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Regulation of phosphatidylinositol turnover in brain synaptoneurosomes: Stimulatory effects of agents that enhance influx of sodium ions

(inositol phosphates/pumiliotoxin/batrachotoxin/tetrodotoxin/saxitoxin)

FABIAN GUSOVSKY, ELIZABETH B. HOLLINGSWORTH, AND JOHN W. DALY

Laboratory of Bioorganic Chemistry, Building 4, Room 212, National Institute of Arthritis, Diabetes, Digestive and Kidney Diseases, National Institutes of Health, Bethesda, MD 20892

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ABSTRACT **Norepinephrine and carbamoylcholine stimulate accumulation of [^{3}H]inositol phosphates from [^{3}H]-inositol-labeled guinea pig cerebral cortical synaptoneurosomes through interaction with α_1-adrenergic and muscarinic receptors, respectively. In addition to such agonists, a variety of natural products that affect voltage-dependent sodium channels can markedly stimulate accumulation of [^{3}H]inositol phosphates. These include (*i*) alkaloids that activate sodium channels, such as batrachotoxin, veratridine, and aconitine; (*ii*) peptide toxins that alter activation or slow inactivation of sodium channels, such as various scorpion toxins from *Leiurus*, *Centruroides*, and *Tityus* species; and (*iii*) agents that cause repetitive firing of sodium channel-dependent action potentials, such as pyrethroids and pumiliotoxin B. Ouabain, an agent that will increase accumulation of internal sodium by inhibition of Na^+,K^+-ATPase, also stimulates formation of [^{3}H]inositol phosphates, as does monensin, a sodium ionophore. Tetrodotoxin and saxitoxin, specific blockers of voltage-dependent sodium channels, prevent or reduce the stimulatory effects of sodium channel agents and ouabain on phosphatidylinositol turnover, while having lesser or no effect, respectively, on receptor-mediated or monensin-mediated stimulation. Removal of extracellular sodium ions markedly reduces stimulatory effects of sodium channel agents, while removal of extracellular calcium ions with EGTA blocks both receptor-mediated and sodium channel agent-mediated phosphatidylinositol turnover. The results provide evidence for a hitherto unsuspected messenger role for sodium ions in excitable tissue, whereby neuronal activity and the resultant influx of sodium will cause activation of phospholipase systems involved in hydrolysis of phosphatidylinositols, thereby generating two second messengers, (*i*) the inositol phosphates, which mobilize calcium from internal stores, and (*ii*) the diacylglycerols, which activate protein kinase C.**

Biogenic amines, acetylcholine analogs, and certain peptides stimulate phosphatidylinositol turnover in brain tissue through interaction with specific receptors (refs. 1–10 and references therein). The mechanism(s) involved in receptor-mediated stimulation of phosphatidylinositol turnover are not well understood. The products of phosphatidylinositol turnover, namely inositol phosphates and diacylglycerides, apparently serve as "second messengers," with the inositol phosphates eliciting mobilization of internal calcium and the diacylglycerides activating protein kinase C (11). An alkaloid, pumiliotoxin B, which causes repetitive firing of neurons and enhances excitation contraction coupling (12, 13), was found to stimulate phosphatidylinositol turnover (14), suggesting that generation of diacylglycerides and inositol phosphates might be the basis for the biochemical and pharmacological activity of this alkaloid. The finding also suggested that nervous activity involving sodium channels might have a regulatory input to the systems involved in phosphatidylinositol turnover. Sodium channel activity *does* appear to provide another stimulatory input to phosphatidylinositol systems, since a variety of agents that enhance sodium channel function have now been shown to stimulate phosphatidylinositol turnover in brain preparations.

