Rapid Communications

Phosphatidic Acid Stimulates Inositol 1,4,5-Trisphosphate Production in Adult Cardiac Myocytes

Thomas Kurz, Robert A. Wolf, and Peter B. Corr

The cellular content of phosphatidic acid can increase in response to several agonists either by phosphorylation of diacylglycerol after phospholipase C-catalyzed hydrolysis of phospholipids or directly through activation of phospholipase D. Although previous findings indicated that the generation of phosphatidic acid was exclusively a means of regulation of the cellular concentration of diacylglycerol, more recent studies have indicated that phosphatidic acid may also directly regulate several cellular functions. Accordingly, the present study was performed to assess whether phosphatidic acid could stimulate cardiac phospholipase C in intact adult rabbit ventricular myocytes. The mass of inositol 1,4,5-trisphosphate [Ins (1,4,5)P$_3$] was determined by a specific and sensitive binding protein assay and by direct mass measurement using anion exchange chromatography for separation of selected inositol phosphates and gas chromatography and mass spectrometry for quantification of inositol monophosphate (IP$_1$), inositol bisphosphate (IP$_2$), inositol triphosphate (IP$_3$), and inositol tetrakisphosphate (IP$_4$). Phosphatidic acid (10$^{-5}$-10$^{-6}$ M) elicited a rapid concentration-dependent increase in Ins (1,4,5)P$_3$ accumulation, with the peak fourfold to fivefold increase at 30 seconds of stimulation; the concentration required for 50% of maximal stimulation was 4.4$\times$10$^{-5}$ M. The time course of individual inositol phosphates indicated a successive increase in the mass of IP$_1$, IP$_2$, IP$_3$, and IP$_4$ in response to stimulation with phosphatidic acid. The production of Ins (1,4,5)P$_3$ in response to phosphatidic acid was not altered in the absence of extracellular calcium or in the presence of extracellular EGTA (10$^{-3}$ M). Thus, these findings indicate that phosphatidic acid is a potent activator of inositol phosphate production in adult ventricular myocytes. Because of the low concentrations required, it is likely that this unique pathway may regulate cardiac function under both physiological and pathophysiological conditions. (Circulation Research 1993;72:701-706)

**Key Words** • inositol phosphates • phospholipase D • calcium • diacylglycerol • protein kinase C

The hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP$_2$) by phospholipase C is an important pathway in hormone-regulated signal transduction in a variety of tissues. The initial products of phospholipase C-mediated hydrolysis of PIP$_2$, inositol 1,4,5-trisphosphate and diacylglycerol, mediate pivotal roles as intracellular second messengers through the mobilization of calcium from intracellular stores and activation of protein kinase C. Although the hydrolysis of PIP$_2$ by phospholipase C and the intracellular effects of the second messengers have been studied extensively, little is known regarding the functional role of phosphatidic acid, which can be synthesized by phosphorylation of diacylglycerol. Phosphatidic acid can increase in a variety of cell types via activation of phospholipase C, by de novo synthesis of phosphatidic acid, or directly through activation of phospholipase D by several agonists. Furthermore, phosphatidic acid generated via these pathways and subsequently released from the plasma membrane may exert secondary effects directly on other cell types.

