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BIOCHEMICAL EFFECTS OF PHOSPHATIDYLCHOLINE TREATMENT IN RATS

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Abstract—We measured several biochemical effects of 10 days of intragastric administration of phosphatidylcholine (10 mmoles/kg) to rats because of the expanding clinical use of chronic phosphatidylcholine treatment for disorders involving impaired cholinergic neurotransmission. The plasma and erythrocyte choline concentrations were increased 3.5-fold, which was the same percent increase as found after an acute treatment with phosphatidylcholine. The lipid and fatty acid compositions of the plasma were also altered; free and total cholesterol levels increased, triglycerides increased, the monoene fatty acids generally decreased, and the diene and tetraene fatty acids generally increased. We found no effect of this treatment on the hepatic microsomal cytochrome P-450 activity or on the N-demethylation of benzphetamine or methamphetamine. Ten days of phosphatidylcholine treatment increased the concentration of choline in the brain but had no effect on the concentration of acetylcholine, the activity of choline acetyltransferase, cholinesterase activity, the apparent K_D or B_{max} of muscarinic receptors, or the fatty acid composition of rat brain lipids. These findings indicate that the largest effect caused by this treatment was an increase in the choline levels. No indication of altered cholinergic metabolism was observed. Further studies of the effects of chronic phosphatidylcholine treatment are required to clarify its therapeutic mechanism of action.

The clinical use of phosphatidylcholine (PCh‖; also referred to as lecithin) in patients with disorders involving impaired CNS cholinergic neurotransmission has grown rapidly in the last few years. Several recent reports have reviewed the clinical use and efficacy of PCh [1–3]. Despite this increasing use of PCh, its biochemical effects have not been well documented. PCh treatment stems mainly from work in Wurtman's and Haubrich's laboratories, in which it was reported that high doses of choline (Ch) or PCh increase the blood Ch level and the concentrations of Ch and acetylcholine (ACh) in the brain (see reviews in Refs. 4 and 5). These investigators concluded that Ch treatment increased the functional activity of cholinergic neurons. In addition, Ch itself is a weak muscarinic agonist, and some of its effects may derive from a direct action of Ch on the receptor [6–10]. We recently reported that PCh treatment supplemented ACh synthesis *in vivo* under conditions of stimulated ACh release and concluded that this effect was due to increased availability of Ch in the brain [11]. Other investigators have also concluded that Ch loading is most effective under conditions of stimulated ACh release [12, 13].

There is little information concerning the biochemical effects of PCh treatment. Most of what has been reported concerns the effects of acute treatments on Ch and ACh concentrations [11, 14, 15]. However, the length of treatment is important in determining the effects of drugs for many reasons, such as cumulative effects of the drug and adaptive mechanisms of the organism. Adaptation may occur within a few days of drug treatment or may take weeks or even months to occur. A single dose of PCh results in increased plasma Ch levels for over 12 hr and increased brain Ch levels for up to 24 hr, depending on the region examined [11]. Prolonged treatment with anticholinesterases, which increase the endogenous ACh concentration [16, 17], or oxotremorine [18], a muscarinic agonist, have been reported to result in downregulation of muscarinic receptors. We have, therefore, examined the effects of large doses of PCh, administered chronically, on the muscarinic receptors of rat brain. It has been reported previously that supplemental dietary Ch increases the nicotinic receptors in brain [19]. We have also measured the endogenous Ch in tissue suspensions to determine whether or not Ch is present at levels high enough to bias the estimated affinity constants of the muscarinic receptors if its presence is ignored. Previous investigations of this subject have concentrated on the effects of PCh treatment on Ch and ACh levels. However, other studies have indicated that lipid treatments have a wide variety of effects, including alterations of lipid content, enzyme activities and hepatic drug metabolism [20–24]. Therefore, we have measured a num-

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‖ Abbreviations: PCh, phosphatidylcholine; ACh acetylcholine; Ch, choline; ChAT, choline acetyltransferase; AChE, acetylcholinesterase; and QNB, 3-quinuclidinyl benzilate.

ber of biochemical parameters as well as the concentrations of ACh and Ch to obtain an overview of the biochemical effects of PCh administration to rats over a 10-day period.

