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Short- and long-term differential effects of neuroprotective drug NS-7 on voltage-dependent sodium channels in adrenal chromaffin cells

[1]Hiroki Yokoo, [1]Seiji Shiraishi, [1]Hideyuki Kobayashi, [1]Toshihiko Yanagita, [1]Shin-ichi Minami, [1]Ryuichi Yamamoto & *,[1]Akihiko Wada

[1]Department of Pharmacology, Miyazaki Medical College, Miyazaki 889-1692, Japan

1 In cultured bovine adrenal chromaffin cells, NS-7 [4-(4-fluorophenyl)-2-methyl-6-(5-piperidinopentyloxy) pyrimidine hydrochloride], a newly-synthesized neuroprotective drug, inhibited veratridine-induced $^{22}Na^+$ influx *via* voltage-dependent Na^+ channels ($IC_{50}=11.4\ \mu M$). The inhibition by NS-7 occurred in the presence of ouabain, an inhibitor of Na^+,K^+ ATPase, but disappeared at higher concentration of veratridine, and upon the washout of NS-7.

2 NS-7 attenuated veratridine-induced $^{45}Ca^{2+}$ influx *via* voltage-dependent Ca^{2+} channels ($IC_{50}=20.0\ \mu M$) and catecholamine secretion ($IC_{50}=25.8\ \mu M$).

3 Chronic ($\geqslant 12$ h) treatment of cells with NS-7 increased cell surface [^{3}H]-STX binding by 86% ($EC_{50}=10.5\ \mu M$; $t_{1/2}=27$ h), but did not alter the K_D value; it was prevented by cycloheximide, an inhibitor of protein synthesis, or brefeldin A, an inhibitor of vesicular transport from the *trans*-Golgi network, but was not associated with increased levels of Na^+ channel α- and β_1-subunit mRNAs.

4 In cells subjected to chronic NS-7 treatment, $^{22}Na^+$ influx caused by veratridine (site 2 toxin), α-scorpion venom (site 3 toxin) or β-scorpion venom (site 4 toxin) was suppressed even after the extensive washout of NS-7, and veratridine-induced $^{22}Na^+$ influx remained depressed even at higher concentration of veratridine; however, either α- or β-scorpion venom, or Ptychodiscus brevis toxin-3 (site 5 toxin) enhanced veratridine-induced $^{22}Na^+$ influx as in nontreated cells.

5 These results suggest that in the acute treatment, NS-7 binds to the site 2 and reversibly inhibits Na^+ channels, thereby reducing Ca^{2+} channel gating and catecholamine secretion. Chronic treatment with NS-7 up-regulates cell surface Na^+ channels *via* translational and externalization events, but persistently inhibits Na^+ channel gating without impairing the cooperative interaction between the functional domains of Na^+ channels.

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Abbreviations: BFA, brefeldin A; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; hNE-Na, TTX/STX-sensitive human neuroendocrine type Na^+ channel α-subunit; KRP, Krebs-Ringer phosphate; NO, nitric oxide; nt, nucleotides SDS, sodium dodecyl sulphate; SSC, saline-sodium citrate; STX, saxitoxin; TTX, tetrodotoxin

Introduction

It has become increasingly evident that noninactivating Na^+ currents (Taylor, 1993) *via* voltage-dependent Na^+ channels (Catterall, 1992) may initiate the detrimental cascade of hypoxia/ischaemia-induced cell injury, such as the massive overflow of glutamate and catecholamines (Toner & Stamford, 1997), the intracellular Ca^{2+} overload *via* reversed operation of Na^+-Ca^{2+} exchanger (Obrenovitch & Richards, 1995; Urenjak & Obrenovitch, 1996), as well as cytotoxic formation of nitric oxide (NO) (Strijbos *et al*., 1996). A few studies have revealed that during hypoxia, the steady-state inactivation of Na^+ currents was shifted to a more hyperpolarizing potential in human cortical neurons (Cummins *et al*., 1993) and rat hippocampus (O'Reilly *et al*., 1997), presumably as the compensatory defensive response against hypoxia-induced neuronal injury. Also, density of cell surface Na^+ channels was fluctuated in brain during hypoxia (Pérez-Pinzón *et al*., 1992; Xia & Haddad, 1994; 1999).

NS-7 [4-(4-fluorophenyl)-2-methyl-6-(5-piperidinopentyloxy) pyrimidine hydrochloride] is a newly-synthesized neuroprotective agent. Previous *in vivo* and *in vitro* studies in cerebral cortex have shown that NS-7 attenuated hypoxia-induced degradation of cytoskeletal protein fodrin (Takagaki *et al*., 1997) and cell injury (Tatsumi *et al*., 1998b) at 10–30 μM, and the cellular mechanisms of NS-7 for neuroprotection are postulated to be attributed to the blockade of voltage-dependent Na^+ and Ca^{2+} channels. In brain, NS-7 bound with a K_i value of 1 μM (Shimidzu *et al*., 1997) to the toxin binding site 2 of the Na^+ channel α-subunit, a major subunit forming the ion-pore and the toxin binding sites 1–5 (Catterall, 1992); NS-7 at 10–30 μM diminished the overflow of glutamate (Shimidzu *et al*., 1997) and dopamine (Itoh *et al*., 1998) caused by veratridine, a toxin that interacts with the site 2 in the transmembrane segment 6 of domain I (IS6) of the Na^+ channel α-subunit (Trainer *et al*., 1996) and activates Na^+ channels (Catterall, 1992). NS-7 suppressed depolarization-elicited Na^+ currents with an IC_{50} of 7.8 μM in NG108-15 cells (Suma *et al*., 1997). Also, in NG108-15 cells, NS-7 decreased Ca^{2+} currents *via* L-, N-, and T-type Ca^{2+} channels with IC_{50} values of 7.3, 4.5, and 17.1 μM, respectively (Suma *et al*., 1997). In cerebrocortical neurons, NS-7 attenuated high K^+- or veratridine-induced activation of NO synthase at 10–30 μM presumably by inhibiting Ca^{2+} influx *via* L- and P/Q-type Ca^{2+} channels (Tatsumi *et al*., 1998a; Oka *et al*., 1999). NS-7

*Author for correspondence; E-mail: akihiko@fc.miyazaki-med.ac.jp

inhibited high K^+-induced dopamine secretion at 10 μM (Itoh *et al*., 1998), but did not affect high K^+-induced glutamate release in brain (Shimidzu *et al*., 1997).

