Published in final edited form as:

J Pharmacol Exp Ther. 2008 August ; 326(2): 604–613. doi:10.1124/jpet.108.138230.

Influence of lipid soluble gating modifier toxins on sodium influx in neocortical neurons

Zhengyu Cao,

Creighton University, School of Medicine, Department of Pharmacology, Omaha, Nebraska

Joju George,

Creighton University, School of Medicine, Department of Pharmacology, Omaha, Nebraska

William H. Gerwick,

Center for Marine Biotechnology and Biomedicine, Scripps Institution of Oceanography, University of California at San Diego, La Jolla, California

Daniel G. Baden,

University of North Carolina at Wilmington, Center for Marine Science Research, Wilmington, North Carolina

Jon D. Rainier, and

University of Utah, Department of Chemistry, Salt Lake City, Utah 84112-0850

Thomas F. Murray

Creighton University, School of Medicine, Department of Pharmacology, Omaha, Nebraska

Abstract

The electrical signals of neurons are fundamentally dependent on voltage-gated sodium channels (VGSCs), which are responsible for the rising phase of the action potential. An array of naturally occurring and synthetic neurotoxins have been identified that modify the gating properties of VGSCs. Using murine neocortical neurons in primary culture, we have compared the ability of VGSC gating modifiers to evoke Na^+ influx. Intracellular sodium concentration ($[\text{Na}^+]_i$) was monitored using the Na^+ sensitive fluorescent dye, sodium-binding benzofuran isophthalate (SBFI). All sodium channel gating modifier compounds tested produced a rapid and concentration-dependent elevation in neuronal $[\text{Na}^+]_i$. The increment in $[\text{Na}^+]_i$ exceeded 40 mM at high concentrations of brevetoxins, batrachotoxin, and the novel lipopeptide, antillatoxin. The maximum increments in neuronal $[\text{Na}^+]_i$ produced by neurotoxin site 2 alkaloids, veratridine and aconitine, and the pyrethroid deltamethrin were somewhat lower with maximum $[\text{Na}^+]_i$ increments of less than 40 mM. The rank order of efficacy of sodium channel gating modifiers was $\text{PbTx-1} > \text{PbTx-desoxydioxolane} > \text{batrachotoxin} > \text{antillatoxin} > \text{PbTx-2} = \text{PbTx-3} > \text{PbTx-3}\alpha\text{-naphthoate} > \text{veratridine} > \text{deltamethrin} > \text{aconitine} > \text{gambierol}$. These data demonstrate that the ability of sodium channel gating modifiers to act as partial agonists is shared by compounds acting at both neurotoxin sites 2 and 5. The concentration-dependent increases in $[\text{Na}^+]_i$ produced by PbTx-2 , antillatoxin, veratridine, deltamethrin, aconitine and gambierol were all abrogated by tetrodotoxin indicating that VGSCs represent the sole pathway of Na^+ entry following exposure to gating modifier neurotoxins.

Introduction

The electrical signals of neurons are fundamentally dependent on voltage-gated sodium channels (VGSCs), which are responsible for the rising phase of the action potential. These ion channels represent the molecular target for an array of natural occurring and synthetic neurotoxins that specifically bind to at least six distinct receptor sites on the sodium channel α subunit (Catterall *et al.*, 2007). These toxins include hydrophilic toxins such as tetrodotoxin, saxitoxin and μ -conotoxin (receptor site 1); lipid soluble alkaloid toxins, including batrachotoxin, veratridine, aconitine and grayanotoxin (receptor site 2); polypeptide toxins such as α -scorpion toxins, sea anemone toxins and some spider toxins (receptor site 3); β -scorpion toxins (receptor site 4); brevetoxins (PbTx) and ciguatoxins originating from the marine dinoflagellates *Karenia brevis* and *Gambierdiscus toxicus* respectively (receptor site 5); and δ -conotoxins (receptor site 6). In addition pyrethroid insecticides act at a site distinct from these better characterized neurotoxin receptor sites on the sodium channel α subunit to enhance channel activity by shifting activation to more negative membrane potentials as well as by inhibiting inactivation (Ruigt *et al.*, 1987). More recently, a structurally unique lipopeptide toxin, antillatoxin, produced by the marine cyanobacterium *Lyngbya majuscula* has been demonstrated to be a potent VGSC activator at yet another distinct receptor site (Berman *et al.*, 1999; Li *et al.*, 2001).

The lipid soluble toxins acting at neurotoxin receptor sites 2 and 5 have been characterized as allosteric modulators of sodium channel function (Catterall *et al.*, 2007). These toxins bind at topologically distinct sites that favor the open state of the sodium channel and display complex allosteric interactions. Batrachotoxin is a neurotoxin that activates site 2 on the α -subunit of VGSC. Batrachotoxin produces a shift in the threshold for VGSC activation to more hyperpolarized membrane potentials resulting in persistent channel opening at resting potential (Wang and Wang, 2003). Batrachotoxin and other site 2 ligands also inhibit VGSC inactivation further contributing to persistent channel opening (Wang and Wang, 2003).

The specific binding of [^{3} H]batrachotoxin to neurotoxin site 2 is sensitive to conformational changes induced by the binding of toxins to other sites on the α -subunit (Catterall *et al.*, 1981). The binding of [^{3} H]batrachotoxin to neurotoxin site 2 is enhanced by the interaction of brevetoxins with site 5 (Sharkey *et al.*, 1987). Using reconstituted VGSC preparations, the most potent brevetoxin analog, PbTx-1, enhanced [^{3} H]batrachotoxin binding greater than five-fold (Trainer *et al.*, 1993). It is noteworthy that the less toxic brevetoxin, PbTx-9, caused only a small increase in [^{3} H]batrachotoxin binding. These results are consistent with brevetoxin analogs possessing distinct efficacies as activators of neurotoxin site 5 (LePage *et al.*, 2003). Lipophilic toxins, in addition to batrachotoxin, that act at neurotoxin site 2 include the alkaloids veratridine and aconitine (Cestele and Catterall, 2000). These ligands interact with neurotoxin site 2 in a mutually exclusive manner and increase Na^+ permeability of neuroblastoma cells to different extents at saturation (Catterall, 1975). Sodium influx studies using rat brain synaptosomes have confirmed that batrachotoxin, veratridine and aconitine activate VGSCs by an interaction with a common receptor site 2, at which batrachotoxin is a full agonist and aconitine and veratridine are partial agonists (Tamkun and Catterall, 1981). These actions of sodium channel activators have been quantitatively described by an allosteric model that assumes toxin high-affinity binding to activated sodium channels with a shift in the conformational equilibrium toward the activated or open state (Catterall, 1977a).

