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Astroglial cell death induced by excessive influx of sodium ions

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Abstract

Na^+ influx has been implicated to play an important role in the mechanisms of neuronal cell damage under ischemia as well as in neurodegenerative disorders. Thus far, however, the effects of Na^+ influx on astrocytic damage have not been studied extensively. In the present study, we have examined the effects of Na^+ influx induced by veratridine (Na^+ channel opener), monensin (Na^+ ionophore), and glutamate (co-transportation with Na^+) on rat cultured astroglial damage. Cells were incubated with bicarbonate buffer with 25 mM glucose containing either 100 μM veratridine, 10 μM monensin, or 1 mM glutamate with or without 1 mM ouabain for 20 h. Cellular damage was evaluated quantitatively by lactate dehydrogenase (LDH) release or 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction. Veratridine, monensin, or glutamate alone did not induce significant astroglial damage. Veratridine and monensin co-incubated with ouabain, which inhibits active extrusion of Na^+ by Na^+,K^+-ATPase, thereby enhances intracellular Na^+ accumulation, caused significant cell death ($P < 0.001$, approximately 50% cell damage), whereas glutamate did not. Na^+-free solution substituted by choline (impermeable cation) attenuated cell damage induced by veratridine and monensin markedly, while Li^+ substitution (permeable cation) rather exacerbated. Nifedipine (100 μM), a blocker of L-type Ca^{2+} channel, reduced veratridine-induced glial damage by 50%. Neither bepridil nor benzamil, a blocker of Na^+–Ca^{2+} exchanger, had any protection. Cyclosporin A (1 or 10 μM), an inhibitor of mitochondrial permeability transition or 10 μM *N*-benzyloxycarbonyl-Val-Ala-Asp-(*O*-methyl)fluoromethyl ketone (zVAD-fmk), which inhibits a broad range of caspases, did not show protective effects.

Keywords: Astrocyte; Ca^{2+}; LDH (lactate dehydrogenase); Mitochondrion; MTT; Na^+,K^+-ATPase; Ouabain

1. Introduction

We previously reported that both veratridine and elevated extracellular K^+ concentration ($[K^+]_o$) elicit increases in the rates of [^{14}C]deoxyglucose phosphorylation (Takahashi et al., 1995) in association with facilitated $^{22}Na^+$ influx into cultured neurons prepared from rat fetal striatum in a tetrodotoxin-sensitive manner (Takahashi et al., 1997). Veratridine causes persistent opening of voltage-sensitive Na^+ channels (Li and White, 1977; Catterall, 1980, 1992) and elevated $[K^+]_o$ also opens these channels by depolarizing the membrane potential. The consequent increases in intracellular Na^+ concentration ($[Na^+]_i$) and/or $[K^+]_o$ stimulate Na^+,K^+-ATPase (enzyme commission 3.6.1.37) that maintains normal transmembrane ionic gradients by consuming approximately one half or more of total ATP generated in the brain at a resting state (Erecinska and Silver, 1989; Clarke and Sokoloff, 1994). Glucose is a sole energy source in the brain. Therefore, both veratridine and increasing $[K^+]_o$ enhance glucose utilization as measured by rates of [^{14}C]deoxyglucose phosphorylation (Sokoloff et al., 1977), like an action potential consisting of rapid Na^+ influx through voltage-sensitive Na^+ channels and delayed efflux of K^+ (Hodgkin and Huxley, 1952) does in association with neuronal activation (Mata et al., 1980; Sokoloff, 1981, 1994; Kadekaro et al., 1985). Interestingly, however, a sustained exposure to veratridine or elevated $[K^+]_o$ causes significant neuronal cell death even with abundant glucose supply (Takahashi et al., 1999).

Under cerebral ischemia, energy failure results in loss of Na^+,K^+-ATPase activity, leading to the elevation of $[K^+]_o$ and membrane depolarization (anoxic depolariza-

