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ON THE MECHANISM OF MUSCARINIC HYDROLYSIS OF CHOLINE PHOSPHOLIPIDS IN THE HEART*

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Abstract—In the heart, choline phospholipids were by far the largest fraction (about 50%) of phospholipids, much larger than that of inositol phospholipids (less than 6%) and phosphatidic acid (0.3%). The choline phospholipids (11 μmol/g) maintained a constant efflux of choline of about 1.5 nmol g^{-1} min^{-1} into the perfusate. Carbachol (10 μM) rapidly enhanced the choline efflux by a muscarinic mechanism, that was independent of mepacrine, an inhibitor of phospholipase A_2, as well as of extracellular Ca^{2+}; the maximum acceleration was reached within 2 min. In contrast, the accumulation of inositol phosphates by carbachol was blocked in the presence of a Ca^{2+}-free perfusion medium. Similar to the carbachol-evoked choline efflux, the increase in tissue content of phosphatidic acid by carbachol was unaffected by infusion of a Ca^{2+}-free, EGTA-containing solution. Sodium oleate (20 μM), an activator of phospholipase D, imitated the effects of carbachol on choline and phosphatidic acid, whereas NaF (5 mM), which has been reported to inhibit phospholipase D, blocked carbachol-evoked efflux of choline.

In conclusion, muscarinic receptor stimulation enhanced the hydrolysis of choline phospholipids presumably via activation of phospholipase D. The immediate formation of choline, phosphatidic acid and presumably diacylglycerol is discussed including its possible physiological importance.

Research on receptor-regulated hydrolysis of membrane phospholipids has been focused on the cleavage of the relatively small fraction of inositol compounds releasing inositol trisphosphate and diacylglycerol [1, 2]. In contrast, the few articles suggesting regulatory mechanisms also for choline phospholipids as the largest fraction of membrane phospholipids gained little attention. Receptor-regulated hydrolysis of these phospholipids was indicated by measuring the release of choline in response to muscarinic agonists [3–5], β-adrenoceptor agonists [6] and phorbol esters [7, 8].

Recently, we proposed that the mobilization of choline and the increase in tissue phosphatidic acid evoked by stimulation of muscarinic receptors in heart and brain was presumably mediated by activation of phospholipase D [5, 9]. Likewise, in hepatocytes, the increase in phosphatidic acid caused by various Ca^{2+}-mobilizing agents has been linked to phospholipase D activity [10].

The present experiments confirm the muscarinic hydrolysis of choline phospholipids and again suggest a role of phospholipase D.

MATERIALS AND METHODS

Hearts isolated from chickens were perfused at 36° as described by Langendorff with Tyrode solution (Na^+, 149.3; K^+, 2.7; Ca^{2+}, 1.8; Mg^{2+}, 1.05; Cl^-, 145.5; HCO_3, 11.9; H_2PO_4, 0.4; (+)-glucose, 5.6 mM). The flow rate was adjusted to about 4 ml min^{-1} g^{-1} wet weight and the solutions were gassed with a mixture of 95% O_2 and 5% CO_2. Drugs were infused after 30 or 40 min of perfusion as indicated in Results. Oleic acid as sodium salt (100 μM) was added to a modified Tyrode solution (0.225 mM Ca^{2+}). This solution, being opaque (due to precipitation of oleic acid–calcium salt), was then filtered through a glass filter with a pore size of 10–16 μm. The clear filtrate contained 20 μM oleic acid (determined by gas chromatography) and 0.18 mM Ca^{2+} (determined by absorption spectrometry); the other constituents of the Tyrode solution were unchanged.

Choline was determined by a chemoluminescence assay [11], which is based on the conversion of choline to betaine and hydrogen peroxide catalyzed by choline oxidase (sensitivity: 1.5 pmol choline in 10 μl perfusate, which was used for the assay without extraction). In samples containing drugs that interfered with the assay (mepacrine, EGTA), choline was determined by a radioenzymatic assay [12]. In selected samples the compatibility of the two methods was tested.

