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INHIBITION OF PURIFIED LYSOSOMAL PHOSPHOLIPASE A_1 BY BETA-ADRENOCEPTOR BLOCKERS

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Abstract—Inhibition of rat liver lysosomal phospholipases is one of the main events that leads to accumulation of tissue phospholipids during drug-induced phospholipidosis. Drug inhibition of lysosomal phospholipase A may occur by direct effects of drugs on the enzyme (or substrate) or by drug-induced increases in intralysosomal pH. Although beta-adrenoceptor blockers have not been reported to cause lipid storage, they do inhibit lysosomal phospholipase A. To investigate the structural requirements for drug inhibition, we studied the effects of six beta-adrenoceptor blockers on purified rat liver lysosomal phospholipase A_1. The agents studied include: propranolol, timolol, metoprolol, practolol, atenolol and the combined alpha and beta adrenoceptor blocking agent, labetalol. The drugs varied by two logs in their abilities to inhibit phospholipase A_1 activity. The relative inhibitory potencies were propranolol > labetalol ≫ timolol > metoprolol ≫ practolol > atenolol. Our studies identify drug hydrophobicity as a key determinant for phospholipase A_1 inhibition. A strong negative correlation was noted between the octanol/water partition coefficients and IC_{50} for phospholipase inhibition ($r = -0.91$). The ability of propranolol to inhibit phospholipase A_1 was identical for the *d, l* and the *d* and *l* stereoisomers.

Drug-induced lipidosis was first reported by a group of Japanese scientists who found that chronic administration of 4,4′-bis(diethylaminothoxy) α,β-diethyldiphenylethane led to the accumulation of lipids in the tissues of humans and experimental animals [1, 2]. Subsequently, a wide variety of cationic amphiphilic drugs (CAD) were shown to induce lipidosis in cultured cells, experimental animals and humans [3–6]. The compounds which have the ability to induce lipidosis share similar chemical structural features consisting of a hydrophobic moiety and an aliphatic side chain having an amino group which is positively-charged at physiological pH. These compounds have been shown to inhibit the activities of several phospholipid-hydrolyzing enzymes [7, 8]. In tissues and cells, accumulation of these drugs in lysosomes and inhibition of lysosomal phospholipases A and C is believed to be the sequence of events that leads to the intracellular accumulation of phospholipids [9]. Although they do not cause lipidosis, beta-adrenoceptor blocking agents inhibit lysosomal phospholipase A [8].

The mechanisms by which the drugs inhibit lysosomal phospholipases are not yet clear. Our prior studies of CAD inhibition of phospholipases using crude soluble lysosomal preparations gave kinetic results which were not consistent with the recognized types of inhibition [7]. This may be due to the presence of the crude enzyme preparation of several different isoenzymes of phospholipase A_1 and to the presence of substantial lysophospholipase activity [10]. Recently, it was reported that antimalarial CAD cause competitive inhibition in a crude lysosomal phospholipase preparation [11].

We recently isolated and purified lysosomal phospholipase A_1 from rat liver [10]. To investigate the structural requirements for drug inhibition of purified lysosomal phospholipase A_1, we selected a group of beta-adrenoceptor blockers since these drugs have a similar structural unit and a similar pharmacological action but differ greatly in their degree of hydrophobicity. A variety of agents are currently available. The beta-blockers studied were propranolol, labetalol, timolol, metoprolol, atenolol and practolol. Labetalol, an agent having alpha- and beta-blocking properties, was also studied. We used the 34,000 mol. wt isoenzyme of lysosomal phospholipase A_1 which represents more than 60% of the recovered phospholipase A activity [10]. Sonicated dispersions of [1-^{14}C]dioleoylphosphatidylcholine were used as the substrate. On the basis of IC_{50} (concentration of drug required to inhibit phospholipase A_1 by 50%), the drugs could be divided into three groups having decreasing inhibitory potential. Our studies demonstrate that hydrophobicity of the respective drugs is an important determinant of their inhibitory potential.

