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SELECTIVE MODIFICATIONS IN THE *DE NOVO* BIOSYNTHESIS OF RETINAL PHOSPHOLIPIDS AND GLYCERIDES BY PROPRANOLOL OR PHENTOLAMINE

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Abstract—The effects of propranolol or phentolamine on the metabolism of phospholipids, diacylglycerol, and triacylglycerol were studied in the bovine retina *in vitro*. Lipid labeling was followed during short-term incubation of intact bovine retinas with [U-^{14}C]glycerol and [1-^{14}C]palmitic acid. Each of these precursors was recovered in the appropriate lipid moiety. Most of the [^{14}C]glycerol appeared progressively in triacylglycerol (TG) through the sequence from phosphatidic acid (PA) to diacylglycerol (DG). Labeled palmitate appeared in much lower quantities than labeled glycerol in all glycerolipids except phosphatidylcholine (PC). Propranolol and phentolamine greatly enhanced the [^{14}C]glycerol specific activities of PA, phosphatidylinositol (PI), and phosphatidylserine (PS), whereas labeling in other glycerolipids was much lower than in controls. The labeling in TG with both precursors was found to be less than 50% of the control values; however, a late increase in DG labeling was observed. The effects of these drugs on broken cell preparations were also described, although lipid synthesis from labeled glycerol in these preparations was only 9% that of intact retinas. It appeared that an amphiphilic cationic structure was necessary to produce these drug effects; propranolol glycol, the hydrophobic moiety of propranolol, did not elicit the same effects. It is suggested that, among other changes, the drugs inhibited phosphatidate phosphohydrolase and redirected the flux predominantly toward PI. Support for the proposed multiple lipid effects elicited by these drugs was provided by the dual changes found in the labeling of DG.

When intact tissues are incubated with radioactive precursors, cellular heterogeneity, subcellular complexity, and the variety of lipid pools involved make it difficult to draw a general scheme showing the flux of labeled precursors through the biosynthetic pathways of phospholipids. The whole retina is a complex organ, but, being a readily available thin slice of neural tissue, it offers many advantages for these studies. In the last few years, mammalian retinas have been used to survey various aspects of membrane lipid metabolism [1–8]. Nonetheless, only scant information on the *de novo* biosynthesis of glycerolipids and the effects of drugs on this pathway is available [9–12].

In this study, radioactive glycerol was used as a marker to observe the biosynthesis of lipids in whole bovine retinas and cell-free preparations. In addition, the labeling by several precursors and the effects of drugs were surveyed, extending previous studies from this laboratory [9–12].

MATERIALS AND METHODS

Chemicals. [U-^{14}C]Glycerol (7.4 mCi/mmole), [1-^{14}C]palmitic acid (55.65 mCi/mmole), [2-^{3}H(N)]glycerol (40 mCi/mmole), Aquasol, and Omnifluor were purchased from the New England Nuclear Corp., Boston, MA, U.S.A. Sterile $Na_2H[^{32}P]O_4$, (69 Ci/g of P), PH 7.4, was obtained from the Comision Nacional de Energia Atomica, Argentina; *dl*-propranolol HCl (Inderal) and phentolamine were gifts from the CIBA Pharmaceutical Co., Summit, NJ, U.S.A.; l-DOPA was obtained from CalBiochem, San Diego, CA, and propranolol glycol was a gift from Dr. Thomas Walle, Medical University of South Carolina, Charleston, SC, U.S.A. All organic solvents and chemicals were analytical reagent grade.

Incubation of retinas. Bovine eyes in ice were obtained from a nearby slaughterhouse. Retinas were dissected under dim light and then incubated (within 2 hr after death) in 7 ml of ionic medium [13], pH 7.3, containing 2 mg/ml of glucose at 37°. The medium was gassed with 5% CO_2 in O_2 and gently shaken during the incubation.

Radioactive precursors. Fifty microliters of sterile aqueous solution of either $Na_2H[^{32}P]O_4$ (40.8 pmoles) or [2-^{3}H(N)]glycerol (630 nmoles) or [U-^{14}C]glycerol (405 nmoles), plus 100 μl of [1-^{14}C]palmitic acid (105 nmoles) complexed with free fatty acid-free bovine serum albumin per retina, was added to the medium.