Na^+ channel α-subunits arise from multiple genes and their alternative splicing (Dietrich *et al*., 1998), whereas the β_1-subunits are structurally similar among various tissues (Makita *et al*., 1994). In adrenal chromaffin cells (embryologically derived from the neural crest), Na^+ channel α-subunit (Yamamoto *et al*., 1997) is homologous to the tetrodotoxin (TTX)/saxitoxin (STX) (site 1 toxin)-sensitive human neuroendocrine type Na^+ channel α-subunit (hNE-Na) (Klugbauer *et al*., 1995). Previous studies showed that veratridine-induced Na^+ influx *via* Na^+ channels, and the subsequent depolarization increases Ca^{2+} influx *via* voltage-dependent Ca^{2+} channels (Wada *et al*., 1985a,b; Lopez *et al*., 1995), thereby triggering veratridine-induced exocytic secretion of catecholamines (Ito *et al*., 1980). We found that chronic treatment of chromaffin cells with the antiepileptic drug valproic acid raised Na^+ channel α- and β_1-subunit mRNA levels, as well as cell surface [^{3}H]-STX binding, thus enhancing veratridine-induced $^{22}Na^+$ influx, $^{45}Ca^{2+}$ influx and catecholamine secretion (Yamamoto *et al*., 1997). Similar observations were made in long-term treatment of chromaffin cells with the anticonvulsant carbamazepine (Yoshimura *et al*., 1998). Also, chronic *in vivo* and *in vitro* treatment with the antiepileptic drug phenytoin (Sashihara *et al*., 1994) and the antiarrhythmic drug mexiletine (Kang *et al*., 1997) caused up-regulation of Na^+ channels, thereby modifying neuronal and cardiac pathophysiologies. Our present study examined whether/how short- and long-term treatment of chromaffin cells with NS-7 might alter $^{22}Na^+$ influx, $^{45}Ca^{2+}$ influx and catecholamine secretion, using veratridine, α-scorpion venom (site 3 toxin), β-scorpion venom (site 4 toxin) and Ptychodiscus brevis toxin-3 (PbTx-3) (site 5 toxin) (Wada *et al*., 1987; 1992; Catterall, 1992). Also, the effects of chronic treatment with NS-7 on cell surface [^{3}H]-STX binding, Na^+ channel α- and β_1-subunit mRNA levels were evaluated.

Methods

Materials

Eagle's minimum essential medium was from Nissui Seiyaku, Tokyo, Japan; α-scorpion venom (Leiurus quinquestriatus quinquesriatus), β-scorpion venom (Centruroides sculpturatus), brefeldin A (BFA), cycloheximide, cytosine arabinoside, ouabain, TTX, and veratridine were from Sigma, St. Louis, MO, U.S.A.; PbTx-3 from Latoxan, Westbury, NY, U.S.A.; calf serum from Nacalai Tesque, Kyoto, Japan; NS-7 was kindly donated from Nippon Shinyaku Co., Ltd., Kyoto, Japan. TRIzol reagent from Life Technologies, Inc., Rockville, MD, U.S.A.; Oligotex-dT30<Super>from Nippon Roche Co., Ltd., Tokyo, Japan; BcaBEST labelling kit from Takara, Kyoto, Japan; ^{22}NaCl (6–17 Ci mmol^{-1}), $^{45}CaCl_2$ (0.5–2 Ci mmol^{-1}), [^{3}H]-STX (20–40 Ci mmol^{-1}), and [α-^{32}P]-dCTP (>4000 Ci mmol^{-1}) from Amersham, Buckinghamshire, U.K. cDNA for human glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was from Clontech Laboratories, Inc., Palo Alto, CA, U.S.A. Plasmids containing hNE-Na cDNA (Klugbauer *et al*., 1995), and rat brain Na^+ channel β_1-subunit cDNA (Oh & Waxman, 1994) were generously donated by Drs F. Hofmann (Technischen Universität München), and Y. Oh (University of Alabama), respectively.

Primary culture of adrenal chromaffin cells and drug treatment

Isolated bovine adrenal chromaffin cells were cultured (4×10^6 dish^{-1}, Falcon; 35 mm in diameter) under 5% CO_2 and 95% air in a CO_2 incubator in Eagle's minimum essential medium containing 10% calf serum (Wada *et al*., 1985b). The culture medium was exchanged with the fresh one every third day, and the cells were used for experiments between 3 and 7 culture days. When the cells were subjected to the chronic treatment with NS-7, it was included in the culture medium with or without cycloheximide or BFA. The culture medium contained 3 μM cytosine arabinoside to suppress proliferation of nonchromaffin cells; when chromaffin cells were further purified by differential plating (Yamamoto *et al*., 1997), concentration-inhibition curves of NS-7 for veratridine-induced influx of $^{22}Na^+$ and $^{45}Ca^{2+}$, and secretion of catecholamines were not changed, as compared to those seen in the cells that were plated by the conventional method.

$^{22}Na^+$ influx, $^{45}Ca^{2+}$ influx, and catecholamine secretion

Cells were washed with Krebs-Ringer phosphate (KRP) buffer (mM)(154 NaCl, 5.6 KCl, 1.1 $MgSO_4$, 2.2 $CaCl_2$, 0.85 NaH_2PO_4, 2.15 Na_2HPO_4, 5 glucose, and 0.5% bovine serum albumin, pH 7.4), and incubated with 2 μCi ^{22}NaCl in 1 ml KRP buffer at 37°C for 5 min with or without veratridine, α- and β-scorpion venom, and PbTx-3 in the absence or presence of NS-7 and ouabain. Previous electrophysiological and $^{22}Na^+$ influx studies showed that the whole venom from Leiurus quinquestriatus quinquestriatus (Catterall, 1976), and that from Centruroides sculpturatus (Meves *et al*., 1982) exert effects similar to those of their major α- and β-scorpion toxin, respectively. To measure $^{45}Ca^{2+}$ influx and catecholamine secretion, cells were incubated with 2 μCi $^{45}CaCl_2$ in 1 ml KRP buffer for 5 min with or without veratridine in the absence or presence of NS-7. Incubation medium was saved in a test tube for catecholamine (epinephrine plus norepinephrine) assay by HPLC (Yamamoto *et al*., 1997), and the cells were washed, solubilized in 10% Triton X-100, and counted for radioactivity (Wada *et al*., 1985b).

[^{3}H]-STX binding

Cells were incubated with 1–25 nM [^{3}H]-STX in 1 ml KRP buffer at 4°C for 15 min in the absence (total binding) and presence (nonspecific binding) of 1 μM TTX, then washed, solubilized, and counted for radioactivity; specific binding was calculated total binding minus nonspecific binding (Wada *et al*., 1987). A mere addition of NS-7 to the binding assay medium *per se* did not alter [^{3}H]-STX binding, as reported previously (Shimidzu *et al*., 1997).

mRNA isolation and electrophoresis

Total cellular RNA was isolated from cells treated with or without NS-7 by acid guanidine thiocyanate-phenol-chloroform extraction, using TRIzol reagent. Poly(A)+ RNA was purified using Oligotex-dT30<Super>, electrophoresed on 1% agarose gel containing 6.3% formaldehyde in the running buffer [40 mM 3-(N-morpholino) propanesulphonic acid, pH 7.2, 0.5 mM EDTA, and 5 mM sodium citrate], then transferred to a nylon membrane (Hybond-N, Amersham) in 20 × saline-sodium citrate (SSC: 1 × SSC = 0.15 M NaCl and 0.015 M sodium citrate) overnight, and cross-linked using a UV cross-linker (Funakoshi, Tokyo, Japan).

