NEUROTRANSMITTERS AND THE PHOSPHOLIPID EFFECT IN RABBIT IRIS MUSCLE

by

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This thesis submitted by Mary Pruitt Owen has been examined and approved by an appointed committee of the faculty of the School of Graduate Studies of the Medical College of Georgia.

The signatures which appear below verify the fact that all required changes have been incorporated and that the thesis has received final approval with reference to content, form and accuracy of presentation.

This thesis is therefore accepted in partial fulfillment of the requirements for the degree of Master of Science.

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DEDICATION

This thesis is gratefully dedicated to Mr. and Ms. Dan Waite and to my parents, without whose support, encouragement and love, it would not have been possible.

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ABBREVIATIONS

The following abbreviations were used throughout this work.

PhA - phosphatidic acid

PhI - phosphatidylinositol

PhC - phosphatidylcholine

PhE - phosphatidylethanolamine

PhS - phosphatidylserine

Lyso-PhC - lysophosphatidylcholine

DPI - diphosphoinositide

TPI - triphosphoinositide

TSH - thyrotropin

CDP - cytidine diphosphate

AMP - adenosine monophosphate

INTRODUCTION

I. Statement of Problem

An enhancement of the incorporation of ^{32}Pi into PhA* and PhI has been shown in a wide variety of tissues in response to a number of extracellular stimuli which include neurotransmitters, hormones, drugs and electrical impulses (see Table I in ref. 53). Although significant contributions have been made towards understanding this "phospholipid effect" and its possible relationship to synaptic neurotransmission and other physiological processes, the molecular mechanism and physiological significance of this effect are still not clear at the present time.

The innervation of the rabbit iris muscle by cholinergic and adrenergic fibers (72) has been shown and the presence of both types of adrenergic receptors has also been demonstrated in this muscle (74, 57). It follows that it would be of interest to show whether neurotransmitters exert any effect on phospholipid metabolism in the rabbit iris muscle. Thus, studies were undertaken to more clearly define the properties and the molecular mechanism underlying the phospholipid effect in this tissue.

II. Review of the Related Literature

There is a widespread phenomenon which has usually been first detected as an increased rate of ^{32}Pi incorporation into PhI, and often

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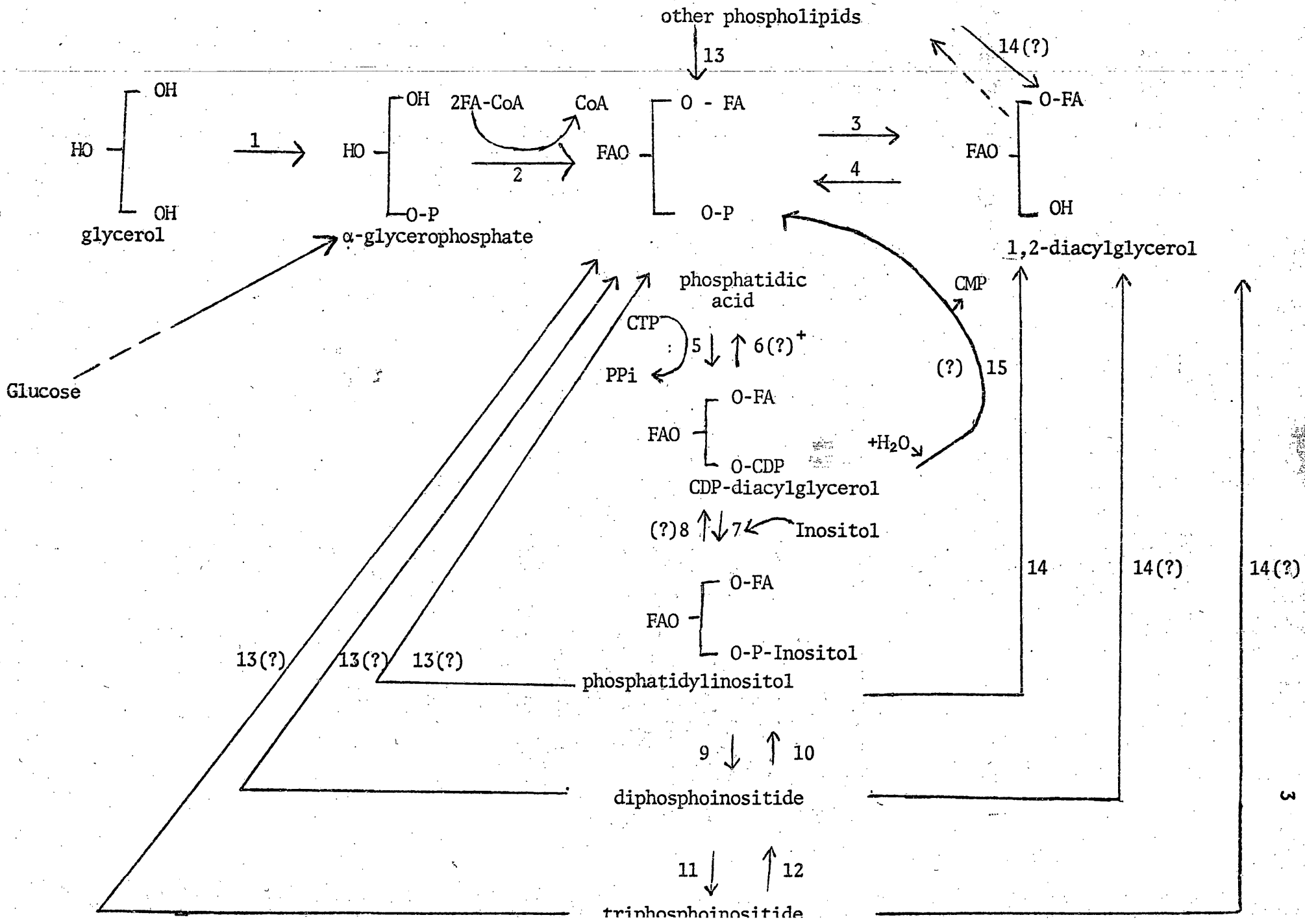
*See Table of Abbreviations for this and all following abbreviations.

also into PhA, which occurs in a tissue within a few minutes after the application of a stimulus and persists for as long as the stimulus is applied. Some of the possible pathways to explain this mechanism are shown in Figure 1. The phenomenon was first discovered in 1953 by Hokin and Hokin (34) as an increase in the incorporation of ^{32}P into the phospholipids of pancreatic slices, *in vitro*, which had been treated with acetylcholine or carbamylcholine. By 1960 this response had been observed in several tissues in response to acetylcholine and in salivary gland in response to adrenaline, anterior pituitary exposed to corticotrophin-releasing factor, thyroid slices treated with thyroid-stimulating hormone, and pancreas treated with pancreozymin (25). Today the list of stimuli and responsive tissues has grown even larger (see Table I in ref. 53).

The fact that the only affected lipids were certain anionic species (PhI and PhA) and that these constituted only a small fraction of the cellular phospholipids stimulated further investigation.

Hokin and Hokin (31) showed that in guinea pig brain cortex the enhanced incorporation of ^{32}Pi and of labelled inositol into PhI was not accompanied by increased incorporation of labelled glycerol, indicating that the phosphorylinositol portion of the molecule was being renewed in response to stimulation but that the diacylglycerol backbone of the molecule was reutilized. They also found that the incorporation of ^{32}Pi into PhA was increased but that there was no equivalent increase in the incorporation of glycerol-1-C^{14} into PhA. Measurements of the phospholipid-P content of the stimulated tissue seemed to confirm the impression that the process under study did not involve an increase in *de novo* synthesis of PhI and PhA. Results from a considerable number of similar


other phospholipids
13
14(?)
OH
HO
OH
glycerol
1
OH
HO
O-P
α-glycerophosphate
2FA-CoA
CoA
2
O - FA
FAO
O-P
3
4
O-FA
FAO
OH
1,2-diacylglycerol
Glucose
phosphatidic acid
CTP
PPi
5
6(?)+
CMP
(?)
15
+H2O
O-FA
FAO
O-CDP
CDP-diacylglycerol
(?)8
7
Inositol
O-FA
FAO
O-P-Inositol
phosphatidylinositol
13(?)
13(?)
13(?)
14
14(?)
14(?)
9
10
diphosphoinositide
11
12
triphosphoinositide
3

studies have now accumulated on a variety of tissues, most of which conform to the same pattern of increased rate of turnover of a cellular pool of PhI of essentially unchanging size (see Table II in ref. 53).

Direct measurements of the specific activities of precursor pools in many tissues have been made which tend to rule out the possibility that an artifactual increase in turnover rate (that is one in which the specific activity of a precursor to the phosphate group of the lipid, e.g. Pi or ATP were to rise after stimulation) should be judged an actual increase in turnover rate (see Table II in ref. 53).

