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PUMILIOTOXIN ALKALOIDS: A NEW CLASS OF SODIUM CHANNEL AGENTS

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Abstract—Pumiliotoxin B (PTX-B) and a variety of congeneric alkaloids and synthetic analogs stimulated sodium flux and phosphoinositide breakdown in guinea pig cerebral cortical synaptoneurosomes. The effects of PTX-B and active congeners and analogs on sodium flux in synaptoneurosomes were potentiated markedly by scorpion venom (*Leiurus quinquestriatus*). In neuroblastoma cells, PTX-B and active congeners had no effect on sodium flux unless synergized by α-scorpion toxin or scorpion venom. Certain inactive congeners, lacking hydroxyl groups in the 6-alkylidene side chain, inhibited sodium flux elicited by PTX-B, scorpion venom, or the sodium channel activator batrachotoxin. Such inhibition appeared different from inhibition by local anesthetics, since pumiliotoxins, unlike local anesthetics, had little or no effect on binding of [^{3}H]batrachotoxinin A benzoate to sodium channels. Thus, it appears likely that some "inactive" congeners bind to the PTX-B binding site, but do not activate sodium channels. In the absence of scorpion venom the stimulation of phosphoinositide breakdown in synaptoneurosomes was consonant with the stimulatory effects of these compounds on sodium flux through voltage-dependent sodium channels.

Pumiliotoxin B (PTX-B), one of a series of congeneric alkaloids from tropical frogs, enhances sodium flux by interaction with a unique modulatory site on the voltage-dependent sodium channel [1]. The pumiliotoxin site exhibits synergistic interactions with two classes of scorpion toxin sites and with a brevetoxin site. PTX-B has no apparent interaction with the other alkaloid site at which batrachotoxin, veratridine and aconitine act to open sodium channels, nor does PTX-B interact with the site at which tetrodotoxin/saxitoxin blocks sodium channels. The alkaloid PTX-B and certain congeners stimulate phosphoinositide breakdown in brain and heart preparations [2, 3]. The stimulation of phosphoinositide breakdown by these alkaloids appears to be physiologically expressed as myotonic and cardiotonic activities [2–7]. It is likely that all of these effects are due to activity on voltage-dependent sodium channels [1, 8].

Structure–activity relationships for stimulation of sodium influx by pumiliotoxin alkaloids in synaptoneurosomes and neuroblastoma cells have now been examined. The results indicate that, while many analogs of PTX-B have very low efficacy as agonists of sodium channels, the addition of scorpion venom (or α-scorpion toxin) reveals agonist activity approaching that of PTX-B itself. Other analogs had no agonist activity even in the presence of scorpion venom and may actually act as antagonists at the PTX-B site. While low concentrations of local anesthetics effectively blocked stimulation of sodium flux by a PTX-B/scorpion venom combination, higher concentrations were required to block stimulation of phosphoinositide breakdown by PTX-B. The results demonstrate that the pumiliotoxin alkaloids are an important new class of substances for investigation of voltage-dependent sodium channels, and suggest that the pumiliotoxin alkaloids can either activate (agonist) or inhibit (antagonist or reverse agonist) the function of voltage-dependent sodium channels.

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MATERIALS AND METHODS

PTX-B and various congeners and synthetic analogs (for structures see Table 1) were isolated or synthesized as reported previously (see Ref. 2 and references therein). The des-18-methyl-pumiliotoxin B (**3**) was synthesized using the approach recently employed for the preparation of (+)-pumiliotoxin A [9]. The synthesis sequence is outlined in Scheme 1 and provided (+)-3, $[\alpha]_D$ +12.2° (c = 0.6, MeOH), in seven steps and 12% overall yield from the known [10] enantiomerically pure ylide (**20**). Details of this sequence will be reported elsewhere. Characterization data for **3** follow: $[\alpha]_D$ +12.2°, $[\alpha]_{577}$ +5.7°, $[\alpha]_{546}$ +7.0°, $[\alpha]_{435}$ +19.7°, $[\alpha]_{405}$ +21.2° (c = 0.6, MeOH); ^{1}H-NMR (500 MHz $CDCl_3$) δ 5.45 (t, *J* = 5.8 Hz, C=CH), 5.23 (t, *J* = 5.8 Hz, C=CH), 3.74 (d, *J* = 11.6 Hz, NCHC=C), 3.71–3.66 (m, 2H, HOCH), 3.06 (t, *J* = 8.3 Hz, 1H, NCH), 2.33 (bs, 1H), 2.28 (d, *J* = 11.7 Hz, NCHC=C), 2.25–1.64 (m, H), 1.61 (s, C=CCH_3), 1.13 (s, COHCH_3),

Table 1. Effects of pumiliotoxin alkaloids and synthetic analogs on sodium flux and phosphoinositide breakdown in guinea pig cerebral cortical synaptoneurosomes

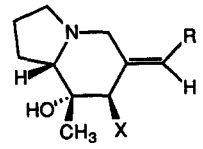
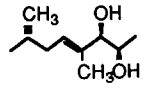
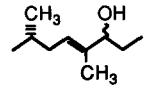
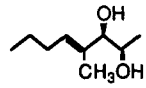
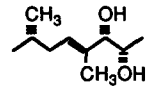
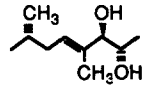
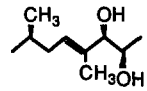
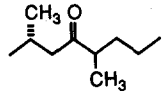
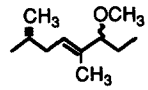
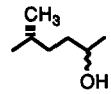
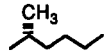
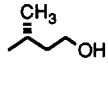
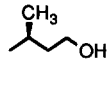
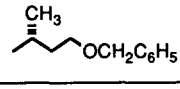
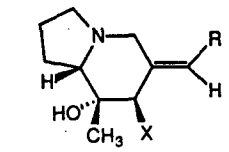
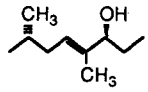
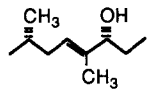
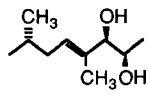
Alkaloid	 R	Sodium flux* (% of response to PTX-B)	Phosphoinositide breakdown† (% of response to PTX-B)
None		27 ± 4	
	Pumiliotoxins X = H		
1. PTX-B		100 + 4	100 ± 14
2. PTX-A		79 ± 8	39 ± 2
3. Des-18-methyl PTX-B		62	52
4. 15,16-Epi PTX-B		66	47 ± 8
5. Erythro PTX-B (16-epi PTX-B)		100 ± 4	92 ± 7
6. 11-Epi PTX-B		12 ± 3	6 ± 2
7. 307F‡		16 ± 4	6 ± 6
8. 321		53 ± 7	12 ± 7
9. 267C		66 + 5	17 ± 1
10. 251D		8 ± 1	17 × 1
11.		11 ± 2	6 ± 4
12.		21 ± 4	8
13.		2 ± 1	1

