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EFFECTS OF DL-PROPRANOLOL ON THE SYNTHESIS OF GLYCEROLIPIDS BY RABBIT IRIS MUSCLE

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Abstract—Propranolol (0.03–0.3 mM), an amphiphilic cationic drug which is used therapeutically as a β-blocker, was found to alter significantly the incorporation of [^{14}C]glucose, [^{14}C]glycerol, [^{14}C]acetate, ^{32}Pi, [^{3}H]cytidine, [^{3}H]inositol, [^{14}C]choline, [^{14}C]ethanolamine and [^{14}C]serine into phospholipids of the iris muscle. Furthermore, it was found to exert a stimulatory effect on the [^{14}C]serine incorporation into phosphatidylserine of the muscle and microsomes. In contrast, sotalol, another β-blocker-but lacking the hydrophobicity of propranolol-exerted no effect on lipid metabolism. Whereas norepinephrine stimulated only the turnover of the phosphate moiety of phosphatidic acid and phosphatidylinositol, in general propranolol caused the following changes: (a) it stimulated by 2- to 6-fold the labelling of phosphatidic acid and phosphatidylinositol from [^{14}C]glucose, [^{14}C]glycerol, [^{14}C]acetate, ^{32}Pi and [^{3}H]inositol, (b) it increased by 5- and 38-fold the incorporation of ^{32}Pi and [^{3}H]cytidine, respectively into CDP-diglyceride, (c) it inhibited appreciably the incorporation of [^{14}C]glucose, [^{14}C]glycerol, [^{14}C]acetate and ^{32}Pi into phosphatidylcholine and phosphatidylethanoalmine. However, while it inhibited significantly the [^{14}C]choline incorporation into the former, it stimulated by 60 per cent the ethanolamine incorporation into the latter phospholipid. These results indicate that propranolol probably redirects phospholipid synthesis *de novo*, by inhibiting phosphatidate phosphohydrolase, such that the increase obtained in the biosynthesis of phosphatidylinositol is accompanied by a corresponding decrease in the synthesis of phosphatidylcholine and phosphatidylethanolamine.

Propranolol also caused a 250 per cent increase in the [^{14}C]serine incorporation into phosphatidylserine of the iris muscle and 28 per cent increase in that of microsomes. The drug appears to stimulate the Ca^{2+}-uptake by muscle and microsomes, which in turn could act to stimulate the Ca^{2+}-catalyzed base-exchange reaction.

In addition the metabolic pathways involved in the biosynthesis of the major phospholipids of the iris, a smooth muscle, are reported for the first time. These pathways were found to be essentially similar to those reported for other tissues.

A number of reports have recently appeared showing that a large variety of amphiphilic cationic drugs, including chlorpromazine and other phenothiazine tranquilizers [1–4], cinchocaine and other local anaesthetics [3–7], morphine [8], fenfluramine and its derivatives [3] and propranolol [6, 7, 9–14], markedly altered phospholipid metabolism in a wide variety of tissues. In general these changes involve redirection of phospholipid synthesis (see Fig. 2 in Discussion) away from triglycerides, phosphatidylcholine (PhC), phosphatidylethanolamine (PhE), and into phosphatidic acid (PhA), phosphatidylinositol (PhI), CDP-diglyceride and diphosphatidylglycerol, and they could be explained in part if the drugs used inhibit phosphatidate phosphohydrolase [3, 4, 7]. Support for this hypothesis comes from the recent report of Brindley and Bowley [3] who showed that amphiphilic cationic drugs such as fenfluramine, cinchocaine and chlorpromazine inhibited liver phosphatidate phosphohydrolase by 50 per cent at concentrations between 0.2 and 0.9 mM.

In previous communications from this laboratory we reported on the adrenergic and cholinergic stimulation of ^{32}Pi labelling of phospholipids in rabbit iris muscle [11–13, 15]. Among these findings was the observation that when the β-adrenergic blocker propranolol was added to irises which were preincubated in Krebs–Ringer containing ^{32}Pi, the labelling of PhA, PhI and CDP-diglyceride was increased by 10-, 2- and 16-fold, respectively, while that of PhC and PhE was inhibited by 90 and 30 per cent respectively. It is interesting to note that this drug is similar in its physicochemical properties to other amphiphilic cationic drugs, which were found to alter phospholipid metabolism, in that it possesses both a hydrophobic region and a substituted amine group which can bear a net positive charge. The amphiphilic nature of these drugs appears to enable them to interact with the phosphatidic acid of membranes which in turn inhibits phosphatidate phosphohydrolase and subsequently the redirection observed in phospholipid metabolism [3, 16].

