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Stimulation of Protein Synthesis by Phosphatidic Acid in Rat Cardiomyocytes

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ABSTRACT. Phosphatidic acid (PA) was observed to stimulate protein synthesis in adult cardiomyocytes in a time- and concentration-dependent manner. The maximal stimulation in protein synthesis (142 ± 12% vs 100% as the control) was achieved at 10 μM PA within 60 min and was inhibited by actinomycin D (107 ± 4% of the control) or cycloheximide (105 ± 6% of the control). The increase in protein synthesis due to PA was attenuated or abolished by preincubation of cardiomyocytes with a tyrosine kinase inhibitor, genistein (94 ± 9% of the control), phospholipase C inhibitors 2-nitro-4-carboxyphenyl *N,N*-diphenyl carbamate or carbonodithioic acid O-(octahydro-4,7-methanol-1H-inden-5-yl (101 ± 6 and 95 ± 5% of the control, respectively), protein kinase C inhibitors staurosporine or polymyxin B (109 ± 3 and 93 ± 3% of the control), and chelators of extracellular and intracellular free Ca^{2+} EGTA or BAPTA/AM (103 ± 6 and 95 ± 6% of the control, respectively). PA at different concentrations (0.1 to 100 μM) also caused phosphorylation of a cell surface protein of approximately 24 kDa. In addition, mitogen-activated protein kinase was stimulated by PA in a concentration-dependent manner; maximal stimulation (217 ± 6% of the control) was seen at 10 μM PA. These data suggest that PA increases protein synthesis in adult rat cardiomyocytes and thus may play an important role in the development of cardiac hypertrophy. *Copyright © 1996 Elsevier Science Inc.* BIOCHEM PHARMACOL 52;11:1735–1740, 1996.

KEY WORDS. heart hypertrophy; mitogen-activated protein kinase; signal transduction; cardiac protein synthesis; phosphatidic acid

Hypertrophy of the heart usually occurs as a compensatory mechanism for the increase in work load or decrease in muscle mass in patients with hypertension or myocardial infarction, respectively [1]. Under certain circumstances, some neurotransmitters and hormones are considered to play a permissive role for the mechanical stimulus or exert a direct effect on the development of cardiac hypertrophy [2, 3]. Because PA† has been shown to stimulate inositol 1,4,5-trisphosphate production in adult cardiomyocytes [4], induce phosphorylation of cardiac proteins [5] and increase both intracellular free Ca^{2+} and cardiac contractile force [6], it is considered to be involved in the process of signal transduction in the myocardium. In this regard it should be noted that thyroid hormone, norepinephrine, angiotensin II or endothelin-1, which are known to induce cardiac hypertrophy [1, 2], were also found to increase PA levels in cardiomyocytes [7–10]. Furthermore, the level of PA in the hypertrophied heart was significantly higher in comparison with the control values [11]. Although a growth factor-like effect has been proposed for PA in human A431 fibroblasts [12], cardiac fibroblasts [13] and mesangial cells [14], no information regarding the influence of PA on protein synthesis in these cell types is available in the literature. In addition, very little is known concerning the growth factor-like actions of PA in adult cardiomyocytes, which do not undergo cell division. The purpose of the present study was to examine the effect of PA on protein synthesis in adult rat cardiomyocytes and to explore possible mechanisms for its action.

METHODS

Chemicals

PA (L-α-phosphatidic acid, from egg yolk lecithin, sodium salt), EGTA, NCDC, D609, genistein, actinomycin D, cycloheximide, staurosporine, verapamil, BAPTA/AM, myelin basic protein and BSA were purchased from the Sigma

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† *Abbreviations:* BABTA/AM, 1,2-bis(*o*-aminophenoxy)ethane-*N,N,N′,N′*-tetraacetic acid tetra(acetoxymethyl) ester; $[Ca^{2+}]_i$, intracellular calcium concentration; D609, carbonodithioic acid O-(octahydro-4,7-methanol-1*H*-inden-5-yl) ester potassium; LPA, lysophosphatidic acid; MAP kinase, mitogen-activatedprotein kinase; NCDC, 2-nitro-4-carboxyphenyl-N,N-diphenyl carbamate; PA, phosphatidic acid; PKC, protein kinase C; PLC, phospholipase C; TrK, tyrosine kinase.