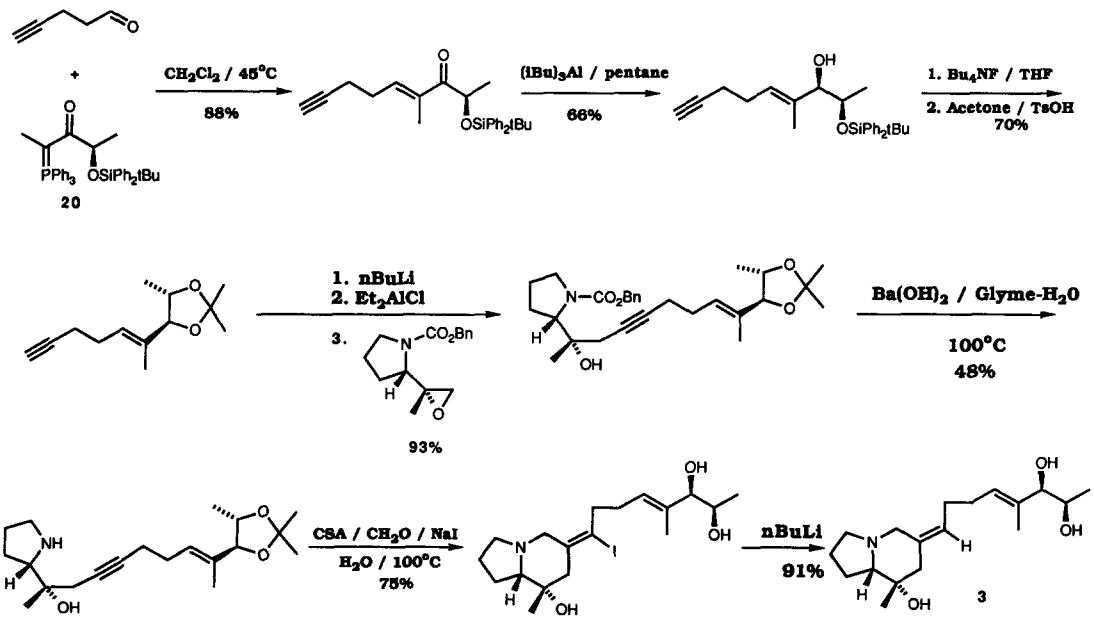
Table 1. *continued*

Alkaloid	 R	Sodium flux* (% of response to PTX-B)	Phosphoinositide breakdown† (% of response to PTX-B)
14.		7 ± 3	6 ± 9
15.		21 ± 1	11 ± 2
	Allopumiliotoxins X = OH		
16. 323B′		98 ± 6	100 ± 17
17. 323B″		79 ± 6	122 ± 3
18. 339A		120 + 4	110 ± 11
19. 267A		22 ± 9	22 ± 5

* Sodium flux was measured in the presence of 2.4 μg/mL scorpion venom. This concentration of scorpion venom elicited a 10-fold stimulation in sodium flux. Pumiliotoxin B (100 μM) elicited a further 3.8-fold stimulation. All pumiliotoxins were tested at 100 μM. Values are means from two experiments or means ± SE from three experiments.

† Phosphoinositide breakdown was measured with pumiliotoxins at 10 μM (data calculated from Ref. 4 except for compounds 3 and 5). Values are means from two experiments or means ± SE from three experiments.

‡ The structure of 307F has been revised (manuscript in preparation).



Scheme 1. Synthesis of des-18-methyl-PTX-B (**3**). Reagents were from standard commercial sources. Glyme = 1,2-dimethoxyethane; Bn = CH_2Ph; and CSA = camphorsulfonic acid.

1.12 (d, $J = 5.3$ Hz, $CHC\underline{H}_3$); ^{13}C-NMR (125 MHz, $CDCl_3$) δ 136.2, 132.1, 127.2, 127.1, 80.8, 72.0, 69.0, 68.4, 54.6, 52.7, 49.0, 27.2, 27.0, 24.4, 23.0, 20.6, 19.0, 13.5; MS (CI, isobutane) m/z 310 (MH), 292, 264, 85; high resolution MS (EI) 309.2272 (309.2304 calcd for $C_{18}H_{31}NO_3$).

Batrachotoxin was isolated as reported previously [11]. α-Scorpion toxin was purified from scorpion venom as described by Catterall [12] except that ion-exchange purification was performed on a Mono Q column [1]. The α-scorpion toxin used in these experiments eluted at 0.4 M ammonium acetate and was a single band of mol. wt ~7000 on sodium dodecyl sulfate (SDS)-urea acrylamide gels as described [13]. Carrier free ^{22}NaCl (25 Ci/mmol) was from Amersham (Arlington Heights, IL) or from New England Nuclear (Boston, MA). [^{3}H]Inositol (14–17 Ci/mmol) and [^{3}H]batrachotoxinin A benzoate (50 Ci/mmol) were from New England Nuclear. Tetrodotoxin, veratridine, ouabain and scorpion venom (*Leiurus quinquestriatus*) were from the Sigma Chemical Co. (St. Louis, MO). Anion-exchange resin, AG1-X8 (formate form) was from Bio-Rad (Richmond, CA), and Hydrofluor, Betafluor, and Filtron X were from National Diagnostics (Sommerville, NJ). Local anesthetics and compounds with marked local anesthetic activity were obtained from the following sources: dibucaine, Ciba-Geigy Corp. (Ardsley, NY); tetracaine, quinacrine, and diphenhydramine, Sigma Chemical Co.; euprocin, Schering AG (Berlin, F.R.G.); bupivacaine, Sterling-Winthrop Research Institute (Rensselaer, NY); etidocaine, Astra Pharmaceutical Products Inc. (Worcester, MA); cocaine, Merck Sharp & Dohme (West Point, PA); and reserpine, Aldrich Chemical Co. (Milwaukee, WI). Diphenhydramine methiodide was prepared by methylation of diphenhydramine [14]. QX-314 and lidocaine were provided by Dr. L-Y. M. Huang, formerly NIMH, Bethesda, MD.

Synaptoneurosomes. Cerebral cortical synaptoneurosomes were prepared by the method of Hollingsworth *et al.* [15]. Brain cortex of Hartley guinea pigs was homogenized in 7–10 vol. of Krebs–Henseleit buffer (pH 7.4) using a glass homogenizer. The homogenate was centrifuged at 1000 *g* for 15 min. The resulting pellet was resuspended in appropriate volume of buffer and used for assay.

Phosphoinositide breakdown in synaptoneurosomes. Phosphoinositide breakdown was measured as previously described [16]. In brief, the synaptoneurosomes (about 50 mg protein) were suspended in 14 mL of fresh Krebs–Henseleit buffer containing 200 μCi [^{3}H]inositol (1 μM). Aliquots of the synaptoneurosome suspension (300 μL) were distributed in 5-mL tubes and incubated at 37° for 60 min. LiCl (final concentration 10 mM) was then added, and 10 min later, stimulatory agents, alone or with blocking agents, were added. The synaptoneurosome suspension was then incubated at 37° for 90 min. After the incubation, the suspension was centrifuged to remove free [^{3}H]inositol and the pellet was resuspended in 1 mL of Krebs–Henseleit buffer, followed by centrifugation. The resulting pellet was mixed with 1 mL of 6% trichloroacetic acid and centrifuged. The supernatant fraction was used for the determination of [^{3}H]inositol phosphate by anion-exchange column chromatography (AG1-X8, formate form) as reported by Berridge *et al.* [17]. The trichloroacetic acid supernatant fraction was added to a column, and the column was washed five times with 3 mL of distilled water to elute [^{3}H]inositol. [^{3}H]Inositol monophosphate was then eluted with 2 mL of 200 mM ammonium formate/100 mM formic acid, and the radioactivity was measured by liquid scintillation spectroscopy after adding 7 mL of Hydrofluor. The trichloroacetic acid precipitate was used for the measurement of incorporation of [^{3}H]inositol into lipids. The pellet was suspended in 0.5 mL of the mixture of 1 M KCl/10 mM inositol and methanol (1 : 1). Chloroform (0.5 mL) was added to the suspension, and lipids were extracted by shaking for 5 min. An aliquot of the chloroform layer (0.2 mL) was transferred to a scintillation vial and evaporated to dryness. Radioactivity in the lipid fraction was measured after adding 4 mL of Betafluor. The results were calculated as cpm inositol phosphate/10,000 cpm lipids [16].

