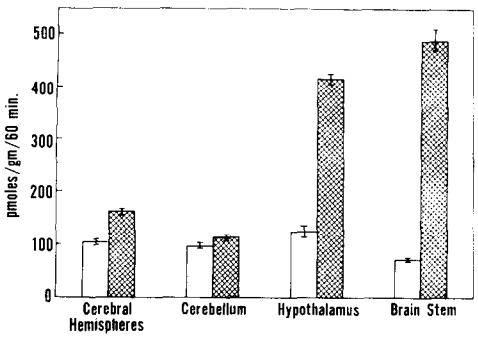

Fig. 2. Methylation of norepinephrine (cross-hatched bars) and α-methylnorepinephrine (open bars) by homogenates of rat brain regions. The substrates were present at 500 μM. Mean values ± standard errors for three rats are shown.

brain regions. These results suggest that the two substrates are not being methylated by the same enzyme, the α-methyl compound being acted on by a relatively ubiquitous enzyme whereas norepinephrine is methylated more rapidly by an enzyme localized in the hypothalamus and brain stem. Rat brain has been shown to contain "nonspecific" methyltransferase that can act on various arylalkylamines [18].

These studies lead us to suggest that α-methylnorepinephrine is a poor substrate for PNMT but that it can be *N*-methylated by another brain enzyme. Thus, the idea that α-methylepinephrine might be a metabolite formed in brain after administration of α-methyldopa is plausible, but the formation of α-methylepinephrine probably would not occur by PNMT action. There is then no basis for expecting that the formation of α-methylepinephrine would occur solely within PNMT-containing neurons or that these epinephrine-forming neurons would be preferentially affected.

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Evidence for a carbachol stimulated phosphatidylinositol effect in heart

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A wide variety of tissues show a selective increase in the incorporation of ^{32}P-inorganic phosphate into phosphatidic acid and phosphatidylinositol in response to certain neurotransmitters and hormones [1, 2]. Cholinergic agents such as acetylcholine and carbachol have been shown to induce this so-called phosphatidylinositol effect in pancreas [3], adrenal medulla [4], smooth muscle [5, 6], synaptosomes [7], avian salt gland [8] and parotid and lacrimal glands [9] by stimulating or acting on muscarinic receptors. Although various regions of cardiac tissue possess muscarinic receptors, evidence for stimulation of ^{32}P-incorporation into phosphatidylinositol and phosphatidic acid by cholinergic agents has not been reported previously. This study was undertaken to examine the effect of the non-

hydrolyzable acetylcholine analogue carbachol on incorporation of ^{32}P into phospholipids in various regions of canine heart.

Methods

Dogs of either sex were anesthetized with surital and halothane, and the heart was removed. Slices of heart were dissected free of fat and large blood vessels and placed in a cold solution containing 124 mM NaCl, 5 mM KCl, 0.3 mM KH_2PO_4, 1.3 mM $MgCl_2$ and 25 mM HEPES* (pH 7.4). Tissue samples from various areas of the heart were sliced into approximately 1 mm^3 pieces using a razor blade in the above solution at 5°. Tissue slices were added to tubes containing 124 mM NaCl, 5 mM KCl, 0.3 mM $^{32}P_i$ (25 μCi), 1.3 mM $MgCl_2$, 25 mM HEPES (pH 7.4), 1.5 mM $CaCl_2$, 1.6 mM cytidine, 1.6 mM myoinositol and 10 mM dextrose. The tubes were incubated for 60 min at 37° and placed in ice. Carbachol, atropine or *d*-tubocurarine was added where indicated, and the tubes were further incubated for up to 30 min at 37°. Three milliliters of cold 5% trichloroacetic acid was added, and the tubes were centrifuged for 10 min at 1500 *g* at 5°. The pellets were washed with 3 ml of water. Finally, the pellets were resuspended in 3 ml of $CHCl_3$–CH_3OH–HCl (20:40:1) and homogenized using a motor-driven all-glass homogenizer at 5°. The homogenizer was washed consecutively with 1.1 ml of $CHCl_3$ and H_2O, and the washings were combined with the homogenate. The tubes were vortexed and centrifuged at 1500 *g* for 10 min. The upper aqueous-methanol phase and the interface were removed by aspiration and the $CHCl_3$ phase was washed with 2 ml of 0.1 N HCl. A 1 ml aliquot of the $CHCl_3$ phase was dried under N_2. The lipids were resuspended in 30 μl of $CHCl_3$–CH_3OH–HCl (60:30:1), and 10 μl aliquots were spotted on silica gel 60 plates (E. Merck) or analyzed for total phospholipid content by the method of Bartlett [10]. Phospholipids were separated on the thin-layer plates using a two-solvent system described by Schacht *et al.* [11]. This system does not routinely separate phosphatidylserine and phosphatidylinositol. Therefore, in some studies, phosphatidylinositol (*rf* .35) and phosphatidylserine (*rf* .35) were separated on silica HL plates (Analtech) in $CHCl_3$–CH_3OH–CH_3NH_2 (65:35:10) by a modification of the method of Harrington *et al.* [12]. Phospholipids were detected by autoradiography or in an I_2 chamber and identified using authentic phospholipid standards. Radioactive phospholipids were scraped from the plates and counted in tritisol. Data were expressed in terms of cpm/mg of total phospholipid.

