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Activation of Sodium Channels and Inhibition of [^{3}H]Batrachotoxinin A-20-α-Benzoate Binding by an *N*-Alkylamide Neurotoxin

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SUMMARY

BTG 502 [(2*E*,4*E*)-*N*-(1,2-dimethyl)-propyl-6-(5-bromonaphth-2-yl)-hexa-2,4-dienamide], a synthetic analog of insecticidal amides isolated from *Piper* species, stimulated $^{22}Na^+$ uptake into mouse brain synaptoneurosomes in the presence of saturating concentrations of *Leiurus quinquestriatus* venom but had no effect on sodium uptake in the absence of venom. In the presence of *Leiurus* venom, half-maximal stimulation was achieved at a BTG 502 concentration of 1.7 μM, whereas maximal stimulation (2.3-fold greater than nonspecific uptake) was observed at 50 μM. In the absence of other modifiers, BTG 502 inhibited batrachotoxin (BTX)-dependent sodium uptake, producing 50% inhibition at 2 μM. In the presence of *Leiurus* venom, BTG 502 was a partial inhibitor of BTX-dependent $^{22}Na^+$ uptake, producing half-maximal inhibition at 1.5 μM. The levels of residual BTX-dependent sodium uptake and maximal BTG 502-dependent sodium uptake measured in the presence of *Leiurus* venom were identical. BTG 502 inhibited the specific binding of [^{3}H]batrachotoxinin A-20-α-benzoate (BTX-B) to the activator recognition site (site 2) of sodium channels in these preparations, producing half-maximal inhibition at 2 μM and maximal inhibition at 30 μM. Equilibrium analysis showed that BTG 502 was an apparent competitive inhibitor of [^{3}H]BTX-B binding, producing a concentration-dependent decrease in the affinity of sodium channels for this ligand without affecting binding capacity. Kinetic analysis demonstrated that BTG 502 slowed the rate of formation of the ligand-receptor complex but did not alter the rate of dissociation of this complex. The effects of BTG 502 on $^{22}Na^+$ uptake and [^{3}H]BTX-B binding are consistent with the action of this compound as an antagonist at the activator recognition site of the voltage-sensitive sodium channel in the absence of *Leiurus* venom and as a partial agonist at this site in the presence of *Leiurus* venom. These results suggest that the *N*-alkylamides represent a novel chemical class of neurotoxins that act at site 2 of the sodium channel.

The insecticidal and medicinal properties of naturally occuring *N*-alkylamides of polyunsaturated alkanoic acids (e.g., pellitorine; Fig. 1) have been known for more than 100 years (1, 2). Recent synthetic efforts have produced analogs (e.g., BTG 502; Figure 1) with improved insecticidal properties (3, 4). Preliminary physiological studies show that insecticidal *N*-alkylamides induce repetitive activity followed by conduction block in housefly nerves (5) and suppress peak sodium current and induce slowly decaying tail currents in voltage-clamped locust nerve cell bodies in culture (6). *N*-Alkylamides also inhibit the VTD-dependent release of acetylcholine from preloaded cockroach central nervous system synaptosomes (7). These results implicate the voltage-sensitive sodium channel as the principal site of action for these compounds.

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The voltage-sensitive sodium channel is known to possess distinct and well characterized binding domains for five classes of neurotoxins (8). In addition, several studies (9–14) have shown that synthetic pyrethroid insecticides and DDT modify sodium channel function by interacting with a sixth binding domain that is allosterically coupled to the binding site for sodium channel activators such as BTX, VTD, and ACN. The present study was undertaken to define the action of *N*-alkylamides in the context of other classes of sodium channel-directed neurotoxins and insecticides. Our results show that BTG 502 acts as a partial agonist at the activator recognition site of sodium channels in mouse brain preparations. A preliminary report of these findings has been presented (15).