Previous studies have suggested that intracellular phosphorylation of diacylglycerol to form phosphatidic acid may be the means whereby the cell controls the level of diacylglycerol. However, more recent studies have demonstrated the potential role for phosphatidic acid as a second messenger per se. These studies have implicated phosphatidic acid in the regulation of DNA synthesis, mobilization of intracellular calcium, stimulation of Na$^+$-Ca$^{2+}$ exchange, and induction of proto-oncogene expression. Several of the effects of phosphatidic acid have been shown to be associated with stimulation of the turnover of phosphoinositides, although the potential for phosphatidic acid to stimulate phospholipase C in cardiac cells has not been characterized previously. The mechanism of the phosphatidic acid effect is unknown, but phospha-
Phosphatidic acid has been shown to stimulate in vitro activity of soluble and membrane-associated phospholipase C.\textsuperscript{37} Accordingly, the purpose of the present study was to assess the influence of exogenous phosphatidic acid on phospholipase C in adult ventricular cardiac myocytes. The questions to be addressed included the following: 1) Does phosphatidic acid increase inositol phosphates in intact myocytes in a concentration-dependent manner? 2) Does the increase involve both inositol trisphosphate (IP\textsubscript{3}) and inositol tetrakisphosphate (IP\textsubscript{4})? 3) Is the effect dependent on extracellular Ca\textsuperscript{2+}? Problems associated with the measurement of phosphatidylinositol turnover in cardiac myocytes using pre-labeling with \textsuperscript{[3H]}myo-inositol were circumvented using two sensitive and specific methods for direct determination of the mass of inositol 1,4,5-trisphosphate and its catabolites. The results demonstrate, for the first time, that phosphatidic acid stimulates inositol phosphate production in intact isolated cardiac ventricular myocytes.

Materials and Methods

Isolation of Adult Ventricular Myocytes

Adult rabbits of either sex, weighing 3–4 kg, were anesthetized with nembutal (50 mg/kg body wt i.v.). After a midventral thoracotomy, the heart was excised rapidly and mounted on a Langendorff perfusion apparatus. The heart was perfused for 4 minutes with a Tyrode’s solution containing (mM) NaCl 118, KCl 4.8, CaCl\textsubscript{2} 1.2, MgCl\textsubscript{2} 1.2, NaHCO\textsubscript{3} 24, KH\textsubscript{2}PO\textsubscript{4} 1.2, and glucose 11, saturated with 95% O\textsubscript{2}-5% CO\textsubscript{2} to yield a pH of 7.4. This procedure was followed by a 4-minute perfusion with a Ca\textsuperscript{2+}-free Tyrode’s solution containing EGTA (100 \textmu M) and a final perfusion for 15–17 minutes with a Tyrode’s solution containing 0.045% collagenase (176 units/mg, type II, Worthington Biochemical Corp., Freehold, N.J.). The calcium concentration in the collagenase-containing Tyrode’s solution was increased sequentially to 100 \textmu M during perfusion. On completion of the perfusion steps, both left and right ventricular tissues were cut into small pieces and placed into two Erlenmeyer flasks, each containing 20 ml fresh enzyme solution. The flasks were shaken in a Dubnoff metabolic shaker at a rate of 100 per minute at 35°C for 10–15 minutes with 95% O\textsubscript{2}-5% CO\textsubscript{2} blowing into each flask. The first harvest of myocytes primarily contained contracted cells and was discarded. Cells from three subsequent harvests were combined and then filtered through a nylon mesh (350 \mu m) using a Tyrode’s solution containing 100 \textmu M calcium. After the cells settled, the surface solution was removed by suction and gradually replaced with a HEPES-buffered solution containing (mM) NaCl 133, KCl 4.8, CaCl\textsubscript{2} 0.5, MgCl\textsubscript{2} 1.2, HEPES 10, KH\textsubscript{2}PO\textsubscript{4} 1.2, and glucose 10 and titrated with NaOH to pH 7.2. Percoll gradients were used to separate elongated myocytes from the rounded, viable population of cells as described previously.\textsuperscript{18} Final cell preparations consisted of up to 85% elongated myocytes.