Preparation and incubation of retinal cell-free homogenates. Retinas were preincubated for 20 min as described above, and homogenized (500 mg wet wt/ml) in 10 mM HEPES†, pH 7.4, with a Potter–Elvehjem homogenizer. Fifty microliters of a 50%

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† Abbreviations: HEPES, 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid; PI, phosphatidylinositol; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine; DG, diacylglycerol; PA, phosphatidic acid; and TG, triacylglycerol.

ethanol solution of propranolol or propranolol glycol with the [^{3}H]glycerol described above was added (5.9 ml/flask), and the cell-free preparations were incubated immediately at 37°; 1 ml aliquots were taken throughout the incubation for analysis.

Extraction of lipids. Lipids were extracted from the tissue with chloroform–methanol by means of a motor-driven Teflon pestle in a Potter–Elvehjem homogenizer [14]. After two additional extractions of the residue, partitioning and washing were carried out in the presence of 0.08% glycerol, when radiolabeled glycerol was the precursor. These procedures and subsequent manipulations were performed under oxygen free-N_2. Overnight storage was at 36°.

Thin-layer chromatography. Diacylglycerols (DG), triacylglycerols (TG), free fatty acids, and total phospholipids were separated on gradient-thickness layers of silica gel G [14]. After development, visualization was attained by brief exposure to I_2 vapor. To quantify the DG and TG, a spray of 2% 2′,7′-dichlorofluorescein in methanol was used, followed by gas–liquid chromatography [14, 15]. Phospholipid classes were isolated by analytical, two-dimensional, thin-layer chromatography [16]. The identity of the phospholipids was confirmed by comparison of relative mobility with pure standards. Methyl esters of lipids were fractionated according to their degree of unsaturation by chromatography of 500 μM layers of Ag^+-impregnated (12%) silica gel G prepared in 30% NH_4OH as previously described [15].

Isolation of hydrophobic and hydrophilic moieties of lipids. Methyl esters were prepared from uneluted thin-layer chromatographic spots corresponding to neutral and polar lipids [14]. Hydrophobic and hydrophilic residues were recovered in the organic and aqueous phases respectively. Quantitative recovery of the corresponding radioactive lipid moiety was checked by additional incubations of retinas with [^{14}C]glycerol or [^{14}C]palmitic acid. Aliquots of lipids containing from 3534 to 277,633 cpm were analyzed. The contamination of both phases ranged between 0.02 and 0.3% of the total radioactivity.

Measurements of radioactivity. The content of ^{32}P was determined with a gas flow counter (Alfa-nuclear, Argentina). ^{14}C- and ^{3}H-labeled samples were counted by a liquid scintillation spectrometer (Packard Tricarb or Beckman LS-250), and the data were corrected for efficiency. Lipids were measured in glass vials containing Omnifluor 4% in toluene, and aqueous samples were mixed with 15 ml Aquasol.

Analytical methods. Lipid P was determined in uneluted thin-layer chromatographic scrapings after digestion by $HClO_4$ [16]; the proteins were measured by the method of Lowry *et al.* [17].

RESULTS

Desaturation and oxidation of [1-^{14}C]*palmitic acid in the isolated bovine retina.* Four retinas were incubated for 60 min in the presence of 0.1 μmole of [1-^{14}C]palmitic acid. Most of the activity was recovered with the saturated fatty acids. In fact, 96.5% of the acyl groups of the total phosphoglycerides was found by Ag^+-impregnated, thin-layer chromatography of the saturated fraction, the remainder being distributed as follows: monoenoic 1.5% and dienoic 2.1%. Under these experimental conditions, the [^{14}C]O_2 that evolved from the incubation flasks amounted to 0.1% of the total ^{14}C uptake in lipids.

Time-course labeling of retina lipids by [U-^{14}C]*glycerol and* [1-^{14}C]*palmitic acid.* The labeled precursors were actively taken up by the retina and incorported into lipids. After 5 min of incubation, the retina had taken up 7% of the lipid radioactivity present after 40 min of incubation. The time–course labeling of the lipid classes was uneven, and remarkable differences were found between the two radioactive precursors.