Materials and Methods

Chemicals. VTD, ScV, and TTX were purchased from Sigma Chemical Co. (St. Louis, MO). ATXII was obtained from Calbiochem-

ABBREVIATIONS: BTG 502, (2*E*,4*E*)-*N*-(1,2-dimethyl)-propyl-6-(5-bromonaphth-2-yl)-hexa-2,4-dienamide; ACN, aconitine; ATXII, *Anemonia sulcata* toxin II; BTX, batrachotoxin; [^{3}H]BTX-B, [^{3}H]batrachotoxinin A 20-α-benzoate; DTM, deltamethrin; HEPES, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; ScV, scorpion (*Leiurus quinquestriatus*) venom; TTX, tetrodotoxin; VTD, veratridine; DDT, 1,1-bis-(*p*-chlorophenyl)-2,2,2-trichloroethane.

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Behring (La Jolla, CA). BTX was a gift from J. Daly, National Institutes of Arthritis, Metabolism and Digestive Disease (Bethesda, MD). BTG 502 was provided by N. Janes, Rothamsted Experimental Station (Harpenden, England). DTM was a gift from J. Martel, Rousel-Uclaf (Romainville, France). Unlabeled BTX-B was provided by G. Brown, University of Alabama at Birmingham. Carrier-free $^{22}NaCl$ was obtained from Amersham Corp. (Arlington Heights, IL). [^{3}H]BTX-B (42.7 Ci/mmol) was purchased from DuPont NEN Research Products (Boston, MA).

Preparation of synaptoneurosomes. Synaptoneurosomes were prepared by the procedure of Brown (16) with minor modifications. The brains of male ICR mice (Blue Spruce Farms, Altamont, NY) were removed immediately following cervical dislocation, rinsed thoroughly in homogenization buffer, and blotted dry on filter paper. For sodium flux studies, the homogenization buffer contained (mM): choline chloride (130), HEPES (30), glucose (5.5), $MgCl_2 \cdot 6\ H_2O$ (3), KCN (3), KCl (10), and ouabain (3). The homogenization buffer used in binding experiments contained (mM): choline chloride (130), HEPES (50), glucose (5.5), $MgCl_2 \cdot 6\ H_2O$) (0.8), and KCl (5.4). The pH of homogenization buffers was adjusted to 7.4 with Tris base. The grey matter was removed from brains, minced in homogenization buffer (1.5 ml/brain), and homogenized by hand using four or five strokes of a Dounce homogenizer. The homogenate was diluted to a final concentration of 1 brain/7.5 ml of homogenization buffer and centrifuged at 1000 × *g* for 15 min. The resulting pellet was resuspended in 3.5 ml (uptake) or 6 ml (binding) of the appropriate homogenization buffer containing 1 mg/ml bovine serum albumin, filtered through nylon mesh, and used immediately for assays. The protein concentration of synaptoneurosomal membranes was estimated by the method of either Lowry *et al.* (17) or Smith *et al.* (18), using bovine serum albumin as the standard.

Sodium uptake assays. The influx of $^{22}Na^+$ into synaptoneurosomes was measured by the procedure of Tamkum and Catterall (19), as modified by Bloomquist and Soderlund (11). BTG 502 or DTM in ethanol (1 μl) was added to the incubation tubes following the addition of the synaptoneurosomal membranes (100 μl; approximately 300 μg of protein). Preliminary experiments established that the amount of ethanol used to deliver lipophilic toxins to the incubation mixture did not affect sodium uptake. Following preincubation of synaptoneurosomes and toxins for 18 min at 20° and then 2 min at 37°, sodium uptake was initiated by the addition of 100 μl of sodium flux buffer (11) containing 150 nCi of $^{22}Na^+$. After incubation at 37° for 15 sec, uptake was terminated by the addition of 3 ml of ice-cold washing buffer (11) and rapid vacuum filtration. Filters were washed with an additional 6 ml of washing buffer, and sodium uptake was quantitated by liquid scintillation counting of the filters using a 4:1 mixture of Betafluor (National Diagnostics, Manville, NJ) and ethylene glycol monomethyl ether as the scintillant. Data points represent at least three separate experiments performed in triplicate using freshly prepared synaptoneurosomes for each assay.