Experimental Protocol

Isolated adult rabbit ventricular myocytes were transferred into HEPES buffer of the following composition (mM): NaCl 133, KCl 4.8, CaCl\textsubscript{2} 1.85, MgCl\textsubscript{2} 1.2, HEPES 10, NaH\textsubscript{2}PO\textsubscript{4} 1.2, taurine 4, and glucose 10, titrated with NaOH to pH 7.2. In experiments in which the calcium was removed, the myocytes were incubated in HEPES buffer as above but without CaCl\textsubscript{2} in the presence or absence of EGTA (1 mM). Phosphatidic acid (1-o-phosphatidic acid, natural, from egg yolk lecithin, sodium salt, Sigma Chemical Co., St. Louis, Mo.) was prepared as stock solutions (10\textsuperscript{3} M) in the corresponding HEPES buffers. The stock solutions were sonicated using a probe-type sonifier (Branson Ultrasonics Corp., Danbury, Conn.) until a homogeneous suspension was obtained. Contamination of phosphatidic acid with lysophosphatidic acid was assessed by thin-layer chromatography using short bed/continuous development (position 5, 60 minutes) and a solvent system of benzene/chloroform/pyridine/formic acid (60/38/4/2.3, by volume). The thin-layer chromatographic plates were stained with Coomassie blue R-250 and scanned with an LKB 2202–enhanced Ultrascan XL densitometer. The contamination of phosphatidic acid (ranging from 10 to 50 \mu g) with lysophosphatidic acid as calculated from a lysophosphatidic acid standard curve on the same plate was 1.26±0.12% (n=5) and less than 1.5% in sonicated phosphatidic acid stock solutions.

Aliquots (900 \mu l, 500,000 cells) of the cell suspension were incubated 10 minutes at 37°C in a water bath before adding 100 \mu l phosphatidic acid or vehicle (HEPES buffer). After a specified time interval, all incubations were terminated by addition of an equal volume of ice-cold 15% (wt/vol) trichloroacetic acid, vortexed for 15 seconds, and placed on ice for 10 minutes. The precipitate was removed by centrifugation, and the supernatant was decanted and placed into 16×125-mm glass tubes coated with silane. Protein precipitate was resuspended in 1 ml distilled water and extracted with an equal volume of ice-cold trichloroacetic acid (15% [wt/vol]). The tubes were vortexed, the precipitate was sedimented by centrifugation, and the supernatants were decanted and combined with the corresponding supernatants from the first step. The trichloroacetic acid was removed by repetitive extraction with 3 ml H\textsubscript{2}O-saturated diethyl ether. Residual ether was removed from the cell extract by lyophilizing the samples to dryness in a Speed-Vac centrifugal evaporator (Savant Instruments Inc., Farmingdale, N.Y.). The recovery of added \textsuperscript{[3H]}IP\textsubscript{3} after trichloroacetic acid extraction was greater than 95%.

Determination of Inositol Phosphates

The mass of inositol phosphates was measured using two different methods. Inositol 1,4,5-trisphosphate was determined by a binding assay using a specific and sensitive binding protein for inositol 1,4,5-trisphosphate. The assay is based on the competition between unlabeled inositol 1,4,5-trisphosphate and a fixed quantity of \textsuperscript{[3H]}inositol 1,4,5-trisphosphate for a limited number of binding sites on a bovine adrenal binding protein preparation. Lyophilized samples were resuspended in 1.5 ml distilled H\textsubscript{2}O and titrated to pH 7.5 with NaHCO\textsubscript{3} (1 M). Aliquots (100 \mu l) of the samples were then assayed directly using a d-myoinositol 1,4,5-trisphosphate H assay system (Amersham Corp., Arlington Heights, Ill.).

The mass of different inositol phosphates, inositol monophosphate (IP\textsubscript{1}), inositol bisphosphate (IP\textsubscript{2}), IP\textsubscript{3}, and IP\textsubscript{4}, was measured in each sample using gas chro-
matography–mass spectrometry procedures developed in our laboratory and described in detail elsewhere. Briefly, lyophilized samples were resuspended in 2 ml Tris buffer (0.01 M, pH 8.5) and titrated to pH 8.5 with NaHCO₃ (1 M). Individual inositol phosphate species (IP₁, IP₂, IP₃, and IP₄) were each separated by ammonium sulfate gradient anion exchange column chromatography. The isolated fractions were dephosphorylated and desalted using barium hydroxide to form solid barium sulfate and volatile ammonium hydroxide. Residual salt was removed by mixed-bed ion exchange chromatography. The myo-inositol derived from each inositol phosphate species was quantified by stable isotope dilution gas chromatography–mass spectrometry of the hexakis(trimethylsilyl) derivative using hexadeceno-my-o-inositol as the internal standard. Recoveries of [³H]IP₁, [³H]IP₂, [³H]IP₃, and [³H]IP₄, after separation by anion exchange chromatography were 99%, 92%, 93%, and 84%, respectively. Incomplete recoveries during subsequent processing steps were corrected by adding hexadeceno-my-o-inositol as the internal standard.