Results and Discussion

Labeling of cardiac phospholipids with ^{32}P. In preliminary experiments, the relative amounts of ^{32}P incorporated into individual phosphilipids in slices of right and left auricles and right and left ventricles were compared. Generally, in auricular as well as in ventricular tissue, ^{32}P-incorporation was highest in phosphatidylinositol 4,5-biphosphate, phosphatidylinositol 4-phosphate, phosphatidylcholine, phosphatidic acid and phosphatidylinositol (Table 1). Incorporation of ^{32}P into phosphatidylserine and phosphatidylethanolamine was relatively low. There were no significant differences in phospholipid labeling between right and left auricular tissue or right and left ventricular tissue. However, ^{32}P-incorporation into phospholipids in right auricular tissue was approximately 2.5-fold higher than in ventricular tissue. This difference could be explained by differences in diffusion and uptake of $^{32}P_i$ in these tissues or by differences in the rate of phospholipid metabolism under these conditions. Santiago-Calvo *et al.* [13] previously reported a similar distribution of ^{32}P-labeling of individual phospholipids in slices of ventrical from guinea pig heart. A comparison between ^{32}P-incorporation into phospholipids in auricle and ventricle was not done in that study.

Effect of carbachol on ^{32}P-incorporation into cardiac phospholipids. In this study, the effect of carbachol on ^{32}P-incorporation into phosphilipids was examined in slices of both atrial (auricular appendage) and ventricular tissue. Specifically, slices of the auricles, which represent a large part of the walls of the atria, were used for the study of carbachol on atrial tissue. Furthermore, atrial was well as ventricular slices were examined because it is well known that, in most species, parasympathetic or cholinergic nerves innervate mainly the atria and the sinoatrial nodes of the heart with sparse innervation to the ventricles [14]. Carbachol also affects heart rate when applied to the atria only [14]. If slices of right auricle were pre-labeled with ^{32}P, the addition of 50 μM carbachol selectively increased ^{32}P-incorporation into phosphatidylinositol and phosphatidic acid, 70 and 80% respectively, without affecting the labeling of other phospholipids (Table 2). This concentration of carbachol was found not to have any effect on ^{32}P-incorporation into any phospholipid in slices of left auricle or right or left ventricle under the same conditions. This selectivity of carbachol to induce a phospholipid effect in slices of right auricle has been observed in six separate heart preparations. Further studies showed that atropine (50 μM), a muscarinic receptor blocking agent, but not the nicotinic blocker, *d*-tubocurarine, completely inhibited the carbachol stimulated phospholipid effect in slices of right auricle. Therefore, the phospholipid effect mediated by carbachol appears to have been due to stimulation of muscarinic receptors. Carbachol was found to half-maximally stimulate ^{32}P-incorporation into phosphatidylinositol and phosphatidic acid at 5–10 μM (Fig. 1). Maximal activation occurred at 20–50 μM. Because carbachol produced these effects at pharmacologically effective concentrations, the phosphatidylinositol effect found here in right atrial tissue

* HEPES, 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid.

Table 1. Incorporation of ^{32}P into phospholipids in slices of right auricle and ventricle

Phospholipid	^{32}P incorporated* (cpm/mg)	
	Right auricle	Right ventricle
Phosphatidylinositol 4,5-biphosphate	21,480 ± 1,890	8,390 ± 772
Phosphatidylinositol 4-phosphate	6,860 ± 493	2,850 ± 208
Phosphatidylcholine	22,610 ± 2,781	8,370 ± 929
Phosphatidylinositol	9,500 ± 589	2,400 ± 168
Phosphatidic acid	12,450 ± 1,369	4,980 ± 552
Phosphatidylserine	150 ± 19	60 ± 24
Phosphatidylethanolamine	660 ± 61	225 ± 30

* Slices of tissue were incubated for 60 min with 0.3 mM [^{32}P]orthophosphoric acid. (see Methods for other conditions). Each value is the mean ± S.E. of triplicate determinations.