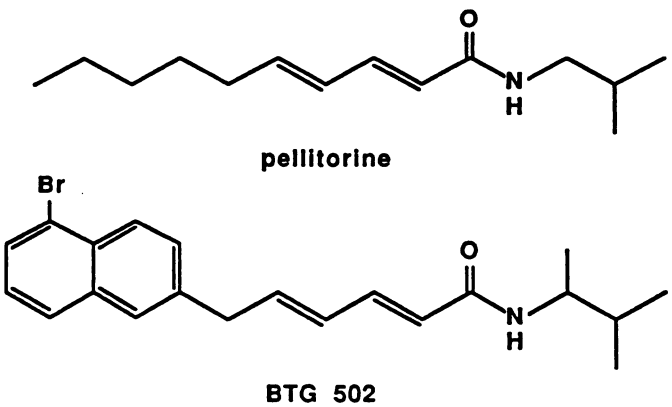

Fig. 1. Structures of a naturally occurring *N*-alkylamide neurotoxin (*top*) and a synthetic analog (*bottom*).

Binding assays. The specific binding of [^{3}H]BTX-B was determined using a modification of the method of Catterall *et al.* (20). Synaptoneurosomes (approximately 160 μg of protein) were incubated with [^{3}H]BTX-B (10–20 nM), unlabeled BTX-B (0–230 nM), and ScV (30 μg) in a total volume of 160 μl of sodium-free binding medium at 37° for 45 min, except for association experiments in which shorter incubation times were employed. Specific binding was defined as the amount of total binding displaced by 500 μM VTD. BTG 502 was added in 0.8 μl of ethanol, an amount that does not affect the binding of [^{3}H]BTX-B (21). After incubation, 120-μl aliquots were applied to Whatman GF/C filters and immediately rinsed (2 × 5 ml) under vacuum with ice-cold washing buffer (20). For dissociation experiments, synaptoneurosomes were preincubated at 37° for 45 min with [^{3}H]BTX-B, ScV, and either BTG 502 or an equal volume of ethanol. Following the addition of either VTD (500 μM) or VTD (300 μM) plus BTG 502 (30 μM), samples were incubated for various times (5–60 min) before isolation of bound ligand by filtration. Bound radioactivity was determined by liquid scintillation counting of the filters in Liquiscint (National Diagnostics). The data presented are means of two to seven replicates using different synaptoneurosome preparations, with triplicate determinations under each experimental condition in each replicate. Curves for linear transformations of data were fitted by least squares regressions.

Results

Effects of BTG 502 on sodium uptake. Our initial experiments surveyed the actions of BTG 502 (30 μM) both alone and in the presence of other sodium channel-directed neurotoxins (Fig. 2). BTG 502 alone did not significantly stimulate sodium uptake over control levels. In the presence of ScV (25 μg), BTG 502 produced a 2.3-fold stimulation of sodium uptake that was completely inhibited by 10 μM TTX. BTG 502 also stimulated sodium uptake in the presence of ATXII (10 μM; data not shown). In the absence of ScV, BTG 502 inhibited sodium uptake stimulated by both VTD (100 μM) and BTX (1 μM). Because the pyrethroid insecticide DTM enhances sodium uptake stimulated by VTD and BTX and inhibits ACN-stimulated uptake (9–11, 13), we examined the interaction between DTM and BTG 502. In the absence of ScV, the combination of DTM (10 μM) and BTG 502 had no stimulatory effect on

