

Muscarinic Stimulation of Phosphatidylinositol Metabolism in Atria

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SUMMARY

Phosphatidylinositol (PtdIns) turnover in murine atria is stimulated by the cholinergic agonist carbachol. Incorporation of either [³²P]phosphate or [*myo*-³H]inositol into PtdIns is increased 40–80% by 30 μM carbachol, but carbachol does not increase the labeling of other major phospholipids. Cholinergic stimulation of PtdIns synthesis is blocked by the muscarinic antagonist atropine. When Ca²⁺ is removed from the extracellular medium, there is a large increase in basal PtdIns synthesis, and carbachol does not produce any further increase in [³²P]phosphate incorporation. Carbachol also stimulates hydrolysis of phosphoinositides as measured by *myo*[³H]inositol 1-phosphate accumulation. A maximal concentration of carbachol causes a 300–400% increase in phosphoinositide breakdown, and half-maximal stimulation occurs at a carbachol concentration of approximately 10 μM. Muscarinic stimulation of inositol phospholipid hydrolysis is seen in left and right atria as well as in ventricular tissue. The effect of carbachol on phosphoinositide hydrolysis is markedly attenuated when extracellular Ca²⁺ is removed. In contrast to most other hormone receptors linked to PtdIns metabolism, there is no evidence that cardiac muscarinic receptors mediate their physiological effects through Ca²⁺ mobilization. Thus, receptor-mediated changes in PtdIns turnover may serve a different function in the heart than in hormone-receptor systems that utilize Ca²⁺ as a second messenger.

INTRODUCTION

The parasympathetic neurotransmitter, ACh,³ regulates atrial rate, nodal conduction, and atrial and ventricular contractility through activation of muscarinic receptors (1). The molecular mechanisms that mediate these parasympathetic responses have not been clearly elucidated. ACh hyperpolarizes atrial cells by increasing potassium permeability (2, 3) and decreases the slow inward current in some preparations (4, 5). Biochemical responses also accompany cardiac muscarinic receptor activation. These include decreased cyclic AMP formation (5–8) and increased cyclic GMP production (8, 9). Although changes in cyclic nucleotide concentrations correlate with physiological responses to muscarinic agonists under some conditions (5, 7, 8), other evidence suggests that such changes cannot fully account for the ionic and physiological effects of ACh in the heart (3, 5, 7, 9).

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³ The abbreviations used are: ACh, acetylcholine; PtdIns, phosphatidylinositol; PtdIns 4P, phosphatidylinositol 4-phosphate; PtdIns 4,5P₂, phosphatidylinositol 4,5-bisphosphate; *myo*-Ins 1P, *myo*-inositol 1-phosphate; *myo*-Ins 1,4P₂, *myo*-inositol 1,4-bisphosphate; *myo*-Ins 1,4,5P₃, *myo*-inositol 1,4,5-triphosphate; TLC, thin-layer chromatography. EGTA, ethylene glycol bis (β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid.

One possibility which has not previously been explored is that changes in the metabolism of inositol phospholipids participate in cardiac physiological responses to ACh. Activation of muscarinic receptors in a variety of tissues (including the central nervous system, glands, and smooth muscle) increases PtdIns turnover (10–14). The hormonally regulated step involved in this response appears to be hydrolysis of PtdIns or phosphorylated PtdIns metabolites by a phospholipase C (13–15). It is not clear whether a similar phenomenon occurs in cardiac tissue. The only available data are those of Sekar and Roufogalis (16), who have reported that carbachol does not significantly enhance [³²P]phosphate incorporation into rat atrial PtdIns, and those of Quist (17), who has reported that cholinergic stimulation of PtdIns synthesis is restricted to the right auricle in the canine heart. There are no reports of muscarinic effects on phosphoinositide breakdown in the heart. We have studied the hydrolysis as well as the synthesis of cardiac inositol phospholipids in order to determine whether muscarinic receptor activation increases cardiac PtdIns turnover. Some of these results have been presented in preliminary form (18, 19).

