Pumiliotoxin Alkaloids: Relationship of Cardiotonic Activity to Sodium Channel Activity and Phosphatidylinositol Turnover

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The cardiotonic activity of pumiliotoxin B (PTX-B, 6-(6′,7′-dihydroxy-2′,5′-dimethyl-(*E*)-4′-octenylidene)-8-hydroxy-8-methyl-1-azabicyclo[4.3.0]nonane) as assessed in guinea pig atrial preparations is markedly dependent on the nature of the 6-alkylidene side chain. Pumiliotoxin A (PTX-A), which differs from PTX-B only in lacking the 7′-hydroxy moiety, is much less active than PTX-B. Alteration in the configuration of the 6′- and/or 7′-hydroxy side chain moieties in synthetic isomers of PTX-B reduces activity, while the lack of such moieties or replacement with methoxy or ketone moieties in PTX-B or PTX-A analogues yields cardiodepressant compounds. PTX-B markedly stimulates phosphoinositide turnover in atrial and brain preparations and sodium influx in brain preparations, while analogues that are cardiac depressant or have low cardiotonic activity have no or minimal effects on such parameters. It is suggested that activation of sodium channels and resultant stimulation of phosphoinositide breakdown play a role in the cardiotonic activity of pumiliotoxin alkaloids.

Pumiliotoxin B (PTX-B) and several congeneric alkaloids from neotropical frogs (Dendrobatidae) have marked cardiotonic activitiy.[1-3] The mechanism(s) involved in both cardiotonic and myotonic activities were initially proposed to be due to effects on calcium mobilization.[2,4] Further studies have indicated that such effects on calcium mobilization are dependent on initial interactions of the alkaloids with function of sodium channels.[3,5] Pumiliotoxin B and a variety of agents known to activate voltage-dependent sodium channels stimulate breakdown of phosphoinositides in brain synaptoneurosomes,[6-9] leading to formation of two classes of second messengers, (i) inositol phosphates, certain of which can mobilize calcium, and (ii) diacylglycerols, which can activate calcium-dependent protein kinase C. The effect of pumiliotoxin on phosphoinositide breakdown in synaptoneurosomes is prevented by specific blockers of voltage-dependent sodium channels, namely, tetrodotoxin and saxitoxin.[6] The present study compares the effect of pumiliotoxin B, related congeneric frog alkaloids, and synthetic isomers and analogues (see Scheme I) on cardiotonic activity in guinea pig atria with effects on sodium-channel activity and on phosphatidylinositol breakdown. The results indicate a strong correspondence of cardiotonic activity with sodium-channel activity and with phospholipid turnover and suggest that such biochemical effects may be predictive of cardiotonic activity for a wide range of agents.

Results and Discussion

Biological Activity. Pumiliotoxin B at 6 μM causes a 3–5-fold increase in force and a 2-fold increase in rate in spontaneously beating guinea pig atrial preparations.[2] All structure–activity comparisons were made after 10-min exposures to each concentration over a range of concentrations for PTX-B, synthetic isomers, congeners, and synthetic analogues. Effects on rate and force of contractures in spontaneously beating guinea pig atria are shown in Figure 1. PTX-B (**1**) was the most potent and efficacious of all compounds tested, with maximal effects on force at 6 μM. Other compounds had maximal effects only at 54 μM except for **16** and **18**, which were more potent, having maximal effects at 18 μM. Some of the data are from a prior study.[2] The marked dependence of cardiotonic activity of alkaloids of the pumiliotoxin class on the nature of the side chain is striking. Absence of the side-chain 7′-hydroxy group in pumiliotoxin A (**2**) results in a marked reduction in activity compared to pumiliotoxin B. However, only the single 6′-hydroxy group is required for cardiotonic activity in the allo series of pumiliotoxins, members of which contain two ring hydroxy groups instead of one. Thus, allopumiliotoxins 323B′ and 323B″ (**16** and **17**), which lack the side-chain 7′-hydroxy group, are at least as active as the allopumiliotoxins 339A and 339B (**18** and **20**), which contain both the 6′- and 7′-hydroxy groups. Alteration of the 6′*S*,7′*R*-threo configuration of the side-chain hydroxy groups of PTX-B to either 6′*R*,7′*R* (**4**) or 6′*S*,7′*S*-erythro (**5**) results in a significant decrease in potency as cardiotonic agents. A range of synthetic analogues (**11–15, 21**) and congeneric alkaloids (**7–10, 19**) either are very weak cardiotonics or have slight cardiodepressant activity. All of these lack the side-chain hydroxy groups of PTX-B. Other features of the side chain of PTX-B may also be important to cardiotonic activity. Thus, the synthetic analogue **6** that has a methyl in a 2′-epi configuration is significantly less active than PTX-B.