Table 2. Effect of carbachol on ^{32}P-incorporation into phosphatidylinositol and phosphatidic acid

	^{32}P incorporated* (cpm/mg)	
	Phosphatidylinositol	Phosphatidic acid
Right auricle		
Control	6,700 ± 445	9,183 ± 868
+ 50 μM Carbachol	11,400 ± 912	16,713 ± 1,203
Left auricle		
Control	6,520 ± 795	8,476 ± 271
+ 50 μM Carbachol	6,610 ± 773	8,740 ± 629
Right ventricle		
Control	2,010 ± 241	2,600 ± 312
+ 50 μM Carbachol	2,150 ± 155	2,850 ± 333
Left ventricle		
Control	2,130 ± 69	2,570 ± 313
+ 50 μM Carbachol	2,050 ± 344	2,480 ± 164

* Results are representative of six separate experiments. Each value is the mean ± S.E. of triplicate determinations.

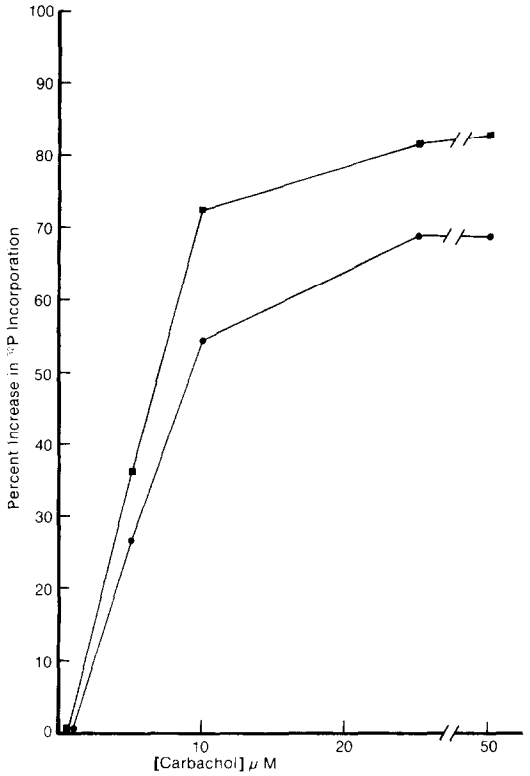

Fig. 1. Stimulation of ^{32}P-incorporation into phosphatidylinositol (●) and phosphatidic acid (■) by carbachol.

probably is physiologically and pharmacologically relevant. A number of investigators have reported that higher or similar concentrations of carbachol induce a phosphatidylinositol effect in other tissues [1, 2, 9].

The absence of a carbachol-stimulated phosphatidylinositol effect in ventricular slices is not surprising, because of the limited parasympathetic innervation of ventricular tissue noted above. However, it was unexpected that the phospholipid effect found here in slices of right auricle was not found in slices of left auricle, because parasympathetic nerves innervate the left and right atria in addition to the sino-atrial nodes. A possible explanation for these results could be that parasympathetic innervation to the right auricle of the right atrium is greater than the left auricle. Therefore, it can not be ruled out that other regions of the left atrium such as the septal region dividing the atria may show a phospholipid effect in response to carbachol or other muscarinic agents. So far this has not been examined.

Presently, the physiological significance of the neurotransmitter or hormonally stimulated phospholipid effect which is found in a wide variety of tissues is unclear [1, 2, 9]. Generally, it has been found that the stimulated increase in the incorporation of ^{32}P into phosphatidylinositol and phosphatidic acid reflects the breakdown of phosphatidylinositol followed by increased synthesis of phosphatidylinositol [15]. Marshall *et al.* [16] recently reported that arachidonic acid was released from phosphatidylinositol during stimulated breakdown in pancreas and that the released arachidonic acid was further synthesized to a prostaglandin. It is anticipated that further studies on the effects of carbachol and cholinergic drugs on phospholipid metabolism and perhaps prostaglandin synthesis in cardiac tissue may further our understanding of the biochemical mechanism by which muscarinic receptors regulate cardiac function.

In summary, incubation of canine heart slices with ^{32}P-inorganic phosphate in physiological medium resulted in the incorporation of ^{32}P into a number of cardiac phospholipids. Carbachol, a non-hydrolyzable acetylcholine analogue, selectively increased ^{32}P-incorporation into phosphatidic acid and phosphatidylinositol in slices of right auricle 80 and 70% respectively. This effect of carbachol was half-maximal at 5–10 μM and was inhibited completely by atropine. Carbachol did not affect labeling of other phospholipids in right auricle and did not affect the labeling of any phospholipid in slices of left auricle or right or left ventricle. These results provide evidence for a muscarinic receptor mediated phospholipid effect which appears to be localized in the right atrial region of canine heart.