MATERIALS AND METHODS

Isolation and purification of rat liver lysosomal phospholipase A_1. Phospholipase A_1 was purified as described earlier by Hostetler *et al.* [10]. To summarize briefly, a composite lysosomal fraction (M + L) was obtained by differential centrifugation of a 20% rat liver homogenate in 0.25 M sucrose containing 5 mM Tris and 2 mM EDTA (pH 7.4). A soluble protein fraction was obtained by three cycles

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of freezing and thawing in dilute buffer followed by sedimentation of the membraneous material. The soluble protein obtained was applied to a hydroxyapatite column attached in series to a DEAE column and washed with 8 mM phosphate containing 20% glycerol. The hydroxyapatite column was detached and the active fractions were eluted from the DEAE column with a linear gradient of 0 to 0.4 M KCl in 8 mM phosphate containing 20% glycerol. The combined active fractions from this step were applied to a Concanavalin A-Sepharose column and eluted with 0.5 M alpha-methylmannoside. The fractions containing phospholipase A activity were combined and concentrated using an Amicon ultrafiltration cell. The partially purified phospholipase A_1 preparation was subjected to a chromatofocusing on Polybuffer PBE 94 exchanger and eluted with pH 4.0 Polybuffer 74 as previously reported [10]. The phospholipase A_1 fraction having an isoelectric point in the region of 5.2 was applied to a column of Ultrogel AcA 44 and eluted with 0.15 M sodium chloride containing 20 mM Tris–HCl (pH 7.2) and 20% glycerol. The column fractions containing the phospholipase A_1 with an approximate molecular weight of 34,000 were combined and stored at −70° in 50% glycerol until use. Protein was estimated by the method of Bradford [12] using rabbit gamma globulins as standard.

Preparation of substrate. Sonicated dispersions of the phospholipid substrate were prepared as follows: chloroform solutions of [1-^{14}C]dioleoylphosphatidylcholine (sp. act. 1 mCi/mmole) were taken to dryness in a nitrogen stream. The lipid was dispersed by vortexing in 5 mM Tris buffer (pH 7.2) containing 20 mM sodium chloride followed by sonication under nitrogen for 20 min using the cuphorn sonicator of a Heat Systems sonicator (model W-225R). After centrifuging at 8500 *g* for 5 min the supernatant fraction contained a dispersion of phosphatidylcholine consisting of a mixture of large multilamellar vesicles and small unilamellar vesicles [13].

Enzyme assays. Incubation medium for the enzyme assay contained 50 mM sodium acetate, pH 4.4, 0.2 mM [1-^{14}C]dioleoylphosphatidylcholine, 1 μg enzyme protein and drugs as indicated in a final volume of 0.200 ml. The incubation was carried out at 37° for 30 min. The reaction product, [1-^{14}C]oleic acid, was isolated by modified Dole extraction according to the method of van den Bosch and Aarsman [14] assayed by liquid scintillation spectrometry as previously described [10]. The concentration of the substrate was varied as noted at fixed drug concentrations. Unless otherwise indicated, the drugs used had the *d*, *l* stereoconfiguration.

Materials. [1-^{14}C]Dioleoylphosphatidylcholine was obtained from the New England Nuclear Corp., Boston, MA. Dioleoylphosphatidylcholine was purchased from Calbiochem, La Jolla, CA, and alpha-methylmannoside, tris(hydroxymethyl)aminomethane and rabbit gamma globulins were purchased from the Sigma Chemical Co., St. Louis, MO. Triton WR-1339 was obtained from Supelco, Bellafonte, PA. DEAE cellulose was obtained from Whatman Chemicals, Clifton, NJ. Biogel HTP (hydroxyapatite) was purchased from Biorad Laboratories, Richmond, CA. Concanavalin A-Sepharose, Polybuffer PBE 94 exchanger and Polybuffer 74 were obtained from Pharmacia Fine Chemicals, Piscataway, NJ. Ultrogel AcA 44 was purchased from LKB Instruments, Rockville, MD. Male rats of the Sprague–Dawley strain were purchased from the Charles River Breeding Laboratories, Wilmington, MA.

Propranolol *d*, *l*; *d* and *l* and practolol were gifts from Ayerst Laboratories Inc., New York, NY. Atenolol was provided by Stuart Pharmaceuticals, Division of ICI Americas Inc., Wilmington, DE. Metoprolol was obtained from the Ciba Pharmaceutical Co., Division of Ciba–Geigy Corp., Summit, NJ. Timolol was provided by Merck Sharp & Dohme Research Lab., Division of Merck & Co., Inc., Rahway, NJ. Labetalol was a gift from Glaxo Group Research Ltd., Ware, UK. All other chemicals used were of analytical grade.

