0026-895X/87/040479-09$02.00/0

MOLECULAR PHARMACOLOGY, 32:479–487

Stimulation of Phosphoinositide Breakdown in Brain Synaptoneurosomes by Agents that Activate Sodium Influx: Antagonism by Tetrodotoxin, Saxitoxin, and Cadmium

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Received January 21, 1987; Accepted July 6, 1987

SUMMARY

Agents that increase intracellular concentrations of Na^+ stimulate phosphoinositide breakdown in guinea pig cerebral cortical synaptoneurosomes. When combined, these agents did not have additive effects on phosphoinositide breakdown but did have additive or greater than additive effects with carbamylcholine. Scorpion venom (*Leiurus quinquestriatus*) and pumiliotoxin B, which induce small increases in influx of $^{22}Na^+$ in synaptoneurosomes, stimulate phosphoinositide breakdown by about 6- and 3-fold, respectively; both effects are inhibited by tetrodotoxin (TTX). Batrachotoxin (BTX) and veratridine, which cause a large increase in influx of $^{22}Na^+$ through activation of voltage-dependent sodium channels, induce a 5- to 6-fold dose-dependent increase in phosphoinositide breakdown, which appears competitively inhibited by 5 μM TTX. BTX- and veratridine-elicited influx of $^{22}Na^+$ into synaptoneurosomes is virtually completely blocked by 5 μM TTX. Agents that block voltage-dependent calcium channels, such as D-600, nifedipine, and Co^{2+}, do not inhibit either influx of $^{22}Na^+$ or stimulation of phosphoinositide breakdown elicited by scorpion venom, pumiliotoxin B, or BTX. Cadmium ions (200 μM), which are known to block TTX-resistant sodium channels, block phosphoinositide breakdown induced by agents that activate sodium influx through sodium channels. Cadmium blocks BTX-induced phosphoinositide breakdown with an IC_{50} value of 48 μM, while blocking BTX-induced $^{22}Na^+$ influx in synaptoneurosomes with a 13-fold lower potency (IC_{50}, 610 μM). In the presence of 0.5 μM TTX, the IC_{50} for Cd^{2+} inhibition of BTX-induced $^{22}Na^+$ influx is now 430 μM. Neither TTX nor Cd^{2+} antagonize neurotransmitter- or monensin-induced phosphoinositide breakdown. It appears that BTX-induced phosphoinositide breakdown in guinea pig synaptoneurosomes is dependent primarily on activation of TTX-resistant, Cd^{2+}-sensitive sodium channels that account for only a small fraction of the total sodium influx induced by BTX in synaptoneurosomes. However, cadmium also may in some way inhibit phosphoinositide breakdown elicited by sodium channel agents at a point subsequent to sodium influx.

Agents that would induce increases in intracellular concentrations of sodium, either through activation of voltage-dependent sodium channels, as with BTX and VT, or inhibition of Na^+/K^+ ATPase, as with ouabain, or directly in the case of the sodium ionophore monensin, cause a stimulation of phosphoinositide breakdown in guinea pig brain synaptoneurosomes (1, 2). ScV (*Leiurus quinquestriatus*), containing an α-scorpion toxin that slows the inactivation of sodium channels (3), and the alkaloid, PTX-B), which induces repetitive firing of sodium channel-dependent action potentials (4), also induce phosphoinositide breakdown in synaptoneurosomes (2). The stimulation of phosphoinositide breakdown induced by ScV and PTX-B can be antagonized in the presence of TTX (1, 2). However, TTX did not block phosphoinositide breakdown elicited by 1 μM BTX or 100 μM VT. The latter alkaloids are well known to stabilize sodium channels in an open state (3). Such results suggest that BTX and VT either stimulate phosphoinositide breakdown through activation of TTX-resistant sodium channels or act via an unexpected mechanism.

Effects of ScV, PTX-B, BTX, and VT on phosphoinositide breakdown have now been further characterized through the use of Cd^{2+} at concentrations that have been shown to block TTX-resistant sodium fluxes in cardiac cells, skeletal myoblasts, and skeletal muscle cells (5). Cadmium ions completely blocked ScV-, PTX-B-, and BTX-induced phosphoinositide breakdown in guinea pig synaptoneurosomes, without affecting receptor- or monensin-mediated responses. The results suggest that activation of TTX-resistant,Cd^{2+}-sensitive sodium channels are primarily responsible for effects of BTX on phosphoinositide breakdown, but that these channels are responsible for only a minor portion of the total sodium flux elicited by BTX in guinea pig brain synaptoneurosomes. It remains possible that cadmium ions also inhibit directly phosphoinositide breakdown elicited by sodium channel agents.

ABBREVIATIONS: BTX, batrachotoxin; VT, veratridine; ScV, scorpion venom; PTX-B, pumiliotoxin B; TTX, tetrodotoxin; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; STX, saxitoxin.

Experimental Procedures

Materials. [^{3}H]Inositol (specific activity 14–17 Ci/mmol) was from New England Nuclear (Boston, MA). ^{22}NaCl (25 Ci/mmol, carrier free) was from Amersham (Arlington Heights, IL). Carbamylcholine, norepinephrine bitartrate, histamine hydrochloride, TTX, ScV (*L. quinquestriatus*), VT, aconitine, and monensin were from Sigma Chemical Co. (St. Louis, MO). BTX was isolated from the poison dart frog *Phyllobates terribilis* (6), and PTX-B was isolated from the poison frog *Dendrobates pumilio* (7). Anion exchange resin (AG 1-X8, formate form) was from Bio-Rad (Richmond, CA). STX was obtained from United States Public Health Service Northeast Research Center. Hydrofluor, Betafluor, and Filtron X were from National Diagnostics (Sommerville, NJ).

Synaptoneurosome preparation. Guinea pig cerebral cortical synaptoneurosomes were obtained as described by Hollingsworth *et al.* (8). Briefly, the cortex of one brain was homogenized in 7–10 volumes of Krebs-Henseleit buffer (pH 7.4) in a glass-glass homogenizer (5 strokes). The suspension was centrifuged at this point at 1000 × *g* for 10 min, the supernatant was decanted, and the pellet was reconstituted in the appropriate volume of buffer to reach a concentration of about 3 mg of protein/ml.