The composition of phospholipids in the heart tissue was determined [13–15]. Heart homogenates (an aliquot with an internal standard added) (chloroform : methanol = 2 : 1) were centrifuged. The addition of 0.1 M KCl (1 : 4) to the clear homogenate yielded a two-phase system. After centrifugation, the lipid phase was dried with N_2.

An aliquot of the lipid phase was spotted onto a silica gel 60 TLC glass plate (Merck, Darmstadt, F.R.G.) and the phospholipids were separated by two-dimensional TLC [14]. Plates were developed in chloroform : methanol : ammonia (25%) (65 : 35 : 5) and subsequently in chloroform : methanol : acet-

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one:acetic acid:water (100:20:40:20:5). Lipids were visualized by iodine staining and identified by co-chromatography with reference substances. Individual phospholipid spots were scraped off and heated in perchloric acid for two hours. Phospholipids were then quantified by determination of phosphorus [16].

Determination of inositol phosphates was carried out as described previously [15, 17]. Hearts weighing about 1.0 g were perfused with Tyrode solution for 10 min and then for 4 hr with Tyrode solution containing [*myo*-^{3}H]-inositol (30 μCi/30 ml; specific activity 12.8 Ci/mmol) in a recirculation system. In some cases Ca^{2+} was omitted from the perfusion medium for the last 40 min of the loading period. Thereafter the hearts were perfused in a non-recirculation system for 40 min with normal or Ca^{2+}-free Tyrode solution containing 10 mM LiCl. Carbachol (10 and 300 μM), when added, was infused throughout the washout period. Gross inspection did not indicate that rate and force of contraction were impaired by the length of the experiments. Hearts were then homogenized in a glass–glass homogenizer in 12 ml chloroform:methanol (1:2). For the estimation of *myo*-inositol phosphates chloroform and water were added to 9 ml of the homogenate to obtain a two-phase system. After centrifugation at 3000 *g* and 4° for 20 min the aqueous (upper) phase was transferred to a column containing 1 g of anion exchange resin (Bio Rad AG 1– X8, 100 – 200 mesh, formate form). The phosphate esters were then eluted by the stepwise addition of solutions containing increasing concentrations of formate as described previously [17]. The 1.0-ml fractions eluted from the columns were counted for radioactivity using a liquid scintillation counter.

The incorporation of [*myo*-^{3}H]-inositol into lipids was investigated using 3 ml of homogenate. Addition of 1 ml chloroform and 1.8 ml 2 M KCl yielded a two-phase system (chloroform:methanol:2 M KCl = 10:10:9). Samples were centrifuged at 3000 *g* and 4° for 20 min. The aqueous (upper) phase and the lipid phase were transferred to separate tubes; the tissue pellet was discarded. The upper phase was washed with approximately 2 ml of the synthetic lower phase and then combined with the original lower phase. Combined lower phases were washed with 2 ml of the synthetic upper phase. After centrifugation, the lower phase was dried in a rotary evaporator and resuspended in 1.5 ml of chloroform/methanol (3:1). A volume of 0.5 ml was given into a plastic tube, and counted after addition of 10 ml of organic counting solution.

The results are expressed as means ± SEM and the significance ($P < 0.05$) was evaluated by Student's *t*-test.

Drugs used in this study were carbachol (carbamyl–choline chloride), oleic acid–sodium salt, mepacrine (quinacrine dihydrochloride) (all Sigma Chemie, München, F.R.G.), EGTA and methylatropine nitrate (Merck, Darmstadt, F.R.G.) and [*myo*-^{3}H]-inositol (DuPont NEN, Dreieich, F.R.G.).

RESULTS

Phospholipid composition of the heart

Choline-containing phospholipids represented more than 50% of the total identified phospholipids (20.9 μmol/g wet weight). The fraction containing phosphatidylcholine and possibly plasmenyl–choline plus 1-*O*-alkyl-2-acyl-sn-glycerophosphocholine was the largest one (49.8%) (Table 1). Inositol phospholipids contribute less than 6% and phosphatidic acid only 0.3%.