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Chemical Co. (St. Louis, MO, U.S.A.); Polymyxin B was purchased from Calbiochem (La Jolla, CA, U.S.A.); L-[2,6-^{3}H]phenylalanine (55 Ci/mmol) was from Amersham Life Science (Oakville, ON, Canada); Joklik medium (containing 194 μM phenylalanine) was purchased from Gibco BRL (Burlington, ON, Canada); [5, 6-^{3}H]uridine (35–50 Ci/mmol) and [γ-^{32}P]ATP (3000 Ci/mmol) were from DuPont (Mississauga, ON, Canada); Collagenase (Type II, 295 U/mg) was from the Worthington Biochemical Co. (Freehold, NJ, U.S.A.); the PhosphoPlus MAPK antibody kit was from New England Biolabs (Mississauga, ON, Canada). All other reagents were of analytical purity from Mallinckrodt (Quebec, Canada).

Isolation of Adult Rat Cardiomyocytes and Protein as well as RNA Synthesis

Cardiomyocytes were isolated from 175–200 g male Spraque-Dawley rats by collagenase digestion followed by suspension in Joklik medium containing 0.1 mM $CaCl_2$ [6, 15]. Quiescent and rod-shaped myocytes comprised over 80% of the total cell population, and no other cell type was visible by light microscopy. The myocytes were washed two times with Joklik medium containing 0.1 mM $CaCl_2$, transferred to test tubes in triplicate to achieve 1×10^6 cells/mL/tube in the same medium and incubated for 20 min at 37°. Ten minutes after the addition of 0.5 μCi of [^{3}H]phenylalanine or 1 μCi of [^{3}H[uridine, different concentrations of PA were added. The reaction was terminated by addition of 1 mL ice-cold 10% trichloroacetic acid at the desired time. The cell suspension was kept on ice for 20 min, washed two times with ice-cold 10% trichloroacetic acid and centrifuged at 4°. In order to avoid the possibility of interference of individual inhibitors on the basal level of [^{3}H[phenylalanine incorporation, a similar concentration of each inhibitor was added in both control and PA-treated groups; thus each experiment had its own control. The data were expressed as the ratio of dpm: PA-treated/control x 100%. The precipitated protein was resuspended in 0.5 mL of 0.5 N NaOH and transferred to scintillation vials. The neutralized aliquots were counted after the addition of 10 mL scintillate (CytoScint, ICN) [15].

Membrane Protein Phosphorylation

Cardiomyocytes (1×10^6 cells/mL) were suspended in phosphate-free HEPES buffer containing (mM): NaCl 110, KCl 2.6, $MgCl_2$ 1.0, HEPES 25 and glucose 11 (pH 7.4). The cells were incubated with 200 μCi [γ-^{32}P]ATP (40 μL) for 1 min at 37°, and then divided into 5 portions (0.2 mL/tube). Either 10 μL water or 0.1, 1, 10, and 100 μM PA plus 25 mM NaF (final concentrations) were added to different tubes. After 10 min of incubation, the reaction was terminated by centrifugation (2000 *g*) at 4° for 1 min. The cell pellet was resuspended in 0.1 mL SDS loading buffer (62.5 mM Tris-HCl pH 6.8, 1% SDS, 10% glycerol, 0.005% bromphenol blue, 5% β-mercaptoethanol) and boiled for 5 min. After cooling the samples, 40-μL aliquots of the samples were loaded onto a 12% polyacrylamide SDS gel [16].

Mitogen-Activated Protein Kinase Assay

Isolated cardiomyocytes were treated with different concentrations of PA (0.01 to 100 μM) for 2 min, harvested in 50 mM β-glycerophosphate, 2 mM EGTA, 0.5% Triton X-100, 1 mM orthophosphate, 1 mM dithiothreitol, 20 μg/mL aprotinin, 0.5 mM phenylmethylsulfonylfluoride and 0.1 mM bacitracin, and samples were run on a 10% acrylamide gel containing 0.25 mg/mL myelin basic protein. After electrophoresis, gels were assayed for MAPK activity according to a modified method of Kameshita and Fujisawa [17].

