Biochemical Pharmacology, Vol. 59, pp. 1163–1171, 2000.

ISSN 0006-2952/00/$–see front matter
PII S0006-2952(00)00239-2

Stimulation of 90- and 70-kDa Ribosomal Protein S6 Kinases by Arginine Vasopressin and Lysophosphatidic Acid in Rat Cardiomyocytes

Yan-Jun Xu, Sung Ouk Kim,* Duan-Fang Liao,* Sidney Katz† and Steven L. Pelech*‡*

*Department of Medicine and †Faculty of Pharmaceutical Sciences, University of British Columbia, Vancouver, B.C., Canada V6T 1Z3

ABSTRACT. Arginine vasopressin (AVP) and lysophosphatidic acid (LPA) have been shown to stimulate protein kinase C (PKC) and mitogen-activated protein (MAP) kinases and the proliferation of vascular smooth muscle cells. However, the actions of these two agents in cardiomyocytes are less well understood. To investigate the signal transduction pathways of AVP and LPA, freshly isolated adult rat cardiomyocytes were examined. Both AVP and LPA induced concentration- and time-dependent stimulation of the phosphotransferase activities of p90 ribosomal S6 kinases (RSK) and their upstream activators, extracellularly regulated kinases (ERK) 1 and 2. The activation of ERK1 and ERK2 by LPA was PKC- and phosphatidylinositol 3-kinase (PI 3-kinase)-dependent. However, AVP-induced activation of RSK2, a downstream substrate of ERK1 and ERK2, was PKC-dependent and PI 3-kinase-independent. AVP and LPA were also observed to increase the phosphotransferase activity of p70 ribosomal protein S6 kinase (p70 S6K) in a time- and concentration-dependent manner. The activation of p70 S6K by LPA and AVP was PI 3-kinase-dependent. PKC was necessary in AVP- but not in LPA-induced activation of p70 S6K. Since RSK and p70 S6K have been implicated in the regulation of translational control of protein synthesis, we concluded that AVP and LPA may stimulate the growth of cardiomyocytes through these two protein kinase cascades. BIOCHEM PHARMACOL **59**;9:1163–1171, 2000. © 2000 Elsevier Science Inc.

KEY WORDS. lysophosphatidic acid; vasopressin; cardiomyocytes; MAP kinase; phosphatidylinositol 3-kinase; RSK; p70 ribosomal S6 kinase

AVP§ is a nine-amino-acid peptide hormone secreted from the pituitary gland. Its antidiuretic and vasoactive effects are well established. Recently AVP has been shown to play important roles in regulating cardiac contractility [1], intracellular free Ca^{2+} concentration [2], atrial natriuretic peptide secretion [3], and protein synthesis [4, 5]. AVP may be involved in the pathogenesis and/or the progression of cardiac hypertrophy, since the plasma concentration of this hormone is elevated in patients with hypertension or congestive heart failure [6, 7]. AVP has been reported to stimulate phospholipase D in the cardiac H9C2 cell line [8], so the cardiac effect of AVP may be mediated partially by LPA. LPA is a water-soluble and bioactive phospholipid existing in serum and cytosol. Serum LPA is released from platelets and fibroblasts in response to thrombin or various growth factors, or LPC can be converted into LPA through the action of plasma lysophospholipase D [9–11]. Cytosolic LPA is a metabolite of PA generated via the action of phospholipase A_2 [12]. LPA receptors have been cloned recently and shown to be expressed in heart tissues [13]. Thus, LPA may regulate cardiac function in an autocrine or paracrine manner.

Protein kinase cascades transfer signals from the cell surface to the nucleus when the extracellular mediators bind to the receptors in the plasma membrane [14]. It has been shown that many growth factors activate MAP kinases, 90-kDa RSK, and p70 S6K. AVP and LPA have been reported to activate ERK in neonatal rat cardiomyocytes [15, 16]. However, the signal transduction pathways of AVP and LPA in adult rat cardiomyocytes have not been examined. Furthermore, the effects of AVP and LPA on the activities of RSK and p70 S6K in cardiomyocytes as well as the role of PI 3-kinase in AVP and LPA signalling have not been described yet. As both LPA and AVP are considered to exert their effects through G protein-coupled receptors, it was interesting to compare the signal transduction pathways of these two agonists in cardiomyocytes.

‡ Corresponding author: Dr. Steven L. Pelech, Department of Medicine, 2nd Floor, Koerner Pavilion, 2211 Wesbrook Mall, University of British Columbia, Vancouver, B.C., Canada V6T 1Z3. Tel. (604) 822-8086; FAX (604) 822-8693; E-mail: spelech@home.com

§ *Abbreviations*: AVP, arginine vasopressin; AII, angiotensin II; ET-1, endothelin-1; ERK, extracellularly regulated kinases; LPA, lysophosphatidic acid; LPC, lysophosphatidylcholine; MAP, mitogen-activated protein; MBP, myelin binding protein; p70 S6K, 70-kDa ribosomal protein S6 kinase; PA, phosphatidic acid; PI, phosphatidylinositol; PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate; PTX, pertussis toxin; and RSK, 90-kDa ribosomal S6 kinase.

Received 16 June 1999; accepted 8 October 1999.