Sodium influx into synaptoneurosomes. The influx of $^{22}Na^+$ induced by various agents was measured by a method based on that of Tamkun and Catterall [18]. Synaptoneurosomes were resuspended at a concentration of 1–2 mg/mL in a buffer containing 50 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES) (pH 7.4 adjusted with 50 mM Tris), 130 mM choline chloride, 5.4 mM KCl, 0.8 mM $MgSO_4$, 5.5 mM glucose and 1 mg/mL bovine serum albumin. Aliquots of 100 μL were incubated with stimulating agents alone or with blocking agents at 37° for 10 min. The $^{22}Na^+$ influx was initiated by adding 150 μL of influx buffer containing ^{22}NaCl (1.3 μCi/mL), 2.66 mM NaCl, 50 mM HEPES, 128 mM choline chloride, 5.4 mM KCl, 0.8 mM $MgSO_4$, 5.5 mM glucose and 1 mg/mL bovine serum albumin After a 10-sec incubation at 37°, the influx was terminated by adding 4 mL of an ice-cold wash buffer containing 5 mM HEPES, 163 mM choline chloride, 0.8 mM $MgSO_4$, 1.8 mM $CaCl_2$ and 1 mg/mL bovine serum albumin, and the synaptoneurosomes were collected on a Gelman GN-6 filter (0.45 μm pore size) and washed twice with 4 mL of ice-cold wash buffer. The filter was then solubilized in Filtron X, and the radioactivity was measured by liquid scintillation spectroscopy. The influx of $^{22}Na^+$ into the synaptoneurosomes through voltage-dependent sodium channels was obtained by subtraction of the influx in the presence of 5 μM tetrodotoxin from that in the absence of tetrodotoxin.

[^{3}H]*Batrachotoxinin A benzoate binding assay.* Incubations were with 50 nM [^{3}H]batrachotoxinin A benzoate, 1 μM tetrodotoxin, 30 μg scorpion venom (*L. quinquestriatus*), and 400 μg of synaptoneurosome protein in a final volume of 250 μL buffer, pH 7.4, containing 130 mM choline chloride, 50 mM HEPES buffer, 5.5 mM glucose, 0.8 $MgSO_4$ and 5.4 mM KCl as described [19]. After 30 min at 37° incubations were terminated by dilution of the reaction mixture with 3 mL of wash buffer and filtration through Whatman GF/c filters. Filters were washed with three 3-mL portions of wash buffer and placed in scintillation vials. Hydrofluor (4 mL) was added and radioactivity determined.

Cell cultures. Neuroblastoma N18 cells were a gift from M. Nirenberg (National Institutes of Health). Cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% fetal bovine serum (FBS) to near confluency in 24-well plates. To label cells to correct for their variable recovery from the wells, cells were cultured for the final 24 hr with [^{3}H]leucine in DMEM/FBS as described [12].

Sodium influx in cultured cells. The procedure was essentially as described by Catterall [12]. Cells were incubated for 30 min at 37° with stimulatory agents alone or with blocking agents in sodium-free incubation buffer, and uptake of $^{22}Na^+$ was measured for 30 sec as described [12] except that stimulating agents were not present during the 30-sec uptake. Protein for each assay was measured as recovered tritium in [^{3}H]leucine-labeled protein and calculated from specific activity of the protein determined in each experiment by the method of Peterson [20].

RESULTS

Sodium flux in synaptoneurosomes. At a concentration of 100 μM, PTX-B elicits about a 4-fold stimulation of sodium flux to guinea pig synaptoneurosomes: an EC_{50} of about 30 μM pertained for this response [1]. The magnitude of the response to PTX-B was greatly augmented in the presence of scorpion venom (Fig. 1). In addition, the EC_{50} was reduced to about 3 μM (see also Ref. 1). The potencies of various pumiliotoxin analogs (for structures see Table 1) were compared to PTX-B by testing their activities at up to 100 μM. Thus, maximal responses may not have been attained in all cases. Pumiliotoxin A (**2**, 100 μM), which differs from PTX-B only in lacking the 15-hydroxy group, elicited a sodium flux about 50% of that elicited by PTX-B (Fig. 1). Even in the presence of scorpion venom, pumiliotoxin A gave a response only 50% of that afforded by PTX-B (Fig. 1). Thus, pumiliotoxin A appears to be both less potent and less efficacious than PTX-B. At 100 μM, the des-18-methyl-PTX-B (**3**) had virtually no effect alone, but in the presence of scorpion venom was 60% as efficacious as PTX-B. Two isomers of PTX-B, 15,16-epi-PTX-B (**4**) and erythro-PTX-B (**5**), were inactive alone at 100 μM, while in the presence of scorpion venom, both of these isomers were about 70% as efficacious as PTX-B, but less potent in stimulating sodium flux (Fig. 1). All activity was lost when the side chain contained a keto group rather than a hydroxy group as in PTX 307F (**7**). Pumiliotoxin 321 (**8**), which differs from pumiliotoxin A in having the 15-hydroxy group replaced with a methoxy group, had no effect alone, and in the presence of scorpion venom exhibited only 25% of the activity of PTX-B (Fig. 1). Pumiliotoxin 267C (**9**), which has a hydroxy group on a shortened side chain, had no effect alone on sodium flux, but was about 50% as efficacious as PTX-B in the presence of scorpion venom. Pumiliotoxin 251D (**10**), lacking the side chain hydroxy group of 267C, had no significant effect on flux either alone or with scorpion venom. The synthetic analog **14** did not stimulate flux alone, and actually inhibited scorpion venom-elicited flux (Fig. 1). Allopumiliotoxin 323B' (**16**), which has the same side chain as pumiliotoxin A, and allopumiliotoxin 267A (**19**), which has the same side chain as pumiliotoxin 251D, had no effect