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Some biochemical characteristics of L1210 cell lines resistant to 6-mercaptopurine and 6-thioguanine and with increased sensitivity to methotrexate

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In experimental animal tumor systems, drug resistance has been demonstrated for all functional classes of anticancer agents [1]. In man, the recurrence or increased growth of leukemias and solid tumors with continuing, unchanged drug treatment suggests that overgrowth of drug-resistant tumor cells is also a therapeutic problem. With L1210 and P388 murine leukemias, we have shown that tumor cells resistant to a single drug or combination of drugs can be controlled in animal models by further treatment with anticancer agents of other classes [1]. Moreover, in some cases, drug-resistant tumor cells exhibit increased sensitivity to alternate drugs.

The 6-mercaptopurine (6MP)* and 6-thioguanine (6TG) resistant L1210 leukemia cells (L1210/6MP, L1210/6TG) used in this investigation are deficient in the purine salvage enzyme, hypoxanthine-guanine phosphoribosyltransferase, EC 2.4.2.8 [2, 3]. Currently, their ability to convert hypoxanthine, guanine, 6MP and 6TG to 5′-ribonucleotide derivatives is 10%, or less, than that of the parent cell line (L1210/0). This suggests that the cells rely heavily on *de novo* purine synthesis.

By inhibiting dihydrofolate reductase (DHFR), EC 1.5.1.3, methotrexate (MTX) depletes the pool of one-carbon units carried by tetrahydrofolate derivatives [4–6] and, as a result, anabolic processes requiring one-carbon units, including the *de novo* synthesis of purines, are inhibited [7, 8]. The toxic effects of an MTX-induced purineless state might be amplified in cells that have a reduced capacity to salvage purine bases. In support of this prediction, Browman and Csullog [9] have reported that DNA synthesis in cell lines with impaired purine salvage was approximately 40% of that in parent drug-sensitive cells after MTX administration to tumor-bearing mice. Although cultured cells resistant to purine analogs did not exhibit collateral MTX sensitivity [9], such *in vitro* sensitivity has been noted in our laboratories [10]. In this investigation, we confirm the increased MTX sensitivity *in vivo* of an L1210 cell line resistant to 6MP, first observed by Law *et al.* [11], and report that a line of L1210 cells resistant to 6TG is similar in response. We have also investigated the biochemical bases for these increases in sensitivity.

The parent L1210 tumor cell line and the cell lines resistant to 6MP and 6TG were maintained by weekly serial passage in female DBA/2 mice as described previously [12]. To test sensitivity of the three tumor cell lines to MTX, 6-methylthiopurine ribonucleoside (6-MeMPR), 6MP or 6TG, cells were harvested from the peritoneal cavities of mice on day 6 or 7 post-implant, counted and inoculated intraperitoneally into healthy 6- to 10-week-old male or female BALB/c × DBA/2 (CDF_1) mice. In each experiment, the inoculum was titrated in 10-fold dilutions down to one cell to determine cell doubling time and to estimate the total body burden of tumor cells at the time of treatment. Tumor cell inoculum sizes, drug treatment schedules and the number of animals treated are given in Table 1. The approximate number of tumor cells alive after the last drug treatment was calculated from the life spans of treated mice (relative to those of control mice), duration of treatment, and cell doubling time [13]. Drug toxicity was based on the absence of gross evidence of leukemia (ascites or splenomegaly) at the death of treated mice.

To determine the intracellular MTX concentration in the three tumor cell lines following treatment of tumor-bearing mice, 3 mg/kg of [3′,5′,9(n)-^{3}H] MTX ([^{3}H]MTX) [9.4 μCi/mg, Amersham/Searle Corp., Arlington Heights, IL, radiochemical purity 95% by high pressure liquid chromatography (HPLC)], dissolved in 2% $NaHCO_3$, was administered subcutaneously to female CDF_1 mice that had received intraperitoneal tumor cell implants (10^5 to 10^6 cells/mouse) 6 days earlier. At 1, 2, 4, 12 and 24 hr after dosing, mice were killed by cervical dislocation, and L1210 cells were harvested from the peritoneal cavity with heparinized syringes. The cells were washed with saline, and erythocytes were lysed with NH_4Cl [14]. The L1210 cells were then resuspended in saline, and a portion was counted with a model Z Coulter Counter. After lysis of the remaining cell suspension, 15 ml of Liquifluor (New England Nuclear Corp., Boston, MA) containing Bio-Solv BBS-3 (Beckman Instruments, Fullerton, CA) (14:1, v/v) was added to 0.1-ml samples and radioactivity was measured with a Packard Tri-Carb liquid scintillation spectrometer (model 3315) equipped with an external standard.

* Abbreviations: 6MP, 6-mercaptopurine; 6TG, 6-thioguanine; L1210/6MP, 6MP-resistant L1210 cell line; L1210/6TG, 6TG-resistant L1210 cell line; L1210/0, parent L1210 cell line; DHFR, dihydrofolate reductase; MTX, methotrexate; 6-MeMPR, 6-methylthipurine ribonucleoside; HPLC, high pressure liquid chromatography; and ILS, increase in life span.