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Role of sodium ion influx in depolarization-induced neuronal cell death by high KCl or veratridine

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Abstract

Intracellular Na^+ concentration plays an important role in the regulation of cellular energy metabolism; i.e., increased intracellular Na^+ concentration stimulates glucose utilization both in cultured neurons and astrocytes. Both high KCl and veratridine, which have been known to cause neuronal damage, elicit increased glucose utilization, presumably via increased intracellular Na^+ concentration. In the present study, we examined the role of intracellular Na^+ influx in the mechanisms of neuronal cell damage induced by high KCl or veratridine assayed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) colorimetric method. Rat primary cultures of striatal neurons were incubated with high KCl (final concentrations: 25, 50 mM) or veratridine (0.1–100 μM) with or without various inhibitors. High KCl depolarizes cell membrane, thus, leading to Na^+ influx through an activation of voltage-sensitive Na^+ channels, while veratridine elicits Na^+ influx by directly opening these channels. After 24-h incubation with elevated $[K^+]_o$ or veratridine, glucose contents in the medium decreased significantly (approximately by 7 mM), but remained higher than 18 mM. High $[K^+]_o$ reduced percent cell viability significantly ($\sim$50% at 25 mM, $\sim$40% at 50 mM $[K^+]_o$, $P < 0.01$), but tetrodotoxin (100 nM) had no protective effect, indicating that Na^+ influx was not essential to high K^+-induced cell death. DL-2-Amino-5-phosponovaleric acid (APV) (1 mM) completely blocked cell death induced by elevated $[K^+]_o$, while 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) (10 μM) did not. In contrast, veratridine ($>$ 10 μM) caused cell damage in a dose-dependent and tetrodotoxin-sensitive manner, but none of APV, CNQX, or bepridil (Na^+–Ca^{2+} exchanger blocker) had any protective effect. Nifedipine (50 $\sim$ 100 μM), however, reduced percent cell damage induced by veratridine.

Keywords: MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide); Ca^{2+}; K^+, extracellular; Tetrodotoxin; Veratridine; Na^+ channel, voltage-sensitive

1. Introduction

Brain requires constant supply of glucose and oxygen to produce ATP (Clarke and Sokoloff, 1994). Approximately one half or more of total ATP generated in the brain at resting state is consumed by Na^+,K^+-ATPase (enzyme commission 3.6.1.37) that is necessary for the maintenance of proper transmembrane ionic gradients (Erecinska and Silver, 1989; Clarke and Sokoloff, 1994). The changes in rates of glucose utilization that occur with functional activation are quantitatively related to the level of functional activity. Indeed, glucose utilization does increase almost linearly with the frequency of action potentials (Kadekaro et al., 1985). Action potentials consist of rapid Na^+ influx through voltage-sensitive Na^+ channels in neurons and delayed efflux of K^+ (Hodgkin and Huxley, 1952). The consequent increases in intracellular Na^+ concentration ($[Na^+]_i$) and/or extracellular K^+ concentration ($[K^+]_o$) stimulate Na^+,K^+-ATPase activity to restore normal ionic gradients, which in turn stimulates energy metabolism and rates of glucose utilization (Mata et al., 1980; Sokoloff, 1981, 1994).

A rise in $[K^+]_o$ is an inevitable result of enhanced neuronal activity (Somjen, 1979). It has been known for long that increased $[K^+]_o$ stimulates energy metabolism in brain (Shinohara et al., 1979), which seems to be mostly, if not all (Erecinska et al., 1991), a reflection of activation of Na^+,K^+-ATPase (Mata et al., 1980; Sokoloff, 1981, 1994). However, neuronal Na^+,K^+-ATPase cannot be activated

