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ADRENERGIC AND CHOLINERGIC STIMULATION OF ^{32}P-LABELING OF PHOSPHOLIPIDS IN RABBIT IRIS MUSCLE

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Abstract— The phospholipid and cholesterol composition of the iris muscle of the rabbit was determined, and the incorporation of ^{32}Pi into the individual phospholipids of irises incubated in Krebs–Ringer bicarbonate buffer that contained glucose in the presence or absence of adrenergic or cholinergic neurotransmitters and their agonists and antagonists was investigated. The results of studies on the characteristics and the effects of norepinephrine and other neuropharmacological agents on the ^{32}P-labeling of phosphatidic acid (PhA), phosphatidylinositol (PhI), phosphatidylcholine (PhC), phosphatidylethanolamine (PhE) and phosphatidylserine (PhS) in the iris muscle are reported. Addition of L-norepinephrine, L-epinephrine, dopamine or acetylcholine (0·003 to 0·3 mM) to irises which were preincubated for 20 min in ^{32}P-Krebs–Ringer without cold phosphate stimulated significantly the isotope incorporation into PhA and PhI, but not PhC. In contrast histamine, noremetanephrine, metanephrine, adrenochrome, 6-hydroxydopamine and eserine (0·003 to 0·3 mM) had a negligible effect on the isotope incorporation. At higher concentrations of norepinephrine (10 mM), labeling of PhA, PhI and PhC was elevated to 989, 630 and 185 per cent of that of the control respectively. It was concluded that in the iris muscle α-receptors and not β-receptors are involved in the stimulatory action of norepinephrine on ^{32}Pi incorporation into phospholipids. This conclusion is based on the following findings. (a) Only α-stimulators, such as norepinephrine and phenylephrine, increased significantly the labeling of PhA and PhI. This effect was blocked by the α-blockers, phenoxybenzamine and phentolamine. (b) Isoproterenol, a β-stimulator, had no effect on the labeling of PhA and PhI. Sotalol and propranolol, both β-blockers, did not block the norepinephrine stimulation of ^{32}Pi incorporation. (c) Cyclic AMP (or dibutyryl cyclic AMP), which has been suggested as a β-receptor mediator, exerted no influence on the phospholipid effect. Atropine blocked completely the acetylcholine-stimulated ^{32}P-labeling of PhA and PhI. At 0·3 mM, phentolamine and propranolol increased by several-fold the isotope labeling of PhA, PhI and, unexpectedly, of CDP-diglyceride and inhibited that of PhC. The properties of the norepinephrine stimulation of ^{32}P-labeling in the iris muscle can be summarized as follows. (a) It is concentration dependent; but this dependence varies with the condition of incubation. (b) It is temperature dependent, but the effect is lost upon freezing and thawing. (c) It can be demonstrated during a period of 3 days of aging at 4°. (d) Glucose is required for maximal stimulation. (e) The phospholipid effect is not specific to a particular subcellular fraction. (f) Addition of norepinephrine brought about a 25 per cent decrease in the level of PhI and a 63 per cent increase in the level of PhA. (g) Time-course studies on the ^{32}P-labeling of phospholipids in the presence and absence of norepinephrine revealed that, in contrast to PhI and PhC, the specific radioactivity of PhA increased with time of incubation (0–90 min) only when the neurotransmitter was added. (h) Direct activation of the enzyme diglyceride kinase by norepinephrine does not appear to be the molecular mechanism underlying the phospholipid effect. Our data suggest that in the iris muscle norepinephrine increases the turnover of PhA and PhI phosphorus by stimulating the hydrolysis of endogenous PhA or PhI, or both, to form more membraneous diglyceride. The latter is then rephosphorylated by diglyceride kinase to form more labeled PhA.