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MATERIALS AND METHODS

Materials. [^{3}H]Inositol (14 Ci/mmol; 1 Ci = 37 GBq) was purchased from Amersham. Batrachotoxin was isolated from the skin of the dart poison frog *Phyllobates terribilis* (15). Pumiliotoxin B was isolated from the skin of the poison frog *Dendrobates pumilio* (16). Scorpion venoms (*Leiurus quinquestriatus, Tityus serrulatus*), carbamoylcholine, norepinephrine, tetrodotoxin, atropine sulfate, veratridine, monensin, and aconitine were from Sigma. AG 1-X8 (100–200 mesh, formate form) anion-exchange resin was from Bio-Rad. Prazosin was provided by Pfizer (Groton, CT). Scorpion (*Leiurus*) toxin (an α-scorpion toxin) was kindly provided by W. Catterall (University of Washington, Seattle). Another scorpion (*Centruroides* suffusus) toxin (toxin II, β-scorpion toxin) was kindly provided by F. Couraud (Institut National de la Santé et de la Recherche Médicale, Marseille, France). The pyrethroids deltamethrin and permethrin were kindly provided by G. Brown (University of Alabama, Birmingham).

Preparation of Synaptoneurosomes. Male Hartley guinea pigs (175–220 g) were decapitated and the brains were rapidly removed. The cerebral cortex was dissected out and synaptoneurosomes were obtained according to Hollingsworth *et al.* (17). Briefly, the cortex of one brain was homogenized in 7–10 vol of Krebs–Henseleit buffer in a glass–glass homogenizer (five strokes). When the nonfiltered preparation was used, the suspension was centrifuged at this point at 1000 × *g* for 10 min, the supernatant was decanted, and the pellet was reconstituted in an appropriate volume of buffer. When a filtered preparation was used, the homogenate was diluted with 35 ml of fresh buffer and filtered first through two layers of nylon material (100 mesh) and then with pressure through 10-μm-pore Millipore filters (LCWP-047). The filtrate was centrifuged at 1000 × *g* for 10 min, the supernatant was decanted, and the pellet was resuspended in an appropriate volume of buffer. The synaptoneurosome preparation contains many synaptosomes with attached resealed postsynaptic entities (neurosomes) (17) and is proving to be a useful, readily obtained, brain preparation for a variety of biochemical studies on cyclic AMP generation (ref. 17 and references therein), phosphatidylinositol breakdown (9, 14), and ion fluxes (18, 19).

Phosphatidylinositol Turnover. The nonfiltered pellet from one guinea pig brain or the combined filtered pellet from two guinea pigs was resuspended in 20 ml of Krebs–Henseleit buffer. Similar results were obtained with nonfiltered and filtered pellets. To the suspension was added 270 μCi of [^{3}H]inositol (1 μM), and the mixture was incubated for 60 min at 37°C with continuous gassing with O_2/CO_2 (95:5, vol/vol). After centrifugation at 1000 × *g* for 10 min and decantation of the supernatant, the labeled synaptoneurosomes were reconstituted in 11–14 ml of fresh buffer containing 10 mM LiCl so that 320-μl aliquots would contain approximately 1 mg of protein. Aliquots were transferred to polypropylene tubes (Falcon 2063) and, after 10 min at room temperature, agents in buffer or buffer alone (20 μl) was added. The tubes were then gassed briefly with O_2/CO_2 (95:5), capped, and placed in a water bath at 37°C. Unless stated otherwise incubations were for 90 min.

The analysis of [^{3}H]inositol metabolites was as described by Berridge *et al.* (1, 20). In brain preparations, the major product of phosphatidylinositol breakdown in the presence of lithium ions is inositol phosphate, with inositol bis- and trisphosphates being very minor (data not shown; see also refs. 1–10). In the present study, total accumulation of [^{3}H]inositol phosphates in inositol-labeled synaptoneurosomes has been used to monitor phosphatidylinositol turnover. Briefly, the tubes were centrifuged for 5 min at 1000 × *g* and the supernatant was discarded. The pellets were washed with 1 ml of fresh buffer. To the washed pellet 0.94 ml of methanol/chloroform (2:1, vol/vol) was added. After 10 min at room temperature, 300 μl of water, 300 μl of buffer, and 300 μl of chloroform were added sequentially to each tube. The extraction of water-soluble metabolites was performed by shaking the tubes manually for 30 sec. The layers were separated by centrifugation and 1 ml of the aqueous upper layer was placed on an AG 1-X8 anion-exchange column (formate form, 0.5–0.8 ml). Free [^{3}H]inositol was eluted with four 3-ml washes of water. [^{3}H]Inositol phosphates were eluted into scintillation vials with 2 ml of 200 mM ammonium formate/100 mM formic acid. Hydrofluor (7 ml; National Diagnostics, Somerville, NJ) was added to each vial for analysis of radioactivity in a scintillation counter.