MATERIALS AND METHODS

Chemicals

Joklik-modified minimal essential medium (Ca^{2+}-free) and collagenase were obtained from GIBCO Life Technologies and the Worthington Biochemical Corp., respectively. Primary antibodies for RSK1 (C-21) were purchased from Santa Cruz Biotechnology. RSK 2-PCT, p70 S6K-PNT, and PI 3-kinase (PI 3-kinase p85 N-SH2) antibodies were obtained from Upstate Biotechnology Inc. Phospho-specific antibody against ERK1 and ERK2 was purchased from New England Biolabs. The horseradish peroxidase conjugate secondary antibodies were obtained from Bio-Rad. AVP, ET-1, PMA, LPA, and bovine MBP were purchased from Sigma-Aldrich Ltd. S6 peptide (RRLSSLRA) was a gift from Kinetek Pharmaceuticals, Inc. PI was obtained from Avanti Polar Lipids. [γ-^{32}P]ATP was obtained from Dupont. TLC plates were purchased from EM SCIENCE. Bisindolymaleimide, Ro-32–0432, LY 294002, and wortmannin were obtained from Calbiochem. PTX was purchased from List Biological Laboratories Inc. Ponceau S was purchased from ICN.

Isolation of Cardiomyocytes

The isolation of adult rat cardiomyocytes was performed as described previously [17]. Male Sprague–Dawley rats (200–250 g) were injected i.p. with heparin (1 U/kg body weight) and anesthetized i.p. with pentobarbital (75 mg/kg, body weight). The hearts were excised and mounted on a Langendorff apparatus. Each heart was perfused initially with calcium-free Joklik medium for 5 min, and then the perfusate was switched to the same solution containing 1% collagenase and 50 μM $CaCl_2$. After recirculation for 15 min, the heart was removed from the cannula, and the atria were excised. The ventricles were cut into small pieces and subjected to a 15-min digestion in fresh collagenase solution in the presence of 4% BSA at 37° in a shaking water bath. The ventricular tissues were aspirated and dispersed gently (twice per min) with a 5-mL transfer pipette. The cell suspension was collected and filtered through a 200-μm nylon mesh. The cells were allowed to settle down by gravity for 15 min. The supernatant was discarded, and the cells were resuspended in Joklik medium containing 0.5 mM Ca^{2+}. The viable rod-shaped myocytes comprised more than 80% of the final cell population.

Cell Treatment and Homogenization

After 1 hr of stabilization of the cells at room temperature, suspended cells (1×10^6) were treated with different concentrations of LPA, or with deionized water or 1% DMSO as a control. The incubation was terminated at the desired times by freezing the cell pellets in liquid nitrogen after centrifugation at 4°. The frozen cell pellets were resuspended in ice-cold homogenizing buffer composed of 20 mM MOPS, 15 mM EGTA, 2 mM Na_2EDTA, 1 mM Na_3VO_4, 1 mM dithiothreitol, 75 mM β-glycerophosphate, 0.1 mM phenylmethylsulfonyl fluoride, 1 μg/mL of aprotinin, 0.7 μg/mL of pepstatin, 1 μg/mL of leupeptin, and 1% Triton X-100. Then the cells were sonicated for 30 sec on ice, and total cell extracts were obtained by centrifugation at 240,000 *g* for 11 min at 4°. The supernatants were stored at −70°. The samples were thawed on ice in a cold cabinet before the assays. Protein concentrations were assayed with Bradford reagent from Bio-Rad.

Western Blotting

Cell lysates were mixed with sample loading buffer (2.5% SDS, 10% glycerol, 50 mM Tris–HCl, pH 6.8, 0.5 M β-mercaptoethanol, and 0.01% bromophenol blue) and boiled for 3 min. After SDS–PAGE in 10% polyacrylamide gels, the proteins were transferred electrophoretically onto nitrocellulose membranes. The proteins on the membranes were stained with Ponceau S to confirm that equivalent amounts of proteins were loaded into each well. Then the membranes were blocked with 5% BSA in Tris-buffered saline (20 mM Tris–HCl, pH 7.5, 0.5 M NaCl, 0.2% Tween-20; TBST). After quickly rinsing the membranes with TBST, they were incubated with primary antibody in TBST for 3 hr with constant shaking at room temperature. The membranes were washed three times for 10 min with TBST and incubated with horseradish peroxidase-conjugated secondary antibody in TBST for 30 min. After washing the membrane three times with TBST, the immunoreactivity was visualized using the Enhanced Chemiluminescence (ECL) system from Amersham and quantitated by densitometric analysis [17].

Immunoprecipitation

Various protein kinases were immunoprecipitated by mixing 500 μg of cell extract protein, 40 μL of protein A–Sepharose (50% slurry), and 6 μL of antibody solution (1 mg/mL) and incubating for 3 hr with constant rotation at 4°. The protein A–Sepharose beads were centrifuged and washed once with NETF buffer [6% Nonidet P-40, 100 mM NaCl, 5 mM EDTA, 50 mM Tris–HCl, pH 7.4, 50 mM NaF], once with 6% Nonidet P-40, twice with NETF, and finally with KII buffer containing 1.25 mM MOPS, 1.25 mM β-glycerophosphate, 0.5 mM EGTA, 7.5 mM $MgCl_2$, and 0.05 mM NaF. Then the immunoprecipitated proteins were used for kinase activity analysis [18].

Phosphotransferase Assays

Phosphotransferase activities of various kinases were assayed from either fast protein liquid chromatography-fractionated extracts, immunoprecipitated proteins (on the beads), or crude extracts. Five microliters of extract or 50% bead slurry was added to 30 μL of reaction buffer containing 25 mM β-glycerophosphate, 20 mM MOPS, pH 7.2, 5 mM EGTA, 2 mM EDTA, 20 mM $MgCl_2$, 1 mM Na_3VO_4, 0.25 mM dithiothreitol, and 4 μg of the appropriate substrate: MBP protein for ERK and S6 peptide for RSK1, RSK2, and p70 S6K. Reactions were started by the addition