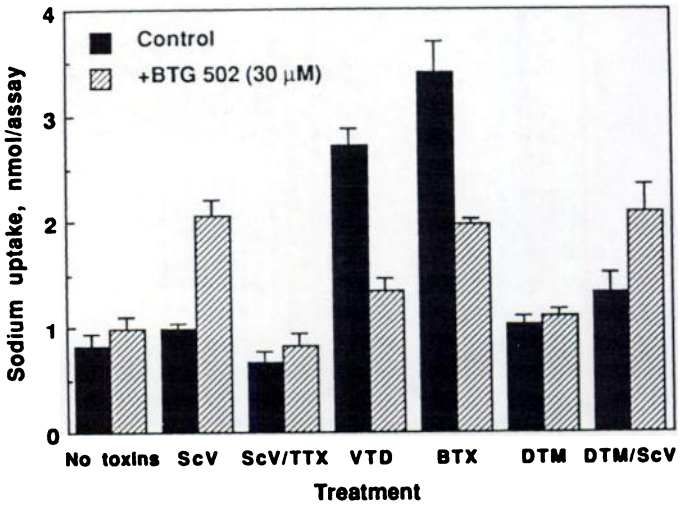

Fig. 2. The effect of sodium channel neurotoxins [ScV (25 μg), TTX (10 μM), VTD (100 μM), BTX (1 μM), and DTM (10 μM)] on total sodium uptake in the absence (■) or presence (▨) of BTG 502 (30 μM). *Bars* represent mean uptake (± standard error) based on at least three replicate experiments.

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sodium uptake. In the presence of ScV, DTM did not modify BTG 502-dependent sodium uptake measured at BTG 502 concentrations of either 30 μM (Fig. 2) or 3 μM (data not shown).

In the presence of a saturating concentration of ScV (25 μg), BTG 502 both stimulated sodium uptake and inhibited BTX-stimulated uptake in a concentration-dependent manner (Fig. 3). Half-maximal stimulation was obtained at a BTG 502 concentration of 1.7 μM, and maximal stimulation was observed at and above 10 μM. BTG 502 was an incomplete inhibitor of BTX (0.3 μM)-dependent uptake, producing maximal (45%) inhibition of BTG 502 concentrations at and above 10 μM. Half-maximal inhibition by BTG 502 was observed at 1.5 μM. The residual level of BTX-dependent sodium uptake observed in the presence of maximally effective concentrations of BTG 502 was equal to that produced by this compound in the presence of ScV but in the absence of BTX.

In incubations without ScV, BTG 502 was an effective inhibitor of BTX (1 μM)-dependent sodium uptake (Fig. 4). In contrast to results obtained in the presence of ScV (Fig. 3), inhibition of BTX-dependent uptake in the absence of ScV (Fig. 4) was nearly complete at high concentrations. The IC_{50} value for BTG 502 as an inhibitor of BTX-dependent uptake in the absence of ScV was calculated from the data in Fig. 4 to be 2 μM, a value close to that obtained for half-maximal inhibition in the presence of ScV (1.5 μM; Fig. 3). The actions of BTG 502 as an inhibitor of VTD-dependent sodium uptake both in the absence and in the presence of ScV were similar to the effects described above on BTX-stimulated uptake (data not shown).

Effects of BTG 502 on [^{3}H]BTX-B binding. Radioligand binding experiments in mouse brain synaptoneurosomes, performed in the presence of a saturating concentration of ScV, showed that BTG 502 was a potent and complete inhibitor of the specific binding of [^{3}H]BTX-B (Fig. 5). BTG 502 produced half-maximal inhibition at 2 μM. The slope of the Hill plot of the displacement of [^{3}H]BTX-B by BTG 502 was 0.83. Scatchard plots of equilibrium saturation experiments performed under the same conditions (Fig. 6; Table 1) demonstrated that BTG 502 produced a concentration-dependent decrease in the affinity of sodium channels for BTX-B, with no significant effect on binding capacity. Kinetic studies were undertaken to define further the action of BTG 502 as an inhibitor of BTX-B binding. Equilibration of membranes with [^{3}H]BTX-B in the presence of BTG 502 (3 μM) had no substantial effect on the subsequent rate of dissociation of the ligand-receptor complex (Fig. 7A; Table 1). Other experiments showed that a high concentration of BTG 502 (30 μM) introduced following the equilibration of membranes with [^{3}H]BTX-B also did not modify the rate of dissociation (data not shown). However, a reduction by BTG 502 in the initial rate of formation of the ligand-receptor complex was evident in association experiments (Fig. 7B; Table 1).