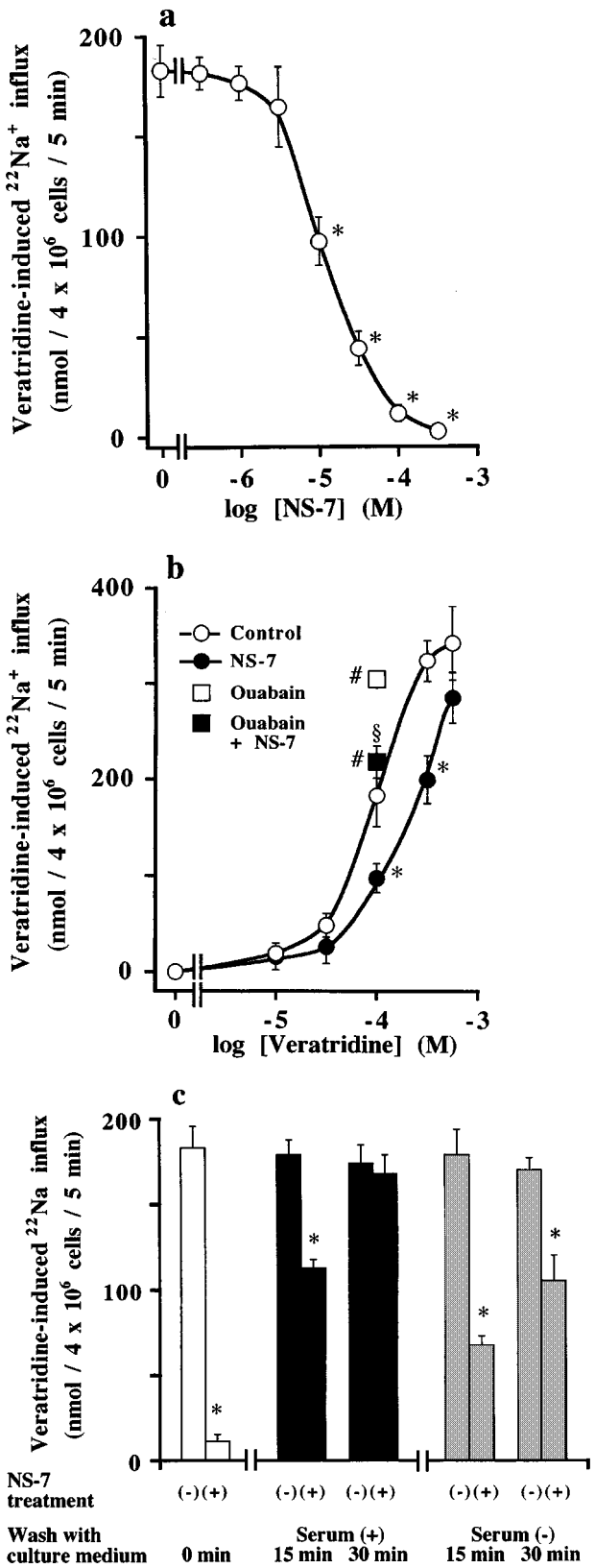

Figure 1 Inhibition of veratridine-induced $^{22}Na^+$ influx by NS-7. Cells were incubated at 37°C for 5 min in 1 ml KRP buffer containing 2 μCi $^{22}NaCl$ (a) with or without 100 μM veratridine in the absence or presence of various concentrations of NS-7, or (b) without or with 10 μM NS-7 in the absence or presence of various concentrations of veratridine. Veratridine (100 μM)-induced $^{22}Na^+$ influx was also measured without or with 10 μM NS-7 in the presence of 100 μM ouabain. Cells were washed, solubilized, and counted for radioactivity. Values at 37°C and ouabain alone were not changed by NS-7, and they were subtracted. (c) Dishes (4×10^6 cells $dish^{-1}$) were treated without (−) or with (+) NS-7 (100 μM for 5 min), washed three times with 1 ml KRP buffer (37°C), then divided into two groups. The one was immediately used for $^{22}Na^+$ influx (0 min, open columns). The other was rinsed in the culture medium supplemented with (closed columns) or without (shaded columns) calf serum for 15 or 30 min in CO_2 incubator, the medium being replaced with the fresh one at 5 min intervals, and subjected to $^{22}Na^+$ influx assay. Veratridine 100 μM. Basal $^{22}Na^+$ influx at 37°C was not changed by NS-7 treatment, and subtracted. Mean ± s.e.mean ($n = 5$). $^*P < 0.05$, compared to veratridine alone; $^{\#}P < 0.05$, compared to 100 μM veratridine alone within each NS-7-nonexposed or -exposed cell group; $\S P < 0.05$, compared between two cell groups.

Northern blot

cDNA fragments of hNE-Na [nucleotides (nt) 435–2666] and β_1-subunit (nt 457–790), prepared according to Yamamoto *et al.* (1997), as well as GAPDH cDNA (1.1 kbp) were labeled with [α-^{32}P]-dCTP using the BcaBEST labelling kit. The membrane was prehybridized, and hybridized with hNE-Na probe for 15 h at 43°C in 6 × SSC, 10 × Denhardt's, 50% formamide, 0.5% sodium dodecyl sulphate (SDS) and 50 μg ml^{-1} salmon sperm DNA; it was washed at 65°C in 2 ×, 1 × and 0.2 × SSC containing 0.1% SDS, each for 30 min twice, and subjected to autoradiography. The same membrane was sequentially hybridized to probes for β_1-subunit and then GAPDH, after it was thoroughly washed to remove the former probe in 0.1% SDS at 100°C. Autoradiogram was quantified by a Bioimage analyser BAS2000 (Fuji Film, Tokyo, Japan).

Statistical methods

All experiments were repeated at least three times (mean ± s.e.mean), and each performed in duplicate, except Northern blot. Significance ($P < 0.05$) was determined by one-way or two-way analysis of variance with *post hoc* mean comparison by the Newman–Keuls multiple range test. Student's *t*-test was used when two means of group were compared.