Western Blotting

Cell lysates were mixed with 20 μL SDS loading buffer and heated at 95° for 5 min. Following electrophoresis through a 7.5% polyacrylamide gel, the protein was transferred to polyvinylidene fluoride membrane in 20% methanol, 25 mM Tris, 130 mM glycine. Membranes were blocked by a 60-min treatment at room temperature with blocking solution (3% BSA in TBS-T (10 mM Tris-HCl pH 7.5, 0.1 M NaCl, 1 mM EDTA, 0.1% Tween-20)). Anti-MAP kinase or anti-phospho-MAP kinase antibody (1:5000 dilution) was added in fresh TBS-T and incubated for 60 min at 37°. Similar conditions were used for the secondary antibody (horseradish peroxidase-conjugated anti-rabbit). The membranes were washed 5 times over 30 min with TBS-T and the horseradish peroxidase was detected using the Amersham Enhanced Chemiluminescence (ECL) system.

Statistical Analysis

Data are presented as mean ± SEM. Comparison of mean values were performed by Student's *t* test. $P < 0.05$ was considered to be significantly different.

RESULTS AND DISCUSSION

Cardiac hypertrophy is characterized by an increase in protein content and cell volume in the absence of cell division [2, 3]. Since adult cardiomyocytes do not divide, an adult cardiomyocyte preparation was chosen for studying the effect of PA on protein synthesis. As shown in Fig. 1, PA enhanced the protein synthesis in cardiomyocytes in a concentration- and time-dependent manner. The maximal increase in protein synthesis (142 ± 12% of the control value) was seen at the 10 μM concentration of PA and was achieved at 60 min after the addition of PA. It should be mentioned that the experimental conditions employed in this study, including the use of [^{3}H[phenylalanine for studying protein synthesis in adult cardiomyocytes, are similar to those used by other investigators [15, 18]. To examine

