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PHOSPHOLIPASE A_2 INHIBITORS

DIFFERENTIAL INHIBITION OF FATTY ACID ACYLATION INTO KIDNEY LIPIDS BY MEPACRINE AND *p*-BROMOPHENACYL BROMIDE

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Abstract—Mepacrine and *p*-bromophenacyl bromide, in addition to their inhibitory effect on lipolysis, are also potent inhibitors of fatty acid acylation into renal medullary lipids. Significant qualitative and quantitative differences in the inhibition by the two drugs were seen. *p*-Bromophenacyl bromide exerted a non-selective effect inhibiting the incorporation of saturated and unsaturated fatty acids into all phospholipid classes and triacylglycerols. In contrast, mepacrine selectively inhibited the incorporation of both saturated and unsaturated acids into phosphatidylcholine, phosphatidylethanolamine and triglycerides, and concurrently markedly enhanced their incorporation into phosphatylinositol. Quantitative analysis of these mepacrine effects, together with the known inhibitory effects of this compound on phospholipase A_2 and phosphatidylinositol-specific phospholipase C, suggests that mepacrine also inhibits phosphatidic acid phosphatase, thereby shunting the flux of phosphatidic acid away from diglyceride formation and into synthesis of phosphatidylinositol.

Mepacrine and *p*-bromophenacyl bromide (*p*-BPB) have been shown to inhibit the release of arachidonic acid and the generation of arachidonate-oxygenated products from blood platelets [1]. Mepacrine has also been shown to inhibit the release of arachidonate, and the generation of its vasoactive products in rat kidney [2] and in the isolated perfused guinea pig lung [3–5]. *p*-BPB has been found to inhibit the activity of snake venom phospholipase A_2 [1, 6] directly, apparently through interaction with specific amino acid residue(s) in the enzyme [6]. The results of several studies suggested that mepacrine inhibits phospholipase A_2 activity [1–5]. However, initial direct measurement of the effect of mepacrine on the activity of snake venom phospholipase A_2 activity did not support this suggestion [1], but more recent results [7] verified it.

In the course of our studies on the biochemical properties of lipolysis and prostaglandin generation in rabbit kidney, we have employed mepacrine and *p*-BPB and have found both compounds to be potent inhibitors of free fatty acid acylation into kidney lipids. After completion of these studies, a paper by Abdel-Latif and Smith [8] appeared which contained studies of a similar nature in the iris.

MATERIALS AND METHODS

Materials. Mepacrine and *p*-BPB were obtained from Sigma (St. Louis, MO). [1-^{14}C]Arachidonic acid (specific activity 55 mCi/mmole), [1-^{14}C]linoleic acid (specific activity 56 mCi/mmole) and [1-^{14}C]stearic acid (specific activity 56.5 mCi/mmole) were obtained from the Radiochemical Centre (Amersham, U.K.). Fatty acid poor bovine serum albumin was from Calbiochem (San Diego, CA). Other reagents were analytical grade.

Preparation and incubation of kidney medulla slices. Rabbits (male, New Zealand white derived, local strain, 2.5–3.0 kg) were sacrificed by air-injection into the heart, both kidneys were removed and medulla slices (approximately 1 mm thick) were quickly prepared. The slices were rinsed with 0.1 M Tris–HCl buffer (pH 8.0) and incubated in the same buffer with various concentrations of mepacrine or *p*-BPB for 10 min at 37° with shaking. Radioactive acid ([-^{14}C]arachidonic, [1-^{14}C]linoleic or [1-^{14}C]stearic) was then added and incubation was continued for 30 min. Mepacrine was dissolved in Tris buffer while *p*-BPB was dissolved in hot ethanol. The ethanol concentration in the incubation medium did not exceed 0.5% and had no effect on the incorporation of fatty acids into the tissue.

The phospholipid fraction was isolated following homogenization of the prelabeled slices, extraction and thin-layer chromatography. The fraction was then treated with phospholipase A_2 (from *Crutalus dirissus terrificus*) or by alkaline hydrolysis. Over 96 and 93% of the incorporated arachidonate and linoleate, respectively, was incorporated into the sn-2 position, whereas 95% of the stearic was at the sn-1 position.

Analysis of radioactivity in lipid classes. Following incubation, the medium was discarded and the slices were rinsed twice with Tris buffer containing bovine serum albumin. Slices (1 mg/ml) were then analyzed for radioactivity in lipid classes. The tissue was homogenized in 20 ml of chloroform–methanol (2:1 by volume) and lipid extraction was carried out as described previously [9]. The extracts were subjected

to thin-layer chromatography for separation of phospholipids (chloroform–methanol–acetic acid–water, 100:20:12.5:4 by volume) and for separation of neutral lipids (petrol ether, 40–60°–diethyl ether–acetic acid, 70:30:3 by volume). Radioactivity of each lipid band was determined by liquid scintillation counting.