It has been demonstrated by a number of workers that cholinergic and adrenergic neurotransmitters stimulate the incorporation of ^{32}Pi into phosphatidic acid (PhA) and phosphatidylinositol (PhI) in a wide variety of cells and tissues, including pancreas [1], brain [2] and its subcellular fractions, mainly the synaptosome [3, 4], sympathetic ganglia [5], pineal [6], parotid [7] and rat vas deferens [8]. The mechanism of the phospholipid effect is still undefined at the molecular, subcellular or cellular levels, although a reasonable amount of progress has been made in this direction in the past few years [9–14]. In a previous communication [15] from this laboratory it was observed that in the iris muscle of the rabbit norepinephrine, other catecholamines, various α- and β-adrenergic receptor blocking agents, and acetylcholine exerted a marked stimulatory effect on the ^{32}Pi incorporation into PhA and PhI and a much lesser effect on phosphatidylcholine (PhC). The increase in the ^{32}P-labeling of PhA and PhI in the presence of norepinephrine or acetylcholine, which ranged from 2- to 6-fold, was found to be time- and concentration-dependent.

The iris muscle of the rabbit is innervated by cholinergic and adrenergic fibers [16], and the presence of both types of adrenergic receptors has also been demonstrated in the same tissue [17, 18]. The primary objectives of the present studies were: (1) to show whether the phospholipid effect in the iris muscle is mediated by α- or β-receptors; and (2) to shed more light on the molecular mechanism underlying this effect.

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MATERIALS AND METHODS

Preparation and incubation of iris muscle. Unanesthetized albino rabbits of either sex, weighing approximately 2 kg, were employed. They were stunned by a blow to the head, exsanguinated and the eyes were enucleated within 15 min of death and placed in Krebs–Ringer bicarbonate buffer, pH 7·4, that contained 11 mM glucose, at 0°. After transportation from the slaughter house (25 min), the iris muscle, which weighed about 36–40 mg, was removed from each eye and placed in the Krebs–Ringer buffer at 0°. In some experiments one or more irises were placed in test tubes containing 1·3 ml of the same buffer, which was gassed with 5% CO_2 in O_2 before use. About 25 μCi of ^{32}Pi was added to each tube and neurotransmitters and pharmacological agents were then added as indicated to give a final volume of 1·5 ml. In other experiments the irises were first preincubated in ^{32}P-Krebs–Ringer without cold phosphate for 20 min, then each was reincubated under various conditions as indicated. After incubation at 37° for 1 hr in a shaking incubator, the irises were washed twice with 5 ml of ice-cold Krebs–Ringer bicarbonate buffer, followed by addition of 5 ml chloroform–methanol (2:1, v/v).

Preparation of subcellular fractions. The irises were homogenized according to the procedure of Clark [19], except that a glass homogenizer was used in the present work, and the various subcellular fractions were isolated by means of conventional differential centrifugation. The nuclear, mitochondrial and microsomal fractions were precipitated at 3000, 8000 and 120,000 *g* respectively.

Assay for diglyceride kinase in the subcellular fractions. Subcellular fractions, equivalent to 2 mg protein, were incubated for 5 min at 37° in a mixture containing: Tris buffer, pH 7·4, 30 mM; $MgCl_2$, 6 mM; and other reagents as indicated. To initiate the reaction, 1·5 μmoles [γ-^{32}P]ATP ($0{\cdot}5 \times 10^6$ cpm) was added to the incubation medium. The final volume was 0·5 ml. The reaction was terminated by the addition of 0·5 ml chloroform–methanol (2:1).

Extraction and isolation of phospholipids. Lipids were extracted from each of the irises, the individual phospholipids isolated from the extracts by means of two-dimensional thin-layer chromatography (TLC) and their radioactivities determined as previously described [14, 15, 20]. In general, we found that one iris contains about 0·5 to 0·6 μmole of lipid phosphorus. In brief, each of the irises was homogenized in 5 ml chloroform–methanol (2:1), the insoluble material was centrifuged at 1000 *g* for 30 min, and the supernatant was washed twice with 0·15 M NaCl. The chloroform-rich layer was concentrated *in vacuo*, and the phospholipids were redissolved in 0·1 ml chloroform–methanol (2:1) and separated into the individual phospholipids by means of two-dimensional TLC employing Silica gel H. In the present work the Silica gel slurry was prepared by mixing 18 g Silica gel H plus 2 g magnesium silicate (Supelcosil 41 A, obtained from Supelco, Inc.) in 65 ml deionized water. Addition of the silicate brought about the complete separation of PhI and phosphatidylserine (PhS). The solvent systems used were the following: solvent A, which consisted of chloroform–methanol (28%, v/v) ammonia (65:25:4, by vol.); and solvent B, which consisted of *n*-butanol–acetic acid–water (6:1:1, by vol.). Lipids were detected by means of iodine vapor, and the phospholipid spots were scraped from the plates, then counted in a Beckman liquid scintillation counter as described previously [20].