Results

Effect of NS-7 on voltage-dependent Na^+ channels

In cultured bovine adrenal chromaffin cells, veratridine causes a persistent influx of $^{22}Na^+$ for at least 5 min, which passes through TTX/STX-sensitive Na^+ channels (Wada *et al.*, 1985a,b; 1987). As shown in Figure 1a, veratridine (100 μM) increased Na^+ influx by 182.9 ± 12.9 nmol over the basal Na^+ influx (18.9 ± 1.7 nmol) ($n = 5$) per 4×10^6 cells per 5 min. NS-7 did not change basal Na^+ influx (18.3 ± 1.8 nmol 4×10^6 $cells^{-1}$ 5 min^{-1}) ($n = 5$), but inhibited veratridine-induced Na^+ influx in a concentration-dependent manner ($IC_{50} = 11.4$ μM).

Veratridine raised $^{22}Na^+$ influx in a concentration-related manner ($EC_{50} = 91.2$ μM) (Figure 1b); the inhibitory effects of NS-7 (10 μM) were attenuated, as the concentrations of veratridine were raised from 100 to 560 μM. Influx of Na^+ elevates the activity of Na^+,K^+ ATPase, whereby Na^+, once it has entered chromaffin cells, is continuously pumped out (Wada *et al.*, 1985a; 1986). Veratridine (100 μM)-induced $^{22}Na^+$ influx was still decreased by NS-7 even in the presence of ouabain at 100 μM (Figure 1b), a concentration at which ouabain completely inhibits the activity of Na^+,K^+ ATPase (Wada *et al.*, 1986).

To examine whether the inhibitory effect of NS-7 on Na^+ channels is reversible, cells were treated without or with 100 μM NS-7 for 5 min, washed three times with KRP buffer, then without or with the culture medium for 15 or 30 min, and

subjected to $^{22}Na^+$ influx assay in the absence of NS-7. When the washing with culture medium was omitted (Figure 1c, open columns, 0 min), veratridine (100 μM)-induced $^{22}Na^+$ influx was remarkably reduced in the NS-7-pretreated cells, compared with nontreated cells. However, rinsing the NS-7-pretreated cells with the calf serum-containing culture medium for 15 and 30 min gradually restored veratridine-induced $^{22}Na^+$ influx to 63 and 97% of the control values, respectively (Figure 1c, closed columns). Our present result may raise the question of whether the highly lipophilic NS-7 (Itoh *et al.*, 1997) was bound to the hydrophobic regions of plasma proteins contained in the calf serum, and was progressively removed from the NS-7-pretreated cells during the 30 min washing. As shown in Figure 1c (shaded columns), when the NS-7-pretreated cells were washed with the calf serum-free culture medium for 15 and 30 min, veratridine-induced $^{22}Na^+$ influx was recovered to 37.8 and 62.0% of the nontreated cells.

Effects of NS-7 on veratridine-induced $^{45}Ca^{2+}$ influx and catecholamine secretion

In cultured bovine adrenal chromaffin cells, veratridine causes a sustained influx of $^{45}Ca^{2+}$ *via* Ca^{2+} channels and catecholamine secretion for at least 5 min, and they were inhibited by Mg^{2+} (20 mM), an inhibitor of voltage-dependent Ca^{2+} channels (Wada *et al.*, 1985a,b; Lopez *et al.*, 1995). As shown in Figure 2, veratridine (100 μM) increased Ca^{2+} influx by 4.8 ± 0.5 nmol over the basal Ca^{2+} influx (0.7 ± 0.2 nmol) ($n=5$) per 4×10^6 cells per 5 min. Veratridine (100 μM) also increased catecholamine secretion by 4.7 ± 0.3 μg over the basal secretion (0.3 ± 0.1 μg) ($n=5$) per 4×10^6 cells per 5 min. NS-7 did not alter the basal values, but suppressed veratridine-induced $^{45}Ca^{2+}$ influx ($IC_{50}=20.0$ μM) and catecholamine secretion ($IC_{50}=25.8$ μM) in a concentration-dependent manner.

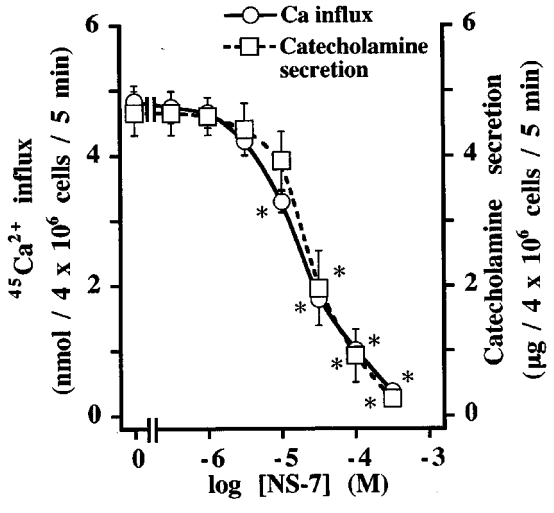

Figure 2 Effects of NS-7 on veratridine-induced $^{45}Ca^{2+}$ influx and catecholamine secretion. Cells were incubated at 37°C with 2 μCi $^{45}CaCl_2$ in KRP buffer without or with 100 μM veratridine for 5 min in the absence or presence of various concentrations of NS-7. Basal Ca^{2+} influx (nmol 4×10^6 cells^{-1} 5 min^{-1}): 0.7 ± 0.2, nontreated cells; 0.7 ± 0.1, NS-7-treated cells. Basal catecholamine secretion (μg 4×10^6 cells^{-1} 5 min^{-1}): 0.3 ± 0.1, nontreated cells; 0.3 ± 0.1, NS-7-treated cells. Catecholamine content (μg 4×10^6 cells^{-1}): 67.5 ± 5.4, nontreated cells; 66.8 ± 6.9, NS-7-treated cells. Basal Ca^{2+} influx and catecholamine secretion were subtracted from the data. Mean$\pm$s.e.mean ($n=5$). $^*P<0.05$, compared to veratridine alone.