METHODS

Incubation. Adult male Swiss-Webster mice (Simenson) were killed by cervical dislocation. Atrial appendages weighing approximately 4 mg (wet weight) per atrium were excised within 30 sec, blotted to remove excess blood, and rinsed in oxygenated (95% O₂, 5% CO₂) Krebs-Hen-

seleit buffer composed of 118 mM NaCl, 4.7 mM KCl, 3.0 mM CaCl₂, 1.2 mM MgSO₄, 1.2 mM KH₂PO₄, 0.5 mM EDTA, 25 mM NaHCO₃, and 10 mM glucose (pH 7.4 at 37°). Atria were transferred to continuously oxygenated vessels containing buffer, and were incubated at 37° in a shaking water bath. Once tissues had been distributed, medium was removed and replaced with fresh medium containing 10–20 μ Ci/ml H₃³²PO₄ (carrier-free, ICN) or 4 μ Ci/ml myo[2-³H]inositol [6.6 Ci/mmol (Amersham) or 15 Ci/mmol (New England Nuclear Corporation)]. After 30 or 60 min of incubation with [³²P]phosphate or myo[2-³H]inositol, drug or control solutions were added. In experiments where myo-Ins 1P accumulation was measured, LiCl (10 mM) was added just prior to drug or control solution addition. At the end of the incubation, tissues were quickly blotted and rinsed with cold saline. Pairs of atria (one left and one right) were transferred to 750 μ l of ice-cold CHCl₃/MeOH (1:2) or frozen in Freon cooled in liquid nitrogen and extracted later.

Phosphatidylinositol analysis. Two hundred microliters of 2 M KCl were added to each sample [final proportions CHCl₃/MeOH/2 M KCl (5:10:4)], and samples were homogenized for 1 min in a glass-glass Duall homogenizer. The further addition of 250 μ l each of CHCl₃ and 2 M KCl yielded a two-phase system. Samples were centrifuged at 1600 \times g at 4° for 20 min. The aqueous upper phase and any interfacial tissue were transferred to a separate tube, the organic lower phase was decanted into a new tube, and the remaining tissue pellet was discarded. Each upper phase was washed with 0.5 ml of synthetic lower phase [lower phase of the two-phase system CHCl₃/MeOH/2 M KCl (10:10:9)], and the resulting lower phase was combined with the original lower phase. Combined lower phases were washed with 0.5 ml of synthetic upper phase [upper phase of the two-phase system CHCl₃/MeOH/2 M KCl (10:10:9)]. Approximately 200 μ g of mouse heart carrier lipid prepared according to the method of Bligh and Dyer (20) were added to each tube. Samples were dried in a rotary evaporator and resuspended in 40 μ l of CHCl₃/MeOH (3:1).

Each sample was spotted onto a silica gel 60 glass plate (20 cm \times 20 cm) (Merck). Phospholipids were separated by 2-dimensional TLC (21). Plates were developed in CHCl₃/MeOH/H₂O/18 M NH₄OH (130:70:8:0.5) for 1 hr. They were removed, air-dried for 10 min, and subsequently developed in CHCl₃/CH₃COCH₃/MeOH/CH₃COOH/H₂O (10:4:2:2:1) for 1 hr. Lipids were visualized by iodine staining. Individual phospholipid spots were scraped into plastic minivials and counted in 3.5 ml of organic counting solution (Amersham)/Triton (2:1) containing 300 μ l of H₂O.

In assays in which myo[³H]inositol incorporation into PtdIns was measured, atria were extracted as above. Combined lower phases were washed twice with 0.5 ml of synthetic upper phase, dried with N₂, and counted in 3.5 ml of organic counting solution/Triton (2:1). When this simplified assay was used, less than 0.05% of the myo[2-³H]inositol contaminated the lower phase, and 100% \pm 1% (mean \pm standard error of the mean, N = 5) of the total lower phase counts were recovered as PtdIns when lipids were separated by 2-dimensional TLC.

Myo-Ins 1P analysis. Samples were homogenized in 1.3 ml of CHCl₃/MeOH/H₂O (5:10:4). A two-phase system was obtained with the addition of CHCl₃ and H₂O to make the solution CHCl₃/MeOH/H₂O (10:10:5). Samples were centrifuged as described above, and 1.0 ml of the upper (aqueous) phase was transferred to a column containing approximately 175 mg of anion exchange resin (Bio-Rad AG 1-X8, 100–200 mesh, formate form). Columns were washed with a total of 10 ml of H₂O to remove [myo-³H]inositol. Labeled myo-Ins 1P was eluted with 8 ml of 200 mM ammonium formate/100 mM formic acid and counted in an equal volume of Liquiscint (National Diagnostics).