METHODS AND MATERIALS

Male, Sprague–Dawley rats (180–200 g) were given PCh (10 mmoles/kg), suspended in 0.45 M NaCl, by intragastric intubation. Control rats were treated with an equal volume of 0.45 M NaCl. For measurement of Ch and ACh in brain regions, the rats were killed by microwave irradiation of the head (5 kW; Gerling-Laboratories, Modesto, CA). Brain regions were dissected, weighed, and immediately homogenized in 5 vol. of cold 15% 1 N formic acid mixed in acetone (v/v) containing [$^2H_9$]Ch and [$^2H_9$]ACh as internal standards. The extraction procedure utilized ion pair extraction with dipicrylamine as described previosly [25]. The concentrations of ACh and Ch were determined by gas chromatography–mass spectrometry (GC–MS) as described by Jenden *et al.* [26].

For other measurements the rats were decapitated and blood was collected from the carcass in heparinized tubes. The blood was placed on ice and centrifuged at 2° for 10 min at 3000 *g*. For measuring Ch-containing lipids, plasma samples were homogenized in 20 vol. of cold $CHCl_3$–CH_3OH (2/1, v/v) containing [$^2H_9$]PCh (labeled in the Ch moiety) as internal standard [27]. Free Ch was liberated from the entire lipid extract by incubation for 1 hr at 100° in 1 N KOH. Quantitative liberation was confirmed by [$^2H_9$]PCh and [$^2H_9$]lysoPCh hydrolysis with [$^2H_4$]Ch added as an internal standard. Ch was extracted and quantitated by GCMS.

For identification of lipids and fatty acids, lipids were extracted by the method of Folch *et al.* [28]. Fractionation by a combination of thin-layer and column chromatography, followed by fatty acid analysis of different lipid components by gas–liquid chromatography, was carried out as described by Menon and Dhopeshwarkar [29].

L(-)[^{3}H]QNB binding was determined by the method of Yamamura and Snyder [30]. Rats were decapitated 6 hr after the last PCh treatment. Striatum, hippocampus and cerebral cortex were rapidly dissected in cold sucrose and gently homogenized in 20 vol. of ice-cold 0.32 M sucrose. This suspension was centrifuged at 2° at 3000 *g* for 10 min. The resulting pellet was discarded, and the supernatant fraction was centrifuged at 2° at 10,000 *g* for 20 min. The resulting crude synaptosomal pellet was suspended in 50 mM phosphate buffer, pH 7.4, and stored frozen at −20° for up to 1 week. Samples (1 ml) were incubated for 60 min at 37° followed by filtration on Whatman GF/B filters and four 5-ml washes with 50 mM phosphate buffer. The filters were placed in scintillation vials containing scintillation fluid (3a70B; RPI) and counted with 39% efficiency. Nonspecific binding was measured in parallel samples containing 1 μM atropine. Protein was determined by the method of Lowry *et al.* [31].

Choline acetyltransferase (ChAT) activity was determined by the method of Fonnum [32]. The activities of plasma cholinesterase (ChE) and erythrocyte and brain acetylcholinesterase (AChE) were measured by the method of Garry and Routh [33].

Washed hepatic microsomes were prepared by ultracentrifugation as described by Cho *et al.* [34]. Microsomal P_{-450} activity was determined by the method of Omura and Sato [35] using 91 $mM^{-1}\,cm^{-1}$ as the extinction coefficient. The rates of N-demethylation of benzphetamine and methamphetamine were determined by measuring the production of formaldehyde as described by Nash [36].

Comparison between two groups were evaluated by Student's *t*-test. An analysis of variance was used to analyze the differences between three groups. When a result was significant at the level $P \leq 0.05$, the individual contrasts were evaluated using the method described by Scheffe [37].

Drugs were from the following sources: paraoxon (ICN Pharmaceuticals, Irvine, CA); and atropine sulfate (J. T. Baker Chemicals, Phillipsburg, NJ). PCh extracted from soybeans was the gift of the Unilever Research Co. (Vlaardingen, Netherlands). This product was stated by the supplier as being at least 80% pure, with the major contaminant being lysophosphatidylcholine. Our own analysis confirmed the presence of 80% PCh and 15% lysophosphatidylcholine.