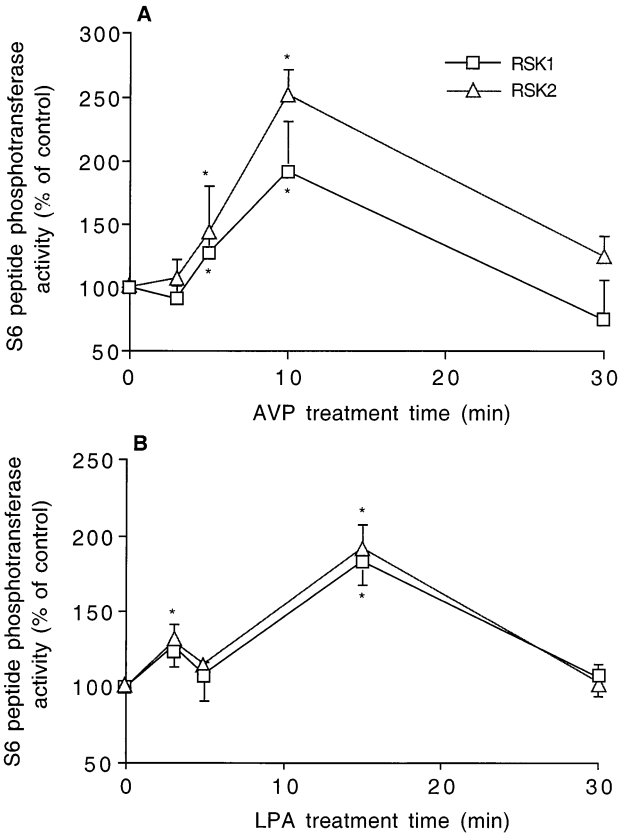

FIG. 1. Time course of AVP (A) and LPA (B) effects on the activation of p90 RSK in cardiomyocytes. The concentrations of AVP and LPA in this experiment were 10 nM and 5 μM. RSK1 and RSK2 proteins were immunoprecipitated with isoform-specific antibodies. The S6 peptide phosphotransferase activities of RSK1 and RSK2 were determined. The values represent averages ± SEM of 5 separate preparations. Key: (*) $P < 0.05$ compared with control values. The absolute values of RSK1 and RSK2 in the control group were 428 ± 123 and 1729 ± 653 cpm/15 min, respectively.

of 50 μM [γ-^{32}P]ATP (2000 cpm/pmol). After incubation at 30° for 15 min, 20 μL of the reaction mixture was deposited on 2 cm^2 of p81 phosphocellulose paper (Whatman). The papers were washed three times with deionized water containing 1% orthophosphoric acid. The radioactivity was measured by scintillation counting [18].

The PI 3-kinase protein was immunoprecipitated from 500 μg of crude cell extracts. The beads were washed three times in lysis buffer (20 mM Tris, pH 7.2, 137 mM NaCl, 1% Triton X-100, 2 mM EDTA, and 10% glycerol) with 50 μM Na_3VO_4. The reaction was initiated by the addition of 40 μL of kinase buffer {30 mM HEPES, pH 7.2, 30 mM $MgCl_2$, 20 μM adenosine, 1 mg/mL of PI, and 50 μM [γ-^{32}P]ATP (10 μCi)}. After incubation for 10 min at room temperature, 30 μL of the reaction mixture was spotted on a TLC plate, which was developed for 2 hr in a solution containing 6.25 mL NH_4OH, 18.75 mL H_2O, 87.5 mL methanol, and 112.5 mL chloroform. After drying the TLC plate, the bands were visualized by autoradiography and quantitated by densitometry [19].

Statistical Analysis

Data are expressed as means ± SEM. The mean values were compared by Student's *t*-test. $P < 0.05$ was considered to be significantly different.

RESULTS

Effects of AVP and LPA on RSK1 and RSK2 Activities

RSK isoforms have been implicated in the control of transcription and protein synthesis. It has been shown previously by this laboratory that RSK1 and RSK2 are highly expressed in the heart [20]. To investigate the role of AVP and LPA in the regulation of these RSK isoforms, freshly isolated adult rat cardiomyocytes were treated with different concentrations of AVP or LPA, and their phosphotransferase activities were determined at various time points following specific immunoprecipitation. As shown in Figs. 1 and 2, AVP and LPA caused time- and concentration-dependent activation of these isozymes. The peak responses of RSK1 and RSK2 to 10 nM AVP (2- to 2.5-fold

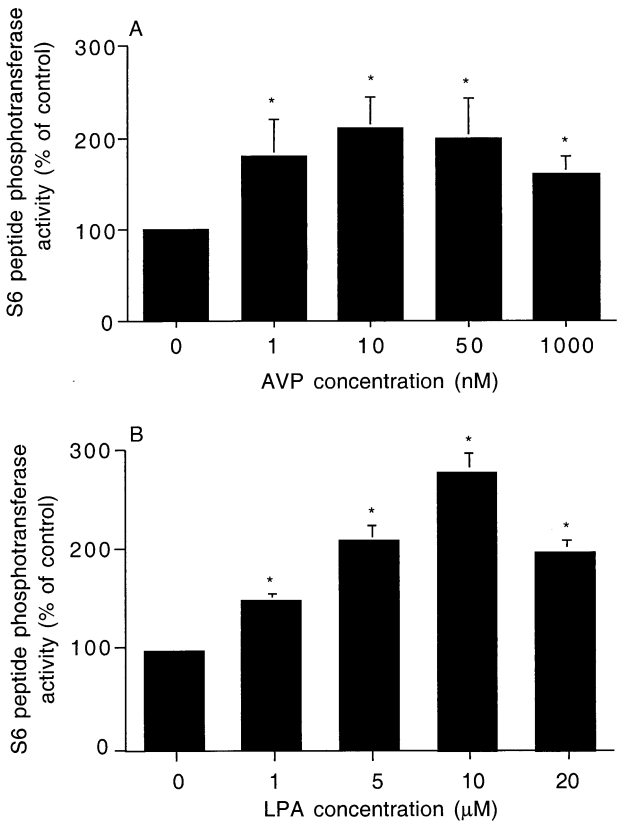

FIG. 2. Concentration-dependent response of RSK2 to AVP (A) and LPA (B). The freshly isolated adult rat cardiomyocytes were treated with different concentrations of AVP or LPA, and the responses were terminated 10 min after the addition of the agonist. RSK2 protein was immunoprecipitated with isoform-specific antibodies. The S6 peptide phosphotransferase activities of RSK2 were determined. The values represent averages ± SEM of 5 separate preparations. Key: (*) $P < 0.05$ compared with control values. The absolute value of RSK2 in the control group was 1980 ± 654 cpm/15 min.