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by increased $[K^+]_o$ because it has high affinity for extracellular K^+, and is already saturated with resting level of $[K^+]_o$ (Sweadner, 1995). Therefore, it has been generally thought that increases in $[Na^+]_i$ caused by secondary Na^+ influx through voltage-sensitive Na^+ channels upon membrane depolarization play a regulatory role in the activation of Na^+,K^+-ATPase in neurons. In ischemia, epilepsy, or cortical spreading depression, $[K^+]_o$ also rises to as high as 50–80 mM (Somjen, 1979). Then, membrane depolarization triggers glutamate release, which plays a pivotal role in cell damage (Choi, 1988, 1995; Ter Horst and Korf, 1997). In addition, it has been widely accepted that Na^+ influx through voltage-sensitive Na^+ channels upon depolarization exacerbates neuronal cell damage in combination with overstimulation of ionotropic glutamate receptors (Lynch et al., 1995; Stys, 1998). Therefore, blockade Na^+ influx through of voltage-sensitive Na^+ channels is a potential therapeutic target for ischemic brain damage as well as epilepsy and other neurodegenerative diseases (Taylor and Meldrum, 1995; Stys, 1998). However, the exact role of Na^+ influx per se in the process of neuronal cell damage in these conditions remains to be elusive, especially when taking it into consideration that high K^+ environment causes only transient opening of voltage-sensitive Na^+ channels and elicits moderate elevations in $[Na^+]_i$ in neurons (Rose and Ransom, 1997).

We previously confirmed that both veratridine, which elicits persistent activation of voltage-sensitive Na^+ channels (Li and White, 1977; Catterall, 1980, 1992), and elevated $[K^+]_o$ facilitate $^{22}Na^+$ influx in a tetrodotoxin sensitive-manner (Takahashi et al., 1996, 1997), and also causes increased rates of [^{14}C]deoxyglucose phosphorylation (Takahashi et al., 1994, 1995b), a reflection of glucose utilization (Sokoloff et al., 1977), in cultured neurons prepared from rat fetal striatum. These results indicate that Na^+ influx through voltage-sensitive Na^+ channels does indeed play an important role in the regulation of energy metabolism in these neurons under physiological conditions. In this context, we addressed two major questions; firstly, whether persistent activation of voltage-sensitive Na^+ channels or high K^+ causes neuronal cell damage in our system, and, if so, secondly, whether high K^+ induced cell death is mediated by Na^+ influx through voltage-sensitive Na^+ channels. The extent of neuronal cell damage was quantitatively assayed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) colorimetric method.

2. Materials and methods

2.1. Animals

Timed-pregnant Sprague–Dawley rats were purchased from Japan SLC (Hamamatsu-shi, Japan). All procedures on animals were in accordance with The Animal Experimentation Guideline of Keio University School of Medicine and approved by the Experimental Committee of Keio University.

2.2. Chemicals

Chemicals were obtained from the following sources: high-glucose (25 mM) Dulbecco's modified Eagle medium, penicillin, and streptomycin from Life Technologies (Grand Island, NY, USA); defined fetal bovine serum from HyClone Laboratories (Logan, UT, USA); Dulbecco's phosphate-buffered saline without Ca^{2+} and Mg^{2+} (PBS), poly-L-lysine, veratridine, aconitine, grayanotoxin III, nifedipine, bepridil, dimethyl sulfoxide (DMSO), MTT, sodium dodecyl sulfate (SDS), DL-2-amino-5-phosponovaleric acid (APV), and *N,N*-dimethyl formamide from Sigma (St. Louis, MO, USA); tetrodotoxin from Calbiochem (San Diego, CA, USA) and ICN Biomedicals (Aurora, OH, USA); 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) from Tocris (Langford Bristol, UK).

2.3. Preparation of neuronal cells

Neuronal cultures were prepared from the striatum of fetal rats on embryonic day 16 as described previously (Takahashi et al., 1995b, 1997). Briefly, striatal tissue was excised and mechanically disrupted by passage through a 22-gauge needle. The dissociated cells were counted and viable cells (1.5×10^6 cells/ml) that excluded trypan-blue were placed in 24-well culture plates (0.4 ml/well) coated with poly-L-lysine (5 μg/ml). Cells were cultured in high-glucose (25 mM) Dulbecco's modified Eagle medium containing 10% (v/v) fetal bovine serum, penicillin (100 U/ml), and streptomycin (100 μg/ml) at 37°C in humidified air with 7% CO_2. Cytosine arabinoside (10 μM) was added 72 h later to produce mitotic arrest of astroglia. Assays were done on 7- to 10-day old cultures. Nutrient medium remained untouched until experiments were undertaken.