RESULTS

Effect of mepacrine and p-*BPB on acylation of arachidonic and linoleic acids into medullary lipids*

Rabbit kidney medulla slices were incubated with radioactive arachidonic acid in the absence or presence of various concentrations of mepacrine (0–1.0 mM) or *p*-BPB (0–0.2 mM). In the absence of the drugs, the incorporated radioactivity was found in the various acylglycerols (91.4%) and arachidonic acid (8.6%) (Table 1). Radioactive arachidonate products (prostaglandins, hydroxy acids) were not detected in the slices but were found in the incubation media [10]. Both mepacrine and *p*-BPB profoundly affected the pattern of incorporation of arachidonate into medullary lipids. Mepacrine exerted a dose-dependent selective inhibitory effect on arachidonate incorporation mainly into phosphatidylcholine and the triacylgycerol fractions, with less pronounced inhibition of incorporation into phosphatidylethanolamine. Concurrently, mepacrine produced a marked increase (150–200%) in the incorporation of arachidonic acid into phosphidylinositol and, to a lesser extent, into diglyceride fractions (Fig. 1). Compared to arachidonic acid, linoleic acid incubated with medulla slices under similar conditions was less efficiently incorporated into cellular lipids, with a higher per cent of the acid remaining unesterified at the end of the incubation period. The effect of mepacrine on incorporation of linoleic acid into medullary lipids was similar to that seen with arachidonic acid. Mepacrine produced a marked decrease in incorporation of linoleic acid into phosphatidylcholine and triacylglycerols and a concurrent increase in the incorporation into phosphatidylinositol (Table 1).

Incorporation of [^{14}C]arachidonic acid into medullary lipids was also dramatically affected by the presence of *p*-BPB. This compound produced a dose-dependent inhibition of arachidonate incorporation into all lipid classes with concurrent accumulation of the free fatty acid (Fig. 2). The accumulated arachidonate appears to reside intracellularly, since subsequent washings with albumin–Tris buffer caused only a slow release of the acid into the medium. No oxygenated products were detected in the slices.

The effect of mepacrine and p-*BPB on acylation of* [1-^{14}C]*stearic acid into medullary lipids*

The effects of mepacrine and *p*-BPB on fatty acid incorporation into the sn-1 position of phospholipids was assayed in experiments with [^{14}C]stearic acid. The cellular uptake of added [^{14}C]stearic acid was much less efficient than that observed for the unsaturated acids. Furthermore, the bulk of the fatty acid which was taken up by the medullary slices remained

Table 1. Effect of mepacrine on the incorporation of radioactive arachidonic and linoleic acids into medulla lipids

Labeled fatty acid added	Addition to incubation medium	Radioactivity in lipids (cpm/g tissue)						
		PI	PC	PE	MG	DG	Fatty acid	TG
[^{14}C]Arachidonate	—	37,880	210,190	68,290	3200	32,540	44,280	119,500
	Mepacrine	96,950	54,950	50,040	4470	43,780	149,220	44,680
	Δcpm	+59,070	−155,240	−18,250	+1270	+11,240	+104,940	−74,820
	% Change	+156	−74	−27	+40	+35	+237	−63
[^{14}C]Linoleate	—	9850	119,250	40,440	3110	28,000	131,180	154,000
	Mepacrine	61,140	43,500	44,680	3530	40,560	249,860	88,770
	Δcpm	+51,290	−75,750	+4240	+420	+12,560	+118,680	−65,230
	% Change	+521	−63	+10	+13	+45	+90	−42

Medulla slices (0.7 g) were pre-incubated in Tris–HCl buffer with or without mepacrine (0.5 mM) for 10 min. Next, 0.9 μCi of [1-^{14}C]arachidonic acid or 0.9 μCi of [1-^{14}C]linoleic acid was added and the incubation continued for 30 min. Following the incubation, the slices were rinsed and analysed for radioactivity distribution in the various lipid fractions. For details, see Materials and Methods. Results are from a single experiment. Similar results were obtained in three additional experiments. Total radioactivity (cpm/g) incorporated into the slices in incubations with [^{14}C]arachidonic was 5.1–5.3 × 10^5 in the absence and 4.5–4.9 × 10^5 in the presence of mepacrine. In incubations with [^{14}C]linoleic, total radioactivity incorporated into the slices was 5.0–5.2 × 10^5 cpm in the absence and 5.3–5.7 × 10^5 in the presence of mepacrine. Abbreviations used: PI, phosphatidylinositol; PC, phosphatidylcholine; PE, phosphatidylethanolamine; MG, monoglycerides; DG, diglycerides; TG, triglycerides. Δcpm denotes the difference between radioactivity in the presence or absence of mepacrine.