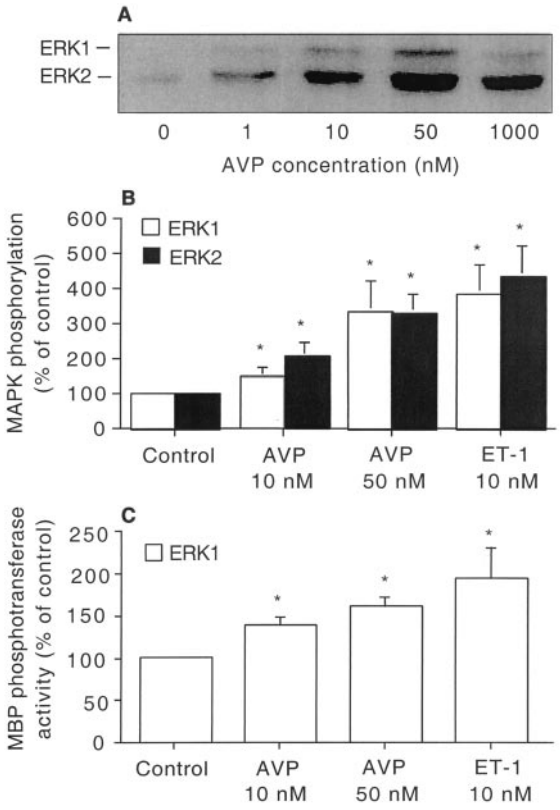

FIG. 3. Effect of AVP on MAP kinase phosphorylation (A and B) and activity (C). The cardiomyocytes were treated with different concentrations of AVP (1–1000 nM) or ET-1 (10 nM) for up to 10 min. MAP kinase phosphorylation was determined with a phospho-specific antibody that recognized phospho-ERK1 and ERK2. The activity of MAP kinase was measured by the MBP phosphotransferase activity after immunoprecipitation with an ERK1-specific antibody. The values represent averages ± SEM of 6 separate preparations. Key: (*) $P < 0.05$ compared with control values. The absolute value of MBP phosphotransferase activity in the control group was 574 ± 150 cpm/15 min.

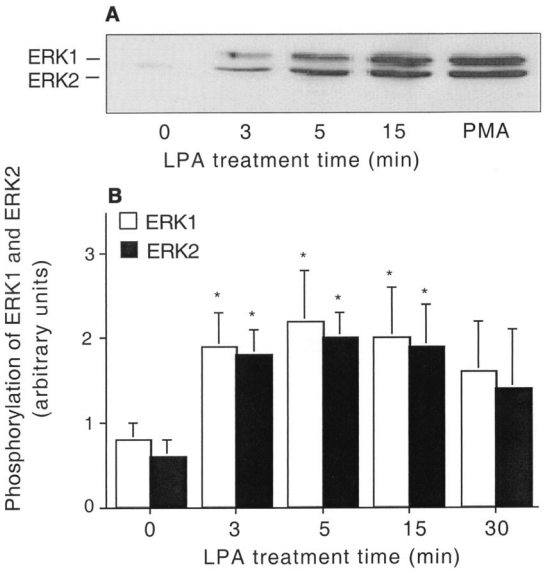

FIG. 4. Effect of 5 μM LPA on the phosphorylation of ERK1 and ERK2 in cardiomyocytes. Phospho-specific antibody was used to recognize phospho-ERK1 and ERK2, and the amount of phospho-ERK1 and ERK2 was determined by western blotting followed by densitometric analysis. Panel A shows a representative immunoblot, and panel B provides the pooled values (averages ± SEM) of densitometric scanning of 42-kDa (ERK2) and 44-kDa (ERK1) MAP kinase bands from 5 separate preparations. Key: (*) $P < 0.05$ compared with control values.

activation) or 5 μM LPA (about 2-fold activation) were achieved in 10–15 min. The peak responses of RSK2 (2-fold) to AVP and LPA were observed with 10 nM and 10 μM, respectively (Fig. 2).

Effects of AVP and LPA on ERK1 and ERK2 Activities

ERK1 and ERK2 are upstream activators of RSK isoforms. Since both AVP and LPA activated RSK, these two agents would be expected to stimulate ERK1 and ERK2. Although AVP and LPA have been shown to activate ERK1 and ERK2 in neonatal rat cardiomyocytes [15, 16], the effects of AVP and LPA on these MAP kinases in adult rat cardiomyocytes, which are fully differentiated, have not been reported. In the present study, an antibody that specifically recognized the dual phosphorylated forms of ERK1 and ERK2 was used to determine the degree of phosphorylation of these MAP kinases. ET-1 and PMA enhanced phosphorylation of ERK1 and ERK2 about 4-fold (Fig. 3) and 6-fold (Fig. 4), respectively. Although this method is an indirect measure of MAP kinase activity, it appeared to correlate well with actual phosphotransferase activity and was highly sensitive (Fig. 3). For example, 50 nM AVP increased phosphorylation of ERK1 and ERK2 by 2-fold and increased phosphotransferase activity of ERK1 by 60%. Five micromolar LPA stimulated the phosphorylation of ERK1 and ERK2 by 2.5-fold within 3–5 min of treatment (Fig. 4).

