ACETYLCHOLINE STIMULATES HYDROLYSIS OF ^{32}P-LABELED PHOSPHATIDIC ACID IN GUINEA PIG SYNAPTOSOMES

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Summary: [^{3}H]-inositol or [^{3}H]-arachidonate was injected intracerebrally into guinea pigs. Labeled nerve endings were incubated with ACh* or CCh, both of which stimulate labeling of PhA and PhI from $^{32}P_i$ by > 100% and 70% respectively. Their addition did not affect the in vivo labeled phosphatidyl-[^{3}H]-inositol or [^{3}H]-arachidonyl-diglyceride and -PhI. Enhanced hydrolysis of [^{3}H]-inositol-PhIP and -$PhIP_2$ in the presence of ACh, CCh or choline was not reversed by atropine. In a two-step experiment, PhA was labeled with $^{32}P_i$, and DNP was added to block further γ-[^{32}P]-ATP formation. Addition of ACh stimulated an atropine-sensitive decrease in [^{32}P]-PhA.

Increased incorporation of $^{32}P_i$ into phospholipids in response to various stimuli has been reported in a number of tissues in vivo as well as in vitro (1,2). The ACh-stimulated turnover of PhA and PhI in brain tissue and in ganglia has been implicated in synaptic transmission (3). Extending previous studies on phospholipid labeling in guinea pig synaptosomes (4) we have examined levels of labeled diglyceride, PhI, PhIP, $PhIP_2$ and PhA of synaptosomal incubations in the presence and absence of cholinergic agents.

MATERIALS AND METHODS

Nerve endings ($NE_{1.1}$) were prepared essentially as described previously (4). A crude nerve ending-mitochondrial (NEM) pellet (800-13,000xg) from a 10% homogenate of guinea pig cerebral cortex, suspended in 0.32 M sucrose was layered over 3 discontinuous density gradient tubes of an SW25 rotor each containing 10 ml of 0.8 M sucrose over 10 ml of 1.1 M sucrose and centrifuged for 150 min at 62,000xg. The band over 1.1 M sucrose was harvested and concentrated by high speed centrifugation.

*Abbreviations: ACh = acetylcholine; CCh = carbamylcholine; DNP = 2,4-dinitrophenol; PhA = phosphatidic acid (1,2-diacyl-sn-glycero-3-phosphate); PhI = phosphatidylinositol; PhIP, $PhIP_2$ = phosphatidylinositol phosphate, -diphosphate; IP, IP_2, IP_3 = inositol mono-, di-, triphosphate.

Incubations were performed at 37° in air with shaking in the following medium (final concentrations): 100 mM sodium glycylglycinate (pH 6.6), 160 mM sucrose, 0.8 mM $MgCl_2$, 1 mM Na-pyruvate, 1 mM Na-fumarate, 1 mM cytidine, 1 mM myo-inositol and 0.1 mM NaH_2PO_4 all in 0.5 ml. Lipids were extracted with an acidified solvent and separated (5) by thin layer chromatography (TLC). ^{32}P-Lipids were located by radioautography and [^{3}H]-lipids were located from the radioautographically established R_f's. Neutral lipids were recovered after removal of phospholipid with activated silica. Diglyceride was separated by TLC after addition of carrier (6) and visualized by I_2-vapors. Radioactive material was scraped and counted by liquid scintillation spectrometry. High voltage electrophoresis of inositol phosphates was carried out with phytic acid hydrolysate as standard (7). Protein was determined spectrophotometrically (8).

^{32}P (carrier free H_3PO_4 in 0.2 N HCl), myo-inositol-2-[^{3}H] (3.09 Ci/mMole) and [^{3}H]-arachidonic acid (4.58 Ci/mMole) were purchased from New England Nuclear (Boston, Mass.), eserine, ACh, CCh from Sigma (St. Louis, Mo.), choline chloride from Merck & Co. (Rahway, N.J.) and atropine from Pierce Chemical (Rockford, Ill.).