Phosphatidylinositol turnover. The procedure is described in detail elsewhere (9). Briefly, the pellet obtained from the synaptoneurosome preparation from one guinea pig cerebral cortex was resuspended in 10–15 ml of fresh buffer containing 1 μM [^{3}H]inositol. Aliquots of 320 μl of the suspension (≈1 mg of protein) were distributed in 5-ml polypropylene tubes and incubated at 37°. After 60 min, 20 μl of 200 mM LiCl were added in each tube. At this time Cd^{2+}, TTX, or other channel blockers were added. Ten min later, receptor agonists or sodium channel agents were added in 20 μl. The final incubation volume was 400 μl. The tubes were gassed briefly with O_2:CO_2, capped, and incubated for 90 min at 37°. At the end of the incubation period the tubes were centrifuged and the tissue was washed with fresh buffer to remove the free [^{3}H]inositol. Then, 1 ml of 6% trichloroacetic acid was added, and the tubes were vortexed and centrifuged. Inositol phosphates were analyzed in the supernatant according to the method of Berridge *et al.* (10). Briefly, anion exchange columns (AG 1-X8, formate form) were used to separate the inositol phosphates. The trichloroacetic acid supernatant was added to the column. After washing four times with 3 ml of water to elute free [^{3}H]inositol, [^{3}H]inositol-1-phosphate was eluted with 2 ml of 200 mM ammonium formate/100 mM formic acid. This eluant was collected in vials. Hydrofluor was added (8 ml) and radioactivity was determined by liquid scintillation spectroscopy. The trichloroacetic acid precipitate (see above) was resuspended in 0.5 ml of a mixture of aqueous 1 M KCl containing 10 mM inositol and methanol (1:1), and 0.5 ml of chloroform was added. The tubes were mechanically shaken for 5 min and then centrifuged in order to separate the two phases. Two hundred-μl aliquots from the chloroform phase were placed in individual scintillation vials and evaporated at room temperature. Betafluor was added and radioactivity determined by liquid scintillation spectroscopy to provide an index of [^{3}H]inositol incorporation into [^{3}H]phosphoinositides. Results are expressed as cpm of [^{3}H]inositol phosphates per 10,000 cpm of radioactivity incorporated into lipids or, alternatively, as percentage of control response.

Sodium influx into synaptoneurosomes. Sodium flux studies were performed essentially as described by Tamkun and Catterall (11) for synaptosomes. Briefly, aliquots of synaptoneurosome suspensions containing approximately 200–300 μg of protein were preincubated in a volume of 100 μl for 10 min at 37° in an incubation buffer containing various test agents. The incubation buffer consisted of 50 mM HEPES (adjusted to pH 7.4 at 36° with 50 mM Tris buffer), 130 mM choline chloride, 5.4 mM KCl, 0.8 mM $MgSO_4$, 5.5 mM glucose, and 1 mg/ml bovine serum albumin. The ^{22}NaCl, 1.3 μCi/ml, was added in a volume of 150 μl of influx buffer containing 2.66 mM NaCl, 50 mM HEPES-Tris (pH 7.4), 128 mM choline chloride, 5.4 mM KCl, 0.8 mM $MgSO_4$, 5.5 mM glucose, 1 mg/ml bovine serum albumin, and 5 mM ouabain. The final volume was 250 μl. The influx buffer contained test agents in the same concentration as was present in the preincubated samples. Influx of $^{22}Na^+$ was stopped after 10 sec by adding 4 ml of cold washing buffer. Samples were immediately collected on a Gelman GN-6 (0.45-μm pore size) filter, and further washed twice with 4 ml of buffer. Washing buffer contained 5 mM HEPES-Tris, 163 mM choline chloride, 0.8 mM $MgSO_4$, 1.8 mM $CaCl_2$, and 1 mg/ml bovine serum albumin (pH 7.4). Filters were dissolved in Filtron-X (National Diagnostics) for liquid scintillation counting (efficiency approximately 99.1%). The specific uptake of $^{22}Na^+$ was determined by subtracting nonspecific uptake obtained in the presence of 5 μM TTX from the total uptake.

Results

Lack of effect of receptor antagonists and calcium channel blockers on phosphoinositide turnover stimulated by sodium channel agents. Various agents that induce an increase in intracellular sodium concentration elicit phosphoinositide breakdown in synaptoneurosomes (Ref. 2 and Table 1). Receptor antagonists at doses that inhibit neurotransmitter-induced phosphoinositide turnover and organic blockers for voltage-sensitive calcium channels are ineffective in blocking responses to sodium agents (Table 1). Co^{2+} at 200 μM did not inhibit phosphoinositide breakdown induced by sodium agents (Table 2).

Effects of combinations of sodium agents and receptor agonists on phosphoinositide breakdown. Combinations of sodium channel agents (ScV + BTX; ScV + PTX-B; BTX + PTX-B) or monensin + ScV were tested on phosphoinositide breakdown (Fig. 1). In each of these cases the responses were not additive. In contrast, the combinations of carbamylcholine + ScV and carbamylcholine + PTX-B resulted in more than additive responses (Fig. 1). A combination of aconitine and carbamylcholine resulted in an additive response (9).

Effects of TTX and STX on phosphoinositide breakdown stimulated by sodium channel agents. Phosphoinositide breakdown induced by ScV, aconitine, and PTX-B is antagonized by a high concentration of TTX (Table 1), suggesting that the effects of these agents are mediated through actions at voltage-dependent sodium channels. However, BTX and VT, alkaloids that induce sodium influx by stabilizing voltage-dependent sodium channels in open states, elicit, at relatively high concentrations, stimulations of phosphoinositide breakdown that are not completely inhibited by high concentrations of TTX (Table 1). Whereas the response to 1 μM BTX is not antagonized by TTX, the response to a lower concentration of BTX is effectively blocked by TTX (Fig. 2A), resulting in a displacement to the right of the dose response curve for phosphoinositide breakdown stimulated by BTX. The stimulation of phosphoinositide breakdown elicited by 100 nM BTX is completely blocked by increasing concentrations of TTX (IC_{50} 2000 nM) and STX (IC_{50} 300 nM) (fig. 2B). Similarly, dose-dependent phosphoinositide breakdown induced by VT is displaced to the right in the presence of 5 μM TTX (Fig. 3A). TTX and STX completely block phosphoinositide breakdown induced by 1 μM VT with IC_{50} values of 300 and 100 nM, respectively (Fig. 3B). TTX also inhibited phosphoinositide breakdown induced by aconitine (IC_{50} 240 nM), PTX-B (IC_{50} 150 nM), and ScV (IC_{50} 250 nM).

Effects of TTX and STX on $^{22}Na^+$ influx stimulated by sodium channel agents. The magnitude of the stimulation of phosphoinositide breakdown elicited by sodium channel agents does not correlate well with the magnitude of stimulation of $^{22}Na^+$ influx elicited by the same agents (Fig. 4). VT and BTX

TABLE 1

Stimulation of phosphoinositide breakdown in guinea pig brain cortical synaptoneurosomes by sodium channel agents and neurotransmitter agonists: effects of neurotransmitter antagonists, organic calcium channel blockers, and TTX

Synaptoneurosomes were labeled for 60 min with [^{3}H]inositol and then incubated with the different agents in the presence of 10 mM LiCl for 90 min. [^{3}H]Inositol phosphates were analyzed as described under Experimental Procedures. Control value was 785 ± 26 cpm/10,000 cpm in lipids. Values represent the mean of at least three experiments ± standard error or are the results of individual experiments performed in triplicate.