2.4. Assay for cell viability

High KCl solution, veratridine and grayanotoxin III in 80–90% ethanol, or aconitine in absolute ethanol was directly added to the original nutrient medium of neuronal cultures. When inhibitors were used, cells were preincubated with them for 20–30 min prior to the addition of KCl or veratridine. CNQX, bepridil, and nifedipine were dissolved in DMSO and then added to the medium. Final concentration of ethanol was 1% v/v and that of DMSO was 0.2% v/v; at these concentrations, neither ethanol nor DMSO had harmful effects on cells (data not shown). Finally, cells that were treated with high KCl, veratridine, grayanotoxin III, or aconitine were kept at 37°C in humidified air with 7% CO_2 for 22–26 h until cell viability assay by MTT reduction.

The MTT assay for cell survival assessment was a modification (Hansen et al., 1989) of the original method by Mosmann (1983). One hundred microliters of sterile MTT solution (5 mg/ml in PBS) was added to each well (final concentration: 1 mg/ml). Cells were further incubated for 2 h for reduction of MTT to form formazan product. Preliminary experiments have shown that the amount of formazan formed increases linearly with the period of incubation time and in our system it reaches plateau within 2 h and remains at the same level up to 6 h of incubation (data not shown). The colored formazan products were extracted by overnight incubation with gentle shaking after addition of 400 μl 20% SDS in 50% *N,N*-dimethyl formamide at a pH of 4.7. After complete extraction, 100 μl of extraction from each well was transferred to a 96-well ELISA plate and the absorbance was measured on a Bio-Rad Benchmark Microplate Reader (Nippon Bio-Rad, Tokyo, Japan) at 570 nm.

In sister cultures, glucose concentrations in the medium were measured by Beckman Glucose Analyzer 2 (Beckman, Tokyo, Japan) after 24-h incubation with high KCl or veratridine.

2.5. Statistical analyses

The survival rates were calculated as percent absorbance (OD_{570}) of appropriate controls with vehicle. Results are expressed as means ± S.E.M. of quadruplicate wells; for each experiment at least two sets of assays were performed on different batches of cell preparations.

Statistical comparisons among the values obtained for each group were made by grouped *t*-test or One-way analysis of variance (ANOVA) followed by Dunnett's test for multiple group comparisons with a single control group. A *P*-value of < 0.05 was considered statistically significant.

3. Results

3.1. Effects of high K^+ and veratridine on glucose contents in medium

We have shown that both high K^+ (Takahashi et al., 1995b) and veratridine (Takahashi et al., 1994) stimulate glucose utilization as measured by rates of [^{14}C]deoxyglucose phosphorylation in cultured rat striatal neurons. Therefore, glucose in the medium had run out by the time of cell viability assay, thus energy failure might have obscured depolarization-induced neuronal cell death. To exclude this possibility, glucose concentration in the medium was directly measured. As shown in Fig. 1 (upper), both high K^+ and veratridine did indeed reduce glucose contents in the medium significantly after a period of 24 h incubation (Dunnett's test for multiple comparisons). Regarding to veratridine, however, it failed to reduce glucose contents at 100 μM, at which concentration, however, veratridine did indeed reduce MMT reduction activity (see below), indicating that cell death occurred regardless of preserved glucose. Maximal reduction in glucose concentration occurred at 25 mM KCl or at 10 μM veratridine, respectively. Nevertheless, the concentrations of remaining glucose were preserved over 320 mg/dl (18 mM) in either condition.