RESULTS

The lysosomal phospholipase A_1 used in our studies has an approximate molecular weight of 34,000 and represents one of the major lysosomal phospholipase isoenzymes in rat liver [10]. Inhibition of lysosomal phospholipase A_1 by beta blockers at a fixed substrate concentration (0.2 mM dioleoylphosphatidylcholine) and various drug concentrations is shown in Fig. 1. On the basis of their ability to inhibit phospholipase A_1, the drugs could be roughly divided into three groups each differing from one another by one to two log concentrations. Propranolol and labetalol are approximately ten times more potent than timolol and metoprolol and one hundred times more potent than atenolol and practolol at a fixed concentration of substrate. The IC_{50}

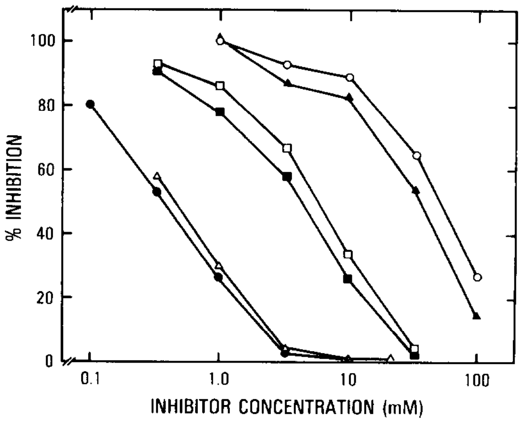

Fig. 1. Effects of various beta-adrenoceptor blockers on rat liver lysosomal phospholipase A_1. The incubation medium contained 50 mM sodium acetate, pH 4.4, 0.2 mM [1-^{14}C] dioleoylphosphatidylcholine, 1 μg enzyme protein and various concentrations of the drugs in a final volume of 0.2 ml. The experimental procedures were as described in Materials and Methods. The results are expressed as a percentage of uninhibited enzyme activity. The drug concentrations shown on the ordinate are given in logarithmic scale. The control enzyme activity was 10.7 ± 0.15 μmoles · hr^{-1} · mg^{-1}. Symbols: (●) propranolol; (△) labetalol; (■) timolol; (□) metoprolol; (▲) practolol and (○) atenolol.

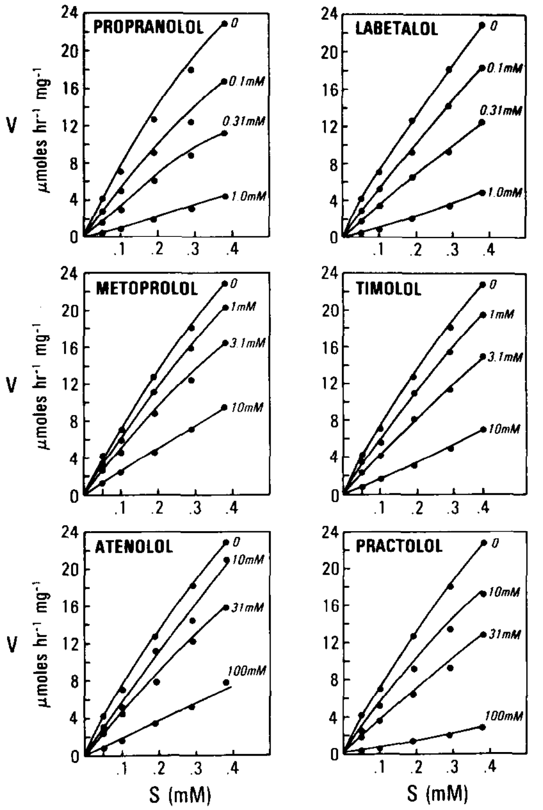

Fig. 2. Studies of lysosomal phospholipase A_1 inhibition by beta-adrenoceptor blockers at various substrate and drug concentrations. The results represent the effects of the increasing substrate concentrations on the reaction velocities of lysosomal phospholipase A_1 in the presence of three fixed concentrations of the respective drugs. The incubation, isolation and measurement of released fatty acid are described in Materials and Methods.

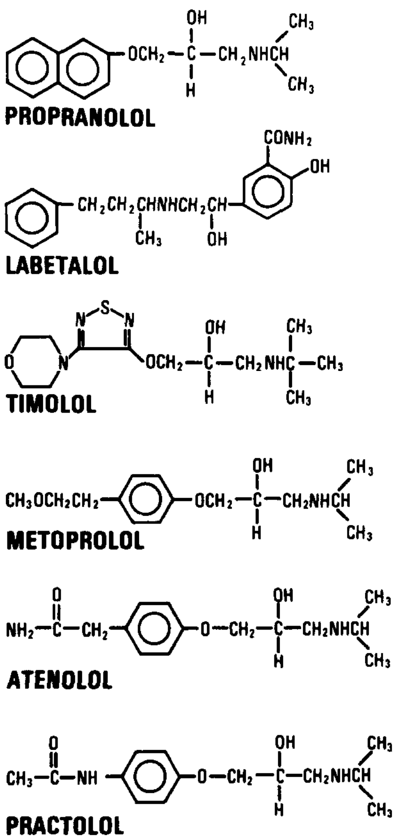

Fig. 3. Structural formulas of beta-adrenoceptor blockers.

values of *d*-propranolol and *l*-propranolol were identical to that of racemic *d*, *l*-propranolol (data not shown).