	No inhibitors	Atropine (10 μM)	Phentolamine (10 μM)	Mepyramine (1 μM)	D-600 (10 μM)	Nifedipine (1 μM)	TTX (5 μM)
				% of control			
BTX (1 μM)	478 ± 27	496 ± 8	523 ± 13	365	458 ± 34	553	410 ± 27
PTX-B (5 μM)	229 ± 11	208, 264	180, 168	288, 270	223, 305	268	120, 110
Aconitine (10 μM)	227 ± 13	205, 254	218, 251	247, 416	264, 368	ND[a]	127, 110
VT (10 μM)	334, 456	359, 536	338, 564	420, 422	347	ND	188, 209
ScV (6 μg/ml)	520 ± 31	517, 440	521, 403	458, 453	615	603 ± 96	158
Carbamylcholine (2 mM)	514 ± 7	120 ± 9	337, 466	414, 544	460, 424	ND	282
Norepinephrine (100 μM)	313 ± 47	450, 340	146 ± 7	505, 379	213 ± 16	275 ± 35	362, 329
Histamine (100 μM)	220 ± 22	ND	ND	59, 85	ND	ND	ND

[a] ND, not determined.

TABLE 2

Effect of divalent inorganic cations on phosphoinositide breakdown induced by sodium channel agents, neurotransmitter agonists, and ionophores in guinea pig brain cortical synaptoneurosomes

Synaptosomes were labeled for 60 min with [^{3}H]inositol and then incubated with the different agents in the presence of 10 mM LiCl for 90 min. [^{3}H]Inositol phosphates were analyzed as described under Experimental Procedures. Control value was 790 ± 29 cpm/10,000 cpm in lipids. Values represent the mean of at least three experiments ± standard error or are the results of individual experiments performed in triplicate.

	Control	Cd^{2+} (200 μM)	Sn^{2+} (200 μM)	Zn^{2+} (200 μM)	Ni^{2+} (200 μM)	Co^{2+} (200 μM)
			% of control			
BTX (1 μM)	486 ± 43	113 ± 11	255	261	355	560 ± 18
PTX-B (5 μM)	263 ± 14	133 ± 13	ND[a]	ND	ND	249, 274
Aconitine (10 μM)	287 ± 30	166 ± 20	ND	ND	ND	ND
VT (10 μM)	576 ± 38	177 ± 33	ND	ND	ND	642 ± 20
ScV (6 μg/ml)	712 ± 12	205 ± 7	ND	ND	ND	757 ± 60
Norepinephrine (100 μM)	441 ± 8	382 ± 18	ND	ND	ND	ND
Carbamylcholine (2 mM)	583 ± 55	953 ± 134	ND	658	ND	ND
Ionomycin (5 μM)	184 ± 19	162 ± 8	ND	ND	ND	ND
Monensin (1 μM)	379 ± 15	356 ± 62	ND	ND	ND	ND

[a] ND, not determined.

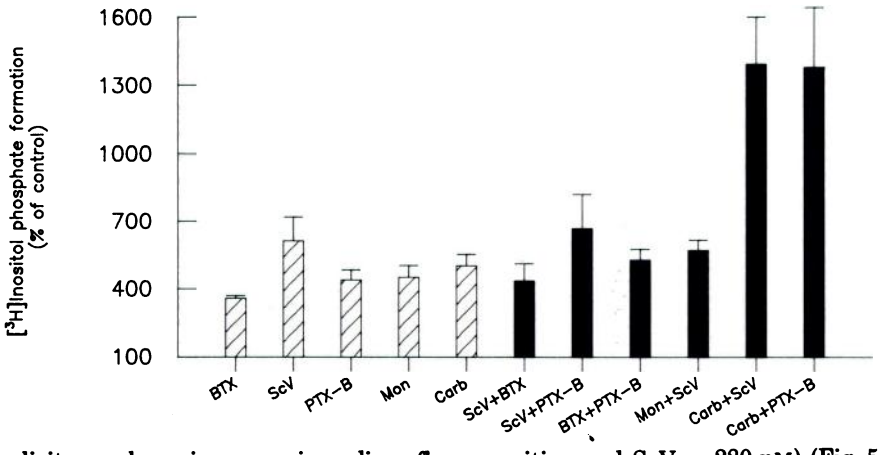

Fig. 1. Effects of combining sodium channel agents and sodium channel agents plus carbamylcholine on phosphoinositide breakdown in guinea pig synaptoneurosomes. Experiments were performed as described under Experimental Procedures. Concentrations of the agents were: BTX, 1 μM; ScV, 6 μg/ml; PTX-B, 10 μM; Monensin (*Mon*), 1 μM; carbamylcholine (*Carb*), 2 mM. The combination of sodium channel agents (ScV + BTX, ScV + PTX-B, BTX + PTX-B, and Mon + ScV) elicited responses that were not different from the responses elicited by the individual agents. The combinations of Carb + ScV and Carb + PTX-B elicited responses that were greater than additive ($p < 0.05$).

elicit very large increases in sodium flux, aconitine and ScV, much smaller increases in sodium flux, and PTX-B, a very small increase. In contrast, the magnitude of the increase in phosphoinositide breakdown elicited by these agents is quite different, with ScV eliciting the largest response, followed by VT, aconitine, BTX, and PTX-B.

TTX and STX are effective and potent in inhibiting sodium fluxes elicited by BTX, VT, and ScV, in contrast to the lower efficacy and potency with which such sodium channel blockers inhibit phosphoinositide breakdown (see above). BTX-induced $^{22}Na^+$ influx into guinea pig cortical synaptoneurosomes (EC_{50} 280 nM) (Fig. 5) was completely blocked by TTX (IC_{50} 19 nM) and STX (IC_{50} 4.0 nM) (Fig. 6A). TTX and STX also block VT-induced $^{22}Na^+$ influx with IC_{50} values of 21 and 5.2 nM, respectively (Fig. 6B). Thus, TTX and STX are from 20- to 100-fold more potent in blocking BTX- and VT-induced $^{22}Na^+$ influx than in blocking BTX- and VT-induced phosphoinositide breakdown. Neither nifedipine (10 μM) nor Co^{2+} (200 μM) inhibited $^{22}Na^+$ influx induced by BTX, ScV, or PTX-B (data not shown). In the presence of 0.5 μM TTX to block TTX-sensitive channels, BTX elicited an apparent biphasic dose response curve for stimulation of sodium influx (Fig. 7), reaching a first plateau at 0.3 μM and a second plateau at 5 μM.