Total phosphorus and Pi were determined as reported by Bartlett [21]. Total cholesterol was determined according to the method of Momose *et al.* [22] using the perchloric acid–phosphoric acid–ferric chloride reagent. Protein was determined as described by Lowry *et al.* [23] with crystalline bovine serum albumin as a standard.

Materials. Norepinephrine (Levophed) and L-isoproterenol were obtained from Winthrop, Sotalol from Mead Johnson (1991-1), phentolamine (Regitine) from Ciba, phenoxybenzamine from S. K. & F. Labs.; the remainder of the neurotransmitters and pharmacological agents were purchased from Sigma Chemical Co. [^{32}P]phosphorus, carrier-free, was obtained from Schwarz/Mann. Adenosine-5-[γ-^{32}P]triphosphate, tetrasodium salt, was purchased from ICN Pharmaceuticals, Inc. All other chemicals were reagent grade.

RESULTS

Phospholipid composition of the iris muscle

As can be seen from Table 1, PhC, phosphatidyl ethanolamine (PhE), PhS and sphingomyelin constituted about 93 per cent of the total phospholipid-P of the iris muscle. In contrast, PhI and PhA, the metabolically active lipids which are mainly affected by neurotransmitters, were less than 5 and 1 per cent respectively.

Effect of different concentrations of various amines on phospholipid labeling

The purposes of preincubating the tissue for 20 min in the ^{32}P-labeled medium were: (a) to label the intracellular precursor pools, and (b) to exclude the possibility that the phospholipid effect is caused by an increase in the permeability to ^{32}Pi in the presence of the neurotransmitters. The amine concentrations used here were similar to those used by Michell and Jones [24] in their studies on the PhI labeling in rat parotid fragments exposed to α-adrenergic stimulation. At 0·3 mM concentrations, the neurotransmitters norepinephrine, epinephrine and dopamine exerted up to 200 per cent stimulation on the ^{32}P-labeling of PhA and PhI; however, at 0·003 mM, the effect was only 10–26 per cent of that of the control (Table 2). In contrast, noremetanephrine, metanephrine and adrenochrome had a much lesser effect. 6-Hydroxydopamine, a drug which is known to destroy sympathetic neurons selectively by causing acute degeneration of adrenergic terminals, inhibited the ^{32}P-labeling of the PhA by more than 20 per cent (Table 2). In contrast to our previous studies [15] in which we observed a stimulatory effect of 1 mM histamine on the ^{32}P-labeling of PhA and PhI, in the present studies we found that 0·3 mM of this amine exerts an inhibitory effect on the ^{32}P-labeling of PhA and PhI. In addition to the difference in concentrations, in the present

Table 1. Phospholipid and cholesterol composition of rabbit iris muscle*

Lipid	P (μmoles/g wet tissue)	P (% 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	

* In this experiment 2·8 g of irises was extracted with chloroform–methanol (2:1), the individual lipids were isolated by means of two-dimensional TLC, and the spots were either digested as was previously described [20] or eluted, and the phospholipid P was determined. In general the lipid phosphorus was lower by 10 per cent when the spots were eluted. Lipid P (1·5 μmoles) was spotted on each TLC plate. The results were obtained from two different experiments.

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

studies we preincubated the slices in ^{32}P-Krebs-Ringer, without cold phosphate, for 20 min in the absence of the amine. The preincubation step appears to bring about formation of more diglyceride in the slices and thus a larger increase in the ^{32}P-labeling of the phospholipids, and subsequently an alteration in their response to the amines. The results we obtained from a number of the following experiments, e.g. the effect of isoproterenol, support this suggestion.

Effect of different concentrations of cholinergic agents on phospholipid labeling

Acetylcholine increased the labeling of PhA and PhI markedly (Table 3). The acetylcholine stimulation was blocked by atropine. Eserine had no effect on the ^{32}P-labeling of phospholipids and, as was reported by other investigators, carbamylcholine was less effective than acetylcholine.