Resting efflux of choline

Resting efflux of choline in the perfused chicken heart was constant throughout the experiment (Fig. 1). Since the resting release of acetylcholine, which is partially hydrolysed to choline, was relatively small, choline phospholipids were found to be the only source for the resting efflux of choline that is quantitatively essential [5].

Similar to previous findings [5], about 40% of the resting efflux of choline was inhibited by mepacrine, a phospholipase A_2 inhibitor (Fig. 1), and was dependent on extracellular Ca^{2+} (Fig. 2).

Mobilization of choline by carbachol

Carbachol (10 μM) accelerated the choline efflux,

Table 1. Phospholipid composition of the chicken heart

	Contents	
	μmol/g wet weight	% of total phospholipid*
Total phospholipids	20.9	100
Lysophosphatidylcholine + sphingomyelin	0.6	2.9
Phosphatidylinositol + phosphatidylserine	1.4	6.7
Phosphatidylcholine†	10.4	49.8
Phosphatidylethanolamine	6.9	33.0
Diphosphatidylglycerol	1.5	7.2
Phosphatidic acid	0.06	0.29

Means of two experiments are shown. The phospholipid composition was nearly identical in the two analysed hearts and was very similar to the values found in cardiac tissue of other species [15]. For extraction and separation of phospholipids see Materials and Methods.

* The sum of the identified phospholipids was set 100%.

† This fraction possibly contains also plasmenylcholine plus 1-*O*-alkyl-2-acyl-sn-glycerophosphocholine.

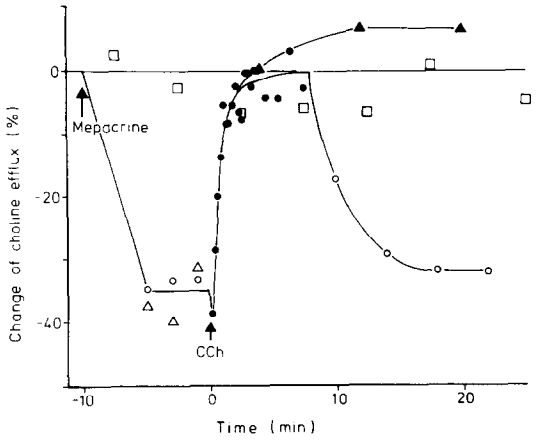

Fig. 1. Time-course and mepacrine-insensitivity of the muscarinic mobilization of choline in the perfused chicken heart. Open squares, perfusion with drug-free Tyrode solution. Mepacrine (30 μM) was infused alone (open triangles and open circles) and, after 10 min, together with carbachol (CCh, 10 μM), which was introduced for 8 min (closed circles) or 20 min (closed triangles). Ordinate, change of choline efflux in percent of efflux measured from -16 to -10 min (100% varied from 0.9 to 1.6 nmol g^{-1} min^{-1}). Abscissa, time in min. Note the rapid response to CCh. Means of 4 experiments each.

an effect that was blocked by 0.3 μM methylatropine, but was not significantly altered ($P < 0.05$) in the presence of mepacrine (Fig. 1; see also [5]) or during infusion of a Ca^{2+}-free Tyrode solution containing EGTA (0.5 mM; Figs. 2 and 3). Thus the target enzyme of the muscarinic mobilization of choline is unlikely to be a Ca^{2+}-regulated and mepacrine-sensitive enzyme, such as phospholipase A_2 and the phosphatidyl inositol-specific phospholipase C (see below).

Figure 1 illustrates the time-course of the muscarinic mobilization of choline. After a latency of a few seconds, the efflux rapidly increased to reach a maximum within 2 min. The elevated efflux was maintained at a plateau in the presence of carbachol or, when the drug was washed out, declined to the control level with a half-time of 2 min.

The results shown in Figs. 1 and 2 support the hypothesis that phospholipase D might be the target enzyme of the muscarinic receptor activation (see Discussion and [5]). Recently, F^- has been reported to inhibit phospholipase D activity in hepatocytes [10]. NaF (5 mM), which was added 20 min before carbachol, blocked the muscarinic mobilization of choline. The choline efflux, which was increased by carbachol to 148 ± 8% (N = 4) in the absence of NaF, remained unaltered (103 ± 4%, N = 3) in its presence. In these experimental series, the hearts were perfused with Ca^{2+}-free Tyrode solution.