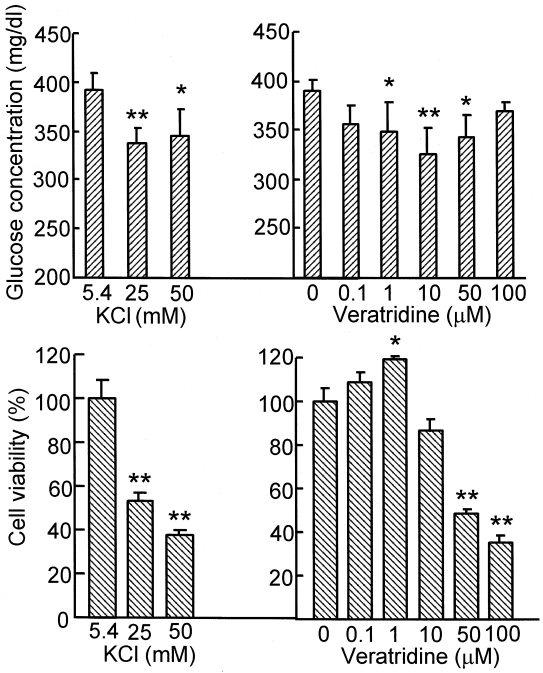

Fig. 1. Glucose concentration in the medium (upper) and percent cell viability (lower) after exposure to high extracellular K^+ and veratridine at indicated concentrations for 24 h. Values are means ± S.E.M. of quadruplicate wells. $^*P < 0.05$; $^{**}P < 0.01$ compared with each control (Dunnett's test for multiple comparisons).

3.2. Effects of high K^+ and veratridine on cell viability assayed by %MTT reduction

Preliminary experiments have verified that MTT reduction is proportional to the number of viable cells that are initially seeded in the plate over the range of $1–5 \times 10^6$ (data not shown). Furthermore, MTT reduction and cell damage induced by glutamate or *N*-methyl-D-aspartate (NMDA) (50–500 μM) assessed by morphological observation with inverted microscopy were well-correlated. Thus, %MTT reduction, which has been thought to be a reflection of mitochondrial activity (Mosmann, 1983; Musser and Oseroff, 1994), was interpreted as rates of cell survival, as expressed percent cell viability. Fig. 1 (lower) shows the effects of elevated $[K^+]_o$ and veratridine on neuronal cell viability. High K^+ elicited significant reduction in cell viability both at 25 and 50 mM ($P < 0.01$, Dunnett's test for multiple comparisons). In contrast, veratridine had a biphasic effect on percent cell survival (i.e.,

%MTT reduction activity) according to its concentrations. At lower concentrations (0.1–1 μM), it appeared to increase percent cell viability (significant increase at 1 μM, $P < 0.05$, Dunnett's test for multiple comparisons), indicating stimulatory effect of veratridine on MTT reduction activity, whereas at higher concentrations (10–100 μM) it decreased (significant decreases at 50 and 100 μM, $P < 0.01$, Dunnett's test for multiple comparisons).

To confirm that veratridine-induced cell damage was mediated by Na^+ influx through voltage-sensitive Na^+ channels, we used two other agents that also cause persistent activation of these Na^+ channels; aconitine and grayanotoxin III. Both of these toxins as well as veratridine act at the neurotoxin receptor site 2 of voltage-sensitive Na^+ channel (Catterall, 1992), and then keep it open, and thus, leading to Na^+ influx from extracellular space. As shown in Fig. 2, both aconitine and grayanotoxin III caused significant cell damage at 500 μM ($P < 0.01$) and 100 μM ($P < 0.05$), respectively.

3.3. Effects of tetrodotoxin on cell damage

Effect of tetrodotoxin, which blocks voltage-sensitive Na^+ channels reversibly (Catterall, 1980), was tested to further examine whether these channels are involved in the mechanism whereby depolarization causes neuronal damage. Firstly, effects of tetrodotoxin at different concentrations (0.001–10 μM) on veratridine-induced neuronal cell damage were examined; veratridine concentration was fixed at 100 μM. As illustrated in Fig. 3, tetrodotoxin showed its maximal protection against veratridine-induced cell death at 0.1 μM. At this concentration, tetrodotoxin showed almost complete protection, but at higher concentrations (1 and 10 μM) its protective effects rather decreased. Thus, we used tetrodotoxin of 0.1 μM in the following experiments.