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tion). Then, membrane depolarization triggers extracellular glutamate release, which plays a pivotal role in ischemic 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 the overstimulation of ionotropic glutamate receptors (Lynch et al., 1995; Stys, 1998). Therefore, blockade of Na^+ influx through voltage-sensitive Na^+ channels can be a potential therapeutic target for ischemic brain damage (Taylor and Meldrum, 1995; Stys, 1998). It is generally believed that astrocytes are less susceptible to ischemic injury and subsequent metabolic inhibition than neurons are (Goldberg and Choi, 1993). This might be related to less Na^+ influx into astrocytes as compared with neurons. In contrast to neurons, astrocytes do not generate action potentials upon membrane depolarization (Barres, 1991; Sontheimer, 1994) even though voltage-sensitive Na^+ channels are also expressed in their membrane (Sontheimer and Ritchie, 1995). In fact, veratridine does indeed elicit increased $^{22}Na^+$ influx into cultured astroglia (Bowman et al., 1984; Takahashi et al., 1997) and further causes increased rates of [^{14}C]deoxyglucose phosphorylation in astroglia (Takahashi et al., 1995). Moreover, Na^+ ionophore monensin, which promotes exchange of extracellular Na^+ and intracellular H^+ (Pressmann and Fahim, 1982) stimulates rates of [^{14}C]deoxyglucose phosphorylation (Yarowsky et al., 1986; Takahashi et al., 1995) both in cultured rat neurons and astroglia. Furthermore, glutamate also stimulates glucose utilization in astroglia, but this effect appears to be mediated by an increased $[Na^+]_i$ secondary to the coupled uptake of Na^+ and glutamate via sodium-dependent glutamate transporters (Pellerin and Magistretti, 1995; Takahashi et al., 1995). Taken together, intracellular Na^+ concentration regulates glucose metabolism both in cultured rat neurons and astroglia and plays a key role in neuronal cell damage. Thus far, however, direct effects of Na^+ influx on astrocytic cell damage have not been focused. Therefore, we addressed the question whether astrocytes are more resistant to Na^+ influx than neurons are. Increasing amount of evidence indicates that astrocytes play important roles in the survival of neurons. Therefore, protection of astrocytes under acute or chronic neurotoxic conditions contributes to neuronal cell survival. In the present study, we have examined the effects of Na^+ influx induced by veratridine (Na^+ channel opener), monensin (Na^+ ionophore), and glutamate (co-transportation with Na^+) on survival of rat cultured astroglia. Cellular damage was evaluated quantitatively by two different methods; the release of cellular lactate dehydrogenase (LDH, enzyme commission 1.1.1.27), reflecting membrane permeability and lysis (Koh and Choi, 1987) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction, a putative indicator of mitochondrial injury, thus cellular viability (Musser and Oseroff, 1994). The abstract has appeared previously (Takahashi et al., 1998).

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 and materials 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); *N*-benzyloxycarbonyl-Val-Ala-Asp-(*O*-methyl) fluoromethyl ketone (zVAD-fmk) from Enzyme Systems (Livermore, CA, USA); all other chemicals from Sigma (St. Louis, MO, USA).

2.3. Preparation of cells

Recently, we have reported the toxic effects of Na^+ influx on cultured neurons prepared from the striatum of fetal rats on embryonic day 16 (Takahashi et al., 1999). To make a direct comparison of the behavior of astrocytes to intracellular Na^+ influx with that of the neurons, astroglia were also prepared from the striatum of fetal rats on embryonic day 16. The detailed procedure was described previously (Takahashi et al., 1995). Briefly, striatal tissue was excised and mechanically disrupted by passage through a 22-gauge needle. Viable cells (2.5×10^5 cells/ml) that excluded trypan-blue were plated in uncoated 75 cm^2 culture flasks and 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. Culture medium was changed every 2 or 3 days until the cultures reached confluence. The flask was then shaken overnight at 37°C to eliminate loosely adherent round cells, conceivably O2A progenitors and/or microglia. The adherent cells were treated with trypsin-EDTA solution diluted 1:5 with Dulbecco's phosphate-buffered saline without Ca^{2+} and Mg^{2+} (PBS) for 1–2 min at 37°C, suspended in four volumes of fresh culture medium, and placed in uncoated 24-well culture plates for cell death assay. It should be noted that these were secondary astrocyte cultures. Culture medium was changed every 3 days, and the cultures were used when confluent (day 28 ± 7).

2.4. Experimental protocol

Just prior to the application of veratridine, monensin, or glutamate, nutrient medium was removed and cell carpets