Pumiliotoxin B at 10 μM causes about a 3-fold increase in phosphoinositide breakdown in guinea pig cerebral cortical synaptoneurosomes.[6] Structure–activity comparisons were done after a 90-min exposure to a 10-μM concentration of PTX-B, congeners, and synthetic isomers and analogues, and the stimulation of phosphoinositide breakdown is reported in Figure 1. PTX-B (**1**) and allopumiliotoxins **16** and **18** caused a 3-fold stimulation of phosphoinositide breakdown and were the most active as cardiotonics. Congeners and analogues that were less active as cardiotonics were less efficacious as stimulants of phosphoinositide breakdown. Cardiodepressant congeners and analogues were inactive or nearly so with respect to stimulation of phosphoinositide breakdown.

The effects of the cardioactive PTX-B (**1**), a congener (PTX-A, **2**) with only slight cardioactivity, and an analogue (**8**) with cardiodepressant activity on phosphoinositide breakdown in mouse atria and on sodium influx in guinea pig synaptoneurosomes are presented in Table I. The

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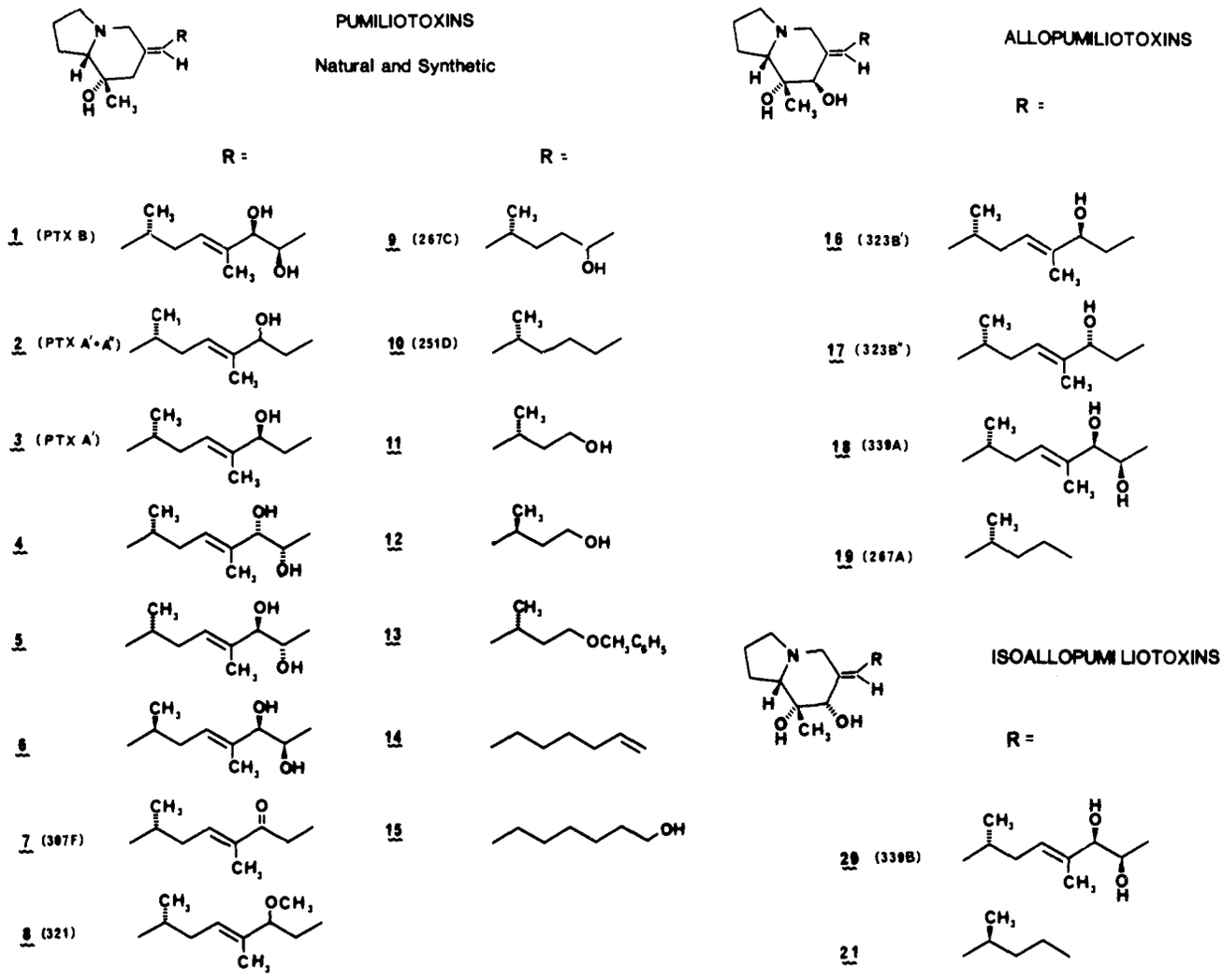
Scheme I



