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CHOLINERGIC STIMULATION OF POLYPHOSPHOINOSITIDE METABOLISM IN BRAIN *IN VIVO*

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Abstract—The effect of carbamylcholine (CARB) on phosphate and inositol incorporation into the polyphosphoinositides (PPI) in regions of rat brain *in vivo* were examined utilizing the techniques of intracisternal administration of radioisotopes and drugs and microwave irradiation fixation. These studies show that PPI incorporation of phosphate and inositol increases 50–100 per cent in response to CARB within 5 min of drug and label administration. These responses were greater in the cerebral cortex and brainstem–midbrain regions than in the cerebellum, and they were blocked by the previous intraperitoneal administration of atropine sulfate. These increases in substrate PPI were not accompanied by detectable increases in PPI concentration, suggesting that PPI turnover was stimulated. CARB also increased isotope incorporation into PPI precursors phosphatidic acid (PA) and monophosphoinoside (MPI) and there were no findings to suggest that the CARB effects on PA and MPI and those on the PPI represent different phenomena. These studies suggest that PPI, like PA and MPI, are associated with membrane events which result from neurotransmitter activation of cholinergic receptors in brain.

Monophosphoinositide (MPI) and its precursor phosphatidic acid (PA) have been implicated repeatedly in neural and secretory membrane processes [1–3]. The other two inositide phospholipids, diphosphoinositide (DPI) and triphosphoinositide (TPI), have received considerably less investigative attention, even though these two polyphosphoinositides (PPI) are potentially the more interesting as membrane constituents because of their relatively unique physicochemical properties. PPI head groups are multiply-charged anions which have a very high affinity for calcium ions (greater than that of EDTA) and exhibit a rapid turnover *in vivo*; PPI hydrophilic/hydrophobic solubility partitions are altered markedly when calcium replaces monovalent phosphate counterions [4–7]. In addition to being few in number, PPI studies have produced contradictory results, leading reviewers to conclude that PPI involvement in neural and secretory membrane phenomena is not well established [3, 7].

The present studies examined the effects of administered cholinergic agonist and antagonist on PPI phosphate and inositol incorporation using a combination of techniques chosen to overcome specific problems associated with studying PPI metabolism (i.e. intracisternal injection of drugs and radioisotopes, short-term drug and radiolabel challenges *in vivo* and microwave irradiation fixation). Similar studies have shown an association between PA and MPI metabolism and neurotransmitter receptor stimulation [8]. The present studies were conducted to determine if such an association could be demonstrated for the PPI, and thus whether the physicochemical properties of the PPI could be included in proposals explaining phosphoinositide functions in neural membranes.

EXPERIMENTAL

Male Sprague–Dawley rats, 35 to 37-days-old, were used for all studies (Zivic-Miller, Allison Park, PA). Radiolabels $NaH_2{}^{32}PO_4$ (500 Ci/mole) and [^{3}H]inositol (3500 Ci/mole) were obtained from New England Nuclear (Boston, MA). Carbamylcholine chloride (Sigma Chemical Co., St. Louis, MO) and radiolabels were injected intracisternally in normal saline under very light ether anesthesia [9]. Atropine sulfate (Sigma Chemical Co.) was administered intraperitoneally (160 mg/kg) 15 min before intracisternal injections. All animals were killed 5 min after intracisternal injection.

Animals were killed by microwave irradiation in a Litton model 70/50 oven delivering 1300 W at 2450 MHz. Each animal received 30–45 sec of irradiation, depending on body weight. The point of adequate fixation was determined by boiling cerebrospinal fluid at the skull lambda. Brains were removed immediately and dissected into cerebellum, cerebral cortex and brainstem (includes midbrain and pons-medulla structures). Microwave fixed brains experienced less than 5 per cent weight loss due to heating, and data are reported per wet weight without correction for this loss (Soukup *et al.*, *J. Neurochem.*, in press).

Phospholipid extractions were based on those of Dawson and Eichberg [10]. Phospholipids except for the PPI were extracted with three homogenizations in 10 vol. chloroform–methanol, 1:1, 2:1 and 2:1. Polyphosphoinositides were then recovered from the residue with three 15-min extractions using 10 vol. chloroform–methanol–hydrochloric acid (2:1:0.25%, v/v) at 37°. The initial neutral pH chloro-

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form: methanol extracts were adjusted to a final chloroform–methanol ratio of 2:1 and washed with 0.2 vol. of 0.73% sodium chloride; then the lower phase was washed with chloroform–methanol–0.58% sodium chloride (3:48:47, v/v) as suggested by Folch *et al.* [11]. The acidified chloroform: methanol extracts containing the PPI were washed with 0.2 vol. of 1 N hydrochloric acid, and then the lower phase was washed with chloroform–methanol–0.05 M calcium chloride (3:48:47, v/v) [11]. The washed lower phases from the neutral and acidified extracts were each dried under nitrogen and redissolved in a known amount of solvent for chromatographic or liquid scintillation procedures (chloroform–methanol, 2:1, for neural extracts; chloroform–methanol–water, 75:25:2, for acidified extracts).