VGSCs are vital for normal CNS functioning and recent studies have additionally shown that intracellular sodium concentration ($[\text{Na}^+]_i$) may act as a signaling molecule. Yu and Salter (1998) reported that increases in intracellular Na^+ increase NMDA receptor mediated whole-cell currents and NMDA receptor single channel activity by increasing both open probability and mean open time of the channel. Using veratridine, these investigators demonstrated that

influx of Na⁺ through a tetrodotoxin (TTX) sensitive VGSC was sufficient to produce potentiation of NMDA-channel activity. Previous studies have moreover found that PbTx-2 augments NMDA receptor-mediated Ca²⁺ influx in both spontaneously oscillating mature and non-oscillatory immature cerebrocortical neurons (Dravid et al., 2005). PbTx-2 also enhanced the effect of bath-applied NMDA on extracellular signal-regulated kinase 2 activation.

We have therefore quantified the potencies and efficacies of an array of lipophilic VGSC gating modifiers by measuring Na⁺ influx in murine neocortical neurons. This was accomplished using the Na⁺ sensitive fluorescent dye, sodium-binding benzofuran isophthalate (SBFI). Application of this assay to primary cultures of neurons afforded a highly quantitative assessment of the increments in neuronal sodium level produced by gating modifier toxins.

Methods

Materials

Trypsin, penicillin, streptomycin, heat-inactivated fetal bovine serum, horse serum and soybean trypsin inhibitor were obtained from Atlanta Biologicals (Norcross, GA, USA). Minimum essential medium, deoxyribonuclease (DNase), poly-L-lysine, cytosine arabinoside, veratridine, aconitine, and deltamethrin were from Sigma (St. Louis, MO, USA), and batrachotoxin from BIOMOL International L.P. (Plymouth Meeting, PA, USA). The fluorescent dye SBFI-AM and pluronic acid F-127 were obtained from Invitrogen Corporation (Carlsbad, CA, USA). Brevetoxins-1, -2 and -3 were isolated and purified from *K. breve* cultures at the Center for Marine Sciences at the University of North Carolina in Wilmington. The semi-synthetic brevetoxin analogs were synthesized as described elsewhere by Purkerson-Parker et al. (2000). Gambierol was synthesized as described by Johnson et al. (2006). Antillatoxin was authentic natural (–)-antillatoxin, isolated as described by Orjala et al (1995). The structures of all gating modifier toxins evaluated are depicted in Figure 1.

Neocortical Neuron Culture

Primary cultures of neocortical neurons were obtained from embryonic day 16 Swiss-Webster mice (LePage et al., 2005). Briefly, pregnant mice were euthanized by CO₂ asphyxiation, and embryos were removed under sterile conditions. Neocortices were collected, stripped of meninges, minced by trituration with a Pasteur pipette and treated with trypsin for 20 min at 37 °C. The cells were then dissociated by two successive trituration and sedimentation steps in soybean trypsin inhibitor and DNase containing isolation buffer, centrifuged and resuspended in Eagle's minimal essential medium with Earle's salt (MEM) and supplemented with 2 mM L-glutamine, 10% fetal bovine serum, 10% horse serum, 100 IU/ml penicillin and 0.10 mg/ml streptomycin, pH 7.4. Cells were plated onto poly-L-lysine-coated 96-well (9 mm) clear-bottomed black-well culture plates (Costar) at a density of 1.5×10^5 cells/cm² and incubated at 37°C in a 5% CO₂ and 95% humidity atmosphere. Cytosine arabinoside (10 μM) was added to the culture medium on day 2 after plating to prevent proliferation of nonneuronal cells. The culture media was changed both on days 5 and 7 using a serum-free growth medium containing Neurobasal Medium supplemented with B-27, 100 I.U./mL penicillin, 0.10 mg/mL streptomycin, and 0.2 mM L-glutamine. Neocortical cultures were used in experiments between 8–9 days *in vitro* (DIV). All animal use protocols were approved by the Institutional Animal Care and Use Committee (IACUC).

Intracellular sodium concentration ([Na⁺]_i) measurement

The cells were washed four times with Lock's buffer (in mM: 8.6 Hepes, 5.6 KCl, 154 NaCl, 5.6 Glucose, 1.0 MgCl₂, 2.3 CaCl₂, 0.0001 glycine, pH 7.4) using an automated cell washer (Biotech instrument Inc., VT, USA). The background fluorescence of each well in the plate was measured and averaged prior to dye loading. Cells were then incubated for 1 h at 37°C