Phosphatidic acid (PA) showed the highest specific activity in the glycerol backbone at 5 min of incubation, followed closely by the specific activity of DG. The specific activity of TG at the same incubation time was lower (Fig. 1), but the accumulation of [^{14}C]glycerol in TG increased as a function of time. Both neutral glycerides contained about 80% of the total labeled glycerol taken up by lipids.

After 15 min of incubation, [^{14}C]glycerol uptake in TG represented about 50%, i.e. about five times

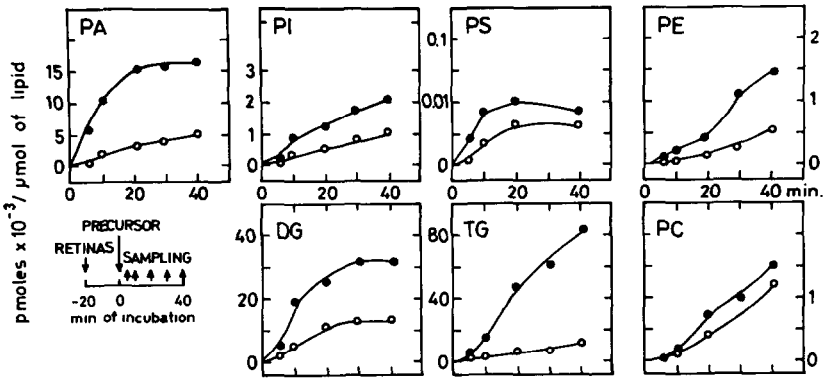

Fig. 1. Time–course modifications in lipid specific activities during incubation of bovine retina with [^{14}C]palmitic acid (○) and [^{14}C]glycerol (●). Retinas were preincubated for 20 min as shown on the left in the experimental outline. Other details are given in Materials and Methods.

Table 1. Effects of α- and β-adrenergic antagonists, DOPA, anoxia and depolarizing concentrations of K^+ on the incorporation of precursors into retina lipids*

Experiment	Precursors	Additions or conditions	Percentage of controls			
			Diacylglycerols	Triacylglycerols	Phospholipids	Residual lipid extract†
A	$^{32}P_i$	DOPA (2 mM)			99	89
		Propranolol + phentolamine			179	305
B	[1-^{14}C]Palmitic acid	DOPA (2 mM)	90	100	96	82
		Propranolol + phentolamine	125	39	125	111
		Propranolol + phentolamine + DOPA (2 mM)	121	45	128	196
		K^+ (55 mM)	101	85	101	63
		Anoxia‡	105	85	107	62
C	[1-^{14}C]Palmitic acid	Propranolol	253	31	148	
		Phentolamine	367	7	120	
	[U-^{14}C]Glycerol	Propranolol	348	31	109	
		Phentoalmine	256	12	105	

* Experiment A was performed by incubating retinas in triplicate with and without drugs for 80 min. At 15 min, 40.8 pmoles of $^{32}P_i$ per retina was added. Experiment B consisted of 65 min of incubation in the presence of 175 pmoles per retina of the labeled fatty acid. Afterward, the retinas were incubated in quadruplicate under the indicated conditions for an additional 60 min. Experiment C was carried out by preincubating retinas in duplicate for 20 min in the presence of drugs. Then, 105 nmoles of [1-^{14}C]palmitic acid and 405 nmoles of [U-^{14}C]glycerol were added per retina and incubation was continued for 40 min. Propranolol and phentolamine in concentrations of 500 μM were present in all experiments. Changes given were calculated from total activity/mass protein.

† Made after lipid extraction [14] by two successive extractions using acidified chloroform–methanol [18] and 2 M KCl [19]; the two extracts were combined.

‡ Incubation under 5% CO_2 in N_2.

the total reached at 5 min. This high rate of labeling had incorporated, at this time, most of the glycerol to be taken up during 90 min of incubation.

At 10 min incubation, the specific activity of DG surpassed the specific activity of PA due to the [^{14}C]glycerol uptake. Moreover, we found a remarkably rapid uptake of [^{14}C]palmitic acid in DG, which reached a maximum at 30 min.