Phosphate analysis. Individual phospholipid spots obtained from TLC plates were scraped into acid-washed glass tubes (16 \times 150 mm). After 0.5 ml of 80% perchloric acid was added, scrapings were heated at 170° for 2 hr. Tubes were vortexed and centrifuged at 1600 \times g for 15 min. Supernatant aliquots were removed for phosphate analysis. These aliquots were adjusted to a volume of 1.0 ml with 80% perchloric acid/H₂O (1:1). Ammonium molybdate (0.5 ml of 2.5%) and ascorbic acid (0.5 ml of 10%) were added to the tubes, and they were boiled for 5 min. Absorbances at 820 nm were measured with a Spectronic 21 (Bausch

and Lomb). The phosphate content was interpolated from a standard curve constructed with standards from 0 to 40 μ moles of KH₂PO₄. Blanks were generally less than 0.030 A, and the 10- μ mole phosphate standard read approximately 0.140 A.

RESULTS

PtdIns is a minor cardiac phospholipid that exhibits a rapid rate of metabolism. Analysis of the phospholipid content of murine atria showed that PtdIns constitutes less than 5% of the total lipid phosphorus (Table 1). Phosphatidylcholine and phosphatidylethanolamine were the major constituents. Although the concentration of PtdIns was low, the PtdIns pool was extensively labeled. After a 60-min incubation with [³²P]phosphate, 67% of the phospholipid radioactivity was in PtdIns, whereas only 29% was in phosphatidylcholine (Table 1). Since the PtdIns concentration in this tissue remained constant (data not shown), the extensive labeling of this phospholipid indicates that PtdIns has a rapid turnover rate.

The nonhydrolyzable ACh analogue, carbachol, stimulated incorporation of [³²P]phosphate into atrial PtdIns (Fig. 1). There was generally a 40–80% increase in PtdIns labeling during a 30-min incubation with 30 μ M carbachol. PtdIns synthesis was also significantly increased by 3 μ M carbachol, but not by 0.3 μ M carbachol (data not shown). Carbachol did not significantly increase [³²P]phosphate incorporation into phosphatidylcholine (Fig. 1), nor into phosphatidylethanolamine, diphosphatidylglycerol, sphingomyelin, or phosphatidylserine (data not shown). When myo[³H]inositol was used to label PtdIns specifically, cholinergic stimulation of PtdIns synthesis was also seen. The average value for myo[³H]inositol incorporation into atrial PtdIns following a 15-min incubation with 30 μ M carbachol was 160 \pm 15% (n = 11, p < 0.01) of the control value. Thus activation of atrial muscarinic recep-

TABLE 1
Phospholipid composition and [³²P]phosphate labeling in mouse atria

For determination of phospholipid composition, excised atria were immediately rinsed in saline, blotted, weighed, and frozen. Phospholipids from three atria were extracted, separated by 2-dimensional TLC, and quantified by phosphate analysis. An aliquot of the lipid extract was used to determine total lipid phosphorus [approximately 18 μ moles of phosphate per milligram (wet weight)]. Values represent mean percentage of total lipid phosphorus \pm standard deviation for two determinations. [³²P]Phosphate labeling of phospholipids was measured after incubating two to four atria with [³²P]phosphate for 60 min. Phospholipids were extracted and separated by 2-dimensional TLC. Individual phospholipid spots were scraped and counted. Values represent mean percentage of total radioactivity recovered in these phospholipids \pm standard error of the mean for six determinations.

Phospholipid	% Total lipid phosphate	% Phospholipid radioactivity
Phosphatidylcholine	44.8 \pm 0.2	29 \pm 1
Phosphatidylethanolamine	26.6 \pm 0.02	3 \pm 1*
Diphosphatidylglycerol	7.3 \pm 0.3	
PtdIns	4.4 \pm 0.2	67 \pm 1
Sphingomyelin	3.7 \pm 1.2	<0.1
Phosphatidylserine	3.7 \pm 0.4	<0.1

* Combined phosphatidylethanolamine and diphosphatidylglycerol radioactivity.

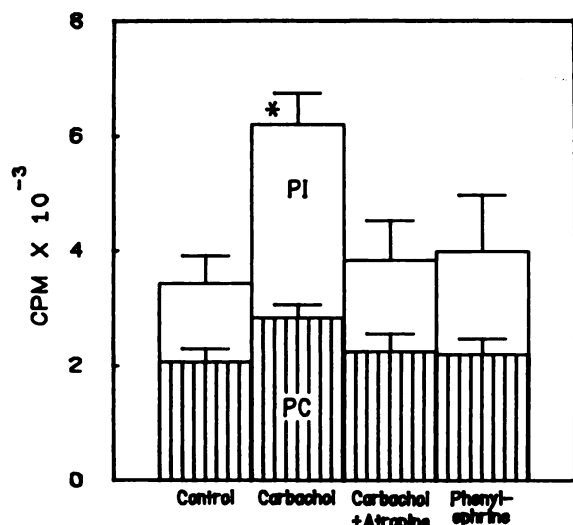


FIG. 1. Muscarinic and alpha-adrenergic effects on [32 P]phosphate incorporation into cardiac PtdIns and phosphatidylcholine