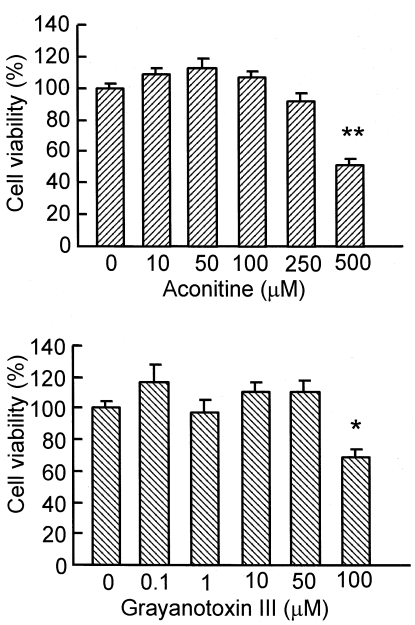

Fig. 2. Effects of aconitine (upper) and grayanotoxin III (lower) on percent viability at indicated concentrations. Values are means ± S.E.M. of quadruplicate wells. $^*P < 0.05$; $^{**}P < 0.01$ compared with each control (Dunnett's test for multiple comparisons).

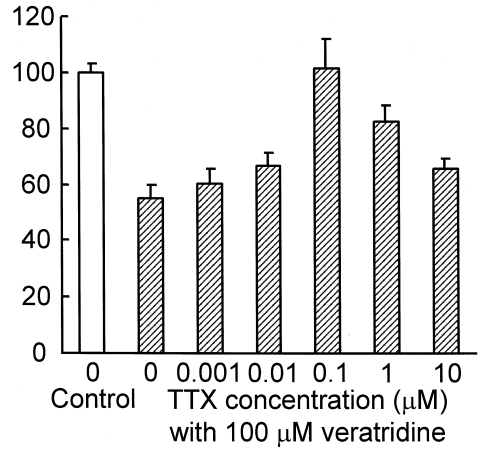

Fig. 3. Concentration effects of tetrodotoxin on veratridine-induced cell death. Values are means ± S.E.M. of quadruplicate wells. Open bar: no veratridine control; hatched bars: veratridine-treated groups (100 μM); TTX: tetrodotoxin.

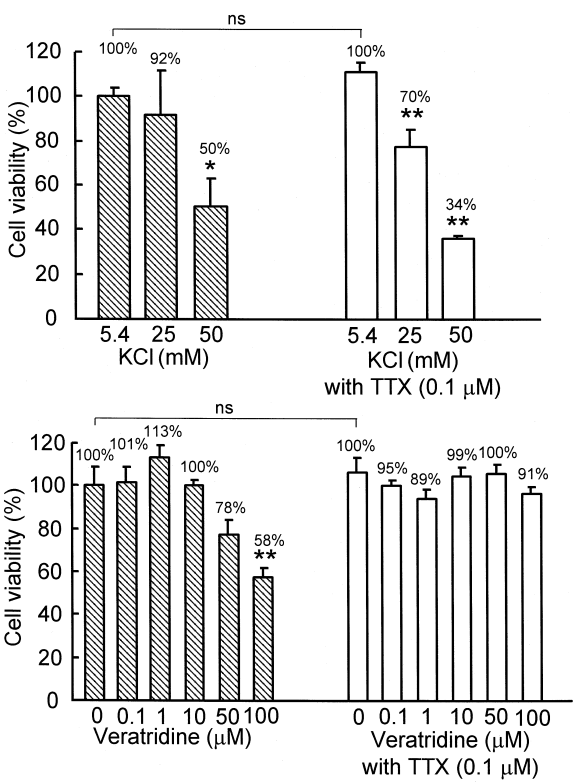

Fig. 4. Effects of tetrodotoxin (0.1 μM) on percent cell viability induced by high extracellular K^+ (upper) and veratridine (lower). Numbers above bars indicate percent cell survival of each control. Values are means ± S.E.M. of quadruplicate wells. TTX: tetrodotoxin; ns, not significantly different (grouped *t*-test). $^*P < 0.05$; $^{**}P < 0.01$ compared with each control (Dunnett's test for multiple comparisons).

As expected, veratridine-induced cell damage was protected completely by addition of 0.1 μM tetrodotoxin (Fig. 4, lower). At this concentration, however, tetrodotoxin did not alter percent cell survival under elevated $[K^+]_o$, indicating that Na^+ influx through voltage-sensitive Na^+ channels is not necessary to high K^+-induced cell damage (Fig. 4, upper).