To throw more light on the molecular mechanism underlying the effects of propranolol on phospholipid metabolism in the iris muscle, a series of experiments was performed to investigate the effects of this drug on the incorporation of a number of radioactive lipid precursors into its phospholipids.† Our results clearly

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† Preliminary reports of this study have appeared [11, 12].

show that: (a) propranolol altered the course of incorporation of labelled glucose, glycerol, acetate, inorganic phosphate, serine, myoinositol, choline, ethanolamine and cytidine into lipids of the iris muscle. The results obtained from these experiments give support to the hypothesis [3, 4, 7] that amphiphilic cationic drugs are inhibitors of phosphatidate phosphohydrolase, (b) propranolol exerted marked effects on the incorporation of serine, ethanolamine and choline into their respective phospholipids by the Ca^{2+}-catalyzed base-exchange reaction.

MATERIALS AND METHODS

Preparation and incubation of iris muscle. Unanaesthetized albino rabbits of either sex, weighing approx 2 kg, were employed. They were stunned by a blow to the head and the eyes 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 of the rabbit eyes 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 general 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 or 4–13 μCi of the other lipid precursors were added to each tube and DL-propranolol and other agents were then added as indicated to give a final volume of 1.5 ml. Following incubation at 37° for 30 min 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 of chloroform–methanol (2:1, v/v). Lipids were extracted from the irises as was described previously [13, 15].

Isolation of microsomes from iris muscle. Rabbit eyes, enucleated immediately after sacrifice were placed in ice-cold saline. After transportation from the slaughter house the irises were removed and placed in 0.25 M sucrose containing 2.5 mM EGTA (pH 7.0). In brief, about 4–8 g (100–200 irises) of tissue are suspended in the same solution to a final volume of 10% (w/v). All the following steps were done at 0–4°. The irises were cut into small pieces with a fine scissor. Ten to fifteen ml volume of suspension at a time was homogenized in a Super Dispax Tissumizer Model SDT-182 (Tekmar Company, Cincinnati, Ohio, U.S.A.) at 14,000 rpm for 30 sec followed by 10 sec (total 40 sec). The pooled homogenate was quickly passed through a 2-fold cheese-cloth under mild water suction to remove most of the connective tissue and debris. The filtrate was then centrifuged at 800 *g* for 20 min. The mitochondria was spun down at 8000 *g* for 20 min and the post-mitochondrial supernatant thus obtained was finally centrifuged at 100,000 *g* for 90 min to pellet out the microsomal fraction. The microsomal pellet was washed twice with 0.25 M sucrose and then homogenized gently in 0.25 M sucrose using a loose-fitting Teflon–glass homogenizer (clearance 0.26 mm). The homogenate was made up to the original volume with 0.25 M sucrose then centrifuged at 100,000 *g* for 90 min. The microsomal fraction was suspended in 0.25 M sucrose (4 ml) and stored overnight at 20°. Protein was determined by the procedure of Lowry *et al.* [17] using bovine serum albumin as standard. The yield of microsomal protein was 1.5–1.7 mg/g tissue. The integrity and purity of the preparation was monitored by electron microscopy and assessment of enzyme markers as previously described [18, 19]. In brief the activity of Ca^{2+}-ATPase (expressed as μmole Pi liberated/mg protein/30 min), acetylcholinesterase (μmole ACh hydrolyzed/mg protein/30 min) and NADPH-cytochrome reductase (change in O.D. at 550 nm/mg protein/30 min) was found to be 3.0, 3.7 and 12.1 in the homogenate and 9.6, 17.2 and 36.0 in the microsomal fraction, respectively. Further evidence for the enrichment of this preparation with microsomes can be seen from the electron micrograph shown in Fig. 1.

Assay for incorporation of [^{14}C]serine and [^{14}C]choline into phospholipids of the microsomal fraction. Under the following conditions of incubation [^{14}C]serine and [^{14}C]choline were found to be incorporated only into PhS and PhC, respectively. Based on these observations the following assay for the Ca^{2+}-catalyzed base-exchange reaction was adopted. The incubation mixture consisted of HEPES buffer (pH 8.5), 50 mM; $CaCl_2$, 2.5 mM; 200–250 μg of microsomal suspension in 0.25 M sucrose and 0.5 μCi of [^{14}C]serine (4×10^{-5} M) or [^{14}C]choline (2×10^{-4} M) in a total volume of 0.5 ml. Whenever the effect of propranolol was studied, the drug was added in 0.1 ml of water and preincubation for 5 min at 37° was performed. The reaction was started by the addition of labelled serine or choline and incubation was carried out for 30 min with shaking before it was terminated by the addition of 0.5 ml of ice-cold