RESULTS

Table I shows a comparison of the effects of two cholinergic agents, ACh and CCh, on the labeling of PhA and PhI from $^{32}P_i$. 10^{-4} M ACh and 10^{-3} M CCh stimulate incorporation of radioactivity into both lipids to the same extent, and their effects are not additive. Atropine blocks the CCh-induced stimulation as is seen with ACh-induced stimulation (4). In contrast, the decrease in labeling of PhIP and $PhIP_2$ is not reversed by atropine. Although less effective on a molar basis, carbamylcholine is not easily hydrolyzed and it therefore does not require the addition of an ACh-esterase blocker.

To measure levels of diglycerides, guinea pigs were injected with [^{3}H]-arachidonate intracerebrally. After 16 hr, nerve endings were prepared and

Table I

Effects of Acetylcholine and Carbamylcholine on Labeling of PhA and PhI

	dpm ^{32}P in lipid			
	PhA	PhI	PhIP	$PhIP_2$
Control	3,330	1,350	4,710	10,030
10^{-4} M ACh*	7,330	2,310	4,020	7,890
10^{-3} M CCh	7,250	2,390	3,980	8,230
10^{-4} M ACh* + 10^{-3} M CCh	6,430	2,210	3,500	7,080
10^{-5} M atropine	3,280	1,410	4,640	10,210
10^{-3} M CCh + 10^{-5} M atropine	3,090	1,280	4,060	7,280

*Eserine sulfate (10^{-4} M) was also present in incubations with ACh.

0.9 mg protein of fraction $NE_{1.1}$ were incubated for 30 min with 25 μC $^{32}P_i$ and lipids separated as described in "Methods". Values in this and subsequent tables are averages of duplicate determinations with variability < ±5%, unless otherwise stated.

incubated as usual. Although PhI constitutes only about 4% of the synaptosomal phospholipids, it contains about 17% of rat brain phosphoglyceride arachidonate (9-11) and in these experiments it accounted for 36% of the labeled lipid. 10^{-3} M CCh had no significant effect on the level of radioactivity of PhI and in the diglyceride pool (Table II). Addition of DNP (10^{-3} M) and prolonged incubation (60 min) did not affect this result.

A direct measure of breakdown of the phosphoinositides was attempted by injecting [^{3}H]-myo-inositol and measuring the labeled lipids after incubation of nerve endings in the presence and absence of added ACh or CCh. Experiments were performed under a variety of conditions: the in vivo pulse was 1 hr, 16 hr and 64 hr; the in vitro incubation time ranged from 5 min to 4 hr; DNP (10^{-3} M) was added to hinder incorporation of labeled inositol in vitro; the concentration of ACh was varied from 10^{-8} M to 10^{-3} M in the presence and absence of 1 mM $CaCl_2$. All of these results resembled those reported in Table III. While there appeared to be degradation of PhI during incubation, this was not stimulated by ACh and there was no enhancement of inositol monophos-

Table II

Effect of Carbamylcholine on Levels of Diglyceride and PhI

Addition	dpm [^{3}H] diglyceride	PhI
[0 - time]	433 ± 100	1,682
None	500 ± 54	1,557
10^{-3} M CCh	489 ± 51	1,577
10^{-3} M CCh + 10^{-4} M atropine	503 ± 29	-

Guinea pigs received 160 µC of [^{3}H]-arachidonic acid intracerebrally 16 hr prior to decapitation. 1.1 mg protein of fraction $NE_{1.1}$ were incubated for 20 min and diglyceride isolated as described in "Methods". Numbers for diglyceride are averages ± s.d. of six values from three experiments. Values for PhI are averages of duplicates from one experiment.

phate release. In contrast, hydrolysis of PhIP and $PhIP_2$ was increased by ACh. This stimulation however was not blocked by atropine. Release of inositol diphosphate was also observed, indicating some diesteratic cleavage of PhIP (Table III) but there was no measurable effect of ACh. Radioactivity in inositol triphosphate was too low to obtain significant data. Further evidence for the lack of specificity of the CCh-action on PhIP and $PhIP_2$ is the result that choline chloride exerts a similar effect on their labeling from $^{32}P_i$ as well as on their levels after in vivo labeling with [^{3}H]-inositol (Table IV).

