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MUSCARINIC RECEPTOR STIMULATED PHOSPHOINOSITIDE TURNOVER IN CARDIAC ATRIAL TISSUE

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Abstract—The properties of muscarinic agonist stimulated phosphoinositide turnover in canine atrial slices were investigated. In slices prelabeled with $^{32}P_i$, carbachol stimulated a 20–30% decrease of ^{32}P-labeled phosphatidylinositol 4′-phosphate (PIP) and phosphatidylinositol 4′,5′-bisphosphate (PIP_2) content within 10–15 sec. This was followed by a resynthesis of these lipids to control levels after 30 sec. Carbachol-stimulated PIP and PIP_2 turnover was followed by a relatively slower increase in ^{32}P incorporation into phosphatidylinositol (PI) and phosphatidic acid (PA) which was maximal after 5–10 min. Carbachol increased ^{32}P-labeling of PA and PI in most regions of right and left atria with equal effectiveness. Muscarinic receptor stimulated increases in PA and PI labeling showed high specificity for certain muscarinic agonists and, unlike most tissues, this muscarinic receptor mediated phospholipid effect was dependent on extracellular calcium. Carbachol did not increase ^{32}P incorporation into PA and PI if Mn^{2+}, Co^{2+}, Mg^{2+}, or La^{3+} was substituted for extracellular Ca^{2+}. Unlike other muscarinic agonists, acetylcholine increased ^{32}P incorporation into phosphatidylcholine in addition to PA and PI. Low concentrations of calcium channel blockers, verapamil, nifedipine or diltiazem, did not block carbachol-stimulated changes in PA and PI labeling in the presence of Ca^{2+}; however, higher concentrations ($\geq 10\,\mu M$) of verapamil increased PA and PI labeling. Ouabain enhanced carabachol-stimulated ^{32}P incorporation into PA but attenuated incorporation into PI. The mechanisms associated with the actions of these agents on phospholipid metabolism and their possible physiological significance are discussed.

Hokin and Hokin in 1953 showed that acetylcholine induces a selective increase in [^{32}P]-labeled inorganic phosphate ($^{32}P_i$) incorporation into phosphatidic acid (PA) and phosphatidylinositol (PI†) in pigeon pancreas by stimulating muscarinic receptors [1]. Since that time, numerous investigators have reported that acetylcholine and certain other muscarinic agonists produce this "phospholipid" effect in a number of tissues regulated by muscarinic receptors [2, 3]. Recent studies suggest that stimulation of muscarinic receptors results in the activation of a polyphosphoinositide phospholipase C or phosphodiesterase which breaks down phosphatidylinositol 4′,5′-bisphosphate (PIP_2) and phosphatidylinositol 4′-phosphate (PIP) to diacylglycerol (DAG) and their respective inositol phosphates, inositol tris- and bisphosphate [4–7]. At least a portion of the DAG produced is synthesized to PA and PI which accounts for the increase in ^{32}Pi labeling of these lipids in response to receptor stimulation of the phospholipid effect [7]. Although cardiac function is regulated by muscarinic receptor stimulation, until recently a muscarinic mediated phospholipid effect in heart had not been reported. Carbachol, a non-hydrolyzable muscarinic agonist, can stimulate an increase of $^{32}P_i$ incorporation into PA and PI in canine atria [8] and into PI in murine atria [9]. These effects of carbachol are blocked by the muscarinic antagonist atropine in both species [8, 9]. Brown and Masters [10] have also shown that, in chick myocytes prelabeled with [^3H]myoinositol, carbachol stimulates an increase in [^3H]inositol phosphate (IP) which is a phosphoinositide breakdown product. However, carbachol-stimulated increases in [^3H]IP are not detectable for at least 10 min in chick myocytes [10], and this rate of phosphoinositide breakdown would be too slow for association of the muscarinic receptor stimulated phospholipid effect with either the negative ionotropic or chronotropic effects caused by activation of these receptors in atria.