Effect of α- and β-adrenergic agonists and antagonists on the ^{32}P-labeling of phospholipids

These studies were undertaken in order to demonstrate whether in the iris muscle the phospholipid effect is mediated by the α- or β-receptors, or by both. As can be seen from Table 4, norepinephrine, an α- and β-receptor stimulator, and phenylephrine, which specifically stimulates adrenergic α-receptors [25], increased appreciably the labeling of PhA and PhI but not PhC. In contrast, isoproterenol, a β-receptor stimulator [25], had no effect on the ^{32}P-labeling of phospholipids. (See the previous section for the possible reasons for the discrepancy from our reported observations with this drug.) This clearly demonstrates, as was shown in the rat parotid [7, 24], that in the iris the stimulation of phospholipid metabolism in the presence of α-agonists involves α-receptors. To support this conclusion we investigated the influence

Table 2. Effect of different concentrations of amine neurotransmitters and their metabolites on labeling of phospholipids in the iris muscle*†

Amine added	Effect of the amines on phospholipid labeling: PhA (% of control)	PhI (% of control)
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

* In the above experiment all the irises 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 test tubes and reincubated for an additional 40 min in 1·5 ml of fresh ^{32}P-labeled Krebs–Ringer in the presence and absence of the amines. The results reported here are averages of two different experiments; each was run in duplicate. In general the variations in this experiment as well as in the following experiments ranged from 0 to 5 per cent between triplicates in the same experiment and between different experiments.

† In this experiment PhC and PhE were analyzed and the values for both ranged between 95 and 110 per cent.

‡ These values correspond to 0·003, 0·03 and 0·3 mM concentrations respectively.

Table 3. Effect of different concentrations of cholinergic agents on labeling of PhA and PhI in the iris muscle*

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

* Conditions of incubation were as described in footnote to Table 2.

† 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.

of α- and β-adrenergic blocking agents on the phospholipid effect (Table 4). Sotalol, a β-blocker, exerted no effect on the norepinephrine-stimulated phospholipid labeling. DL-Propranolol, a β-receptor blocker, did not block the norepinephrine-stimulated phospholipid effect; however, at 0·3 mM concentration it increased the ^{32}P-labeling of PhA and PhI by 10- and 2-fold respectively. Furthermore, it inhibited almost completely the ^{32}P-labeling of PhC. In contrast the drug 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. The effect of higher concentrations (0·3 mM) of propranolol and phentolamine on various phospholipids in the iris muscle revealed a dramatic increase in the ^{32}P-labeling of PhA, PhI and, unexpectedly, of CDP-diglyceride but not PhE, and a significant inhibition of PhC (Table 5). In Table 5 one can see the high level of ^{32}P-labeling of the various phospholipids under our conditions of incubation. Further evidence that α-receptors are involved in the phospholipid effect comes from the present studies on phenoxybenzamine, an α-receptor blocker. As can be seen from Table 4, this drug abolished the norepinephrine-stimulated ^{32}P-labeling of phospholipids. In accord with our previous studies on cyclic AMP [15], dibutyryl cyclic AMP had no influence on the phospholipid effect.

Studies on properties and mechanism of the phospholipid effect

The second objective we sought in the present studies was to learn more about the properties and the molecular mechanism underlying the phospholipid effect. In all of the following experiments, unless indicated otherwise, Krebs–Ringer containing cold phosphate was used and the irises were not preincubated. The following are some of the experiments we have undertaken.

Effect of glucose and its intermediates on the phospholipid effect. When glucose, pyruvate, glycerol, fructose 1,6-diphosphate or α-glycero-P were included in the Krebs–Ringer, only glucose increased significantly

Table 4. Effect of different concentrations of α- and β-adrenergic agonists and antagonists on the labeling of phospholipids in the iris muscle*

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

* Conditions of incubation were the same as described in footnote to Table 2, except that the agonists were added in the final 20 min of incubation, while the antagonists were added immediately after preincubation of the irises.

† These values correspond to 0·003, 0·03 and 0·3 mM concentrations respectively.

Table 5. Effect of propranolol and phentolamine on the incorporation of ^{32}Pi into phospholipids of the iris muscle*

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

* These data are taken from an experiment similar to that described in Table 4.

† PhS was identified by incubating irises in Krebs–Ringer medium containing DL-serine[1-^{14}C]. All the radioactivity was found in the PhS spot. Furthermore, in order to show whether the PhS contained any PhI, the irises 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 t.l.c. and through the use of [^{3}H]cytidine as precursor. More than 95% of the stimulatory effect of the drugs was found in this compound.

the ^{32}P-labeling of PhA and PhI and the stimulatory effect of norepinephrine.