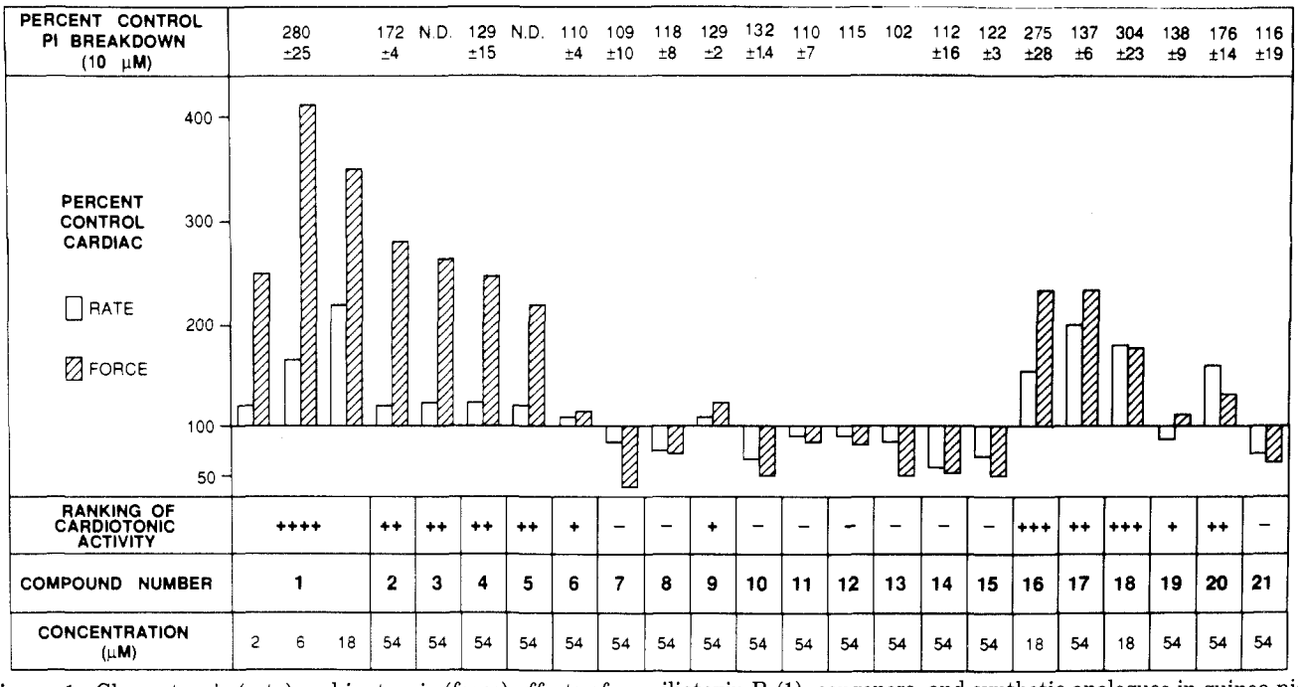


Figure 1. Chronotropic (rate) and inotropic (force) effects of pumiliotoxin B (1), congeners, and synthetic analogues in guinea pig atria and stimulation of phosphoinositide (PI) breakdown in guinea pig brain synaptoneurosomes. Dose-dependent effects of pumiliotoxin B (1) on atria are shown. Effects of other agents are for 54 μM except for 16 and 18, which show maximal effects at 18 μM. Values are averages for two to six experiments. Effects on phosphoinositide breakdown in synaptoneurosomes are given as means ± SEM (n = 3) or are individual values at a concentration of 10 μM. N.D. = not determined.