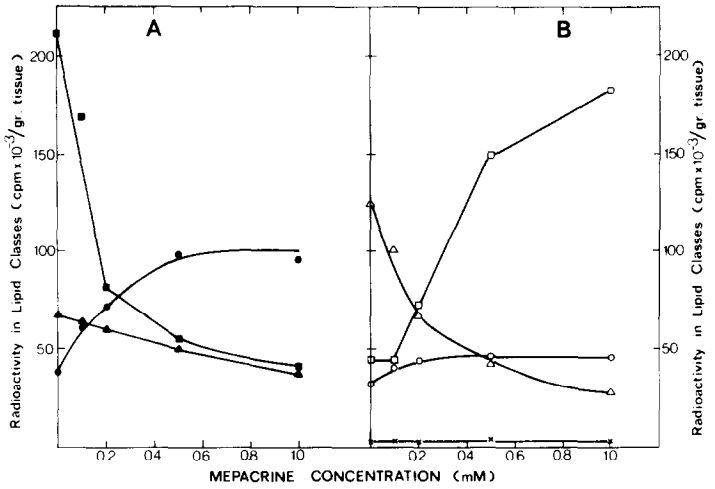

Fig. 1. Effect of mepacrine on the incorporation of labeled arachidonic acid into medullary lipids. Medulla slices (0.7 g) were pre-incubated in 0.1 M Tris–HCl (pH 8.0) in the presence of different concentrations of mepacrine (0–1.0 mM) for 10 min at 37° with shaking. Following that, 0.9 μCi of [1-^{14}C]arachidonic acid was added and incubation continued for 30 min. The medulla slices were then isolated, rinsed and the tissue lipids analysed for radioactivity in (panel A) phosphatidylinositol (●—●), phosphatidylethanolamine (▲—▲), phosphatidylcholine (■—■), and in (panel B) monoglycerides (×—×), diglycerides (○—○), triglycerides (△—△) and arachidonic acid (□—□). Results are means of three experiments. Standard errors were less than 7%.

unesterified (Table 2). Mepacrine at a concentration of 0.1 mM inhibited the incorporation of [^{14}C]stearic acid into phosphatidylcholine, phosphatidylethanolamine and triacyglycerols, whereas the incorporation into phosphatidylinositol was increased (Table 2). At higher mepacrine concentrations, both the uptake of [^{14}C]stearic acid into medullary slices and, in parallel, the incorporation of stearic acid into all lipid fractions were reduced. *p*-BPB markedly inhibited the incorporation of [^{14}C]stearic acid into all lipid classes except for the diacylglycerol fraction where the incorporation was only marginally reduced.

DISCUSSION

Mepacrine and *p*-BPB have been shown to inhibit arachidonate release and generation of prostaglandins and thromboxane in several systems [1–4]. The two compounds were suggested to exert their effects by inhibiting phospholipase A_2 activity. Initially, direct demonstration of such effects on purified snake venom phospholipase A_2 was seen with *p*-BPB but not with mepacrine [1, 6]. More recently, Vigo *et al.* [7] did show that mepacrine at concentrations of 0.4–1.6 mM, produced dose-dependent inhibition of snake venom or pancreatic phospholipase A_2. This report shows that the two compounds also have a

Table 2. Effect of mepacrine and *p*-BPB on the incorporation of radioactive stearic acid into medulla lipids

Addition to incubation medium	Radioactivity (cpm)					
	PI	PC	PE	DG	Stearic acid	TG
—	1610	1650	1250	2730	123,040	2390
0.1 mM mepacrine	1930	1010	1100	2400	115,820	1150
% Change	+20	−39	−12	−12	−6	−52
0.1 mM *p*-BPB	680	810	540	2170	127,540	1350
% Change	−58	−51	−57	−20	+4	−44

Medulla slices (0.3 g) were pre-incubated in 0.1 M Tris–HCl buffer (pH 8.0) with various concentrations of mepacrine or *p*-BPB for 10 min at 37° with shaking. Next, 0.5 μCi of [^{14}C]stearic acid was added and the incubation continued for 30 min. The slices were then rinsed and analysed for radioactivity in the lipid fractions. For details, see Materials and Methods. Results given are for a single experiment. Similar results were obtained in two additional experiments. Total radioactivity (cpm) incorporated into the slices was 1.21–1.40 × 10^5 in the absence or presence of the added compounds. For abbreviations, see Table 1.