with dye loading buffer (100 μ l/well) containing 10 μ M SBFI-AM and 0.02% Pluronic F-127. After 1 h incubation in dye loading medium, cells were washed five times with Locke's buffer, leaving a final volume of 150 μ l in each well. The plate was then transferred to the plate chamber of a FLEXstation™ II (Molecular Devices, Sunnyvale, CA, USA). Cells were excited at 340 nm and 380 nm and Na⁺-bound SBFI emission was detected at 505 nm. Fluorescence readings were taken once every 5 s for 60 s to establish the baseline and then 50 μ l of neurotoxin containing solution (4x) was added to each well from the compound plate at the rate of 26 μ l/s, yielding a final volume of 200 μ l/well. The cells were exposed to the VGSC gating modifiers for another 240 s. Full in situ calibration of the SBFI fluorescence ratio was performed as described previously (Diarra *et al.*, 2001) using calibration media containing (in mM): 0.6 MgCl₂, 0.5 CaCl₂, 10 HEPES, Na⁺ and K⁺ such that [Na⁺]+[K⁺]=130, 100 gluconate, and 30 Cl⁻ (titrated with 10 mol/L KOH to pH=7.4). Gramicidin D (5 μ M) (Na⁺ ionophore), monensin (10 μ M) (Na⁺/H⁺ carrier) and ouabain (100 μ M) (Na⁺/K⁺-ATPase inhibitor) were added to equilibrate the intracellular and extracellular sodium concentration. After 5 washes, the Locke's buffer was replaced by 150 μ l sodium containing calibration solution (0–130 mM). The plate was then loaded onto the FLEXstation™ chamber for recording of emitted fluorescence during excitation at 340 nm and 380 nm.

Data analysis

The raw emission data at each excitation wavelength were exported to an Excel work sheet and corrected for background fluorescence. The SBFI fluorescence ratios (340/380) versus time were then analyzed, and time-response and concentration-response graphs generated using Graphpad Prism software. The EC₅₀ and maximum response values for VGSC gating modifiers were determined by non-linear regression analysis using a logistic equation. In select experiments SBFI fluorescence ratios were analyzed by one-way analysis of variance. A Dunnet's post hoc analysis was performed to compare these values for different treatments.

Results

Calibration of [Na⁺]_i in neocortical neurons

To quantify VGSC gating modifier-induced [Na⁺]_i elevation in neocortical neurons, the relationship between the ratiometric SBFI signal and [Na⁺]_i was established in situ. This in situ calibration is required inasmuch as SBFI, similar to the Ca²⁺ indicator fura-2, displays different spectral properties in an intracellular environment than in a cell-free system. The emitted fluorescence intensities were recorded during excitation at 340 and 380 nm and were converted to a ratio (340/380) following background correction (Fig. 2a). To convert the ratio of emitted SBFI signals into a [Na⁺]_i value, the following equation was used.

$$[\text{Na}^+] = \beta K_d [(R - R_{\min}) / (R_{\max} - R)] \quad (1)$$

Where β is the ratio of the fluorescence of the free (unbound) dye to bound dye at the second excitation wavelength (380 nm), K_d is the apparent dissociation constant of SBFI for Na⁺, R is the background-subtracted SBFI fluorescence ratio, and $R_{\min}$ and $R_{\max}$ are, respectively, the minimum and maximum fluorescence values. The data points relating [Na⁺]_i to R were fitted by a three-parameter hyperbolic equation having the form

$$R = R_{\min} + [a([\text{Na}^+]) / (b + [\text{Na}^+])] \quad (2)$$

Where a and b are constants and equal to $R_{\max} - R_{\min}$ and βK_d , respectively (Diarra *et al.*, 2001). These data relating [Na⁺]_i to R (Fig. 2.b) were well described ($r^2=0.987$) by Equation 2. The derived parameters were $R_{\min}=1.460 \pm 0.012$, $a=2.950 \pm 0.029$ and $b=39.14 \pm 1.26$.

The value for $R_{\min}$ obtained by this method was identical to the value of $R_{\min}$ derived experimentally at $[Na^+] = 0$ mM. The corresponding values for $R_{\max}$ and βK_d were therefore $R_{\max} = 4.41 \pm 0.04$ and $\beta K_d = 39.14 \pm 1.26$ mM.

Another method to derive the parameters for calibration of SBFI ratios is by a Hanes plot (Donoso *et al.*, 1992; David *et al.*, 1997; Diarra *et al.*, 2001). We therefore compared the values of $R_{\max}$ and βK_d obtained from a Hanes plot to those derived from the three-parameter hyperbolic fit. The equation was rearranged to generate a Hanes plot such that

$$\begin{aligned} [Na^+]/(R - R_{\min}) = & \{ [Na^+] / (R_{\max} - R_{\min}) \} \\ & + [\beta K_d / (R_{\max} - R_{\min})] \end{aligned} \quad (3)$$

As shown in Fig. 2.c, a plot of $[Na^+]/(R - R_{\min})$ vs. $[Na^+]_i$ yields a straight line ($r^2 = 0.9965$). The slope $\{1/(R_{\max} - R_{\min})\}$ provides a means to estimate of $R_{\max}$ while the intercept on the abscissa is equal to $-\beta K_d$. The value for $R_{\min}$ was obtained from the experimental data. The values of $R_{\max}$ and βK_d calculated from Hanes plot were 4.35 ± 0.04 and 39.37 ± 1.42 mM, respectively and were not significantly different from the values derived from the three-parameter hyperbolic fit that were 4.41 ± 0.04 ($R_{\max}$) and 39.14 ± 1.26 mM (βK_d).

VGSC gating modifiers elevate intracellular sodium concentration

Given the role of intracellular sodium as a putative regulator of NMDA receptor mediated signaling, it was important to quantify VGSC gating modifier-induced elevation of $[Na^+]_i$ (Yu, 2006). We therefore assessed gating modifier-induced elevation of $[Na^+]_i$ in neocortical neurons loaded with SBFI. Fluorescence emitted during excitation at 340 nm was unaffected by changes in $[Na^+]_i$. As depicted in Fig. 3, exposure to PbTx-2 (300 nM) produced a rapid decrease in SBFI fluorescence emitted by excitation at 380 nm whereas emitted fluorescence during excitation at 340 nm was unaffected. The lack of effect on fluorescence emission after excitation at 340 nm indicates that PbTx-2 did not produce significant cell swelling in neocortical neurons. The in situ SBFI calibrations revealed that the basal $[Na^+]_i$ concentration in DIV 9 cerebocortical neurons was 10.3 ± 0.22 mM. This value is in agreement with the 8.9 mM concentration determined in cultured hippocampal neurons (DIV 14–21) (Rose and Ransom, 1997). This basal level of $[Na^+]_i$ was decreased slightly, although significantly ($P < 0.05$), by exposure to 1 μ M tetrodotoxin to a value of 9.37 ± 0.17 mM.