In this investigation the rate of the muscarinic receptor stimulated phospholipid effect was examined in canine right atrial tissue prelabeled with $^{32}P_i$. The regional distribution, the cation requirements, and the agonist specificity of the muscarinic receptor stimulated changes in phosphoinositide metabolism were also investigated. These studies show that carbachol stimulates a relatively rapid breakdown of polyphosphoinositides in atrial tissue which suggests that this carbachol-mediated effect may have important functional significance in this tissue.

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† Abbreviations: PI, phosphatidylinositol; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*′-tetraacetic acid; PIP, phosphatidylinositol 4′-phosphate; PIP_2, phosphatidylinositol 4′,5′-bisphosphate; IP_2, inositol bisphosphate; IP_3, inositol trisphosphate; PA, phosphatidic acid; DAG, diacylglycerol; PC, phosphatidylcholine; and HEPES, 4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid.

MATERIALS AND METHODS

Materials. [^{32}P]-Labeled inorganic phosphate (10–25 Ci/mmole) was purchased from ICN Biomedicals

Inc., Irvine, CA. Phospholipid standards, carbamylcholine chloride, carbamyl-β-methylcholine chloride, arecoline hydrobromide, physostigmine, acetylcholine chloride, methacholine chloride, pilocarpine hydrochloride, ouabain octahydrate and atropine sulfate were purchased from the Sigma Chemical Co., St. Louis, MO. Solvents used for phospholipid analysis and chromatography were HPLC grade. Silica gel 60 plates (0.25 mm thick) were obtained from Brinkmann Instruments Co., Westbury, NY. Verapamil, diltiazem and nifedipine were gifts from the Knoll Pharmaceutical Co., Whippany, NJ, Marion Laboratories Inc., Kansas City, MO, and Pfizer Inc., New York, NY, respectively.

Labeling studies. Mongrel dogs of either sex were anesthetized with Surital i.v., and the hearts were removed immediately. Heart tissue was dissected free of excess fat and large vessels and placed in a solution containing 124 mM NaCl, 5 mM KCl, 0.3 mM KH_2PO_4, 0.05 mM $CaCl_2$, 1.3 mM $MgCl_2$, 25 mM HEPES, pH 7.4, and 5 mM dextrose at 5°. Tissues from various regions of atria were sliced into approximately 1 mm pieces using a razor blade in the above solution at 5°. Slices of tissue (combined blotted weight 30–40 mg) were added to tubes usually containing 0.5 ml of buffer A which had a composition of 125 mM NaCl, 1.3 $MgCl_2$, 25 mM HEPES, pH 7.4, 5 mM KCl, 0.3 mM $^{32}P_i$ (25 μCi), 5 mM dextrose, 1.0 mM $CaCl_2$, 1.6 mM cytidine and 1.6 mM myoinositol. In some experiments, the composition of the medium and the incubation time were varied as described in the text. Reactions were stopped with 3.0 ml of cold 5% trichloroacetic acid and the pellets were further washed with 3.0 ml of water [8]. Phospholipids were extracted as previously described with minor modifications [8]. Pellets were suspended with 2.5 ml of chloroform–methanol–HCl (20:40:1) and homogenized with a motor-driven all-glass homogenizer. Homogenates were transferred back to the tubes and the homogenizer was washed consecutively with 1.0 ml each of chloroform and water and the washings were combined with the homogenate. The tubes were vortexed and centrifuged at 500 *g* at 5° for 10 min. The aqueous-methanol phase and the protein interface were removed by aspiration, and the chloroform phase was washed with 2 ml of 0.1 N HCl. Aliquots (1 ml) of the chloroform phase were dried under nitrogen. The dried lipids were resuspended with 40 μl of chloroform–methanol–HCl (60:30:1), 20-μl aliquots were spotted on silica gel 60 plates (E. Merck), and a 10-μl aliquot was assayed for total phospholipid phosphorous by the method of Barlett [11]. Plates were first developed for 10 cm in chloroform–methanol–water–30% NH_3 (25:35:7.5:2.5) and dried at room temperature. The plates were then developed for 15 cm in the same direction with chloroform–methanol–methylamine (65:35:10). Phospholipids were detected by autoradiography overnight with XAR-5 X-ray film (Kodak) and in an iodine chamber. The identity of the phospholipids was determined by running authentic standards. R_f values for phosphatidylcholine, PA, PI, PIP and PIP_2 were 0.566, 0.34, 0.244, 0.127 and 0.055 respectively. Phospholipids were scraped from the plates and counted in 8 ml of Tritisol. Values plotted represent cpm/50 μg of total phospholipid. All assays were performed in triplicate, and the mean values were plotted. Standard deviations where not indicated were no greater than 10%.