Table I. Effect of Pumiliotoxin B (1), Pumiliotoxin A (2), and a Synthetic Analogue (8) on Phosphatidylinositol (PI) Breakdown in Mouse Atria and on Specific Sodium Influx in Guinea Pig Cerebral Cortical Synaptoneurosomes

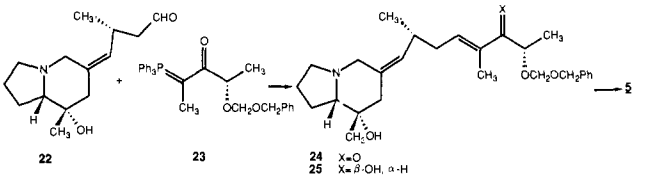
compd	PI breakdown (mouse atria),[a] % of control	specific $^{22}Na^+$ influx (synaptoneurosomes),[b] nmol/mg protein
none	100 ± 10	0.29 ± 0.05
1	150 ± 6*[c]	3.05 ± 0.22*
2	114 ± 11	0.72 ± 0.02*
8	92 ± 15	0.29 ± 0.03

[a] Compounds were tested at 10 μM concentration. Incubations were for 30 min at 37 °C (see Experimental Section for details). Results are averages of two to four determinations ± SEM. Stimulation by 1 was blocked with 5 μM tetrodotoxin. [b] Compounds were tested at 100 μM concentration. Incubations were stopped after 10 s. Nonspecific influx of $^{22}Na^+$ in the presence of tetrodotoxin has been subtracted (see Experimental Section for details). Results are averages of two to five determinations ± SEM. Stimulation by 1 was blocked with 5 μM tetrodotoxin. [c] $p < 0.005$ vs respective control.

results suggest a correspondence between cardiotonic activity and stimulatory effects on phosphoinositide breakdown and sodium influx for these three alkaloids: PTX-B (1) is quite active, while 2 has minimal effects and 7 is devoid of activity. The stimulatory effects of PTX-B both on phosphoinositide breakdown in mouse atria and on sodium influx in guinea pig synaptoneurosomes were blocked by tetrodotoxin (Table I). The present data provide further support for the proposal[6] that agents, such as PTX-B, that enhance sodium influx will thereby trigger increased breakdown of phosphoinositides leading to formation of inositol phosphates that can mobilize calcium from internal stores and to formation of diacylglycerides that can activate protein kinase C. A correlation between sodium influx elicited by various agents and triggering of phosphoinositide breakdown has been established for brain preparations.[6-8] For PTX-B and a variety of other sodium-channel agents, blockade of influx of sodium by tetrodotoxin prevented activation of phosphoinositide breakdown.[6,9] Elevation of internal sodium would be expected to result in heightened activity of Na^+-Ca^{2+} transport systems. Such calcium could be responsible for increasing phosphoinositide breakdown. In heart, the enhanced sodium influx and the resultant phosphoinositide breakdown and calcium mobilization would appear likely to be the basis for the cardiotonic activity of pumiliotoxin B and related compounds. Concomitantly, formation of diacylglycerides and resultant activation of protein kinase C may also increase cardiotonic activity, since phorbol esters, which directly activate protein kinase C, are cardiotonic.[10]

Chemistry. Isolation and characterization of PTX-B (1), PTX-A (2), and other naturally occurring pumiliotoxins (7–10), allopumiliotoxins (16–19), and isoallopumiliotoxins (20) have been described.[11-14] The syntheses of (6′*S*)-PTX-A (3),[15] the 6′*S*,7′*S* analogue (4) of PTX-B,[16] the 2′*S* epimer (6) of PTX-B,[16] PTX analogues 11–15[2] and

Scheme II



21[17] have been described. The 6′*R*,7′*S* analogue (5) of PTX-B was prepared from the known optically active intermediates aldehyde 22[16] and ylide 23[18] derived, respectively, from L-proline and L-lactic acid (see Scheme II). Wittig condensation of these intermediates provided the *E* enone 24 in 80% yield. As would be anticipated from our earlier model studies,[19] reduction of this α′-(benzyloxy)methoxy enone with $LiAlH_4$ occurred with high erythro selectivity to provide 25 (*E*:*T* > 95:5). Deprotection with lithium and ammonia then gave the desired erythro analogue 5 in 83% yield.