All sodium channel gating modifier compounds tested produced a rapid and concentration-dependent elevation in neuronal $[Na^+]_i$ (Figure 4.a-j). The increment in $[Na^+]_i$ exceeded 40 mM at high concentrations of PbTx-1 (100 nM), PbTx-2 (1 μ M), PbTx-3 (1 μ M), PbTx-desoxydioxolane (1 μ M), batrachotoxin (100 nM) and antillatoxin (1 μ M). The maximum increments in neuronal $[Na^+]_i$ produced by PbTx-3 α -naphthoate, veratridine, aconitine, deltamethrin and gambierol were somewhat lower with maximum increments of less than 40 mM. Among the neurotoxin site 2 and site 5 compounds depicted in Fig. 4, aconitine displayed the lowest efficacy with maximum increments of $[Na^+]_i$ of less than 15 mM (Fig. 4.j) (see Fig. 8a for gambierol time-response data). The distinct efficacies of the array of gating modifier toxins examined are illustrated in Fig. 5 depicting the concentration-response data fit by a three-parameter logistic equation. These data indicate that the relative efficacies of sodium channel gating modifiers as stimulators of Na^+ influx differ substantially. The relative potencies (EC_{50} values) and efficacies of all compounds tested are summarized in Table 1. PbTx-1 was the most efficacious compound with PbTx-desoxydioxolane, batrachotoxin and antillatoxin having comparable maximum responses. The rank order of efficacy of sodium channel gating

modifiers was PbTx-1 > PbTx-desoxydioxolane > batrachotoxin > antillatoxin > PbTx-2 = PbTx-3 > PbTx-3 α -naphthoate > veratridine > deltamethrin > aconitine > gambierol. The relative efficacies of the site 2 compounds batrachotoxin, veratridine and aconitine are congruent with those originally reported by Catterall (1977a,b) in which batrachotoxin was characterized as a full-, and veratridine and aconitine partial-agonists at this site.

Although all of the $[Na^+]_i$ responses to gating modifier compounds reported herein were derived from DIV 7–9 cerebrocortical neurons, we have found robust responses to these compounds as early as DIV 2. Both PbTx-2 and antillatoxin evoke maximum $[Na^+]_i$ responses in DIV 2 cerebrocortical neurons that are comparable to those found in DIV 7–9 neurons (data not shown).

Tetrodotoxin (TTX) antagonism of sodium channel gating modifier-induced elevation of $[Na^+]_i$

To confirm the role of VGSCs in gating modifier-induced elevation of $[Na^+]_i$ in neocortical neurons, we examined the influence of TTX (1 μ M) on this response. As shown in Fig. 6, the concentration-dependent increases in $[Na^+]_i$ produced by antillatoxin, PbTx-2, veratridine, deltamethrin, aconitine and gambierol were all abrogated by TTX. These data suggest that the observed gating modifier-induced elevation of $[Na^+]_i$ is dependent on the activation of VGSCs. Due to the limited availability of the brevetoxin analogs, we only tested TTX with PbTx-2 inasmuch as this is the most abundant naturally occurring brevetoxin and all brevetoxin analogs tested have been shown to inhibit the binding of [^{3} H]PbTx-3 to neurotoxin site 5 (Gawley et al., 1995; Michelliza S et al., 2007).

Brevetoxin enhancement of deltamethrin-induced increase in $[Na^+]_i$

Although the multiple neurotoxin receptor sites on VGSCs are topologically distinct, there are strong allosteric interactions between them. Pyrethroids have been shown to increase the binding of [^{3} H]batrachotoxin to neurotoxin site 2 of VGSCs and this effect is enhanced by brevetoxins interacting with neurotoxin site 5 (Lombet *et al.*, 1988; Trainer *et al.*, 1993; Li *et al.*, 2001). We therefore assessed the ability of a subthreshold concentration of PbTx-2 (10 nM) to enhance the effect of the pyrethroid deltamethrin on $[Na^+]_i$ in neocortical neurons. As shown in Fig. 7, a concentration of PbTx-2 (10 nM), that was inactive alone, produced a significant potentiation of deltamethrin (1 μ M) induced elevation of $[Na^+]_i$. These data are consistent with the previously demonstrated positive allosteric interaction between the site 5 ligand brevetoxin and pyrethroids.

Inhibition of PbTx-1-induced increase in neuronal $[Na^+]_i$ by the low-efficacy partial agonist gambierol

We have previously reported that gambierol binds to neurotoxin site 5 on the VGSC, albeit with modest affinity ($K_i = 4.8 \mu$ M), and acts as a functional antagonist of this site on neuronal VGSCs (LePage et al., 2007). As depicted in Fig. 5, gambierol acts as a low-efficacy partial agonist to stimulate Na^+ influx in neocortical neurons. The time-response relationships for gambierol-induced elevation of neuronal $[Na^+]_i$ are depicted in Fig. 8.a. Inasmuch as the maximum increment in $[Na^+]_i$ produced by gambierol was only 4 mM, this polyether ligand is less than 11% as effective as PbTx-1 as an activator of VGSCs. Given its low efficacy, we performed a titration with gambierol in the presence of a fixed concentration of the full agonist PbTx-1 (30 nM). As depicted in Fig. 8.b, titration with gambierol produced a concentration-dependent reduction in the PbTx-1-induced integrated SBFi response. These data are consistent with gambierol and PbTx-1 interacting in a mutually exclusive manner with a common recognition site on VGSCs. A similar antagonistic effect for a low-efficacy agonist at neurotoxin site 2 has previously been demonstrated; aconitine, a low-efficacy agonist, was shown to inhibit the batrachotoxin-induced increase in Na^+ permeability in neuroblastoma cells

(Catterall, 1977a). Considered together these results demonstrate that the ability of neurotoxins to act as partial agonists is shared by compounds acting at both neurotoxin site 2 and 5 of the VGSC.