Polyphosphoinositide determination. For determination of PIP and PIP_2 content, 1 to 1.5 g (wet weight) of sliced atrial tissue was suspended in 2.5 ml of buffer A. The tubes were preincubated for 25 min at 37° with shaking. After this preincubation, either 500 μM carbachol in buffer or buffer was added to the tubes. After 15 or 30 sec further incubation, the reactions were stopped with 2.5 ml of cold 5% trichloroacetic acid. The tubes were centrifuged at 1500 *g* for 10 min and pellets were washed with 5 ml of H_2O. Five milliliters of chloroform–methanol–HCl (20:40:1) was added to the tubes, and the pellets were homogenized for 10 sec at a setting of 8 with a polytron. All steps in the extraction procedure were performed at 5°. After 20 min, 2 ml of chloroform and 1 ml of H_2O were added to the tubes. After vortexing, the tubes were centrifuged at 1500 *g* for 10 min. The upper phase was removed, and the chloroform phase and the protein interface were thoroughly mixed with 5 ml of 0.1 N HCl and centrifuged for 1500 *g* for 10 min. The upper phase was removed, and 2.5 ml of the $CHCl_3$ phase was transferred to tubes. The lipids were dried under nitrogen and resuspended with 0.065 ml of chloroform–methanol–HCl (6:3:0.1) and 0.05 ml was spotted over 2 cm on silica gel 60 plates. Plates were developed as in the isotope labeling studied above, except that the plates were developed 13 cm in the first solvent and 19 cm in the second solvent. In some studies, extracted heart phospholipids prelabeled with $^{32}P_i$ were spotted on top of the lipids for further confirmation of the identity of separated PIP and PIP_2 by autoradiography. PIP and PIP_2 could be visualized in a u.v. light box after spraying the plates with 0.001% Rhodamine 6G. Lipids corresponding to PIP and PIP_2 were scraped from the plates and assayed for phosphorous content [11]. The absorbance of silica gel blanks of equal area were subtracted from the sample values. PIP and PIP_2 contents were estimated by assuming a phospholipid molecular weight of 770 daltons and by dividing values for PIP and PIP_2 by 2 and 3, respectively, to correct for polyphosphate content. Total phospholipid content was estimated by assaying 0.01 ml of the lipid extract for phosphorous [11].

RESULTS

Time course of ^{32}P incorporation into phospholipids. In preliminary studies, the rate of ^{32}Pi incorporation into atrial slices was examined. Labeling of PA, PIP and PIP_2 was found to be maximal after a 30-min incubation (Fig. 1). These phospholipids are relatively minor in terms of total phospholipid content (Table 1) [6] and therefore have probably attained isotopic equilibrium with [γ-^{32}P]ATP. In support of this assumption England and Walsh [12] reported that $^{32}P_i$ incorporation into [γ-^{32}P]ATP reaches equilibrium with a similar rapid time course in rat heart. In contrast, labeling of PC and PI did not plateau after 120 min of incubation. For most subsequent

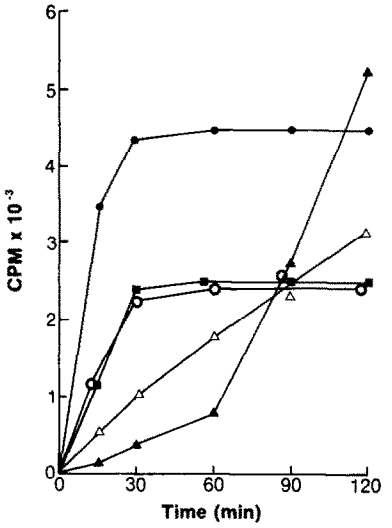

Fig. 1. Time course of ^{32}P incorporation in PA (■), PI (△), PIP (○), PIP_2 (●) and PC (▲) in atrial slices. Results are representative of three separate experiments.