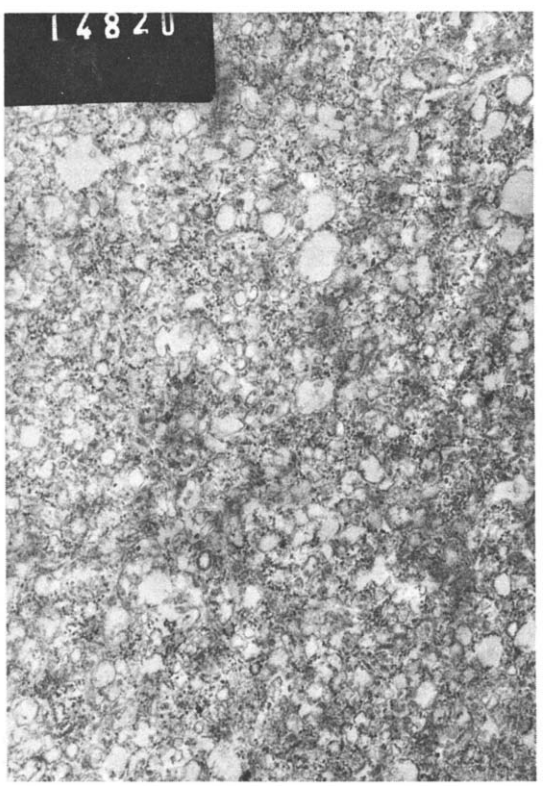

Fig. 1. Electron micrograph of a microsomal fraction prepared from the rabbit iris muscle. Magnification: 5,200.

10% trichloroacetic acid. The content of the tubes was then filtered directly on a nitrocellulose membrane filter (HA 0.45 μm, Millipore Corp. Bedford, Mass.) which has been pre-washed once with 3 ml of 5% TCA. The filter was washed with 20 volumes of ice-cold 5% TCA containing 0.02 M unlabelled respective bases. The filter disc was then dried in an oven at 60° for 30 min. The dried filters were placed in glass vials containing 10 ml scintillation liquid (0.4% PPO and 0.015% POPOP) in toluene–ethanol, 600:378 v/v) and counted. The results are expressed as cpm/mg protein/30 min (or nmole/mg protein/30 min). The zero-time values of approximately 400 cpm were routinely subtracted.

$^{45}Ca^{2+}$ uptake by iris muscle. Iris muscle obtained from right eye served as control while that of left eye was used for experimental. Irises were incubated in presence or absence of propranolol for 15 min at 37° in Krebs–Ringer bicarbonate medium. At the end of preincubation 0.1 ml of $^{45}Ca^{2+}$ (2.5 μCi) was added and incubation was continued for another 30 min. The total volume of the reaction mixture was 2 ml. The slices were quickly transferred to tubes containing 5 ml of ice-cold Ca^{2+}-free Krebs–Ringer solution. The tubes were stirred and the slices left for 30 min before being transferred into tubes containing 5 ml of the same buffer. The slices were washed twice and each time they were allowed to stay in solution for 10 min. Finally they were transferred to scintillation vials containing 1 ml of NCS tissue solubilizer. The slices were digested at 50° for 24 hr with occasional shaking. Ten ml of scintillation liquid was added and the vials counted.

$^{45}Ca^{2+}$ uptake by iris microsomes. The reaction mixture for the $^{45}Ca^{2+}$ uptake consisted of the following (mM); KCl, 100; imidazole–histidine buffer (pH 6.8), 30; ammonium oxalate, 5; sodium azide, 5; $MgCl_2$, 5; $CaCl_2$, 0.02 (0.66 μCi) in a final volume of 0.5 ml. Propranolol was added as indicated. The incubation mixture was preincubated for 5 min at 37° and the reaction was started by the addition of 100 μg of microsomal proteins. After 15 min of incubation at 37° the reaction mixture was filtered through 0.45 μm membrane filters (Millipore Corporation). The filters were washed twice with 6 ml of 0.25 M sucrose. The filters were dried at 60° and their radioactive contents determined. The zero time values were routinely substracted.

Isolation of lipids. The individual lipids were isolated from the total lipid extract by means of two-dimensional t.l.c. and their radioactive contents were determined as described previously [13, 15].