Chromatography. Phospholipids from neutral chloroform: methanol extracts were separated by two-dimensional thin-layer chromatography [12] using Silica gel G plates (Redicoats, Supelco, Inc.) developed with chloroform–methanol–4 N ammonium hydroxide (130:70:10, v/v) as the first solvent system and chloroform–acetone–methanol–glacial acetic acid–water (100:40:20:20:10, v/v) as the second system.

PPI from acidified extracts were separated by thin-layer chromatography on Silica gel H-R (E. Merck Co.) using chloroform–methanol–4 N ammonium hydroxide (100:60:17) (see Ref. 13). Phospholipids were located for quantitative recovery with iodine vapors.

Analytical procedures. Phosphate analyses were conducted on extract aliquots or on samples adsorbed onto Silica gel by ashing with 10 N sulfuric acid and 0.5 N perchloric acid at 165° for 1.5 h, followed by colorimetric quantitation of phosphomolybdenum complexes reduced with ascorbic acid [14–16].

Phospholipids separated by chromatography were scraped from thin-layer plates and radioisotope incorporation was determined by liquid scintillation spectrometry. Incorporation into PA and MPI was quantitated as PA plus MPI by counting an unchromatographed aliquot of the washed neutral pH extract, as no other phospholipids incorporate significant amounts of phosphate during the 5-min challenge period.

RESULTS

Carbamylcholine (CARB) (60 nmoles/g of brain) stimulate both [^{32}P]orthophosphate and [^{3}H]inositol incorporation in TPI, DPI and MPI in whole brain by 35–80 per cent (Table 1). Phosphate incorporation into PA was similarly increased, as previously reported [8]. In brain regions (Table 2) CARB effects on phosphoinositide phosphate incorporation were greater in cerebral cortex and brainstem, where both the PPI and PA/MPI were stimulated, than in cerebellum where only PA/MPI increases were significant. Inositol incorporation was affected significantly in brainstem but not in cerebellum; the per cent changes in cerebellum tended to be elevated but variable with larger than usual standard errors of the mean. Inositol incorporation into phosphoinositides of cerebral cortex was not sufficient during the 5-min challenge to reliably quantitate.

In both brain region and whole brain studies, there was a tendency for CARB stimulations as per cent of control to be less for DPI than for TPI or PA plus MPI, and greater for inositol than for phosphate. On a molar basis more phosphate than inositol radiolabel was incorporated into all inositides.

Atropine sulfate, injected intraperitoneally (160 mg/kg body wt) 15 min before the intracisternal administration of CARB, blocked the effects of CARB on phosphoinositide phosphate incorporation in all three brain regions (Table 3). Atropine alone had no effect on inositide phosphate metabolism.

No differences in the endogenous concentrations of TPI or DPI were seen between control and CARB-treated animals in any of the present studies, and PPI specific activities expressed as radiolabel per mole of lipid exhibited the same patterns as data expressed per g of brain. Typical concentrations for TPI were 480 ± 50 nmoles/g in brainstem, 300 ± 30 nmoles/g in cerebellum and 290 ± 30 nmoles/g in

Table 1. Effects of carbamylcholine on [^{32}P]orthophosphate and [^{3}H]inositol incorporation into phosphoinositides of whole brain*

Drug	Lipid	[^{32}P]orthophosphate % Control	N	P	[^{3}H]inositol % Control	N	P
Saline	TPI	100 ± 4	40		100 ± 10	18	
CARB		140 ± 6	40	0.001	178 ± 11	15	0.001
Saline	DPI	100 ± 9	40		100 ± 10	18	
CARB		136 ± 10	40	0.01	145 ± 11	15	0.005
Saline	MPI	100 ± 10	15		100 ± 10	18	
CARB		185 ± 11	10	0.001	180 ± 11	15	0.005
Saline	PA	100 ± 10	15				
CARB		195 ± 12	10	0.001			

* These data represent three experiments in which animals received [^{32}P]orthophosphate and three experiments in which both [^{32}P]orthophosphate and [^{3}H]inositol were administered. CARB animals also received 60 nmoles carbamylcholine chloride/g of brain, and all animals were killed 5 min after intracisternal administration of drug and/or radioisotope. Data are expressed as the mean ± the standard error of the mean. Animals typically received 25 μCi [^{32}P]orthophosphate and 40 μCi [^{3}H]inositol. Control values for ^{32}P- and ^{3}H-incorporation as μCi/g of brain were: TPI, 35 ± 2 ^{32}P and 4.1 ± 0.4 ^{3}H; DPI, 6.3 ± 0.6 and 1.4 ± 0.1; MPI, 18 ± 2 and 19 ± 2; and PA, 11 ± 1 ^{32}P.

