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Changes in brain polyphosphoinositide metabolism induced by cationic amphiphilic drugs *in vitro*

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Propranolol, which contains both a hydrophobic moiety and a basic amino group, is a cationic amphiphilic drug. A number of such drugs in chronic clinical use are known to induce phospholipidosis in mammals, characterized by occurrence in tissues of cytoplasmic inclusion bodies with properties of lysosomes and containing polar lipids [1–4]. Propranolol, an agent with β-adrenergic receptor blocking and other pharmacological properties, which is widely used in the treatment of hypertension and is of possible utility in schizophrenia [5, 6], may have the potential to be a lipidosis-inducing drug, since it can profoundly alter the pattern of phospholipid metabolism and induce acidic lipid accumulation in tissues *in vitro* [7–14].

The principal effects of propranolol in pineal gland are to stimulate *de novo* synthesis of the liponucleotide phosphatidyl-CMP (CDP-diacylglycerol) and its acidic metabolic products, phosphatidylinositol and phosphatidylglycerol, and to reduce labeling of phosphatidylcholine. In brain cortex mince, on the other hand, the main phospholipid showing increased labeling is phosphatidic acid [11, 12], although decreased incorporation of $^{32}P_i$ into phosphatidylcholine is also characteristic of the action of propranolol in this preparation.

In the course of the study on propranolol-induced alterations of the labeling pattern of phospholipids in rat tissues *in vitro*, we obtained evidence that in addition to the changes described earlier [11, 12] the labeling of polyphosphoinositides from $^{32}P_i$ in preparations of brain is markedly enhanced. This effect of propranolol on brain was compared with those on other tissues and of other cationic amphiphilic drugs on brain cortex. The influence on polyphosphoinositide labeling of different treatments of the tissue preparation and of additions to the incubations has also been investigated.

Materials and methods

Materials. Materials were obtained from the following suppliers: [^{32}P]orthophosphate and [2-^{3}H]myoinositol from New England Nuclear, Boston, MA; (±)-propranolol from Ayerst Laboratories, Inc., New York, NY; haloperidol from McNeil Laboratories, Inc., Fort Washington, PA; pimozide from Janssen Pharmaceutical N.V., Beerse, Belgium; *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES) and chloroquine from the Sigma Chemical Co., St. Louis, MO; and diacylglycerol from pig liver lecithin from Serdary Research Laboratories, London, Ontario, Canada. 4,4'-Bis(diethylaminoethoxy)α,β-diethyldiphenylethane was a gift from Dr. J. Eichberg, Houston, TX. Silica gel H thin-layer plates were purchased from Analtech, Inc., Newark, DE. Silica gel G-60 EM precoated plates were from Brinkmann Instruments, Inc., Westbury, NY. Kodak X-omat RP film was used for radioautography. Adult male rats were provided by Charles River Breeding Laboratories, Wilmington, MA.

Methods. Rats were decapitated and their brains removed immediately. Cerebral cortices were carefully freed of underlying white matter and a mince was prepared with a McIlwain tissue chopper. The mince was suspended at a concentration of approximately 40 mg wet tissue/ml in an incubation medium containing 144 mM NaCl, 6 mM KCl, 5 mM glucose, 2.5 mM $CaCl_2$ and 1.2 mM $MgCl_2$, buffered to pH 7.4 with 50 mM HEPES. Whenever the mince was washed, homogenized, or dialyzed, the same incubation medium was used. The incubations were carried out with 10 mg of mince together with 5–10 μCi $^{32}P_i$, plus drugs and lipid precursors when indicated in a final volume of 0.5 ml for 1 hr at 37° in air. Propranolol was used at a concentration of 0.1 or 0.2 mM and other drugs at 0.2 mM. Myoinositol and choline, when added, were 1 mM; diacylglycerol (as sonicate) and cytidine were 0.1 mM. The reactions were terminated by the addition of 7.5 ml chloroform–methanol (2:1, v/v). The extraction, washing and separation of lipids were essentially as described by Smith and Hauser [15].

The method of Hauser and Eichberg [16] was used when extraction of polyphosphoinositides from the tissue residue was desired. Polyphosphoinositides obtained in this way were separated by one-dimensional chromatography using two successive solvent systems [16]. Lipids were visualized by radioautography, scraped from the plates, and counted in toluene scintillation fluid. Radioactivity data were normalized to 1×10^7 cpm added to the incubation mixture.