Experimental Section

(8*S*,8a*S*)-8-Hydroxy-8-methyl-6(*Z*)-[6′-oxo-2′(*R*),5′-dimethyl-7′(*S*)-[(benzyloxy)methoxy]-4′(*E*)-octenylidene]-octahydroindolizidine (24). A carefully degassed solution of aldehyde 22[16] (34.4 mg, 0.145 mmol), ylide 23[18] (98 mg, 0.20 mmol), and CH_2Cl_2 (0.5 mL) was heated at reflux under an Ar atmosphere for 66 h. Concentration and purification of the residue by column chromatography (silica gel, 40:1:0.1 $CHCl_3$–MeOH–NH_4OH) gave 51 mg (80%) of enone 24 as a chromatographically homogeneous yellow viscous oil: $[\alpha]^{25}_D$ −5° (*c* 2, MeOH); IR (film) 3200–3600, 1680 cm^{-1}; ^1H NMR (250 MHz, $CDCl_3$) δ 7.32 (br s, Ph), 6.57 (br t, *J* = 7.6 Hz, CH=CCO), 5.06 (br d, *J* = 9.7 Hz, =CH), 4.92 (q, *J* = 6.8 Hz, C*H*OR), 4.71 and 4.81 (d, *J* = 7.2 Hz, OCH_2O), 4.59 (br s, CH_2Ph), 3.74 (br d, *J* = 11.8 Hz, H-5α), 3.00–3.10 (m, H-3α), 2.70–1.50 (m), 1.76 (br s, =CMe), 1.36 (d, *J* = 6.9 Hz, ROCH*Me*), 1.11 (s, C-8 Me), 1.05 (d, *J* = 6.6 Hz, CH*Me*); MS (isobutane CI), *m*/*z* 442 (MH), 306, 304, 193, 107, 91; MS (EI), *m*/*z* 441.2862 (441.2879 calcd for $C_{27}H_{39}NO_4$).

(8*S*,8a*S*)-8-Hydroxy-8-methyl-6(*Z*)-[6′(*R*),7′(*S*)-dihydroxy-2′(*R*),5′-dimethyl-4′(*E*)-octenylidene]octahydroindolizidine (5). To a suspension of $LiAlH_4$ (4 mg, 0.1 mmol) in Et_2O (5 mL) was added dropwise a solution of enone 24 (44.1 mg, 0.10 mmol, in 1 mL of a 2:1 mixture of Et_2O and THF) at −20 °C. After 1 h, the reaction mixture was allowed to warm to room temperature, solid $Na_2SO_4 \cdot H_2O$ and $CHCl_3$ (10 mL) were added, and the resulting mixture was stirred for 30 min. After filtration using Celite, the filtrate was concentrated to afford a crude sample of diol ether 25, which was immediately dissolved in a mixture of THF (4 mL) and liquid NH_3 (4 mL). Small pieces of lithium metal were added until the blue color persisted for 1 h, and the reaction was then quenched with excess NH_4Cl. The resulting mixture was allowed to warm to room temperature and filtered through Celite, and the filtrate was concentrated and purified by column chromatography (silica gel, 5 g, 10:1:0.1 $CHCl_3$–MeOH–NH_4OH) to give 27 mg (83%) of 5 (contaminated with <3% of PTX-B by 250-MHz ^1H NMR analysis) as a yellow oil: $[\alpha]^{25}_D$ +19° (*c* 1, MeOH); IR (film) 3100–3600, 1461, 1380, 1317, 1090, 970 cm^{-1}; ^1H NMR (250 MHz, $CDCl_3$, 3.3 mg/0.5 mL) δ 5.42 (br t, *J* = 7.2 Hz, CH_2CH=), 5.09 (br d, *J* = 9.9 Hz, CHCH=), 3.8–3.9 (m, H-5α, CHOHCHOH), 3.05–3.15 (m, H-3α), 1.68–2.60 (m), 1.62 (br s, $=CCH_3$), 1.14 (d, *J* = 6.1 Hz, $CHCH_3$), 1.14 (s, C-8 CH_3), 1.01 (d, *J* = 6.6 Hz, $CHCH_3$); MS (EI), *m*/*z* 323 (MH), 194, 166, 70; high-resolution MS (EI), *m*/*z* 323.2469 (323.2460 calcd for $C_{19}H_{33}NO_3$).