Pairs of atria were incubated for 30 min with [32 P]phosphate and stimulated with drug or vehicle for an additional 30 min. Drug concentrations were 30 μ M. Data are expressed as mean counts per minute incorporated into PtdIns or phosphatidylcholine per pair of atria \pm standard error of the mean for five pairs of atria. The total bar height represents counts per minute in PtdIns (PI) and the hatched portion represents counts per minute in phosphatidylcholine (PC). * $p < 0.05$ when compared with control with one-way analysis of variance and the Dunnett test.

tors stimulates PtdIns synthesis as measured by either [32 P]phosphate or *myo*[3 H]inositol incorporation.

Cholinergic stimulation of PtdIns synthesis appears to be mediated through activation of muscarinic receptors, since the effect of carbachol was blocked by the specific muscarinic antagonist, atropine (Fig. 1). Phenylephrine, an α -agonist, did not enhance [32 P]phosphate incorporation into atrial PtdIns or phosphatidylcholine (Fig. 1).

In most systems, the hormonal effects on PtdIns metabolism are Ca^{2+} -independent (10, 13, 14, 22), although there are systems in which the response requires extracellular Ca^{2+} (12, 22–24). We examined the Ca^{2+} dependence of the atrial PtdIns response by comparing the response to carbachol in normal medium and medium from which Ca^{2+} was omitted. As shown in Fig. 2, 30 μ M carbachol failed to stimulate [32 P]phosphate incorporation into PtdIns in the absence of extracellular Ca^{2+} . An unexpected finding was that removal of extracellular Ca^{2+} caused a large increase in basal [32 P]phosphate incorporation into PtdIns. Incorporation of [32 P]phosphate into phosphatidylcholine and the other major phospholipids was not stimulated by removal of Ca^{2+} (data not shown). Thus, phosphatidylinositol synthesis in the heart appears to be uniquely sensitive to changes in extracellular Ca^{2+} concentration.

Studies that have carefully characterized hormonal effects on PtdIns metabolism show that increased PtdIns synthesis is often preceded by hormonal stimulation of phosphoinositide breakdown. Such studies are often difficult because the decreases in PtdIns, PtdIns 4P, and PtdIns 4,5P₂ content are usually exceedingly small (10, 14, 25). Also, although pulse-chase experiments can be used to monitor the disappearance of label in these

inositol phospholipids, it is not always possible to remove radioactive precursor totally. Berridge *et al.* (25) have recently described a novel technique for monitoring phospholipase C-mediated inositol phospholipid hydrolysis by measuring accumulation of *myo*-Ins 1P. Under normal conditions, *myo*-Ins 1P is rapidly dephosphorylated to *myo*-inositol. However, the addition of millimolar concentrations of lithium inhibits *myo*-Ins 1-phosphatase (26) and allows the accumulation of *myo*-Ins 1P. This water-soluble *myo*-Ins 1P can be separated from *myo*-inositol with anion exchange chromatography (24, 25, 27).

We used this method to investigate cholinergic effects on phosphoinositide hydrolysis in the heart. When water-soluble extracts from *myo*[3 H]inositol-labeled atria were applied to anion exchange columns, a peak of radioactivity was eluted with 200 mM ammonium formate/100 mM formic acid (Fig. 3, A), a solution which elutes *myo*-Ins 1P (24, 27). The radioactivity in this *myo*-Ins 1P peak was enhanced when tissues were treated with LiCl, and treatment with carbachol in the presence of LiCl caused a 3- to 4-fold further increase (Fig. 3). These results indicate that carbachol stimulates cardiac phosphoinositide breakdown by activating a phospholipase C. The finding that carbachol had no measurable effect on accumulation of *myo*-Ins 1P in the absence of LiCl is consistent with the idea that *myo*-Ins 1P is rapidly converted to *myo*-Ins.