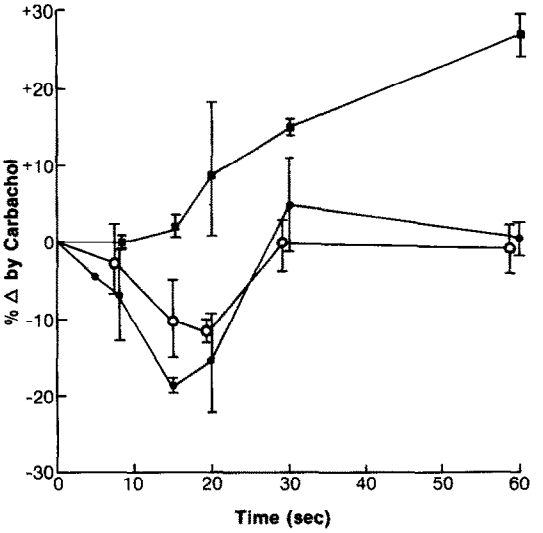

Fig. 2. Effect of carbachol on ^{32}P-labeled PIP_2 (●), PIP (○) and PA (■) in atrial slices prelabeled with ^{32}Pi. Results are expressed as the means ± SD of at least three separate experiments.

Table 1. Effects of carbachol on polyphosphoinositide content in atrial slices

		Polyphosphoinositide content	
Incubation time (sec)	Carbachol (μM)	PIP (μg/g)	PIP_2 (μg/g)
15	0	15.08 ± 2.0	3.86 ± 0.26
15	500	10.45 ± 1.6	2.53 ± 0.25
30	0	15.45 ± 1.4	3.56 ± 0.08
30	500	15.15 ± 1.2	3.76 ± 0.22

Tissue slices were preincubated for 25 min at 37°; then either buffer or carbachol was added and the tissues were incubated further for 15 or 30 sec. Results are expressed as μg of polyphosphoinositide/g (wet weight of tissue slices), and values represent the means ± SD of three separate experiments performed in triplicate.

studies, tissue slices were preincubated for 60 and 90 min to ensure maximal labeling of PIP and PIP_2.

Effect of carbachol on ^{32}P-labeled phospholipids. In atrial slices, prelabeled 60 min with $^{32}P_i$, carbachol stimulated a 10 and 18% decrease in labeled PIP and PIP_2, respectively, within 15 sec (Fig. 2). Carbachol did not stimulate any change in labeled PA, PI or PC after a 15-sec incubation nor were there any changes in the controls after this time (Fig. 2 or not shown). Following this initial carbachol-stimulated decrease in labeled PIP and PIP_2, levels of these polyphosphoinositides returned to control levels after 30 sec and remained at control levels for at least 30 min. After 15 sec, carbachol stimulated an increase in labeled PA (Figs. 2 and 3) which plateaued after 5–10 min of incubation. Carbachol stimulated a delayed increase in PI labeling which became measurable after 2.5 min of incubation and plateaued after 10–15 min of incubation (Fig. 3). Carbachol had no effect on PC labeling under these conditions. Atropine (50 μM) inhibited the carbachol-stimulated effects on the labeling of the other phospholipids.