PA displayed the highest specific activity of all the phospholipids incubated with [^{14}C]glycerol, followed by phosphatidylinositol (PI). Phosphatidylcholine (PC), phosphatidylethanolamine (PE), and phosphatidylserine (PS) showed much lower specific activities.

In all lipids, but mainly in PA and TG, the specific activity of [^{14}C]palmitate was less than that of [^{14}C]glycerol. Only in PC did both precursors yield similar labeling patterns (Fig. 1). The specific activity of the [^{14}C]acyl group in PC increased 300 times from 5 to 30 min, whereas in PE the enhancement was only 10-fold (Fig. 1).

Modifications of retinal lipid labeling from radiolabeled precursors under different conditions and in the presence of drugs. Table 1 shows typical results from experiments designed to compare the effects of alpha- and beta-adrenergic receptor blocking drugs on the labeling of different moieties in the lipids of isolated bovine retina. An increase in ^{32}P uptake in phosphoglycerides and in the residual lipid extract was produced by the simultaneous addition of propranolol and phentolamine, whereas DOPA did not produce any significant rise in ^{32}P uptake.

Labeling by [^{14}C]palmitate in diacylglycerol was not modified during 60 min of incubation (Table 1, experiment B), even with exposure of the retina to drugs, high K^+ concentration, or anoxia. Propranolol plus phentolamine, however, decreased the uptake of palmitate in triacylglycerol and increased it in the phospholipids. Again, under depolarizing concentrations of K^+ or anoxia, phospholipid labeling remained unchanged, and a slight decrease in TG labeling was observed. Propranolol or phentolamine, with either [^{14}C]palmitate or [^{14}C]glycerol as precursors, yielded enhanced labeling of DG and diminished labeling of TG when the retinas were incubated with drugs for 60 min (Table 1, experiment C).

The addition of 500 μM propranolol increased the labeling of PA with [U-^{14}C]glycerol or with [1-^{14}C]palmitic acid (Table 2). The largest effect produced by the drug took place during the 5-min period after the addition of the precursors. We observed a larger increase in radioactive acyl chains with respect to the glycerol moiety. The labeling of PI was also enhanced; the maximum [U-^{14}C]glycerol specific activity for this phospholipid occurred 10 min after the addition of the precursor. Another acidic lipid, PS, displayed enhanced labeling from the two precursors. A decrease in [1-^{14}C]palmitic acid, however, was observed after 30 min of incubation in these phospholipids. Whereas, at the beginning of incubation, *dl*-propranolol decreased DG labeling mainly in the glycerol backbone, after 30 min of incubation a 2-fold increase in uptake of both precursors had taken place. Specific activities in PC, PE and TG were inhibited drastically.

In the presence of 500 μM phentolamine, the

Table 2. Effects of propranolol on the incorporation of radiolabeled precursors into bovine retina lipids*

Lipid	5 (minutes of incubation)		10 (minutes of incubation)		30 (minutes of incubation)	
	[U-^{14}C]Glycerol	[1-^{14}C]Palmitic acid	[U-^{14}C]Glycerol	[U-^{14}C]Palmitic acid	[U-^{14}C]Glycerol	[1-^{14}C]Palmitic acid
	(pmoles/mg protein)		(pmoles/mg protein)		(pmoles/mg protein)	
PA	82.9 (630)	8.0 (888)	114.5 (491)	13.7 (360)	140.5 (395)	20.0 (229)
PI	11.7 (367)	1.1 (220)	51.7 (466)	5.5 (117)	78.5 (245)	4.2 (30)
PS	1.1 (183)	0.7 (700)	1.9 (130)	0.3 (50)	2.9 (181)	0.9 (75)
DG	3.9 (18)	5.9 (74)	15.8 (24)	12.3 (71)	225.4 (213)	80.9 (175)
PC	0.4 (1)	1.2 (75)	1.0 (5)	2.8 (15)	9.9 (77)	5.4 (9)
PE	1.3 (8)	0.2 (28)	9.5 (50)	13.7 (334)	36.6 (50)	3.9 (26)
TG	0.7 (5)	0.3 (30)	2.1 (4)	0.6 (14)	30.3 (10)	9.0 (37)

* *dl*-Propranolol (500 μM) was present for a 20-min incubation period prior to the addition of the radiolabeled compounds. Figures in parentheses are percentage changes relative to controls.