Acetylcholine was the first agonist to be shown by Hokin and Hokin (34) to provoke a phospholipid response and is still by far the most fully studied. It was shown that the phospholipid response was blocked by atropine, a drug which prevents those effects of acetylcholine which are mediated through muscarinic receptors. Activation of the muscarinic receptors produces relatively slow responses to acetylcholine such as slowing of heart beat or contraction of intestinal smooth muscles. Other effects of acetylcholine which are mediated through the faster-acting nicotinic receptors are at the skeletal neuromuscular junction, autonomic ganglia and adrenal medulla. These nicotinic receptors are inhibited by tubocurarine.

Schacht and Agranoff (63) showed that the phospholipid effect in synaptosomes was sensitive to atropine but insensitive to tubocurarine. Jones and Michell (40) demonstrated the sensitivity of the PhI response in the rat parotid gland to atropine and insensitivity to tubocurarine. They also showed the PhI response could be evoked by acetyl β-methyl choline, an agonist which only stimulates muscarinic receptors. In

sympathetic ganglia, Larrabee and Leicht (46) have reported that both acetylcholine-induced and electrically-induced PhI responses are tubocurarine-sensitive and that the PhI response and nervous transmission through the ganglia are inhibited in a parallel fashion by the drug. They did not report the effect of atropine. However, in recent preliminary experiments Michell and Brown (53) showed that the acetylcholine-stimulated PhI response of rat superior cervical ganglia is tubocurarine-insensitive.

Hokin and Sherwin (33) in 1957 were the first to detect an increase in the incorporation of ^{32}P into phospholipids in response to adrenergic stimuli. They also showed that this phospholipid effect was prevented by dibenamine and ergotamine, compounds which produce blockade of α-adrenergic receptors. At that time effective β-blocking agents were not available. During the next fifteen years, responses to adrenergic stimuli were reported in many tissues and the characteristics of all of them were consistent with mediation by α-receptors. However, in none of them was a direct attempt made to access β-receptor involvement. This was recently done for cerebral cortex (23), parotid gland (54,55), vas deferens (12) and pineal gland (24). In each case the data indicated mediation by α-receptors.

Stimuli, other than the cholinergic and adrenergic stimuli, such as glucose (20), hormones (36,22) and electrical impulses (75) have been found to provoke enhanced metabolism of PhA and PhI.

Secretin (30), prostaglandin E_1 (47) and wheat germ lectin (50) are examples of types of stimuli which exert their effects on cells through interaction with cell surface receptors, but which have so far been found to have little effect on phospholipid metabolism. This would

seem to indicate that the PhI response cannot be regarded simply as a nonspecific response of cells to agents which interact with cell-surface receptors.

The concept that the effects of many hormones and neurotransmitters on cellular metabolism are mediated through the production and actions of intracellular second messengers is now established for cyclic AMP and widely accepted for cyclic GMP and Ca^{2+}. It was therefore natural that the effects of these agents on phospholipid metabolism would be tested.

In 1966 De Torrontegui and Berthet (15) demonstrated in rat liver that the incorporation of inositol into PhI was considerably stimulated by adrenalin but not by dibutyryl cyclic AMP. Then in 1967 Sandhu and Hokin (62) showed an increase in the incorporation of ^{32}P into PhA and PhI in parotid gland slices which were stimulated with epinephrine. They found no increased synthesis of PhA and PhI when dibutyryl cyclic AMP was used as a stimulus. So far in most systems studied cyclic AMP, dibutyryl cyclic AMP or theophylline (which raises intracellular cyclic AMP concentrations by inhibiting cyclic AMP phosphodiesterase) have not been shown to mimic the effects of other stimuli on phospholipid labeling (8,11,15,62,66).

Results from experiments using cyclic GMP derivatives to test for activity in modifying phospholipid metabolism are few and contradictory. In thyroid, Macchia and Varrone (49) found dibutyryl cyclic GMP to have no detectable effect on phospholipid synthesis. In cerebral cortical synaptosomes, however, Torch (70) recently reported effects on PhA and PhI labeling similar to those of acetylcholine with 0.1-5.0 μM dibutyryl cyclic GMP.

Hokin (29) found the acetylcholine-stimulated increased incorporation of ^{32}P into the phospholipids of pigeon pancreas slices to be insensitive to the omission of Ca^{2+} from the incubation medium. Trifaro (73) demonstrated similar results in bovine adrenal medulla. However, Zor, et al. (77) demonstrated the need for the presence of Ca^{2+} in the incubation medium for the TSH stimulation of ^{32}P incorporation into phospholipids in dog thyroid slices.

Much time and considerable effort has been, and is being, spent in order to identify the earliest biochemical event involved in the phospholipid response. It is thought that this identification will throw much light on the possible function of the response.

Reactions (Figure 1) which have so far been proposed as candidates for the reaction which is under the control of the applied stimulus are the synthesis of PhA by 1,2-diacylglycerol kinase (reaction 4), the breakdown of PhA by phosphatidate phosphatase (reaction 3), the breakdown of PhI by cleavage of the glycerol-phosphate bond (reaction 14), the breakdown of PhI by cleavage of the phosphate inositol bond (reaction 13 or 8) and the conversion of TPI to DPI by TPI-phosphomonoesterase (reaction 12).

In 1959 Hokin and Hokin (32,35) proposed the stimulation of the enzyme 1,2-diacylglycerol kinase as an explanation of the acetylcholine-stimulated labelling of PhA in a brain microsomal fraction. However, later experiments by Durell and Sodd (17,18) indicated that the response of these "microsomes" was dependent on the presence of metabolically viable synaptosomes, rather than on the single enzyme diacylglycerol kinase. In 1971 Lapetina and Hawthorne (43) found that acetylcholine

had no stimulatory effect on diglyceride kinase of isolated nerve-ending particles or of nerve-ending membranes of rat cerebral cortex obtained after osmotic shock.

Schacht and Agranoff (64,65) postulated the stimulation of hydrolysis of phosphatidic acid by cholinergic agents in guinea pig synaptosomes.

In 1974 Hokin-Neaverson demonstrated that in pancreas exposed to acetylcholine or pancreozymin there was a decrease in the concentration of PhI and an increase in that of PhA (37). Banschbach, et al. (5) showed an increase in the level of diglyceride in the same tissue upon stimulation by acetylcholine.

Kemp, Hübscher and Hawthorne (41) have demonstrated the presence in animal tissues of a Ca^{2+}-dependent phospholipase C type activity which degrades PhI to form inositol monophosphate and diglyceride. Subsequently when Dawson and his co-workers reported the formation of inositol 1,2-cyclic phosphate and inositol 1-phosphate from PhI in the soluble supernatant fraction of thyroid and liver homogenates, they suggested that the pathway might be involved in the responses of PhI metabolism to stimulatory agents in various tissues (14). Lapentina and Michell detected a membrane-bound enzyme in rat cerebral cortex which has similar properties (45). They have speculated that the function of the PhI breakdown might be to generate inositol-cyclic phosphate at the plasma membrane as a second messenger (44,53).

Recently Hokin-Neaverson (38) has found inositol to be the water-soluble product of acetylcholine-stimulated breakdown of PhI in mouse pancreas. This has led her to suggest that the acetylcholine-stimulated reaction is PhI $\longrightarrow$ PhA + inositol. She believes this might occur by either phospholipase D activity or through complete or partial reversal

of the cytidine nucleotide pathway of PhI biosynthesis.

Although many attempts in the past, such as addition of acetylcholine to tissue slices or electrical stimulation of tissue slices, to alter the labeling of DPI and TPI have failed, more recent evidence indicates that such changes may occur. In 1965 Palmer and Rossitor showed no significant change in the incorporation of inorganic ^{32}P into DPI and TPI of cat brain slices (56) stimulated by acetylcholine. Hokin (28) also showed a lack of effect in sympathetic or vagal ganglia. While acetylcholine did not stimulate synthesis of TPI, it has been shown to stimulate TPI hydrolysis in irides (2). In 1969 Pumphrey (58) found no significant change in the labeling of DPI and TPI in brain slices which had been stimulated electrically. However, in 1972 Schacht and Agranoff (63) observed a decreased labeling of polyphosphoinositides with ^{32}Pi in guinea-pig brain cortex subfractions incubated with acetylcholine. Then in 1973 White and Larrabee (75) reported a specific reduction in the labeling of TPI in rat vagus nerve after electrical stimulation for 3 hours. More recently White, et al. (76) reported that stimulation of vagus nerve for 30 minutes increased phosphate incorporation into all the phospholipids studied, but the increase was significant only in the phospholipids TPI and DPI.