were washed twice with prewarmed PBS supplemented with 25 mM D-glucose (i.e., the same glucose concentration as the nutrient medium). Then, 0.4 ml of standard Na^+ solution containing 110 mM NaCl, 5.4 mM KCl, 1.8 mM $CaCl_2$, 0.8 mM $MgSO_4$, 0.9 mM NaH_2PO_4, 44 mM $NaHCO_3$, and 25 mM D-glucose with or without 1 mM ouabain and various inhibitors, which had been prewarmed at 37°C and equilibrated with 7% CO_2 for adjustment of pH to 7.4, was added to each well. Na^+–Ca^{2+} exchanger inhibitor, bepridil or benzamil (Kaczorowski et al., 1989), L-type voltage-gated Ca^{2+} channel inhibitor, nifedipine (Janis et al., 1987), and a caspase inhibitor, zVAD-fmk, were dissolved in dimethyl sulfoxide (DMSO), and then added to the assay solution during the preincubation period of 30–60 min. Final concentration of DMSO was 0.2% v/v; at this concentration, DMSO had no harmful effects on cells (data not shown). To examine the effects of Na^+ removal, Na^+ was replaced with choline (impermeable cation) and/or Li^+ (permeable cation) in some experiments. Detailed ionic compositions were described in Table 1. After preincubation period with salt solution with or without inhibitors, veratridine or monensin dissolved in 80–90% ethanol or glutamate in water was added. Cells were kept at 37°C in humidified air with 7% CO_2 for 20 h until cell viability assay.

2.5. Evaluation of cell damage by LDH release and MTT reduction activity

Cellular damage was evaluated quantitatively by colorimetric assay based on the conversion of tetrazolium salt, MTT to blue formazan, and was confirmed by the amount of LDH released into the solution. LDH activity was measured by a method based on the simultaneous reduction of NAD^+ to NADH through the conversion of lactate to pyruvate in the presence of LDH. Five μl of supernatant was transferred to each well of 96-well ELISA plate and the enzymatic reaction was begun with the addition of 100 μl of reaction mixture supplied with Sigma LDH diagnostic kit (LD-L 10, Asuka-Sigma, Tokyo, Japan). The reaction was run at room temperature with light-protection for 60 min and was terminated by the addition 12 μl of LDH inhibitor, oxamate (15 mM in final concentration). The addition of oxamate was regulated in the same order and speed as the addition of reaction mixture to ensure the equal reaction times. Produced NADH in each well was measured by absorbance at a wavelength of 340 nm (OD_{340}) by Bio-Rad Benchmark Microplate Reader (Nippon Bio-Rad, Tokyo, Japan). Four wells of appropriate control from sister culture were treated with 0.4 ml of 1% Triton-X 100 in PBS and the average of LDH activities (OD_{340}) of quadruplicate wells was considered as maximal LDH release. In previous experiments, it was found that the variability in maximal LDH release among different wells in a given batch was sufficiently small (i.e., coefficient of variation = 5%) that released LDH activity from each well was divided by maximal LDH release and was considered as percent cell death in each well.

MTT reduction assay for cell survival assessment, a putative marker of cellular redox state reflecting mitochondrial activity (Musser and Oseroff, 1994), was a modification (Hansen et al., 1989) of the original method by Mosmann (1983). After 5 μl aliquots for LDH assay were sampled, assay solution was gently sucked-up, and then 0.2 ml of MTT assay solution (1 mg/ml in PBS) supplemented with 25 mM glucose was added to each well at the point of cell death evaluation. 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 2–3 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 after addition of 0.2 ml 20% sodium dodecyl sulfate in 50% *N*,*N*-dimethyl formamide adjusted to 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 at 570 nm. The survival rates were calculated as percent absorbance (OD_{570}) of appropriate control with vehicle, then (100-survival rate) was thought to be percent cell death in each well.

Table 1
Ionic composition of assay solutions (mM)

	Standard Na^+	Na^+ removal	
		Choline substitution	Lithium substitution
NaCl	110	–	–
Choline chloride	–	110	–
LiCl	–	–	110
$NaHCO_3$	44	–	–
Choline bicarbonate	–	44	44
NaH_2PO_4	0.9	–	–
KH_2PO_4	–	0.9	0.9
KCl	5.4	5.4	5.4
$CaCl_2$	1.8	1.8	1.8
$MgSO_4$	0.8	0.8	0.8
Glucose	25	25	25

2.6. Statistical analyses

Statistical comparisons among the values of percent cell death 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, when applicable. A *P*-value of < 0.05 was considered statistically significant. For each experiment, at least three sets of assays were performed on