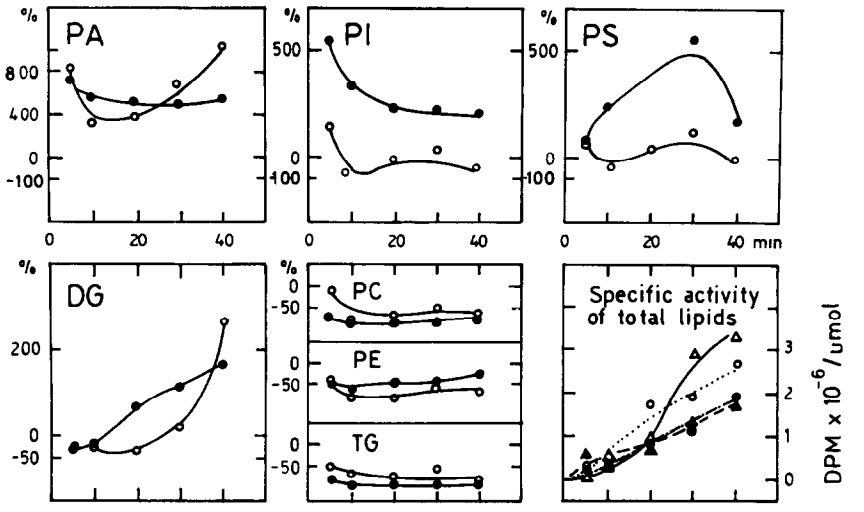

Fig. 2. Effects of phentolamine on the labeling of retinal lipids by [^{14}C]palmitic acid (○) and by [^{14}C]glycerol (●). Phentolamine (500 μM) was incubated with retinas for 20 min prior to the addition of precursors. On the lower right side, the specific activities of total lipids are given for controls (· · · ● · · ·) and phentolamine (- - ▲ - -) using [^{14}C]glycerol as precursor and for controls (· · · ○ · · ·) and phentolamine (—△—) using [^{14}C]palmitic acid as precursor. Other numbers are percentages of specific activities of controls.

labeling of acidic phospholipids with [U-^{14}C]glycerol increased several-fold. Whereas PA and PI showed their maximum values at the beginning of incubation, PS increased up to about 30 min (Fig. 2).

Radioactive fatty acid labeling in PA increased 10-fold after 5 min of incubation, whereas PI labeling increased only 2-fold over the control value. Throughout the incubation we found slight changes in the specific activities of palmitate in PI and PS. Changes in DG, PC and PE labeling with both precursors were similar to those promoted by propranolol. TG synthesis was found to be less depressed by phentolamine. Figure 2 also shows no changes in the specific activities for total lipids as a function of incubation time, with either palmitic acid or glycerol as precursors. Propranolol was also not able to modify the total lipid labeling (data not shown).

[2-^{3}H(N)]*Glycerol uptake in lipids from cell-free retinal homogenates.* Labeled glycerol incorporation was reduced drastically when homogenates were used instead of intact retinas. After 30 min of incubation, lipids of homogenates took up only 9% of