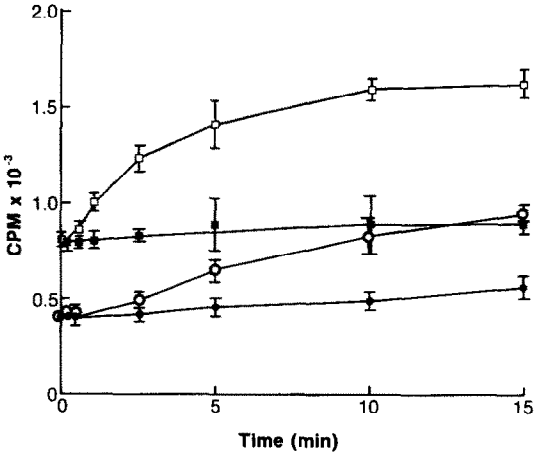

Fig. 3. Effect of carbachol on ^{32}P-labeled PA and PI in atrial slices prelabeled with ^{32}Pi. Key: PA (■) and PI (●) in the absence of carbachol; PA (□) and PI (○) in the presence of 250 μM carbachol. Results are representative of at least three separate experiments and are expressed as the means ± SD of triplicate determinations.

Effect of carbachol on PIP and PIP_2 content. It was found that PIP and PIP_2 content accounted for only 0.04 and 0.1% of the total phospholipid content in atria (Table 1). However, by using 1 to 1.5 g of tissue for each determination, carbachol could be shown to decrease PIP and PIP_2 content approximately 30% after 15 sec of incubation. After 30 sec of incubation, PIP and PIP_2 content had returned to control levels. In previous studies (Quist and Powell, unpublished observations), carbachol was found to have no measurable effect on PI, PC, cardiolipin, or phosphatidylethanolamine content under similar conditions.

Muscarinic agonist specificity. From the above studies, it is clear that carbachol-stimulated increases

Table 2. Effects of muscarinic agonists on the phosphorylation of phospholipids

Agonist (500 μM)	Phospholipids (% change in labeling) PI	PA	PC
Carbachol	56.6 ± 3	52.6 ± 8	4.6 ± 5
Acetylcholine and 100 μM physostigmine	62.25 ± 12	68.5 ± 19	20.5 ± 6
Methacholine	43.4 ± 5	44.0 ± 8	5.4 ± 4
Bethanechol	19.5 ± 3	18.0 ± 2	4.6 ± 1
Arecoline	18.0 ± 6	14.3 ± 6	−2.5 ± 3
Pilocarpine	3.5 ± 4	5.5 ± 2	0.23 ± 4
Oxotremorine	6.0 ± 8	3.0 ± 3	−1 ± 1

Right atrial slices were prelabeled for 60 min with ^{32}Pi before the addition of agonists and were then incubated for 30 min further with the above agents. Data points represent the means ± SD of at least three separate experiments.

in ^{32}P incorporation into PA and PI are secondary to the breakdown of PIP and PIP_2 to DAG and inositol phosphates. To further characterize these muscarinic receptor stimulated changes, the effects of various muscarinic agonists were examined. At 500 μM, carbachol, acetylcholine and methacholine stimulated ^{32}P incorporation into PA and PI by approximately 50% (Table 2). The effect of acetylcholine was the greatest and acetylcholine, unlike the other two agents, increased ^{32}P incorporation into PC. The pathway for this effect of acetylcholine on PC labeling was blocked by atropine but not by 10^{-4} M hexamethonium, a nicotinic cholinergic receptor antagonist (not shown), and therefore results from stimulation of muscarinic receptors. Bethanechol and arecoline were partially effective, whereas pilocarpine or oxotremorine were completely ineffective in increasing ^{32}P incorporation into PA and PI. A similar muscarinic agonist specificity for stimulating phosphoinositide turnover has been reported in brain nerve endings [13] and human astrocytoma cells [14].

Regional specificity. In a previous study [8], carbachol was reported to increase ^{32}P incorporation into slices of canine right, but not left, auricle. However, other regions of the atria were not examined in the study [8]. A more extensive examination of various regions of canine atria revealed that 100 μM carbachol increased ^{32}P incorporation into PA and PI approximately 60% in slices from all atrial regions except for the outermost part of the left auricle which contains a higher proportion of connective tissue and adipocytes (not shown). For these studies, right and left atria were subdivided into four equal areas each. There was no evidence that seven of the various areas of right or left atria were more sensitive to carbachol than others.