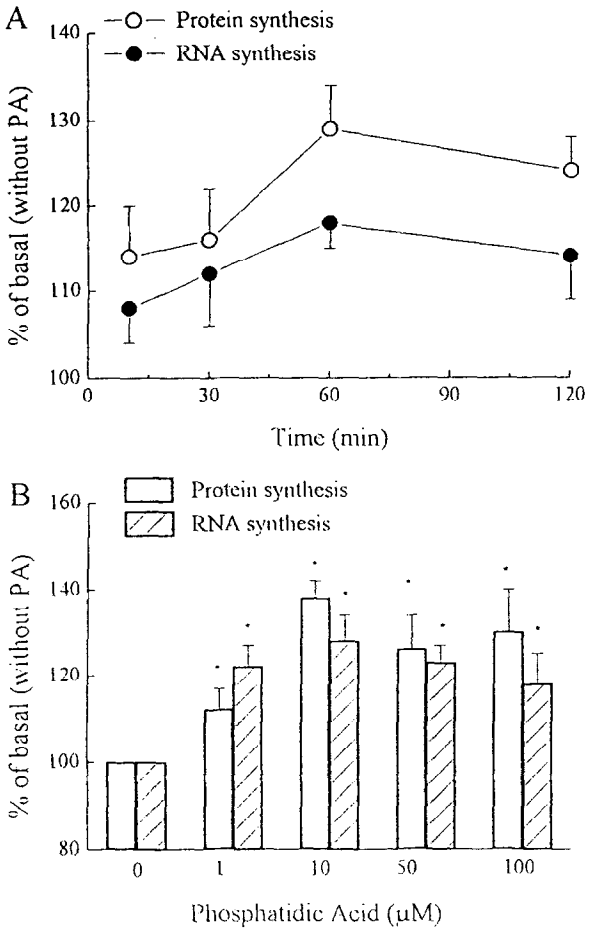

FIG. 1. Phosphatidic acid (PA)-induced protein synthesis and RNA synthesis in adult rat cardiomyocytes. The time-course effects (panel A) were measured at 10, 30, 60 and 120 min after the addition of 10 μM PA; an equivalent volume of the vehicle was used for the control group. The responses at different concentrations (panel B) were measured at 60 min after the addition of PA (1–100 μM). The data are from 6 separate preparations. *P < 0.05 compared with the control values in the absence of PA. The absolute values of [^{3}H]phenylalanine and [^{3}H]uridine incorporation in the absence of PA per 0.5 × 10^6 cells at 10 min were 6596 ± 394 dpm and 4348 ± 244 dpm, respectively.

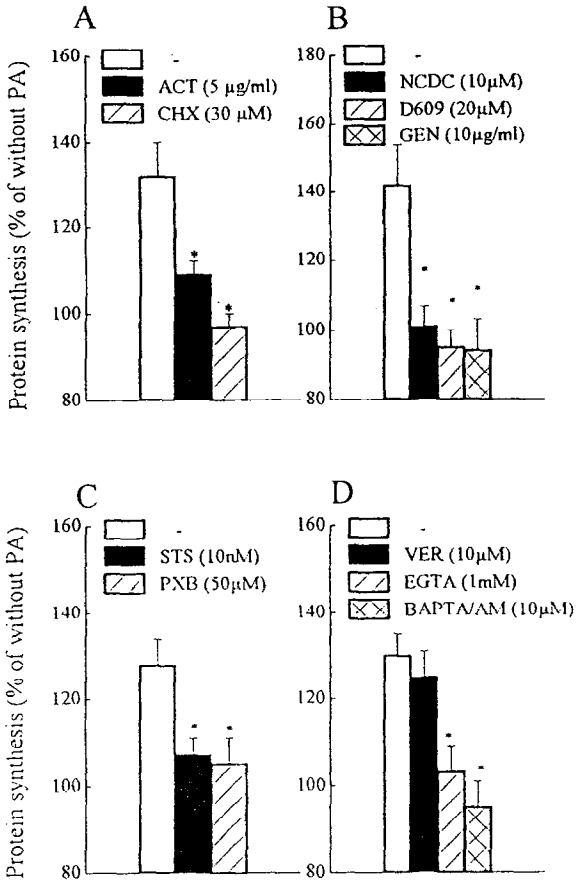

FIG. 2. Effect of transcription or translation inhibitors (panel A), phospholipase C or tyrosine kinase inhibitors (panel B), protein kinase C inhibitors (panel C) and Ca^{2+} channel blocker in chelators (panel D) on the phosphatidic acid (PA)-induced protein synthesis in rat cardiomyocytes. The data are from 6 separate preparations. *P < 0.05 compared to the control values in the absence of inhibitors. The absolute value of [^{3}H[phenylalanine incorporation per 0.5 × 10^6 cells by the untreated control was 4370 ± 239 dpm. Incorporation by each inhibitor-treated sample (0.5 × 10^6 cells) in the absence of PA stimulation were 3921 ± 315 dpm (ACT, actinomycin D); 3535 ± 516 dpm (CHX, cycloheximide); 1922 ± 44 dpm (NCDC); 4194 ± 586 dpm (D609); 2176 ± 443 dpm (GEN, genistein); 6476 ± 438 dpm (STS, staurosporine); 5231 ± 262 dpm (PXB, polymyxin B); 7667 ± 619 dpm (BAPTA/AM); 3648 ± 363 dpm (EGTA) and 5923 ± 579 dpm (VER, verapamil).

whether the increase in protein synthesis by PA occurs at the level of transcription or translation, RNA synthesis was measured by [^{3}H]uridine incorporation. The profile of increased RNA synthesis by PA was similar to that observed for protein synthesis (Fig. 1). Furthermore, the increase in protein synthesis in response to PA was partially blocked by the inclusion of either actinomycin-D (107 ± 4% of the control value), an inhibitor of transcription [15], or cycloheximide (105 ± 6% of the control value), an inhibitor of translation [19] (Fig. 2, panel A). These data indicate that PA may stimulate both transcription and translation processes for accelerating protein synthesis in cardiomyocytes.