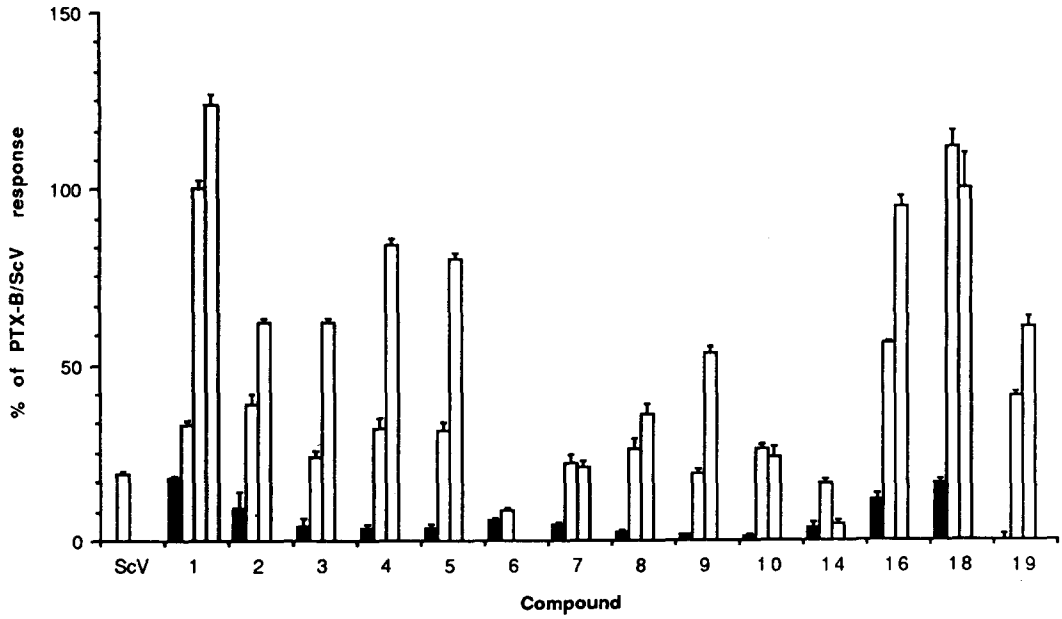


Fig. 1. Stimulation of sodium flux by pumiliotoxin B (PTX-B) (**1**), pumiliotoxin A (**2**), and other pumiliotoxins and synthetic analogs in guinea pig cerebral synaptoneurosomes (see Table 1 for structures). Sodium influx experiments were in the absence (solid bars, 100 μM) or presence (open bars from left to right, 1, 10, 100 μM for PTX-B; 100 μM for **6**, and 10 and 100 μM for other pumiliotoxins) of scorpion venom (ScV, 3 μg/mL). Data are means ± SE (N = 3) and are presented as a percent of the response elicited by a combination of PTX-B (10 μM) and ScV (3 μg/mL). This combination elicited a sodium flux of 13.7 ± 0.3 nmol/mg protein. Basal sodium flux was less than 0.1 nmol/mg protein.

alone, but in the presence of scorpion venom elicited a marked stimulation of sodium flux.

In another set of experiments the complete series of pumiliotoxin alkaloids and synthetic analogs had been screened at 100 μM for effects on scorpion venom-induced sodium flux under slightly different conditions (Table 1, see footnotes). Many of the compounds caused significant stimulation of sodium flux in synaptoneurosomes. However, some pumiliotoxins and synthetic analogs (**6**, **7**, **10**, **11**, **13**, **14**) inhibited flux elicited by scorpion venom. The most effective in this regard was analog **13**, which contains an omega-benzyloxy moiety in the side chain. Such inhibition may be due to "reverse agonist" activity, at a PTX-B site on the sodium channel, or may merely reflect a "local anesthetic-like" activity of the inhibitory pumiliotoxin alkaloids. Local anesthetic-like activity is used in the sense of blockade of voltage-dependent sodium channels. One assay for local anesthetic-like activity is the inhibition of binding of [^{3}H]batrachotoxinin A benzoate to a site on voltage-dependent sodium channels in synaptoneurosomes [19, 21]. PTX-B, pumiliotoxin A, 15,16-epi-PTX-B, erythro-PTX-B, 11-epi-PTX-B, pumiliotoxins 307F, 321 and 267C, allopumiliotoxins 323B′ and 267A and synthetic analog **14** at 100 μM had no significant inhibitory effects on binding of [^{3}H]batrachotoxinin A benzoate in synaptoneurosomes (data not shown). Pumiliotoxin 251D and the synthetic analog **13**, which along with the synthetic analog **14** were the most effective in reducing scorpion venom-elicited sodium flux (Table 1), had IC_{50} values versus [^{3}H]batrachotoxinin A benzoate binding at about 100 and 180 μM, respectively (data not shown). Thus, as determined by this assay, local anesthetic-like activity of pumiliotoxins probably has only a minor role in the inhibition of sodium flux even by 251D and synthetic analog **13**.

A number of less active or inactive alkaloids and synthetic analogs, including 251D and analog **13**, were tested for dose-dependent inhibition of sodium flux, elicited either by a PTX-B/scorpion venom combination or by batrachotoxin (Fig. 2). Several of the pumiliotoxins, namely 11-epi-PTX-B (**6**), 251D (**10**) synthetic analog **14** and allopumiliotoxin 267A (**19**), were equally effective in inhibiting flux elicited by either PTX-B/scorpion venom or batrachotoxin. Pumiliotoxin 307F (**7**) and synthetic analog **13** were somewhat more effective versus the PTX-B/scorpion venom combination than versus batrachotoxin, while pumiliotoxin 321 (**8**) was somewhat more effective versus batrachotoxin than versus PTX-B/scorpion venom. While in some cases, the less active/inactive alkaloids caused a somewhat selective antagonism of PTX-B, the results do not provide convincing evidence for a "reverse agonist" activity of pumiliotoxins at the PTX-B site, which might be expected to yield very selective antagonism of PTX-B-elicited flux compared to batrachotoxin-elicited flux. However, since scorpion venom was present in such experiments, the "agonist" activity of some of the pumiliotoxins, such as 267A, would be revealed in the presence of venom and would complicate interpretations. The flux elicited in synaptoneurosomes by PTX-B alone was relatively small, rendering quantitative studies on inhibition by the less active/inactive alkaloids of flux elicited by PTX-B alone difficult.

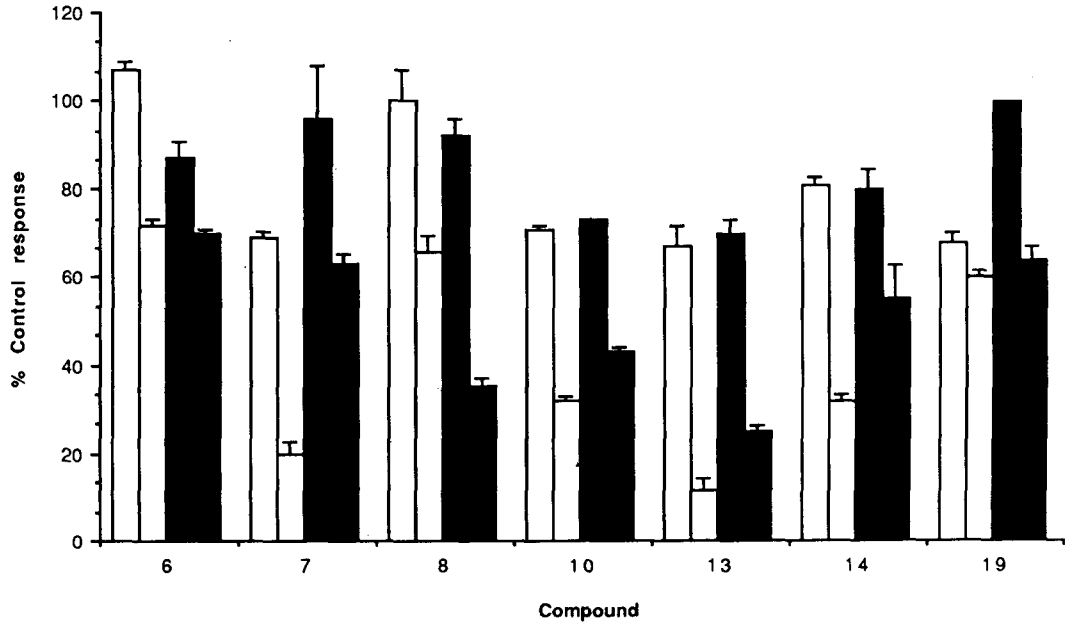


Fig. 2. Inhibitory effects of pumiliotoxins and synthetic analogs on sodium flux elicited by a pumiliotoxin B (PTX-B)/scorpion venom combination (open bars) or by batrachotoxin (solid bars) (see Table 1 for structures). Alkaloids and analogs were tested as inhibitors from left to right at 10 and 100 μM. PTX-B was at 10 μM, scorpion venom at 3 μg/mL and batrachotoxin at 1 μM. Data are means ± SE (N = 3). The value for sodium flux with the combination was as given in the legend of Fig. 1. Batrachotoxin elicited a sodium flux of 12.7 ± 0.7 nmol/mg protein.