To investigate in more detail the effects of various drug concentrations on reaction velocity, we incubated sonicated phosphatidylcholine at five different substrate concentrations without additions and with three different fixed drug concentrations; the results are shown in Fig. 2. Based on the data in Fig. 2, we calculated the IC_{50} of the various beta blockers at [1-^{14}C]dioleoylphosphatidylcholine concentrations ranging from 0.05 to 0.4 mM. As shown in Table 1, propranolol and labetalol were most effective as phospholipase A_1 inhibitors with IC_{50} values of 0.29 ± 0.06 and 0.30 ± 0.04 respectively. Fifteen to twenty times more timolol and metoprolol and 103 and 156 times more practolol and atenolol were required to inhibit phospholipase A_1 by 50% than that observed with propranolol and labetalol. Interestingly, the drugs which were most hydrophobic (highest octanol/water partition coefficient) were the most effective inhibitors.

Table 1. Inhibition of purified lysosomal phospholipase A_1 : IC_{50} of various beta blockers

Drug	IC_{50}* (mM)	Log octanol/water partition coefficient†
Propranolol	0.29 ± 0.06	3.65
Labetalol	0.30 ± 0.04	2.39‡
Timolol	4.5 ± 0.56	2.10
Metoprolol	6.1 ± 1.4	2.15
Practolol	31.0 ± 8.1	0.79
Atenolol	46.8 ± 9.0	0.23

* Concentration of drug required to reduce the uninhibited rate by 50%. Data were calculated from results in Fig. 2 and represent the IC_{50}, mean ± S.D. at five different substrate concentrations.

† Adapted from Ref. 15.

‡ Personal communication: J. Bradshaw and D. E. Bays, Glaxo Groups Research Ltd., Ware, England, 1983.

DISCUSSION

The structural formulas of the beta blockers used in these studies are shown in Fig. 3. With the exception of labetalol, all possess a substituted isopropanol having an isopropylamino or a tertiary butyl group as well as a hydrophobic ring structure. They differ mainly in the bulk and the substitution of the ring system. Substituents that reduce the hydrophobicity of the ring tend to reduce the inhibitory potency of the compounds (timolol), and the presence of a charged group in the hydrophobic region greatly lowers the inhibitory potential (atenolol and practolol).

To examine the role of hydrophobicity group in determining inhibition, we plotted the log of the octanol/water partition coefficient, a measure of hydrophobicity, against the IC_{50}, a measure of the relative potency of the drugs in inhibiting the enzyme. Linear regression analysis of the data by least squares fit indicates an excellent negative correlation between the log octanol/water partition coefficient of these compounds and their relative inhibitory potencies ($r = -0.91$). Thus, lipid solubility appears to be a major determinant of a beta adrenergic blocker inhibition of lysosomal phospholipase A_1. Bulk of the aromatic ring system of the drugs has also been shown to play a significant role in determining inhibition of rat liver phosphatidate phosphohydrolase [16].

It has been demonstrated that CAD form complexes with phospholipids; these CAD–phospholipid complexes are suggested to be less susceptible to hydrolysis by phospholipases [5, 17]. The present studies do not directly support or exclude this possibility. However, our recent experiments with lysosomal phospholipase A preparations using neutral and acidic phospholipid substrates indicate that much more inhibitor is required to block acidic phospholipid breakdown [13]. Since acidic phospholipids would be expected to form complexes with CAD more readily than neutral phospholipids [17, 18], this suggests the possibility that CAD–enzyme interactions may also be a significant factor in the inhibition mechanism(s). Kunze *et al.* [19] previously demonstrated binding of a CAD to a Ca^{2+}-dependent, neutral phospholipase A_2 by equilibrium gel filtration. However, CAD interaction with lysosomal phospholipase A_1 has not yet been studied due to the lack of large amounts of pure enzyme which would be required for this type of binding study.

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