Like many growth factors, PA has been shown to stimulate the γ-isoform PLC in adult cardiomyocytes [20]. The activation of PLC generates two second messengers, inositol 1,4,5-trisphosphate and diacylglycerol [4]; the former stimulates the release of Ca^{2+} from the sarcoplasmic reticulum while the latter activates PKC. To test whether PA-induced protein synthesis is related to the activation of PLC, two PLC inhibitors, namely D609 and NCDC [21, 22], were used. As shown in Fig. 2 (panel B), the PA-induced protein synthesis was not stimulated in the presence of either D609 or NCDC (101 ± 6% and 95 ± 5% of the control values, respectively). These data indicate that the PA-induced protein synthesis may be dependent on the activation of PLC. Since tyrosine phosphorylation is essential for activation of the γ-isoform of PLC [23], genistein, a TrK inhibitor [24], was employed to examine whether TrK

is involved in the PA-induced protein synthesis. Figure 2, panel B, shows that the response to PA of protein synthesis was abolished by the inclusion of genistein (94 ± 9% of the control value). If activation of TrK by PA leads to the phosphorylation of tyrosine, certain protein(s) in the membrane should be phosphorylated. Figure 3 shows that PA caused phosphorylation of a cell surface protein of approximately 24 kDa. It should be noted that we demonstrated that PA causes an increase in the $[Ca^{2+}]_i$ in cardiomyocytes [6]. In this study, we observed that the PA-induced protein synthesis is blocked by the pretreatment of cells with BAPTA/AM (95 ± 6% of the control value) or EGTA (103 ± 6% of the control value), intracellular or extracellular Ca^{2+} chelators, respectively [7, 16], but not by verapamil (125 ± 6% of the control value), a well known L-type Ca^{2+} channel blocker (Fig. 2, panel D).

The PA-induced DNA synthesis was dependent upon the activation of PKC in cortical astrocytes [25] and mesangial cells [14], but was not affected by the down-regulation of PKC in vascular smooth muscle cells [26]. In cardiomyocytes, PA has been shown to stimulate PKC by activating the PLC pathway [4, 7]. To investigate the role of PKC in the PA-induced protein synthesis by cardiomyocytes, the cells were preincubated with staurosporine, a non-specific PKC inhibitor [27], or polymyxin-B, a relatively specific PKC inhibitor [28]. As shown in Fig. 2, panel C, the PA-induced response was partially blocked by staurosporine (109 ± 3% of the control value) and completely blocked by polymyxin B (97 ± 3% of the control value). The strong inhibitory effect of polymyxin B is probably due to its ability to decrease $[Ca^{2+}]_i$ in addition to the inhibition of PKC [29].

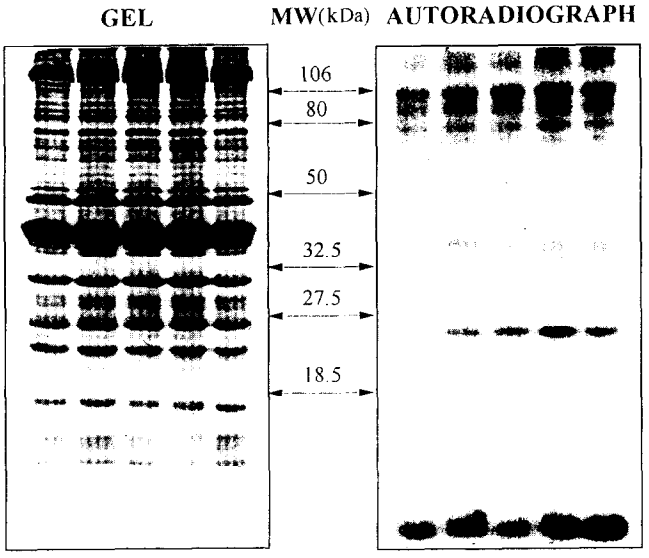

FIG. 3. Phosphatidic acid (PA)-induced surface protein phosphorylation in rat cardiomyocytes. Left panel: Coomassie brilliant blue staining; right panel: autoradiograph of the same gel indicating protein phosphorylation. Either 10 μL water or 0.1, 1, 10 and 100 μM PA were added to different lanes (from the left side). A similar pattern was obtained in two other experiments.