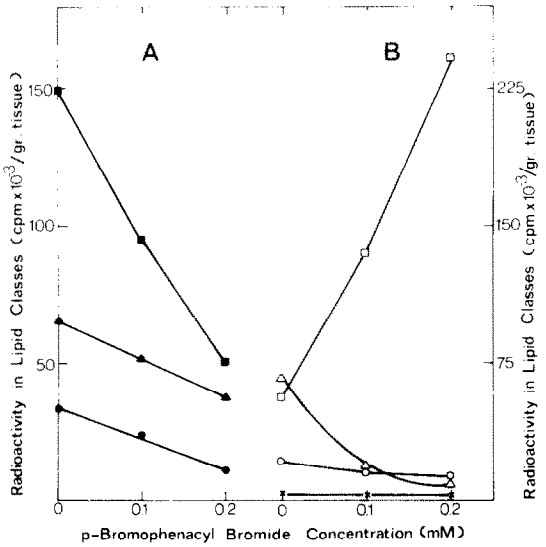

Fig. 2. Effect of *p*-BPB on the incorporation of labeled arachidonic acid into medullary lipids. Medulla slices (0.7 g) were pre-incubated in Tris–HCl buffer (pH 8.0) in the presence of different concentrations (0–0.2 mM) of *p*-BPB for 10 min at 37° with shaking. Following that, 0.9 μCi of [1-^{14}C]arachidonic acid was added and the incubation continued for 30 min. Slices were isolated and tissue lipids analysed for radioactivity. For details see Materials and Methods. Symbols of the plots are the same as in Fig. 1. Results are means of three experiments. Standard errors were less than 7%.

marked effect on lipid metabolism in inhibiting free fatty acid incorporation into cellular lipids.

p-BPB exerted a non-selective inhibitory effect on the incorporation of both polyunsaturated fatty acids, arachidonic and linoleic acids, and of saturated stearic acid into all lipid classes. The compound is an alkylating agent and may be expected to react with, and modify, many cellular compounds, and affect numerous biochemical processes, as indeed suggested from studies with platelets [11, 12]. The use of *p*-BPB in this study was intended only for contrast with the results obtained with mepacrine.

Mepacrine was found to exert a selective effect on the incorporation of fatty acids into tissue phospholipids and acylglycerols. The compound markedly depressed incorporation of the unsaturated fatty acids, linoleic and arachidonic, into phosphatidylcholine and triglycerides, concurrent with a dramatic increase in their incorporation into phosphatidylinositol. Similarly, mepacrine also inhibited the incorporation of [^{14}C]stearic acid into phosphatidylcholine and triglycerides, whereas incorporation into phosphatidylinositol was increased.

Abdel-Latif and Smith [8] have recently reported results on the effects of mepacrine on the incorporation of [^{14}C]arachidonate into glycerolipids of the rabbit iris. The major effects of mepacrine observed in their studies are similar to ours: (1) a dose-dependent decrease in arachidonate incorporation into phosphatidylcholine, phosphatidylethanolamine and triglyceride fractions and increased incorporation into phosphatidylinositol and to a lesser extent into diglycerides. Abdel-Latif and Smith [8] also showed a substantial increase in the incorporation into phosphatidic acid. (2) The decreased incorporation into phosphatidylethanolamine is less pronounced than that into phosphatidylcholine. The two studies do differ in the results on the effect of mepacrine on the intracellular level of unesterified arachidonic acid. In kidney medulla slices, mepacrine produced a dose-dependent rise in intracellular-free arachidonate (over 200% increase at 0.5 mM, Fig. 1, Table 1), whereas in the iris smooth muscle, only a marginal increase (up to 8%) in free arachidonate was seen [8]. The reason for this difference is not known; it may relate to tissue differences between the iris and the kidney.

The synthesis of acylglycerols occurs via two types of mechanisms. The first type involves either: (1) transacylation between two lysophospholipid molecules, e.g. according to the sequence lyso PC + lyso PC → PC + glycerol-3-phosphorylcholine; or (2) acyl transfer from an appropriate donor to a lysophospholipid. Both transacylation and acyltransferase reactions are involved in phospholipid metabolism; their participation in triglyceride formation has not, however, been shown. Since mepacrine exerted a marked inhibitory effect on fatty acid incorporation into triacylglycerol, it argues against a possible effect of mepacrine on transacylation or acyltransferase reactions.

