanism, which remains to be elucidated. Our results show an irreversible increase in $[Ca^{2+}]_i$ after treatment with veratridine. Glutamate release was also observed after veratridine addition. Elevation of $[Ca^{2+}]_i$ and extracellular glutamate both play important roles in neuronal death. Thus, cellular death induced by veratridine is likely to be mediated by an increase in $[Ca^{2+}]_i$ and glutamate release.

Veratridine induces amino acid release, which is independent of extracellular Ca^{2+}. A mechanism has been proposed which involves reversal of the uptake of amino acids by transporters (Nicholls and Attwell, 1990; Attwell et al., 1993; Levi and Raiteri, 1993). Glutamate, aspartate, glycine, taurine, alanine and GABA are reported to be transported by Na^+-dependent transporters in the brain (Kanai and Hediger, 1992; Smith et al., 1992; Jursky et al., 1994; Sakata et al., 1997). The existence of a Na^+-dependent glutamine transporter is suggested even though a specific transporter has not yet been identified (Tamarappoo et al., 1997). Thus, we suggest that the veratridine-induced release of these amino acids is, at least in part, mediated by the reversal of the transporters. In our study, the removal of Ca^{2+} enhanced the veratridine-induced release of amino acids. It should be noted that similar results have been reported for some amino acids, but not for non-amino acid neurotransmitters (Levi et al., 1980; Bernath, 1992). The removal of divalent cations has been shown to cause a large rise in $[Na^+]_i$ (Sorimachi et al., 1992) and a concomitant decrease in the Na^+ gradient, providing a potential explanation for the phenomenon described above.

T-477, a novel neuronal Na^+ and Ca^{2+} channel blocker, inhibited all the veratridine-induced changes in cultured rat hippocampal neurons in a concentration-dependent manner. In contrast, diltiazem did not prevent the cellular swelling and death induced by veratridine. Diltiazem is a specific blocker of L-type Ca^{2+} channels (IC_{50}: 11 μM), while T-477 inhibits, not only the L-type, but also the neuronal Q-, R-, and N-type Ca^{2+} channels (IC_{50}: 45–74 μM; Kobayashi et al., 1997). Thus, we can rule out the participation of L-type Ca^{2+} channels in the cellular injury induced by veratridine. The participation of neuronal Ca^{2+} channels in veratridine-induced cellular injury is still unclear. However, IC_{50} values of T-477 at neuronal Ca^{2+} channels (45–74 μM) were more than 10 times greater than those for veratridine-induced cellular swelling and death (4.1 and 3.6 μM, respectively), suggesting that a Ca^{2+} channel blocking action is not the mechanism by which T-477 prevents veratridine-induced cellular injury.

The mechanism T-477 inhibits veratridine-induced neuronal swelling and increase in $[Na^+]_i$ is most certainly due to the blocking action of this drug on voltage-dependent Na^+ channels. IC_{50} values of T-477 for veratridine-induced amino acid release under normal conditions were higher than those under the Ca^{2+}-free conditions although veratridine induced a smaller release of amino acids under normal conditions than under Ca^{2+}-free conditions. The IC_{50} value of T-477 for the veratridine-induced increase in $[Ca^{2+}]_i$ was higher than that for the $[Na^+]_i$ increase. These results suggest that amino acid efflux under normal conditions is likely to be mediated not only by the reversal of the transporters due to the increased $[Na^+]_i$ but also by exocytosis or other unknown Ca^{2+}-dependent pathways due to $[Ca^{2+}]_i$ increase. In contrast, amino acid release under Ca^{2+}-free conditions is probably mediated by the reversal of the transporters. Thus, inhibition of veratridine-induced amino acid release by T-477 is most likely mediated, at least in part, by the inhibition of the reversal of the transporter due to the blockade of Na^+ channels. The IC_{50} value of T-477 for veratridine-induced $[Ca^{2+}]_i$ increase was more than three times higher than that for Ca^{2+} channels, suggesting that T-477 may not inhibit the increase in $[Ca^{2+}]_i$ by a Ca^{2+} channel blocking action but by an indirect effect, by a Na^+ channel blocking action. However, the pathway for the increase in $[Ca^{2+}]_i$ after veratridine addition, namely, the activation of Na^+ channel is unknown. The mechanism by which T-477 prevents cellular death induced by veratridine is likely to be the inhibition of the increase in both glutamate release and $[Ca^{2+}]_i$ due to the blockade of Na^+ channel as described above. In summary, T-477 inhibits veratridine-induced neuronal injury most likely through its Na^+ channel blocking action.

Our study suggests that T-477 may inhibit the opening of Na^+ channels and the ensuing cascade of events in ischemia, even though this represents only one of many events in ischemia. Thus, the present study leads us to speculate that the mechanism of protective effect of T-477 against cerebral ischemia in rats (Ishii et al., 1996) is, at least in part, due to its blockade of Na^+ channels. This speculation is also supported by some reports of a protective effect of tetrodotoxin in a brain ischemia model (Prenen et al., 1988; Lysko et al., 1994). We also speculate that the protective effect of T-477 on brain edema (Okuyama et al., 1999) is not only the secondary result of the inhibition of cellular death but is also in part due to the Na^+ channel blocking action, which inhibits the development of cytotoxic edema due to the swelling of cerebral cells (Klatzo, 1967).

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