Protein kinases that mediate phosphorylation cascades are required for signal transduction between the membrane and nucleus during the development of cardiac hypertrophy [30]. The stimulation of MAP kinase has been shown to be coupled to PKC activation in many systems and is known to participate in the development of cardiac hypertrophy [30]. MAP kinase also participates in signal transduction into the nucleus [31]. The results in Fig. 4 indicate that PA activates MAP kinase in cardiomyocytes in a time- and concentration-dependent manner. Both MAP kinase phosphorylation and enzymatic activity were monitored over a time period of 20 min following treatment with PA (10 μM). Peak responses (217 ± 6% of control with 10 μM PA) were obtained within 2 min and returned to the basal level at 20 min. It should be noted that activation of the 44-kDa isoform of MAP kinase became apparent at 2 min of incubation with PA (Fig. 4A). These effects of PA in adult cardiomyocytes are consistent with results obtained with cardiac fibroblasts [13]. It has already been established that different hypertrophic agonists activate MAPK as well as increase the formation of PA in adult rat cardiomyocytes [32]. These findings suggest that PA may serve as a second messenger in the regulation of cell growth in cardiomyocytes. It can be argued that the responses to PA observed in this study may be due to some contamination and/or the formation of LPA. However, this does not seem to be the case because 0.1 to 10 μM LPA did not affect the protein synthesis in adult cardiomyocytes under the experimental conditions employed in this study (data not shown). Furthermore, the results from our laboratory [6] and by others [33] have shown a minimal contamination (<1%) of PA by LPA. Similar conclusions regarding different effects of PA were made by other investigators [4, 33].

Because the protein synthesis in the cell is dependent upon the intracellular pool of free amino acids, it is possible that the PA-induced increase in protein synthesis in cardiomyocytes may be due to an effect of PA on the uptake of radiolabelled phenylalanine used in this study. Although we have not measured the action of PA on [^{3}H]phenylalanine uptake in adult cardiomyocytes, it is unlikely that this mechanism could serve to promote protein synthesis by PA. This view is based on the fact that we have employed 200 μM phenylalanine in the Joklik medium for studying protein synthesis, under which conditions the equilibration of [^{3}H]phenylalanine is rapid and optimal [34]. It should also be noted that the PA-induced increase in protein synthesis was coupled with increased uptake of [^{3}H]uridine in the cardiomyocytes; this is indicative of a concomitant increase in RNA synthesis. Furthermore, it would be difficult to explain the inhibition of the PA-induced increase in protein synthesis due to different agents that are known to affect PLC, PKC, and TrK as well as both translation and transcription sites. Nonetheless, it is understood that the exact mechanisms for the increase in protein synthesis by PA in cardiomyocytes remain to be investigated.