Table 2. Effects of carbamylcholine on [^{32}P]orthophosphate and [^{3}H]inositol incorporation into phosphoinositides of brain regions*

Region	Drug	Lipid	[^{32}P]orthophosphate incorporation			[^{3}H]inositol incorporation		
			% Control	N	P	% Control	N	P
Brainstem	Saline	TPI	100 ± 8	24		100 ± 10	10	
	CARB		145 ± 9	24	0.005	180 ± 14	10	0.001
	Saline	DPI	100 ± 10	25		100 ± 11	10	
	CARB		133 ± 8	24	0.01	165 ± 15	10	0.001
	Saline	PA + MPI	100 ± 9	24		100 ± 10	10	
	CARB		160 ± 11	24	0.001	184 ± 13	10	0.001
Cerebellum	Saline	TPI	100 ± 8	24		100 ± 10	10	
	CARB		123 ± 9	24	NS	140 ± 20	10	NS
	Saline	DPI	100 ± 10	24		100 ± 9	10	
	CARB		116 ± 9	24	NS	130 ± 15	10	NS
	Saline	PA + MPI	100 ± 9	24		100 ± 11	10	
	CARB		135 ± 11	24	0.05	150 ± 30	10	NS
Cerebral cortex	Saline	TPI	100 ± 11	24		ND	10	
	CARB		170 ± 13	24	0.001	ND	10	
	Saline	DPI	100 ± 11	24		ND	10	
	CARB		148 ± 10	24	0.005	ND	10	
	Saline	PA + MPI	100 ± 13	24		ND	10	
	CARB		167 ± 12	24	0.001	ND	10	

* These data represent two experiments in which animals received both [^{32}P]orthophosphate and [^{3}H]inositol simultaneously and two experiments in which animals received [^{32}P]orthophosphate only. Data are expressed as the mean ± standard error of the mean; NS indicates not significantly different from control values, ND indicates not detectable (see Results).

Animals typically received 40 μCi [^{32}P]orthophosphate and 20 μCi [^{3}H]inositol. Control values for ^{32}P- and ^{3}H-incorporation as μCi/g of brain were: (1) in brainstem, TPI, 60 ± 6 ^{32}P and 3.9 ± 0.6 ^{3}H; DPI, 18 ± 2 and 1.8 ± 0.2; MPI, 25 ± 2 ^{3}H; and PA plus MPI, 68 ± 7 ^{32}P; (2) in cerebellum, TPI, 50 ± 5 and 2.1 ± 0.2; DPI, 20 ± 2 and 1.1 ± 0.1; MPI, 50 ± 6 ^{3}H; and PA plus MPI, 69 ± 7 ^{32}P; and (3) in cerebral cortex (phosphorus values only), TPI, 10 ± 1; DPI, 3.1 ± 0.4; and PA plus MPI, 15 ± 2.

Table 3. Effects of carbamylcholine and atropine on [^{32}P]orthophosphate incorporation into phosphoinositides of brain regions*

Brain region	Drug	TPI			DPI			PA + MPI		
		% Control	N	P	% Control	N	P	% Control	N	P
Brainstem	Saline	100 ± 10	11		100 ± 9	11		100 ± 9	11	
	CARB	150 ± 11	10	0.002	131 ± 10	10	0.03	159 ± 12	10	0.001
	Atropine	97 ± 10	10		85 ± 13	10		78 ± 14	10	
	CARB + atropine	103 ± 12	11		111 ± 12	11		90 ± 18	11	
Cerebral cortex	Saline	100 ± 9	11		100 ± 10	11		100 ± 10	11	
	CARB	165 ± 13	10	0.001	146 ± 11	10	0.005	173 ± 13	10	0.001
	Atropine	114 ± 9	10		116 ± 13	10		111 ± 15	10	
	CARB + atropine	112 ± 12	11		121 ± 18	11		119 ± 13	11	
Cerebellum	Saline	100 ± 9	11		100 ± 11	11		100 ± 10	11	
	CARB	130 ± 9	10	0.05	119 ± 13	10		150 ± 16	10	0.02
	Atropine	120 ± 13	10		114 ± 16	10		111 ± 14	10	
	CARB + atropine	110 ± 10	10		109 ± 12	10		110 ± 13	10	

* Atropine sulfate (160 mg/kg) was administered intraperitoneally 15 min before intracisternal injection of carbamylcholine and/or [^{32}P]orthophosphate. These data represent the sum of two separate experiments. Data are expressed as the mean ± the standard error of the mean. Amounts of [^{32}P]orthophosphate administered and incorporated were similar to values for Table 2.

cerebral cortex. DPI concentrations were 120 ± 12 nmoles/g in brainstem, 130 ± 14 nmoles/g in cerebellum and 100 ± 10 nmoles/g in cerebral cortex.