Effects of long-term treatment with NS-7 on $^{22}Na^+$ influx caused by veratridine, α- and β-scorpion venom, and PbTx-3

Cells were treated for 12 h without or with 100 μM NS-7, washed with KRP buffer, and used for $^{22}Na^+$ influx assay. Veratridine (100 μM)-induced $^{22}Na^+$ influx was significantly inhibited in the NS-7-pretreated cells, compared to nontreated

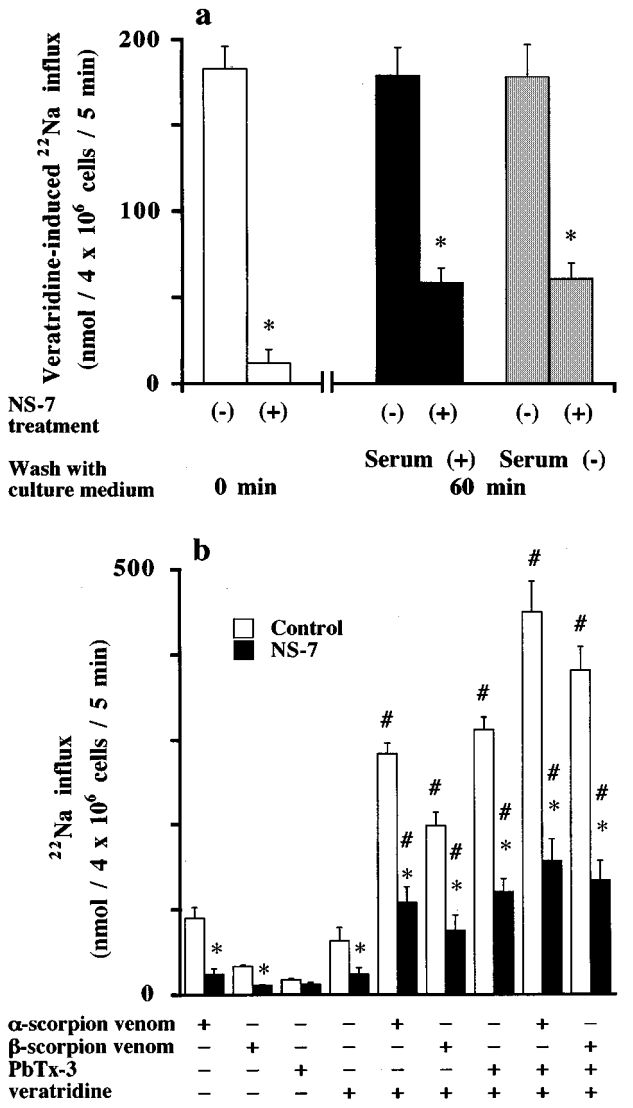

Figure 3 Effects of chronic treatment with NS-7 on $^{22}Na^+$ influx caused by veratridine, α-and β-scorpion venom, and PbTx-3. (a) Dishes (4×10^6 cells dish^{-1}) were treated without (−) or with (+) NS-7 (100 μM for 12 h), washed three times with 1 ml KRP buffer (37°C), then divided into two groups. The one was immediately used for $^{22}Na^+$ influx assay (0 min, open columns), whereas the other was rinsed with the culture medium supplemented with (closed columns) or without (shaded columns) calf serum for 60 min in CO_2 incubator, the medium being replaced with the fresh one every 10 min, and subjected to $^{22}Na^+$ influx assay. Veratridine 100 μM. Basal values were not changed by NS-7, and subtracted. Mean$\pm$s.e.mean ($n=3$). $^*P<0.05$, compared to nontreated cells. (b) Cells were treated without (open columns) or with (closed columns) NS-7 (100 μM for 12 h), washed with KRP buffer and calf serum-containing culture medium as in (a), and $^{22}Na^+$ influx was measured at 37°C for 5 min, using veratridine (30 μM), α-scorpion venom (5 μg ml^{-1}), β-scorpion venom (50 μg ml^{-1}) and PbTx-3 (1 μM). Basal $^{22}Na^+$ influx was subtracted from the data. Mean$\pm$s.e.mean ($n=3$). $^*P<0.05$, compared with cells nonexposed to NS-7; $^\#P<0.05$, significant enhancement by venom or PbTx-3 of veratridine-induced $^{22}Na^+$ influx.

cells (Figure 3a, open columns, 0 min). Even when the NS-7-pretreated cells were washed for 60 min with either calf serum-containing culture medium or calf serum-free culture medium, veratridine-induced $^{22}Na^+$ influx remained suppressed by 67% in each case, in contrast to the reversible inhibitory effect of NS-7 in its short-term treatment (Figure 1c). As shown in Figure 1b, the acute inhibitory effect of 10 μM NS-7 on veratridine (100 μM)-induced $^{22}Na^+$ influx was reversed by higher concentrations (560 μM) of veratridine. However, when cells were treated for 12 h without or with 10 μM NS-7, and washed for 60 min, veratridine (560 μM)-induced $^{22}Na^+$ influx was still depressed by 51% in NS-7-pretreated cells (167.6 ± 10.3 nmol 4×10^6 cells^{-1} 5 min^{-1}), compared to nontreated cells (342.6 ± 18.3 nmol 4×10^6 cells^{-1} 5 min^{-1}) ($n = 3$).

We evaluated whether/how chronic treatment with NS-7 may alter $^{22}Na^+$ influx, using α-scorpion venom, β-scorpion venom and PbTx-3, because these toxins bind to their respective sites of Na^+ channel α-subunit (Catterall, 1992; Trainer *et al*., 1994; Yuhi *et al*., 1994; Rogers *et al*., 1996), and potentiate veratridine-induced $^{22}Na^+$ influx in adrenal chromaffin cells (Wada *et al*., 1987; 1992). As shown in Figure 3b, either α- or β-scorpion venom *per se* increased $^{22}Na^+$ influx (Cahalan, 1975; Catterall, 1976; Meves *et al*., 1982), whereas PbTx-3 had little effect by itself on $^{22}Na^+$ influx (Baden, 1989), as reported previously. In NS-7-pretreated cells, $^{22}Na^+$ influx caused by α- or β-scorpion venom was significantly lowered, compared with nontreated cells. Either α- or β-scorpion venom, or PbTx-3, however, augmented veratridine-induced $^{22}Na^+$ influx in a more than additive manner in nontreated and NS-7-pretreated cells, but the absolute values of $^{22}Na^+$ influx remained depressed in NS-7-pretreated cells. In addition, PbTx-3 in combination with α- or β-scorpion venom strikingly enhanced veratridine-induced $^{22}Na^+$ influx even in NS-7-pretreated cells, as in nontreated cells.

Effect of long-term treatment with NS-7 on cell surface [^{3}H]-STX binding

To characterize cell surface Na^+ channels, cells were treated without or with 100 μM NS-7 for up to 96 h, and subjected to [^{3}H]-STX binding assay (Figure 4a). Chronic ($\geqslant$12 h) treatment with NS-7 increased [^{3}H]-STX binding in a time-dependent manner, reaching the almost maximum 82% increase at 72 h. When cells were treated for 24 h with 1–100 μM NS-7, the increasing effect of NS-7 on [^{3}H]-STX binding was concentration-dependent ($EC_{50} = 10.5$ μM) (Figure 4b). NS-7 treatment (100 μM for 24 h) elevated the B_{max} from 58.6 ± 4.7 to 82.4 ± 5.9 fmol 4×10^6 cells^{-1} without altering the K_D value (4.6 ± 0.5 nM, nontreated cells; 4.9 ± 0.7 nM, NS-7-treated cells; $n = 5$) (Figure 4c).