Hendrickson and Reinertsen have reported results from experiments comparing the ion affinities of DPI and TPI (26). Their measurements were actually made with the water-soluble deacylation products derived from DPI and TPI, but results were extrapolated back to the lipids themselves. They concluded that TPI had a considerably higher affinity for Ca^{2+} than DPI, presumably because it could use the two phosphate

groups on the inositol ring to form a chelate with divalent metal ions. They proposed that DPI/TPI interconversion might itself be adequate to markedly modify Ca^{2+} binding at membrane surfaces and also to control intracellular concentrations of free Ca^{2+}. They set up a hypothetical model of axonal membrane function and it was clearly demonstrated that the amount of Ca^{2+} bound to inositides, and presumably to membranes containing them, would indeed be markedly lowered by TPI $\longrightarrow$ DPI conversion. They proposed that these changes could conceivably bring about a reorganization of the membrane with a resulting change in Na^{+} and K^{+} permeability (27). In 1972 the studies of Buckley and Hawthorne (10) using erythrocyte membranes confirmed that inositide interconversions can lead to changes in Ca^{2+} binding by membranes. They found an increase in high-affinity binding when membrane PhI was largely converted to DPI and TPI; their data do not, however, reveal the relative contributions of the PhI $\longrightarrow$ DPI and DPI $\longrightarrow$ TPI conversions.

Although many researchers have contributed towards understanding the "phospholipid effect", at the present time, its molecular mechanism and physiological significance remain unknown.

MATERIALS AND METHODS

I. Materials

A. Radioactive Compounds

The following radioactively labeled compounds were used in the studies reported here: [^{32}P] orthophosphate, carrier-free (Schwarz/Mann); adenosine-5-[γ-^{32}P] triphosphate tetrasodium salt (ICN Pharmaceuticals, Inc.); DL-serine [1-^{14}C] (Amersham/Seale); [^{3}H] inositol, [^{3}H] cytidine and acetic anhydride [^{3}H] (New England Nuclear).

B. Non-radioactive Compounds

The non-radioactively labeled compounds used in the studies reported here were: Norepinephrine (Levophed) and L-isoproterenol (Winthrop); Sotalol (Mead Johnson - 1991 - 1); phentolamine (Regitine) (Ciba); phenoxybenzamine (S.K. and F. Labs); atropine (Merck); L-epinephrine (Calbiochem); 6-hydroxydopamine (Aldrich); and normetanephrine, metanephrine, and iproniazid phosphate (Regis). The following compounds were obtained from Sigma Chemical Company: acetycholine, phenylephrine, dibutyryl cyclic AMP, DL-propranolol, eserine, carbamylcholine, adrenochrome, dopamine, histamine and 1,2-dipalmitin. Silica Gel G and Silica Gel H were purchased from Brinkmann Instruments, Inc.; 2,5-diphenyloxazole (PPO) and 1,4-bis-(5-phenyloxazol-2-yl) benzene (POPOP), from Packard Instrument Co.; and magnesium silicate (Supelcosil 41A) from Supelco, Inc.

II. Animals

Albino rabbits of either sex, weighing approximately 2 kg, provided by R.C. Ranch Rabbit Processing Plant and Palmetto Growers Co-operative were used throughout the study.

III. Preparation of Irides

The rabbits were stunned by a blow to the head and exsanguinated. The eyes were enucleated within 15 min of death and placed in a Krebs-Ringer bicarbonate buffer, pH 7.4, that contained 11 mM glucose, chilled in ice. After transportation from the slaughter house (approximately 25 min), the irides, which weighed about 36-40 mg each, were removed from the eyes and placed in Krebs-Ringer buffer chilled in ice.

IV. Incubation of Irides

The Krebs-Ringer buffer incubation medium in all experiments was gassed with 5% CO_2-95% O_2 before use. All the incubations were done at 37°C unless otherwise specified. The incubations were carried out in a shaking water bath. Iproniazid phosphate, a monoamine oxidase inhibitor, and ascorbic acid, an antioxidant, were used in incubations with norepinephrine. In each experiment the incubation was terminated by washing the irides twice with 5 ml of ice-cold Krebs-Ringer bicarbonate buffer and then transferred to 3 ml of chloroform-methanol (2:1, v/v). The specific details of each incubation used will be discussed under "Results".

V. Preparation of Subcellular Fractions of Iris Muscle

The irides were homogenized by the procedure of Clark (13) using a glass homogenizer. The various subcellular fractions were isolated by means of conventional differential centrifugation. The nuclear, mitochondrial and microsomal fractions were precipitated at 1,000, 8,000 and 120,000 g respectively. All centrifugations were done in a Spinco Model L2-65 B ultracentrifuge maintained at 4°C, using Spinco 30 or 40 rotors.

VI. Extraction of Phospholipids and Cholesterol from Iris Muscle

Each iris was homogenized in 6 ml chloroform-methanol (2:1), the insoluble material removed by centrifugation (1000 g for 30 min), and the supernatant washed twice with 1.5 ml of 0.15 M NaCl. The chloroform layer was concentrated *in vacuo* and the total lipid fraction redissolved in 0.1 ml chloroform-methanol (2:1).

VII. Thin-layer Chromatography of Total Lipid Extract

Individual phospholipids and cholesterol were isolated from the total lipid extract by means of two-dimensional thin-layer chromatography using a modification of the method of Rouser, *et al.* (61). A silica gel slurry was prepared by mixing 18 g of Silica Gel H plus 2 g magnesium silicate in 65 ml deionized water, and spread on glass plates (20 x 20 cm) in a layer 0.25 mm thick. The solvent systems used were the following:

1. Chloroform-methanol-28% ammonia (65:25:4, v/v).
2. n-butanol-acetic acid-water (6:1:1, v/v).

The plates were activated at 110° C for one hour and spotted with 0.1 ml of the lipid extract.

Roberts (59) identified the phospholipid spots separated by this chromatographic system using radioactive precursors. The cholesterol spot location was identified by following the migration of a cholesterol standard.

After development in the first dimension, the plates were air dried and then placed in the second solvent system such that it ran at right angles to the direction of the first solvent system. The lipids on the completed chromatogram were visualized using iodine vapor. For analysis, the phospholipid spots and the cholesterol spot were either scraped from the plate with a razor blade or a modified Hirsch funnel and eluted by the procedure of Skipski (68).

VIII. Measurement of Phospholipid Radioactivity

When the total radioactivity of a particular phospholipid spot was to be counted, the silica gel was scraped from the plate directly into a scintillation vial and suspended in 10 ml of the following scintillation fluid: 0.4% 2,5-diphenyloxazole (PPO) and 0.015% 1,4-bis-(5-phenyloxazol-2-yl) benzene (POPOP) in toluene. All measurements were made using a Beckman Model LS-230 liquid scintillation counter.

IX. Phosphate Assay

Total phosphorus and Pi were determined as reported by Bartlett (7).

X. Protein Assay

Protein was determined by the method of Lowry, et al. (48) using crystalline bovine serum albumin as a standard.

XI. Marker Enzyme Assays

A. NADPH cytochrome C reductase activity was measured by the method described by Sottocasa, et al. (69).

B. $[Mg^{2+} + Ca^{2+}]$-dependent ATPase activity was measured by the enzyme's ability to split ATP as has been outlined by Meissner (52).

C. Succinic dehydrogenase activity was assayed by the spectrophotometric method described by Bonner (9).

XII. Extraction of Diglycerides from Iris Muscle

The extraction of diglycerides from irides was performed by using the procedure of Folch, et al. (21). The chloroform layer was concentrated in vacuo, and the total lipid fraction redissolved in 0.1 ml chloroform-methanol (2:1).

XIII. Thin-layer Chromatography and Assay of Diglyceride

Quantitation of diglycerides was accomplished by a modification of the method described by Banschbach, et al. (6). 500-µm Silica Gel G plates were activated at 110° C for one hour and spotted with 0.1 ml lipid extract. Then they were developed in benzene: chloroform: methanol (80:15:5, v/v). After development in this solvent system the plates were air dried and then the lipids were visualized in iodine vapor. The phospholipids and monoglycerides were scraped from the plates and discarded. The remaining lipid, which included the diglycerides, was extracted with ether:methanol (9:1, v/v), concentrated and evaporated under a stream of nitrogen in 1 ml conical centrifuge tubes. Then pyridine (50 µl), [^{3}H] acetic anhydride (20 µl) (this stock acetic anhydride was prepared by adding 1.5 ml of unlabeled acetic anhydride

to a 25 mCi vial of [^{3}H] acetic anhydride), and perchloric acid (0.7 µl) were added to the lipid extract. The tubes were mixed and after 40 minutes the reaction was stopped by the addition of 0.4 ml of distilled water. Diglyceride acetate and cholesterol acetate were extracted with heptane (0.5 ml). The [^{3}H] acetylated diglyceride was then separated from the total acetylated lipid extract by means of one-dimensional thin-layer chromatography. The same type of Silica Gel G plates were used and activated as in the first isolation. The plates were then developed in heptane: ether: acetic acid (80:20:2, v/v). The acetylated diglyceride was visualized by iodine vapor, scraped into a test tube and extracted with ether:methanol (9:1, v/v). Unlabeled carrier acetylated diglyceride was added to the [^{3}H] acetylated diglyceride to aid visualization on the thin-layer plates. After evaporation of the ether:methanol solvent, [^{3}H] acetate in the acetylated diglyceride was counted in a Beckman liquid scintillation counter using a toluene-PPO (0.4%)-POPOP (0.04%) scintillation cocktail. The diglyceride acetates were quantified by comparison of counts incorporated into the sample diglycerides versus counts incorporated into known amounts of dipalmitin standard. Decomposition of the radioactive acetic anhydride caused extra counts to migrate with the diglyceride acetate spot. This "background" was corrected for by running one blank reaction for each thin-layer plate used.