Materials. CDP-diglyceride, derived from egg lecithin, was purchased from Serday Research Laboratories, London, Ontario. This preparation gave several spots when it was run in the above t.l.c. system and thus prior to use it was purified by means of silicic acid chromatography [20]. Its purity was ascertained by means of two-dimensional t.l.c. and chemical analysis [20]. Furthermore, when [^{3}H]cytidine was used as precursor all the radioactivity was found in the spot corresponding to CDP-diglyceride. [1,3-^{14}C]Glycerol (10–20 mCi/m-mole); [1,2-^{14}C]acetic acid (1–3 mCi/m-mole); [1-^{14}C]DL-serine (10–25 mCi/m-mole); [^{14}C-U]L-serine (>135 mCi/m-mole); [2-^{3}H]myo-inositol (1–5 Ci/m-mole); [methyl-^{14}C]-choline (30–50 mCi/m-mole); [1,2-^{14}C]-ethanolamine (1–5 mCi/m-mole); [5-^{3}H]-cytidine (20–30 Ci/m-mole); [^{14}C-U]D-glucose (1–5 mCi/m-mole) and ^{45}Ca (21.8 Ci/g Ca) were purchased from New England Nuclear. [^{32}P]-Phosphorus, carrier free, was obtained from Schwartz–Mann. DL-Propranolol was obtained from Ayerst and Sotalol (1991-1) was from Mead Johnson. All other reagents were reagent grade.

RESULTS

Effect of different concentrations of norepinephrine, propranolol and sotalol on the ^{32}P-labelling of iris phospholipids. The information sought from the following experiments was to show if there was similarity in the action of the neurotransmitter norepinephrine and each of the β-blockers propranolol and sotalol on the turnover of phospholipids in the iris muscle. As can be seen from Table 1 norepinephrine at concentrations between 3 and 300 μM increased appreciably the ^{32}P incorporation into PhA and PhI but not into PhC. Furthermore while propranolol (30–300 μM) stimulated the ^{32}P-labelling of PhA and PhI, it inhibited significantly that of PhC. In contrast sotalol, which possesses a polar moiety in its hydrophobic region, exerted no effect on phospholipid metabolism. These data indicate firstly that the effects of norepinephrine and the amphiphilic amine propranolol on phospholipid metabolism are different, which could suggest that the two phenomenan might be results of two different responses in the lipid metabolism of cells [1, 4, 6]. Secondly the finding that propranolol was effective in altering phospholipid metabolism could be due to the fact that it possesses both

Table 1. Effect of different concentrations of norepinephrine, propranolol and sotalol on the ^{32}P-labelling of iris phospholipids*

Drug added	Effect of drug on phospholipid metabolism		
	PhA (% of control)	PhI (% of control)	PhC (% of control)
L-Norepinephrine	117 ± 3; 176 ± 5; 220 ± 7†	122 ± 2; 153 ± 6; 193 ± 5	102 ± 5; 105 ± 7; 104 ± 7
DL-Propranolol	111 ± 4; 133 ± 6; 968 ± 15	106 ± 3; 129 ± 7; 212 ± 10	67 ± 5; 54 ± 6; 9 ± 2
Sotalol	95 ± 5; 92 ± 6; 92 ± 5	90 ± 8; 98 ± 3; 103 ± 2	103 ± 3; 102 ± 4; 101 ± 5

* In the above experiment all the irises were first preincubated for 20 min in 15 ml of ^{32}P-labelled Krebs Ringer, then each muscle was transferred into test tubes and reincubated for an additional 40 min in 1.5 ml of fresh ^{32}P-labelled Krebs–Ringer in the presence or absence of the drug. The values represent mean ± S.E.M. for three experiments.

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

Table 2. Effects of propranolol on the incorporation of various radioactive lipid precursors into phospholipids of the iris*

Lipid precursor	Amount of radioactivity (μCi)	Effects of propranolol on phospholipid metabolism (% of control)					
		PhA	PhI	PhS	PhC	PhE	CDP-diglyceride
[^{14}C-U]D-Glucose	10		359 ± 8		22 ± 4	69 ± 5	
[1,3-^{14}C]Glycerol	6	452 ± 15	182 ± 7		79 ± 10	70 ± 6	
[1,2-^{14}C]Acetate	13	600 ± 16	384 ± 20		47 ± 6	67 ± 8	
^{32}Pi	25	478 ± 13	234 ± 9	105 ± 6	17 ± 2	73 ± 8	516 ± 49
[5-^{3}H]Cytidine	12.5						3800 ± 300
[Me-^{14}C]Choline	4				35 ± 3		
[2-^{3}H]Myo-inositol	4		215 ± 6				
[1-^{14}C]DL-Serine	4			354 ± 10			
[^{14}C-U]Serine	4			326 ± 11			
[1,2-^{14}C]Ethanolamine	4					162 ± 7	

* One to three irises were placed in test tubes which contained Krebs–Ringer bicarbonate buffer and the radioactive lipid precursor as indicated in a final volume of 1.5 ml. The reaction mixtures were incubated in the presence and absence of propranolol (0.3 mM) at 37° for 30 min. The individual phospholipids were isolated by means of two-dimensional t.l.c. and their radioactivities determined. The values represent mean ± S.E.M. for three experiments.

a hydrophobic region and a substituted amine group which can bear a net positive charge.