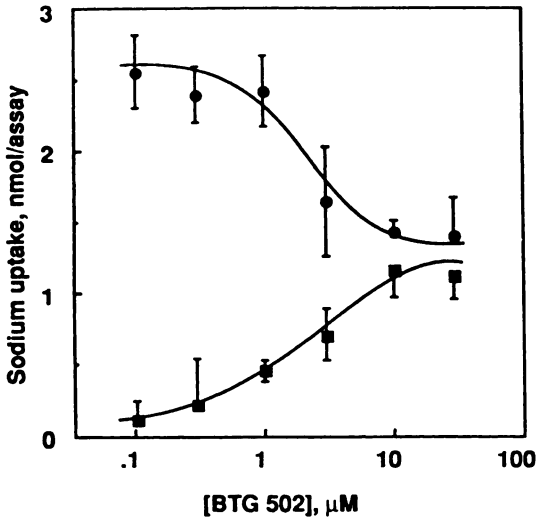

Fig. 3. Concentration-dependent activation of sodium uptake (■) and inhibition of BTX-dependent sodium uptake (●) by BTG 502 in the presence of ScV (25 μg). Values are means (± standard errors) of three (without BTX) or four (with BTX) replicate experiments and are corrected for sodium uptake measured in the presence of ScV alone.

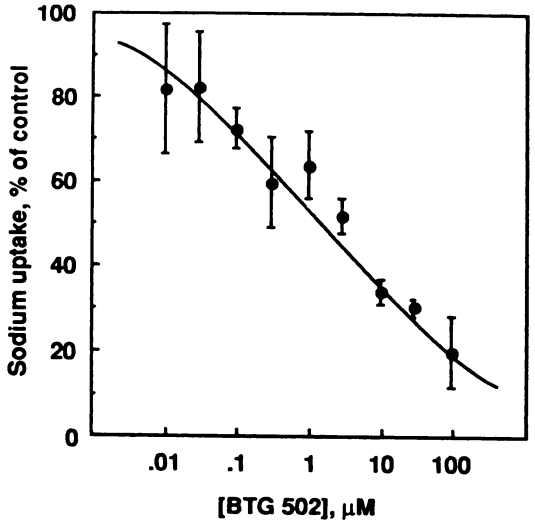

Fig. 4. Concentration-dependent inhibition of BTX-dependent sodium uptake by BTG 502 in the absence of ScV. Values are means (± standard errors) of at least three replicate experiments and are corrected for nonspecific uptake measured in the absence of toxins.

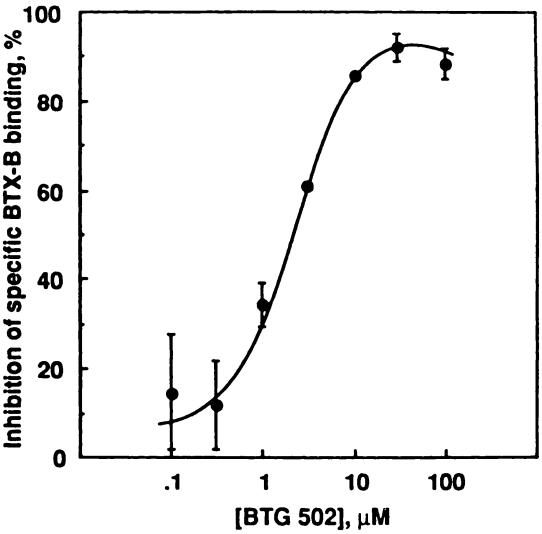

Fig. 5. Concentration-dependent inhibition of [^{3}H]BTX-B (20 nM) binding by BTG 502 measured in the presence of ScV (30 μg); data are means (± standard errors) of two replicates.