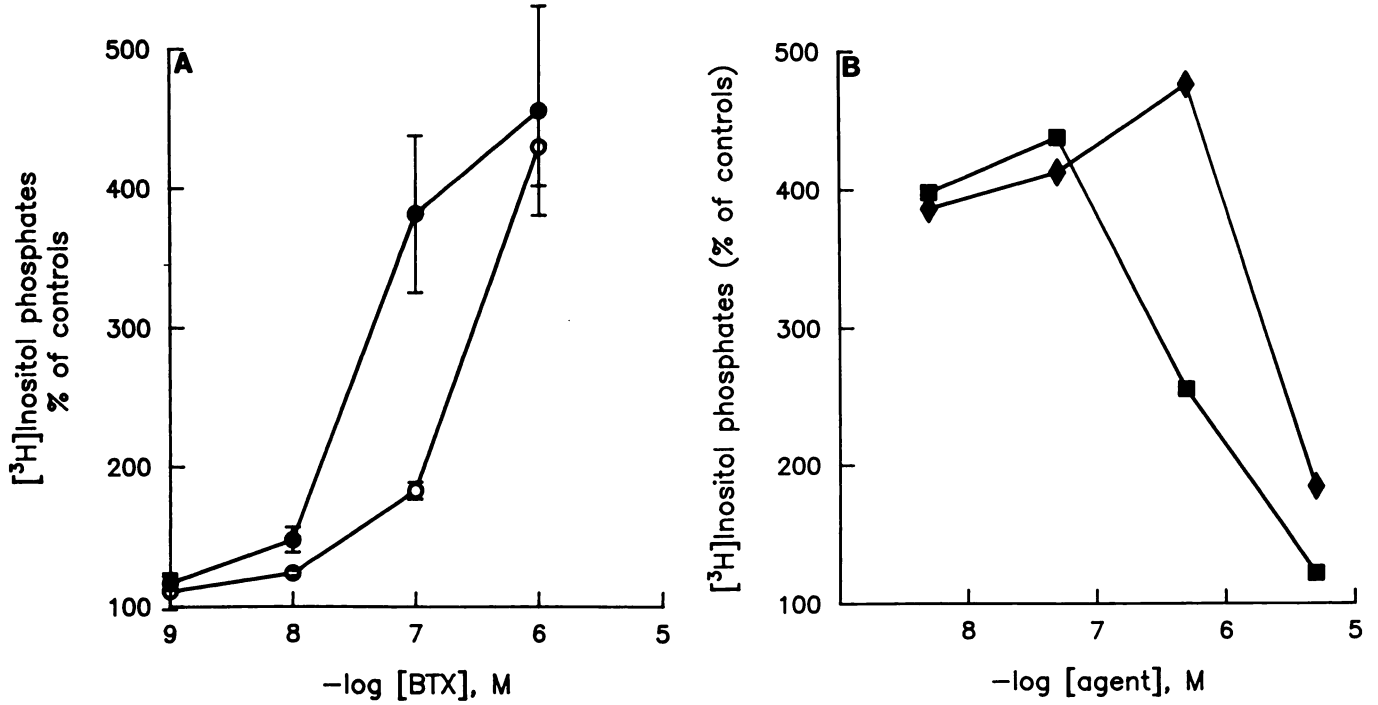

Fig. 2. A. Dose-dependent stimulation of phosphoinositide breakdown in guinea pig synaptoneurosomes for BTX in the absence (○) and presence (●) of 5 μM TTX. Each *point* is the average value (±SEM) of at least three experiments, each one performed in triplicate. B. STX (■) and TTX (♦) inhibitory curves for phosphoinositide breakdown stimulated by 100 nM BTX in guinea pig synaptoneurosomes. *Curves* are from representative experiments performed in triplicate. The experiment was repeated at least three times and yielded similar results. Synaptoneurosomes were labeled for 60 min with [^{3}H]inositol and then incubated with the different agents in the presence of 10 mM LiCl for 90 min. [^{3}H]Inositol phosphates were analyzed as described in Experimental Procedures. Control values were 783 ± 23 cpm/10,000 cpm in lipids.

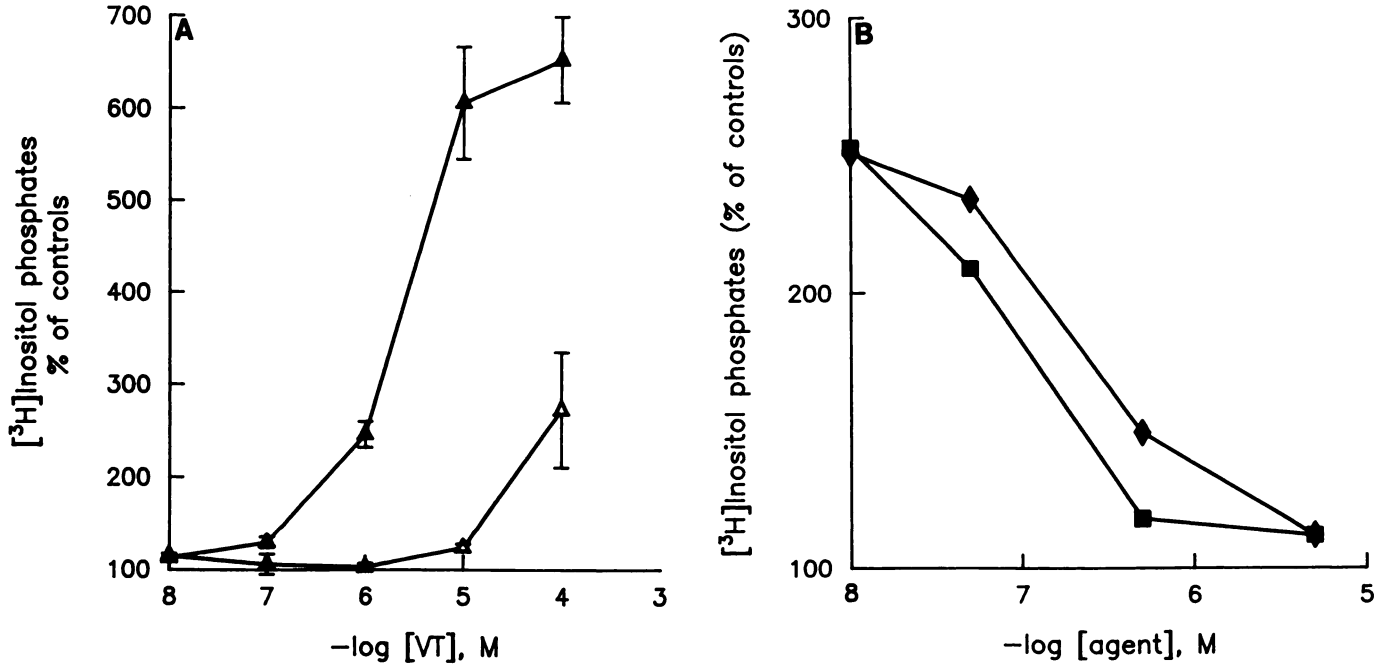

Fig. 3. A. Dose-dependent stimulation of phosphoinositide breakdown in guinea pig synaptoneurosomes for Vt in the absence (▲) and presence (△) of 5 μM TTX. Each *point* is the average value (±SEM) of at least three experiments, each one performed in triplicate. B. STX (■)- and TTX (♦)-inhibitory curves for phosphoinositide breakdown stimulated by 1 μM VT in guinea pig synaptoneurosomes. Curves are from representative experiments performed in triplicate. The experiment was repeated at least three times and yielded similar results. Synaptoneurosomes were labeled for 60 min with [^{3}H]inositol and then incubated with the different agents in the presence of 10 mM LiCl for 90 min. [^{3}H]Inositol phosphates were analyzed as described in Experimental Procedures. Control values were 796 ± 33 cpm/10,000 cpm in lipids.