Discussion

Based on the original work of Hodgkin and Huxley (1952) with squid axons, a single action potential was calculated to change minimally the Na^+ electrochemical gradient (Hille, 1991). The situation in mammalian neurons with fine axons, dendrites and spines is, however, much different due to greater surface to volume ratios. Thus, a single action potential may elevate $[\text{Na}^+]_i$ substantially (Hille, 1991). Using two-photon imaging to measure Na^+ transients in spines and dendrites of CA 1 pyramidal neurons in hippocampal slices, Rose *et al.* (1999) demonstrated action potential-induced $[\text{Na}^+]_i$ increments reached values of 3–4 mM following a train of just 20 action potentials. The influence of synaptic activity on $[\text{Na}^+]_i$ in apical dendrites and spines was subsequently demonstrated in rat hippocampal slice preparations by Rose and Konnerth (2001). These authors demonstrated that $[\text{Na}^+]_i$ increased by 30–40 mM during short bursts of synaptic stimulation and reached 100 mM during a long-term potentiation protocol. The influence of $[\text{Na}^+]_i$ dynamics on NMDA receptor function has been demonstrated in hippocampal neurons where elevation of $[\text{Na}^+]_i$ increased the open probability of NMDA receptors (Yu and Salter, 1998; Yu, 2006). An increment of $[\text{Na}^+]_i$ of 10 mM was sufficient to produce significant increases in NMDA receptor single channel activity. This Na^+ -dependent regulation of NMDA receptor function was moreover shown to be controlled by Src family kinase-induced phosphorylation of the receptor (Yu and Salter, 1998; Yu, 2006).

Lipid soluble toxins acting at neurotoxin site 2 and 5 have been shown to affect gating and to be allosterically coupled. Catterall has provided evidence that these neurotoxins induce conformational changes that alter the equilibrium between open and closed/inactivated states of the sodium channel (Catterall, 1977a, b, 1980). This work further indicated that the site 2 toxins aconitine, veratridine, grayanotoxin and batrachotoxin act as full or partial agonists and cause persistent activation of sodium channels that can be quantitatively fit by an allosteric model (Catterall, 1977a; 1980).

We have previously demonstrated that a group of site 5 ligands including both naturally occurring and semi-synthetic brevetoxin analogs produce different maximum responses at saturating concentrations (Lepage *et al.*, 2003). These previous studies used alterations in $[\text{Ca}^{2+}]_i$ in intact neurons to demonstrate that the brevetoxin PbTx-1 was a full agonist, whereas PbTx-3 and its analogs were partial agonists. In the present study these data were extended by directly comparing the efficacies of an array of gating modifier toxins as stimulators of Na^+ influx in cerebrocortical neurons. Measurement of $[\text{Na}^+]_i$ transients in neocortical neurons represents a more direct measure of sodium channel activation than the $[\text{Ca}^{2+}]_i$ transients used previously (Lepage *et al.*, 2003).

To explore the relative efficacies of gating modifier toxins, we used SBFI-loaded neocortical neurons to evaluate their respective concentration-response relationships. All neurotoxins tested evoked a rapid and concentration-dependent increase in neuronal $[\text{Na}^+]_i$. In agreement with the original observations of Catterall (1975, 1978), our results with the site 2 activators batrachotoxin, veratridine and aconitine indicate that these compounds produce concentration-dependent elevations of $[\text{Na}^+]_i$ in neocortical neurons with markedly distinct efficacies. Veratridine and aconitine were partial agonists, while batrachotoxin acted as a full agonist at neurotoxin site 2 in neocortical neurons. The naturally occurring brevetoxins PbTx-1, PbTx-2 and PbTx-3 also produced rapid and concentration-dependent increments in $[\text{Na}^+]_i$ with PbTx-1 proving to be the most potent and efficacious toxin. The primacy of PbTx-1 as an activator of Na^+ influx in neocortical neurons suggests that this compound is a full agonist at

neurotoxin site 5. The lower maximum responses to PbTx-2 and PbTx-3, accordingly, suggests that these compounds are partial agonists at neurotoxin site 5, inasmuch as a previous study has shown that all three toxins interact with the VGSC in a mutually exclusive manner (Gawley *et al.*, 1995). The greater efficacy of PbTx-1 at neurotoxin site 5 relative to other naturally occurring brevetoxins accords with the results of Trainer *et al.* (1993), who showed PbTx-1 to be the most effective brevetoxin in enhancing the binding of [³H]batrachotoxin to rat brain VGSCs. It has been suggested that the greater conformational flexibility in the backbone structure of PbTx-1 as compared to that of PbTx-2 may underlie its efficacy as a modulator of [³H]batrachotoxin binding to neurotoxin site 2 (Cestele *et al.*, 1995). The present results with the brevetoxin analog PbTx-3 α -naphthoate are also in agreement with our earlier studies monitoring brevetoxin-induced Ca²⁺ influx in cerebellar granule neurons in that this compound acts as a partial agonist in stimulating Na⁺ influx in neocortical neurons (LePage *et al.*, 2003). The brevetoxin analog PbTx-desoxydioxolane displayed an efficacy comparable to that of PbTx-1, but was more than 15-fold less potent.

In addition to the demonstration of partial agonism of site 2 and site 5 gating modifiers, we now report that antillatoxin has an efficacy at VGSCs that is comparable to PbTx-2 and PbTx-3. Antillatoxin-induced neurotoxicity and Ca²⁺ influx are abrogated by tetrodotoxin indicating that this compound is an activator of VGSCs (Li *et al.*, 2001). The ability of antillatoxin to activate VGSCs was directly demonstrated by the TTX-sensitive stimulation of ²²Na⁺ influx in cerebellar granule neurons (Li *et al.*, 2001). These results were confirmed and extended in the present report showing that antillatoxin is a potent and efficacious stimulator of [Na⁺]_i transients in neocortical neurons. Its efficacy was comparable to that of the site 5 ligands PbTx-2 and PbTx-3. The EC₅₀ value of antillatoxin of 78.9 nM was moreover similar to the previously reported value of 98.2 nM as a stimulator of ²²Na⁺ influx. Antillatoxin therefore appears to represent a novel activator of VGSCs whose site of action remains to be established (Li *et al.*, 2001).