RESULTS

Carbamoylcholine and norepinephrine stimulate phosphatidylinositol turnover in [^{3}H]inositol-labeled guinea pig cerebral cortical synaptoneurosomes (Table 1). The effects of carbamoylcholine are completely blocked by a muscarinic antagonist, atropine, while effects of norepinephrine are completely blocked by a specific α_1-adrenergic antagonist, prazosin (data not shown). Tetrodotoxin caused a 20–35% inhibition of these receptor-mediated responses (Table 1). Saxitoxin (0.5 μM) in one experiment caused 40% inhibition of the carbamoylcholine response and a 9% inhibition of the norepinephrine response. Such inhibitions suggest that receptor-mediated stimulation of phosphatidylinositol breakdown in brain preparations involves to some extent influx of sodium ions through voltage-dependent sodium channels.

A variety of agents that are known to affect the state or function of voltage-dependent sodium channels enhance accumulation of [^{3}H]inositol phosphate in synaptoneurosomes (Table 1). The time courses for responses to a receptor agonist, carbamoylcholine, to scorpion (*Leiurus*) venom, and to pumiliotoxin B are shown in Fig. 1. The concentration dependency of the effect of purified scorpion (*Leiurus*) toxin on phosphatidylinositol breakdown is shown in Fig. 2. The concentration of 2 nM for half-maximal effect is commensurate with the potency of scorpion toxin in enhancing sodium fluxes and allosterically affecting sodium channels in brain preparations (21, 22).

Tetrodotoxin, a specific blocker of sodium channels, antagonizes the responses to scorpion toxins, pumiliotoxin B, deltamethrin, and aconitine (Table 1). However, tetrodotoxin even at 5 μM was not particularly effective versus batrachotoxin and veratridine responses. Saxitoxin, another specific blocker of sodium channels, in one experiment at 0.5 μM blocked the response to batrachotoxin (0.1 μM) by 50% and the response to veratridine (10 μM) by 100% (data not shown). The potency of tetrodotoxin and of another sodium channel blocker, saxitoxin, as antagonists of scorpion venom-elicited phosphatidylinositol turnover was ascertained (Fig. 3). Tetrodotoxin had an IC_{50} of about 300 nM, indicating that the sodium channel was relatively insensitive

Table 1. Effects of receptor agonists and agents that modify intracellular sodium on phosphatidylinositol turnover in guinea pig cerebral cortical synaptoneurosomes and inhibition by tetrodotoxin

Agent	Conc.	Site of action or effect	[^{3}H]Inositol phosphate, % of control	Inhibition by tetrodotoxin, %
Receptor agonists				
Carbamoylcholine	2 mM	Muscarinic receptor	326 ± 31	33, 36
Norepinephrine	100 μM	α_1-Adrenergic receptor	217 ± 31	23, 22
Sodium channel agents				
Batrachotoxin	0.1 μM	Activates channel	146	53, 75*
Veratridine	10 μM	Activates channel	141	22*
Aconitine	10 μM	Activates channel	190 ± 32	92 ± 8
Scorpion toxins				
Leiurus venom	1 μg/ml	Slows inactivation	145 ± 9	98, 74
Leiurus (α)	2 nM	Slows inactivation	171	80 ± 19
Centruroides II	10 nM	Slows inactivation	141 ± 3	100, 100
Tityus venom	0.2 μg/ml	Slows inactivation	209	73, 74
Pumiliotoxin B	5 μM	Repetitive firing	155 ± 6	94 ± 4
Deltamethrin	5 μM	Repetitive firing	152 ± 13	88 ± 5
Other agents				
Ouabain	1 μM	Na^+,K^+-ATPase inhibitor	122 ± 1	66 ± 3
Monensin	1 μM	Sodium ionophore	156 ± 4	3, 5
Potassium ions	40 mM	Depolarizing agent	152 ± 4	46, 38