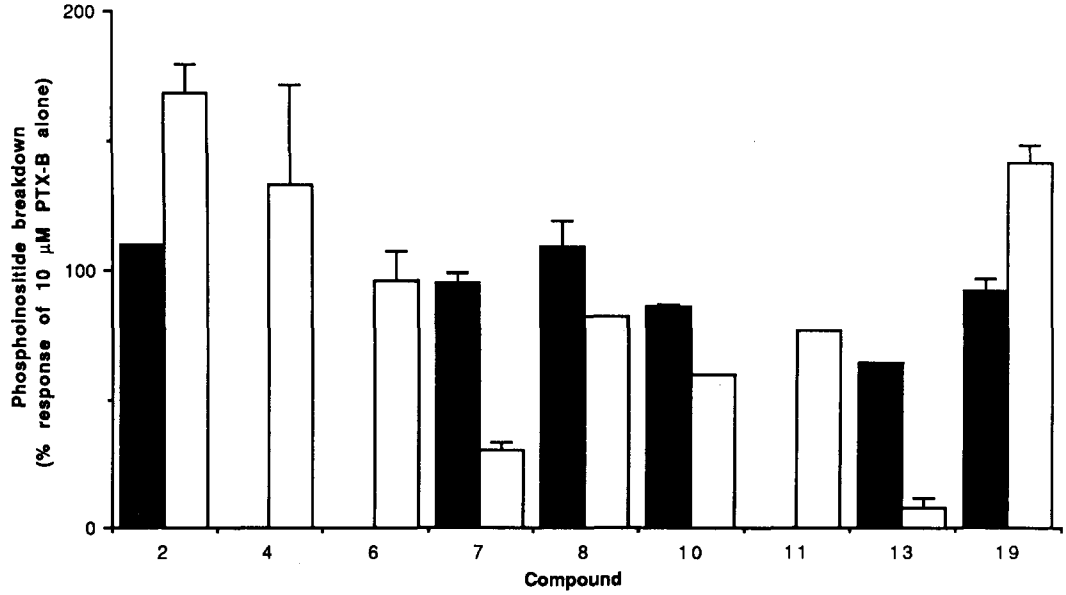


Fig. 3. Effects of pumiliotoxins and synthetic analogs on phosphoinositide breakdown elicited by pumiliotoxin B (10 μM) (see Table 1 for structures). Concentrations of pumiliotoxins and synthetic analogs were 10 (solid bars) and 100 μM (open bars). Data are means ± SE (N = 3) or from a single experiment done in triplicate and are presented as a percent of the response elicited by PTX-B (10 μM). PTX-B elicited a 307 ± 20% increase in [^{3}H]inositol phosphate. Control values without agent were 553 ± 31 cpm/10,000 cpm lipids.

Certain pumiliotoxins and synthetic analogs were found to inhibit phosphoinositide breakdown elicited by PTX-B alone (Fig. 3). The most effective were pumiliotoxin 307F and synthetic analog **13**, which at 100 μM caused 80 and 95% inhibition, respectively, of the response to 10 μM PTX-B. Certain pumiliotoxins, namely PTX-A, 15,16-epi-PTX-B and allopumiliotoxin 267A, increased the phosphoinositide breakdown elicited by 10 μM PTX-B (Fig. 3). This is not unexpected since these compounds stimulate phosphoinositide breakdown alone at 100 μM (see below).

A potent local anesthetic, dibucaine, inhibited sodium influx elicited by a PTX-B/scorpion venom combination, or batrachotoxin with equal potency (Fig. 4A). Dibucaine also inhibited the stimulation of phosphoinositide breakdown elicited by PTX-B, PTX-B/scorpion venom, scorpion venom alone, or batrachotoxin. But in these cases the potency of dibucaine differed markedly (Fig. 4B). The slopes of the inhibition curves versus phosphoinositide breakdown suggest multiple sites of action of dibucaine except in the case of PTX-B/scorpion venom combination where the slope is consonant with a single site.

A comparison of the IC_{50} values of several local anesthetics versus sodium flux elicited by either the PTX-B/scorpion venom combination or by batrachotoxin reveals an excellent correlation ($r = 0.945$) (Fig. 5). The pumiliotoxin analogs (251D, 307F, **13**, and **14**) had IC_{50} values consonant with this correlation.

Phosphoinositide breakdown in synaptoneurosomes. Stimulatory effects of pumiliotoxin alkaloids and synthetic analogs on phosphoinositide breakdown were assessed in the absence of scorpion

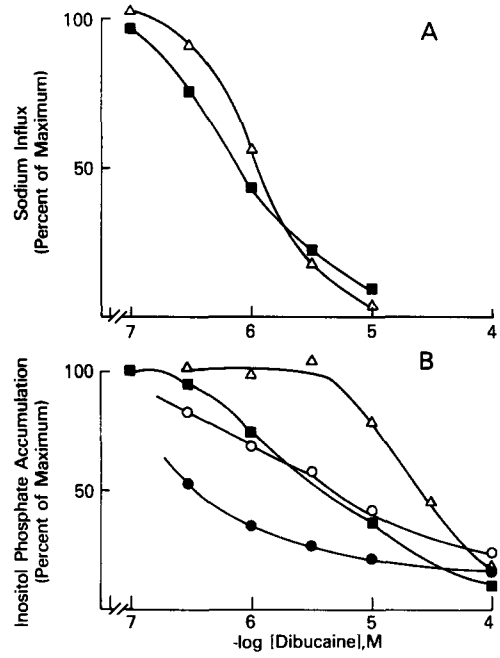


Fig. 4. Effects of the local anesthetic dibucaine on responses to pumiliotoxin B (10 or 100 μM) (●), a pumiliotoxin B (10 μM)/scorpion venom (3 μg/mL) combination (△), scorpion venom (3 μg/mL) (○) or batrachotoxin (1 μM) (■). (A) Effects on sodium flux. The value for sodium flux with the combination was as given in the legend to Fig. 1 and with batrachotoxin as in the legend of Fig. 2. PTX-B (100 μM) elicited a sodium flux of 3.5 ± 1 nmol/mg protein. (B) Effects on phosphoinositide breakdown. PTX-B (10 μM) elicited a 307 ± 20% increase in [^{3}H]inositol phosphate; the combination, a 580 ± 44% increase; scorpion venom a 610 ± 60% increase; and batrachotoxin, a 487 ± 45% increase. Data are means (N = 3).