Effects of cycloheximide and BFA treatment on NS-7-induced up-regulation of [^{3}H]-STX binding

Because rise of [^{3}H]-STX binding caused by NS-7 treatment developed gradually ($t_{1/2} = 27$ h), we examined whether NS-7-induced up-regulation of cell surface Na^+ channels may require the translational and cell surface trafficking events. Figure 4d shows that treatment with cycloheximide, an inhibitor of protein synthesis, reduced *per se* [^{3}H]-STX binding by 30% at 10 μg ml^{-1}, a concentration that blocks synthesis of proteins by 95% (Craviso *et al*., 1995), and remarkably diminished the increasing effect of NS-7 on [^{3}H]-STX binding. BFA is an inhibitor of guanine nucleotide-exchange protein for ADP-ribosylation factor 1 (Morinaga *et al*., 1997), a monomeric GTPase; thus, BFA blocks cell surface incorporation of newly-synthesized ion channels/receptors from the *trans*-Golgi network, but has no effect on the endocytic internalization of ion channels/receptors from plasma membrane (Schonhorn & Wessling-Resnick, 1994; Shimkets *et al*., 1997; Staub *et al*., 1997; Hirasawa *et al*., 1998). As shown in Figure 4d, exposure to BFA by itself lowered [^{3}H]-STX binding by 36%, and significantly attenuated the rise of [^{3}H]-STX binding caused by NS-7.

Effects of long-term treatment with NS-7 on Na^+ channel α- and β_1-subunit mRNA levels

Because up-regulation of [^{3}H]-STX binding by NS-7 was dependent on the translational and cell surface targeting events, we measured whether NS-7 treatment could increase the steady-state levels of Na^+ channel α- and β_1-subunit mRNAs (Figure 5). cDNA probes for hNE-Na and β_1-subunit hybridized to α ($\sim$9.4 Kb)- and β_1 ($\sim$1.5 Kb)-subunit mRNAs, respectively, as reported previously (Oh & Waxman, 1994; Klugbauer *et al*., 1995; Yamamoto *et al*., 1997). When the levels of α- and β_1-subunit mRNAs were normalized against those of GAPDH mRNA, NS-7 (100 μM) treatment did not elevate α-subunit mRNA (112.6 ± 7.1, 108.6 ± 6.4, 118.1 ± 18.7, and 103.2 ± 17.8% of levels in nontreated cells), and β_1-subunit mRNA (91.0 ± 9.4, 107.4 ± 15.9, 110.4 ± 12.2, and 111.7 ± 10.1% of levels in nontreated cells) at 3, 6, 12 and 24 h ($n = 3$).

Discussion

Effects of short-term treatment with NS-7 on voltage-dependent Na^+ channels, veratridine-induced $^{45}Ca^{2+}$ influx and catecholamine secretion

In adrenal chromaffin cells, NS-7 inhibited veratridine-induced $^{22}Na^+$ influx *via* the human neuroendocrine type Na^+ channels (Klugbauer *et al*., 1995) with the IC_{50} value of 11.4 μM; this potency is comparable to those of previous binding studies in brain ($K_i = 1$ μM) and cardiac ($K_i = 13$ μM) Na^+ channel subtypes (Shimidzu *et al*., 1997). NS-7 also attenuated Na^+ currents ($IC_{50} = 7.8$ μM) in NG108-15 cells (Suma *et al*., 1997), where Na^+ channel isoform(s) expressed has not been specified.

Inhibition of veratridine-induced $^{22}Na^+$ influx by NS-7 could be overcome by increasing concentrations of veratridine. Veratridine binds to site 2, which is located at the transmembrane IS6 of brain Na^+ channel α-subunit (Trainer *et al*., 1996). Thus, NS-7 shares site 2 with veratridine in Na^+ channels of adrenal chromaffin cells, consistent with previous studies where NS-7 almost entirely displaced [^{3}H]-batrachotoxinin A 20-α-benzoate binding from the site 2 of brain and cardiac Na^+ channels (Shimidzu *et al*., 1997). In our present study, the inhibitory effect of a brief (5 min) exposure to NS-7 on veratridine-induced $^{22}Na^+$ influx was gradually, but completely reversed by washing the NS-7 (100 μM for 5 min)-pretreated cells for 30 min. This result suggests that the washing can remove NS-7 from the IS6 of Na^+ channel α-subunit, thereby enabling veratridine to bind to the site 2 and gate Na^+ channels.

Several lines of evidence have documented that the massive release of catecholamines induced by hypoxia/ischaemia causes neuronal injury in corpus striatum and hippocampus, and the prior depletion of endogenous catecholamines prevents hypoxia/ischaemia-induced histological and metabolic aberra-