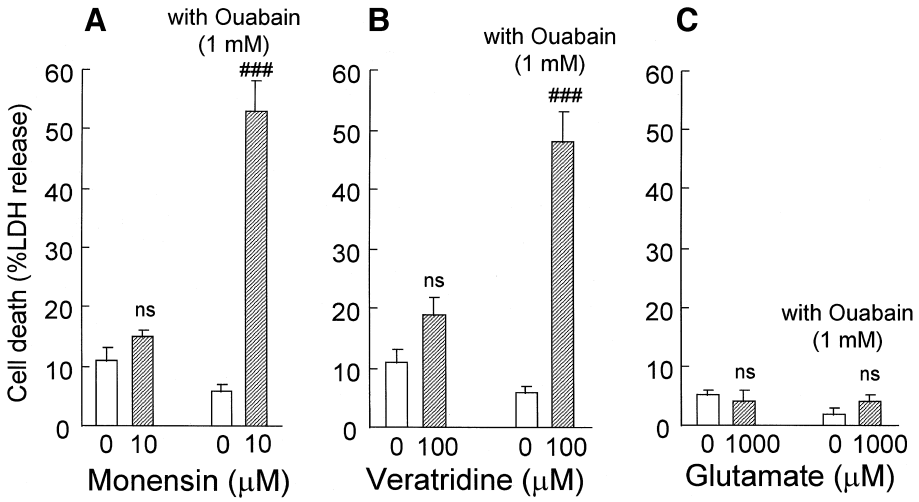

Fig. 1. Effects of monensin (A), veratridine (B), and glutamate (C) with or without 1 mM ouabain on astroglial cell death (%) as assayed by LDH release after 20 h. Values are means ± S.E.M. of quadruplicate wells. Open bars, vehicle only; hatched bars, monensin (A), veratridine (B), and glutamate (C) at indicated concentrations. ns, Not significant; ###, $P < 0.001$ compared with each control (grouped *t*-test).

different batches of cell preparations, and a set of representative data was presented in each figure or table.

3. Results

3.1. Effects of monensin, veratridine, and glutamate on astroglial damage

Fig. 1 shows astroglial cell damage as assayed by LDH release. Monensin (10 μM), veratridine (100 μM), or glutamate (1 mM) alone did not cause statistically significant cellular damage. Veratridine and monensin co-incubated with 1 mM ouabain, which inhibits active extrusion of Na^+ by Na^+,K^+-ATPase, thereby enhances intracellular Na^+ accumulation, caused significant cell death ($P < 0.001$, 48% and 53%, respectively), whereas glutamate did not. Ouabain at this concentration, which is sufficient to inhibit astroglial Na^+,K^+-ATPase (Walz and Hertz, 1982), did not have any deleterious effects on cultured astroglia for a period of 20-h incubation.

Time course of cellular damage was evaluated by both LDH release and MTT assay. Fig. 2 shows astroglial cell death up to 38-h incubation with monensin (10 μM) or veratridine (100 μM) co-incubated with 1 mM ouabain assayed by the two methods. Both monensin and veratridine caused astroglial cell damage linearly with the incubation period. Note that percent cell death assayed by MTT is greater than that by LDH release at each time point,

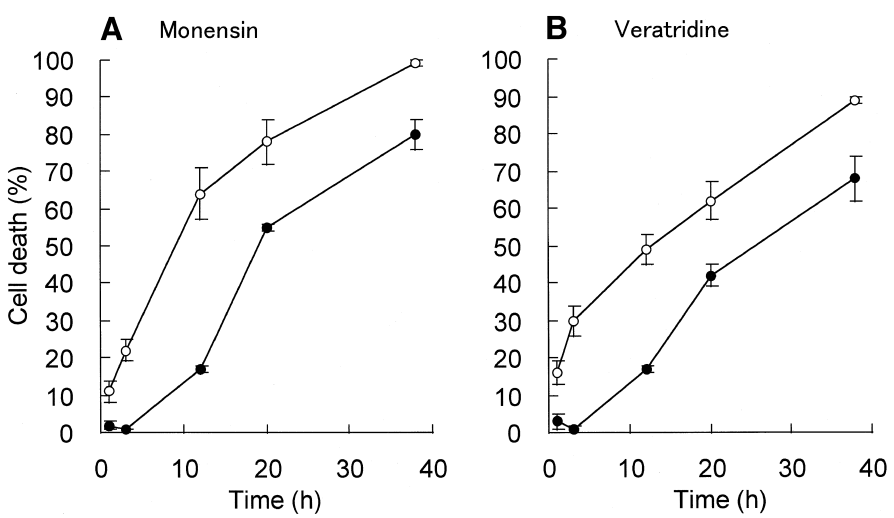

Fig. 2. Time course of astroglial cell damage (%) induced by 10 μM monensin (A) and 100 μM veratridine (B) co-incubated with 1 mM ouabain. Values are means ± S.E.M. of quadruplicate wells. Open circles, assayed by MTT reduction; closed circles, assayed by LDH release.