Accumulation of phosphatidic acid by carbachol and by oleic acid

If muscarinic receptor activation leads to stimulation of phospholipase D, one might expect an increase in phosphatidic acid in parallel to the

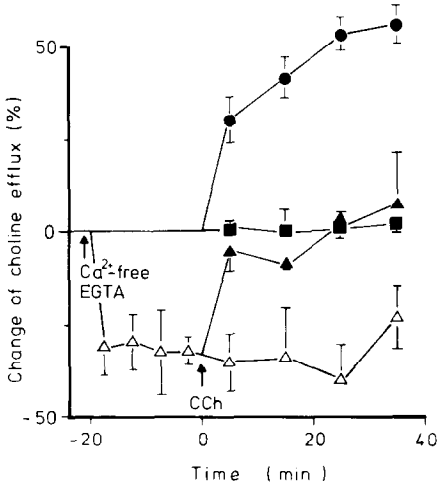

Fig. 2. The effects of methylatropine and of Ca^{2+}-free, EGTA-containing Tyrode solution on the muscarinic mobilization of choline (open triangles), perfusion with Ca^{2+}-free Tyrode solution containing EGTA (0.5 mM). Carbachol (CCh; 10 μM) was infused at zero-time for 40 min in normal Tyrode solution (closed circles) or in Ca^{2+}-free, EGTA-containing solution (closed triangles). Methylatropine (0.3 μM) was infused from the beginning of the perfusion and, at zero-time, in combination with CCh (closed squares). Ordinate, change of choline efflux in percent of efflux before drug addition (100% varied from 1.1 to 2.2 nmol g^{-1} min^{-1}). Abscissa, time in min. Means ± SEM of 3–5 experiments.

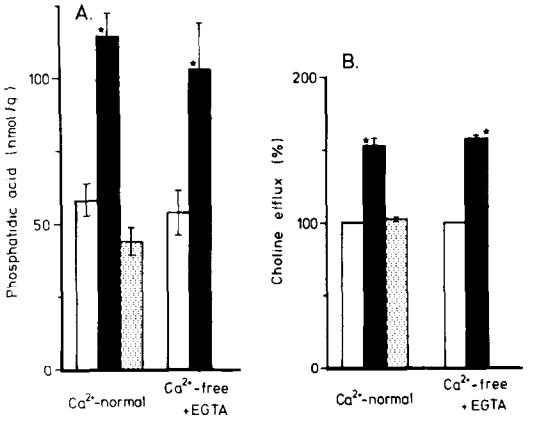

Fig. 3. The effects of carbachol on tissue content of phosphatidic acid and on choline efflux. Chicken hearts were perfused for 80 min. At the end of the perfusion, tissue content of phosphatidic acid (A) and choline efflux (B) were determined. Carbachol (10 μM; black columns) was infused from 40 to 80 min, methylatropine (0.3 μM; stippled columns) from the beginning of the experiment (Fig. 2) and Ca^{2+}-free, EGTA-containing Tyrode solution from 20 to 80 min (2nd pair of columns in A and B). Columns indicate (A) tissue content of phosphatidic acid in nmol/g and (B) choline efflux in percent of control (open columns; 100% values varied from 0.6 to 1.8 nmol g^{-1} min^{-1}). Means ± SEM of 3–9 experiments. * Significantly different from control.

observed accelerated efflux of choline. Indeed, carbachol (10 μM) markedly increased the tissue content of phosphatidic acid (57 ± 7 nmol/g, N = 8) at normal Ca^{2+} and also, when a Ca^{2+}-free solution containing EGTA (0.5 mM) was infused (Fig. 3). A comparative evaluation of the absolute increases is of limited value, because both phosphatidic acid as well as choline are rapidly metabolized. Nevertheless, the increase in tissue phosphatidic acid (about 50 nmol/g) was of the same order of magnitude as the total evoked efflux of choline during infusion of carbachol (about 25 nmol/g; see Fig. 2 "area under the curve").