XIV. Statistical Analysis of Data

Where appropriate, Student's t test for unpaired data was employed to evaluate changes in experimental data. Values were considered significantly different from control when $P \leq 0.05$.

RESULTS

I. Effects of Neurotransmitters on Phospholipid Metabolism of Iris Muscle

A. Phospholipid and Cholesterol Composition of the Iris Muscle

Table 1 shows that PhC, PhE, PhS and sphingomyelin constitute about 94 per cent of the total phospholipid-P of iris muscle. In contrast, PhI and PhA account for less than 5 and 1 per cent respectively.

B. Effect of Amines on Phospholipid Labeling

At 0.3 mM concentrations, the biogenic amines, norepinephrine, epinephrine and dopamine, exerted between 141 and 247 per cent stimulation on the ^{32}P-labeling of PhA and PhI; however at 0.003 mM, the effect was only 110-126 per cent of that of control (Table 2). In contrast, normetanephrine, metanephrine and adrenochrome had a lesser effect. 6-Hydroxydopamine inhibited the ^{32}P-labeling of the PhA by from 19 to 33 per cent. Histamine exerted a similar inhibitory effect (16 to 23 per cent) on the ^{32}P-labeling of PhA.

C. Effect of Different Concentrations of Cholinergic Agents on Phospholipid Labeling

Acetylcholine markedly increased the labeling of PhA and PhI (Table 3). The acetylcholine stimulation was blocked by atropine, a muscarinic cholinergic receptor blocking agent. Eserine (0.03 mM) was added to the acetylcholine incubation media to prevent its hydrolysis by acetylcholinesterase. Control studies demonstrated that eserine

TABLE 1: Phospholipid and cholesterol composition of rabbit iris muscle

2.8 g of irides were extracted with chloroform-methanol (2:1). 1.5 µmoles lipid P was spotted per TLC plate. The individual lipids were isolated by means of two-dimensional TLC and the spots were either digested directly or eluted and digested, and the phospholipid P determined. In general the lipid phosphorus was lower by 10 per cent when the spots were eluted. The results were obtained from 2 different experiments. (from Abdel-Latif, A.A., Mary P. Owen, and James L. Matheny, Biochem. Pharmacol. Vol. 25, p 463, 1976).

+ This value represents total cholesterol in the lipid extract before TLC analysis. After TLC isolation and elution of the cholesterol spot, the amount was 75 per cent of this value.

Lipid	µmole P/g wet tissue	P as % of total lipid P
PhC	4.81	41.00
PhE	3.80	32.40
PhS	0.99	8.4
PhI	0.57	4.86
PhA	0.063	0.54
Lyso-PhC	0.058	0.49
Sphingomyelin	1.43	12.2
Total Cholesterol[+]	2.98 mg/g tissue	——

TABLE 2: Effect of different concentrations of biogenic amines and their metabolites on ^{32}P-labeling of phospholipids in the iris muscle[+]

All the irides were first preincubated for 20 min in 15 ml of ^{32}P-labeled Krebs-Ringer from which cold phosphate was omitted. Then each muscle was transferred into individual test tubes and reincubated for an additional 40 min in 1.5 ml of fresh ^{32}P-labeled Krebs-Ringer either in the presence or absence of the amines. The results reported here are the averages of two different experiments which were run in duplicate. The variations in this experiment ranged from 0 to 5 per cent between duplicates in the same experiment and between different experiments (from Abdel-Latif, A.A., Mary P. Owen, and James L. Matheny, Biochem. Pharmacol., Vol. 25, p 463, 1976).

+ PhC and PhE were analyzed and the values for both upon addition of the amines and their metabolites ranged between 95 and 110 per cent of their control value.

‡ These values correspond to 0.003, 0.03 and 0.3 mM concentrations respectively.

Amine Added	Effect of the amines on phospholipid labelling expressed as % of control	
	PhA	PhI
L-Norepinephrine	115; 141; 247‡	117;133;196
Normetanephrine	89; 94; 102	109;117;120
L-Epinephrine	126; 195; 244	132;178;200
Metanephrine	104; 142; 138	120;151;140
Adrenochrome	89; 108; 94	132;141;145
Dopamine	110; 177; 185	126;135;141
6-Hydroxydopamine	76; 67; 81	97; 95;108
Histamine	84; 76; 77	115;117;113

TABLE 3: Effect of cholinergic agents on ^{32}P-labeling of PhA and PhI in the iris muscle

Conditions of incubation were as described for Table 2 except that cholinergic agents rather than amines were used (from Abdel-Latif, A.A., Mary P. Owen, and James L. Matheny, Biochem. Pharmacol., Vol. 25, p 464, 1976).

+ Eserine (0.03 mM) was added to each of the incubation mixtures in order to protect acetylcholine from hydrolysis by acetylcholinesterase.

‡ These values correspond to 0.003, 0.03 and 0.3 mM concentrations respectively.

Cholinergic agent added	Concentration of each agent (mM)	Effect of the cholinergic agents on phospholipid labelling expressed as % of control	
		PhA	PhI
Acetylcholine+	0.003;0.03;0.3	140;151;205‡	175;196;229
Eserine	0.03	101	103
Carbamylcholine	0.003;0.03;0.3	126;128;141	137;151;182
Atropine	0.003;0.03;0.3	90; 87; 75	105;107;113
Acetylcholine + atropine	0.003;0.03;0.3	102;113;136	119;145;132

alone had no effect on the ^{32}P-labeling of the phospholipids. Carbamylcholine was less effective than acetylcholine in the stimulation of labeling.

II. Adrenergic Receptor Specificity of Phospholipid Effect in Iris Muscle

A. Effect of α- and β- Adrenergic Agonists and Antagonists and Dibutyryl Cyclic AMP on the ^{32}P-labeling of Phospholipids

Norepinephrine, an α- and β- receptor stimulator, and phenylephrine, which is a more specific stimulator of adrenergic α-receptors (39), increased appreciably the labeling of PhA and PhI but not PhC (Table 4). In contrast, isoproterenol, a β-receptor stimulator (39), had no effect on the ^{32}P-labeling of phospholipids. Sotalol, a β-blocker, had no effect on the norepinephrine-stimulated phospholipid labeling. DL-propranolol, a β receptor blocker, did not block the norepinephrine-stimulated phospholipid effect at the lower concentrations, however, at 0.3 mM concentration it increased the norepinephrine-stimulated ^{32}P-labeling of PhA and PhI by approximately 5 and 2-fold respectively. Furthermore, it inhibited almost completely the ^{32}P-labeling of PhC. Phentolamine, an α-adrenergic blocker, inhibited the norepinephrine-stimulated phospholipid effect at lower concentrations (0.003 and 0.03 mM) and as with propranolol, it inhibited the ^{32}P-labeling of PhC. Higher concentrations (0.3 mM) of propranolol and phentolamine produced a dramatic increase in the ^{32}P-labeling of PhA, PhI and CDP-diglyceride, a significant inhibition of PhC but no effect on PhE (Table 5). Phenoxybenzamine, an α-receptor blocker, abolished the norepinephrine-stimulated ^{32}P-labeling of phospholipids. Dibutyryl cyclic AMP had no influence on the phospholipid effect.

TABLE 4: Effect of different concentrations of α- and β- adrenergic agonists and antagonists and dibutyryl cyclic AMP on the ^{32}P-labeling of phospholipids in iris muscle

The conditions of incubation were the same as described for Table 2, except that the agonists were added in the final 20 min of incubation, and the antagonists were added immediately after pre-incubation of the irides. (from Abdel-Latif, A.A., Mary P. Owen, and James L. Matheny, Biochem. Pharmacol., Vol. 25, p 464, 1976).

+ These values correspond to 0.003, 0.03 and 0.3 mM concentrations respectively.