Effect of propranolol on the incorporation of labelled glucose, glycerol, acetate and inorganic phosphate into phospholipids of the iris muscle. To throw more light on the molecular mechanism underlying the action of propranolol on phospholipid metabolism a number of radioactive lipid precursors were employed in the following experiments (Table 2). The drug stimulated significantly the incorporation of labelled glucose, glycerol, acetate and Pi into PhA and PhI and inhibited their incorporation into PhC and PhE. These findings are in accord with the hypothesis that cationic amphiphilic drugs alter phospholipid metabolism by inhibiting phosphatidate phosphohydrolase [3, 4]. Inhibition of this regulatory enzyme results in accumulation in the cell of CDP-diglyceride and a corresponding decrease in diacylglycerol synthesis. The finding that propranolol increased by several-fold the incorporation of ^{32}Pi and [^{3}H]cytidine into the CDP-diglyceride and inhibited [^{14}C]choline incorporation into PhC gives further support to this hypothesis. No effort was made in the present work to localize the distribution of radioactivity in the phospholipid molecule when [^{14}C]glucose and [^{14}C]glycerol were employed as precursors. Brindley and Bowley, working with liver slices reported that [1,3-^{3}H]glycerol was incorporated almost exclusively into the glycerol backbone of the lipids [3].

Effect of propranolol on the incorporation of [^{3}H]inositol into PhI of the iris muscle and microsomal fraction. As can be seen from Table 2 the drug also increased the incorporation of [^{3}H]inositol into PhI by almost 200 per cent of that of the control. Inositol is incorporated into PhI via the CDP-diglyceride pathway and the concentration of inositol is rate-limiting in this reaction [20]. Thus the increase in the incorporation of [^{3}H]inositol (Table 2) could be due to the increase in the propranolol-stimulated CDP-diglyceride pool. This was demonstrated through the experiments summarized in Table 3. Thus when propranolol was included in the reaction mixture there was a 19-fold increase in the incorporation of [^{3}H]cytidine into CDP-diglyceride and addition of unlabelled inositol brought it down to 53 per cent of that of the control. There was little incorporation of [^{3}H]cytidine in the absence of the drug. It was of interest to show whether propranolol was increasing the labelling of PhI by stimulating the CDP-diglyceride-inositol phosphatidyltransferase (E.C. 2.7.8.11). Results from these experiments revealed that the drug exerted a negligible effect on this pathway (Table 4). Thus it can be concluded from the above experiments that propranolol stimulates the formation of CDP-diglyceride. The problem arises as to whether this stimulation is caused (a) by the increase in phosphatidate, due to inhibition of phosphatidate phosphohydrolase, or (b) by stimulation of phosphatidate cytidyltransferase (E.C.2.8.8.41).

Effect of propranolol and calcium on the incorporation of [^{14}C]serine, [^{14}C]ethanolamine and [^{14}C]choline into phospholipids of the iris muscle and microsomal fraction. When [^{14}C]choline was the precursor, propranolol inhibited its incorporation into PhC by almost 65 per cent of that of the control (Table 2). This could be due to the inhibition by the drug of phosphatidate phosphohydrolase. However, when [^{14}C]serine and [^{14}C]ethanolamine were employed as precursors, the drug increased their incorporation

Table 3. Effect of the addition of inositol on the propranolol-stimulated incorporation of [^{3}H]cytidine into CDP-diglyceride of the iris muscle*

Additions	[^{3}H]Cytidine incorporated into CDP-diglyceride (cpm)
Control	76 ± 10
Inositol (0.15 mM)	43 ± 7
Propranolol (0.3 mM)	1445 ± 56
Propranolol (0.3 mM) + inositol (0.15 mM)	765 ± 37

* Conditions of incubation and analysis of phospholipids were as described under Table 2 except that only 1.8 μCi of [^{3}H]cytidine was used. The values represent mean ± S.E.M. for three experiments.