Synaptoneurosomes were labeled with [^{3}H]inositol, incubated with the agents for 90 min unless otherwise noted, and assayed for [^{3}H]inositol phosphates. Tetrodotoxin was at 5 μM. Values are means ± SEM for three experiments or are means for representative individual experiments in which each measurement was made in triplicate.
*A 15-min incubation. Less inhibition occurred with the 90-min incubation.

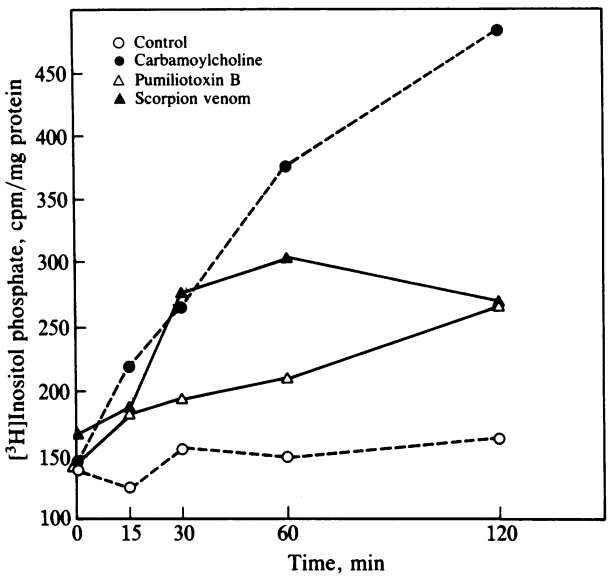

FIG. 1. Time courses for accumulation of inositol phosphates. Synaptoneurosomes labeled with [³H]inositol were incubated with no agent (○), 2 mM carbamoylcholine (●), 5 μM pumiliotoxin B (△), or scorpion (*Leiurus*) venom at 0.6 μg/ml (▲) for the times indicated. [³H]Inositol phosphate was separated and analyzed. Values are means from a representative experiment; each measurement was in triplicate. SEM is less than symbol size.

to this blocker (see ref. 23 for discussion of tetrodotoxin sensitivity of sodium channels). Saxitoxin had an IC_{50} of about 3 nM, consonant with its potency as a ligand for sodium channel sites in rodent synaptosomes (ref. 24, see also ref. 23). The concentrations of agents that are known to enhance sodium channel function were selected on the basis of their known potencies at sodium channels. The alkaloids batrachotoxin, veratridine, and aconitine are well known as agents that activate sodium channels (see ref. 25), and all caused phosphatidylinositol turnover (Table 1). Batrachotoxin and veratridine have EC_{50} values of 40 nM and 4 μM, respectively (data not shown), and are thus relatively more potent in causing phosphatidylinositol breakdown than was

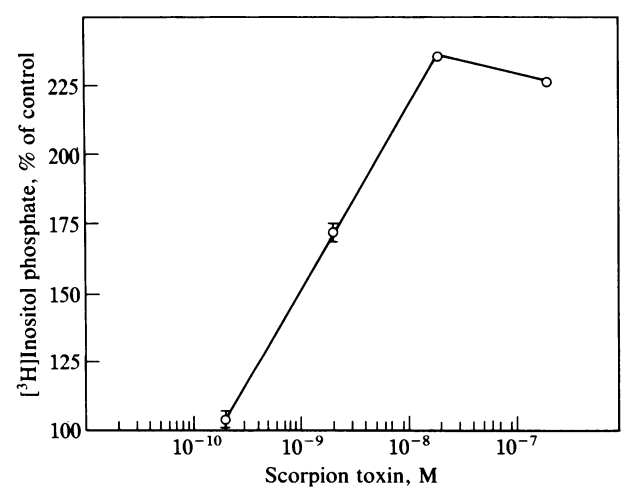

FIG. 2. Dose–response curve for stimulation of phosphatidylinositol turnover by α-scorpion toxin from *Leiurus quinquestriatus*. Synaptoneurosomes labeled with [³H]inositol were incubated with the α-scorpion toxin for 90 min and [³H]inositol phosphate was separated and analyzed. Values are means from a representative experiment; each measurement was in triplicate. Error bars (SEM) less than symbol size are not shown.