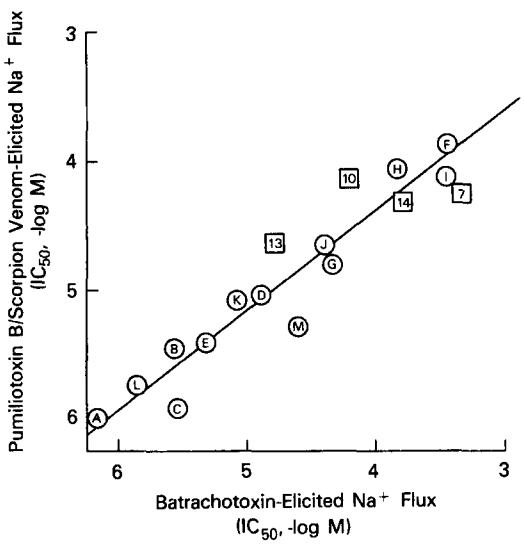


Fig. 5. Relationship between inhibitory effects of local anesthetics (circles) and pumiliotoxin alkaloids (squares) on sodium influx elicited by the pumiliotoxin B (10 μM)/scorpion venom (3 μg/mL) combination or by batrachotoxin (1 μM). Local anesthetics: (A) dibucaine; (B) tetracaine; (C) euprocin; (D) bupivacaine; (E) quinacrine; (F) QX 314; (G) diphenhydramine; (H) lidocaine; (I) diphenhydramine methiodide; (J) cocaine; (K) etidocaine; (L) reserpine; and (M) promethazine. Pumiliotoxins: **7** (307F); **10** (251D); **13**; and **14**.

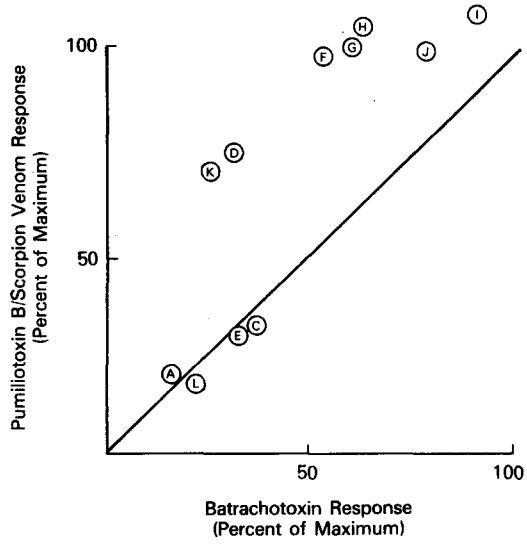


Fig. 6. Relationship between inhibitory effects of local anesthetics on phosphoinositide breakdown elicited by pumiliotoxin B (10 μM)/scorpion venom (3 μg/mL) or by batrachotoxin (1 μM). Local anesthetics (see legend of Fig. 5 for key to letters) were at 100 μM. Data are means (N = 3). For responses for the agents, see the legend to Fig. 4.

venom, since the venom itself elicits near maximal sodium-channel-dependent stimulation of breakdown of phosphoinositides [8]. Only a few of the pumiliotoxin alkaloids had marked effects on phosphoinositide breakdown under these conditions (Table 1). These were pumiliotoxin A (**2**), which gave 39% of the PTX-B response, des-18-methyl-PTX-B (**3**), which gave 52% of the PTX-B response, 15,16-epi-PTX-B (**4**, 47%), and erythro-PTX-B-β (**5**), allopumiliotoxin 323B′ (**16**), 323B″ (**17**) and 339A (**18**), which gave responses nearly equivalent to that of PTX-B (Table 1). Pumiliotoxin 267C (**9**), 251D (**10**), and allopumiliotoxin 267A (**19**) gave a stimulation of phosphoinositide breakdown only 17–22% of that elicited by PTX-B. Stimulation of phosphoinositide breakdown by PTX-B and other sodium channel agents has been shown to be dependent on stimulation of sodium influx [8, 16]. Thus, the low activity of many compounds in stimulating phosphoinositide breakdown (Table 1) is not surprising, since all of the pumiliotoxins and synthetic analogs that were tested with the exception of PTX-B caused no or very little stimulation of sodium flux when tested in the absence of scorpion venom (Fig. 1).

A comparison of percent inhibition by a series of local anesthetics versus phosphoinositide breakdown elicited by either the PTX-B/scorpion venom combination or batrachotoxin reveals a poor correlation (Fig. 6). However, two subsets of local anesthetics are apparent. The first subset was nearly equally effective against PTX-B/scorpion venom and batrachotoxin as had been the case for inhibition of sodium flux by most local anesthetics (Fig. 5) and includes quinacrine, diphenhydramine and its methiodide, and cocaine. Another subset of local anesthetics was significantly less potent against phosphoinositide breakdown stimulated by PTX-B/scorpion venom than against batrachotoxin-stimulated phosphoinositide breakdown. This subset included more classical anesthetics, such as dibucaine, tetracaine, and lidocaine.

The IC_{50} values for local anesthetics versus batrachotoxin-elicited sodium influx and versus batrachotoxin-elicited phosphoinositide breakdown show a significant correlation (Ref. 22, $r = 0.725$).

Sodium flux in neuroblastoma cells. In contrast to synaptoneurosomes, PTX-B alone has no effect on sodium flux in neuroblastoma cells and requires synergism by α-scorpion toxin to reveal agonist activity [1]. Pumiliotoxin A, other pumiliotoxins and synthetic analogs also had no effect on sodium flux in neuroblastoma cells in the absence of α-scorpion toxin (data not shown). Concentration–response relationships for effects of PTX-B and PTX-A on sodium flux were compared in the presence of either α-scorpion toxin or scorpion venom (Fig. 7). Scorpion venom was more effective than the purified α-scorpion toxin at synergizing both PTX-B and pumiliotoxin A responses even when the α-scorpion toxin was present at saturating concentrations. Enhanced synergism by scorpion venom compared to scorpion toxin was not unique to PTX-B, as veratridine-stimulated sodium flux was also markedly greater (2-fold) when synergized by venom as compared to synergism with toxin (data not shown). At 100 μM, the magnitude of the response to PTX-A was 23% of that of PTX-B in the presence of the α-scorpion toxin and 33% in the presence of scorpion venom. This stimulation is roughly similar to that seen in synaptoneurosomes, where PTX-A at 100 μM gave a