RESULTS

Since we have reported previously [11] that rat plasma and erythrocytes attain peak levels of Ch 6 hr after a single oral administration of PCh, all rats were killed 6 hr after the last dose of PCh. To determine if 10 days of oral PCh treatment (10 mmoles/kg) would alter hepatic drug metabolism, we measured the activity of the cytochrome P_{-450} system and the rates of N-demethylation of a secondary and a tertiary analog of amphetamine. These rates were the same in liver microsomes prepared from control and chronically treated rats (Table 1).

Plasma and erythrocyte Ch levels rose to about the same extent (300–350% of control) in rats 6 hr after acute or chronic treatment with PCh (Table 2). The increase was highly significant by analysis of variance ($F_{2,22} = 53.0$ and 88.4 for plasma and erythrocytes respectively; $P < 0.001$ in both cases). Scheffe's test [37] showed that, for both, the choline levels after acute and chronic PCh treatment were significantly higher than control ($P < 0.001$ in all cases), while there was no significant difference between acute and chronic treatments. The activity of ChE in plasma was slightly higher in chronically treated rats than in controls ($P < 0.05$), but there was no difference in the activity of erythrocyte AChE.

Chronic PCh treatment altered the lipid composition of the plasma: there were increases in the concentrations of Ch-containing lipids, total lipid and free and total cholesterol (Table 3). The fatty acid composition of the plasma lipids was also altered by chronic PCh treatment (Table 4). In general, there was a tendency for monoenes to decrease and for dienes and tetraenes to increase, but none of the differences were significant.

The concentrations of the Ch in the striatum cortex and hippocampus were increased by both acute and chronic PCh treatments (Table 5). Differences

Table 1. Effect of oral PCh treatment on liver microsomal cytochrome P-450 activity and N-demethylation of benzphetamine and methamphetamine*

	Cytochrome P-450 (nmoles/g liver)	N-Demethylation (nmoles H_2CO/min/nmole cytochrome P-450)	
		Benzphetamine	Methamphetamine
Experiment 1			
Control	14 ± 1	6.1 ± 0.3	2.6 ± 0.2
PCh	15 ± 2	5.5 ± 0.6	2.6 ± 0.2
Experiment 2			
Control	18 ± 2	4.9 ± 0.4	2.6 ± 0.2
PCh	18 ± 2	5.2 ± 0.4	2.9 ± 0.3

* Values are means ± S.E.M. of triplicate determinations. PCh (10 nmoles/kg) was given orally for 10 days.

Table 2. Blood choline levels and cholinesterase activities 6 hr after acute or chronic (10 days) oral PCh (10 mmoles/kg) treatment*

	Plasma Ch (μM)	Erythrocyte Ch (μM)	Plasma Ch-containing lipids (μM)	Plasma ChE (μmoles/min/ml)	(μmoles/min/ml)
Control (N = 9)	11.4 ± 1.1	11.5 ± 1.2	829 ± 45	0.81 ± 0.05	5.6 ± 0.9
Acute (N = 11)	34.3 ± 2.0†	39.4 ± 1.8†	ND‡	ND	ND
Chronic (N = 5)	36.7 ± 2.7†	40.0 ± 2.3†	1069 ± 65§	1.10 ± 0.12‖	4.7 ± 0.9

* Values are means ± S.E.M.
† $P < 0.001$.
‡ Not determined.
§ $P < 0.01$.
‖ $P < 0.05$.

Table 3. Rat plasma lipid levels in controls and after 10 days of oral PCh (10 mmoles/kg)*

	Total lipid (mg/ml)	Free cholesterol (mg/100 ml)	Total cholesterol (mg/100 ml)	Triglyceride (mg/100 ml)
Control (N = 4)	3.2 ± 0.4	3.3 ± 1.1	35.4 ± 2.9	70.7 ± 28.8
PCh (N = 5)	4.2 ± 0.2†	7.6 ± 1.8†	49.6 ± 4.6†	104.6 ± 17.6

* Concentrations were determined as described in Methods and Materials. Values are means ± S.E.M.
† $P < 0.05$.