Calcium dependence. The Ca^{2+} dependence of carbachol-stimulated changes in ^{32}P incorporation into PA and PI ion canine right atria [15] was examined further. The calcium specificity was determined by substituting 1 mM concentrations of various divalent metal cations for Ca^{2+} in the external medium. When Mn^{2+}, Mg^{2+} or Co^{2+} was substituted for Ca^{2+}, carbachol did not stimulate ^{32}P incorporation into PA or PI after 30 min of incubation (not shown). Substitution of 1 mM $LaCl_3$ for Ca^{2+} completely abolished ^{32}Pi incorporation into all phospholipids in these tissue slices.

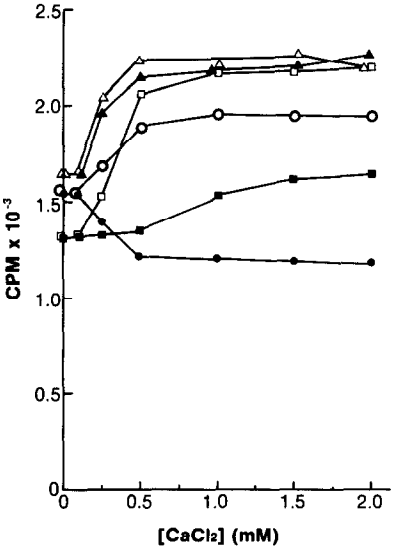

Fig. 4. Ca^{2+} concentration dependence of the phospholipid effect. Key: incorporation of ^{32}P into PI (●), PA (■) and PC (▲) in the absence of carbachol; incorporation of ^{32}P into PI (○), PA (□) and PC (△) in the presence of 100 μM carbachol. Results are representative of three separate experiments.

The Ca^{2+} concentration dependence was assessed by preincubating slices for 90 min with ^{32}Pi before the addition of carbachol. Extracellular Ca^{2+} was found to have carbachol-dependent and -independent effects on phospholipid labeling (Fig. 4). In the absence of carbachol, calcium half-maximally decreased PI and increased PC labeling at approximately 0.25 mM. This probably is a reflection of Ca^{2+} inhibition of the synthesis of PI from PA [16]. Under this condition, more PA is directed towards PC synthesis with a resultant increase in ^{32}P incorporation into PC. Over this calcium concentration range (0.1 to 2 mM), PIP, PIP_2 and PA labeling were not affected significantly. In the presence of carbachol, PA and PI labeling were half-maximally stimulated in presence of approximately 0.25 mM extracellular

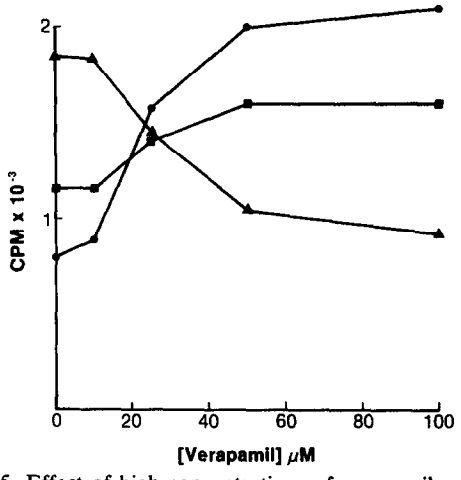

Fig. 5. Effect of high concentrations of verapamil on ^{32}P incorporation into PA (■), PI (●) and PC (▲) in atrial slices.

$CaCl_2$ (Fig. 4). Carbachol did not have any effect on labeled PIP, PIP_2 or PC at any of these calcium concentrations.