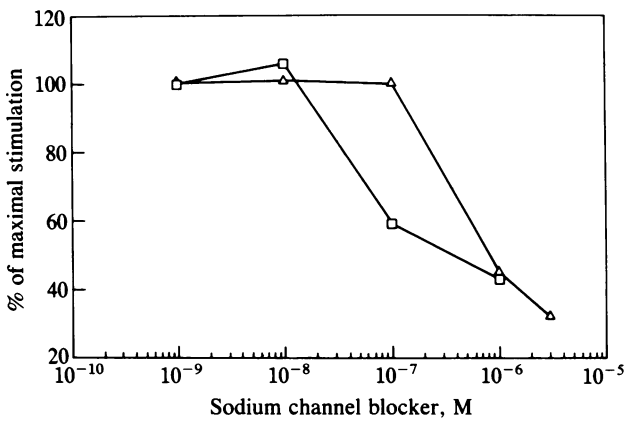

FIG. 3. Dose–response curves for inhibition of scorpion venom-elicited phosphatidylinositol turnover by tetrodotoxin and saxitoxin. Synaptoneurosomes labeled with [³H]inositol were incubated with scorpion venom (1 μg/ml) from *Leiurus quinquestriatus* in the presence of tetrodotoxin (△) or saxitoxin (□) at various concentrations. Values are means from a representative experiment; each measurement was in triplicate. SEM is less than symbol size.

expected on the basis of their potency in activating sodium channels (see ref. 22). Toxins in venoms from scorpion species of the genera *Leiurus*, *Centruroides*, and *Tityus* either enhance activation or slow inactivation of sodium channels (see ref. 25). These toxins or crude venom enhanced phosphatidylinositol turnover in a tetrodotoxin-sensitive manner. Pyrethroids (26) and pumiliotoxin B (13) cause repetitive firing of excitable cells—i.e., cause repetitive opening of voltage-dependent sodium channels. The molecular mechanisms involved are as yet poorly defined. The active pyrethroid deltamethrin stimulated phosphatidylinositol turnover (Table 1), while a less active analog, permethrin, had no effect (data not shown). Tetrodotoxin blocked the response to deltamethrin. Pumiliotoxin B had similar stimulatory effects, which were sensitive to blockade by tetrodotoxin. In addition to blockade by tetrodotoxin or saxitoxin, the stimulation of phosphatidylinositol turnover by sodium channel agents, such as batrachotoxin, scorpion venom, and pumiliotoxin B, were reduced by 63–78% in sodium-free medium (Table 2). The response to veratridine was reduced by only 44%, suggestive of the involvement of other mechanisms in the effects of this agent on phosphatidylinositol turnover. The response to carbamoylcholine also was reduced by 66% in sodium-free medium.

Ouabain, a classical inhibitor of the Na^+,K^+-ATPase that "pumps" sodium out of cells, stimulated phosphatidylinositol turnover. The stimulation by ouabain was reduced by tetrodotoxin. Monensin, a sodium ionophore (27), enhanced phosphatidylinositol turnover and, in this case, tetrodotoxin had no effect on the response. Depolarization of synaptoneurosomes (see ref. 28) by potassium ions stimulated turnover of phosphatidylinositol (Table 1). This stimulation was reduced by tetrodotoxin, suggesting that depolarization *per se* was not responsible but that a depolarization-elicited influx of sodium ions was involved. Removal of extracellular calcium with EGTA blocked completely both receptor and sodium channel activator-mediated responses (Table 2; see also ref. 2). High concentrations of magnesium to block transmitter release did not reduce stimulation of phosphatidylinositol elicited by a sodium channel agent, scorpion venom. In the presence of normal 1.18 mM magnesium ions, scorpion (*Leiurus*) venom (1 μg/ml) caused a 2.3-fold increase in phosphatidylinositol turnover above control, whereas it caused a 2.1-fold increase in turnover in the presence of 30 mM magnesium above that seen with a 30 mM magnesium control (data not shown). It should be noted that