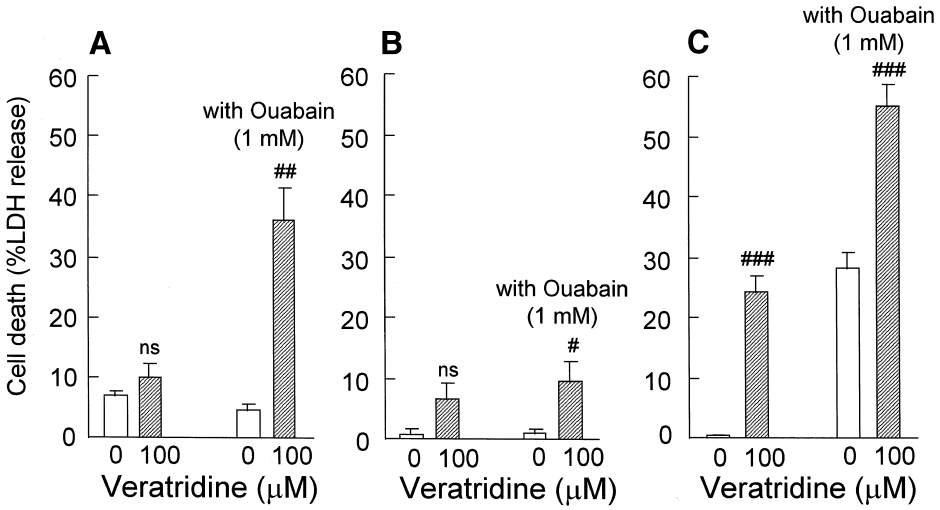

Fig. 3. Veratridine-induced astroglial cell death under standard Na^+ concentration (A), choline substitution (B), and Li^+ substitution (C) with or without 1 mM ouabain. Values are means ± S.E.M. of quadruplicate wells. Open bars, vehicle only; hatched bars, veratridine at indicated concentration. ns, Not significant; #, $P < 0.05$; ##, $P < 0.01$; ###, $P < 0.001$ compared with each control (grouped *t*-test).

indicating that mitochodrial dysfunction may precede disruption of cellular cytoplasmic membrane.

3.2. Effects of Na^+ removal on veratridine- or monensin-induced astroglial cell death

To examine whether Na^+ influx per se causes astroglial cell death when co-inbubated with ouabain, Na^+ was replaced with two cations, i.e., choline (impermeable) and Li^+ (permeable). Both veratridine- and monensin-induced astroglial damages were suppressed markedly by choline substitution (Figs. 3B and 4B). Li^+ substitution, however, rather exaggerated cellular damage (Figs. 3C and 4C).

3.3. Role of L-type Ca^{2+} channels on astroglial cell death induced by Na^+ influx

The above-described results indicate that influx of cationic ions plays a key role in the astroglial cell death in this model. Irrespective of their entry routes, i.e., voltage-sensitive Na^+ channels or Na^+ ionophore, once cations enter into astroglia, membrane depolarization follows. It has been established that Ca^{2+} influx plays a central role in the neuronal cell death process (Choi, 1988, 1995). Astrocytes express various kinds of Ca^{2+} channels (MacVicar, 1984; Sontheimer, 1994). Therefore, the secondary influx of extracellular Ca^{2+} through voltage-gated Ca^{2+}

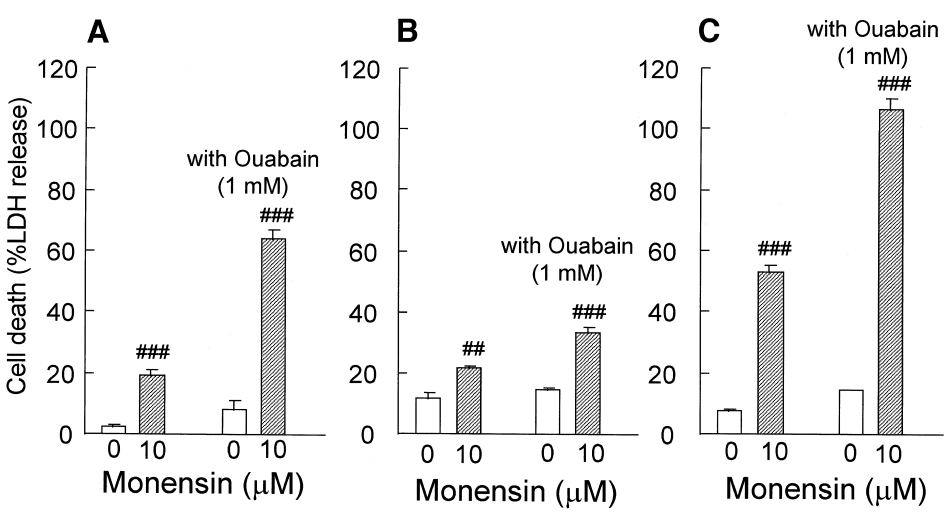

Fig. 4. Monensin-induced astroglial cell death under standard Na^+ concentration (A), choline substitution (B), and Li^+ substitution (C) with or without 1 mM ouabain. Values are means ± S.E.M. of quadruplicate wells. Open bars, vehicle only; hatched bars, monensin at indicated concentration. ##, $P < 0.01$; ###, $P < 0.001$ compared with each control (grouped *t*-test).