Effects of AVP and LPA on p70 S6K Activity

A role of p70 S6K in the regulation of cell growth has been proposed [21]. As shown in Figs. 5 and 6, AVP and LPA caused time- and concentration-dependent activation of p70 S6K. Ten nanomolar AVP stimulated p70 S6K (2- to 2.5-fold) at 5 min (Fig. 5A). Five micromolar LPA stimulated this kinase by 1.5-fold within 5 min, and this activation was sustained for a longer period than that seen with AVP (Fig. 5B). The maximum activation of p70 S6K was induced by 10 nM AVP and 5 μM LPA (Fig. 6).

Effect of AVP and LPA on PI 3-Kinase Activity

PI 3-kinase has been implicated in LPA signalling in renal cells [22]. To investigate whether AVP and LPA stimulate PI 3-kinase in adult rat cardiomyocytes, we measured the activity of PI 3-kinase before and after treatment with AVP or LPA. As shown in Fig. 7, 10 nM AVP stimulated PI 3-kinase activity significantly at 5 min and reached a peak

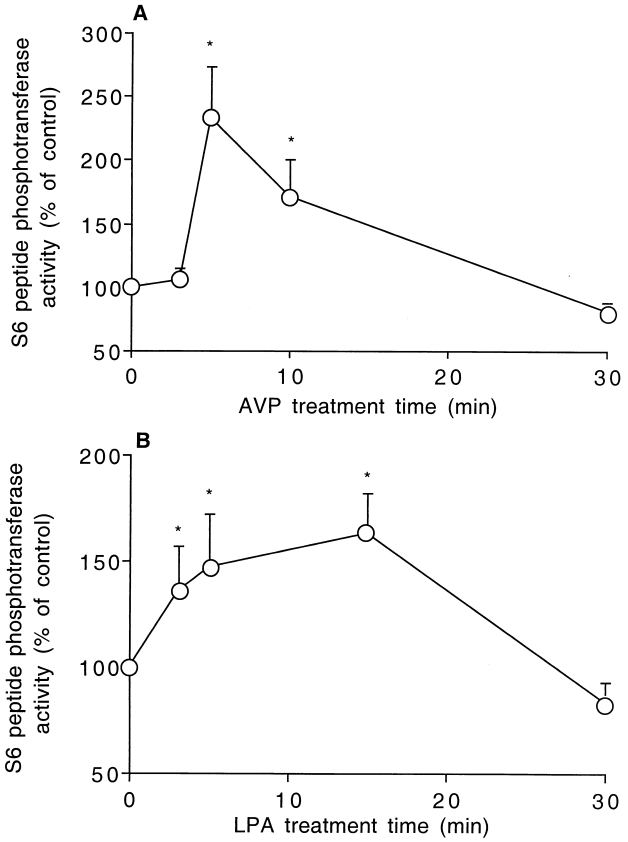

FIG. 5. Time course of AVP (A) and LPA (B) effects on the phosphotransferase activity of p70 S6K in cardiomyocytes. Ten nanomolar AVP and 5 μM LPA were used in these experiments. The phosphotransferase activity of p70 S6K towards a synthetic peptide (S6 peptide) was measured following specific immunoprecipitation of this kinase. The averages ± SEM of 6 separate experiments are shown. Key: (*) $P < 0.05$ compared with control values. The absolute value of the control group was 432 ± 124 cpm/15 min.

at 15 min (2-fold increase). Five micromolar LPA stimulated PI 3-kinase significantly at 3 min (2-fold higher than basal level); the peak response was achieved at 5 min (7-fold higher than basal level) and was largely reversed by 15 min.

Roles of G Proteins, PKC, and PI 3-Kinase in AVP- and LPA-Induced Activation of RSK and p70 S6K Pathways

AVP and LPA are known to exert their effects through G protein-coupled receptors [16]. To test whether this was the case in adult rat cardiomyocytes, PTX was used to block $G_{i\alpha}$ protein function. The stimulating effects of AVP and LPA on RSK2 and p70 S6K were abolished, indicating that these two agonists bind to PTX-sensitive G_i proteins (Fig. 8). To test whether AVP-induced activation of RSK and p70 S6K was mediated by PKC and PI 3-kinase, specific inhibitors were used in the present study. The response of RSK2 to 10 nM AVP was inhibited significantly (Fig. 9; $P < 0.05$) by bisindolymaleimide (50 nM) and Ro-32–0432 (500 nM), which are PKC inhibitors [23], but was not affected (Fig. 9) by wortmannin (10 nM) or LY 294002 (10 μM), which are PI 3-kinase inhibitors [18, 24]. However, high concentrations of LY 294002 (50–200 μM) blocked the action of AVP (Fig. 10). This demonstrated that AVP-evoked activation of RSK was mediated through PKC, but may not have been via the p85-associated isoform of PI 3-kinase. In contrast, the activation of p70 S6K by 10 nM AVP was blocked significantly ($P < 0.05$) by preincubation of cells with similar concentrations of PI 3-kinase inhibitors and PKC inhibitors (Fig. 9). The response of ERK to LPA was inhibited by wortmannin (10 nM) [18, 24] and bisindolymaleimide (50 nM) [23] (Fig. 11A). Therefore, the activation of ERK1 and ERK2 by LPA appeared to be mediated by PKC and the p85 isoform of PI 3-kinase. The activation of p70 S6K by LPA was blocked by preincubating the cells with 10 nM wortmannin, but not by 50 nM bisindolymaleimide (Fig. 11B), indicating that LPA-induced activation of p70 S6K was mediated mainly by PI 3-kinase but perhaps not by PKC (Fig. 12).