Pyrethroids are synthetic insecticide compounds that resemble the natural pyrethrin toxins. Their primary molecular target as insecticides and neurotoxins is the VGSC (Trainer *et al.*, 1997). Pyrethroids such as deltamethrin shift the VGSC activation voltage to more negative values and inhibit inactivation. These activities result in a persistent activation of VGSCs as reflected in an enhancement of ²²Na⁺ influx similar to other gating modifiers (Lombert *et al.*, 1988). We demonstrate here that deltamethrin produces a concentration-dependent stimulation of Na⁺ influx in neocortical neurons with an efficacy of 0.53.

The lowest efficacy neurotoxin evaluated was gambierol (efficacy = 0.11). Gambierol is a polyether ladder toxin derived from the marine dinoflagellate *Gambierdiscus toxicus*, which also produces the potent site 5 activator ciguatoxin (LePage *et al.*, 2005, 2007). Gambierol has been shown to block voltage-gated K⁺ channels at depolarized membrane potentials under voltage clamp conditions in *Xenopus* oocytes (Cuypers *et al.*, 2008). Whether the development of K⁺ channel block by gambierol requires depolarizing steps, however, remains to be determined. At the resting membrane potential of cerebellar granule cells we have previously shown that, unlike ciguatoxin, gambierol has no effect on intracellular Ca²⁺ concentration and is without neurotoxic action in these cells (LePage *et al.*, 2007). By directly monitoring [Na⁺]_i transients in the present studies, we have found gambierol to be a low efficacy activator of VGSCs. These data are in agreement with those of Louzao *et al.* (2006) who showed that gambierol acts as a partial agonist at site 5 of VGSCs in a neuroblastoma cell line. The involvement of neurotoxin site 5 in the Na⁺ influx produced by gambierol reported herein was demonstrated by the ability of gambierol to produce a concentration-dependent inhibition of PbTx-1-induced elevation of [Na⁺]_i in neocortical neurons. These data parallel the original observations of Catterall (1977a) demonstrating partial agonism at neurotoxin site 2 in that treatment of cells with a combination of a good activator (batrachotoxin) and a poor activator

(aconitine) resulted in an inhibition of the response to the good activator by the poor activator. Thus, a low-efficacy agonist can be treated as an inhibitor of a high-efficacy agonist. Our demonstration of gambierol inhibition of PbTx-1-induced Na^+ influx therefore parallels the earlier findings of Catterall, and further demonstrates that the ability of neurotoxins to act as partial agonists is shared by compounds acting at neurotoxin sites 2 and 5. An additional parallel for site 2 and 5 gating modifier compounds documented herein is in regards to the maximum increment in $[\text{Na}^+]_i$ produced by the respective full agonists, batrachotoxin and PbTx-1. The similarity in the maximum responses to batrachotoxin and PbTx-1 may reflect the partial overlap of neurotoxin sites 2 and 5 on S6 segments of the VGSC α -subunit (Wang and Wang, 2003). Receptor mapping studies have found that both site 2 and site 5 ligands interact with residues in the S6 segments of domain I on the VGSC α -subunit (Wang and Wang, 2003).

Thus, when considered together, the lipophilic gating modifier toxins (brevetoxins, batrachotoxin, veratridine, aconitine, gambierol and deltamethrin) as well as the novel lipopeptide antillatoxin are all capable of exerting a TTX-sensitive elevation of $[\text{Na}^+]_i$ in neocortical neurons. Although these sodium channel activators display differing efficacies, they are all able to produce $[\text{Na}^+]_i$ increments of 4–60 mM. These $[\text{Na}^+]_i$ increments are therefore sufficient to increase NMDA receptor channel activity. Sodium channel gating modifier toxins are therefore all potentially capable of upregulating NMDA receptor signaling (Dravid et al., 2005).

Acknowledgments

This work was supported in part by NIH grants ES10594 to DGB, GM56677 to JDR and NS053398 to WHG and TFM.

ABBREVIATIONS

DIV	days in vitro
HEPES	N-(2-hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid)
NMDAR	N-methyl-D-aspartic acid receptor
PbTx	brevetoxin
SBFI	sodium-binding benzofuran isophthalate
TTX	tetrodotoxin
VGSC	voltage-gated sodium channel

REFERENCES

- Berman FW, Gerwick WH, Murray TF. Antillatoxin and kalkitoxin, ichthyotoxins from the tropical cyanobacterium *Lyngbya majuscula*, induce distinct temporal patterns of NMDA receptor-mediated neurotoxicity. *Toxicon* 1999;37:1645–1648. [PubMed: 10482399]
- Catterall WA. Cooperative activation of action potential Na^+ ionophore by neurotoxins. *Proc Natl Acad Sci U S A* 1975;72:1782–1786. [PubMed: 1057169]
- Catterall WA. Activation of the action potential Na^+ ionophore by neurotoxins. An allosteric model. *J Biol Chem* 1977a;252:8669–8676. [PubMed: 925017]
- Catterall WA. Membrane potential-dependent binding of scorpion toxin to the action potential Na^+ ionophore. Studies with a toxin derivative prepared by lactoperoxidase-catalyzed iodination. *J Biol Chem* 1977b;252:8660–8668. [PubMed: 72754]
- Catterall WA. Neurotoxins that act on voltage-sensitive sodium channels in excitable membranes. *Annu Rev Pharmacol Toxicol* 1980;20:15–43. [PubMed: 6247957]