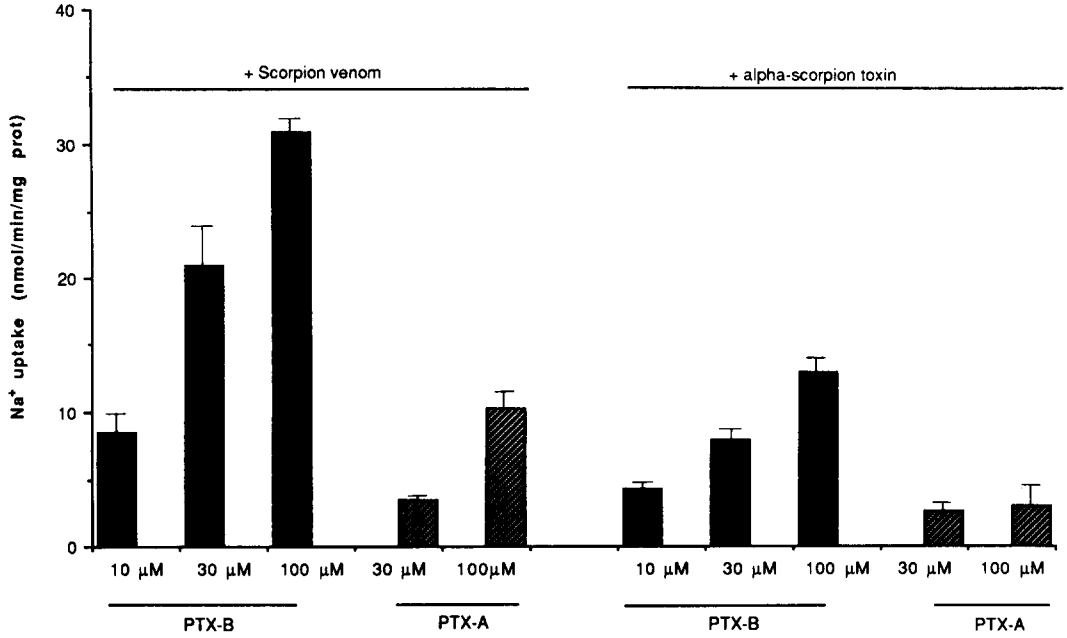


Fig. 7. Effects of pumiliotoxin B (PTX-B) and pumiliotoxin A (PTX-A, **2**) on sodium flux in neuroblastoma cells in the presence of scorpion venom (4 μg/mL) or α-scorpion toxin (3 nM). Data are means ± SE (N = 3).

response 50% of that of 100 μM PTX-B in the presence of scorpion venom (Fig. 1). Other pumiliotoxins and synthetic analogs were tested for stimulation of sodium flux in neuroblastoma cells in the presence of scorpion venom (Table 2). In nearly all cases the maximal response was elicited at 100 μM and was only a fraction of the response elicited by 100 μM PTX-B. Pumiliotoxin A, 307F (**7**) and allopumiliotoxin 339A (**18**) were the most efficacious, causing a maximal stimulation of 32–88% of that to PTX-B. Pumiliotoxin 251D (**10**) was virtually inactive. These results (Table 2) are similar to those obtained

Table 2. Effects of pumiliotoxins and synthetic analogs on sodium flux in neuroblastoma cells (see Table 1 for structures)

Compound	Maximum stimulation of flux* (% of PTX-B)		Inhibition† (IC_{50}, μM) of:	
	+ Scorpion toxin	+ Scorpion venom	PTX-B-elicited flux	BTX-elicited flux
1 (PTX-B)	100	100		⩾100
2 (PTX-A)	23	33	7	⩾100
4		10	50	>100
5		25	>100	>100
6	10	17	50	>100
7		32	10	70
8		20	10	27
9		26	>100	⩾100
10		<10	50	~100
11		15	>100	>100
13		27	1	1
15		22	35	⩾100
16		21	>100	>100
18		88	⩾100	⩾100
19		24	10	>100

* Sodium flux was measured in the presence of 3 nM α-scorpion toxin or with 4 μg/mL scorpion venom, and is expressed relative to flux elicited by 100 μM PTX-B, set equal to 100. PTX-B (100 μM) elicited a sodium flux of 13.4 ± 1.1 nmol/min/mg protein with α-scorpion toxin and 31 ± 1 with scorpion venom. All compounds were tested at 100 μM. Values are averages for three or more experiments.

† The IC_{50} for inhibition of sodium flux elicited by either 33 μM PTX-B or 1 μM batrachotoxin (BTX) was determined and is an average estimated from three or more experiments.

in synaptoneurosomes (see Fig. 1, Table 1) with the exception of 11-epi-PTX-B (**6**), 307F (**7**) and synthetic analog **13**. In synaptoneurosomes, these compounds were virtually inactive, whereas in neuroblastoma cells they were 22–32% as effective as PTX-B at stimulating Na^+ influx into neuroblastoma cells. However, it should be noted that lower levels of activity (22% of that of PTX-B) represent influx of only 2-fold or less over background, and the compounds ought to be considered only marginally active. Compared to PTX-B, certain pumiliotoxins, namely erythro-PTX-B (**5**) and allopumiliotoxins 323B′ (**16**) and 339A (**18**) appear somewhat more efficacious in synaptoneurosomes (Fig. 1, Table 1) than in neuroblastoma cells (Table 2).

Several of the pumiliotoxins inhibited sodium flux elicited by a PTX-B/α-scorpion toxin combination or by a batrachotoxin/α-scorpion toxin combination in neuroblastoma cells (Table 2) as had been the case in synaptoneurosomes (Fig. 2). PTX-B at 100 μM did not affect batrachotoxin/α-scorpion toxin stimulated flux, nor did pumiliotoxin A (**2**), 267C (**9**), the synthetic analog **15**, or allopumiliotoxin 339A (**18**). Pumiliotoxin A (**2**) did inhibit the response to the PTX-B/α-scorpion toxin combination with an estimated IC_{50} of 7 μM. Several other pumiliotoxins and synthetic analogs also inhibited responses to the PTX-B/α-scorpion toxin combination. In all cases except one, these compounds, namely 307F (**7**), 321 (**8**) and synthetic analog **15**, were more potent versus the PTX-B/α-scorpion toxin combination than versus the batrachotoxin/α-scorpion toxin combination (Table 2). The exception was synthetic analog **13**, which was very potent (IC_{50} 1 μM) versus either combination. In synaptoneurosomes this analog was also potent and relatively nonselective as an "antagonist" with an IC_{50} value of about 20 μM against PTX-B/scorpion venom and about 40 μM versus batrachotoxin (Fig. 2). It should be noted that in neuroblastoma cells pumiliotoxin 321 (**8**) was about 3-fold more potent against the PTX-B/α-scorpion toxin than against batrachotoxin/α-scorpion toxin (Table 2). In synaptoneurosomes, however, it was several fold *less* potent against PTX-B/scorpion venom than against batrachotoxin (Fig. 2). Whether differences in assays or differences between neuroblastoma cells and synaptoneurosomes account for such lack of clear correspondence will require further investigation.