The small peaks of tritium eluted at B and C in Fig. 3 probably represent *myo*-Ins 1,4P₂ and *myo*-Ins 1,4,5P₃, respectively (24, 27). These are phospholipase C hydrolysis products of the polyphosphoinositides, PtdIns 4P and PtdIns 4,5P₂. The radioactivity in both peaks was increased by carbachol in the presence of LiCl. The finding that carbachol increased Peak C suggests that

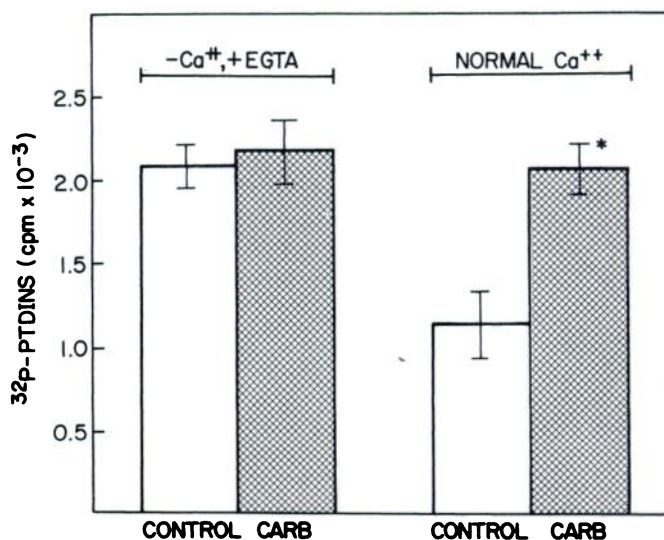


FIG. 2. Effects of calcium removal on [32 P]phosphate incorporation into murine atria PtdIns

Pairs of atria were incubated with [32 P]phosphate in normal medium or medium without Ca^{2+} and with 0.5 mM EGTA ($-\text{Ca}^{2+}$, +EGTA) for 30 min and stimulated with 30 μ M carbachol or vehicle for an additional 30 min. Values represent mean counts per minute incorporated into PtdIns per pair of atria \pm standard error of the mean for five pairs of atria. * $p < 0.05$ when compared with control with the two-sample *T*-test.

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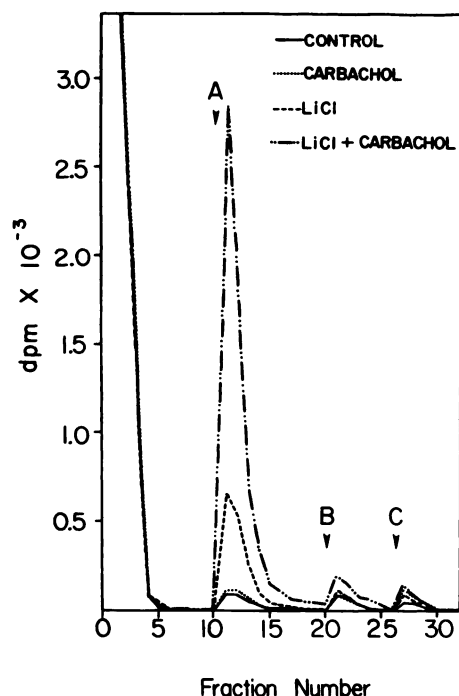


FIG. 3. Anion exchange chromatography of water-soluble atrial extracts

Pairs of atria were incubated for 60 min with [*myo*-³H]inositol and for an additional 30 min with 100 μ M carbachol or vehicle in the absence or presence of LiCl. Aqueous extracts prepared as described under Methods were applied to anion exchange columns and were eluted with water or with 200 mM ammonium formate/100 mM formic acid (A), 450 mM ammonium formate/100 mM formic acid (B), or 1 M ammonium formate/100 mM formic acid (C). Fractions (1.0 ml) were collected and counted. Each curve represents averaged values from four pairs of atria.

carbachol stimulates PtdIns 4,5P₂ hydrolysis. The observed increase in Peak B may be due to PtdIns 4P hydrolysis or may alternatively reflect *myo*-Ins 1,4,5P₃ dephosphorylation or *myo*-Ins 1P crossover. Further metabolic studies of PtdIns 4P and PtdIns 4,5P₂ are necessary to define precisely their respective roles in the phosphoinositide response to carbachol.

Cholinergic stimulation of phosphoinositide hydrolysis is blocked by atropine but not by the specific nicotinic antagonist *d*-tubocurarine (Fig. 4). These findings indicate that the response is mediated through activation of muscarinic receptors. A half-maximal response occurs at a carbachol concentration of about 10 μ M (Fig. 5). Carbachol concentrations that cause *myo*-Ins 1P accumulation are similar to those that stimulate [³²P]phosphate incorporation into PtdIns.

