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CATIONIC AMPHIPHILIC DRUGS PERTURB THE METABOLISM OF INOSITITIDES AND PHOSPHATIDIC ACID IN PHOTORECEPTOR MEMBRANES

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Summary: Incubation of purified bovine photoreceptor rod outer segments with [γ-^{32}P]ATP resulted in the labeling of phosphatidylinositol 4-phosphate (PIP) and phosphatidic acid (PA) with little labeling of phosphatidylinositol 4,5-bisphosphate (PIP_2). Propranolol inhibited in a dose-dependent manner the labeling of PA and enhanced that of PIP. Various cationic amphiphilic drugs also were tested for these effects. Propranolol had the same effects on high-speed rat brain particulate material. While this particular preparation displayed more labeling of PIP_2, propranolol was ineffective, as it was on retinal PIP-kinase. Ca^{2+}-activated polyphosphoinositide phosphodiesterase activity in nerve-ending membranes also was inhibited by propranolol. It is concluded that cationic amphiphilic drugs can inhibit diacylglycerol kinase and the polyphosphoinositide phosphodiesterase and stimulate the phosphatidylinositol-kinase (but not PIP-kinase).

Cationic amphiphilic drugs (CAD; e.g. propranolol) redirect the de novo biosynthesis of lipids towards the formation of inositides in several tissues, including the retina (1-6). This redirection is the result of stimulation of the biosynthetic pathway and inhibition of phosphatidic acid phosphohydrolase (2,7-9). Retinal polyphosphoinositides (PPI) are increased both in mass and in labeling by various precursors when retinas are exposed

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ABBREVIATIONS USED

CAD = cationic amphiphilic drugs
PA = phosphatidic acid
PI = phosphatidylinositol
PIP = phosphatidylinositol 4-phosphate
PIP_2 = phosphatidylinositol 4,5-bisphosphate

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to propranolol. In fact, microsomal fractions from retinas incubated with propranolol show a net synthesis of phosphatidic acid (PA) (7,10).

Recently, inositides have been implicated in light transduction in the neural retina of vertebrates (11) and in the photoreceptors of invertebrates (12-16). Light-induced changes in these lipids also have been reported in intact mammalian retina (17-19). When CAD are administered over prolonged periods to man, they disorganize photoreceptor membranes causing retinal degeneration (20). We, therefore, have investigated the mechanism of CAD action on the metabolism of inositides and PA in rod outer segments from bovine retina.

MATERIALS AND METHODS

Preparation of membrane fractions -- Bovine eyes were obtained from a local abattoir, placed on ice and transported to the laboratory in a light-tight box. Retinas were removed under dim red light, and rod outer segments were prepared by differential and sucrose gradient centrifugation (21). All handling was done at 0-4°C and under dim red light. High speed retinal supernatant was prepared as described previously (22). Nerve-ending membranes, prelabeled with ^{32}P, were prepared from rat forebrain as described elsewhere (23). After decapitation, the high speed particulate material was prepared by rapidly removing the forebrains from five rats and placing them in 40 ml ice-cold 0.32 M sucrose. After homogenization, 4 ml were taken and diluted to 25 ml with 10 mM Tris/HCl (pH 7.4) and placed on ice for 30 min. After this, the material was centrifuged for 45 min at 100,000 x g, the supernatant decanted and the pellet resuspended in 10 mM Tris/HCl (pH 7.4).

Incubations -- Rod outer segments (about 40 µg of protein) were incubated at 37°C under dim red light for 5 min in a buffer containing 10 mM Tris/HCl (pH 7.4), 3 mM $MgCl_2$, 50 µM [γ-^{32}P]ATP (ICN Radiochemicals, Irvine, CA; 3-10 µCi/mmol) and various other additives as indicated, in a final volume of 250 µl. After 15 min preincubation, ^{32}P-labeled nerve ending membranes were incubated for an additional 5 min at 37°C in a buffer containing 30 mM HEPES/NaOH (pH 7.4), 0.25 mM EGTA, 1 mM ATP and other additives (final vol, 250 µl) (23,24). High speed particulate material (about 20 µg protein) was incubated for 5 min at 37°C in a buffer containing 10 mM Tris/HCl (pH 7.4), 0.25 mM EGTA, 3 mM $MgCl_2$, 100 µM [γ-^{32}P]ATP (3-10 µCi) and additives as indicated (final vol, 200 µl). All drugs were obtained from Sigma Chemical Co., St. Louis, MO, with the exception of propranolol and betacor (cetamolol = 2-[2-[3-(1,1-Dimethylethyl)amino-2- hydroxy-propoxy]phenoxy]-N-methylacetamide hydrochloride), which were gifts from Ayerst Laboratories, New York, NY.

Lipid Analysis -- Incubations were stopped with 1.5 ml chloroform:methanol (1:2 by vol), 20 µg of the Folch fraction enriched in inositides (Sigma) were added and lipids were extracted under acidic conditions and separated by thin layer chromatography. Lipids were visualized by autoradiography and iodine staining, scraped into vials and scintillation counted (23,25).
Protein Analysis -- Protein content was analyzed spectrophotometrically (26) using bovine serum albumin as a standard.