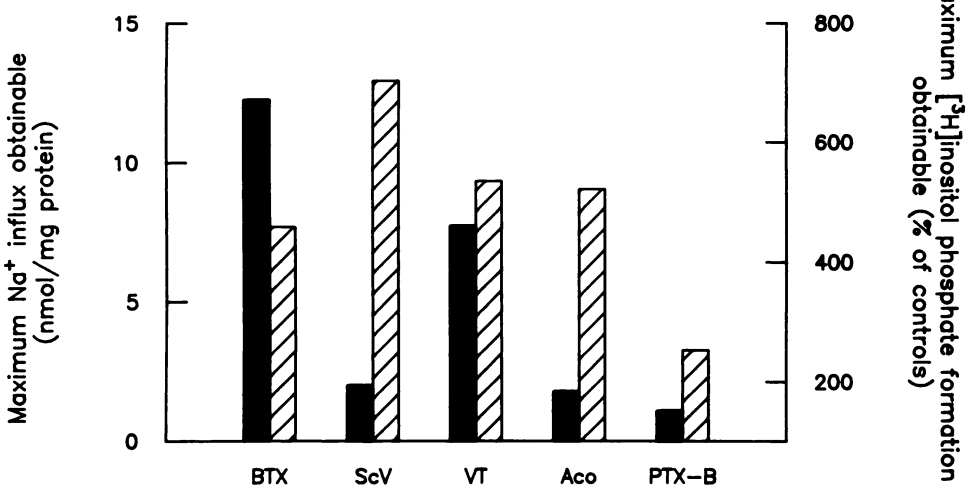

Fig. 4. Maximal stimulation of sodium uptake and of phosphoinositide breakdown (■) obtained in guinea pig synaptoneurosomes by agents that interact with voltage-dependent sodium channels. The concentrations of the different agents used to study sodium uptake stimulation were: BTX, 1 μM; ScV, 60 μg/ml; VT, 100 μM; aconitine (*Aco*), 100 μM; and PTX-B, 100 μM. The concentrations of the different agents used to study phosphoinositide breakdown stimulation were: BTX 1 μM; ScV, 6 μg/ml; VT, 10 μM; Aco, 100 μM; and PTX-B, 5 μM. Experiments were performed as described under Experimental Procedures.

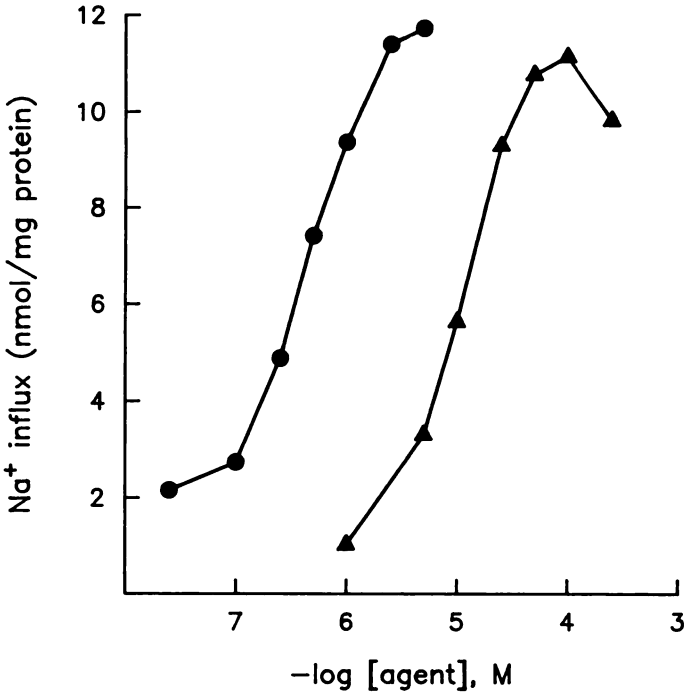

Fig. 5. Dose-dependent stimulation of $^{22}Na^+$ uptake by BTX (○) and VT (▲) into guinea pig synaptoneurosomes. Synaptoneurosomes were incubated for 10 min with the toxins and $^{22}Na^+$ uptake was measured for 10 sec as described under Experimental Procedures. Curves are from representative experiments which were repeated at least three times with similar results. Control value was 0.17 nmol/mg of protein.

Effect of cadmium ions on stimulation of phosphoinositide breakdown and sodium flux elicited by sodium channel agents. Cadmium ions (200 μM) inhibited the stimulatory effects of BTX, ScV, VT, and aconitine, but not the stimulatory effects of neurotransmitters or ionophores on phosphoinositide turnover (Table 2). Other divalent cations at 200 μM were less effective in blocking BTX-induced phosphoinositide breakdown ($Zn^{2+} \geq Sn^{2+} > Ni^{2+}$), and Co^{2+} was inactive (Table 2). Cadmium ions inhibited ScV-induced phosphoinositide breakdown and $^{22}Na^+$ influx with IC_{50} values of 80 μM and 260 μM, respectively (Fig. 8). Cadmium ions inhibited BTX-induced phosphoinositide breakdown with an IC_{50} of 48 μM (Fig. 9A). Cadmium was less potent in inhibiting BTX-induced $^{22}Na^+$ influx into synaptoneurosomes (IC_{50} 610 ± 26 μM). In the presence of 0.5 μM TTX, Cd^{2+} was more potent and inhibited sodium influx elicited by 1 μM BTX with an IC_{50} of 430 ± 52 μM (Fig. 9B).

Discussion

Various neurotransmitters stimulate phosphoinositide breakdown in brain slices and synaptoneurosomes, suggesting that phosphoinositide turnover is important to signal transduction and subsequent generation of second messengers in the central nervous system (see Refs. 12 and 13). In addition to neurotransmitters, agents that cause increases in intracellular sodium can elicit phosphoinositide breakdown in brain synaptoneurosomes (2). Increases in intracellular sodium can be elicited by alkaloids that activate voltage-dependent sodium channels, such as BTX, VT, and aconitine, by toxins that delay inactivation of sodium channels such as the α-scorpion toxin from *L. quinquestriatus*, by compounds that induce repetitive firing of sodium channels, such as PTX-B and certain pyrethroids. Increases in intracellular sodium can also be elicited by the sodium ionophore, monensin, or by an inhibitor of Na^+/K^+ ATPase, ouabain. All such agents stimulate phosphoinositide turnover in guinea pig brain synaptoneurosomes (1, 2). In contrast to neurotransmitter-induced phosphoinositide breakdown, which varies considerably with brain region, sodium agents induce similar increases in phosphoinositide breakdown in synaptoneurosomes from several regions of the guinea pig brain (9). The mechanism whereby increases in intracellular sodium lead to increases in phosphoinositide breakdown is not yet defined. However, such regulation by sodium of phosphoinositide breakdown is conceivably of great relevance since sodium channels and sodium influx are responsible for activity of many neuronal systems.

Combinations of sodium agents did not have additive effects on phosphoinositide breakdown, suggesting that all of these agents stimulate the hydrolysis of the same pool of phosphoinositides. Conversely, carbamylcholine in combination with ScV or PTX-B induced more than additive responses. Such synergistic responses are at this point unexplained, but indicate distinct interactive mechanisms of action.