To observe the possible role of phosphohydrolases in PhA labeling, oxidative phosphorylation was terminated after 60 min of in vitro incubation with $^{32}P_i$ by the addition of $2x10^{-3}$ M DNP. After 10 min, ACh was added and the samples were incubated for an additional 20 min. While no significant changes in ^{32}P-content of PhI could be detected, label in PhA decreased, and this decrease was augmented by the presence of 10^{-4} M ACh (Table V). 10^{-5} M Atropine abolished the stimulatory action of ACh on hydrolysis of the [^{32}P]-PhA

DISCUSSION

While the physiological significance of cholinergic effects on lipid

Table III

Effect of Acetylcholine *in vitro* on Phosphoinositides and Inositol Phosphates Labeled *in vivo* with [^{3}H]-*myo*-Inositol

Additions	dpm [^{3}H]				
	PhI	PhIP	$PhIP_2$	IP	IP_2
[0 - time]	47,220 ±2,010	4,700 ± 220	2,050 ± 140	67 ± 5	33 ± 2
Control	45,480 ±1,440	1,720 ± 170	1,070 ± 60	189 ±13	64 ±23
10^{-4} M ACh	44,980 ±5,520	1,500 ± 160	720 ± 20	207 ±11	55 ±14
10^{-4} M ACh + 10^{-4} M atropine	44,910 ±1,240	1,260 ± 150	700 ± 90	232 ±10	48 ±20

One guinea pig was injected intracerebrally with 300 µC [^{3}H]-*myo*-inositol 16 hr prior to decapitation.

1.3 mg protein of fraction $NE_{1.1}$ were incubated for 15 min. Numbers are averages ± s.d. of triplicate incubations. Numbers for lipids represent the total fraction, numbers for inositol phosphates are dpm of a 40 µl aliquot of a 500 µl incubation. Dpm in IP_3 were not sufficiently high above background to give reliable values.

Table IV

Effects of Carbamylcholine and Choline on PhIP and $PhIP_2$

Additions	Exp I: cpm [^{32}P]			Exp II: dpm [^{3}H]		
	PhA + PhI	PhIP	$PhIP_2$	PhI	PhIP	$PhIP_2$
[0 - time]	-	-	-	2,245	409	289
None	7,720	5,430	11,430	2,370	328	95
10^{-3} M CCh	13,190	3,550	8,110	2,309	226	61
10^{-3} M Choline chloride	7,220	3,790	8,700	2,208	158	72
10^{-3} M Choline + 10^{-4} M Atropine	6,630	3,440	8,080	-	-	-

Exp I: 1.3 mg protein of fraction $NE_{1.1}$ were incubated for 30 min with 35 µC $^{32}P_i$ as described in "Methods".

Exp II: One guinea pig was injected intracerebrally with 75 µC [^{3}H]-*myo*-inositol 16 hr prior to decapitation. 0.6 mg protein of fraction $NE_{1.1}$ were incubated for 15 min.

Table V

Effect of Acetylcholine on Hydrolysis of [^{32}P]-PhA

Additions	PhA hydrolyzed (dpm) Exp I	Exp II
None	497	775
10^{-4} M ACh	840	1,246
10^{-4} M ACh + 10^{-5} M atropine	528	546

Fraction $NE_{1.1}$ was incubated with $^{32}P_i$ as described in "Methods". (Exp I: 1 mg protein and 25 μC. Exp II: 0.9 mg protein and 35 μC). After 60 min $2x10^{-3}$ M 2,4-dinitrophenol (final concentration) was added to all samples. At 70 min the above additions were made and hydrolysis of PhA was measured from 70-90 min.