DISCUSSION

The stimulation of sodium flux by the alkaloid PTX-B was markedly synergized by α-scorpion toxin or by a scorpion venom that contained α-scorpion toxin ([1], see also Figs 1 and 7). Indeed, in neuroblastoma cells, α-scorpion toxin (or scorpion venom) is required for PTX-B to elicit a response. Thus, as previously described [1], an allosteric relationship between the PTX-B site and the α-scorpion toxin site appears to exist for voltage-dependent sodium channels. The PTX-B site also interacts positively with the β-scorpion toxin site, and with the brevetoxin site, but apparently not with the other alkaloid site at which batrachotoxin, veratridine and aconitine act [1]. Scorpion venom enhanced not only PTX-B activity, but revealed stimulatory effects of congeners and analogs that alone showed little stimulation of sodium flux in synaptoneurosomes and no measurable effect at all in neuroblastoma cells (Fig. 1, Tables 1 and 2). Certain congeners and analogs of PTX-B that were inactive in eliciting sodium flux, even in the presence of scorpion venom, reduced responses to a PTX-B /scorpion venom combination, to scorpion venom alone, or to batrachotoxin (Fig. 2, Table 2). Such inhibitory effects could reflect either antagonist or "reverse agonist" interactions at a PTX-B binding site or to a local anesthetic-like activity at other sites. We have tried to distinguish antagonist/reverse agonist activity from local anesthetic activity, based on the observations that local anesthetics have marked inhibitory effects on the binding of [^{3}H]batrachotoxin A benzoate, which correlate with inhibition of activation of sodium channels by batrachotoxin [18, 19, 21, 22]. The pumiliotoxin alkaloids showed either no or very weak inhibition of [^{3}H]batrachotoxin A benzoate binding (see Results). Thus, it appears unlikely that local anesthetic-like activity explains inhibition of sodium flux by these compounds. This may not be completely true for pumiliotoxin 251D (**10**) and the synthetic analog **13**, since both of these compounds significantly inhibited binding of [^{3}H]batrachotoxinin A benzoate to sodium channels, albeit at rather high concentrations. Limited supplies of **13** prevented a more detailed analysis of its inhibition of sodium flux in synaptoneurosomes (Fig. 2) and neuroblastoma cells (Table 2). For PTX-B and other congeners that showed little or no local anesthetic-like activity against binding of [^{3}H]batrachotoxinin A to sodium channels, it appears likely that their activities reflect binding to the PTX-B site. Some alkaloids, like PTX-B, and allopumiliotoxins 323B and 339A, are agonists causing activation of sodium flux. Others, such as pumiliotoxin A, may be antagonists, blocking binding and action of PTX-B, while having no effect of their own. Some compounds in this group, such as pumiliotoxin A, can be converted to agonists through synergistic effects of α-scorpion toxin. Finally, a third group may be "reverse agonists," causing an inhibition of activation of sodium channels even when elicited by agents, such as α-scorpion toxin or batrachotoxin, that act by binding to other sites on sodium channels.

Certain of the pumiliotoxins were better inhibitors of PTX-B/scorpion venom-elicited flux than batrachotoxin-elicited flux in synaptoneurosomes (Fig. 2), while nearly all of the tested compounds were better inhibitors of PTX-B/α-scorpion toxin-elicited flux than batrachotoxin/α-scorpion toxin-elicited flux in neuroblastoma cells. The only exception was alkaloid 321 (**8**), which was somewhat more potent versus the batrachotoxin-elicited flux than versus PTX-B/α-scorpion toxin-elicited flux in synaptoneurosomes (Fig. 2). In contrast, it was 3-fold more potent versus PTX-B/α-scorpion toxin-elicited flux than against batrachotoxin/α-scorpion toxin-elicited flux in neuroblastoma cells (Table 2). Local anesthetics had similar inhibitory effects on sodium elicited by either a PTX-B/-scorpion venom combination or by batrachotoxin in synaptoneurosomes (Fig. 4A). Further studies will be required to determine the basis for

inconsistencies between effects of pumiliotoxins on sodium flux in synaptoneurosomes and neuroblastoma cells. However, to a large extent similar results pertain for most of the pumiliotoxins in both preparations. The results with PTX-B and pumiliotoxin A in the presence of either purified α-scorpion toxin or scorpion venom (Fig. 7, Table 2) suggest that there are other factors in scorpion venom besides α-scorpion toxin that augment responses to pumiliotoxins.

The structure–activity profile for stimulation of sodium flux by pumiliotoxin alkaloids and synthetic analogs is very similar to the cardiotonic activity of these compounds [2, 5]. The effects of pumiliotoxin alkaloids on phosphoinositide breakdown in synaptoneurosomes (Table 1) appear consonant with effects on sodium flux elicited by the alkaloids alone, as opposed to their effects in synergy with scorpion venom (Fig. 3). It would appear that in the pumiliotoxin series that two hydroxy groups in the threo *R,R*-configuration in alkylidene side chain are optimal for activity at sodium channels, whereas activity of the allopumiliotoxin series, which contain an additional ring hydroxy group, is effectively expressed even in compounds that have only one hydroxy group in either the *R*- or *S*-configuration in the alkylidene side chain. In the presence of α-scorpion toxin, these structural requirements are no longer as stringent, and isomers of PTX-B with threo-15*S*,16*S*- or erythro-15*R*,6*S*-hydroxy groups in the side chain have significant activity. The absence of the 18-methyl group in des-18-methyl-PTX-B reduced activity somewhat, while inversion of that methyl in 11-epi-PTX-B virtually eliminated activity (Fig. 2). Congeners and synthetic analogs that have no hydroxy group in the alkylidine side chain had only modest stimulatory effects on sodium flux even in the presence of scorpion venom. Indeed, some markedly inhibited the sodium flux elicited by scorpion venom, pumiliotoxin B or batrachotoxin.

In synaptoneurosomes, stimulation of sodium flux by a variety of agents including batrachotoxin, scorpion venom, and pumiliotoxin B leads to an increase in rates of phosphoinositide breakdown [8, 16]. The degree of stimulation of sodium influx by pumiliotoxin congeners and analogs corresponds well to stimulation of phosphoinositide breakdown. In contrast, sodium flux induced by pumiliotoxins when synergized by scorpion venom does not correspond to phosphoinositide breakdown elicited by the alkaloids in the absence of scorpion venom. Clearly, phosphoinositide breakdown, while dependent on sodium flux [8, 14], does not correlate quantitatively with the magnitude of sodium flux in synaptoneurosomes (see Ref. 23).

Pumiliotoxins that inhibited sodium flux elicited by the PTX-B/scorpion venom combination (Fig. 2) also inhibited phosphoinositide breakdown elicited by PTX-B (Fig. 3). Local anesthetics also have inhibitory effects on both sodium flux and phosphoinositide breakdown (see Ref. 22). Most local anesthetics were more effective against batrachotoxin-elicited phosphoinositide breakdown than versus breakdown elicited by the PTX-B/scorpion venom combination (Fig. 6). This is in marked contrast to the correlation in inhibitory effects of local anesthetics versus sodium flux elicited by PTX-B/scorpion venom and by batrachotoxin (Figs 4A and 5). Only a few pumiliotoxins have been examined versus sodium flux elicited by either PTX-B/scorpion venom or batrachotoxin. Most are more potent inhibitors versus responses to PTX-B/scorpion venom (Fig. 2, Table 2).

The present data extend the definition of a PTX-B site on voltage-dependent sodium channels in synaptoneurosomes and neuroblastoma cells. Such a site appears allosterically coupled to the α-scorpion toxin site. In synaptoneurosomes, synergism with α-scorpion toxin (or venom) enhances the agonist activity of PTX-B and reveals agonist activity for a number of other pumiliotoxin alkaloids. In neuroblastoma cells, expression of agonist activity for PTX-B and other pumiliotoxin alkaloids is expressed *only* in the presence of α-scorpion toxin (or venom). The inhibition of PTX-B-elicited sodium flux by several structurally similar pumiliotoxins and synthetic analogs appears likely to be due to competition as "partial agonists," for example in the case of pumiliotoxin A, or as antagonists or even "reverse agonists" in the case of some pumiliotoxins. For some congeners and synthetic analogs, inhibition of flux may be due to some extent to local anesthetic-like activity at another site. There is a remarkable sensitivity of the activity of PTX-B to minor alterations in the stereochemistry of the side chain substituents. As yet no alterations have led to an analog more active than PTX-B, but instead minor alterations usually yield compounds with partial agonist or antagonist/reverse agonist activities.

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