Table 2
Effects of nifedipine on veratridine- and monensin-induced astroglial damage (% cell death)

	Nifedipine concentration (μM)		
	0	10	100
No veratridine	7 ± 1	4 ± 1	10 ± 1
100 μM veratridine	65 ± 4[a]	48 ± 2[a]	34 ± 8[b,c]
No monensin	5 ± 1	1 ± 0	2 ± 2
10 μM monensin	58 ± 5[d]	46 ± 1[d]	44 ± 5[d]

Values are means ± S.E.M. of quadruplicate wells.
[a] $P < 0.001$ as compared with no veratridine control at each concentration of nifedipine (grouped *t*-test).
[b] $P < 0.05$ as compared with no veratridine control at each concentration of nifedipine (grouped *t*-test).
[c] $P < 0.01$ as compared with no nifedipine with 100 μM veratridine (Dunnett's test for multiple comparisons).
[d] $P < 0.001$ as compared with no monensin control at each concentration of nifedipine (grouped *t*-test).

channels upon membrane depolarization might also be essential to astroglial death. Thus, protective effect of the L-type Ca^{2+} channel inhibitor, nifedipine (Janis et al., 1987) was tested. As shown in Table 2, nifedipine inhibited veratridine-induced astroglial damage dose-dependently. At 100 μM, it suppressed veratridine-induced cell damage approximately by half. Nifedipine also showed protective tendency against monensin-induced astroglial death, but did not reach statistical significance.

3.4. Role of Na^+–Ca^{2+} exchanger on astroglial cell death induced by Na^+ influx

Another mechanism that has been proposed for extracellular Ca^{2+} entry accompanied with intracellular Na^+ accumulation is a reverse operation of Na^+–Ca^{2+} exchanger (Stys, 1998). Two inhibitors of Na^+–Ca^{2+} exchanger, bepridil and benzamil (Kaczorowski et al., 1989) were applied. As shown in Table 3, neither of these was protective against veratridine- or monensin-induced astroglial death.

3.5. Effects of inhibition of mitochondrial permeability transition and caspase

Recently, mitochondrial dysfunction has been focused as a trigger of various types of cell damage. In the present study, enhanced Na^+ influx under loss of Na^+,K^+-ATPase function caused diminished MTT reduction activity and eventually led to cytoplasmic membrane disruption as described above. MTT reduction is a reflection of a cellular redox state, thus mitochondrial function. In this context, we examined the effects of cyclosporin A that inhibits mitochondrial permeability transition (MPT) (Gunter and Pfeiffer, 1990), and a caspase inhibitor, zVAD-fmk, that acts downstream or upstream of MPT-related cell death (Keller et al., 1998) on monensin-induced astroglial death.

Table 3
Effects of Na^+–Ca^{2+} exchanger inhibitors on veratridine- and monensin-induced astroglial damage (% cell death)

	Bepridil concentrations (μM)			Benzamil concentrations (μM)		
	0	10	100	0	50	100
No veratridine	7 ± 2	22 ± 1	91 ± 4	5 ± 1	11 ± 1	35 ± 2
100 μM veratridine	52 ± 6[a]	55 ± 4[a,b]	100 ± 4[b]	51 ± 3[a]	77 ± 2[a,c]	86 ± 4[a,c]
No monensin	5 ± 1	17 ± 1	78 ± 3	5 ± 1	11 ± 1	35 ± 2
10 μM monensin	58 ± 5[d]	80 ± 4[d,e]	86 ± 4[e]	58 ± 5[d]	73 ± 6[d]	62 ± 4[f]

Values are means ± S.E.M. of quadruplicate wells.
[a] $P < 0.001$ as compared with no veratridine control at each concentration of bepridil or benzamil (grouped *t*-test).
[b] $P < 0.01$ as compared with no bepridil with 100 μM veratridine (Dunnett's test for multiple comparisons).
[c] $P < 0.01$ as compared with no benzamil with 100 μM veratridine (Dunnett's test for multiple comparisons).
[d] $P < 0.001$ as compared with no monensin control at each concentration of bepridil or benzamil (grouped *t*-test).
[e] $P < 0.01$ as compared with no bepridil with 10 μM monensin (Dunnett's test for multiple comparisons).
[f] $P < 0.01$ as compared with no monensin control at each concentration of bepridil or benzamil (grouped *t*-test).