Results and discussion

Incubation in air of cerebral cortex mince with 0.1 mM propranolol caused a large increase in the incorporation of $^{32}P_i$ into phospholipids as compared to controls (Table 1). When the medium was equilibrated and the incubation performed with 95% O_2–5% CO_2, total incorporation was increased, but the changes induced by propranolol remained the same. Washing the mince with incubation medium to reduce endogenous P_i enhanced its ability to incorporate $^{32}P_i$ into phospholipids more than 2-fold and retained its susceptibility to the action of propranolol. Dialysis of the mince reduced its ability to incorporate $^{32}P_i$ as compared to unwashed mince but did not lower the incorporation in the presence of the drug. Homogenization of the washed preparation reduced incorporation consider-

Table 1. Effect of different physical treatments of brain cortex mince on incorporation of $^{32}P_i$ into phospholipids*

Treatment	Total phospholipids (cpm × 10^{-3}/10 mg tissue)		Polyphosphoinositides (cpm × 10^{-3}/10 mg tissue)			
	Control	Propranolol	Control	% of Total	Propranolol	% of Total
	8.7 ± 2.6	14.8 ± 2.7	3.4 ± 1.4	39	6.3 ± 0.9	43
Washing	21.5 ± 3.6	35.8 ± 4.5	11.8 ± 1.5	55	21.6 ± 2.5	60
Washing and homogenization	9.0 ± 1.9	14.2 ± 1.1	5.0 ± 0.7	56	7.9 ± 0.4	56
Dialysis	5.1 ± 0.8	13.9 ± 3.5	2.6 ± 0.1	51	8.3 ± 0.3	60

* Brain cortex mince was incubated and lipids were extracted as described in Materials and Methods. Propranolol was 0.1 mM. The mince was washed twice with incubation medium or dialyzed against 50 volumes of incubation medium for 1.5 hr with changes of the medium at intervals of 30 min. The results are means ± S.D. of three observations. All values obtained in the presence of propranolol were significantly different from control values, $P < 0.02$.

ably, presumably owing to the less efficient energy metabolism as compared with that in preparations containing intact cells. Although, expressed as a percentage, no stimulation of labeling of acidic phospholipids by propranolol was obtained, relative labeling of neutral lipids was reduced, confirming the direct action of the drug without mediation by cell surface receptors.

Radioautograms of lipids of brain cortex mince, separated by two-dimensional thin-layer chromatography, showed the presence of intense radioactivity in the area near the origin. The location of the unknown radioactive lipid on the chromatogram excluded the possibility of its being phosphatidyl-CMP which might have been expected to be present in analogy with findings in pineal gland [7–11]. The radioactivity eluted from this area did not cochromatograph with lysophosphatidylcholine, lysophosphatidic acid or lysophosphatidylinositol. We have identified this material as polyphosphoinositides with diphosphoinositide as the major constituent by cochromatography with authentic samples in several solvent systems and by identification of the alkaline methanolysis products [17].

The presence of polyphosphoinositides can be attributed to the fact that the method used for washing the neutral lipid extract [15] involves the use of dilute HCl in order to retain acidic lipids completely in the lower phase. Under these conditions, about 70% of diphosphoinositide (phosphatidylinositol-4-phosphate) and about 30% of triphosphoinositide (phosphatidylinositol-4,5-biphosphate) are obtained as compared to the amounts obtained by the method for extraction of total polyphosphoinositides [16]. Suitable corrections were made in all instances where the tissue residue was not further extracted with acidified solvents.

The altered basal incorporation into total phospholipids produced by manipulation of the mince resulted in even higher labeling of polyphosphoinositides as a percentage of the total (Table 1). The enhanced labeling of phospholipids triggered by propranolol was mirrored in polyphosphoinositides which accounted for a significantly greater percentage of the increase than other lipids in washed or dialyzed as well as unwashed mince. Since the effect of propranolol may manifest itself by enhancing precursor incorporation into polyphosphoinositides and thus limiting precursor availability for biosynthesis of other phospholipids, it was studied in the presence of diffusible substrates. Propranolol had no effect on the labeling of phosphatidylinositol [12], and hence the results are expressed as the ratio of radioactivity incorporated into polyphosphoinositides to that incorporated into phosphatidylinositol (Table 2). Addition of cytidine and inositol, essential factors for phosphatidylinositol and polyphosphoinositide biosynthesis, enhanced the ratio significantly, but addition of choline or diacylglycerol, which might favor neutral phospholipid biosynthesis, did not divert the label from polyphosphoinositides (Table 2). The magnitude of the polyphosphoinositide effect brought about by propranolol could thus be influenced to some extent by the quantity of certain precursors in the medium. Studies with [^{3}H]inositol indicate that propranolol enhances the labeling of polyphosphoinositides without affecting the labeling of phosphatidylinositol, yielding ratios of radioactivity (polyphosphoinositides/phosphatidylinositol) for control and propranolol-treated mince of 0.19 ± 0.03 and 0.66 ± 0.01 respectively.