3.4. Involvement of glutamate receptor overstimulation

Both veratridine and high $[K^+]_o$ depolarizes membrane potential. Depolarization triggers synaptic vesicular release of neurotransmitter under preserved cellular ATP concentration. Cell damage induced by elevated $[K^+]_o$ might be mediated by such intrinsic glutamate release from cultured neurons. To test this possibility, we applied NMDA or non-NMDA receptor antagonist. NMDA receptor antagonist, APV (1 mM) showed almost complete protection against high K^+-induced neuronal cell damage or rather enhanced %MTT reduction (Fig. 5, upper). By contrast, non-NMDA receptor antagonist, CNQX (10 μM) showed no protection (Fig. 5, lower). Veratridine facilitates Na^+ influx into neurons and results in consequent membrane depolarization, which might also lead to intrinsic glutamate release. Neither APV nor CNQX, however, protected veratridine-induced neuronal cell damage (Fig. 6), indicating that Na^+ influx causes cell death without involvement ionotropic glutamate receptors in our system.

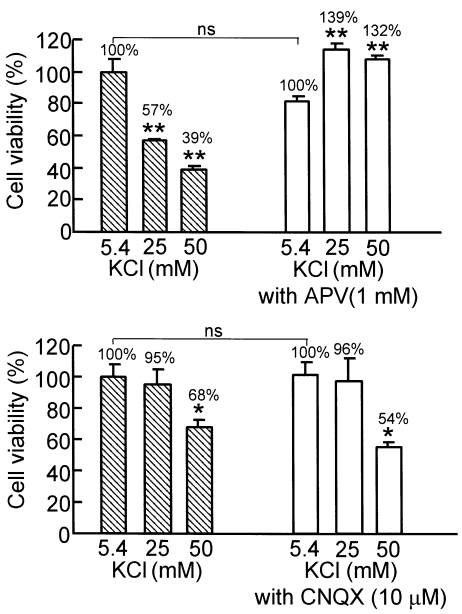

Fig. 5. Effects of APV (1 mM, upper) and CNQX (10 μM, lower) on high extracellular K^+-induced cell damage. Numbers above bars indicate percent cell survival from each control. Values are means ± S.E.M. of quadruplicate wells. ns: not significantly different (grouped *t*-test). $^*P < 0.05$; $^{**}P < 0.01$ compared with each control (Dunnett's test for multiple comparisons).

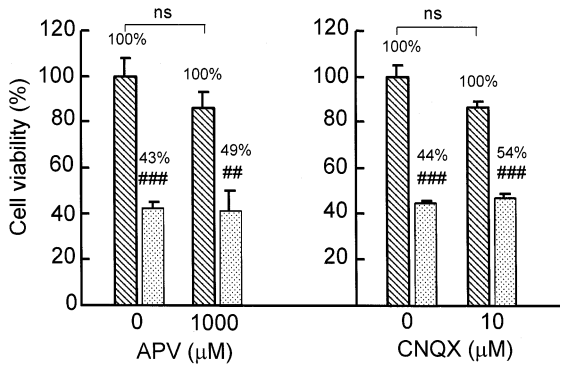

Fig. 6. Effects of APV (1 mM) and CNQX (10 μM) on veratridine-induced cell damage. Hatched bars: no veratridine controls; dotted bars: 100 μM veratridine. Numbers above bars indicate percent cell survival from each control. Values are means ± S.E.M. of quadruplicate wells. ns: not significantly different; ##, $P < 0.05$; ###, $P < 0.01$ as compared with each control (grouped *t*-test).

3.5. Veratridine-induced cell damage and Ca^{2+} influx

It has been established that Ca^{2+} influx into neuron is the pivotal stage of cell damage process (Choi, 1988, 1995; Ter Horst and Korf, 1997). Besides the entry of Ca^{2+} through ionotropic glutamate receptors, as examined above, at least three other pathways of Ca^{2+} influx associated with Na^+ influx have been proposed (Stys, 1998); (1) reverse operation of Na^+–Ca^{2+} exchanger, (2) voltage-dependent Ca^{2+} channels, and (3) non-selective Ca^{2+} entry through voltage-sensitive Na^+ channels (Edwards, 1982; Santana et al., 1998). Therefore, veratridine-induced cell damage could be mediated by secondary influx of Ca^{2+} from extracellular space. To test this possibility, we performed the following experiments.