To determine if any of the above Ca^{2+}-dependent effects occurred through entry of Ca^{2+} into the cells via Ca^{2+} channels, the effects of various Ca^{2+} entry blockers on ^{32}P-labeled phospholipids were examined in the presence and absence of 100 μM carbachol. Nifedipine (1 μM), diltiazem (10–100 μM) and verapamil (<10 μM) had no effect on ^{32}P-labeled phospholipids in the presence and absence of carbachol (not shown). These concentrations of channel blockers have been reported to be effective in blocking Ca^{2+} entry through channels in the heart [17] and, therefore, entry of Ca^{2+} through these channels probably does not account for the effects of calcium on phospholipid labeling. It was found however that higher concentration of verapamil (10–100 μM) inhibited PC labeling and increased PI and PA labeling by a carbachol-independent mechanism (Fig. 5). This effect of verapamil resembles the inhibitory effect which drugs such as propranolol and chlorpromazine have on phosphatidate phosphohydrolase [18]. Inhibition of this enzyme predictably would redirect synthesis of PA towards PI [18]. It remains to be determined if the effects of verapamil reported here are related to its greater cardiac depressant effects relative to diltiazem and nifedipine [17].

The effect of increasing intracellular Ca^{2+}, by inhibiting $Na^+ + K^+$-ATPase activity with ouabain, on phosphorylation of phospholipids was examined. Inhibition of $Na^+ + K^+$-ATPase leads to an increase in intracellular Na^+ with a resultant secondary increase in the exchange of intracellular Na^+ for extracellular Ca^{2+} [19]. Addition of ouabain 10 min prior to the addition of carbachol markedly decreased the ability of carbachol to increase ^{32}P incorporation into PI (Table 3) while increasing incorporation into PA. This finding could be explained by inhibition of synthesis of PA to PI by intracellular Ca^{2+} [16]. It was surprising that PC labeling was not increased by carbachol in the presence of ouabain because it would be expected that more PA would be available for PC synthesis. Therefore, it appears that carbachol directs synthesis of DAG from PIP and PIP_2 breakdown towards the synthesis of PA and phosphoinositides. Mechanisms could include inhibition of phosphatidate phosphohydrolase and/or cholinephosphotransferase.

DISCUSSION

Very few studies have been done to investigate the mechanism of muscarinic receptor stimulated phosphoinositide turnover in atria. The results of the present investigation demonstrate that the initial event in carbachol stimulated phosphoinositide turnover is the rapid breakdown of PIP and PIP_2 (Fig. 2 and Table 1) within 10–15 sec. Presumably this carbachol-stimulated decrease in labeled and unlabeled PIP and PIP_2 results from stimulation of a phosphodiesterase activity which hydrolyzes these phospholipids to DAG and inositol phosphates [6, 7]. It was further observed that labeled and unlabeled PIP and PIP_2 returned to control levels after 30 sec. Similar receptor mediated transient changes in ^{32}P-labeled PIP and PIP_2 have been reported in platelets [20], parotid glands [5], and in other tissues [7]. Smith *et al.* [21] reported that angiotensin II stimulates phosphoinositide breakdown and resynthesis simultaneously in cultured arterial muscle cells. The rapid breakdown and resynthesis of PIP and PIP_2 observed here (Fig. 2 and Table 1) suggests that carbachol stimulates a similar mechanism in atria. The transient breakdown in PIP and PIP_2 was quickly followed by an increase in ^{32}P incorporation into PA or PI (Fig. 3) and can be explained by the sequential synthesis of these phospholipids from the DAG released from PIP and PIP_2 breakdown [6, 7]. We have also reported similar carbachol-stimulated changes in ^{32}P-labeled phosphoinositides and PA in myocytes isolated from canine atria by collagenase digestion [22]. The carbachol-stimulated changes in PIP and PIP_2 in canine atrial slices or myocytes are rapid enough to be associated with the negative chronotropic and/or inotropic effects caused by muscarinic receptor stimulation. For instance, vagal nerve stimulation or stimulation by acetylcholine produces maximal changes in rate and contractility in rabbit atria within 5–15 sec [23]. Further studies are in progress to determine the physiological function(s) associated with muscarinic stimulated changes in atrial tissue and myocytes.