The second mechanism for acylglycerol synthesis involves *de novo* synthesis from glycerol-3-phosphate and fatty acids with intermediate formation of phosphatidic acid which is then metabolized by two distinct routes (Fig. 3). In the first route, phosphatidic acid is converted into diglycerides, which either serve as a precursor for triglycerides or for the biosynthesis of phospholipids (phosphatidylcholine, phosphatidylethanolamine) by reacting with the corresponding CDP-derivative of the bases. In the other metabolic route, phosphatidic acid is converted to CDP-diglyceride, which serves as a precursor for the synthesis of phosphatidylinositol and other lipids (e.g. phosphatidylglycerol). Intermediate formation of diglycerides in this metabolic route is not involved. Lapetina *et al.* [13] have recently observed that mepacrine (quinacrine) stimulates [^{14}C]arachidonate incorporation into the phosphatidylinositol fraction in platelets. From their data they suggested that mepacrine exerts its effect by stimulation of phosphatidic acid conversion to CDP-diglyceride. Based on this explanation, the observed decrease in arachidonic acid incorporation into diglyceride-derived lipids (triacylglycerol, phosphatidylcholine, phosphatidylethanolamine) should not exceed the observed increase in incorporation into phosphatidylinositol. However, analysis of our data (Fig. 1, Table 1) shows that the combined decrease in the radioactivity incorporated into the diglyceride-derived lipids is 2.7 to 4.2-fold higher than the increase in incorporation into phosphatidylinositol, thus arguing against the mechanism for mepacrine effect as suggested by Lapetina *et al.* [13].

Another mechanism which may explain the results we obtained involves inhibition by mepacrine of only certain acylhydrolases. Mepacrine inhibits phospholipase A_2 and may also inhibit renal triglyceride lipase. However, Lapetina *et al.* [14] have also

recently reported that mepacrine does not inhibit phosphatidylinositol-specific phospholipase C in platelets. This observation, together with (1) mepacrine inhibition of phospholipase A_2; (2) inhibition of phosphatidic acid-specific phospholipase A_2 as shown by Lapetina and collaborators in platelets [13–15]; and (3) inhibition by mepacrine of diacylglycerol acyltransferase as suggested by Abdel-Latif and Smith [8], provides a basis for a mechanism in which mepacrine inhibits the turnover of fatty acids in triacyglycerols, phosphatidylcholine and phosphatidylethanolamine, but does not affect the hydrolysis and turnover of fatty acids in phosphatidylinositol. In this case, a higher portion of the newly synthesized phosphatidic acid, containing incorporated [^{14}C]arachidonate, will be directed towards generation of phosphatidylinositol, as indeed was observed by us (Fig. 1, Table 1) and others [8]. However, more recently, detailed studies by Hofmann *et al.* [12] have shown that mepacrine is also a potent inhibitor of phosphatidylinositol-specific phospholipase C in human platelets, rendering the suggested mechanism outlined above less likely.

An alternative mechanism we propose for the observed effects of mepacrine involves inhibition of phosphatidic acid phosphatase (phosphohydrolase) (Fig. 3). Such inhibition will bring about redirection of glycerolipid synthesis away from synthesis of triglycerides, phosphatidylcholine and phosphatidylethanolamine and towards the production of phosphatidylinositol. Abdel-Latif and Smith [8] have also concluded that this mechanism is the most likely to explain the effect of mepacrine on arachidonate incorporation into glycerolipids, and have cited some unpublished data from their laboratory which further supports the mechanism.

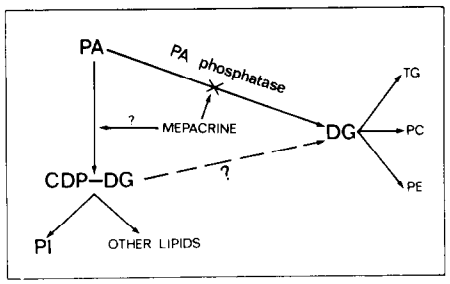

Fig. 3. Suggested mechanism for mepacrine inhibition of medullary *de novo* acylglycerol synthesis. Abbreviations used: PA, phosphatidic acid; DG, diglycerides; CDP-DG, cytidine diphosphate diglyceride; PC, phosphatidylcholine; PE, phosphatidylethanolamine; TG, triglycerides; PI, phosphatidylinositol. X denotes inhibition.

Finally, an additional point which needs clarification is the substantial decrease by mepacrine of fatty incorporation into phosphatidylcholine in contrast to the incorporation into phosphatidylethanolamine, which is only slightly reduced. This difference may be due to apparent differences in the kinetic parameters for biosynthesis of phosphatidylcholine and phosphatidylethanolamine from precursor diglyceride [16], which indicates a higher rate of phosphatidylethanolamine formation.

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