Since Quist (17) reported that muscarinic stimulation of cardiac [³²P]phosphate incorporation into cardiac PtdIns is limited to the right auricle in canine tissue, we were concerned that our protocol of pairing left and right atria might obscure any difference between them. However, when we compared responses in left and right atria, we found that they were very similar (Table 2). Moreover, murine ventricular slices prelabeled with *myo*[³H]inositol responded to 100 μ M carbachol with a large increase in *myo*-Ins 1P accumulation (Table 2). Thus,

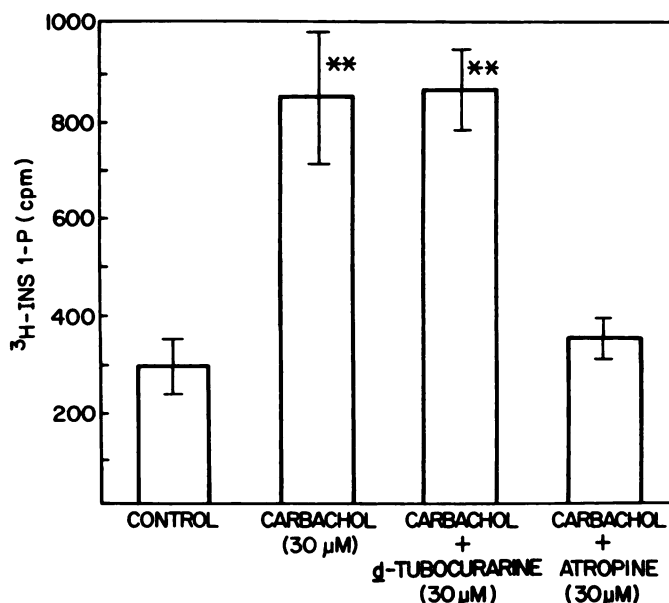


FIG. 4. Effects of muscarinic and nicotinic antagonists on phosphoinositide hydrolysis

Pairs of atria were incubated for 30 min with *myo*[³H]inositol and for an additional 30 min with drug or vehicle. *Myo*-Ins 1P was analyzed as described under Methods. Values represent mean counts per minute of *myo*-Ins 1P per pair of atria \pm standard error of the mean for four pairs of atria. ***p* < 0.01 when compared with control by one-way analysis of variance and the Dunnett test.

muscarinic receptor activation stimulates PtdIns hydrolysis in a variety of regions of murine heart.

We also investigated the Ca²⁺ dependency of the muscarinic increase in phosphoinositide hydrolysis. Muscarinic stimulation of *myo*-Ins 1P accumulation was vir-

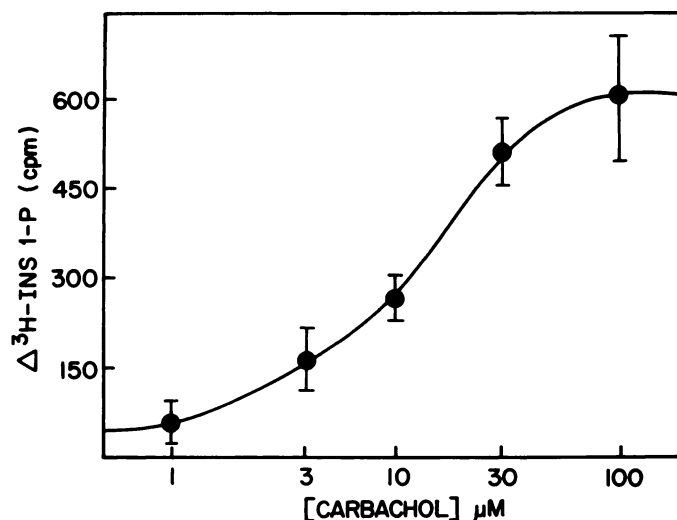


FIG. 5. Dose dependence of carbachol stimulation of phosphoinositide hydrolysis

Pairs of atria were incubated with *myo*[³H]inositol for 30 min and incubated for an additional 30 min with carbachol or vehicle. *Myo*-Ins 1P was analyzed as described under Methods. Values represent mean increases above control in counts per minute of *myo*-Ins 1P per pair of atria \pm standard error of the mean for five pairs of atria.