The order of potencies for stimulation of phosphoinositide breakdown by the alkaloids BTX, VT, and aconitine (9) is

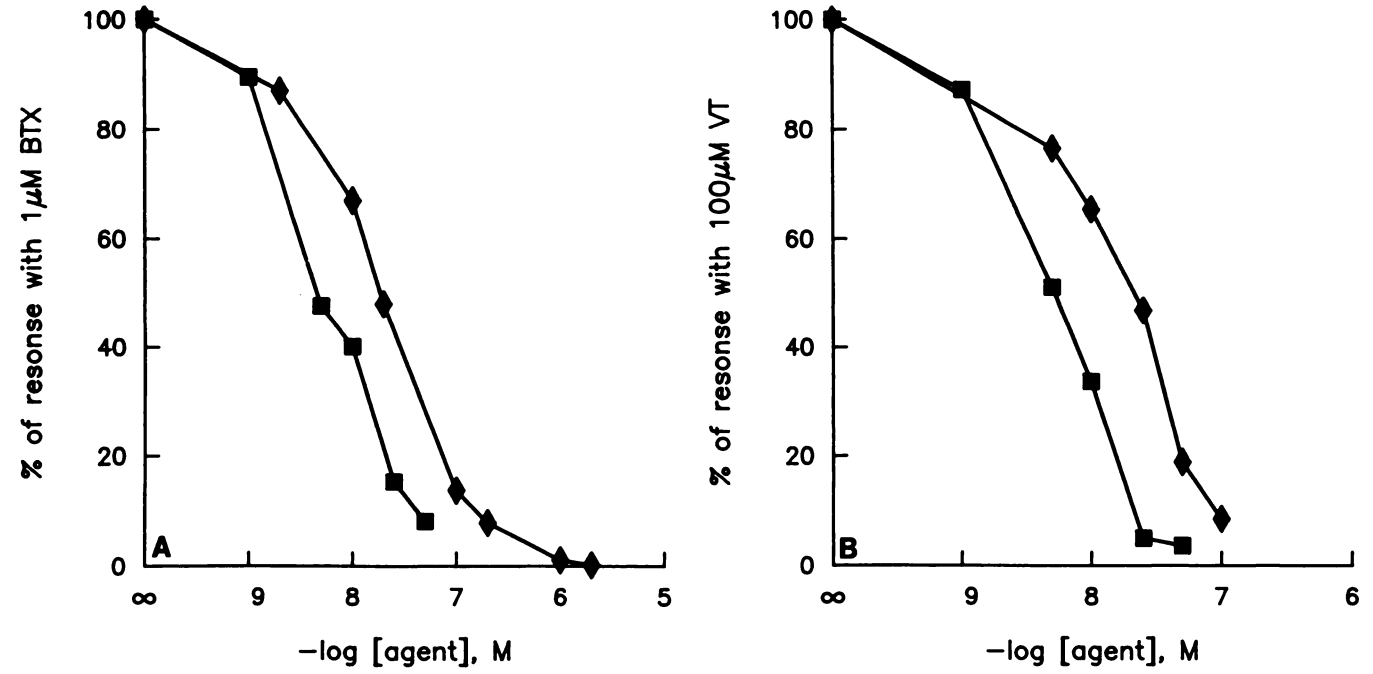

Fig. 6. Dose-response curves for STX (■) and TTX (♦) inhibition of $^{22}Na^+$ influx induced by 1 μM BTX (A) and 100 μM VT (B). Synaptoneurosomes were incubated for 10 min with the toxins and $^{22}Na^+$ uptake was measured for 10 sec as described under Experimental Procedures. *Curves* are from representative experiments which were repeated at least three times with similar results.

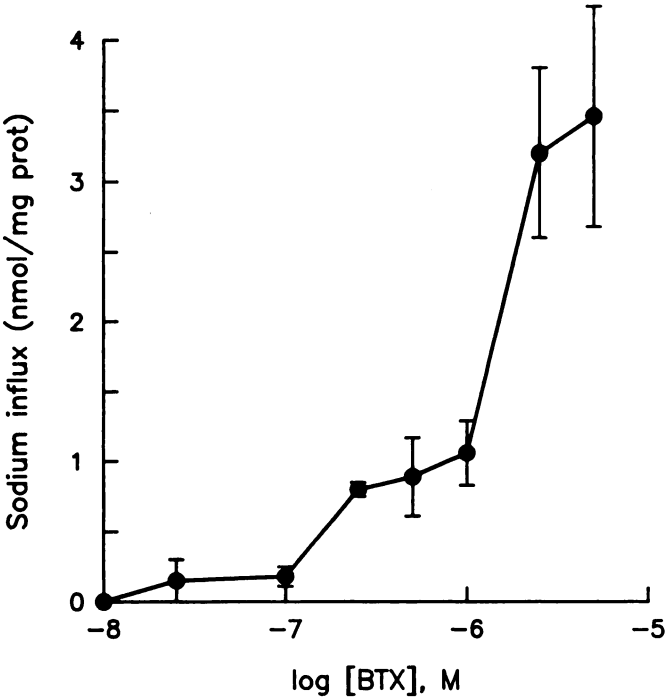

Fig. 7. Dose response curve for BTX stimulation of $^{22}Na^+$ influx in guinea pig synaptoneurosomes in the presence of 0.5 μM TTX. Experiments were performed as described under Experimental Procedures. Control value was 0.20 ± 0.1 nmol/mg of protein.

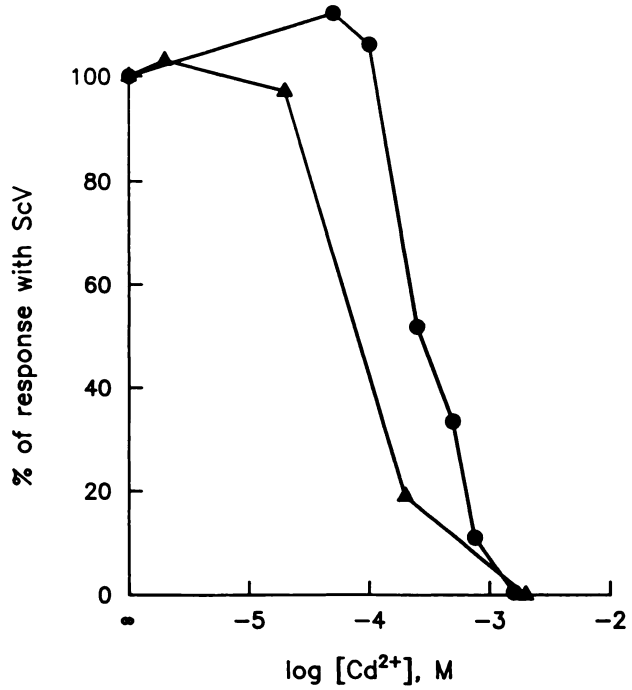

Fig. 8. Dose response curves for Cd^{2+} inhibition of phosphoinositide breakdown (▲) and $^{22}Na^+$ uptake (○) induced by ScV (*L. quinquestriatus*) in guinea pig synaptoneurosomes. Concentrations of ScV used were as follows: for studies on phosphoinositide breakdown, 6 μg/ml; for studies on sodium uptake, 60 μg/ml. Experiments were performed as described under Experimental Procedures. *Curves* are from representative experiments which were repeated at least three times with similar results.