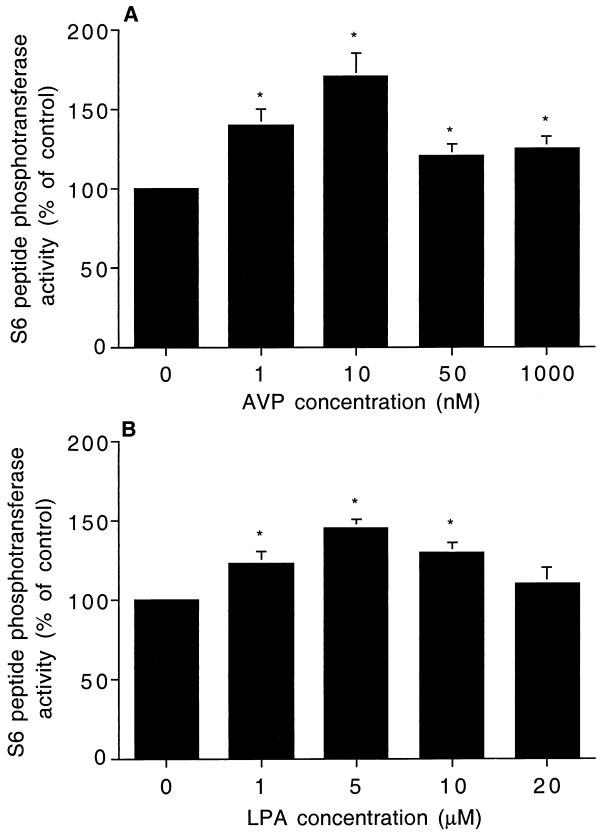

FIG. 6. Concentration–response studies of AVP (A) and LPA (B) effects on the phosphotransferase activity of p70 S6K in cardiomyocytes. The phosphotransferase activity of p70 S6K towards a synthetic peptide (S6 peptide) was measured following specific immunoprecipitation of this kinase. The reaction was terminated at 10 min after treatment. The averages ± SEM of 6 separate experiments are shown. Key: (*) $P < 0.05$ compared with control values. The absolute value of the control group was 510 ± 150 cpm/15 min.

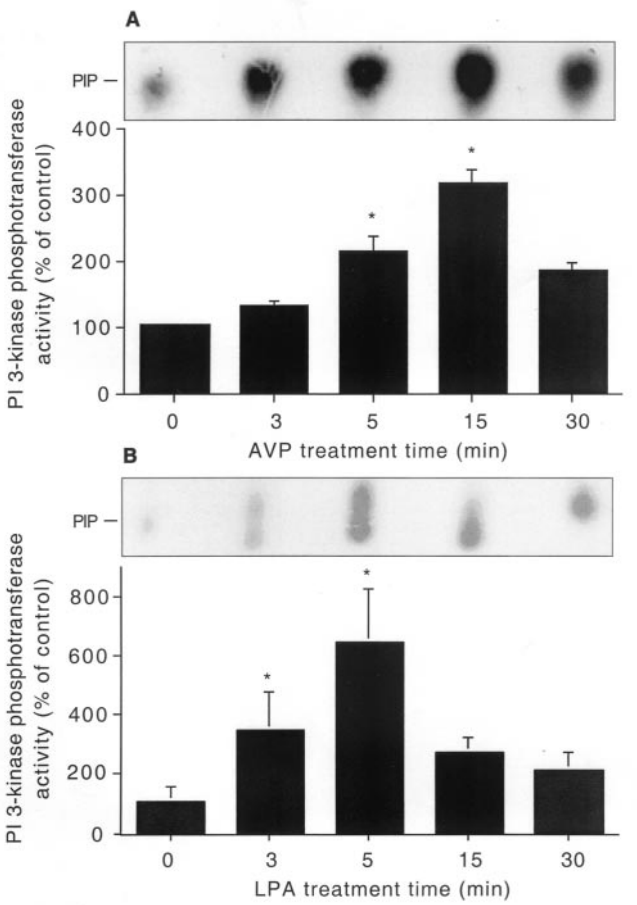

FIG. 7. Effects of 10 nM AVP (A) and 5 μM LPA (B) on the activation of PI 3-kinase in cardiomyocytes. The PI 3-kinase activity was determined following immunoprecipitation by a specific antibody. The radioactivity incorporated into PI 3-phosphate (PIP) was quantitated following TLC resolution from PI. The bar graph represents the averages ± SEM of 4 separate experiments. The inserts are representative experimental traces. Key: (*) $P < 0.05$ compared with control values.

DISCUSSION

We have demonstrated for the first time that AVP and LPA caused time- and concentration-dependent activation of the S6 kinases RSK and p70 S6K in cardiomyocytes by PKC- and PI 3-kinase-dependent and -independent routes. It has been shown previously that AVP stimulates ERK1 and ERK2 activities in neonatal rat cardiomyocytes at 100 nM [5], but not at 1 μM [16]. In our hands, a bell-shaped response of AVP was evident in the activation of RSK2 and p70 S6K. Walker *et al.* [1] reported that AVP exerts a positive inotropic effect at lower concentrations (50–100 pg/mL) and a negative inotropic effect at higher concentrations. The mechanisms for the bell-shaped response or the biphasic effect of AVP are not clear. In vascular smooth muscle, two binding sites of the AVP V_1-receptor with two different affinities have been identified [25]. The activation of lower affinity binding sites at the micromolar concentration range could buffer its high affinity (nanomolar range) stimulating effects. Activation of ERK1 and ERK2 may lead