Table 4
Effects of cyclosporin A and zVAD-fmk on veratridine- and monensin-induced astroglial damage (%cell death)

	Cell death assayed by LDH release (%)			Cell death assayed by MTT reduction (%)		
	Vehicle	Veratridine (100 μM)	Monensin (10 μM)	Vehicle	Veratridine (100 μM)	Monensin (10 μM)
Control	5 ± 0	20 ± 3	38 ± 3	0 ± 4	36 ± 4	72 ± 3
DMSO	3 ± 3	19 ± 2	40 ± 2	0 ± 3	26 ± 3	69 ± 2
Cyclosporin A (1 μM)	9 ± 0	19 ± 1	39 ± 4	0 ± 4	30 ± 3	81 ± 2[a]
Cyclosporin A (10 μM)	8 ± 1	22 ± 1	53 ± 12	6 ± 4	36 ± 5	83 ± 2[a]
vZAD-fmk (10 μM)	14 ± 1[b]	23 ± 3	36 ± 1	1 ± 1	32 ± 2	80 ± 2

Values are means ± S.E.M. of quadruplicate wells.
[a] $P < 0.05$ as compared with monensin-treated control without inhibitors (Dunnett's test for multiple comparisons).
[b] $P < 0.01$ as compared with vehicle-treated control without inhibitors (Dunnett's test for multiple comparisons).

As shown in Table 4, however, cyclosporin A (1 or 10 μM) or zVAD-fmk (10 μM) did not have any protection against monensin- or veratridine-induced astroglial death.

4. Discussion

It has been known that astrocytes are more resistant to ischemic insult than neurons are. At present, however, the exact mechanisms of astroglial resistance to ischemia remain to be elusive (Goldberg and Choi, 1993). One possible explanation is that the extent of Na^+ influx to astrocytes is less than that to neurons because of lower density of voltage-sensitive Na^+ channels expressed in astrocytes (Sontheimer and Ritchie, 1995). In fact, veratridine that opens these channels did not cause astroglial damage, while it causes neuronal cell damage at the same concentration after 20-h incubation (Takahashi et al., 1999). Nevertheless, the present study does not necessarily support this idea because astroglia were equally resistant to monensin-induced Na^+ influx that occurs without the presence of voltage-sensitive Na^+ channels. Conversely, veratridine did indeed elicit astroglial death when Na^+,K^+-ATPase activity was inhibited by ouabain. These observations are in sharp contrast to those that cultured neuron are quite vulnerable to veratridine and monensin even without an inhibition of Na^+,K^+-ATPase (Catterall, 1980; Takahashi et al., 1999). A novel finding of the present study is that astroglia have strong resistance to Na^+ influx only when Na^+,K^+-ATPase activity is maintained. Under complete ischemia, ATP production halts and Na^+,K^+-ATPase dysfunction follows. In the area of penumbra, however, glucose supply is relatively maintained in contrast to lack of oxygen supply. Therefore, ATP production continues through anaerobic glycolysis (Obrenovitch, 1995). In fact, in astrocytes, glycolytic energy production predominates oxidative phosphorylation (Robinson et al., 1998). This notion also supports the idea that astrocytes are more resistant to ischemia than neurons because astrocytic Na^+,K^+-ATPase can operate and keep extruding sodium ions actively (Swanson et al., 1997). Only when ATP synthesis is completely abolished, Na^+,K^+-ATPase will stop functioning. An application of ouabain, a specific inhibitor of Na^+,K^+-ATPase, stimulates such conditions. Surprisingly, however, the present results indicate that astrocytes can survive for more than 20 h even when Na^+,K^+-ATPase activity is completely inhibited unless mandatory Na^+ influx is induced (i.e., co-application of veratridine or monensin).