Table 4. Effects of propranolol and CDP-diglyceride on the incorporation of [^{3}H]myo-inositol into phosphatidylinositol of the iris microsomal fraction*

Additions	[^{3}H]Inositol incorporated into phosphatidylinositol (cpm)
Control	153 ± 10
Propranolol (0.15 mM)	218 ± 17
CDP-diglyceride (0.15 mM)	3090 ± 187
CDP-diglyceride (0.15 mM) + propranolol (0.15 mM)	3327 ± 220

* The basic incubation medium for the incorporation of [^{3}H]inositol into PhI of the microsomes consisted of the following (mM); Tris–HCl buffer, pH 7.5, 80; $MgCl_2$, 10; ATP, 1.5; [^{3}H]inositol, 2.3 μCi; and microsomes equivalent to 2 mg protein were added in a final volume of 1.0 ml. Time of incubation was 1 hr. The reaction was terminated by adding 0.5 ml of 10% TCA. The values represent mean ± S.E.M. for three experiments.

into their respective phospholipids by 254 and 60 per cent respectively (Table 2). Since it is well established that in animal tissues serine is incorporated into phosphatidylserine (PhS) through the Ca^{2+}-catalyzed base-exchange reaction [21, 22] it was of interest to show whether this effect of propranolol could be due to the stimulation of this pathway. In a number of preliminary experiments we have demonstrated that cytidine and cytidine nucleotides including CMP, CDP, CTP and CDP-diglyceride exerted no effect on the [^{14}C]serine incorporation into PhS of the iris or its microsomal fraction. These studies indicate that [^{14}C]serine, as in other animal tissues, enters into PhS by the Ca^{2+}-catalyzed base-exchange reaction. Since it is well known that Ca^{2+} is required for the latter reaction [21–26] the problem arises as to how does propranolol stimulate the base-catalyzed exchange reaction. The following experiments were designed to answer this question. As can be seen from Table 5 addition of Ca^{2+} to the muscle, which contains bound Ca^{2+}, increased the [^{14}C]serine incorporation by 42 per cent while in the microsomes the increase was 89 per cent. In contrast propranolol increased the [^{14}C]serine incorporation by up to 79 per cent in the muscle and only 28 per cent in the microsomes. The effect of the drug plus Ca^{2+} on the serine incorporation was less than additive. These data suggest that propranolol could be acting on the base-exchange reaction through Ca^{2+}. Thus when EGTA (0.05 mM) was added to the incubation medium there was almost no incorporation of [^{14}C]serine into phospholipids of the microsomes (Table 5). Although the muscles were homogenized in sucrose–EGTA the finding that there was an appreciable amount of labelling in the control suggests the presence of bound Ca^{2+} in these particles.

When [^{14}C]choline was the precursor, Ca^{2+} increased the labelling of PhC by 134 per cent and addition of propranolol, either alone or with Ca^{2+} had no influence on the labelling (Table 6). Thus of the three bases investigated in the present work, incorporation of serine and ethanolamine into their respective phospholipids was stimulated by the drug while that of choline was inhibited. In contrast while in microsomes the drug increased by only 28 per cent the serine incorporation into PhS it had no effect on that of choline. This could be due to the presence of high concentrations of bound Ca^{2+} in these particles.

If propranolol stimulates the Ca^{2+}-catalyzed base-exchange reaction by making more Ca^{2+} available to it, then one would expect the drug to act by increasing either the release or uptake of this ion by

Table 5. Effects of Ca^{2+} and propranolol on the incorporation of [^{14}C]serine into phosphatidylserine of the iris muscle and microsomal fraction*

Additions	[^{14}C]serine incorporated into PhS of muscle		[^{14}C]serine incorporated into PhS of microsomes	
	Total radioactivity incorporated (cpm)	% of control	Total radioactivity incorporated (cpm)	% of control
Control	22035 ± 1400	100	50533† ± 3200 (1.6)††	100
EGTA (0.05 M)			850 ± 30	
EGTA (0.05 mM) + propranolol (0.15 mM)			840 ± 27	
Ca^{2+} (2.5 mM)	31210 ± 1900	142	95600 ± 3900 (3.05)	189
Propranolol (0.15 mM)	39200 ± 1100	178	64635 ± 3400 (2.06)	128
Ca^{2+} (2.5 mM) + propranolol (0.15 mM)	44732 ± 2100	203	137284 ± 5400 (4.38)	272

* Incubation conditions for incorporation of [^{14}C]serine into the muscle were as described under Table 2 except that Ca^{2+} was omitted from the Krebs–Ringer buffer. The observation that there was [^{14}C]serine incorporation in the control indicates the presence of endogenous Ca^{2+} in the tissue. Incubation conditions for microsomes were as follows: 200–250 μg of protein in 0.25 M sucrose were preincubated for 5 min at 37° in a mixture containing 50 mM HEPES buffer, pH 8.5, 2.5 mM $CaCl_2$ in a final volume of 0.5 ml. Propranolol was added as indicated. [^{14}C]Serine (4×10^{-5} M) was added and the incubation was continued for an additional 30 min. The rate of incorporation into phospholipids was assayed as described under Methods. The values represent mean ± S.E.M. for six experiments.