Table 4. Fatty acid composition of plasma lipids in controls and after 10 days of oral PCh (10 mmoles/kg)*

Fatty acids	Control (N = 4)			PCh (N = 5)		
	PL	TG	CE	PL	TG	CE
Saturates	55.9 ± 1.6	30.8 ± 1.4	23.8 ± 5.5	49.6 ± 3.6	27.7 ± 3.0	20.4 ± 2.2
Monoenes	14.5 ± 1.5	38.9 ± 3.4	27.4 ± 6.3	12.5 ± 2.3	27.7 ± 3.5	18.9 ± 5.7
Dienes	20.8 ± 2.3	25.5 ± 4.0	32.4 ± 1.2	27.7 ± 3.0	39.9 ± 4.8	34.6 ± 3.6
Tetraenes	2.8 ± 1.3		14.3 ± 9.2	4.4 ± 2.8		21.2 ± 8.7

* Abbreviations: PL, phospholipids; TG, triglycerides; and CE, ceramide. Units = percent of total fatty acids. Values are means ± S.E.M.

Table 5. Rat brain region choline and acetylcholine concentrations after acute or chronic (10 days) oral PCh (10 mmoles/kg) treatment*

	Choline (nmoles/g)			Acetylcholine (nmoles/g)		
	Striatum	Hippocampus	Cortex	Striatum	Hippocampus	Cortex
Control (N = 11)	29.9 ± 3.5	30.0 ± 2.9	20.1 ± 1.6	99.5 ± 3.1	30.4 ± 0.8	19.7 ± 0.6
Acute (N = 8)	37.0 ± 1.9	38.2 ± 4.6	31.1 ± 2.7	96.9 ± 4.1	32.1 ± 1.6	20.8 ± 1.2
Chronic (N = 4)	50.3 ± 6.7	41.5 ± 3.3	28.4 ± 1.0	89.5 ± 9.2	31.5 ± 0.7	18.5 ± 0.4

* Values are means ± S.E.M. Rats were killed by microwave irradiation 6 hr after the last PCh treatment.

Table 6. Rat brain region ChAT and AChE activities in controls and after 10 days of PCh (10 mmoles/kg) treatment*

	ChAT (nmoles/mg protein/hr)			AChE (μmoles/mg protein/hr)		
	Cortex	Hippocampus	Striatum	Cortex	Hippocampus	Striatum
Control (N = 5)	32.8 ± 1.5	45.8 ± 2.0	121.4 ± 12.1	1.51 ± 0.10	1.69 ± 0.39	7.09 ± 0.73
PCh (N = 5)	34.9 ± 1.4	48.3 ± 2.1	122.5 ± 12.1	1.27 ± 0.28	1.49 ± 0.25	6.53 ± 0.27

* Values are means ± S.E.M.

Table 7. Fatty acid composition of rat brain lipids in controls and after 10 days of oral PCh (10 mmoles/kg)*

Fatty acids	PCh (N = 5)				Control (N = 4)			
	TL	PCh	PS	PE	TL	PCh	PS	PE
Saturates	51.7 ± 2.3	66.7 ± 4.4	70.3 ± 5.0	42.0 ± 2.6	51.5 ± 1.4	65.6 ± 4.9	66.1 ± 9.8	40.5 ± 5.1
Monoenes	23.2 ± 0.09	27.6 ± 2.2	15.2 ± 1.8	16.9 ± 1.2	23.3 ± 1.3	27.4 ± 2.9	15.0 ± 2.8	16.3 ± 1.3
Dienes	0.8 ± 0.1	0.9 ± 0.2	Tr	0.3 ± 0.2	1.0 ± 0.2	1.1 ± 0.2	Tr	0.3 ± 0.04
Trienes	0.4 ± 0.04	0.01 ± 0.05	0.5 ± 0.05	0.4 ± 0.08	0.4 ± 0.03	Tr	Tr	0.4 ± 0.04
Tetraenes	12.5 ± 1.5	3.2 ± 1.1	8.0 ± 2.0	13.9 ± 1.0	12.6 ± 0.9	4.1 ± 1.4	8.9 ± 3.0	14.4 ± 2.6
Pentaenes			0.2 ± 0.2	0.3 ± 0.3			0.7 ± 0.6	0.3 ± 0.3
Hexaenes	8.0 ± 1.5	0.3 ± 0.3	4.8 ± 2.4	6.5 ± 1.7	7.9 ± 1.2	0.7 ± 0.6	7.4 ± 4.8	8.4 ± 3.2
Unidentified†	3.4 ± 0.3	0.5 ± 0.2	Tr	18.6 ± 2.4	2.9 ± 1.2	0.6 ± 0.07	0.2 ± 0.05	18.5 ± 3.3