It has been reported that excessive intracellular Na^+ influx causes astrocytic cell death by several other investigators. Recently, David et al. (1996) have found that α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA) receptor agonist, which also elicits Na^+ influx, causes acute astrocytic cell death only when an inhibitor of AMPA receptor desensitization, cyclothiazide was used. They have also found that AMPA receptor agonist with cyclothiazide elicits Ca^{2+} influx into astrocytes and they speculate that this Ca^{2+} influx plays an important role in the cell death mechanisms. However, the precise entry route of Ca^{2+} or the downstream pathway to cell death has not yet been clarified. Matsuda et al. (1996, 1998) have focused delayed astroglial death induced by exposure to low extracellular Ca^{2+} condition. They have found that transient exposure to low extracellular Ca^{2+} condition accelerates the forward mode of Na^+–Ca^{2+} exchange and leads to increased $[Na^+]_i$. They have concluded that these increases in $[Na^+]_i$, then, induce the reverse operation of Na^+–Ca^{2+} exchange and finally lead to cell death by increased intracellular Ca^{2+} concentration. The present study, however, did not support the significant role of the reverse operation of Na^+–Ca^{2+} exchanger at least in acute phase of astroglial cell death by increased $[Na^+]_i$. Our results rather indicate a possible role of L-type voltage-dependent Ca^{2+} channels in Na^+-induced astrocytic death because nifedipine showed a partial inhibition of cell death by veratridine and monensin. We speculate that membrane depolarization by initial Na^+ influx causes secondary activation of voltage-dependent Ca^{2+} channels. However, nifedipine even at the highest concentration (100 μM, see results) showed only partial inhibition. In addition to L-type, other voltage-dependent Ca^{2+} channels might be involved. In fact, anoxic injury of cultured astrocytes is only partly dependent on Ca^{2+} influx via L-type channels (Haun et al., 1992) and a role of T-type as well as L-type channels has been reported (Fern, 1998). Another possible mechanism by which nifedipine inhibited veratridine-induced astroglial death in the present study is that nifedipine at high concentration might have acted on voltage-sensitive Na^+ channels and inhibited either Na^+ entry or nonselective Ca^{2+} entry (Catterall, 1980; Edwards, 1982) or both through these channels. Pauwels et al. (1990) have reported that nifedipine inhibits both [^{3}H] Batrachotoxinin A 20-α-benzoate binding and veratridine-induced cell death, indicating that Na^+ channels can be blocked by a Ca^{2+} channel blocker. Nevertheless, the exact route of Ca^{2+} entry and further downstream pathway after Ca^{2+} entry to astrocytic cell death should be examined.

Matsuda et al. (1998) have found that this form of delayed glial cell death is at least partially apoptotic and can be protected by FK506 and cyclosporin A, both of which acted as the calcineurin inhibitor. The acute astroglial death in the present study was not protected by cyclosporin A or zVAD-fmk, a caspase inhibitor. Ample evidence has been accumulated to support that mitochondrial dysfunction triggers caspase-medicated neuronal cell death in various neurological disorders (Green and Reed, 1998). In contrast, however, the exact roles of mitochondrial dysfunction and/or caspase-mediated cell death in astroglial cell damage remain to be clarified.

In our study, glutamate alone or even with ouabain did not induce astroglial damage. Astroglia take up glutamate

extensively by Na^+-dependent co-transporters: i.e., two molecules of Na^+ is co-transported with one molecule of glutamate (Pines et al., 1992; Storck et al., 1992). Thus, the exact reason why such Na^+ load associated with glutamate application did not induce glial damage with the inhibited extrusion of Na^+ by Na^+,K^+-ATPase is not readily understood. This passive transportation system is dependent on steep inwardly-directed Na^+ gradient across cytoplasmic membrane. Therefore, one possible explanation is that glutamate transport per se is slowed when Na^+,K^+-ATPase is inhibited, because gradual increases in $[Na^+]_i$ occurs spontaneously when ouabain is applied (Rose et al., 1998); thus, glutamate-induced excessive Na^+ influx may not occur. In fact, it has been reported that glutamate uptake by astrocytes is markedly reduced under energy failure like ischemic conditions (Attwell et al., 1993), which exacerbates increases in glutamate concentration in extracellular space, then leads to neuronal damage, but maybe negligible astroglial damage because of lack of Na^+ influx into astroglia.

In addition to increases in extracellular glutamate concentration, increasing $[K^+]_o$ occurs following Na^+,K^+-ATPase dysfunction under metabolic suppressions. In neurons, increased $[K^+]_o$ elicits Na^+ influx through voltage-sensitive Na^+ channels, because elevated $[K^+]_o$ depolarizes cell membrane, thereby opens these channels. However, in astroglia, high KCl application does not elicit Na^+ influx or rather seems to suppress it (Takahashi et al., 1997). These results are also in accordance with the fact that astroglia are resistant to ischemic conditions because excessive Na^+ influx is not induced by increases in either extracellular K^+ or glutamate concentration.

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