Antagonist or agonist added	Concentration of each agent (mM)	Effect of the antagonists and/or agonists on phospholipid metabolism expressed as % of control		
		PhA	PhI	PhC
L-Norepinepinephrine	0.003;0.03;0.3	117;176;220+	122;153;193	102;105;104
Phenylephrine	0.003;0.03;0.3	121;162;191	102;120;163	103;102;101
Isoproterenol	0.003;0.03;0.3	92;100;109	98;102;105	98; 99;101
Sotalol	0.003;0.03;0.3	95; 92; 92	89; 98;103	103;102;101
Norepinephrine + Sotalol	0.003;0.03;0.3	107;169;225	119;147;182	98; 96;102
DL-Propranolol	0.003;0.03;0.3	98;133;968	106;129;212	67; 54; 9
Norepinephrine + Propranolol	0.003;0.03;0.3	129;212;1312	119;129;439	109; 60; 9
Phentolamine	0.003;0.03;0.3	89;123;295	112;137;200	90; 45; 19
Norepinephrine + Phentolamine	0.003;0.03;0.3	90;114;277	98;123;243	80; 76; 35
Phenoxybenzamine	0.003;0.03;0.3	103; 97; 90	100;122;147	103; 97; 69
Norepinephrine + Phenoxybenzamine	0.003;0.03;0.3	109; 95; 93	91; 95; 84	91; 87; 85
Phentolamine + Propranolol	0.003;0.03;0.3	102;160;1164	132;175;331	47; 38; 4
Norepinephrine + Phentolamine + Propranolol	0.003;0.03;0.3	106;228;871	139;198;180	100; 56; 6
Dibutyryl Cyclic AMP	0.003;0.03;0.3	97; 89; 87	100; 95; 93	101; 96; 90

TABLE 5: Effects of adrenergic blockade on the incorporation of ^{32}Pi into phospholipids of iris muscle+

+ See Table 4 for description of experimental protocal.

‡ PhS was identified by incubating irides in Krebs-Ringer medium containing DL-serine [1-^{14}C]. All the radioactivity was found in the PhS spot. To show whether the PhS spot contained any PhI, the irides were incubated in Krebs-Ringer medium containing [^{3}H] inositol. The results from this experiment showed that the PhS spot contained no [^{3}H] inositol-labeled phospholipids. The CDP-diglyceride was identified by means of two-dimensional TLC using [^{3}H] cytidine as the precursor. More than 95% of the stimulatory effect of the drugs seen in CDP-diglyceride + PhS was found in CDP-diglyceride (from Abdel-Latif, A.A., Mary P. Owen, and James L. Matheny, Biochem. Pharmacol., Vol. 25, p 465, 1976).

Drug added	Concentration (mM)	Activity found in the various phospholipids (cpm/spot)				
		PhA	PhI	PhC	PhE	CDP-diglyceride + PhS‡
None		19,645	69,953	32,486	10,372	2,498
DL-Propranolol	0.3	190,174	148,131	2,965	8,923	39,068
Phentolamine	0.3	58,045	140,341	6,338	11,645	14,322

III. Molecular Mechanism Underlying the Phospholipid Effect

In all these experiments, unless otherwise indicated, ^{32}Pi was added to Krebs-Ringer which contained cold phosphate and the irides were not preincubated.

A. Time-course of the Norepinephrine Mediated Phospholipid Effect in Iris Muscle

In the absence of norepinephrine the labeling of PhA increased gradually until 30 min after the start of incubation (Figure 2). After 30 min the labeling of PhA showed no further increase with time. In contrast to PhA, the labeling of PhI increased with time until it reached its maximum after 60 min of incubation. When norepinephrine was added the stimulation occurred immediately after the start of incubation and the labeling of PhA and PhI increased considerably with time up to 60 min of incubation. At 60 min the addition of norepinephrine had increased the labeling of both phospholipids by approximately 400%.

B. Effect of Glucose or Its Intermediates on the Phospholipid Effect

When glucose, pyruvate, glycerol, fructose-1,6-diphosphate or α-glycero-P were included in the Krebs-Ringer buffer, only glucose increased significantly the ^{32}P-labeling of PhA and PhI and the stimulatory effect of norepinephrine.

C. Effect of Incubation Temperature on the Phospholipid Effect

In studies on the effect of temperature on ^{32}P labeling of the iris muscle without stimulation by norepinephrine only PhA was significently labeled at 0^{o} (Table 6). PhI contained a negligible amount of radioactivity and PhC contained none. At $15^{o}C$, PhA labeling increased

FIGURE 2: Time-course of the norepinephrine mediated phospholipid effect in iris muscle

Each iris was placed in 1.4 ml of Krebs-Ringer bicarbonate buffer, pH 7.4, containing 11 mM glucose and ^{32}Pi (25 μCi). l-norepinephrine (1 mM) was added as indicated to give a final volume of 1.5 ml. Following incubation at 37°C for various specified intervals, the irides were washed with Krebs-Ringer solution, the lipids extracted, separated into their individual components by means of two-dimensional TLC, and the various phospholipids counted. The results are averages of a triplicate experiment and the values agreed within 0-5 per cent (from Abdel-Latif, A.A., Life Sciences, Vol. 15, p 967, 1974).

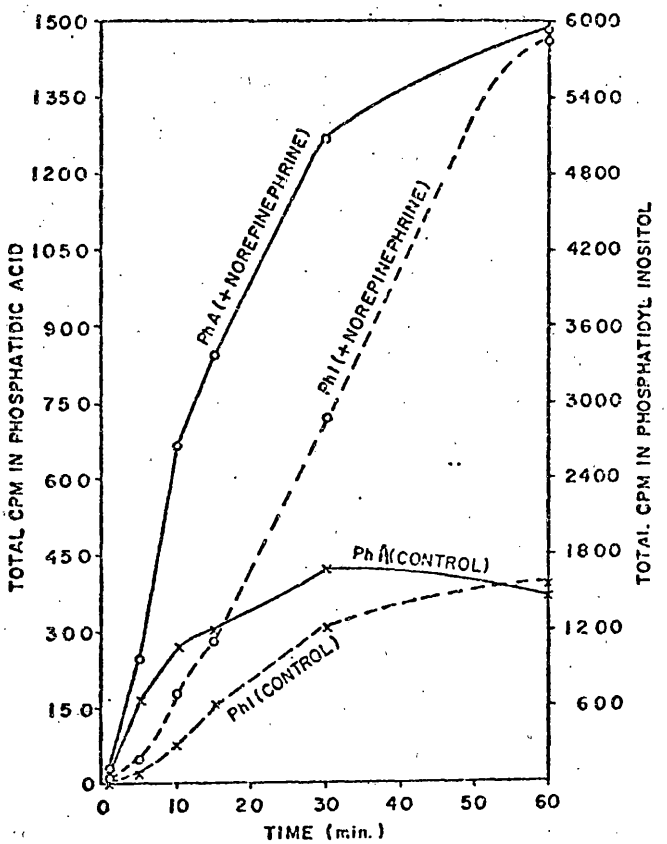

1500
1350
1200
1050
900
750
600
450
300
150
0
TOTAL CPM IN PHOSPHATIDIC ACID
6000
5400
4800
4200
3600
3000
2400
1800
1200
600
TOTAL CPM IN PHOSPHATIDYL INOSITOL
PhA (+ NOREPINEPHRINE)
PhI (+ NOREPINEPHRINE)
PhA (CONTROL)
PhI (CONTROL)
0
10
20
30
40
50
60
TIME (min.)

TABLE 6: Effects of incubation temperature on the ^{32}P-labeling of phospholipids in the presence and absence of norepinephrine

Individual irides were placed in separate tubes containing 1.3 ml Krebs-Ringer (with cold phosphate) and 25 μCi ^{32}Pi with norepinephrine (0.5 mM) as indicated. Final volume was 1.5 ml and time of incubation was 30 min (from Abdel-Latif, A.A., Mary P. Owen, and James L. Matheny, Biochem. Pharmacol., Vol. 25, p 465, 1976).

+ Activity (cpm) in the phospholipid in the absence and presence of norepinephrine respectively.

‡ This radioactivity was insignificant and thus was not used in computing the % increase in PhI with temperature.

Temperature	CPM/Spot			Effect of norepinephrine on phospholipid labelling expressed as % of control		
	PhA	PhI	PhC	PhA	PhI	PhC
0°C	63 (67)+	3‡ (3)	0(0)	106	100	—
15°C	1134(1478)	168(224)	0(0)	135	133	—
22°C	2947(4358)	1395(1893)	37(31)	148	136	—
37°C	5187(9462)	11505(16715)	1384(1425)	182	145	103

by 18 fold, PhI labeling increased significantly, and PhC still showed no radioactivity. When incubated at 22°C, the level of radioactivity in PhI increased by 8-fold while that of PhA increased less than 3-fold. PhC still contained little radioactivity. The increase in the labeling of PhA, PhI and PhC when the incubation temperature was 37°C instead of 22°C was approximately 2-, 8- and 37- fold respectively. In summary, the stimulatory effect of norepinephrine on ^{32}P labeling in the phospholipids increased gradually with temperatures over the range studied.