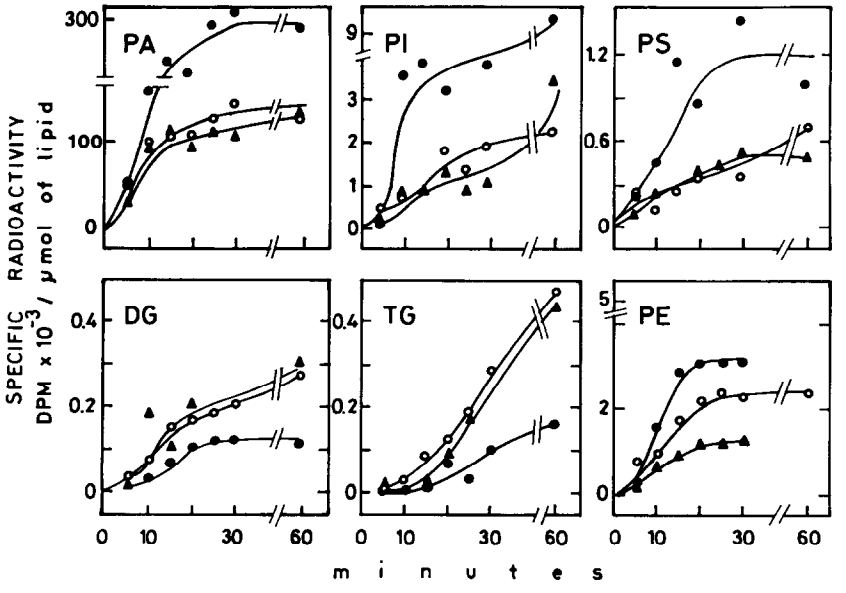

Fig. 3. [2-^{3}H(G)]Glycerol uptake in glycerolipids from retinal cell-free homogenates in the presence of *dl*-propranolol or propranolol glycol. Homogenates were made in 10 mM HEPES, pH 7.3, after preincubation (20 min) of retinas. Subsequently, suitable aliquots were incubated with radioactive glycerol as controls (—○—), 500 μM *dl*-propranolol (—●—) or 500 μM propranolol glycol (—▲—). Samples were taken at various incubation times. Further details are given in the text.

the [^{14}C]glycerol incorporated into lipids from the whole retina (Table 3). As in whole retinas, PA attained the highest specific activity (Fig. 1), followed by PE and PI. PC, on the other hand, incorporated very small amounts of [^{14}C]glycerol as compared to the labeling of monoacylglycerols (data not shown). We also found that homogenization reduced the specific activities of DG and TG.

Effects of propranolol and propranolol glycol on lipid synthesis from [2-^{3}H(N)]*glycerol in retinal homogenates.* We found that the effect exerted by propranolol on lipid synthesis also took place in cell-free preparations of the retina, either by enhancing the specific activities of PA, PI, and PS or by lowering the specific activities of DG and TG.

In contrast, these effects were not seen when propranolol glycol was added (Fig. 3). Except for PE, the specific activities of every lipid were the same as those of the controls. However, whereas PE labeling increased up to about 15 min of incubation with propranolol, an opposite effect was exerted by its metabolite.

The uptake of labeled glycerol in whole retinas or homogenates is shown in Table 3. In entire retinas, we observed an accumulation of about 20 pmoles of glycerol in acidic phospholipids due to propranolol. This value was similar to the decrease in labeling in neutral glycerides and PC. In contrast, the stimulation of acidic phospholipids in homogenate was greater than the decrease in radioactivity in neutral lipids. Thus, a larger increase in glycerol uptake (as a result of addition of the drug) was evidenced in this system.

DISCUSSION

The uptake of radiolabeled glycerol was used as an indicator of *de novo* biosynthesis, and palmitate was used as a marker of acylation. Short labeling experiments showed that no significant recycling of the precursors took place. Our results agreed with previous observations that [^{14}C]glycerol is an efficient precursor of glycerolipids in the isolated retina [6–10]. After passing through PA–DG, the major portion of the [^{14}C]glycerol accumulated in the TG pool; furthermore, palmitate labeling actively increased as a function of incubation time and, eventually, reached the lower levels of [^{14}C]glycerol labeling.

The labeling sequence reported in previous studies using other tissues [20] showed that most of the palmitate uptake in TG represents the fatty acid introduced during biosynthesis, rather than during turnover.

In diacylglycerols and in PC, [^{14}C]palmitate incorporation was similar to that of glycerol. Since the saturated fatty acid was slowly introduced into PA and PE, there were differences in the acylation of PC and PE labeled with [^{14}C]palmitate. These differences indicated that a high degree of selectivity in the cell membrane influenced the rate of the reaction. PC is known to be located in the outer leaflet of the plasma membrane, whereas PE exists mainly in the inner leaflet [21].