Table 2. Phosphatidylinositol turnover in guinea pig cerebral cortical synaptoneurosomes: Effect of sodium-free and calcium-free (EGTA) medium

Stimulant	Conc.	Inhibition, %	
		Na^+-free	Ca^{2+}-free (EGTA)
Carbamoylcholine	2 mM	68 ± 5	100
Batrachotoxin	1 μM	66 ± 9	100
Veratridine	10 μM	44	—
Scorpion venom	1 μg/ml	63	100
Pumiliotoxin B	5 μM	71 ± 22	100

Synaptoneurosomes were labeled with [^{3}H]inositol, incubated with agents for 90 min, and assayed for [^{3}H]inositol phosphates. The sodium-free medium was 130 mM choline chloride/2.5 mM $CaCl_2$/0.8 mM $MgSO_4$/5.4 mM KCl/5.5 mM glucose/50 mM Hepes/23 mM Tris buffer, pH 7.4. The calcium-free medium contained no calcium (normally 2.5 mM $CaCl_2$), and 1 mM EGTA was present. Values are means ± SEM for three experiments or are means of triplicate measurements for individual experiments.

increasing magnesium chloride to 30 mM alone caused a 1.7-fold increase in the basal rate of phosphatidylinositol turnover. The mechanism involved is unclear. Combination of a receptor agonist, carbamoylcholine (2 mM), which caused a 4.0-fold stimulation, and a sodium channel agent, scorpion (*Leiurus*) venom (1 μg/ml), which caused a 2.3-fold stimulation, had nearly additive stimulatory effects (5.1-fold) on phosphatidylinositol turnover (data not shown).

DISCUSSION

The control of phosphatidylinositol turnover has been considered to be receptor-mediated through an, as yet, undefined calcium-dependent mechanism. In brain slices or vesicular preparations from brain activation of muscarinic, α_1-adrenergic, H_1-histamine, 5-HT_2-serotonin, neurotensin, and vasopressin receptors can elicit turnover of phosphatidylinositol (1–10). Depolarizing concentrations of potassium and veratrine were reported to increase phosphatidylinositol turnover in rat cerebral cortical slices (2), but the mechanism(s) involved were not investigated. The present results suggest a control mechanism for phosphatidylinositol metabolism linked to intracellular levels of sodium ions. Thus, a variety of agents that activate, enhance activation, slow inactivation, or cause repetitive activity of voltage-dependent sodium channels stimulate phosphatidylinositol turnover in guinea pig cerebral cortical vesicular preparations. The blockade or reduction in responses by tetrodotoxin, saxitoxin, or sodium-free medium provides strong evidence for the importance of voltage-dependent sodium channels and sodium ions in this regulatory mechanism. In some instances tetrodotoxin is relatively ineffective even at high concentrations (Table 1), suggesting the involvement of tetrodotoxin-insensitive sodium channels (see ref. 23). This is particularly true for batrachotoxin and veratridine and requires further study. Saxitoxin completely blocked the veratridine response and partially blocked the batrachotoxin response. The various agents that enhance phosphatidylinositol turnover do not have a common mode of action on sodium channels, but all would enhance influx of sodium into nervous tissue. The alkaloids batrachotoxin, veratridine, and aconitine interact, in that order of potency, with voltage-dependent sodium channels to cause opening or activation (see ref. 25). These are potent depolarizing agents. The peptide toxins, such as the toxins of the three scorpion species, interact with sodium channels to either enhance activation or slow inactivation (see ref. 25). These interact at different sites on the sodium channel and cause minimal depolarization. Pyrethroids (26) and pumiliotoxin B (12, 13) cause repetitive firing of action potentials in nerve with minimal steady state depolarization.