Firstly, the effect of a Na^+–Ca^{2+} exchanger blocker, bepridil (Kaczorowski et al., 1989), was examined on veratridine-induced cell damage. As shown in Table 1, bepridil did not protect veratridine-induced cell death at indicated concentrations (0.05–5 μM). At higher concentrations (50–100 μM), however, it appeared to be harmful to our neurons and also enhanced cell damage. Within 30 min of addition of 50–100 μM of bepridil, for example,

Table 1
Effects of bepridil on veratridine-induced neuronal damage (percent cell survival)

	Bepridil concentration (μM)			
	0.00	0.05	0.5	5
No veratridine	100 ± 9	109 ± 8	81 ± 2	61 ± 11[a]
100 μM veratridine	45 ± 10[b]	51 ± 10[b]	42 ± 6[c]	35 ± 9
% control	45	47	52	57

Values are means ± S.E.M. of quadruplicate wells.
[a] $P < 0.01$ as compared with control (no veratridine, no bepridil) (Dunnett's test for multiple comparisons).
[b] $P < 0.01$, [c] $P < 0.001$ as compared with no veratridine control (grouped *t*-test).

Table 2
Effects of nifedipine on veratridine-induced neuronal damage (percent cell survival)

	Nifedipine concentration (μM)			
	0	10	50	100
No veratiridine	100±5	99±5	80±2[a]	67±2[b]
100 μM veratridine	44±2[e]	51±3[e]	62±3[d]	55±4[c]
% control	44	51	78	82

Values are means±S.E.M. of quadruplicate wells.
[a]$P < 0.05$, [b]$P < 0.01$ as compared with control (no veratridine, no nifedipine) (Dunnett's test for multiple comparisons).
[c]$P < 0.05$, [d]$P < 0.01$, [e]$P < 0.001$ as compared with each no veratridine control (grouped *t*-test).

neurons were severely damaged by microscopic observation (data not shown). These results did not support the positive role of Na^+–Ca^{2+} exchanger in veratridine-induced cell death.

Secondly, L-type voltage-dependent Ca^{2+} channel blocker, nifedipine (Janis et al., 1987), was tested. Nifedipine itself reduced cell viability dose-dependently (10–100 μM), but it also attenuated veratridine-induced percent cell damage (Table 2). These results support the involvement of Ca^{2+} influx in veratridine-induced neuronal damage.

Regarding the third possibility, we attempted to reduce the veratridine-induced cell damage by removing Ca^{2+} from the assay medium. Incubation with Ca^{2+}-free solution for 24 h, however, reduced cell viability significantly (by 30–40%, data not shown). Therefore, protective effect was not able to be evaluated precisely.

4. Discussion

The results of the present study have confirmed that increased $[K^+]_o$ and veratridine stimulates glucose consumption by cultured striatal neurons, as observed in the reduction of glucose contents in the medium. These results are in consistent with our previous observation of the stimulatory effects of elevated $[K^+]_o$ and veratridine on rates of [^{14}C]deoxyglucose phosphorylation in cultured neurons in a short term of period (Takahashi et al., 1994, 1995b). On the other hand, longer incubation under the same condition has caused significant neuronal cell damages despite preserved glucose contents in the medium. These results indicate that elevated $[K^+]_o$ and veratridine, both of which have already been confirmed to facilitate increased Na^+ influx in our system (Takahashi et al., 1996, 1997), cause neuronal cell death which is not associated with energy failure.

Numerous studies (Ashton et al., 1990; Pauwels et al., 1990; Schramm et al., 1990; Ramnath et al., 1992; Lakics et al., 1995; Maroto et al., 1996) as well as ours, but not all of them (Dargent et al., 1996), have reported neurotoxic effects of veratridine. For example, Dargent et al. (1996) have reported that veratridine does indeed have toxic effects on rat cerebellar granule cells while it has no harmful effects on neuronal cells prepared from cerebral hemisphere or striatum of fetal rats. They speculate that veratridine induced rapid disappearance of Na^+ channels from the surface of hemispheric or striatal cells; i.e., channel internalization (Dargent et al., 1994) based the observation of the [^{3}H]saxitoxin-binding assay on these cells. These results imply that the failure of veratridine-induced cell damage on striatal neurons is ascribed to absence of Na^+ influx into these cells. One can, therefore, reasonably speculate that Na^+ influx per se is responsible for neuronal cell damage induced by veratridine. In fact, in our system, not only veratridine but grayanotoxin III and aconitine caused neuronal damage, and tetrodotoxin completely abolished veratridine-induced neuronal damage. These results clearly demonstrates neurotoxic effects of Na^+ influx per se through voltage-sensitive Na^+ channels. Therefore, we further tried to define the mechanisms by which Na^+ influx leads to cell death.