Effect of varying the temperature on the phospholipid effect. Studies on the effect of temperature on the ^{32}P-labeling of the iris muscle showed that only PhA was significantly labeled at 0 (Table 6). While PhI had a negligible amount of radioactivity, PhC contained none. When the temperature was raised to 15°, PhA labeling increased by almost 18-fold, PhI increased significantly, and PhC still showed no radioactivity. In contrast, when the temperature was raised from 15° to 22°, the level of radioactivity in PhI increased by 8-fold while that of PhA was less than 3-fold. Again PhC contained little radioactivity. The increase in the labeling of PhA, PhI and PhC when the temperature was raised from 22° to 37° was found to be around 2-, 8- and 37-fold respectively. The stimulatory effect of norepinephrine on phospholipid labeling increased gradually with temperature (Table 6).

Effect of freezing and thawing on the phospholipid effect. When the irises 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 slices which were frozen and thawed. Thus, values for PhA obtained from a typical experiment showed that, in contrast to the normal slices where norepinephrine (0·3 mM) stimulated the PhA labeling from $2{\cdot}59 \times 10^4$ to $6{\cdot}68 \times 10^4$ cpm, in the frozen and thawed slices it stimulated the labeling from 192 to 198 cpm.

Effect of norepinephrine on the ^{32}Pi incorporation into phospholipids of rabbit iris muscle—subcellular distribution. To localize the site of the phospholipid effect and thus gain more insight into the molecular mechanism as well as the physiological role of this effect in the iris muscle, the irises were first incubated in Krebs–Ringer buffer containing ^{32}Pi in the presence and absence of norepinephrine (10^{-2} M) for 1 hr, then subfractionated by means of differential subfractionation into their respective subcellular fractions. We used a higher concentration of norepinephrine because in our previous studies [15] we observed maximal stimulation at this concentration. Furthermore, PhC is also stimulated at this concentration. All the subcellular fractions were found to be susceptible to the stimulatory effect of norepinephrine, and in general PhA and PhI and, to a lesser extent, PhC were the phospholipids most affected (Table 7).

Effect of aging on the phospholipid effect. To investigate the stability of the phospholipid effect in the iris muscle, the action of norepinephrine (0·5 mM) on the ^{32}P-labeling of phospholipids was investigated in irises which were kept at 4° for periods up to 3 days (Fig. 1). Maximal stimulation for PhA and PhI labeling was observed after 1 day, then it decreased gradually with aging. Interestingly, the level of ^{32}P-labeling in all of the phospholipids was highest after 3 days of aging. Thus, in irises which were dissected 1 hr prior to incubation, the radioactivities (cpm/spot) which were found in PhA, PhI and PhC were 6,429, 10,557 and 10,055; in irises which were aged for 3

Table 6. Effect of varying the temperature on the labeling of phospholipids in the presence and absence of norepinephrine*

Temperature	Activity (cpm/spot) PhA	PhI	PhC	Effect of norepinephrine on phospholipid labeling (% of control) PhA	PhI	PhC
0	63 (67)†	3‡ (3)	0 (0)	106	100	
15	1,134 (1,478)	168 (224)	0 (0)	135	133	
22	2,947 (4,358)	1,395 (1,893)	37 (31)	148	136	
37	5,187 (9,462)	11,505 (16,715)	1,384 (1,425)	182	145	103

* One iris was placed in each tube which contained 1·3 ml Krebs–Ringer (with phosphate) and 25 μCi ^{32}Pi. Norepinephrine (0·5 mM) was added as indicated. Final volume was 1·5 ml and time of incubation was 30 min.

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

‡ This radioactivity is insignificant and thus cannot be used in computing the % increase in PhI with temperature.

Table 7. Effect of norepinephrine on the incorporation of ^{32}Pi into phospholipids of subcellular fractions isolated from incubated iris muscle*

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

* In this experiment 23 irises were placed in each of two 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° for 1 hr, the irises were homogenized and subfractionated by means of differential centrifugation as described under Methods.

† Activity in the control (cpm/spot).