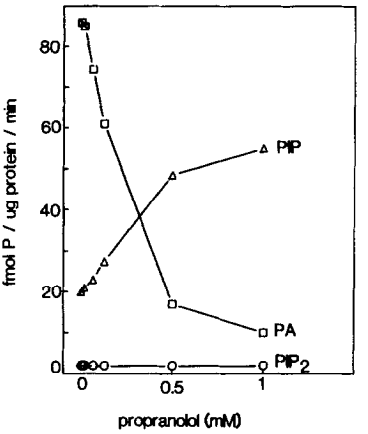

Fig. 1 Effect of various concentrations of propranolol on labeling of phospholipids in rod outer segments. Rod outer segments were incubated, as described in Materials and Methods, in the presence of various concentrations of propranolol. The values indicate the mean (n=2) of radiotracer recovered as PIP_2, PIP and PA.

RESULTS and DISCUSSION

When propranolol was added to an incubation of bovine retinal rod outer segments with Mg^{2+} and [γ-^{32}P]ATP, there was a concentration-dependent stimulation of radiotracer incorporation into phosphatidylinositol 4-phosphate (PIP) (Fig. 1). In contrast, the labeling of phosphatidylinositol 4,5-bisphosphate (PIP_2) under these conditions was very small and not affected by the presence of propranolol (Fig. 1). Rod outer segments have a very low PIP-kinase activity, which would explain the small amount of radiotracer recovered as PIP_2 (data not published). For this reason the analysis of PIP_2 was omitted from further study on rod outer segments. The effect of various CAD on PIP labeling in rod outer segments is shown in Table I. At 1 mM, tetracaine was about as potent as propranolol; chloroquine, lidocaine, procaine and betacor were ineffective.

The only other phospholipid that was labeled under these conditions was PA. Propranolol caused a dose-dependent inhibition of radiotracer incorporation into this phospholipid (Fig. 1). Comparison of the effect of various CAD revealed the potency ranking: propranolol > tetracaine > chloroquine >> lidocaine = procaine = betacor = 0. While propranolol was the most potent

TABLE I. THE EFFECT OF VARIOUS CATIONIC AMPHIPHILIC DRUGS ON THE LABELING OF PIP AND PA IN ROD OUTER SEGMENTS

	PIP	PA
	(fmol/μg protein/min)	
Experiment 1		
Control	11.93 ± 0.34	173.24 ± 8.70
Propranolol	27.72 ± 0.23	8.40 ± 0.17
Chloroquine	11.03 ± 0.51	65.97 ± 3.14
Tetracaine	28.82 ± 0.83	35.50 ± 1.35
Lidocaine	12.34 ± 0.62	154.91 ± 6.06
Procaine	11.46 ± 0.35	169.76 ± 3.29
Experiment 2		
Control	7.15 ± 0.18	110.96 ± 6.36
Propranolol	18.86 ± 0.18	12.50 ± 1.05
Betacor	7.17 ± 0.36	93.46 ± 3.30

Rod outer segments were incubated in the presence of 1 mM of various substances as described in Materials and Methods. Values are mean ± S.E.M. (n = 3).

substance tested with regard to the inhibition of PA labeling and the stimulation of PIP labeling, chloroquine and tetracaine each exerted a similar effect on the incorporation of radiotracer into PA, but not on the labeling of PIP (Table I). This could dissociate the effects of CAD on PIP and PA labeling.

To test whether the effect of propranolol on PIP and PA is related through the phosphodiesteratic generation of diacylglycerol, ^{32}P-prelabeled nerve-ending membranes (23,24) were used. When Ca^{2+} was added to the incubation of nerve-ending membranes, there was a loss of radiotracer from prelabeled PIP_2 and PIP, but not from phosphatidylinositol (PI) (Table II). The presence of propranolol prevented this loss of label from the PPI (Table II). Although a slight degradation of PA was possible in this preparation (27), propranolol had no visible effect (Table II). Also, the addition of propranolol produced no visible effect in [γ-^{32}P]ATP-prelabeled rod outer segments (data not shown). Because these membranes were incubated under non-phosphorylating conditions (23), there was no vis-

TABLE II. EFFECT OF PROPRANOLOL ON POLYPHOSPHOINOSITIDE PHOSPHODIESTERASE

Additives		Lipids (amount of radiotracer recovered; cpm)			
Ca^{2+}	Propranolol	PIP_2	PIP	PI	PA
-	-	999.7 ± 33.8	2712.0 ± 80.0	1682.3 ± 48.7	3832.7 ± 57.2
+	-	758.3 ± 24.6	1795.0 ± 37.3	1632.7 ± 39.9	3951.7 ± 103.6
+	+	967.7 ± 30.3	2604.3 ± 66.9	1630.0 ± 36.1	3756.7 ± 48.9

^{32}P-prelabeled nerve ending membranes were incubated, as described in Materials and Methods, in the absence (-) or presence (+) of 1mM $CaCl_2$ and 1 mM propranolol. Values are the mean are the mean ± S.E.M. (n = 3).

ible increase in the amount of label recovered as phospholipids. These data may indicate that the observed propranolol-induced enhancement of PIP labeling and the inhibition of PA labeling in rod outer segments is due to an inhibition of the PPI phosphodiesterase. However, this would imply that propranolol had a similar effect on the labeling of PIP_2.