TABLE 2

Carbachol effects on Myo-Ins 1P accumulation in left atria, right atria, and ventricle

Mouse ventricles were removed, rinsed in Krebs-Henseleit buffer, sliced into small pieces (10–15 mg), and distributed into incubation vessels. Left atria, right atria, and ventricular slices were incubated for 30 min with $myo[^3H]$ inositol and for an additional 30 min with LiCl and 100 μM carbachol or vehicle. Myo-Ins 1P was analyzed as described under Methods. Values represent means \pm standard error of the mean for (*N*) determinations. ** $p < 0.01$ when compared with control with the Mann-Whitney rank sum test.

Tissue	Myo-Ins 1P	
	Control	Carbachol
	cpm/mg frozen wt	
Left atria	28.6 \pm 3.2 (4)	103.2 \pm 8.6** (4)
Right atria	27.5 \pm 0.8 (4)	132.4 \pm 4.0** (4)
Ventricle	3.4 \pm 0.3 (7)	26.9 \pm 1.8** (8)

tually abolished in atria that were switched to Ca^{2+} -deficient medium 5 min prior to addition of lithium and drug (Fig. 6). These results are consistent with the Ca^{2+} requirement for muscarinic stimulation of [^{32}P]phosphate incorporation into PtdIns in our preparation.

DISCUSSION

Muscarinic effects on ion permeability and cyclic nucleotide metabolism in cardiac tissue have been studied

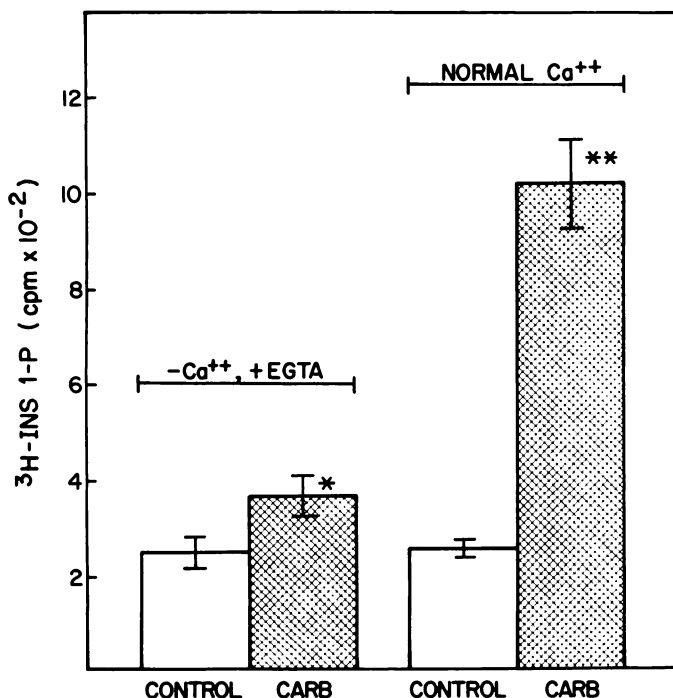


FIG. 6. Effects of Ca^{2+} removal on phosphoinositide hydrolysis

Pairs of atria were incubated with $myo[^3H]$ inositol in normal medium for 25 min and in normal medium or medium without Ca^{2+} and with 0.5 mM EGTA (- Ca^{2+} , +EGTA) for an additional 35 min. Carbachol (100 μM) or vehicle was added for the final 30 min. Myo-Ins 1P was analyzed as described under Methods. Values represent mean myo-Ins 1P counts per minute per pair of atria \pm standard error of the mean for four pairs of atria. * $p < 0.05$, ** $p < 0.01$ when compared with control with the two-sample *T*-test.

extensively, but little is known about the hormonal effects on cardiac PtdIns metabolism. We have found that activation of muscarinic receptors stimulates PtdIns turnover in murine atrial and ventricular tissue. This phospholipid effect is specific for PtdIns, since the labeling of other phospholipids is not affected by carbachol. It is also Ca^{2+} -sensitive, since the response is diminished when extracellular Ca^{2+} is removed.

Our results on PtdIns synthesis are consistent with those of Quist (17), who recently reported that carbachol causes Ca^{2+} -dependent stimulation of phosphate incorporation into PtdIns in the canine heart. However, in his system, the response was not seen in all regions of the heart, but was limited to the right auricle. Sekar and Roufogalis (16) have also presented preliminary data concerning PtdIns responses in the heart. In their preparation, phenylephrine, but not carbachol, stimulated PtdIns synthesis. Neither of these reports demonstrated muscarinic effects on phosphoinositide hydrolysis.