Oleic acid, which is a well-known activator of phospholipase D (see Discussion), also enhanced the tissue content of phosphatidic acid and the increase in choline efflux at reduced Ca^{2+} (0.18 mM; Fig. 4).

Muscarinic mobilization of inositol phosphates

The experiments were carried out to test the possibility that the above-described increases in choline and in phosphatidic acid evoked by carbachol were caused primarily by the well-known "PI response", i.e. the hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP_2) into diacylglycerol (eventually phosphorylated to phosphatidic acid) and inositol trisphosphate (IP_3) (degraded to inositol phosphate) via activation of a specific, Ca^{2+}-dependent phosphodiesterase (phospholipase C; see Discussion).

Hearts were perfused with [*myo*-^{3}H]-inositol for 4 hr in a recirculation system and then washed out for 40 min. At the end of the experiment, the mean radioactivity of the total phospholipids was 11,500 cpm/g wet weight (mean of all experiments carried out with normal Tyrode solution). When Ca^{2+} was omitted during the last 40 min of the loading period and during the washout period, the incorporation of label was slightly, but significantly greater

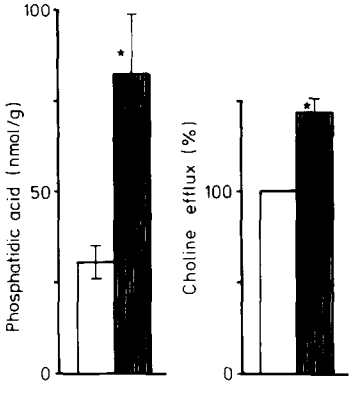

Fig. 4. The effects of oleic acid on tissue content of phosphatidic acid and on choline efflux. For details of similar experiments see Fig. 3. Sodium oleate (20 μM; stippled columns) was infused from 40 to 80 min at low Ca^{2+} (0.18 mM). Columns indicate (A) tissue content of phosphatidic acid in nmol/g or (B) choline efflux in percent of control (open columns; 100% equals 1.2 nmol g^{-1} min^{-1}). Means ± SEM of 4 experiments each. * Significantly different from control.

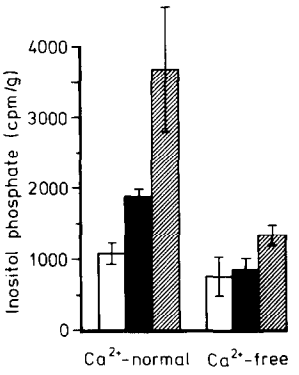

Fig. 5. The Ca^{2+}-dependence of the muscarinic mobilization of inositol phosphate (IP_1). Chicken hearts were perfused for 4 hr with (*myo*-^{3}H)-inositol and then for 40 min with normal (left columns) or Ca^{2+}-free (right columns) Tyrode solution containing LiCl. In some experiments, carbachol (CCh) in concentrations of 10 μM (black columns) or 300 μM (hatched columns) was added after the loading period. Open columns, control. Aqueous extracts containing various inositol phosphates were applied to anion exchange columns. Fractions (1.0 ml) were collected and counted. Columns indicate radioactivity in cpm/g (area under the curve of ion exchange chromatogram). Means ± SEM of 3–4 experiments each.

(14,519 cpm/g; $P < 0.05$) than with normal Tyrode solution.

Figure 5 shows that carbachol (10 and 300 μM) enhanced the formation of inositol phosphate (IP_1) in the presence of Li^+. These effects caused by either concentration were blocked in the Ca^{2+}-free medium. Similar responses were observed for inositol bisphosphate (IP_2) and inositol trisphosphate (IP_3). Of the phosphoinositides labelled in the lipid phase (100%), 12.5 ± 2.4% (N = 3) were mobilized as inositol phosphates ($IP_1 + IP_2 + IP_3$) in response to carbachol at the same concentration (10 μM), that had been used for the above-described experiments on choline mobilization. In the presence of a Ca^{2+}-free medium, carbachol was ineffective (−1.6 ± 2.4%, N = 3). As shown above (Figs. 2 and 3), the muscarinic hydrolysis of choline phospholipids was not reduced by omission of Ca^{2+}, even when EGTA was added. In conclusion, the "phosphoinositide cycle" (see Discussion) was not involved in the muscarinic hydrolysis of choline phospholipids.