D. Results of Freezing and Thawing on the Phospholipid Effect

When irides were frozen and thawed four times, they lost almost all of their ability to incorporate ^{32}Pi into phospholipids. Furthermore, norepinephrine (0.3 mM) exerted little effect on the ^{32}P-labeling of phospholipids in the irides which were frozen and thawed. Thus, values for PhA obtained from a typical experiment showed that, in contrast to the control irides where norepinephrine (0.3 mM) stimulated the PhA labeling from 2.59×10^4 cpm (control tissues without norepinephrine) to 6.68×10^4 cpm, in the frozen and thawed tissues, it stimulated the labeling from 192 cpm (control tissues without norepinephrine) to 198 cpm.

E. Subcellular Localization of the Norepinephrine Mediated Phospholipid Effect

In an attempt to localize the site of the phospholipid effect, irides were first incubated in Krebs-Ringer buffer containing ^{32}Pi in the presence and absence of norepinephrine (10 mM) for 1 hr, homogenized, then subfractionated by means of differential centrifugation into their respective subcellular fractions. A high concentration

TABLE 7: Subcellular localization of the norepinephrine mediated phospholipid effect

Irides (23 per flask) were placed in control and experimental 125-ml stoppered conical flasks containing 3 ml of complete ^{32}P-Krebs-Ringer. Norepinephrine (10 mM) was added to the experimental flask and after incubation at 37^{o} for 1 hr, the irides were homogenized and subfractionated by means of differential centrifugation as described in the text (from Abdel-Latif, A.A., Mary P. Owen, and James L. Matheny, Biochem. Pharmacol., Vol. 25, p 466, 1976).

+ Activity in the control (cpm/spot).

‡ Activity in the presence of norepinephrine (cpm/spot).

§ Stimulatory effect of norepinephrine expressed as % of control.

* The marker enzymes assayed were: NADPH-cytochrome reductase designated as A, [Mg^{2+} + Ca^{2+}]-dependent ATPase designated as B and Succinic dehydrogenase designated as C.

** NADPH-cytochrome reductase unit = increase in O.D. at 550 nm/mg protein/hr.

*** [Mg^{2+} + Ca^{2+}]-dependent ATPase unit = amt. which splits 1 μmole ATP/mg of protein/hr.

**** Succinic dehydrogenase unit = amt. which reduces $K_3Fe(CN)_6$ at 20^{o}C. This is expressed as the decrease in O.D. at 400 nm/mg of protein/hr.

***** The enzyme activities were not determined in the original homogenate.

Fraction	Marker Enzymes*			Stimulatory effect of norepinephrine (% of control)		
	A**	B***	C****	PhA	PhI	PhC
Original homogenate	*****	———	———	954⁺;9,440‡;(989)§	6,854;43,185;(630)	3,768;6,974;(185)
Nuclear fraction	1.02	2.54	0	1,040;7,946;(764)	7,726;44,349;(574)	4,305;6,977;(162)
Mitochondrial fraction	4.5	2.58	1.22	882;7,992;(906)	7,028;46,060;(655)	3,980;7,285;(183)
Microsomal fraction	7.35	14.40	0	1,440;8,832;(613)	9,375;54,435;(581)	5,125;9,309;(182)

of norepinephrine was employed to assure maximal stimulation. Preliminary experiments showed that PhA, PhI and PhC were stimulated at this concentration. The subcellular fractions were assayed using appropriate enzyme markers. All subcellular fractions were found to contain ^{32}P-labeled PhA, PhI and PhC in the absence of norepinephrine. Norepinephrine increased ^{32}P-labeling of PhA and PhI and, to a lesser extent PhC (Table 7).

F. Phospholipid Effect After In Vitro Aging of the Irides

The action of norepinephrine (0.5 mM) on the ^{32}P-labeling of phospholipids was investigated in irides which were stored at 4^{o} for periods up to 3 days (Figure 3). Maximal stimulation for PhA and PhI labeling was observed after 1 day, and decreased gradually with aging. The level of ^{32}P-labeling without norepinephrine stimulation was highest after 3 days of aging. For example, in irides which were isolated 1 hr prior to incubation, the radioactivities (cpm/spot) found in PhA, PhI and PhC were 6,429, 10,577 and 10,055; in contrast, irides which were aged for 3 days showed radioactivities of 7,313, 14,947 and 24,287 respectively.

G. Effect of Norepinephrine on PhA and PhI Levels in Iris Muscle

Incubation of irides in media containing increasing concentrations of norepinephrine resulted in dose-dependent decreases in the level of PhI and dose-dependent increases in the level of PhA as compared to control tissues (Table 8). The PhC and PhE levels did not change under the same conditions.

FIGURE 3: Phospholipid effect after in vitro aging of the irides

The effect of norepinephrine on the ^{32}Pi incorporation into PhA, PhI and PhC of irides, which were isolated and stored at 4^{o} in Krebs-Ringer for 2, 24, 48 and 72 hr prior to incubation for 40 min at 37^{o} in the presence and absence of norepinephrine (0.5 mM) is expressed as per cent of control (from Abdel-Latif, A.A., Mary P. Owen, and James L. Matheny, Biochem. Pharmacol., Vol. 25, p 466, 1976).

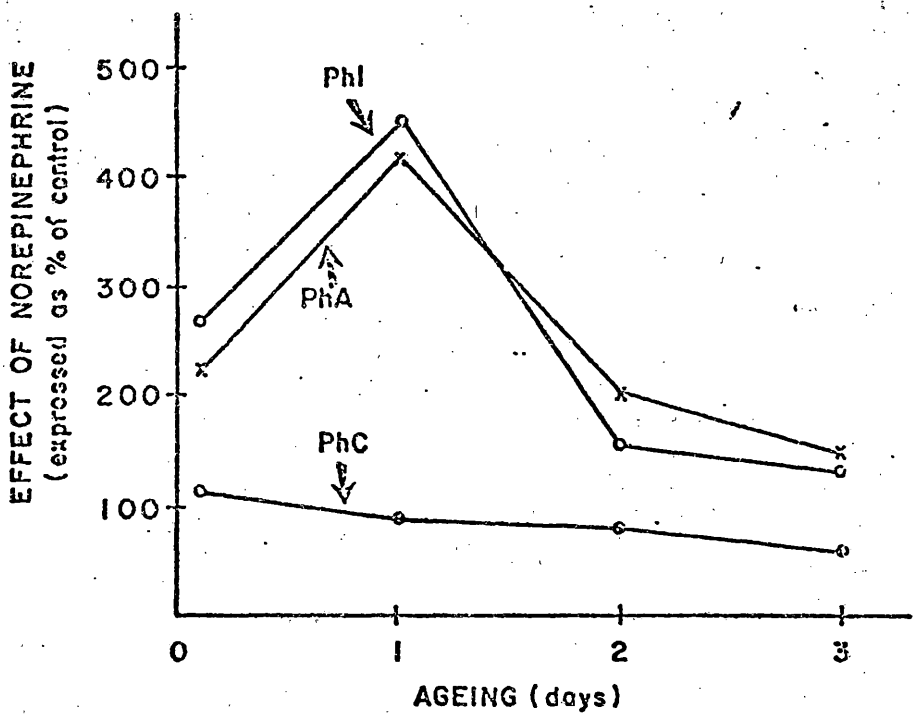

EFFECT OF NOREPINEPHRINE
(expressed as % of control)
500
400
300
200
100
PhI
PhA
PhC
0
1
2
3
AGEING (days)

TABLE 8: Effect of norepinephrine on PhA and PhI levels in iris muscle

The results are averages of two different experiments which were run in duplicate. Ten irides per flask were incubated at 37° for 1 hr with norepinephrine added as indicated (from Abdel-Latif, A.A., Mary P. Owen, and James L. Matheny, Biochem. Pharmacol., Vol. 25, 467, 1976).

+ The phospholipid spots were scraped from the plates, digested in perchloric acid and their phosphate contents determined according to the procedure of Bartlett (7).

Agent added	Concentration (mM)	(μmoles of phospholipid-P^+/g wet tissue)	
		PhI	PhA
Control	——	0.650	0.063
Norepinephrine	0.003	0.610	0.074
Norepinephrine	0.03	0.533	0.083
Norepinephrine	0.3	0.490	0.103

H. Effect of Norepinephrine on Diglyceride Kinase Activity in Subcellular Fractions of Iris Muscle

When norepinephrine was added to the subcellular fractions incubated with [γ-^{32}P] ATP, there was no increase in the ^{32}P-labeling of PhA and PhI over that obtained when norepinephrine was not added to the incubation mixture.

I. Effect of Norepinephrine on the Rate of Incorporation of ^{32}Pi Into Iris Phospholipids

The specific radioactivities of PhI and PhC increased with time in the absence of norepinephrine; however the specific radioactivities of these two phospholipids increased even more in the presence of norepinephrine. The specific radioactivity of PhA increased only in the presence of the neurotransmitter (Table 9).

J. Effect of Norepinephrine on Diglyceride Level in Iris Muscle

The diglyceride level of iris muscle remained constant throughout the 80 min incubation period (Table 10).

Norepinephrine (0.003 mM or 0.3 mM) had no effect on the level of diglyceride (Table 11).