The sum of the incorporation of [^{32}P]orthophosphate into other major brain phospholipids (phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine and sphingomyelin) at 5 min after intracisternal injection amounted to approximately 1 per cent of the radiolabel incorporated into phosphatidic acid and the phosphoinositides, even though the phosphoinositides comprise less than 10 per cent of total brain phospholipids (cf. Ref. 8).

DISCUSSION

The present studies show that CARB, a cholinergic agonist in rat brain [17, 18], stimulated both [^{32}P]orthophosphate and [^{3}H]inositol incorporation into PPI as well as into MPI. These effects were blocked by atropine, a cholinergic antagonist in rat brain [17, 18]. The stimulations were not likely to result from CARB effects on pools of ATP or other phosphoinositide precursors since CARB produces no detectable changes in nucleotide specific activity in this system [8], and both phosphate and inositol incorporations were increased. Therefore, the data indicate that cholinergic neurotransmitter receptor activation is necessary for CARB effects on PPI metabolism. This conclusion is supported by the observed tendency for CARB to exert greater effects on the PPI in cerebral cortex and brainstem than in cerebellum, since this pattern is consistent with densities of cholinoceptive neurons in rat brain [19–21]. Similar findings for CARB and atropine effects on PA and MPI metabolism have been reported in the central nervous system (CNS) *in vivo* [8] and in numerous peripheral neural and secretory tissues *in vitro* [2, 3]. Cholinergic effects on the PPI of rabbit iris muscle have been recently reported [22]; however, the present studies are the first clear demonstration in brain of an association between the PPI and specific neurotransmitter receptor activation.

Regarding the nature of the CARB effect, there were no changes produced in endogenous PPI concentrations when increased isotope incorporations were observed, suggesting an increase in PPI turnover (increased biosynthesis and biodegradation) rather than a net PPI synthesis. Similar findings have been reported for stimulations of PA and MPI *in vivo* [8, 23] and *in vitro* [23–27]. The effects of cholinergic agents on rabbit iris muscle PPI metabolism differed in that a net reduction in TPI was observed [22]. The difference could possibly be due to a compromised anabolic metabolism *in vitro* in this preparation.

Earlier studies of phosphoinositide metabolism *in vivo* failed to detect any PPI response to CARB [8], and studies of PPI have produced conflicting reports of increases, decreases and no changes in metabolism *in vitro* in response to cholinergic agonists [28–32]. PPI studies in certain systems *in vitro* are complicated by altered ATP metabolism (a direct PPI precursor), and by the fact that some anabolic enzymes are particulate bound while others are soluble. These problems are illustrated by the observation of Hawthorne and Kai [7] that in brain homogenates the rate of PPI degradation is 100 times the rate of synthesis. PPI studies *in vivo* must use a rapid tissue fixation technique to prevent post-mortem PPI degradation [10, 33, 34] and must examine drug effects on PPI metabolism before the PPI equilibrate with administered radioisotopes, which in rat brain is approximately 15 min, for intracisternally administered orthophosphate [35]. Therefore, the present studies were conducted *in vivo*, PPI metabolism was examined 5 min after label administration and microwave irradiation fixation techniques were used to prevent post-mortem loss [36–39]. The present success in demonstrating PPI stimulation by administered drugs is attributed to this combination of techniques. A similar approach was used by Schacht and Agranoff [40] to demonstrate that PPI incorporation of phosphate was increased during pentylenetetrazol-induced convulsions in goldfish brain *in vivo*.

Several investigators have proposed different roles for PPI than for MPI in excitable tissues, due in part to the paucity of studies relating PPI to cell functions [3, 7]. In the present studies the response of the PPI and MPI to CARB and atropine were very similar, and since the PPI and MPI are directly related metabolically, the question arises as to whether the PPI response to CARB and those of MPI are part of the same phenomena. This possibility is supported by the data showing that CARB stimulated inositol incorporation into the PPI. Thus, an association between at least part of the PPI and MPI stimulations by CARB is indicated in brain. There are numerous other neural and secretory tissues which exhibit increased MPI and PA metabolism in response to neurotransmitter agonists and secretogogues, but in which the PPI have not been examined systematically. Evidence associating the PPI with such cellular functions would require the inclusion of their unique physicochemical properties, e.g. changing calcium affinities and solubility partitions, in proposed mechanisms for phosphoinositide involvement in neural and secretory processes.

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