Firstly, the involvement of glutamate receptor was examined because veratridine depolarizes membrane potential, and thus might induce intrinsic glutamate release. However, neither NMDA nor non-NMDA receptor antagonist protected veratridine-induced cell damage. These results are in accordance with those by others (Ramnath et al., 1992; Dargent et al., 1996), but not by Schramm et al. (1990) who have observed that NMDA receptor antagonist reduced the damage of cerebellar granule cells by 10 μM veratridine.

By contrast, it is of interest that high $[K^+]_o$-induced cell damage was completely protected by APV considering that both high $[K^+]_o$ and veratridine seem to facilitate Na^+ influx into our striatal neurons. Possibly it might be ascribed to differences in the duration of open state of voltage-sensitive Na^+ channels and in the extent of resultant increases in $[Na^+]_i$; veratridine causes persistent opening of these channels and increases in $[Na^+]_i$ in contrast to transient opening and moderate increases in $[Na^+]_i$ by high $[K^+]_o$ (Catterall, 1992; Rose and Ransom, 1997). Regarding high $[K^+]_o$-induced cell damage, therefore, depolarization-induced glutamate release rather than Na^+ influx played a more significant role. These results are in accordance to those by others (Schramm et al., 1990; Ramnath et al., 1992; Takahashi et al., 1995a).

Secondly, possible role of transmembrane Ca^{2+} influx was examined because Na^+ influx can also elicit Ca^{2+} influx by several different mechanisms. With regard to white matter damage under ischemic conditions, Ca^{2+} influx associated with Na^+ influx through voltage-sensitive Na^+ channels have been focused because the densities of Na^+ channels in axon are much higher than those in neuronal cell bodies (Stys, 1998). Thus far, there is not any cogent evidence that Na^+ influx causes cell death without concomitant flux of Ca^{2+}. Ca^{2+} influxes can occur as a result of (1) reverse operation of Na^+–Ca^{2+} exchanger,

(2) voltage-dependent Ca^{2+} channels, or (3) non-selective Ca^{2+} entry through voltage-sensitive Na^{+} channels. The present study does not support the first possibility because bepridil, Na^{+}–Ca^{2+} exchanger blocker (Kaczorowski et al., 1989), had no protective effect on veratridine-induced cell damage. Secondly, nifedipine (L-type Ca^{2+} channel blocker) had somehow protective effect, but not completely. Regarding the third possibility, it has been reported that Ca^{2+} influx can occur through voltage-sensitive Na^{+} channels and that veratridine even alters channel selectivity (Catterall, 1980; Edwards, 1982; Santana et al., 1998). Therefore, we tried to reduce veratridine-induced neuronal damage by Ca^{2+} removal, but the results were equivocal because of the toxic effects of Ca^{2+} free solutions. Lakics et al. (1995) have also reported that veratridine-induced cell death is independent of external Ca^{2+} concentration in accordance with our results, whereas Pauwels et al. (1990) have found that veratridine failed to produce lactate dehydrogenase release (i.e., cell damage) in the absence of exracellular Ca^{2+}. In our system, L-type Ca^{2+} channel showed only partial protection against veratridine-induced cell death. Therefore, some other mechanisms must operate subsequent to veratridine-induced Na^{+} influx. Involvement of Ca^{2+}, especially release of Ca^{2+} from internal storage cannot be denied. Notwithstanding partial protection by nifedipine or tetrodotoxin against veratridine-induced neuronal cell death, the present study still support the therapeutic usage of voltage-dependent Ca^{2+} and Na^{+} channel blockers as well as NMDA receptor antagonist against neuronal damage under depolarizing conditions such as ischemia, hypoglycemia, or epilepsy.

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