This study demonstrates for the first time that PA stimu-

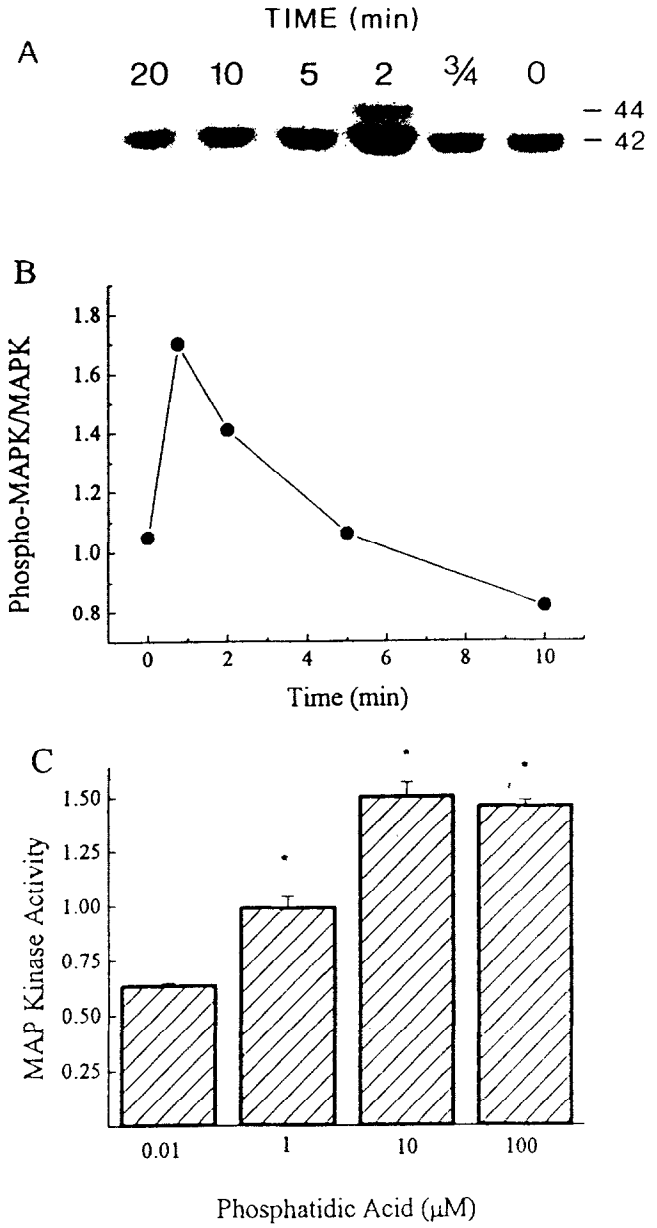

FIG. 4. MAPK activation of phosphatidic acid (PA) in adult rat cardiomyocytes. Panel A: Time course of MAP kinase activation by 10 μM PA as measured using the activity gel assay. Panel B: Time course of MAP kinase phosphorylation induced by 10 μM PA. Phosphorylated MAP kinase and total MAP kinase protein were detected by western blot analysis. The data are presented as the ratio of phosphorylated and total MAP kinase at each time point. Panel C: MAP kinase activity was measured in extracts obtained at 2 min after PA (0.01, 1, 10, 100 μM) treatment using the activity gel assay. The data are from 3 separate preparations and represent the combined densitometric values obtained for both 42- and 44-kDa MAP kinase bands.

lates protein synthesis and RNA synthesis by affecting the signal transduction mechanisms in adult rat cardiomyocytes. Such a signal transduction cascade may include the activation of PLC leading to the elevation of the $[Ca^{2+}]_i$ level and an increase in the PKC activity. As a result, MAPK is activated, which in turn may phosphorylate certain sites to accelerate the processes of transcription and translation for protein synthesis in cardiomyocytes. It can be argued that the observed reduction in PA-induced protein synthesis by several inhibitors employed in this study may be a consequence of a cytotoxic effect of the inhibitors. This does not seem to be the case because the concentration of each inhibitor used in the present study was within the range employed by other investigators; secondly, the cell viability (percentage of rod-shaped cells) was not affected by the presence of individual inhibitors. Furthermore, similar concentrations of these inhibitors were present in the control groups. Nonetheless, it should be pointed out that the use of pharmacological inhibitors can only provide a clue regarding signal transduction pathways of PA-induced protein synthesis. Further studies are needed to test the effect of PA on the biochemical activities of TrK, PLC and PKC. It should also be mentioned that the *in vitro* results reported in this study by employing 10 μM concentration of PA may be of some physiological significance under *in vivo* conditions, because the concentration of PA in cardiomyocytes is considered to be about 20 μM [6]. Thus PA can be seen to serve as a signal transducer for the induction of cardiac hypertrophy due to certain hormones, which are known to increase its content in the myocardium [1, 2, 7–10, 35].

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