* Abbreviations: TL, triglycerides; PCh, phosphatidylcholine; PS, phosphatidylserine; and PE, phosphatidyletheanolamine. Units = percent of total fatty acids. Values are means ± S.E.M.

† Possibly vinyl ethers.

Table 8. Effects of treatment with phosphatidylcholine for 10 days on muscarinic receptors*

	K_D (pM)	B_{max} (pmoles/mg protein)	Protein (μg/assay)
Striatum			
Control	21.1 ± 1.8	1.69 ± 0.11	36 ± 4
PCh	18.8 ± 1.4	1.85 ± 0.17	25 ± 4
Hippocampus			
Control	17.0 ± 1.8	1.12 ± 0.06	47 ± 7
PCh	16.7 ± 0.7	1.11 ± 0.01	48 ± 3
Cortex			
Control	25.4 ± 3.4	0.90 ± 0.13	106 ± 18
PCh	27.2 ± 3.3	0.94 ± 0.07	103 ± 19

* Rats were treated with phosphatidylcholine (10 mmoles/kg; i.g.) for 10 days. Six hours after the last treatment, the rats were decapitated and the striatum, hippocampus and cerebral cortex were dissected. Synaptosomes were prepared from the three brain regions, and L(-)[^{3}H]QNB binding was measured as described in Methods and Materials. Values are means ± S.E.M. of five treated and four control (treated daily with an equal volume of NaCl) samples.

among control, acute and chronic treatment groups were significant in all areas (striatum: $F_{2,20} = 18.03$, $P < 0.001$: hippocampus: $F_{2,20} = 4.69$, $P < 0.05$; cortex: $F_{2,20} = 17.24$, $P < 0.001$). Evaluating individual contrasts by Scheffe's test, the effect of chronic treatment was significant compared to control only in the striatum ($P = 0.01$), while the effect of acute treatment was significant only in cortex. In no region did the effects of acute and chronic treatments differ significantly. There were no changes in the ACh concentrations in these three regions in either acutely or chronically treated rats. Chronic PCh treatment had no effect on the activities of AChE or ChAT in the striatum, cortex or hippocampus (Table 6). There were also no significant effects of chronic PCh treatment on the fatty acid composition of four lipids in rat brain (Table 7).

There were no significant differences in either the apparent affinity (K_D) or the maximum number of binding sites (B_{max}) of muscarinic receptors in the striatum, hippocampus or cortex from rats treated 4 days (data not shown) or 10 days (Table 8) with PCh (10 mmoles/kg). The concentration of Ch was measured in samples run in parallel to the binding experiments. The Ch concentrations in the lysed synaptosomal suspensions ranged from 0.4 to 2.7 μM (Table 9). There were no significant differences between the Ch concentrations of samples prepared from control or PCh-treated rats.

DISCUSSION

Treatment of rats for 10 days with very high doses of PCh had surprisingly little effect on the biochemical parameters that we have investigated. In agreement with our previous conclusions, the results of this investigation are consistent with the conclusion that PCh is hydrolyzed within the GI tract, resulting in large increases in the concentration of Ch in the plasma but only a small increase in the plasma lipid concentration. The largest change in the plasma, other than Ch, was the increase in the free cholesterol concentration. This observation warrants study in human subjects undergoing long-term PCh therapy because of the role of cholesterol in disorders such as atherosclerosis and its effect on membrane associated processes, including membrane fluidity [38, 39]. Such changes may be especially important in the aged, the group receiving the most attention as recipients of PCh for the treatment of Alzheimer's Disease. The alterations of plasma fatty acids also warrant attention since many investigators have reported metabolic effects of alterations of the plasma fatty acid composition [40]. In this regard, Melancon *et al.* [41] have suggested that the fatty acids in PCh may account for the therapeutic effects of lecithin in Friedreich's ataxia.