consonant with their potencies in triggering sodium influx. However, at 1 μM, BTX-elicited phosphoinositide breakdown was not effectively blocked by TTX (Table 1). Similarly, at 100 μM, VT-elicited phosphoinositide breakdown was not effectively blocked by TTX (Table 1). Stimulation of phosphoinositide breakdown by BTX was abolished in sodium-free media (2), indicating that sodium influx is a requisite for the BTX effect on phosphoinositide breakdown and suggesting that BTX-sensitive, TTX-resistant channels are involved. The dose response curves for BTX- and VT-stimulated phosphoinositide

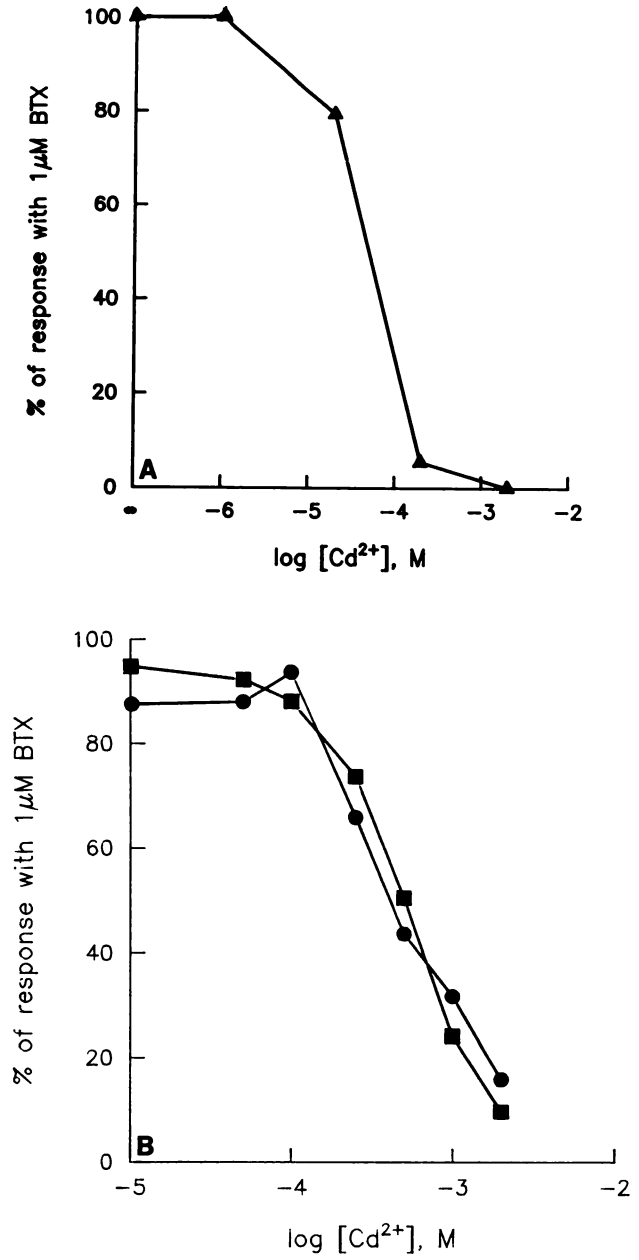

Fig. 9. A. Dose-dependent inhibition by Cd^{2+} of 1 μM BTX-induced phosphoinositide breakdown in guinea pig synaptoneurosomes. B. Dose-dependent inhibition by Cd^{2+} of BTX-induced $^{22}Na^{+}$ influx in synaptoneurosomes. ■, stimulation by 1 μM BTX; O, stimulation by 1 μM BTX in the presence of 0.5 μM TTX. Experiments were performed as described under Experimental Procedures. See the text for details. *Curves* are from representative experiments which were repeated at least three times with similar results.

breakdown were shifted to the right in the presence of 5 μM TTX (Figs. 2A and 3A). Thus, TTX inhibits the stimulatory effects of BTX and VT on phosphoinositide breakdown in an apparent competitive manner and the inhibition is overcome at high concentrations of the alkaloids. It is noteworthy that, although the rank order of potencies for BTX, VT, and aconitine to stimulate $^{22}Na^{+}$ influx into synaptoneurosomes is the same as the rank order of potencies in inducing phosphoinositide breakdown (9), the absolute EC_{50} values are about 1 order of magnitude lower for the phosphoinositide response. Similarly, ScV is more potent in stimulating phosphoinositide turnover than in stimulating $^{22}Na^{+}$ influx (Ref. 1 and data not shown). Experiments on phospholipid breakdown and sodium flux are, of necessity, performed under quite different conditions: sodium influx experiments are carried out in low sodium medium in the presence of ouabain (see Experimental Procedures), whereas the phosphoinositide breakdown experiments are carried out in the presence of 10 mM LiCl. It is thus possible that effects of sodium channel agents on function of sodium channels might differ quantitatively under such dissimilar conditions. Similarly, effects of blocking agents might differ quantitatively under such dissimilar conditions.

ScV-, PTX-B, and aconitine-elicited phosphoinositide breakdown was inhibited by TTX (Table 1). TTX is, however, less potent in blocking phosphoinositide breakdown than sodium influx induced by these agents (data not shown).

All of the sodium channel agents increase sodium flux in synaptoneurosomes and cause a stimulation of phosphoinositide breakdown. TTX blocks sodium influx in all cases but is less potent in blocking phosphoinositide breakdown. The magnitude of maximal influx of sodium with each agent does not correlate with the magnitude of the stimulation of phosphoinositide breakdown, and in all cases the sodium channel agent is more potent in stimulating phosphoinositide breakdown than in stimulating sodium flux. Several hypotheses can be advanced to rationalize these results. Two are as follows: (i) a subpopulation of TTX-insensitive sodium channels accounting for a small fraction of total sodium channels is responsible for the phosphoinositide breakdown; or (ii) only a small fraction of maximal sodium flux in all compartments is necessary to stimulate fully phosphoinositide breakdown, and TTX cannot fully block the final small fraction for agents such as BTX. We find the first hypothesis more attractive and have carried out experiments to test it. The potency of TTX (IC_{50} 2 μM) and STX (IC_{50} 300 nM) for inhibition of 100 nM BTX-induced phosphoinositide breakdown was low, and it was suspected, as proposed above, that TTX-resistant channels might be selectively responsible for mediation of the stimulatory effects of BTX on phosphoinositide breakdown. However, TTX and STX at 2 μM virtually completely abolished $^{22}Na^{+}$ influx induced by BTX and VT in synaptoneurosomes (Fig. 6), suggesting that such TTX-resistant channels could be responsible for only a small percentage of BTX- or VT-elicited sodium flux. In order to test the possibility of a minor component of TTX-insensitive channels, the ability of BTX to stimulate sodium influx at a relatively high TTX concentration (0.5 μM as compared to an IC_{50} value of 19 nM) was evaluated. TTX at 0.5 μM inhibited greater than 90% of BTX-stimulated sodium influx (Fig. 6A). Under such conditions, BTX induced a biphasic dose-dependent sodium influx curve (Fig. 7) suggestive of a two-component system. It appears likely that at least one component represents sodium flux through TTX-insensitive channels, while the second component seen at high concentrations of BTX may represent a residual flux through TTX-sensitive channels, even in the presence of 0.5 μM TTX (see below). At higher concentrations of TTX, the amount of sodium influx, although measurable, was too small for meaningful experiments on dose-dependent blockade by cadmium ions.