Discussion

Previous physiological (5, 6) and biochemical (7) studies of the action of *N*-alkylamides in insect neuronal preparations

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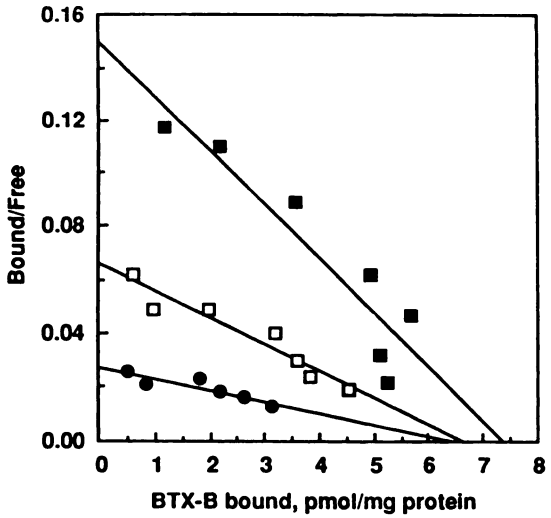

Fig. 6. Scatchard analyses of the displacement of [^{3}H]BTX-B (10 nM) by increasing concentrations of BTX-B, in the absence and presence of BTG 502, measured in the presence of ScV (30 μg). Data in the absence of BTG 502 (■) and in the presence of 3 μM BTG 502 (□) are means of seven replicate experiments; data in the presence of 10 μM BTG 502 (●) are means of three replicates.

TABLE 1

Calculated equilibrium and kinetic constants for the binding of [^{3}H] BTX-B to mouse brain sodium channels in the absence and presence of BTG 502

Treatment	K_D[a]	B_{max}[a]	k_{-1}[b]	k_{obs}[c]
	nM	pmol/mg of protein	min^{-1}	
Control	50	7.4	0.07	0.13
BTG 502 (3 μM)	101	6.6	0.08	0.07
BTG 502 (10 μM)	235	6.4		

[a] Calculated from regression analyses of data in Fig. 6.
[b] Dissociation rate constant, calculated from regression analyses of data in Fig. 7A.
[c] Slope of normalized radioligand association curve, calculated from regression analyses of data in Fig. 7B.

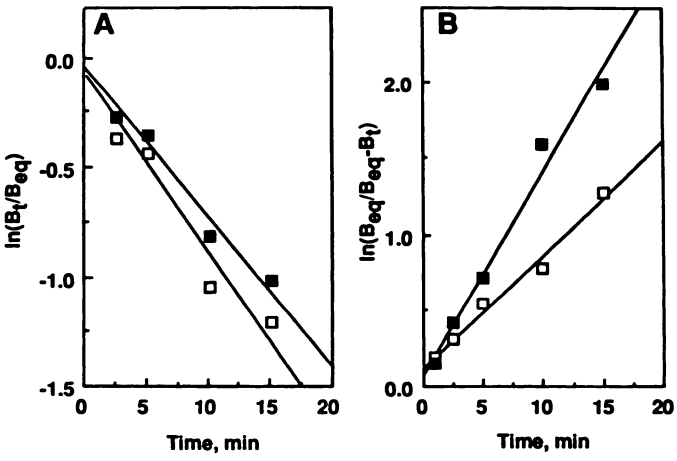

Fig. 7. Kinetic analysis of the effect of BTG 502 on the binding of [^{3}H] BTX-B measured in the presence of ScV (30 μg). A, Dissociation curves for [^{3}H]BTX-B (20 nM) in the absence (■) and presence (□) of BTG 502 (3 μM). B, Linear transformations of association curves for [^{3}H]BTX-B (10 nM) in the absence (■) and presence (□) of BTG 502 (3 μM). Data are means of three to five replicates.

implicate the voltage-sensitive sodium channel as the target site for these compounds. However, these studies do not provide insight into the molecular domain of the sodium channel that participates in the binding and action of *N*-alkylamides. It is now known that the voltage-sensitive sodium channel has at least five distinct neurotoxin recognition sites (8), site 1, which binds sodium channel blockers such as TTX and saxitoxin; site 2, which binds sodium channel activators such as VTD, BTX, ACN, and the grayanotoxins; site 3, which binds ATXII, *Leiurus quinquestriatus* toxin, and other α-polypeptide toxins from North African scorpion venoms; site 4, which binds the β-polypeptide toxins isolated from American scorpion venoms; and site 5, which binds the brevetoxins and ciguatoxins. In addition to these five sites, additional but less well characterized sites have been proposed for pyrethroid insecticides and DDT analogs (9–13), pumiliotoxins (22), and a polypeptide toxin isolated from *Goniopora* corals (23). The results of our studies with mouse brain preparations provide evidence for the action of BTG 502, a potent synthetic *N*-alkylamide, at site 2, the activator recognition site of the sodium channel.