Ouabain, as an inhibitor of the Na^+,K^+-ATPase serving to pump sodium out of cells, would increase intracellular sodium ions. Monensin, as a sodium ionophore, increases intracellular sodium (29). Thus, both of these agents would, on the basis of their known biochemical mechanism of action, be expected to increase intracellular sodium ions, and both enhance phosphatidylinositol turnover. Depolarizing concentrations of potassium ions have stimulatory effects on phosphatidylinositol turnover that again appear dependent on sodium channel function, since tetrodotoxin partially antagonizes the response. The relationship of the present results to an α-latrotoxin-elicited accumulation of inositol phosphates in pheochromocytoma PC12 cells (30) deserves investigation. Such cells do contain voltage-dependent sodium channels, and it is possible that activation of such channels is involved in the stimulatory effects of α-latrotoxin.

Depolarization by sodium channel activators is known to cause release of neurotransmitters that might then stimulate phosphatidylinositol turnover. Indeed, high concentrations of potassium cause accumulations of inositol phosphates in sympathetic ganglia, and it was suggested that release of an unidentified stimulatory peptide was involved (31). However, in guinea pig cerebral cortical preparations, high concentrations of magnesium ions, which should block evoked transmitter release, do not reduce the responses to sodium channel agents. The inhibition of responses by EGTA (Table 2) is not unexpected in view of the calcium dependency of phospholipase C, the enzyme(s) involved in phosphatidylinositol turnover (32): dependency of receptor-elicited phosphatidylinositol turnover on extracellular calcium is well documented in a variety of systems, including brain slices (2). Recently, electrical stimulation of [^{3}H]inositol-labeled rat sciatic nerve was reported to have no significant effect on phosphatidylinositol turnover (33), but lithium ions were not present to block the phosphatidylinositol cycle.

Tetrodotoxin, in addition to blocking phosphatidylinositol turnover elicited by sodium channel activators, also reduces receptor-mediated turnover to some extent (Table 1). It appears possible that in brain preparations part of the basal and receptor-mediated turnover is due to spontaneous activity of voltage-dependent sodium channels. The response to carbamoylcholine also is reduced in sodium-free medium (Table 2). The involvement of sodium ions in receptor-mediated phosphatidylinositol turnover requires further study. It is possible that activation of muscarinic receptors not only inhibits cyclic AMP generation and evokes phosphatidylinositol breakdown but also directly or indirectly causes activation of sodium channels (see ref. 34). Tetrodotoxin had no effect on neurotensin-elicited phosphatidylinositol turnover in rodent brain slices (8).

The mechanism involved in sodium ion or sodium channel modulation of phosphatidylinositol turnover is unclear. It is tempting to speculate that sodium ion might enhance the regulation of phosphatidylinositol turnover through interaction with guanine nucleotide regulatory proteins (35). A pertussis toxin-sensitive guanyl nucleotide regulatory protein is necessary for receptor-mediated phosphatidylinositol turnover in some cells (36). A related pertussis toxin-sensitive N_i protein subserves inhibitory input to adenylate cyclase (37), and such inhibitory inputs to adenylate cyclase are markedly affected by sodium ions (38, 39). A role for sodium-dependent mobilization or transport of calcium cannot be excluded. Extracellular calcium is clearly required for activation of phosphatidylinositol metabolism in brain preparations (2).

The significance of an input to phospholipid turnover linked to neuronal or muscle activity is considerable. Any condition leading to enhanced sodium channel activity would then result in generation of inositol phosphates and

diacylglycerides. The latter, through activation of protein kinase C, could affect sodium channel function (40), potassium channel function (41), or calcium channel function (42). It is tempting to speculate that activation of sodium channel leading to phosphatidylinositol turnover and a resultant protein kinase C-catalyzed phosphorylation of sodium channels is a self-regulated homeostatic cycle in electrically excitable cells.

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