Recently, the presence of TTX-resistant sodium channels was documented in different excitable cells by biochemical (5) and electrophysiological (14) procedures. In such cases the channels were sensitive to blockade by Cd^{2+}. The TTX-resist-

ant channels (5) were also sensitive to Zn^{2+} and to higher concentrations of TTX. Cadmium ions blocked the phosphoinositide breakdown elicited by sodium channel agents in guinea pig synaptoneurosomes (Table 2, Figs. 8 and 9). Cadmium ions also blocked increases in $^{22}Na^+$ influx elicited by sodium channel agents in synaptoneurosomes. Organic blockers of voltage-dependent Ca^{2+} channels proved to be ineffective as inhibitors of BTX-induced phosphoinositide breakdown (Table 1, Ref. 9). In addition, Co^{2+} (200 μM), an inorganic calcium channel blocker, was also ineffective in blocking the stimulation of phosphoinositide breakdown by sodium channel agents (Table 2). Thus, it seems unlikely that inhibition by cadmium ions of phosphoinositide breakdown is due to blockade of calcium channels. Instead, the results are consonant with the hypothesis that BTX induces sodium influx through a subset of TTX-insensitive channels that are sensitive to blockade by cadmium ions. Such channels then must either be closely associated with the inositol-labeled phospholipids that act as substrate for sodium-regulated breakdown or be directly associated with phospholipase C. In addition, such channels must represent only a small fraction of total channels sensitive to BTX. As opposed to TTX and STX, cadmium was more potent in blocking BTX-induced phosphoinositide turnover than in blocking BTX-induced sodium influx (Fig. 9). Thus, when cadmium has eliminated 95% of the BTX-elicited phosphoinositide breakdown, only 10% of the sodium flux is blocked. It may be that this 10% or less represents sodium flux through the TTX-insensitive channels associated with phosphoinositide breakdown. Cadmium inhibition of BTX-induced $^{22}Na^+$ influx was tested in two different experimental conditions: (i) 1 μM BTX in the absence of TTX and (ii) 1 μM BTX in the presence of 0.5 μM TTX. In the absence of TTX, BTX-induced sodium influx is inhibited with much lower potency by Cd^{2+} (IC_{50} 610 μM) than is phosphoinositide breakdown (IC_{50} 50 μM). Since, in the presence of 0.5 μM TTX, a biphasic stimulation by BTX is apparent (Fig. 7), a concentration of BTX corresponding to activation of mainly the first component was tested for Cd^{2+} sensitivity. The sensitivity of BTX-stimulated sodium influx to cadmium, albeit different, was not greatly affected in the presence of a 0.5 μM TTX. It is possible that, even at 0.5 μM TTX, there is still a considerable amount of TTX-sensitive flux that contributes to the influx measured. Conversely, in other cells (5), TTX-resistant channels show an IC_{50} for cadmium of 200 μM which is in the same order of magnitude as the value for synaptoneurosomes (430 μM). It is possible that the different conditions used to assess phosphoinositide breakdown and sodium influx (see above) are responsible for significant differences in the potency of cadmium in blocking phosphoinositide breakdown ($IC_{50} \approx 50$ μM) and blocking TTX-insensitive sodium flux ($IC_{50} \approx 400$ μM). Alternatively, it is possible that cadmium may inhibit directly phosphoinositide breakdown elicited by sodium channel agents at a site or sites beyond the initial sodium influx. It should be noted, however, that the response to the sodium ionophore monensin is not affected by cadmium ions, supporting the proposal of TTX-insensitive, cadmium-sensitive sodium channels, rather than a direct inhibitory effect on sodium-elicited phosphoinositide breakdown.

ScV-induced phosphoinositide breakdown was only 3-fold more sensitive to cadmium blockade than ScV-induced sodium influx in synaptoneurosomes (Fig. 8). ScV stimulates flux to a much lesser extent than BTX (Fig. 4). Since ScV has effects primarily on inactivation of channels, it should affect flux primarily through those channels in which spontaneous rates for opening in synaptoneurosomes are high. Therefore, it appears possible that the sodium channels that are associated with sodium-sensitive phosphoinositide breakdown have higher spontaneous activity than the majority of sodium channels in guinea pig synaptoneurosomes.

In summary, stimulation of $^{22}Na^+$ influx and stimulation of phosphoinositide breakdown in synaptoneurosomes induced by agents that activate sodium channels do occur in parallel, but a variety of differences are apparent. (i) The phosphoinositide breakdown effect is about 1 order of magnitude more sensitive to sodium channel agents. It remains possible that differences in conditions used to evaluate the two responses might account for such differences in sensitivity to sodium channel agents. Similarly, it remains possible that only a small fraction of total sodium flux for each agent is sufficient to fully activate phosphoinositide breakdown. However, if such is true, then Cd^{2+} cannot be inhibiting phosphoinositide breakdown through inhibition of sodium flux since it is more potent versus the former than the latter. (ii) TTX and STX are about 20–100 times more potent in inhibiting $^{22}Na^+$ uptake than in inhibiting phosphoinositide breakdown elicited by sodium channel agents. (iii) Cd^{2+} is about 13 times more potent in inhibiting phosphoinositide breakdown than in inhibiting $^{22}Na^+$ uptake induced by BTX, but it was only about 3 times more potent in inhibiting phosphoinositide breakdown than in inhibiting $^{22}Na^+$ uptake induced by ScV. The results can be rationalized by proposing that the cadmium-sensitive, TTX-insensitive sodium flux is intimately linked to phosphoinositide breakdown in synaptoneurosomes. The results could alternatively be explained by proposing a direct inhibitory action of cadmium on some transducer involved in coupling of action of sodium channel agents to phosphoinositide breakdown. However, this seems unlikely due to the fact that the stimulatory effect of monensin on phosphoinositide breakdown is unaffected by Cd^{2+}. Cd^{2+} also had no effects on neurotransmitter-elicited phosphoinositide breakdown. We therefore propose that a subpopulation of cadmium-sensitive, TTX-resistant sodium channels may be closely related to sodium-sensitive phosphoinositide breakdown in the central nervous system. TTX-insensitive sodium channels have been demonstrated electrophysiologically in the nervous system (14). The TTX-resistant channels in synaptoneurosomes must also be proposed to have high rates of spontaneous activity and, thereby, would be primarily involved in increases in sodium flux evoked by agents that delay inactivation of active channels, such as ScV and perhaps PTX-B. Such channels would account for only a small amount (<10%) of the flux elicited by agents that cause opening of sodium channels, such as BTX and VT. The association in the central nervous system of such a subset of TTX-insensitive, cadmium-sensitive sodium channel with phospholipases modulating phosphoinositide breakdown suggests an important functional role for such channels. The nature of sodium-sensitive effectors of phosphoinositide breakdown is unknown. It is attractive to speculate on the possible involvement of a guanyl nucleotide-regulatory protein, analogous to the sodium-sensitive N_i protein involved in inhibitory input to adenylate cyclase. However, it is also possible that a sodium-calcium transporter might be involved and that the effector is calcium taken into

the cell by such a transporter as a result of increases in internal sodium elicited by the sodium channel agents, monensin or ouabain.

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