The mass of inositol phosphates was expressed as picomoles per milligram protein. Protein measurements were made by a modification of the method of Lowry et al. using bovine serum albumin as the standard.

**Statistical Analysis**

Data were subjected to either nonpaired Student's t test or analysis of variance followed by Scheffe's F test as appropriate. Criterion for significance was p<0.05. Data are expressed as mean±SEM.

**Results**

Phosphatidic acid (1 μM) rapidly increased the level of inositol 1,4,5-trisphosphate in isolated adult rabbit myocytes (Figure 1). Inositol 1,4,5-trisphosphate mass increase from control values of 6.1±1.9 to 27.8±6.8 pmol/mg protein at 15 seconds. Inositol 1,4,5-trisphosphate accumulation was maximal after 30 seconds of stimulation (35.3±5.4 pmol/mg protein), followed by a rapid decline by 45 seconds (9.2±1.3 pmol/mg protein). After 60 seconds, the level of inositol 1,4,5-trisphosphate returned to control values. The time course of inositol 1,4,5-trisphosphate accumulation is consistent with an action of phosphatidic acid to stimulate phospholipase C-catalyzed hydrolysis of PIP₂ to generate IP₃ and 1,2-diacylglycerol.

A characteristic feature of agonist-induced PIP₂ hydrolysis is the rapid and transient accumulation of IP₃, which is phosphorylated to IP₄ and a more gradual and sustained accumulation of IP₂ and IP₃. Therefore, inositol phosphate metabolites were measured in rabbit myocytes exposed to phosphatidic acid for selected time intervals (Figure 2). Stimulation of the cells with phosphatidic acid (1 μM) resulted in a rapid increase in the total mass of IP₃, which was followed with a delay of approximately 15 seconds by a significant increase in IP₄. A significant accumulation of IP₂ and IP₄ in response to stimulation with phosphatidic acid was observed after 60 and 90 seconds, respectively (Figure 2). The sequential production of IP₁, IP₃, IP₄, and IP₅ after stimulation of myocytes with phosphatidic acid suggests phosphorylation of IP₁ to IP₃, and sequential dephosphorylation of IP₅ to IP₄ and IP₃.

The effect of phosphatidic acid to stimulate the formation of inositol 1,4,5-trisphosphate in rabbit myocytes was concentration dependent (Figure 3). The concentration–response curve for inositol 1,4,5-trisphosphate production was assessed by determination of the level of inositol 1,4,5-trisphosphate produced after 20 seconds of stimulation by selected concentrations of phosphatidic acid. The stimulation time of 20 seconds was chosen to avoid a variable response because of the rapid decline in IP₃ after the 30-second interval (Figure 1). A significant increase in inositol 1,4,5-trisphosphate was observed at concentrations of phosphatidic acid as low as 0.1 μM. Maximum inositol 1,4,5-trisphosphate levels were reached at concentrations of phosphatidic acid of 1 μM. The concentration–response curve for inositol 1,4,5-trisphosphate production after phosphatidic acid stimulation expressed as a percentage of the maximum response is shown in the inset of Figure 3. The concentration of phosphatidic acid providing the half-maximal response was 4.4×10⁻⁸ M. To determine whether the increase in inositol 1,4,5-trisphosphate production in response to phosphatidic acid is due to contaminating lysosphatidic acid, we stimulated cardiac myocytes with 1.5×10⁻⁸ M lysosphatidic acid (i.e., the calculated contaminating concentration in 10⁻⁸ M phosphatidic acid; see “Materials and Methods”) for 20 seconds. Lysosphatidic acid did not increase inositol 1,4,5-trisphosphate production (control, 5.18±0.21 pmol/mg protein; 1.5×10⁻⁸ M lysosphatidic acid, 4.89±0.98 pmol/mg protein; n=4), demonstrating that in rabbit cardiac myocytes the increase in inositol 1,4,5-trisphosphate production by
The increase in inositol 1,4,5-trisphosphate in response to phosphatidic acid was independent of extracellular Ca\(^{2+}\). Adult ventricular myocytes were transferred to calcium-free HEPES buffer in the presence or absence of EGTA (1 mM) and then stimulated with phosphatidic acid (1 \(\mu\)M). The increase in inositol 1,4,5-trisphosphate after 20 seconds of stimulation with phosphatidic acid was not altered in the absence of extracellular calcium, even in the presence of EGTA (Figure 4). Although the basal levels of inositol 1,4,5-trisphosphate in cells were slightly but not significantly lower in the absence of extracellular Ca\(^{2+}\), the response of the myocytes to stimulation with phosphatidic acid was comparable to that seen in the presence of extracellular calcium.