† cpm/mg of microsomal protein/30 min.

†† nmoles/mg of microsomal proteins/30 min.

Table 6. Effects of Ca^{2+} and propranolol on the incorporation of [^{14}C]choline into phosphatidylcholine of the microsomal fraction*

Conditions	[^{14}C]choline incorporated into PhC	
	Total radioactivity incorporated (cpm)	% of control
Control	8250† ± 250 (0.98)††	100
$CaCl_2$ (2.5 mM)	19330 ± 1300 (2.2)	234
Propranolol (0.15 mM)	8250 ± 270 (0.98)	100
$CaCl_2$ (2.5 mM) + propranolol (0.15 mM)	20772 ± 1200 (2.24)	252

* Conditions of incubation were as described under Table 5 except that the concentration of choline was 2×10^{-4} M. The values represent mean ± S.E.M. for three experiments.

† cpm/mg of microsomal protein/30 min.

†† nmoles/mg of microsomal proteins/30 min.

the tissue. A number of cationic drugs are known to increase the release or uptake of Ca^{2+} by various tissues [27]. Efforts to show if propranolol increases the $^{45}Ca^{2+}$ release from either iris muscle or microsomes were unsuccessful (Lakshmanan and Adel-Latif, unpublished work). However we found that propranolol at 0.15 mM and 1 mM concentrations increased the $^{45}Ca^{2+}$ uptake into the iris muscle by 32 and 74 per cent, and into the microsomal fraction by almost 6- and 7-fold respectively (Table 7). Thus the drug could be stimulating [^{14}C]serine incorporation by increasing Ca^{2+}-uptake.

DISCUSSION

In the present study, we have added the iris muscle to the tissues which show redirection of glyceride and phospholipid synthesis by amphiphilic cationic drugs. Thus propranolol provoked an enhancement in the labelling of PhA and PhI from labelled glucose, glycerol, acetate and ^{32}Pi and a corresponding decrease in the synthesis of PhC and PhE (Table 2). These data could be interpreted in the light of recent reports [3, 4, 7] on the effects of a number of amphiphilic cationic drugs, including local anaesthetics, on phospholipid synthesis in various tissues. These drugs appear to exert their effect on phospholipid metabolism by inhibiting phosphatidate phosphohydrolase [3, 4, 16]. Our studies, which are summarised in Fig. 2, are in accord with this hypothesis. Thus, by blocking the formation of 1,2-diglyceride there is an increase in phosphatidate which in turn leads to an increase in the CDP-diglyceride pool and subsequently in PhI. The enhancement in CDP-diglyceride synthesis was demonstrated by employing ^{32}Pi and [^{3}H]cytidine as precursors (Tables 2 and 3). Further work is required to show whether the drug exerts a stimulatory effect on phosphatidate cytidyltransferase, since it is possible that an increase in its activity could bring about a corresponding decrease in the formation of 1,2-diglyceride (Fig. 2). However this is unlikely in the light of the report by Brindley and Bowley [3] who reported that at concentrations of 0.2 to 0.9 mM a number of amphiphilic amines inhibited phosphatidate phosphohydrolase by 50 per cent. Furthermore propranolol does not appear to exert an effect on the enzyme CDP-diglyceride-inositol phosphatidyltransferase since it showed no effect on the incorporation of [^{3}H]inositol into PhI of the microsomal fraction when CDP-diglyceride was added (Table 4). Thus one can conclude that this drug acts to redirect lipid synthesis by inhibiting phosphatidate phosphohydrolase (Fig. 2). This conclusion is supported by the decrease in the labelling of PhE and PhC when a number of precursors were used including [^{14}C]choline (Tables 2 and 6). The mode of action of the amphiphilic cations on phosphatidate phosphohydrolase is not clear at the present time. However, it is conceivable that the inhibition is caused by a physical interaction between the cations and the phosphatidate resulting in the neutralization of the negatively charged phosphate group, which may be accompanied by the displacement of bivalent cations [28]. The molecular mechanism underlying the action of propranolol on phospholipid metabolism in the iris muscle is different from that of norepinephrine (Table 1). They could be different in that whereas the former increases the synthesis of PhI *de novo*, the latter provokes a rapid turnover of only the phosphorylinositol group of the PhI molecule [29]. Furthermore, the effect of propranolol on phospholipid metabolism does not appear to be related to its well known β-blocker activity. Thus sotalol, another β-blocker but its structure is different from that of propranolol in that its hydrophobic