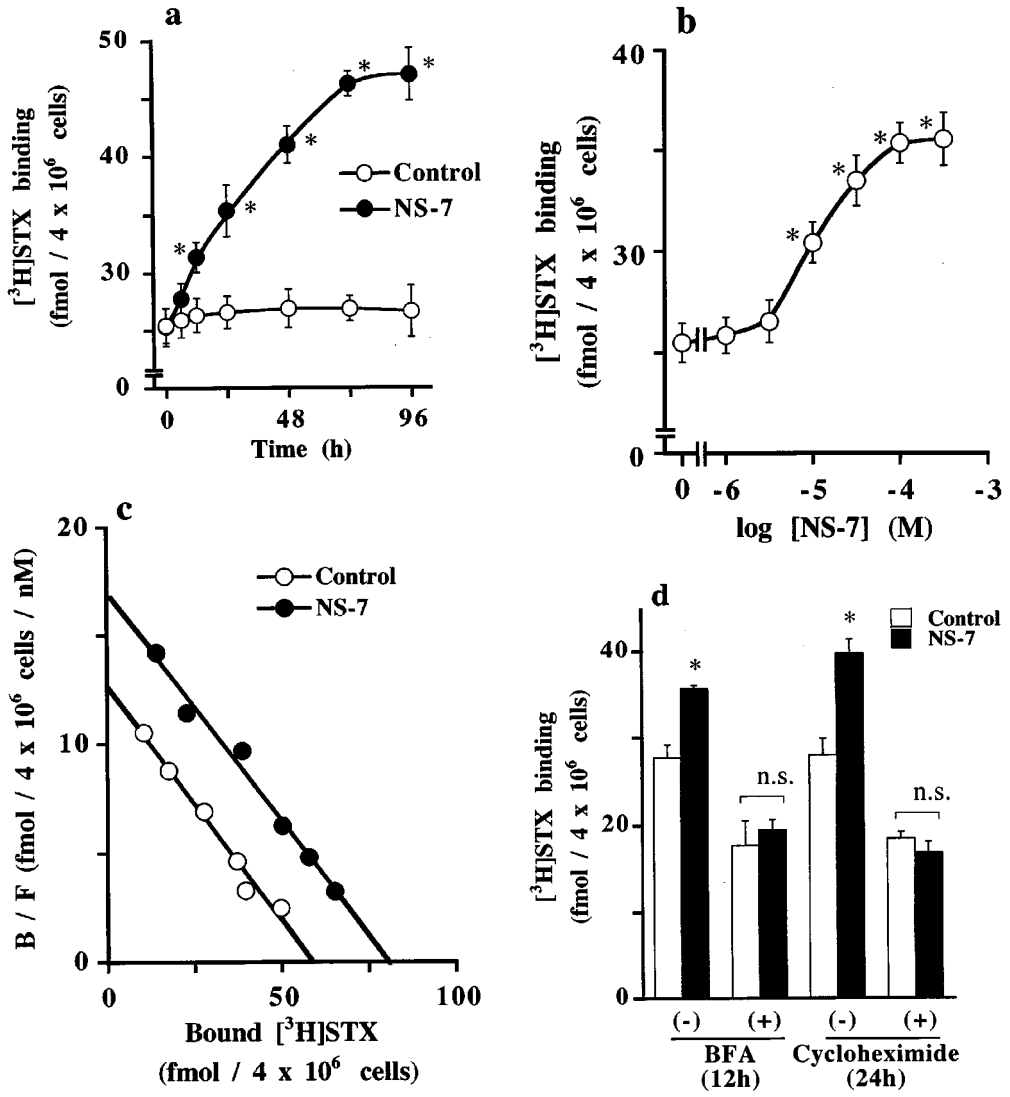

Figure 4 Effect of chronic treatment with NS-7 on [^{3}H]-STX binding. (a) Cells were treated without or with 100 μM NS-7 for the indicated periods, washed with KRP buffer (Figure 3a, legend), and subjected to [^{3}H]-STX binding assay. Mean$\pm$s.e.mean ($n=5$). *$P<0.05$, compared to nontreated cells. (b) Cells were treated without or with 1–300 μM NS-7 for 24 h, washed as in (a), and used for [^{3}H]-STX binding. Mean$\pm$s.e.mean ($n=5$). *$P<0.05$, compared to nontreated cells. (c) Scatchard plot of [^{3}H]-STX binding to the cells treated without or with 100 μM NS-7 for 24 h. Data is the typical one from four independent experiments with similar results. (d) Cells were treated without or with 100 μM NS-7 for the indicated periods in the absence or presence of 10 μg ml^{-1} BFA or 10 μg ml^{-1} cycloheximide, then washed as in (a), and used for [^{3}H]-STX binding. Mean$\pm$s.e.mean ($n=5$). *$P<0.05$, compared to NS-7-nontreated cells. n.s., no significant difference from BFA or cycloheximide alone.

tions (Obrenovitch & Richards, 1995; Urenjak & Obrenovitch, 1996). In our present study, NS-7 decreased veratridine-induced $^{45}Ca^{2+}$ influx ($IC_{50}=20.0$ μM) and secretion of epinephrine and norepinephrine ($IC_{50}=25.8$ μM). Previous studies showed that veratridine caused exocytic secretion of catecholamines in adrenal glands (Ito *et al*., 1980), and it was dependent on veratridine-induced $^{45}Ca^{2+}$ influx *via* voltage-dependent Ca^{2+} channels in cultured adrenal chromaffin cells (Wada *et al*., 1985a,b; Lopez *et al*., 1995). Thus, NS-7 inhibits veratridine-induced exocytic secretion of catecholamines by decreasing veratridine-induced Ca^{2+} influx. A previous intracerebral microdialysis study showed that NS-7 (10 μM) attenuated veratridine-induced release of dopamine in striatum (Itoh *et al*., 1998). Toner and Stamford (1997) revealed that hypoxia/hypoglycaemia increased the secretion of dopamine by an exocytic mechanism, and its exocytosis was accelerated by veratridine, while being diminished by Na^+ channel antagonists (e.g. TTX) in caudate nucleus slices. In spinal cord slices, ischaemia increased the overflow of norepinephrine in a Ca^{2+}-independent manner, presumably *via* the reversed operation of a Na^+-dependent plasma membrane norepinephrine transporter, and the non-exocytic release of norepinephrine was blocked by Na^+ channel antagonists (Uchihashi *et al*., 1998). Therefore, the blockade of Na^+ channels by NS-7 may have neuroprotective effects that can be mediated *via* attenuation of exocytic and non-exocytic secretion of catecholamines.

Effects of long-term treatment with NS-7 on voltage-dependent Na^+ channels

Quantitative and qualitative changes of Na^+ channels and their subunit mRNAs have been observed in various noxious insults, such as epilepsy (Lombardo *et al*., 1996; Gastaldi *et al*., 1997) and hypoxia (Pérez-Pinzón *et al*., 1992; Cummins *et al*., 1993; Xia & Haddad, 1994; 1999; Urenjak & Obrenovitch,

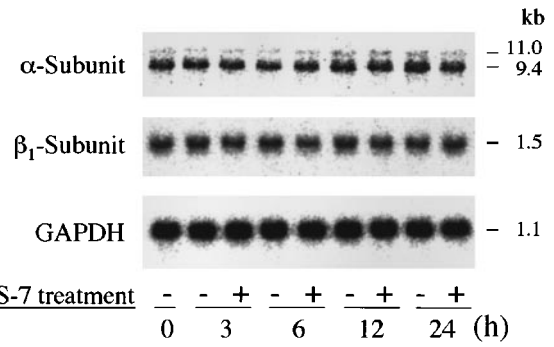

Figure 5 Lack of effect of NS-7 treatment on Na^+ channel α- and β_1-subunit mRNA levels. Cells were treated with (+) or without (−) 100 μM NS-7 for the indicated periods, after which poly(A)+ RNA was extracted, electrophoresed (5 μg each lane) on 1% agarose gel, and transferred to a membrane. The membrane was sequentially hybridized to each ^{32}P-labelled cDNA probe for hNE-Na (top), rat brain β_1-subunit (middle), and GAPDH (bottom), after removal of the former probe. One typical data from three separate experiments with similar results.

1996; O'Reilly *et al*., 1997), as well as during long-term treatment with phenytoin (Sashihara *et al*., 1994), valproic acid (Yamamoto *et al*., 1997), mexiletine (Kang *et al*., 1997) and carbamazepine (Yoshimura *et al*., 1998).