The rapid onset of action of either propranolol or phentolamine drastically modified retinal lipid metabolism. Thus, the increasing incorporation of ^{32}P, [^{14}C]glycerol, and [^{14}C]palmitate into lipids by the drugs was in agreement with previous reports which described the effect of propranolol on ^{32}P uptake in pineal gland lipids [22, 23] and in iris muscle [24]. When other precursors were used, however, stimulation of the biosynthetic route led to PI rather than to an enhancement of the ^{32}P turnover. On the other hand, the increase in [^{14}C]glycerol incorporation found in PS after the addition of either propranolol or phentolamine took place while both nitrogen-containing phospholipids and DG were depressed. Thus, it is likely that the precursor had not gone through DG, PE, and then PS by the base exchange reaction of L-serine by ethanolamine [25]. Moreover, the labeling of PS followed closely that of PI. The possibility that a route for PS synthesis from PA operates in the retina has been suggested previously [9, 11] and, recently, such linkage has been shown in rat brain microsomes [26]. Alternatively, a secondary effect of propranolol, e.g. releasing membrane-bound Ca^{2+} [24, 27], may stimulate the base exchange reaction to synthesize PS starting from a specific PE pool [25]. Although we found that PE synthesis from [^{14}C]glycerol was inhibited, the amount of label present did not rule out such a possibility.

In addition, we showed that both drugs exerted dual changes on DG synthesis. These data could be explained in terms of an additional source of this lipid, other than PA hydrolysis. A stimulated metabolic step from PI to DG cannot be excluded, since the amount of label in this phospholipid supports this possibility.

Comparison of the amount of radioactive glycerol

Table 3. Differential effect of propranolol on biosynthesis of lipids in whole retina and in retinal homogenates

	PA + PI + PS	DG + PC + TG	PE	Total
Whole retina*				
Control	28.4	22.1	2.4	52.9
Propranolol (500 μM)	50.9	0.60	0.40	51.90
Homogenates†				
Control	1.95	0.44	1.17	3.56
Propranolol (500 μM)	4.4	0.13	1.91	6.44

* Whole retinas were incubated as described in Materials and Methods. Five μCi [U-^{14}C]glycerol/retina was present for 5 min. Values are in pmoles [U-^{14}C]glycerol per mg protein.

† Retinal homogenate (25 mg protein) was incubated with 5 μCi [2-^{3}H]glycerol for 10 min as described in Materials and Methods. Values are in pmoles [2-^{3}H]glycerol per mg protein.

in the entire retina and in the retinal homogenates further supports the view that the stimulation of PA synthesis in the retina is an additional effect elicited by these drugs [7, 12]. Moreover, the accumulation of PA in microsomes from propranolol-treated retinas showed an overall enhancement of the acyl groups with a predominance of palmitate (unpublished). Thus, our observations also suggested that the acylation of saturated fatty acid in PA was enhanced.

Since propranolol and phentolamine were seen to exert similar effects on lipid metabolism, we concluded that these changes were not related to their adrenergic antagonist properties. The changes were most likely elicited by the entire molecule, particularly because propranolol glycol, which is the major propranolol metabolite produced in the nervous tissue [28] and is devoid of the isopropylamine group, did not mimic the lipid effect of propranolol. The site of this drug action appears to be intracellular, because the *de novo* biosynthetic enzymes of glycerolipids are on the endoplasmic reticulum. One explanation that fits our observations is that, if propranolol or phentolamine enter the cytoplasmic leaflet of the membrane, certain membrane-bound metabolic sequences will be altered. One change seen in retinal lipid metabolism was the inhibition of phosphatidate phosphohydrolase, which was elicited by some common property of these drugs; we found that the ionizable amine function was essential for this action. Further support for our hypothesis is the fact that fenfluoramine and its derivatives act by inhibiting phosphatidate phosphohydrolase [29], as do other cationic amphiphilic drugs [23, 24, 30, 31], including several with local anesthetic properties [32].

Our results suggest that propranolol or phentolamine elicited other changes in the retinal lipid metabolism, in addition to inhibiting phosphatidate phosphohydrolase. Also, the high *de novo* biosynthetic rate of retinal phospholipids and the action of the cationic amphiphilic drugs imply that this highly dynamic pathway may, in fact, be shifted at a branch regulatory site. Moreover, as yet unidentified intracellular moderators might, under physiological conditions, produce an action similar to that which modifies the availability of polar lipid classes for cellular membrane biogenesis or renewal.

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