Because there was little labeling of PIP_2 in rod outer segments, the effect of propranolol was tested in a preparation enriched in PIP-kinase. High speed supernatant from bovine retina contains PIP-kinase (22), which is assumed to be a soluble enzyme (28). In the absence and presence of 1 mM propranolol, 41.66 ± 2.61 and 48.54 ± 0.74 fmol PIP_2/μg protein/min(± SEM, n=3), respectively, were formed. PIP-kinase, therefore, appeared to be unaffected by the presence of propranolol. A high speed particulate rat forebrain fraction also was tested, because it exhibited PIP-kinase activity. Propranolol had no apparent effect on the labeling of PIP_2 in rat brain particulate material; PIP labeling was stimulated (Table III).

The effect of propranolol on the labeling of PA also was seen in rat brain particulate material (Table III). Membranes were incubated in the presence of 0.25 mM EGTA to completely inhibit phosphodiesteratic activity against the PPI. Such an approach was not feasible with the rod outer segments, because of the high Ca^{2+}-content of the discs, which makes a con-

TABLE III. EFFECT OF PROPRANOLOL ON THE LABELING OF PHOSPHOLIPIDS IN HIGH SPEED BRAIN PARTICULATE MATERIAL

Additives		Lipids (fmol/μg protein/min)		
Ca^{2+}	Propranolol	PIP_2	PIP	PA
-	-	4.94 ± 0.45	42.13 ± 3.30	23.11 ± 1.89
-	+	4.88 ± 0.14	89.67 ± 5.37	8.47 ± 0.83
+	-	3.31 ± 0.31	25.07 ± 2.90	29.33 ± 3.54
+	+	3.18 ± 0.17	40.93 ± 1.20	7.95 ± 0.60

High speed brain particulate material was incubated, as described in Materials and Methods, in the absence (-) or presence (+) of 0.75 mM $CaCl_2$ and 1 mM propranolol. The values are the mean ± S.E.M. (n = 3).

trol less precise. After addition of propranolol, PA labeling was inhibited to an extent similar to that in rod outer segments (Tables I and III). This inhibition of PA labeling occurred independent of the presence or absence of Ca^{2+} (Table III). The addition of Ca^{2+} had a slight stimulatory effect on the labeling of PA in brain high speed particulate material (Table III) similar to the effect seen in nerve-ending membranes (23). The labeling of PA in the absence of Ca^{2+} indicated that there is diacylglycerol accessible to its kinase. The propranolol-induced inhibition of PA labeling in both rod outer segments and brain particulate material is probably best interpreted as an inhibition of the diacylglycerol kinase. Although studies on whole retina suggest that propranolol enhances the conversion of PA to PI and subsequently to the PPI, the latter explanation for these observations seems less appropriate because PI labeling was not detected in rod outer segments or particulate material from rat brain. The absence of PI labeling, therefore, dissociates the effects of propranolol on PIP and PA in rod outer segments and high speed brain particulate material. Taken together, the data presented here indicate that CAD can inhibit diacylglycerol-kinase and the PPI-phosphodiesterase and can stimulate the PI-kinase (but not PIP-

kinase). We conclude that CAD can exert pleiotropic effects on phospholipid metabolism, which does not necessarily need to have one single underlying primary effect. The CAD-elicited shift in the biosynthesis of glycerolipids towards acidic phospholipids should be investigated as a possible underlying cause of side effects or of impaired signaling systems. In intact bovine retinas there are pronounced alterations in inositol lipids during in vitro incubation with proporanolol, including changes in PIP_2 mass (6).

Although the generally employed beta-adrenergic antagonistic potency of propranolol can be found at concentrations several orders of magnitude lower than necessary for the enzymatic effects observed here, the lipophilicity of these drugs merits some caution. For this reason, this study also has included betacor, which seems to be a more specific beta-adrenergic receptor antagonist with fewer membrane perturbing effects than propranolol (29). Our data support this contention; betacor elicited fewer of these effects than the other drugs studied here. During prolonged treatment with propranolol (and also in acute types of exposure) the exact local drug concentration in the plasma membrane can at best be estimated. The interference with various enzymes in the inositide cycle may involve cellular activation by ligands which utilize the inositide cycle to transduce this signal. Therefore, consideration of propranolol as a specific antagonist (e.g. beta-receptor) is an insufficient account of the information available; rather, membrane perturbing effects may be responsible for the newly reported effects. Moreover, because of these effects, propranolol may be useful as a tool to further our understanding of inositol lipid metabolism.

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