labeling appears to be well-accepted, the biochemical mechanism remains unclear. In guinea pig synaptosomes, the synthetic reaction sequence leading to the labeled PhA and PhI after conversion of $^{32}P_i$ to γ-[^{32}P]-ATP by intrasynaptosomal mitochondria (12,13) is: (I) 1,2-diglyceride + ATP → PhA + ADP; (II) PhA + CTP → CDP-diglyceride + PP_i; (III) CDP-diglyceride + myo-inositol → PhI + CMP. Since the glycerol backbone of these lipids does not turn over as does the phosphate moiety (14), there must be a source of preformed (presumably membrane-bound) diglyceride. A possible site of the ACh effect on the above sequence is at the diglyceride kinase step (I), proposed by Hokin (15), but a purified kinase preparation is not affected by ACh (16). Durell et al. (17) postulated that diglyceride is a rate-limiting substrate, and that an ACh-stimulation of phosphodiesteratic cleavage of PhI or PhIP and $PhIP_2$ yields diglyceride, thus increasing [^{32}P]-PhA formation. The release of inositol phosphates from a prelabeled crude NEM-pellet from guinea pig cortex by ACh (18) is cited as supporting evidence. A number of other agents reportedly also increased this release (19). We find that PhIP and $PhIP_2$ are degraded during incubation of nerve-endings in vitro and that their hydrolysis is indeed enhanced by ACh. However, this effect lacks specificity since atropine fails to block the ACh action and choline will also exert a similar stimulation

(Table IV). This effect may well be mediated *via* phosphomonoesterases catalysing $PhIP_2 \rightarrow PhIP \rightarrow PhI$, since we failed to find an ACh-stimulation on the release of $[^3H]$-IP_2 (Table III). There was only little hydrolysis of PhI (Table III) under various conditions which included also the presence and absence of Ca^{++} (1 mM), variations in the ACh-concentration and incubation of a microsomal fraction (replacing NE 1.1). There were also no significant changes of $[^3H]$-IP release in the presence of ACh. These results strongly argue against the possibility that phosphodiesteratic cleavage of the inositides mediates the ACh effect (18,19,20).

Further evidence against involvement of PhI-phosphodiesterase in the ACh-stimulated phospholipid labeling comes from our determinations of diglyceride labeled *in vivo* (Table II). Since PhI is particularly high in arachidonate, this fatty acid must be present in a diglyceride pool derived from its phosphodiesteratic cleavage (9). In fact, no change was detected in $[^3H]$-diglyceride after incubation with CCh.

That PhA itself might serve as a substrate for ACh-stimulated hydrolysis has been considered (4). Since the labeled PhA pool in synaptosomes equilibrates with the precursor $[^{32}P]$-ATP within 1 hr, the addition of DNP to block oxidative phosphorylation after the labeling period allows us to observe hydrolysis in this pool as well as of PhI, PhIP, and $PhIP_2$ independently of synthetic processes. Labeled PhIP and $PhIP_2$ decrease by 50% 2-3 min after DNP addition, while labeled PhI levels remain unaffected during the 30 min incubation. Labeled PhA *decreases*, and ACh accelerates this breakdown by 60-70%. This ACh effect is blocked by the addition of atropine. Increased formation of PhI from PhA was not observed under these conditions. Conversion of PhA to lyso-PhA was also excluded by TLC analysis.

The results do not support an atropine-sensitive ACh action on phosphoinositide phosphodiesterase(s) in isolated guinea pig synaptosomes. We suggest that ACh enhances ^{32}P-phospholipid labeling by increasing a specific diglyceride pool through stimulation of phosphatidic acid phosphohydrolase,

and that the decrease in PhIP and $PhIP_2$ is a non-specific concomitant. The "ACh effect" has in these experiments been dissociated from the "inositide effect". The latter may not be directly related to the primary action of ACh.

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