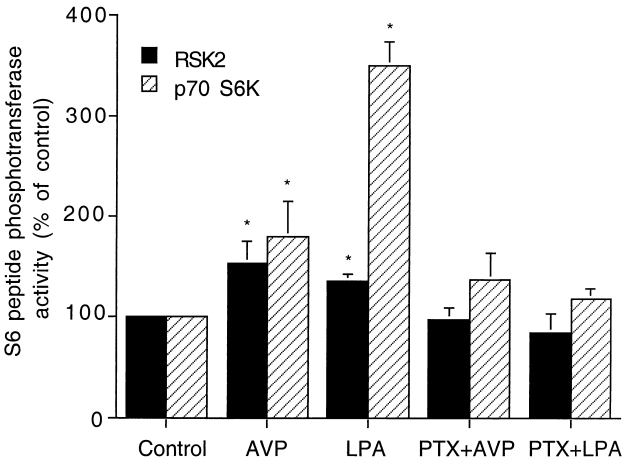

FIG. 8. Effects of PTX on AVP- and LPA-induced activation of RSK2 and p70 S6K in cardiomyocytes. PTX (100 ng/mL) was preincubated with cardiomyocytes for 10 min at 37°; then AVP (10 nM) or LPA (5 μM) was added to each sample except for the control groups. The bar graph represents pooled values (averages ± SEM) from 5 separate preparations. Key: (*) $P < 0.05$ compared with control values. The absolute values of RSK2 and p70 S6K were 3450 ± 236 and 1450 ± 75 cpm/15 min, respectively.

to the phosphorylation and activation of RSK isoforms. We also noticed that the maximal activation of RSK2 was observed at 10 nM AVP, a concentration that only marginally activated the MAP kinases. This observation indicated that the total activation of RSK2 could be induced

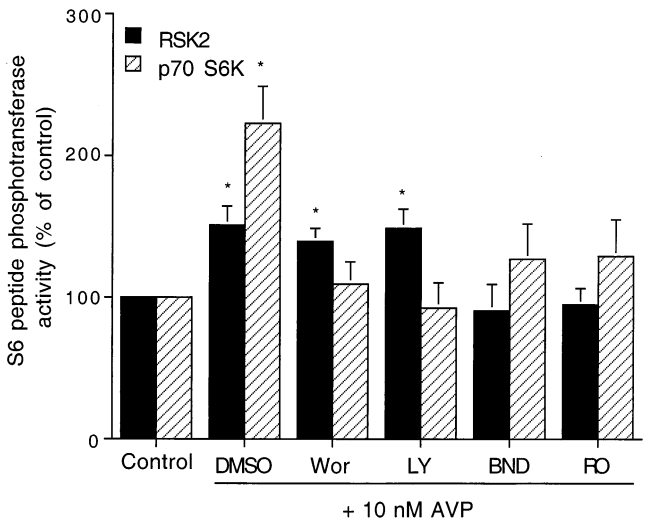

FIG. 9. Effects of wortmannin (Wor), LY 294002 (LY), bisindolymaleimide (BND), and Ro-32–0432 (RO) on AVP-induced activation of RSK2 and p70 S6K in cardiomyocytes. The cardiomyocytes (1×10^6 cells/mL) were preincubated with 10 μL of 1% DMSO, 10 nM Wor, 10 μM LY, 50 nM BND, or 500 nM RO for 10 min at 37°; then AVP (10 nM) was added to each sample for 10 min except for the control group. The bar graph represents pooled values (averages ± SEM from 5 separate preparations). Key: (*) $P < 0.05$ compared with control values. The absolute values of RSK2 and p70 S6K were 2020 ± 310 and 1208 ± 78 cpm/15 min, respectively.

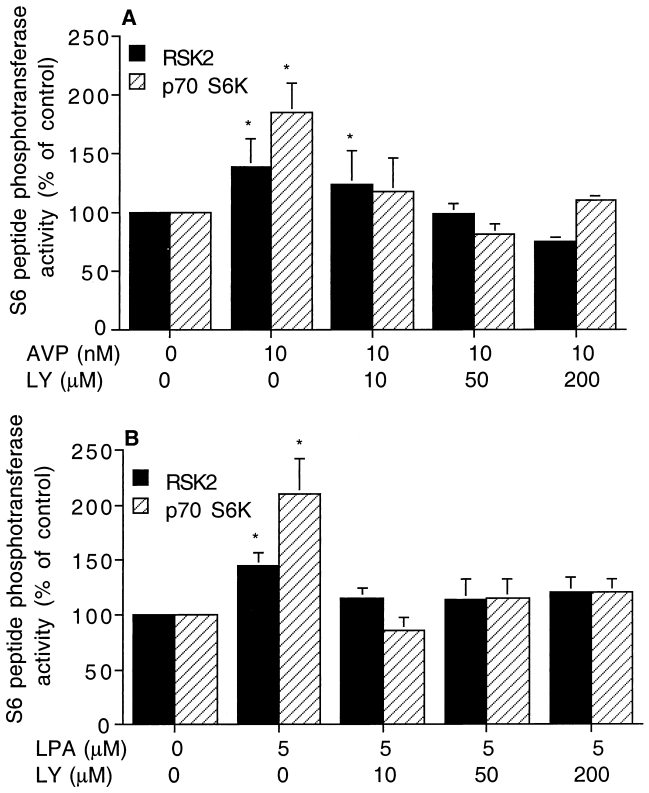

FIG. 10. Effects of different concentrations of LY 294002 (LY) on AVP (A)- and LPA (B)-induced activation of RSK2 and p70 S6K in cardiomyocytes. The cardiomyocytes (1×10^6 cells/mL) were preincubated with 10, 50, and 200 μM LY for 10 min at 37°; then AVP (10 nM) was added to each sample for 10 min except for the control group. The bar graph represents pooled values (averages ± SEM from 5 separate preparations). Key: (*) $P < 0.05$ compared with control values. The absolute values of RSK2 and p70 S6K were 3337 ± 267 and 1450 ± 64 cpm/15 min, respectively.