**Discussion**

This is the first report demonstrating that phosphatidic acid stimulates inositol phosphate production in adult cardiac myocytes, leading to a marked increase in IP\(_3\) and IP\(_4\). The time course of inositol 1,4,5-trisphosphate generation and the successive formation of IP\(_2\), IP\(_3\), and IP\(_4\) are comparable to results reported previously from our laboratory\(^{23}\) and others\(^{22-24}\) with other more classical activators of phospholipase C.

Recent reports have suggested that exogenous phosphatidic acid may stimulate phospholipase C in various cultured cell systems. Moolenaar et al.\(^{11}\) have observed that phosphatidic acid can increase intracellular calcium, accumulation of \([H^\text{3}]-\text{inositol phosphates, and the expression of c-fos and c-myc proto-oncogenes in A431 cells. Likewise, phosphatidic acid can stimulate inositol phosphate generation in 3T3 fibroblasts, neonatal islet cells, mesangial cells, and parathyroid cells. As with these other cells, in adult cardiac myocytes, phosphatidic acid–mediated generation of inositol 1,4,5-trisphosphate is not affected by the absence of extracellular calcium. This finding indicates that the stimulation of inositol 1,4,5-trisphosphate production by phosphatidic acid is not simply a consequence of a calcium-ionophoretic action of this hydrophobic phospholipid. The mechanisms by which phosphatidic acid stimulates phospholipase C activity have not yet been determined. Phosphatidic acid stimulates in vitro activity of

**FIGURE 3.** Bar graph showing inositol 1,4,5-trisphosphate accumulation after 20 seconds of stimulation with selected concentrations of phosphatidic acid in adult rabbit ventricular myocytes. Stimulation with phosphatidic acid was at 37°C in HEPES buffer ([Ca\(^{2+}\)], 1.85 mM; pH 7.2) in the absence or presence of phosphatidic acid (10\(^{-6}\)–10\(^{-5}\) M) for 20 seconds, after which the mass of inositol 1,4,5-trisphosphate was determined by a specific binding protein assay. The dose–response curve for inositol 1,4,5-trisphosphate (IP\(_3\)) production after phosphatidic acid stimulation (expressed as percentage of the maximum response) is shown in the inset. Each concentration represents the mean ± SEM of three separate experiments. Statistical significance was assessed by analysis of variance followed by Scheffe’s F test for comparing treated groups (*p<0.05, **p<0.01).
platelet-associated phospholipase C, indicating that phosphatidic acid may interact directly with the enzyme or alter the lipid environment of the substrate to increase susceptibility to phospholipase C attack.\textsuperscript{17} Alternatively, phosphatidic acid has been shown to stimulate phosphatidylinositol-4-phosphate kinase activity.\textsuperscript{25} Activation of this enzyme may result in a faster replenishment of the PIP\textsubscript{2} pool accessible for enzymatic breakdown by phospholipase C. An additional possibility, suggested by Murayama and Uji,\textsuperscript{8} is that phosphatidic acid may interact with a specific membrane receptor, thereby triggering multiple effector systems including inhibition of adenyl cyclase and activation of phospholipase C. This hypothesis is based on the finding that the ability of exogenous phosphatidic acid to inhibit adenyl cyclase activity in 3T3 fibroblasts is GTP dependent, sensitive to inactivation by pertussis toxin, and blocked by antagonists of phosphatidic acid. This hypothesis requires additional support, since exogenous phosphatidic acid may in fact gain access to intracellular phospholipase C, which involves formation of diacylglycerol at the plasma membrane, transbilayer movement of diacylglycerol, and intracellular rep sparing of phosphatidic acid.\textsuperscript{26}