Chronic (⩾12 h) treatment of adrenal chromaffin cells with 100 μM NS-7 elevated the number of [^{3}H]-STX binding sites by 86% without altering the K_D value. The increasing effect of NS-7 treatment on [^{3}H]-STX binding was concentration-dependent (EC_{50} = 10.5 μM). NS-7 treatment did not elevate Na^+ channel α- and β_1-subunit mRNA levels at 3, 6, 12 and 24 h, when cell surface [^{3}H]-STX binding progressively increased with NS-7 treatment. However, the rise of [^{3}H]-STX binding caused by NS-7 treatment was completely prevented by the coincident treatment with cycloheximide. Taken together, these results may imply that NS-7 treatment stimulates translational rather than transcriptional steps of Na^+ channel synthesis, thereby causing up-regulation of cell surface Na^+ channels, in agreement with the gradual development ($t_{1/2}$ = 27 h) of NS-7-induced up-regulation of Na^+ channels. Also, concurrent treatment with BFA totally abolished the increase in [^{3}H]-STX binding caused by NS-7 treatment. Previous studies documented that BFA treatment (2.5–10 μg ml^{-1} for 2–36 h) inhibited cell surface externalization of transferrin receptors (Schonhorn & Wessling-Resnick, 1994), α_{1B}-adrenoceptors (Hirasawa *et al*., 1998), and renal epithelial Na^+ channels (Shimkets *et al*., 1997; Staub *et al*., 1997) from the *trans*-Golgi network, but did not change the endocytic internalization of these receptors/ion channels from plasma membrane. Thus, these correlative results raise the possibility that chronic treatment with NS-7 causes up-regulation of cell surface Na^+ channels by accelerating translational event of Na^+ channel synthesis from existing mRNA and/or cell surface externalization from the *trans*-Golgi network rather than by retarding internalization of Na^+ channels from plasma membrane. In addition, up-regulation of Na^+ channels by NS-7 treatment may be attributed to the *de novo* synthesis and cytoplasmic delivery of protein(s) that promotes cell surface trafficking of Na^+ channels from the *trans*-Golgi network. Little is known however, as to the molecular machinery that regulates intracellular trafficking of ion channels/receptors including Na^+ channels (Green & Miller, 1995; Sheng & Kim, 1996).

In adrenal chromaffin cells, previous studies showed that chronic treatment with valproic acid (>24 h) (Yamamoto *et al*., 1997) or carbamazepine (5 days) (Yoshimura *et al*., 1998) caused parallel up-regulations of cell surface [^{3}H]-STX binding and veratridine-induced $^{22}Na^+$ influx, thus enhancing veratridine-induced $^{45}Ca^{2+}$ influx and catecholamine secretion. The up-regulation of [^{3}H]-STX binding caused by valproic acid or carbamazepine was dependent on *de novo* synthesis of protein(s), as seen in the up-regulation of Na^+ channels caused by chronic (⩾12 h) treatment with NS-7. In the NS-7 (100 μM for 12 h)-pretreated cells, however, veratridine-induced $^{22}Na^+$ influx remained suppressed in a noncompetitive manner even after the extensive 60 min washout of NS-7. Because NS-7 and veratridine competed with each other for binding at site 2 during the acute treatment with NS-7, $^{22}Na^+$ influx should be activated by other mechanisms that do not involve the site 2. One strategy to gate Na^+ channels may be the use of depolarizing concentrations of K^+, because 55 mM high K^+ depolarized plasma membrane is to an extent comparable with 10–100 μM veratridine in adrenal chromaffin cells (Fenwick *et al*., 1982; Friedman *et al*., 1985; López *et al*., 1995). In adrenal chromaffin cells, current amplitude and density of Na^+ channels were similar to those of Ca^{2+} channels; however, Ca^{2+} channels were little inactivated, whereas Na^+ channels were inactivated within 1 ms, as evidenced by patch-clamp recordings (Fenwick *et al*., 1982). These distinct inactivation kinetics of Na^+ and Ca^{2+} channels may be related to the fact that high K^+ (56 mM for 1 min) increased $^{45}Ca^{2+}$ influx, but failed to cause an appreciable amount of $^{22}Na^+$ influx in adrenal chromaffin cells (Wada *et al*., 1985b). In our present study, the $^{22}Na^+$ influx caused by α- or β-scorpion venom was lowered by chronic treatment with NS-7. Either α- or β-scorpion venom, or PbTx-3, however, remarkably augmented veratridine-induced $^{22}Na^+$ influx even in NS-7-pretreated cells, as in nontreated cells (Wada *et al*., 1987, 1992; Yuhi *et al*., 1994). Multiple lines of evidence have documented that α-scorpion toxin, a polypeptide toxin, binds to the extracellular loop between IVS3 and IVS4 (Rogers *et al*., 1996), and PbTx-3, a lipophilic polyether toxin, interacts with the transmembrane segment between IS6 and IVS5 (Trainer *et al*., 1996), although the exact location of the site 4 for polypeptide β-scorpion toxin is unknown (Catterall, 1992). Because cooperative enhancement of $^{22}Na^+$ influx caused by the site 2–5 toxins occurs in a Na^+ channel isoform-specific manner (Cestéle *et al*., 1995), chronic treatment with NS-7 does not alter the pharmacological properties on Na^+ channels, but inhibits gating of Na^+ channels caused by veratridne, α- or β-scorpion venom. STX has been shown to bind to the extracellular loop between IS5 and IS6 of brain Na^+ channel α-subunit (Noda *et al*., 1989; Satin *et al*., 1992). In brain Na^+ channels reconstituted into phospholipid vesicles, however, removal of β_1- (but not β_2-) subunit reduced the abilities of Na^+ channels to bind to [^{3}H]-STX and to increase $^{22}Na^+$ influx in response to veratridine (Messner & Catterall, 1986; Messner *et al*., 1986). Although the precise molecular mechanisms of Na^+ channel gating remain largely unknown (Catterall, 1992), most straightforward interpretation of our present results may be that although chronic treatment with NS-7 causes up-regulation of cell surface Na^+ channels, NS-7, a highly lipophilic drug (Itoh *et al*., 1997), binds tightly to the membrane lipids, thus producing the long-lived inhibition of toxin-induced gating of Na^+ channels.

Previous studies showed that the partition coefficient of NS-7 between octanol and phosphate buffer (pH 6.8) was 81.3 (Itoh *et al*., 1997); because of the lipophilic property of NS-7, about 50% of total amount (8 mg kg^{-1}) of NS-7 administered intravenously in rat was highly concentrated at 5 min in brain P_2 fraction, where Na channels were located (Shimidzu *et al*.,

1997). They also found that during 2 h after the intravenous injection of NS-7 (10 mg kg^{-1}) into the rat, the striatal extracellular concentrations of NS-7 were maintained at 1.1–1.4 μM, the concentrations being comparable with those of NS-7 in the extracellular milieu in our present study that caused up-regulation of Na^+ channels. Our present study showed that chronic treatment with NS-7 up-regulates Na^+ channels, but displays persistent inhibitory effect on Na^+ channel gating, thus exerting long-lasting neuroprotective effect.

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