Biological Effects. Cardiotonic Activity. Atria were removed from male 250–300-g Hartley-strain guinea pigs and

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suspended at 37 °C in a 20-mL organ bath with Tyrode's solution aerated with a 95:5 mixture of O_2–CO_2 gas.[20] After equilibration for at least 1 h, cumulative dose–response curves for effects on rate and force of spontaneous contractions were determined by serial additions of the alkaloid in methanol, with at least 10 min between additions.[2] Such experiments were repeated two to six times.

Synaptoneurosomes. Preparation of synaptoneurosomes was as described.[21] Briefly, the cerebral cortex of one guinea pig 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 decanted, and the pellet reconstituted in an appropriate volume of buffer to reach a concentration of about 3 mg of protein/mL.

Phosphoinositide Breakdown in Synaptoneurosomes. Phosphoinositide breakdown was measured as described.[22] Briefly, the synaptoneurosome pellet from one guinea pig was resuspended in 10–15 mL of fresh buffer containing 1 μM [^{3}H]inositol (10 μCi/mL). Aliquots of 320 μL of the suspension (~1 mg of protein) were distributed in 5-mL polypropylene tubes and incubated at 37 °C. After 60 min, 20 μL of 200 mM LiCl was added in each tube. Ten minutes later, pumiliotoxin B, analogues, or congeners 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 °C. 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. 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 × 1 mL of 200 mM ammonium formate–100 mM formic acid. This eluant was collected in vials. Hydroflour was added (8 mL) and radioactivity 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 microliter 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 percent of control response.

Sodium Influx in Synaptoneurosomes. Sodium flux studies were carried out essentially as described by Tamkun and Catterall[23] 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 °C in an incubation buffer containing various test agents. The incubation buffer consisted of 50 mM HEPES (adjusted to pH 7.4 at 36 °C 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 (BSA). 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 BSA, 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 s by adding 4 mL of cold washing buffer. Samples were immediately collected on a Gelman filter (GN-6, 0.45-μm pore size, Gelman Sciences, Inc., Ann Arbor, MI) 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 BSA (pH 7.4). Filters were dissolved in Filtron-X (National Diagnostics, Sommerville, NJ) for liquid scintillation counting (efficiency approximately 99%). The uptake of $^{22}Na^+$ through sodium channels was determined by subtracting nonspecific uptake obtained in the presence of 5 μM tetrodotoxin from the total uptake.

The protein content of the synaptoneurosome preparation was determined by precipitation with 6% trichloroacetic acid, centrifugation, and assay of the resulting pellet in 1 M NaOH using the method of Lowry et al.[24]

Phosphoinositide Breakdown in Mouse Atria. Male mice (20–25 g) were decapitated, and hearts were rapidly dissected. Right and left atrial tissue was excised and incubated at 37 °C for 60 min in 1 mL of Krebs–Henseleit buffer (pH 7.4) containing 1 μM [^{3}H]inositol (10 μCi/mL). LiCl was then added (final concentration 10 mM), and after 15 min, agents were added in 50 μL. Incubations were for 30 min at 37 °C. At the end of the incubation, the tissue was blotted, rinsed twice with fresh buffer, and homogenized in 1 mL of 6% trichloroacetic acid. After centrifugation, [^{3}H]inositol phosphates were analyzed in the supernatant by anion-exchange chromatography as described for synaptoneurosomes. Results are expressed as percent of control.

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4-*N*-Hydroxy-L-2,4-diaminobutyric Acid. A Strong Inhibitor of Glutamine Synthetase

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Analogues of glutamic acid were synthesized and evaluated for their inhibitory activity toward glutamine synthetase (EC 6.3.1.2; GS). The title compound, 4-*N*-hydroxy-L-2,4-diaminobutyric acid (NH-DABA), showed a potent inhibitory activity against GS from both sheep brain and soybean. The inhibition is competitive with respect to glutamic acid and the K_i values of sheep brain GS and soybean GS for NH-DABA are 0.007 mmol and 0.021 mmol, respectively. The activity of inhibition is comparable to those of L-methionine sulfoximine and 2-amino-4-(hydroxymethylphosphinyl)butyric acid (phosphinothricin).

Glutamine synthetase (GS; L-glutamate; ammonia ligase; EC 6.3.1.2) is widely distributed in microorganisms, animal tissues, and higher plants, and catalyzes the formation of glutamine from glutamic acid and ammonia.[1] In the

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