TABLE 9: Effect of norepinephrine on the rate of incorporation of ^{32}Pi into the iris phospholipids

The irides were preincubated in Krebs-Ringer containing ^{32}Pi (8 μCi/ml) for 20 min at 37°. The irides (10/flask) were then transferred to 3 ml fresh Krebs-Ringer containing ^{32}Pi and incubated in the presence and absence of norepinephrine (2.5 mM) at various time intervals as indicated. The radioactivities and phosphate content of PhA, PhI and PhC were then determined and their specific radioactivities computed. The results are averages of two different experiments (from Abdel-Latif, A.A., Mary P. Owen, and James L. Matheny, Biochem. Pharmacol., Vol. 25, p 467, 1976).

+ Control

‡ In the presence of norepinephrine (2.5 mM).

Time of incubation (Min)	Specific radioactivity of phospholipids (CPM/μmole P) x 10^{-4}		
	PhA	PhI	PhC
0	100+ (91)‡	24.9 (25)	3.2 (3.7)
5	100 (147)	31.5 (33.6)	3.4 (3.8)
10	95 (190)	39.6 (44.6)	3.8 (3.95)
15	96 (228)	46.5 (92.4)	4.4 (6.1)
20	111 (246)	62.3 (117.3)	5.3 (9.1)
30	111 (342)	78.4 (167)	6.9 (12.9)
60	115 (333)	122.6 (187)	19 (24)
90	109 (441)	129 (229.8)	24 (41)

TABLE 10: Effect of norepinephrine and time of incubation on total diglyceride level in iris muscle in vitro

Each iris was incubated for the specified time at 37° in 1.5 ml Krebs-Ringer bicarbonate buffer, pH 7.4, with 11 mM glucose. The buffer contained norepinephrine as indicated.

+ The control value is the diglyceride level obtained from irides before incubation.

Norepinephrine (mM conc.)	Time of incubation (Min)	DG level (% of control)+
0	0	100
1	5	100
1	10	90
1	15	97
1	20	98
1	25	103
1	30	97
1	35	103
1	40	90
1	80	97
0	80	106

TABLE 11: Effect of norepinephrine on total diglyceride level in iris muscle in vitro

Each iris was incubated for 40 min at 37° in 1.5 ml Krebs-Ringer bicarbonate buffer, pH 7.4, with 11 mM glucose. The buffer contained norepinephrine as indicated.

n.s. - not significantly different from control.

Norepinephrine	Diglyceride Conc.			P
(mM conc.)	(μ Moles)			
	Mean	SD	n	
0	0.020	±.004	10	——
0.003	0.015	±.005	10	n.s.
0.3	0.018	±.004	10	n.s.

DISCUSSION

The phospholipid composition of iris muscle was found to be similar to that of heart and skeletal muscle (67). PhC accounted for approximately 41 per cent of the total phospholipid P, PhE for 32 per cent, sphingomyelin for 12 per cent and PhS for 8 per cent. PhA and PhI accounted for only 0.5 and 5 per cent respectively (Table 1). There is little information on phospholipid metabolism in iris muscle. When the irides were incubated in ^{32}P-Krebs-Ringer at different time intervals, PhA was labeled first and, as the labeling of this phospholipid decreased with time, that of PhI increased (Figure 2). This observation, coupled with the results obtained from the temperature experiment (Table 6) in which the PhA/PhI activity ratios at 0^{o}, 15^{o}, 22^{o} and 37^{o} were approximately 21, 6.8, 2.1 and 0.5 respectively, suggested a central role for PhA in phospholipid biosynthesis in the iris muscle. The results are similar to those found in liver (42).

Iris muscle of rabbit is innervated by cholinergic and adrenergic fibers (72) and consists of dilator and sphincter muscles. Both muscles respond to adrenergic and cholinergic stimuli. Dilator muscle responds to and is under primary control of the adrenergic (sympathetic) system, whereas the sphincter muscle is primarily under the control of the cholinergic (parasympathetic) system. Norepinephrine, predominately an α-receptor agonist, induces contraction of the smooth muscle of the

iris while isoproterenol, a β-receptor agonist, causes relaxation (51).

The cholinergic neurotransmitter acetylcholine was found to increase the ^{32}P-labeling of PhA and PhI in the iris. The acetylcholine stimulation was blocked by atropine, a muscarinic cholinergic receptor blocking agent. Carbamylcholine was less effective than acetylcholine in the stimulation of phospholipid labeling (Table 3).

The rationale for preincubating the tissue in the experiments with amines, cholinergic agents and α- and β- adrenergic agonists and antagonists for 20 min in the ^{32}P-labeled medium was (1) to label the intracellular precursor pools and (2) to exclude the possibility that the phospholipid effect was caused by an increase in permeability to ^{32}Pi in the presence of the agents. However, by using this ^{32}P-preincubation step, the phospholipid effect was obtained at lower concentrations of the neurotransmitters or neuropharmacological agents than in preliminary experiments where this ^{32}P-labeled medium pre-incubation step was not used (1). Also the preincubation step seems to bring about an alteration in the phospholipid response to some amines. The preincubation step might increase the susceptibility of the enzymes involved in the phospholipid effect to the agents.

At 0.3 mM concentrations the biogenic amines norepinephrine and epinephrine exerted between 196 and 247 per cent stimulation on the ^{32}P-labeling of PhA and PhI. Dopamine (0.3 mM) exerted between 141 and 185 per cent stimulation. In contrast, normetanephrine, metanephrine and adrenochrome had a much lesser effect. 6-Hydroxydopamine inhibited the ^{32}P-labeling of PhA by approximately 20 per cent. Histamine (0.3 mM)

exerted an inhibitory effect on the ^{32}P-labeling of PhA and PhI. In preliminary experiments (1), run without ^{32}P-preincubation, histamine (1 mM) exerted a stimulatory effect on the ^{32}P-labeling of PhA and PhI. Also an increase in the ^{32}P-labeling of PhC was observed at the higher concentrations (1-10 mM) of cholinergic or adrenergic neurotransmitters used in the preliminary experiments (without ^{32}P-preincubation). Only the labeling of PhA and PhI was affected in the ^{32}P preincubation experiments with concentrations of 0.03 to 0.3 mM neurotransmitters (Table 2).

Adrenergic α-receptor stimulators, e.g. phenylephrine, increased significantly the ^{32}P-labeling of PhA and PhI. In contrast adrenergic β-stimulators (0.003, 0.03, 0.3 mM) such as isoproterenol had little influence on the ^{32}Pi incorporation into phospholipids (Table 4). (In preliminary experiments in which there was no ^{32}P-labeled medium preincubation (1), isoproterenol (1 mM) stimulated ^{32}P-labeling of the phospholipids.) Experiments with ^{32}P-labeled medium preincubation demonstrated in the iris as has been shown in other tissues (12, 23, 54, 55) that the stimulation of phospholipid metabolism in the presence of α-agonists involves α-receptors. To support this conclusion the influence of α- and β- adrenergic blocking agents were investigated. Sotalol, a β-blocker, had no effect on the norepinephrine-stimulated phospholipid labeling. DL-propranolol, also a β receptor blocker, did not block the norepinephrine-stimulated phospholipid effect at the lower concentrations. However, at 0.3 mM concentration it increased the norepinephrine-stimulated ^{32}P-labeling of PhA and PhI by approximately 5 and 2-fold respectively. Furthermore, it inhibited almost completely

the ^{32}P-labeling of PhC. Phentolamine, an α-adrenergic blocker, inhibited the norepinephrine-stimulated phospholipid effect at lower concentrations (0.003 and 0.03 mM) and as with propranolol, it inhibited the ^{32}P-labeling of PhC (Table 4). Higher concentrations (0.3 mM) of propranolol and phentolamine produced a dramatic increase in the ^{32}P-labeling of PhA, PhI and CDP-diglyceride, a significant inhibition of PhC, but no effect on PhE (Table 5). This observed stimulatory effect of propranolol and phentolamine is believed to be non-specific and not related to their ability to block adrenergic receptors. In a more detailed study on the effect of propranolol, Abdel-Latif and Smith (in press), have shown that it increases the incorporation of serine into PhS and also the incorporation of [^{3}H] cytidine into CDP-diglyceride by more than 10 fold. Eichberg, et al. (19) showed that several substances with widely differing potencies as β-adrenergic receptor blocking agents including DL-propranolol, D-propranolol, dichloroisoproterenol and alprenolol, but not sotalol, increased the ^{32}P incorporation into pineal acidic phospholipids. β-Receptor stimulation has been suggested to be mediated by cyclic AMP (60). Results from the preliminary studies (1) and the present studies on the iris showed that neither cyclic AMP nor dibutyryl cyclic AMP were involved in the phospholipid effect (Table 4). Phenoxybenzamine, an α-receptor blocker, abolished the norepinephrine-stimulated ^{32}P-labeling of phospholipids (Table 4). Thus, the stimulation of ^{32}P-labeling of PhA and PhI by the neurotransmitter norepinephrine, an α- and β-receptor stimulator, appears to be mediated by α- and not β-receptors.