DISCUSSION

About thirty years elapsed after the Hokins had detected the receptor-mediated turnover of phospholipids [18, 19], until its physiological role in the mechanism of Ca^{2+}-mobilization evoked by certain neurotransmitters and hormones was acknowledged (for reviews see Refs. 20, 21). The original observation that muscarinic receptor activation enhanced the incorporation of $^{32}P_i$ into phosphatidylinositol and phosphatidic acid (for review see Ref. 20) plus the fact that inositol trisphosphate exhibited the characteristics of a second messenger [1, 2] may explain why the research on receptor-regulation of phospholipid hydrolysis was, and still

is, focused almost exclusively on the inositol phospholipid subspecies.

Apart from occasional speculations (e.g. Ref. 22), a systematic search for receptor-mediated hydrolysis of choline phospholipids began by studying the mobilization of choline from phospholipids [3–6] and by analyzing the source of labelled diacylglycerol [23, 24], phosphatidic acid [9, 10] and arachidonic acid [25].

The present study confirms the recent assumption [5] that muscarinic receptor activation enhances the hydrolysis of choline phospholipids presumably by stimulating phospholipase D, which cleaves the terminal diester bond of phosphatidylcholine and forms phosphatidic acid plus choline. The evidence is based on the following observations obtained in the isolated chicken heart.

1. The muscarinic mobilization of choline from phospholipids in the heart was not inhibited by mepacrine, which blocks phospholipase A_2. There is no evidence that mepacrine inhibits also phospholipase D. Own experiments excluded an inhibition of phospholipase D in the *in vitro* assay (unpublished).

2. Moreover, the muscarinic mobilization of choline was totally independent of extracellular Ca^{2+}, as perfusion with a Ca^{2+}-free, EGTA-containing solution failed to reduce the effect of carbachol. It is well-known that phospholipase D activity is not dependent on Ca^{2+} [26] in contrast to phospholipase A_2 [27] and phosphatidylcholine-specific phospholipase C [28]. It deserves mention that the β-adrenoceptor-mediated mobilization of choline was blocked at low Ca^{2+} and by mepacrine [6].

3. Muscarinic receptor activation increased both the tissue content of phosphatidic acid and the rate of choline efflux. The molecular extent of both effects was in the same range (nmol/g). Nevertheless, the question had to be answered conclusively whether the increase in phosphatidic acid was directly or indirectly linked to the well-known muscarinic hydrolysis of inositol phospholipids, which has also been frequently described in heart preparations [15, 29, 30]. This problem is important, because phosphatidic acid has long been recognized as a key metabolite of the receptor-activated "phosphoinositide cycle" or, recently-called, "PIP_2 cycle" [20]. The phosphodiesteratic cleavage of these phospholipids leads to the formation of diacylglycerol, which subsequently may be phosphorylated to phosphatidic acid.

We have measured the muscarinic formation of inositol phosphate in the perfused chicken heart, which is the standard preparation of this study. The response was blocked, when the heart was perfused with a Ca^{2+}-free solution, a condition under which the extracellular Ca^{2+}-concentration was still 24 μM [5]. A similar Ca^{2+}-dependency was observed in cardiac tissue of other species [15]. Since the phosphoinositide-specific phospholipase C is known to be Ca^{2+}-sensitive [20], it is suggested that the reduction or omission of extracellular Ca^{2+} for at least 20 min (Figs. 3 and 5) inhibited the formation of inositol phosphates by lowering the intracellular concentration of Ca^{2+} [29]. Under the same condition [5], and even when EGTA was added (this study), the muscarinic mobilization of choline and the accumulation of phosphatidic acid were unaltered. These results clearly indicate, that the "phosphoinositide cycle" was not involved in the observed effects on choline and phosphatidic acid.