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

§ Stimulatory effect of norepinephrine expressed as % of control.

¶ The activities of the following enzyme markers were determined: (1) NADPH-cytochrome reductase and (Mg^{2+} + Ca^{2+})-dependent ATPase for the microsomal fraction; and (2) succinic dehydrogenase for the mitochondrial fraction. The values obtained for the nuclear, mitochondrial and microsomal fractions were respectively: NADPH-cytochrome reductase, 1·02, 4·5 and 7·35 units, where the latter is defined as the increase in O.D. at 550 nm/mg protein per hr; [Mg^{2+} + Ca^{2+}]-dependent ATPase, 2·54, 2·58 and 14·40 units, where the latter is defined as that which splits 1 μmole ATP/mg of protein per hr; and succiniedehydrogenase, 0, 1·22 and 0 units, where the latter is determined as that which reduced $K_3Fe(CN)_6$ at 20° and is expressed as the decrease in O.D. at 400 nm/mg of protein/hr.

days, the radioactivities were 7,313, 14,947 and 24,287 respectively. The increase in the ^{32}P-labeling of the phospholipids could be due to the enzymatic hydrolysis of the latter to form more diglycerides with aging.

Effect of norepinephrine on PhA and PhI levels in iris muscle. In a recent paper Hokin-Neaverson [26] showed that acetylcholine caused a net decrease in PhI and a net increase in PhA in mouse pancreas. It was of interest to show if norepinephrine has any effect on the level of these lipids in the iris muscle. We found that norepinephrine (up to 0·3 mM) can indeed bring about a decrease in the level of PhI and an increase in PhA (Table 8). Thus at 0·3 mM of norepinephrine the levels of PhI and PhA were 75 and 163 per cent of that of the control respectively.

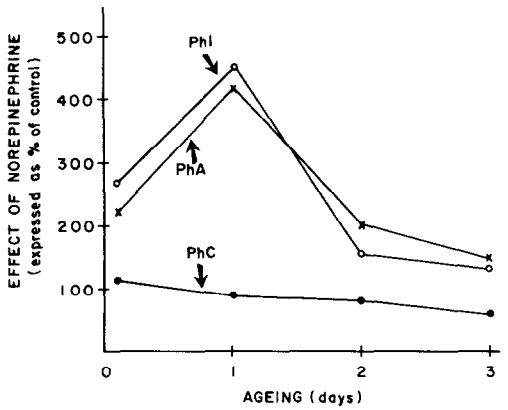

Fig. 1. Effect of norepinephrine, expressed as per cent of control, on ^{32}Pi incorporation into PhA, PhI and PhC of irises which were isolated and kept at 4° in Krebs Ringer for 2, 24, 48 and 72 hr prior to incubation for 40 min at 37° in the presence and absence of norepinephrine (0·5 mM).

Effect of norepinephrine on diglyceride kinase activity in the subcellular fractions of the iris muscle. The finding that the phospholipid effect was distributed in all the subcellular fractions (Table 7), coupled with the fact that the level of PhA was increased in the norepinephrine-stimulated iris muscles (Table 8), suggested to us that norepinephrine could be acting by stimulating the diglyceride kinase. The results from these studies showed that direct activation of diglyceride kinase by norepinephrine does not appear to be the molecular mechanism underlying the phospholipid effect.

Effect of norepinephrine on the rate of incorporation of ^{32}Pi into the iris phospholipids. Results of studies on the effect of norepinephrine on the rate of ^{32}Pi incorporation into PhA, PhI and PhC are summarized in Table 9. While the specific radioactivities of PhI and PhC increased with time both in the presence and absence of norepinephrine, that of PhA increased only in the presence of the neurotransmitter. This could suggest either that there are two pools of PhA and only one of them is being stimulated by norepinephrine, or that the latter could be stimulating the enzymatic hydrolysis of specific phospholipids, e.g. PhA or PhI, or both, to form more membraneous diglycerides.

DISCUSSION

An advantage of working with the iris muscle is that it is an instant tissue slice whose dimensions are such that it can be immersed in an appropriate medium and various parameters of metabolism studied

Table 8. Effect of norepinephrine on PhA and PhI levels in the iris muscle*

Agent added	Conc (mM)	PhI (μmoles phospholipid-P/g wet tissue)†	PhA (μmoles phospholipid-P/g wet tissue)†
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

* In this experiment each flask contained 10 irises. Each experiment was run in duplicate at 37° for 1 hr. Norepinephrine was added as indicated. The results are averages of two different experiments.