- Catterall WA, Beress L. Sea anemone toxin and scorpion toxin share a common receptor site associated with the action potential sodium ionophore. *J Biol Chem* 1978;253:7393–7396. [PubMed: 29897]
- Catterall WA, Cestele S, Yarov-Yarovoy V, Yu FH, Konoki K, Scheuer T. Voltage-gated ion channels and gating modifier toxins. *Toxicon* 2007;49:124–141. [PubMed: 17239913]
- Catterall WA, Morrow CS, Daly JW, Brown GB. Binding of batrachotoxinin A 20- α -benzoate to a receptor site associated with sodium channels in synaptic nerve ending particles. *J Biol Chem* 1981;256:8922–8927. [PubMed: 6114956]
- Cestele S, Ben Khalifa RB, Pelhate M, Rochat H, Gordon D. Alpha-scorpion toxins binding on rat brain and insect sodium channels reveal divergent allosteric modulations by brevetoxin and veratridine. *J Biol Chem* 1995;270:15153–15161. [PubMed: 7797499]
- Cestele S, Catterall WA. Molecular mechanisms of neurotoxin action on voltage-gated sodium channels. *Biochimie* 2000;82:883–892. [PubMed: 11086218]
- Cuyper E, Abdel-Mottaleb Y, Kopljar I, Rainier JD, Raes AL, Snyders DJ, Tytgat J. Gambierol, a toxin produced by the dinoflagellate *Gambierdiscus toxicus*, is a potent blocker of voltage-gated potassium channels. *Toxicon*. 2008 in press.
- David G, Barrett JN, Barrett EF. Spatiotemporal gradients of intra-axonal $[Na^+]$ after transection and resealing in lizard peripheral myelinated axons. *J Physiol* 1997;498(Pt 2):295–307. [PubMed: 9032679]
- Diarra A, Sheldon C, Church J. In situ calibration and $[H^+]$ sensitivity of the fluorescent Na^+ indicator SBFI. *American Journal of Physiology-Cell Physiology* 2001;280:C1623–C1633. [PubMed: 11350758]
- Donoso P, Mill JG, O'Neill SC, Eisner DA. Fluorescence measurements of cytoplasmic and mitochondrial sodium concentration in rat ventricular myocytes. *J Physiol* 1992;448:493–509. [PubMed: 1593474]
- Dravid SM, Baden DG, Murray TF. Brevetoxin augments NMDA receptor signaling in murine neocortical neurons. *Brain Res* 2005;1031:30–38. [PubMed: 15621010]
- Gawley RE, Rein KS, Jeglitsch G, Adams DJ, Theodorakis EA, Tiebes J, Nicolaou KC, Baden DG. The relationship of brevetoxin 'length' and A-ring functionality to binding and activity in neuronal sodium channels. *Chem Biol* 1995;2:533–541. [PubMed: 9383457]
- Hille, B. Ionic channels of excitable membranes. Sunderland, Massachusetts: Sinauer associates Inc; 1991.
- Hodgkin AL, Huxley AF. Currents carried by sodium and potassium ions through the membrane of the giant axon of *Loligo*. *J Physiol* 1952;116:449–472. [PubMed: 14946713]
- Inoue M, Hirama M, Satake M, Sugiyama K, Yasumoto T. Inhibition of brevetoxin binding to the voltage-gated sodium channel by gambierol and gambieric acid-A. *Toxicon* 2003;41:469–474. [PubMed: 12657316]
- Johnson HW, Majumder U, Rainier JD. Total synthesis of gambierol: Subunit coupling and completion. *Chem Eur J* 2006;12:1747–1753.
- LePage KT, Baden DG, Murray TF. Brevetoxin derivatives act as partial agonists at neurotoxin site 5 on the voltage-gated Na^+ channel. *Brain Res* 2003;959:120–127. [PubMed: 12480165]
- LePage KT, Dickey RW, Gerwick WH, Jester EL, Murray TF. On the use of Neuro-2a neuroblastoma cells versus intact neurons in primary culture for neurotoxicity studies. *Critical Rev in Neurobiol* 2005;17:27–50. [PubMed: 16307526]
- Li WI, Berman FW, Okino T, Yokokawa F, Shioiri T, Gerwick WH, Murray TF. Antillatoxin is a marine cyanobacterial toxin that potently activates voltage-gated sodium channels. *Proc Natl Acad Sci U S A* 2001;98:7599–7604. [PubMed: 11416227]
- Lombet A, Mourre C, Lazdunski M. Interaction of insecticides of the pyrethroid family with specific binding sites on the voltage-dependent sodium channel from mammalian brain. *Brain Res* 1988;459:44–53. [PubMed: 2844361]
- Louza MC, Cagide E, Vieytes MR, Sasaki M, Fuwa H, Yasumoto T, Botana LM. The sodium channel of human excitable cells is a target for gambierol. *Cell Physiol Biochem* 2006;17:257–268. [PubMed: 16791001]
- Michelliza S, Abraham WM, Jacocks HM, Schuster T, Baden DG. Synthesis, modeling, and biological evaluation of analogues of the semisynthetic brevetoxin antagonist beta-naphthoyl-brevetoxin. *Chembiochem* 2007;17:2233–2239. [PubMed: 18000915]