4. The results with NaF and with oleic acid support the conclusion that the muscarinic hydrolysis of choline phospholipids was due to stimulation of phospholipase D. This enzyme was detected in synaptic membranes of the brain [31, 32] and in heart tissue [33]. Unfortunately, no selective activators or inhibitors are available yet. However, it has been reported that oleic acid activated [32] and F^- inhibited phospholipase D [10]. Indeed, 20 μM of oleate increased both choline efflux and tissue content of phosphatidic acid and 5 mM of NaF blocked the efflux of choline evoked by carbachol. Both experiments were carried out using low Ca^{2+} or Ca^{2+}-free solutions.

Recently a receptor-mediated accumulation of phosphatidic acid and choline in isolated rat hepatocytes has been described that was suggested to be also caused by a phospholipase D mechanism [10]. It was found that the rapid increase of phosphatidic acid levels evoked by 10 nM vasopressin occurred within 2 min and preceded that of diacylglycerol. These results, among others of this study [10], suggested that Ca^{2+}-mobilizing hormones mainly increased phosphatidic acid levels by a mechanism that involves a guanine nucleotide binding protein coupled to phospholipase D.

Taken together, phospholipase D is the most likely target enzyme for muscarinic agonists in the receptor-mediated hydrolysis of choline phospholipids. However, the conclusion is based on the accumulation of various indirect evidence pointing in the same direction. More conclusive evidence is required. This seems especially necessary as we have not yet identified the precise source of choline, be it plasmenylcholine (plasmalogen), 1-*O*-alkyl-2-acyl-*sn*-glycerophosphocholine or a specialized pool of phosphatidylcholine. The various choline phospholipids seem to be hydrolyzed by more-or-less specific, and only partially characterized, subtypes of phospholipases [28].

The receptor-mediated hydrolysis of choline phospholipids may be a ubiquitous phenomenon present in many organs of the body. So far, it has been found in heart tissue (present study; [3–6]), in rat hepatocytes [10], in rat striatal slices [34], and in rat brain *in vivo* [35, 36].

The physiological role of muscarinic hydrolysis of choline phospholipids is, more or less, a matter of speculation. Alterations in the phospholipid composition may modulate general properties of the membrane (e.g. fluidity, surface charge, plasticity) or activities of membrane-bound proteins (e.g. receptors, G-proteins, enzymes, ion-channels). The immediate phosphatidylcholine metabolites formed by activation of phospholipase D are phosphatidic acid and choline.

Phosphatidic acid was shown to mobilize intracellular Ca^{2+} [37, 38] and has properties of a Ca^{2+}-ionophore [39]. These effects may play a crucial role in smooth muscle contraction [40] or in positive inotropic responses of the heart [41]. Formation of phosphatidic acid in the sarcolemmal membrane via

phospholipase D activity markedly increased Na^+–Ca^{2+}-exchange rates [42, 43]. Some experimental evidence indicates that a phospholipase D mechanism may be involved in the "paradoxical" positive inotropic effect of carbachol [5, 44].

Moreover, an even more important consequence of the formation of phosphatidic acid may be that diacylglycerol, the endogenous activator of protein kinase C, is formed from phosphatidic acid due to the ubiquitous presence of phosphatidic acid phosphohydrolase [10]. Under the conditions of the phospholipase D assay, with the modification that the phosphohydrolase activity was not inhibited, the newly-formed phosphatidic acid occurred as diacylglycerol [45]. These considerations raise the intriguing possibility that muscarinic receptor stimulation activates protein kinase via two mechanisms: activation of polyphosphoinositide phosphodiesterase (phospholipase C) or of phosphatidylcholine phospholipase D.

Free choline, as the second product of phospholipase D activity, is present in all body fluids and serves as precursor for acetylcholine and for phospholipids [46]. When release and turnover of acetylcholine are increased, extracellular choline may become rate-limiting for the synthesis of acetylcholine in the brain (reviewed by Ref. 47). It has been suggested that, in the brain, the muscarinic mobilization of choline from phospholipids (possibly via a phospholipase D mechanism) may increase the availability of choline and may therefore represent a positive feedback regulation of acetylcholine synthesis [4, 36, 48].

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