The results thus clearly show that propranolol selectively causes enhanced incorporation of precursors into polyphosphoinositides in brain cortex mince. However, the observed changes in $^{32}P_i$ or [^{3}H]inositol labeling of diphosphoinositides and triphosphoinositides need not necessarily indicate changes in turnover of individual phosphoinositides. Rather they may be indicative of changes in the turnover of the total inositol phospholipid pool [18].

The ^{32}P radioactivity ratio of diphosphoinositide to triphosphoinositide in control brain cortex was 2.2 ± 0.2. Propranolol had no significant effect on this ratio (2.8 ± 0.3), even though it caused an approximately 2-fold increase in the labeling of total polyphosphoinositides.

Table 2. Effect of lipid precursors on $^{32}P_i$ incorporation into polyphosphoinositides of brain cortex mince in the presence of propranolol*

Additions	Ratio of radioactivity Polyphosphoinositides / Phosphatidylinositol
None	2.2 ± 0.2
Choline	2.5 ± 0.2
Diacylglycerol	1.9 ± 0.4
Cytidine	3.8 ± 0.1†
Myoinositol	3.9 ± 0.5‡

* All incubations contained 0.1 mM propranolol. Choline and myoinositol were 1 mM. Cytidine and diacylglycercol (added as sonicate in water) were 0.1 mM. Incubations, extractions of phosphatidylinositol and polyphosphoinositides, and isolations were as described in Materials and Methods. The results are means ± S.D. of three observations.

† $P < 0.005$, compared to the ratio obtained without addition.

‡ $P < 0.01$.

Table 3. Effect of propranolol on polyphosphoinositide labeling in different rat tissues*

Tissue	Control (cpm × 10^{-3} in polyphosphoinositides/10 mg tissue)	Propranolol
Brain		
Cortex	11.5 ± 1.7	25.4 ± 2.8†
Stem	4.3 ± 0.5	8.3 ± 1.1‡
Kidney cortex	21.0 ± 4.0	29.0 ± 5.0
Lung	0.5 ± 0.1	0.7 ± 0.3
Liver	0.4 ± 0.1	0.6 ± 0.1

* All incubations contained 10 mg of washed tissue mince. Propranolol was 0.1 mM. The incubation, extraction, and separation of polyphosphoinositides were as described in Materials and Methods. The results are means ± S.D. of three observations.

† $P < 0.001$, compared to control value.

‡ $P < 0.02$.

In order to investigate the possibility that the drug-induced elevation in polyphosphoinositide metabolism may be a generalized phenomenon occurring also in tissues other than brain, the effect of propranolol was studied in minces of kidney, liver and lung (Table 3). The latter was included because propranolol, administered systemically, reaches particularly high levels in this organ [19]. In these tissues, the percentage of ^{32}P appearing in polyphosphoinositides is lower than in brain, the order being brain cortex (40%) > kidney (12%) > lung (7%) > liver (5%).

Among the tissues studied, the enhancement of labeling in polyphosphoinositides was evident only in brain. When grey and white matter were compared, considerably less incorporation occurred in white matter. The stimulation by propranolol was substantially greater in grey matter, even though polyphosphoinositides are preferentially localized in myelin, where they constitute a major, relatively stable pool [16]. Thus, the central nervous system is the tissue most susceptible to the action of propranolol on lipid metabolism. These results may be important in view of the fact that propranolol can pass the blood-brain barrier [20], can be accumulated in cerebral cortex [21], and could therefore be readily effective in influencing brain lipid metabolism *in vivo*. Studies to examine this possibility are in progress.

To determine the possible relationship between the structure of the drug and the observed polyphosphoinositide effect, the influence of several compounds with similar physico-chemical characteristics on the labeling of phosphoinositides was studied in brain cortex (Table 4). 4,4'-Bis(diethylaminoethoxy)α,β-diethyldiphenylethane, chloroquine, chlorpromazine, and fenfluramine enhanced the labeling ratio of polyphosphoinositides/phosphatidylinositol, although not as much as did propranolol. The first two of these have been shown to induce lipidosis in mammals after injection [2]. The ratios in the experiments with haloperidol and pimozide were unchanged from control, but haloperidol, in contrast to pimozide, increased labeling of all phosphoinositides equally. Both had effects on the incorporation of ^{32}P into other phospholipids [12]. Thus, it appears that the size and degree of substitution of the hydrophobic group are positively correlated with the ability of the drug to influence the disposition of label in phospholipids. In this context it is pertinent that, in guinea pig cortex slices, results on phosphoinositide labeling from ^{32}P in the presence of chlorpromazine, haloperidol or pimozide were similar to ours, except that chlorpromazine also increased the specific activity of phosphatidylinositol [22].