Studies to elucidate the molecular mechanism underlying the phospholipid effect yielded the following observations, which indicate that certain enzymic pathways are being stimulated to produce the phospholipid effect: (a) In the presence of norepinephrine maximal stimulation was obtained when glucose, rather than pyruvate, glycerol, fructose-1,6-diphosphate or α -glycero-P, was used as a substrate. (b) The phospholipid effect was temperature-dependent with maximal stimulation occurring at 37° (Table 6). (c) Both the control ^{32}Pi incorporation and norepinephrine stimulation was lost upon freezing and thawing. (d) The ^{32}Pi incorporation either increased or remained unchanged when the irides were aged up to 3 days at 4°. The norepinephrine stimulation increased after 1 day of aging but decreased gradually during days 2 and 3 (Figure 3). (e) The phospholipid effect was nonspecific with respect to subcellular distribution (Table 7) as has been shown for rat brain cortex clices (4).

Reactions which have so far been proposed to be responsible for or involved in the phospholipid effect are:

(a) the synthesis of PhA by 1,2-diacylglycerol kinase,

(b) the breakdown of PhA by phosphatidate phosphatase,

(c) the breakdown of PhI by cleavage of the glycerol-phosphate bond (phospholipase C),

(d) the breakdown of PhI by cleavage of the phosphate-inositol bond (phospholipase D or through complete or partial reversal of the cytidine nucleotide pathway of PhI biosynthesis) and

(e) the conversion of TPI to DPI by TPI-phosphomonoesterase (Figure 1).

When norepinephrine was added to the subcellular fractions derived from irides incubated with [γ-^{32}P] ATP, there was no increase in the ^{32}P-labeling of PhA and PhI over that obtained when norepinephrine was not added to the incubation mixture. These results showed that direct activation of the enzyme diglyceride kinase by norepinephrine does not appear to be the molecular mechanism underlying the phospholipid effect. Lapetina and Hawthorne (43) found that acetylcholine had no stimulatory effect on diglyceride kinase of isolated intact nerve-ending particles or of nerve-ending membranes of rat cerebral cortex obtained after osmotic shock.

A recent paper (37) showed that acetylcholine caused a net decrease in PhI and a net increase in PhA in mouse pancreas. The present studies showed that the addition of norepinephrine (0.3 mM) brought about a 25 per cent decrease in the level of PhI and a 63 per cent increase in the level of PhA of iris muscle (Table 1).

Banschbach, et al. (5) showed a net increase in the level of diglyceride in mouse pancreas upon stimulation by acetylcholine. No increase in the level of diglyceride was observed in iris muscle upon stimulation by norepinephrine (Table 10 and 11). Even though no increase in diglyceride level was observed, this does not rule out the possibility that more diglyceride is being formed, which is then rapidly converted to PhA.

The finding that the specific radioactivity of PhA remained almost constant between 0 and 90 min of control incubation, but increased in the presence of norepinephrine, while that of PhI and PhC increased with time in control and was further stimulated by norepinephrine (Table 9)

suggests the following possibilities. (1) There are two pools of PhA, only one of which is responsive to norepinephrine. (2) The neurotransmitter stimulates the hydrolysis of the cold (endogenous) PhA and PhI to form more diglyceride and the diglyceride is then rephosphorylated in the presence of diglyceride kinase to form more labeled PhA.

Abdel-Latif, et al. (3) have demonstrated a pronounced phosphatidic acid effect in sympathetically denervated rabbit iris muscle in response to addition of small amounts of norepinephrine (3 μM). This suggests a post junctional site for the phospholipid effect.

Insofar as the physiological significance of the phospholipid effect is concerned, one of the lines of evidence which has been thought to dissociate the PhI response from immediate involvement at the cell surface in receptor activation or secretory processes has been the demonstration that the newly-synthesized PhI in stimulated cells is not localized at the cell surface. However, PhI synthesis is a secondary response (16,44) and measurement of the subcellular distribution of newly labeled PhI is not equivalent to measurement of the initial stimulation site. It is more likely that this response is a composite measurement of the intracellular distribution of the enzymes which catalyze the final steps of PhI biosynthesis and of the activity of the PhI exchange proteins which distribute newly synthesized PhI throughout the membranes of the cell. Abdel-Latif, et al. (2) have recently shown that in the iris muscle, acetylcholine (0.05 mM) appeared to bring about the phospholipid effect by stimulating the hydrolysis of TPI to DPI which subsequently leads to an increase in PhI and PhA labeling. Hendrickson and Reinertsen have proposed that TPI $\longrightarrow$ DPI

conversion and vice-versa could conceivably bring about a reorganization of the membrane with a resulting change in Na^+ and K^+ permeability (27). Torda (71) envisions the receptor to which acetylcholine binds as an inhibitory regulatory subunit of the enzyme catalysing the tri ——→ diphosphoinositide conversion (triphosphoinositide phosphomonoesterase). Thus, the phospholipid effect may be directly connected with the process of synaptic transmission per se, or it may represent an alteration in the properties of the membrane following transmission of the nerve impulse. It must be realized that though considerable information has been elucidated on this effect, at present, the specific molecular mechanism and physiological significance of this effect are still not completely understood.

SUMMARY

1. The phospholipid composition of the iris muscle was found to be similar to that of heart and skeletal muscle (67). PhA and PhI accounted for only 0.5 and 5 per cent of the total phospholipid respectively.

2. Acetylcholine (0.3 mM) increased the ^{32}P-labeling of PhA by 105 per cent and PhI by 129 per cent. This stimulatory effect was abolished upon the addition of atropine. Carbamylcholine was less effective than acetylcholine in stimulating ^{32}P-labeling of PhA and PhI.

3. At 0.3 mM concentrations, norepinephrine, epinephrine and dopamine exerted up to 200 per cent stimulation on the ^{32}P-labeling of PhA and PhI. In contrast, normetanephrine, metanephrine and adrenochrome had a much lesser effect. 6-Hydroxydopamine and histamine exerted an inhibitory effect on the ^{32}P-labelling of PhA and PhI.

4. Only α-stimulators, e.g. phenylephrine, increased significantly the ^{32}P-labeling of PhA and PhI. The norepinephrine-stimulated phospholipid effect was blocked by α-blockers such as phentolamine (0.003, 0.03 mM) and phenoxybenzamine (0.003, 0.03, 0.3 mM).

5. β-Stimulators such as isoproterenol had little influence on the ^{32}Pi incorporation into phospholipids. β-Blockers such as sotalol (0.003, 0.03, 0.3 mM) and propranolol (0.003, 0.03 mM) did not block the norepinephrine-stimulated phospholipid effect.

6. Dibutyryl cyclic AMP had no effect on the ^{32}P-labeling of PhA and PhI.

7. At higher concentrations (0.3 mM) phentolamine and propranolol increased by several-fold the labelling of PhA, PhI, CDP-diglyceride and inhibited ^{32}P incorporation into PhC. This observed effect of propranolol and phentolamine is not thought to be a result of drug reaction with adrenergic receptors.

8. The following observations indicate that certain enzymic pathways are being stimulated to produce the phospholipid effect. (a) In the presence of norepinephrine maximal stimulation was obtained when glucose, rather than pyruvate, glycerol, fructose-1,6-diphosphate or α-glycero-P, was used as a substrate. (b) The phospholipid effect was temperature-dependent with maximal stimulation occurring at 37^{o}. (c) Both the ^{32}Pi control incorporation and norepinephrine stimulation were lost upon freezing and thawing. (d) The ^{32}Pi incorporation either increased or remained unchanged when the irides were aged up to 3 days at 4^{o}. In contrast norepinephrine stimulation increased after one day of aging but decreased gradually during days 2 and 3. (e) The phospholipid effect was nonspecific with respect to subcellular distribution.

9. Norepinephrine had no effect upon diglyceride kinase activity in subcellular fractions of the iris muscle.

10. Norepinephrine (0.3 mM) brought about a 25 per cent decrease in the level of PhI and a 63 per cent increase in the level of PhA.

11. Norepinephrine (0.003, 0.3, 1.0 mM) did not increase the level of diglyceride in iris muscle.

12. The specific radioactivities of PhI and PhC increased with time in the absence of norepinephrine; however, the specific radioactivities of these two phospholipids increased even more in the presence of norepinephrine. The specific radioactivity of PhA increased only in the presence of the neurotransmitter.

13. No increase in diglyceride level upon norepinephrine stimulation was obtained, however, the possibility that the neurotransmitter stimulates the hydrolysis of the cold (endogenous) PhA and PhI to form more diglyceride, which is then rapidly rephosphorylated in the presence of diglyceride kinase to form more labeled PhA cannot be ruled out from the present experimental data.

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