† In this experiment the phospholipid spots were scraped off the plates, then digested in perchloric acid and their phosphate contents determined according to the procedure of Bartlett [21]. The levels of PhC and PhE did not change under the same conditions.

without further dissection. This characteristic of the iris, coupled with the finding in the present studies that the phospholipid effect can still be demonstrated after 3 days of aging (Fig. 1), has facilitated our work and made it rather easy to obtain very reproducible data. The composition of phospholipids of the iris muscle was found to be similar to those of liver, kidney and spleen [27]. Thus, PhC accounted for about 41 per cent of the total phospholipid P, PhE for 32 per cent, and sphingomyelin and PhS for 12 and 8 per cent respectively. PhA and PhI accounted for only 5 and 0·5 per cent respectively. There is little information on phospholipid metabolism in the iris muscle. Previously we showed that when the iris muscle was 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 [15]. This observation, coupled with the results obtained from the temperature experiment (Table 6) in which the PhA/PhI ratios at 0°, 15°, 22° and 37° were 21, 6·7, 2·1 and 0·5, respectively, suggests a central role for PhA in phospholipid biosynthesis in the iris muscle, and the results are similar to those found in liver [28].

The iris consists of dilator and sphincter muscles and both contain α- and β-adrenergic receptors [18]. Norepinephrine, predominantly an α-receptor agonist, induces contraction of the smooth muscle of the iris, while isoproterenol, a β-receptor agonist, causes relaxation [29]. The findings that both adrenergic and cholinergic neurotransmitters enhanced the ^{32}P-labeling of PhA and PhI (Tables 2 and 3 respectively) could suggest a physiological role for the phospholipid effect. In contrast, their metabolites (Table 2) or histamine, which has not yet been shown to function as a neurotransmitter [30], exerted little effect on the ^{32}Pi incorporation. It is interesting to note that an increase in the ^{32}P-labeling of PhC was observed only at higher concentrations (1–10 mM) of cholinergic or adrenergic neurotransmitters [15]. Thus, in the present studies, when lower concentrations of the neurotransmitters (0·03 to 0·3 mM) were added to irises which were preincubated in ^{32}P-Krebs–Ringer for 20 min, only the labeling of PhA and PhI was affected. Initially the objective from the preincubation step was to label the intracellular precursor pools and to exclude the possibility that the phospholipid effect is caused by an increase in the permeability to ^{32}Pi in the presence of the neurotransmitter. However, the results from our present studies show that this step is required in obtaining this effect at lower concentrations of the neurotrans-

Table 9. Effect of norepinephrine on the rate of incorporation of ^{32}Pi into the iris phospholipids*

Time of incubation (min)	Specific radioactivity of phospholipids (cpm/μmole P $\times 10^{-4}$) 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)

* In this experiment the irises were preincubated in Krebs–Ringer containing ^{32}Pi (8 μCi/ml) for 20 min at 37°. The irises (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 reported in this table are averages of two different experiments.

† Control.

‡ In the presence of norepinephrine.