- Orjala J, Nagle D, Gerwick WH. Antillatoxin, an Exceptionally Ichthyotoxic Cyclic Lipopeptide from the Marine Cyanobacterium *Lyngbya majuscula*. *J. Am. Chem. Soc* 1995;117:8281–8282.
- Rose CR, Konnerth A. NMDA receptor-mediated Na⁺ signals in spines and dendrites. *J Neurosci* 2001;21:4207–4214. [PubMed: 11404406]
- Rose CR, Kovalchuk Y, Eilers J, Konnerth A. Two-photon Na⁺ imaging in spines and fine dendrites of central neurons. *Pflugers Arch* 1999;439:201–207. [PubMed: 10651018]
- Rose CR, Ransom BR. Regulation of intracellular sodium in cultured rat hippocampal neurones. *J Physiol* 1997;499(Pt 3):573–587. [PubMed: 9130155]
- Ruigt GS, Neyt HC, Van der Zalm JM, Van den Bercken J. Increase of sodium current after pyrethroid insecticides in mouse neuroblastoma cells. *Brain Res* 1987;437:309–322. [PubMed: 2449265]
- Sharkey RG, Jover E, Couraud F, Baden DG, Catterall WA. Allosteric modulation of neurotoxin binding to voltage-sensitive sodium channels by *Ptychodiscus brevis* toxin 2. *Mol Pharmacol* 1987;31:273–278. [PubMed: 2436034]
- Tamkun MM, Catterall WA. Ion flux studies of voltage-sensitive sodium channels in synaptic nerve-ending particles. *Mol Pharmacol* 1981;19:78–86. [PubMed: 6259514]
- Trainer VL, McPhee JC, Boutelet-Bochan H, Baker C, Scheuer T, Babin D, Demoute JP, Guedin D, Catterall WA. High affinity binding of pyrethroids to the alpha subunit of brain sodium channels. *Mol Pharmacol* 1997;51:651–657. [PubMed: 9106631]
- Trainer VL, Moreau E, Guedin D, Baden DG, Catterall WA. Neurotoxin binding and allosteric modulation at receptor sites 2 and 5 on purified and reconstituted rat brain sodium channels. *J Biol Chem* 1993;268:17114–17119. [PubMed: 8394327]
- Wang SY, Wang GK. Voltage-gated sodium channels as primary targets for diverse lipid-soluble neurotoxins. *Cellular Signalling* 2003;15:151–159. [PubMed: 12464386]
- Yu XM. The role of intracellular sodium in the regulation of NMDA-receptor-mediated channel activity and toxicity. *Mol Neurobiol* 2006;33:63–80. [PubMed: 16388111]
- Yu XM, Salter MW. Gain control of NMDA-receptor currents by intracellular sodium. *Nature* 1998;396:469–474. [PubMed: 9853755]
- Yu XM, Salter MW. Src, a molecular switch governing gain control of synaptic transmission mediated by N-methyl-D-aspartate receptors. *Proc Natl Acad Sci U S A* 1999;96:7697–7704. [PubMed: 10393883]

Fig. 1.
Structures of sodium channel gating modifiers.

Fig. 2.

Full calibration of Na⁺-binding benzofuran isophthalate (SBFI) at 37°C. Neocortical neurons cultured in a 96-well plate were exposed to the calibration solution containing eight sodium concentrations from 0–130 mM (*see methods*). The emitted fluorescence during excitation at 340 nm and 380 nm were recorded and converted to the ratio of 340/380 after background subtraction. (a). The relationship between eight different [Na⁺]_i and SBFI ratio values. (b) A three-parameter hyperbolic fit (Eq. 2) of [Na⁺] vs. mean ratio values (R) obtained from 3 calibration experiments ($r^2=0.987$) (c) Hanes plot (Eq. 3) of calibration data show a linear regression fit of the data ($r^2=0.997$).

Fig. 3. Representative emitted fluorescence intensities before and after application of 300 nM PbTx-2. Emitted fluorescence during excitation at 340 nm (diamond) was insensitive to 300 nM PbTx-2 exposure, whereas emitted fluorescence during excitation at 380 nm (circle) decreased after application of 300 nM PbTx-2. Arrow indicates time of addition of PbTx-2.

Fig. 4.

Time-response relationships for VGSC gating modifier-induced $[Na^+]_i$ changes in neocortical neurons. $[Na^+]_i$ responses after exposure to different concentrations of PbTx-1 (a), PbTx-2 (b), PbTx-3 (c), PbTx-desoxydioxolane (d), PbTx-3- α -naphthoate (e) antillatoxin (f), deltamethrin (g), batrachotoxin (h), veratridine (i) and aconitine (j). These data were obtained from at least four different cultures performed in duplicate.

Fig. 5. Nonlinear regression analysis of the peak SBFI (340/380) response versus the concentration of brevetoxins (PbTx-1, PbTx-2, PbTx-3, PbTx-desoxydioxolane, PbTx-3- α -naphthoate), batrachotoxin, antillatoxin, veratridine, deltamethrin, aconitine and gambierol.

Fig. 6. Effects of TTX on PbTx-2 (a), antillatoxin (b), veratridine (c), aconitine (d), deltamethrin (e) and gambierol (f) induced elevation of $[Na^+]_i$.

Fig. 7. PbTx-2 (10 nM) enhances deltamethrin (1 μ M) induced elevation of neocortical $[Na^+]_i$. Each data point represents the mean of seven determinations.

Fig. 8.

a. Time-response relationships for gambierol-induced $[Na^+]_i$ changes in neocortical neurons. These data were obtained from three different cultures performed in duplicate. b. Nonlinear regression analysis of gambierol inhibition of the integrated SBFI response [area under the curve (AUC)] to PbTx-1 (30 nM) in neocortical neurons. Histogram represents the responses to PbTx-1 (30 nM) and gambierol (10 μ M) alone. Each data point is the mean ($\pm$ SEM) of 6 determinations.

Table 1

Potencies and efficacies of sodium channel gating modifiers as stimulators of Na⁺ influx in cerebrocortical neurons.

Gating Modifier	EC50 (95% CI)	Efficacy
	(nM)	
PbTx-1	44.6 (29–70)	1.0
PbTx-desoxydioxolane	682.5 (471–990)	0.96
Batrachotoxin	9.9 (5.4–18)	0.91
Antillatoxin	78.9 (58–107)	0.90
PbTx-2	95.0 (68–132)	0.81
PbTx-3	87.4 (54–142)	0.81
PbTx-3 α -naphthoate	4,986 (2,617–9,499)	0.76
Veratridine	536.7 (358–806)	0.67
Deltamethrin	1,180 (926–1,455)	0.53
Aconitine	2,841 (1,884–4,286)	0.35
Gambierol	305.8 (115–816)	0.11