At present very little is known about the properties of muscarinic receptor mediated changes in phosphoinositide turnover in atria. Unlike a number of tissues in which receptor mediated changes in phosphoinositide turnover are Ca^{2+} independent, atrial and ventricular tissue require extracellular calcium [9, 10, 15]. The results of the present investigation indicate that the effect is relatively specific for Ca^{2+}. Although the site of action of calcium is unknown, it could be required as a cofactor for polyphosphoinositide phosphodiesterase which is presumably located on the cytoplasmic surface of the sarcolemmal membrane [24]. This would imply that Ca^{2+} in the extracellular medium can enter into myocytes at least in submicromolar concentrations to satisfy the minimal Ca^{2+} requirements reported

Table 3. Effect of ouabain on the phosphorylation of phospholipids in the presence and absence of carbachol

Agents	Phospholipids (% change in labeling)		
	PI	PA	PC
Carbachol (250 μM)	56.6 ± 3.2	52.6 ± 8.5	16.6 ± 11
Ouabain (200 μM)	−5.5 ± 6.0	25.0 ± 9.4	16.3 ± 10
Carbachol (250 μM) and ouabain (200 μM)	19.0 ± 3.6	93.3 ± 12	1.2 ± 8

Right atrial slices were preincubated with ^{32}Pi for 50 min before any additions. At 50 min ouabain or buffer was added, and the tubes were incubated for a further 10 min before the addition of either buffer or carbachol. The reaction was stopped after 90 min total incubation time. Data represent the means ± SD of four separate experiments, and these changes are relative to controls.

for this enzyme [24]. Calcium entry or channel blockers, nifedipine, diltiazem or verapamil, failed to block the ability of carbachol to increase ^{32}P incorporation into PA and PI and, therefore, it is unlikely that extracellular calcium enters the myocytes by Ca^{2+} channels. In contrast, alpha$_1$ adrenergic receptor stimulated changes in phosphoinositide turnover in cultured rat myocytes [25] or canine cardiac slices [26] are Ca^{2+} independent. The difference in the Ca^{2+} requirements of muscarinic and alpha$_1$ adrenergic receptors suggests that these receptors may be coupled to phosphoinositides by different mechanisms. Such a difference might explain the opposite functional effects mediated by these receptors; however, further studies are warranted to examine this possibility.

The present study also demonstrated that muscarinic receptor stimulated phosphoinositide turnover was highly specific for certain muscarinic agonists (Table 2). The results of this investigation extend the observation of Brown and Brown [27] that the partial muscarinic agonist oxotremorine was ineffective in increasing phosphoinositide breakdown in isolated chick embryonic myocytes. Identical muscarinic agonist specificities for stimulation of phosphoinositide breakdown have been reported in brain nerve endings [13] and astrocytoma cells [14]. In these preparations [13, 14], and especially in heart, the mechanism for this agonist specificity remains unclear. However, the purpose of this study was to increase our limited knowledge of the properties of muscarinic receptor stimulated phosphoinositide turnover in cardiac tissue to aid in determination of the physiological response associated with this event.

Another difficult challenge in cardiac tissue will be to resolve how muscarinic receptor mediated PIP and PIP_2 breakdown can elicit a physiological effect. A number of possible mechanisms associated with receptor stimulated polyphosphoinositide breakdown have been proposed in other tissues [6, 7] and in heart [28]. In a number of tissues [6, 7] except for heart [28], it has been shown that IP_3 released from PIP_2 can stimulate the release of Ca^{2+} from nonmitochondrial stores. Although in heart tissue IP_3 does not release Ca^{2+} from sarcoplasmic reticulum [28], this does not rule out a possible role for IP_3 or IP_2 in modulating other membrane proteins such as those involved in Ca^{2+} or K^+ channel regulation. Alternatively, DAG produced from polyphosphoinositide breakdown could activate protein kinase C [7] or be deacylated by a lipase to release arachidonic acid and thus alter cardiac function. Lastly, alteration of membrane PIP and PIP_2 content could affect the conformation of other membrane proteins. Polyphosphoinositides are highly acidic phospholipids and bind tightly to membrane proteins, and it has been proposed that changes in the content of these lipids in erythrocyte membranes regulate $Ca^{2+} + Mg^{2+}$-ATPase [29] or shape and deformability [30]. A large investment of research effort will be required to understand which mechanisms are operable in cardiac tissue.

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