Table 7. Effect of propranolol on the Ca^{2+}-uptake into the iris muscle and microsomal fraction*

Conditions	Muscle		Microsomes
	Total radioactivity bound (cpm)	Ca^{2+} bound (nmoles)	cpm/mg protein
Control	6428 ± 400 (6)†	34.1	3330 ± 496 (7)
Propranolol (0.15 mM)	8486 ± 570 (3)	45	22634 ± 1970 (5)
Propranolol (1 mM)	11191 ± 733 (3)	59.4	29850 ± 3235 (8)

* Conditions for the Ca^{2+} uptake into muscle and microsomes are described in the Method section. The values represent mean ± S.E.M. for the number of experiments indicated in brackets.

† Number of experiments.

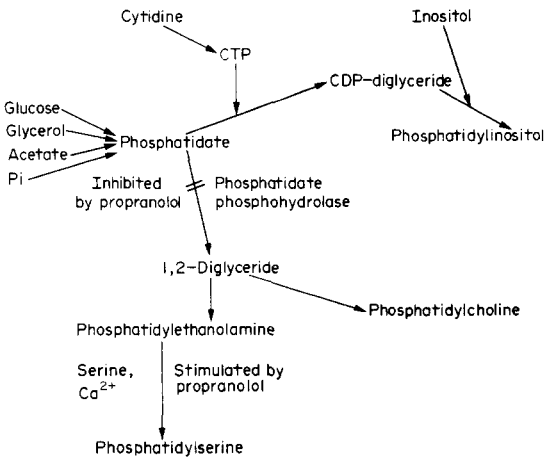

Fig. 2. Effect of propranolol on the metabolism of phospholipids in the iris muscle.

region possesses a more polar group, had no effect on phospholipid metabolism (Table 1) (see also compound S1204 in [3]).

The other major finding in the present work is the observation that propranolol stimulated the incorporation of [^{14}C]serine into PhS of the iris muscle and its microsomal fraction (Tables 2 and 5, Fig. 2). The only mechanism available for the formation of PhS in mammalian tissues is a Ca^{2+}-catalyzed base-exchange reaction, which involves an exchange of free L-serine with the polar moiety of phospholipids [21, 22, 30–33]. This reaction has also been shown to result in the formation of PhE [31] and PhC [23–26]. Kanfer and his collaborators [34] have recently succeeded in preparing a "solubilized" base-exchange enzyme from rat brain which has phospholipase D activity (phosphatidylcholine phosphatidohydrolase, EC.3.1.4.4). This reaction is Ca^{2+}-dependent and thus one can conclude that propranolol acts on the [^{14}C]serine incorporation into PhS by facilitating the movement of Ca^{2+} to make it more available for this reaction. That this hypothesis is feasible is supported by the finding that the drug increased significantly the uptake of $^{45}Ca^{2+}$ both into the muscle and its microsomal fraction (Table 7). These conclusions could also apply to ethanolamine and choline, however the fact that the drug inhibited their *do novo* synthesis puts them in a different category. Thus in the microsomal fraction propranolol exerted no effect on the [^{14}C]choline incorporation into PhC (Table 6) but it inhibited its incorporation into that of the muscle (Table 2). It has been reported that the incorporation of serine, choline and ethanolamine into their respective phospholipids could be catalyzed by different base-exchange enzymes [33]. The variations in the susceptibility of these enzymes to propranolol could account for the differences obtained with serine, ethanolamine and choline incorporation.

Thus at concentrations between 0.03 and 0.3 mM propranolol, which can be classified as an amphiphilic cationic drug, appears to interfere with phospholipid metabolism in the iris muscle in the following manner: (a) by inhibiting phosphatidate phosphohydrolase, an enzyme thought to have a regulatory function in controlling phospholipid synthesis [35–37], it causes a redirection in the *de novo* synthesis of phospholipids (b) by enhancing the availability of Ca^{2+} in the muscle, it increased the [^{14}C]serine incorporation into the PhS through the Ca^{2+}-catalyzed base-exchange reaction by more than 2-fold. It will be important now to show whether these effects can be demonstrated *in vivo*, and if so how are they related to its therapeutic use or side effects.

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