mitters. This appears to hold true also for the neuropharmacological agents. This step could either serve to form more diglyceride in the slices or could increase the susceptibility of those enzymes involved in the phospholipid effect to the neurotransmitters. Under these experimental conditions the following findings, which could support a physiological role for the phospholipid effect, were observed. (1) Acetylcholine at concentrations of the order of 0·003 mM, but not eserine (0·03 mM), increased the ^{32}P-labeling of PhA and PhI by 40 and 75 per cent respectively. This effect was abolished upon the addition of atropine (Table 3). (2) Norepinephrine and epinephrine stimulated the ^{32}P-labeling of PhA and PhI markedly and this stimulatory effect appears to be mediated through α- and not β-receptors (Table 4). This conclusion is supported by the following observations. (a) Only α-stimulators, e.g. phenylephrine, increased significantly the ^{32}P-labeling of PhA and PhI, and the phospholipid effect was blocked by α-blockers such as phentolamine and phenoxybenzamine. (b) β-Stimulators such as isoproterenol had little influence on the ^{32}Pi incorporation into phospholipids, and β-blockers such as sotalol and propranolol did not block the norepinephrine-stimulated phospholipid effect. Adrenergic stimulation of the ^{32}P-labeling of phospholipids has been shown to be mediated through α-adrenergic receptors in rat parotid [7, 24], rat brain [13] and rat vas deferens [8]. (c) β-Receptors have been suggested to be mediated by cyclic AMP [31]. Results from our previous [15] and present studies on the iris showed that neither cyclic AMP nor dibutyryl cyclic AMP had any effect on the phospholipid effect. At higher concentrations (0·3 mM), phentolamine and propranolol increased by several-fold the labeling of PhA and PhI, and, unexpectedly, of CDP-diglyceride (Table 5). Furthermore, both drugs inhibited ^{32}Pi incorporation into PhC. This observed effect of propranolol and phentolamine could be due to their marked influence on phospholipid metabolism and is probably not related to their ability to block adrenergic receptors. In a more detailed study on the effect of propranolol we have shown that it increases the incorporation of serine into PhS; similarly, it increased the incorporation of [^{3}H]cytidine into CDP-diglyceride by more than 10-fold (A. Abdel-Latif and J. Smith, unpublished observations). Eichberg *et al.* [6] 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 ^{32}Pi incorporation into pineal acidic phospholipids.

In our studies on the molecular mechanism underlying the phospholipid effect we have shown the following. (a) In the presence of norepinephrine maximal stimulation is obtained when glucose is used as substrate. (b) The phospholipid effect is temperature-dependent with maximal stimulation occurring at 37° (Table 6). (c) Both the ^{32}Pi incorporation and norepinephrine stimulation are lost upon freezing and thawing. (d) The ^{32}Pi incorporation either increased or remained unchanged when the irises were aged up to 3 days at 4° (Fig. 1). In contrast, the norepinephrine stimulation increased after 1 day of aging but decreased gradually during days 2 and 3. (d) The phospholipid effect is nonspecific with respect to subcellular distribution (Table 7). These observations indicate clearly that certain enzymes are being stimulated to produce the phospholipid effect. The enzymes which have been implicated in the phospholipid effect are diglyceride kinase [32], PhA-phosphatase [12] and PhI-inositolphosphohydrolase [11]. The fact that diglyceride kinase [33], PhA phosphatase [14, 34] and PhI-inositolphosphohydrolase [35] are widely distributed in the cell could explain the nonspecific distribution of the phospholipid effect in the subcellular fractions of the iris muscle (Table 7). This finding is in accord with previous observations on the nonspecific distribution of the phospholipid effect in the subcellular and cellular [14] fractions of rat brain cortex slices. The finding that addition of norepinephrine brought about a 25 per cent decrease in the level of PhI and a 63 per cent increase in the level of PhA (Table 8) could suggest that this neurotransmitter could be acting by stimulating PhI-inositolphosphohydrolase and subsequently conversion of PhI into 1,2-diglyceride. Hokin-Neaverson *et al.* reported recently that addition of acetylcholine to slices from mouse pancreas decreased the level of PhI and increased that of PhA [26] as well as that of diglyceride [36]. The finding that the specific radioactivity of PhA remained almost constant between 0 and 90 min of incubation, in contrast to that in the presence of norepinephrine, while that of PhI and PhC increased with time (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. The latter is then rephosphorylated in the presence of diglyceride kinase to form more labeled PhA. Our data support the latter possibility.

Insofar as the physiological significance of the phospholipid effect is concerned, our feeling is that it could represent an alteration in the properties of the post-synaptic membrane following transmission of the nerve impulse. The function of this alteration could be to bring about the original conformation of the post-synaptic membrane. These alterations could correspond to the dephosphorylation (to form diglyceride) and rephosphorylation of the post-synaptic membrane. The latter could simply be functioning as a repair process. To shed more light on the physiological function of this effect we are investigating the effect of chemical denervation, surgical denervation and electrical stimulation of the sympathetic nerve on phospholipid metabolism in the iris muscle. Insofar as the molecular mechanism underlying the phospholipid effect is concerned, our data indicate an increase in 1,2-diglyceride, which could be caused by norepinephrine stimulation of either PhA-phosphatase or PhI-inositolphosphohydrolase, or both, since diglyceride kinase was shown not to be involved.

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