In a number of systems, hormonal stimulation causes inositol phospholipid hydrolysis by activation of a phospholipase C, and the increase in PtdIns synthesis is believed to be secondary to this initial event (13–15, 22). Since there is a variety of mechanisms by which hormones can alter PtdIns synthesis (22), it is essential to use hormonal effects on phosphoinositide breakdown as the basis for comparison of PtdIns responses in different systems. Our observation that carbachol increases formation of myo-Ins 1P, a product of phospholipase C hydrolysis of phosphoinositides, provides good evidence that the cardiac PtdIns response is mechanistically similar to hormonal stimulation of PtdIns turnover in other tissues.

One theory which has recently gained acceptance is that the polyphosphoinositides, PtdIns 4P and PtdIns 4,5P₂, are the primary substrates for hormonally activated phospholipase C (20, 28). According to this theory, PtdIns disappears because it is used as a substrate for polyphosphoinositide resynthesis rather than hydrolyzed by a phospholipase C. Our observations are not inconsistent with this theory, since increased polyphosphoinositide hydrolysis would also result in myo-Ins 1P accumulation in our experiments. The finding that carbachol increased the radioactivity in the peak that coelutes with myo-Ins 1,4,5P₃ suggests that that PtdIns 4,5P₂ hydrolysis may be an early event in the hormonal PtdIns response in the heart.

There is presently some controversy concerning the Ca^{2+} dependency of phosphoinositide responses. In tissues such as the parotid gland, hormonal stimulation of phosphoinositide metabolism is not reduced when extracellular Ca^{2+} is removed (17). In contrast, the response in the heart as well as that in other tissues (12, 22–24) is sensitive to extracellular Ca^{2+} concentrations. Although the PtdIns response in the heart exhibits an absolute requirement for extracellular Ca^{2+} , it is doubtful that the response is mediated by Ca^{2+} mobilization. First, only a low concentration of extracellular Ca^{2+} (0.125 mM) is needed to support carbachol-stimulated phosphoinositide hydrolysis.⁴ Second, preliminary experiments in our

⁴ S. L. Brown and J. H. Brown, unpublished observations.

laboratory have shown that the Ca^{2+} ionophore A23187 does not mimic the response to carbachol.⁴ Third, the inhibitory effects of ACh on cardiac rate and contractility are consistent with decreased, rather than enhanced, Ca^{2+} mobilization.

The concentrations of carbachol needed to stimulate phosphoinositide metabolism are higher than agonist concentrations that inhibit cyclic AMP formation or elicit physiological responses in the heart (6–9). However, relative insensitivity to agonist is characteristic of the PtdIns response in a number of tissues (11, 13) and may indicate that the PtdIns response is closely linked to receptor occupation. In [³H]quinuclidinyl benzilate competition experiments with membranes from murine atria, we find that carbachol recognizes two states of muscarinic receptors with K_{app} values of approximately 1 μM and 80 μM .⁴ The lower affinity state appears to predominate in intact cardiac cells (29). Thus the carbachol concentration for half-maximal receptor occupation is probably at least as high as that for half-maximal phosphoinositide hydrolysis.

The relationship between this cardiac phospholipid effect and other biochemical responses is not known. A popular theory is that hormonal stimulation of PtdIns metabolism causes Ca^{2+} mobilization (12–14, 22). It is unlikely that the phosphoinositide response serves this function in the heart, since there is no evidence that activation of cardiac muscarinic receptors leads to Ca^{2+} mobilization. An alternate possibility is that muscarinic stimulation of phosphoinositide hydrolysis leads to changes in protein phosphorylation. In support of this, diacylglycerol, a product of phospholipase C hydrolysis of inositol phospholipids, is an activator of the Ca^{2+} -activated, phospholipid-dependent protein kinase (30). Another possibility is that phosphoinositide hydrolysis generates other intracellular signals such as arachidonic acid metabolites or cyclic GMP. Further investigations are needed to explore the proposed relationships with biochemical events and ultimately with physiological responses.

The demonstration that PtdIns metabolism in the heart is specifically regulated by muscarinic receptor activation provides a new direction for research into possible mediators of cardiac parasympathetic responses. Much additional work is needed to prove that the response occurs in myocytes and to understand how enhanced PtdIns metabolism might lead to or result from previously described muscarinic responses in the heart. Nevertheless, our data suggest that this rapidly metabolized inositol phospholipid may play a critical role in determining cardiac parasympathetic responses to drugs and hormones.

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