Activation of sodium channels by BTG 502 was dependent on the presence of ScV in the assay medium. In the presence of ScV, BTG 502 activated sodium uptake and partially inhibited BTX-dependent sodium uptake. The partial inhibition of BTX-dependent activation under these conditions and the identical levels of BTG 502-dependent sodium uptake obtained in the presence and absence of BTX provide evidence that BTG 502 acts as a partial agonist with respect to BTX at site 2 of the sodium channel. Because the effect of ScV on BTG 502-dependent activation was mimicked by ATXII, we conclude that allosteric effects of ScV observed in our assays are due to the α-polypeptide toxin present in this venom. In the absence of ScV, stimulation of sodium uptake was not detectable and inhibition of BTX-dependent sodium uptake was extensive. These results are consistent with the action of BTG 502 as an antagonist at site 2 in the absence of ScV.

Our findings also identify differences between BTG 502 and other sodium channel activators. In the absence of ScV, BTX is defined as a full agonist at site 2, and other activators act as partial agonists of varying maximal efficacy depending on the source of sodium channels assayed (11, 19, 24, 25). In assays with mammalian brain preparations, two allosteric effects of site 3 neurotoxins on the action of established sodium channel activators are typically observed; first, site 3 neurotoxins increase the potency of all activators and, second, site 3 neurotoxins increase the efficacy of partial agonists (11, 19). With BTG 502, the latter effect was observed but the potency of BTG 502 (measured as the concentration producing half-maximal inhibition of BTX-dependent sodium uptake) was not affected by ScV. BTG 502 also differed from other site 2 neurotoxins in that it failed to exhibit allosteric interactions, at either saturating or subsaturating concentrations, with the pyrethroid insecticide DTM.

The effects of BTG 502 on the binding of [^{3}H]BTX-B are also consistent with an action at site 2. BTG 502 inhibited the binding of [^{3}H]BTX-B in a competitive fashion, reducing the affinity of sodium channels for this ligand in a concentration-dependent manner without significantly affecting binding capacity. Kinetic studies showed that the reduction in affinity was due to a reduction in the rate of formation of the ligand-receptor complex rather than an increase in the dissociation

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rate. Moreover, the magnitude of the effect of 3 μM BTG 502 on association rates was sufficient to account for the magnitude of the decrease in affinity obtained at the same concentration of BTG 502 in equilibrium saturation experiments. The failure of high concentrations of BTG 502 to modify the rate of dissociation of [^{3}H]BTX-B also tends to rule out a negative allosteric mechanism. This result differentiates the actions of BTG 502 from those of known allosteric inhibitors of [^{3}H]BTX-B binding (e.g., anticonvulsants and local anesthetics), which reduce the affinity of sodium channels for this ligand by increasing the rate of dissociation of the ligand-receptor complex (26, 27). The competitive inhibition of [^{3}H]BTX-B binding by BTG 502 also distinguishes the latter compound from pumiliotoxins, which also activate sodium uptake and interact allosterically with α-toxins but fail to displace [^{3}H]BTX-B (22).

In summary, we have demonstrated that an *N*-alkylamide neurotoxin activates sodium channels through an interaction at site 2 of the sodium channel. This compound differs substantially in chemical structure from other site 2 neurotoxins and, therefore, represents a new chemical class of sodium channel activators. Preliminary experiments suggest that toxic analogs of BTG 502 also activate sodium uptake in the presence of ScV and inhibit the binding of [^{3}H]BTX-B, whereas nontoxic analogs are generally ineffective in these *in vitro* assays.[1] Further studies with a wider structural variety of *N*-alkylamides are required to define these structure-activity relationships and determine whether actions of these compounds at site 2 of the voltage-sensitive sodium channel are sufficient to account for their neurotoxic effects *in vivo*.

Acknowledgments

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