Regardless of the mechanism whereby phosphatidic acid regulates the activity of phospholipase C, the ability of phosphatidic acid to stimulate the production of the second messengers inositol 1,4,5-trisphosphate and, presumably, diacylglycerol in adult cardiac ventricular myocytes is likely to have very important implications for cell function. First, the ability of exogenous phosphatidic acid to activate phospholipase C accounts for the biological effects of this phospholipid that mimic the responses evoked by hormones known to stimulate phospholipase C. Although release of phosphatidic acid from platelets has been shown on stimulation with thrombin,\textsuperscript{8} further studies are required to establish a role for phosphatidic acid as an extracellular regulator of cardiac cell function. A second possible role for phosphatidic acid may be linked to intracellular production of phosphatidic acid from diacylglycerol via phosphorylation after hormone activation of phospholipase C. Newly formed phosphatidic acid could then enhance the turnover of PIP\textsubscript{2} through intracellular activation of phospholipase C, providing a positive feedback mechanism that could sustain a hormonal response. This, however, remains to be established. Further studies will be required to assess which specific subform(s) of phospholipase C is activated by phosphatidic acid or whether the effect is ubiquitous for all subforms of the enzyme.\textsuperscript{27} Third, agonist-induced activation of phospholipase D to yield phosphatidic acid directly may serve as an alternative pathway by which receptor agonists could activate phospholipase C-mediated cleavage of PIP\textsubscript{2}. Potential hormones for such an alternative pathway are growth factors, muscarinic and \alpha\textsubscript{adrenergic} agonists, and endothelin, which have been shown to activate both phospholipase D-catalyzed production of phosphatidic acid and formation of IP\textsubscript{3}.\textsuperscript{6,7,21,28–33} Fourth, phosphatidic acid production within the sarcolemma of cardiac myocytes might result from the action of an extracellular phospholipase D. Such humoral phospholipase D activity has been detected in human plasma.\textsuperscript{34} The effects of exogenously applied phospholipase D have been investigated in isolated tissues and subcellular fractions. For example, exogenous phospholipase D increased the contractility of neonatal rat ventricular tissue\textsuperscript{35} and produced positive inotropy in rabbit papillary muscle.\textsuperscript{36} Philipson and Nishimoto\textsuperscript{16} demonstrated that exogenous phospholipase D stimulated Na\textsuperscript{+}-Ca\textsuperscript{2+} exchange in cardiac sarc olemmal vesicles, which was accompanied by an increase of phosphatidic acid in the sarcolemma. Depending on the intracellular and extracellular Na\textsuperscript{+} and Ca\textsuperscript{2+} concentrations and the membrane potential, this bidirectional transport mechanism could elevate cytosolic calcium and thereby increase contractility. In view of the findings in the present study, stimulation of phospholipase C may be another important mechanism by which phosphatidic acid could increase myocardial contractility. Inositol 1,4,5-trisphosphate mobilizes calcium from the sarcoplasmic reticulum,\textsuperscript{37} whereas stimulation of protein kinase C by diacylglycerol may also increase cytosolic calcium by phosphorylating sarcolemmal calcium channels.\textsuperscript{38}
In summary, the present study has clearly demonstrated that phosphatidic acid is a potent activator of cardiac inositol phosphate production. Future studies pertaining to the functional significance of this novel mechanism will be required. It is evident, however, that phosphatidic acid may serve a key regulatory role in potentiating the phospholipase C-mediated generation of intracellular second messengers involved in regulation of cardiac cells.

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