Di- and triphosphoinositides have high affinity for Ca^{2+}, so that interconversion of phosphoinositide pools might modulate membrane-bound Ca^{2+} levels and thus help control free and bound intracellular Ca^{2+} concentrations [16, 23]. In view of the fact that a number of cationic amphiphilic drugs influence the uptake and binding of Ca^{2+} [24, 25] as well as polyphosphoinositide metabolism, the compounds should be useful for obtaining a better understanding of the interrelationship between polyphosphoinositides, Ca^{2+} pools, and the regulation of membrane functions in nervous tissue.

In summary, propranolol and some other cationic amphiphilic drugs are able to enhance labeling of polyphosphoinositides in rat cerebral cortex preparations *in vitro*. The extent of this drug-induced labeling is partly regulated by the availability of cytidine and inositol. The increase in polyphosphoinositide metabolism appears to be limited to neural tissues and is greater in grey than in white matter.

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Table 4. Effect of cationic amphiphilic drugs on incorporation of $^{32}P_i$ into phosphoinositides of cerebral cortex mince*

Additions	Polyphosphoinositides (cpm × 10^{-3}/10 mg tissues)	Phosphatidylinositol	Ratio of radioactivity: Polyphosphoinositides / Phosphatidylinositol	Increase over control (%)
None	6.3 ± 0.7	4.5 ± 0.3	1.4 ± 0.4	
Propranolol	23.0 ± 2.0†	3.6 ± 0.8	6.4 ± 2.0†	370
4,4'-Bis-(diethylaminoethoxy)-α,β-diethyldiphenylethane	18.5 ± 0.5†	4.5 ± 0.2	4.1 ± 0.6†	203
Chlorpromazine	11.5 ± 1.2†	3.6 ± 0.5	3.2 ± 0.3‡	135
Fenfluramine	21.0 ± 2.2†	8.4 ± 0.1†	2.4 ± 0.1†	76
Chloroquine	8.2 ± 0.8‡	3.9 ± 0.3	2.1 ± 0.1‡	51
Pimozide	6.4 ± 1.1	4.0 ± 0.3	1.6 ± 0.2	17
Haloperidol	8.7 ± 0.9‡	6.7 ± 0.1†	1.3 ± 0.1	−6

*All drugs were used at a concentration of 0.2 mM. The incubations and extractions of phosphoinositides were as described in Materials and Methods. The results are means ± S.D. of three to nine observations.

† $P < 0.01$, compared to the value obtained without addition.

‡ $P < 0.05$.

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Quantitative correlation between secretion and cellular content of catecholamines and dopamine-β-hydroxylase in cultures of adrenal medulla cells*

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Essentially all of the catecholamines (CA) and dopamine-β-hydroxylase (DBH) of the bovine adrenal medulla is present in the chromaffin vesicles [1–3]. Osmotic lysis of the vesicles results in the total release of the CA and solubilization of about 50 per cent of the DBH activity [4]; the remainder of the DBH is tightly bound to the membrane and requires a detergent for solubilization [5]. The concurrent release of CA and DBH upon nicotinic stimulation of isolated perfused adrenal glands has been demonstrated in several laboratories [6–10] and has been used to support the hypothesis that secretion occurs by exocytosis. However, there is disagreement about the quantitative relationship between the relative amounts of CA and DBH secreted and that present in the cell. Viveros *et al.* [6] using isolated bovine adrenal glands found a parallel increase in the amounts of CA and DBH released; the ratio of DBH:CA in the perfusate was similar to the DBH:CA ratio in subcellular fractions of the gland. Similarly, Ito *et al.* [7] using perfused guinea pig adrenal glands found that a variety of secretagogues caused the release of DBH and CA in very nearly the same ratio as that found in the soluble fraction of chromaffin vesicles. Aunis *et al.* [8] and Serck-Hanssen *et al.* [9] also found a parallel increase in the amounts of CA and DBH released upon stimulation, but the ratios of DBH:CA in the perfusates were considerably less than that of the soluble content of isolated chromaffin vesicles. They attributed this to both a release of CA and DBH from vesicles having a lower than average DBH:CA ratio and to partial inactivation of the DBH that was released. On the other hand, Dixon *et al.* [10] found a poor correlation between the amounts of CA and DBH released in stimulated perfused adrenal glands; the ratio of DBH:CA in the perfusate was variable and much less than that of the soluble content of isolated chromaffin vesicles. They concluded that the release of CA was quantal but that the release of DBH was not necessarily coupled to it.

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