Table 9. Choline concentrations in synaptosomal suspensions used in muscarinic binding experiments*

Synaptosome source	Choline concentration	
	μM	nmoles/mg protein
Hippocampus		
Control	0.67 ± 0.19	13.3 ± 2.0
PCh	0.61 ± 0.11	12.4 ± 1.7
Striatum		
Control	0.55 ± 0.09	16.5 ± 1.8
PCh	0.57 ± 0.14	22.6 ± 2.8
Cortex		
Control	2.02 ± 0.51	21.4 ± 1.0
PCh	2.06 ± 0.27	25.8 ± 2.0

* Tissue was prepared as described in the legend to Table 8. Choline was measured by GC–MS as described in Methods and Materials. Values are means ± S.E.M. of five treated and four control samples.

Our observations that this treatment does not appear to alter drug-metabolizing systems in the liver are encouraging. However, since PCh is often administered in conjugation with other drugs, further studies should be directed towards studying the effect of PCh administration on the pharmacokinetics and metabolism of other drugs. The increase in plasma ChE activity after chronic PCh treatment is also noteworthy. Since the factors regulating plasma ChE are not known, it is difficult to determine the mechanism underlying this effect.

We found no effect of PCh administration on the fatty acids in the brain. The major effect of PCh treatment that we observed in the brain was an increase in the concentration of Ch. This result is similar to our findings after acute treatment with PCh [11] in which the concentration of Ch was increased but the concentration of ACh was unaffected unless the system was stressed by drugs that increase the release of ACh. It is clear that even a long-term increase in the brain Ch concentration does not increase the concentration of ACh under normal conditions. The effects of chronic PCh treatment on the ACh-depleting effects of drugs that stimulate ACh turnover, such as atropine, remain to be examined.

Pharmacological treatments which chronically increase agonist binding to muscarinic receptors have been reported to lead to downregulation of muscarinic receptors [16–18]. Ch has been reported to act both as a weak muscarinic agonist [6–10] and as a precursor of ACh leading to increased levels of ACh [4, 5]. Either of these actions may increase activity at muscarinic receptors and could cause the development of subsensitivity. PCh treatment results in increased concentrations of Ch in the plasma and in brain regions for 12–24 hr [11]. However, there was no effect of 4 or 10 days of PCh treatment on the apparent affinity or maximum number of L(-)[^{3}H] QNB binding sites in any of the three brain regions examined. Apparently, there was less agonist receptor activation following PCh treatment than following infusion with oxotremorine or chronic treatment with anticholinesterase drugs. This result is consistent with studies indicating that Ch itself is only a weak muscarinic agonist [9, 10]. These results also indicate that, if ACh release is enhanced during PCh treatment, the synaptic ACh concentration is not increased to the same extent as after anticholinesterase treatment. From previous reports it seems most likely that Ch loading treatment has its greatest effects on stressed cholinergic neurons and only a modest effect under normal conditions [11]. Therefore, under the conditions of the present ex-

periments we would perhaps not expect to see receptor changes. The immunity of muscarinic receptors to downregulation during Ch loading may be critical for the subtle effects which are observed in certain diseases.

We measured the concentration of Ch in the samples used for L(-)[^{3}H]QNB binding for two reasons: (1) treatment with PCh may have altered the Ch concentration in these samples to a great enough extent to alter the measured binding isotherm, and (2) the Ch concentration in these suspensions has never been reported so it was not known whether or not endogenous Ch may interfere with measurements of Ch or QNB binding. First, we found that there was no difference in the Ch concentrations in the dilute tissue suspensions prepared from control or PCh-treated rats that were used in the binding studies. Second, we found only micromolar quantities of Ch present in the incubation medium. This is probably too low to have a significant effect on binding experiments unless there is a subpopulation of receptors with a high affinity for Ch.

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