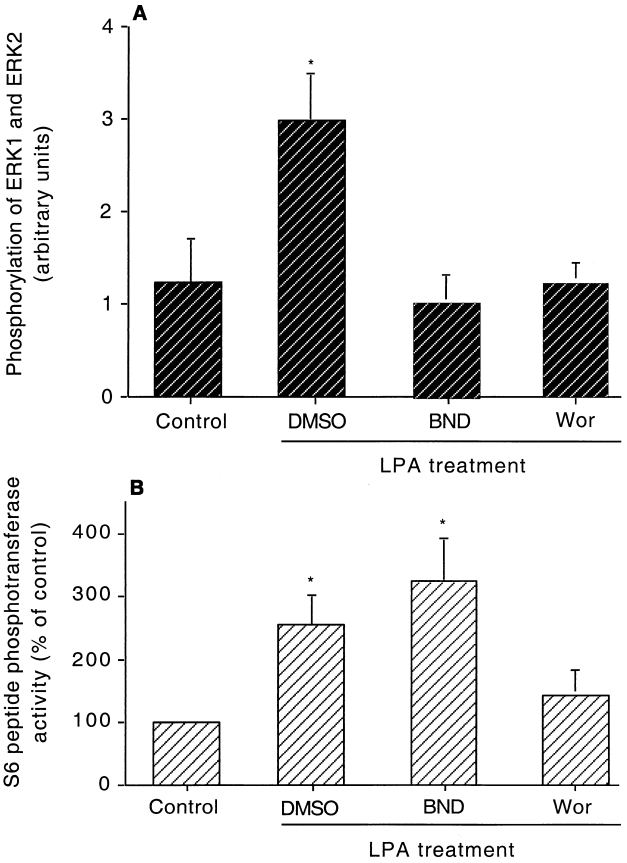

FIG. 11. Effects of bisindolymaleimide (BND) and wortmannin (Wor) on LPA-induced activation of ERK1/ERK2 (A) and p70 S6K (B) in cardiomyocytes. Cardiomyocytes (1×10^6 cells/mL) were preincubated with 10 μL of 1% DMSO as a vehicle control, then with 50 nM BND or 10 nM Wor for 10 min, and finally LPA (5 μM) was added to the cells for an additional 10 min prior to harvesting. In panel A, a phospho-specific antibody was used to recognize phospho-ERK1 and ERK2, and the amount of phospho-ERK1 and ERK2 was determined by western blotting (averages ± SEM of 6 experiments). The S6 peptide phosphotransferase activity (averages ± SEM of 6 experiments) of immunoprecipitated p70 S6K kinase is shown in panel B. Key: (*) $P < 0.05$ compared with control values. The absolute value of p70 S6K in the control group was 530 ± 230 cpm/15 min.

either by partial activation of ERK1 and ERK2 or by other unknown pathways. The activation of RSK2 by AVP was mediated by PKC in adult rat cardiomyocytes, which is consistent with the ERK data from neonatal rat cardiomyocytes [15]. LPA has been shown to stimulate RSK2 in fibroblasts [26]. AII was observed previously to stimulate RSK1 in cardiomyocytes [27], but the knowledge of its effect on RSK2 in cardiomyocytes was limited. In the present study, LPA was observed for the first time to stimulate both the RSK1 and RSK2 isoforms in cardiomyocytes.

LPA has been shown previously to stimulate ERK1 and ERK2 in neonatal rat cardiomyocytes at a single concentration of 100 μM [16]. Here, we demonstrated that LPA stimulated ERK1 and ERK2 activity in adult rat cardiomyocytes at physiological concentrations, about 1–5 μM [9, 22]. LPA has been known to stimulate PKC in several cell types. LPA may be able to activate PKC through: (i) lipid products of the PI 3-kinase catalyzed reaction [10]; (ii) altering membrane lipid properties [28]; and (iii) LPA conversion to phosphatidic acid and subsequent increases in the level of the PKC activator diacylglycerol. PKC is known to activate ERK1 and ERK2 indirectly [29]. McLees *et al.* [30] reported that LPA-evoked activation of these MAP kinases was blocked by the inhibition of PKC activity in an endothelial cell line. Taken together, it appears that PKC activation is important for both AVP- and LPA-induced activation of the ERK1/ERK2-RSK1/RSK2 pathways. It seems that PI 3-kinase plays a role in the activation of these MAP kinases, which could be due to the requirement of PI 3-kinase for Ras-dependent Raf-1 activation [31]. Daub *et al.* [32] reported that LPA-induced activation of MAP kinases in fibroblasts is blocked by a PI 3-kinase inhibitor. Ferby *et al.* [33] reported that 1 μM wortmannin inhibits MAPK activation through a mechanism independent of p85/p110-type PI 3-kinase. Stephens [34] reported

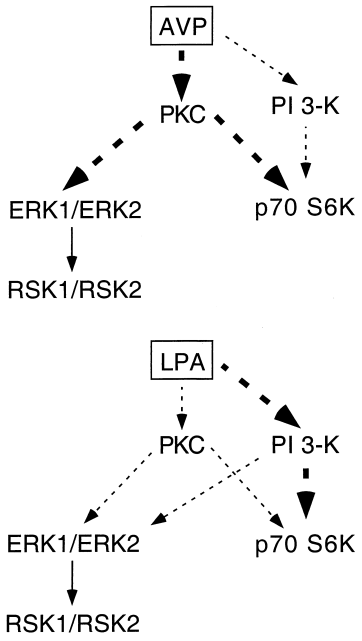

FIG. 12. Summary of the signal transduction pathways of AVP and LPA in freshly isolated adult rat cardiomyocytes. The dark lines represent the major routes.

that the IC_{50} of wortmannin for inhibition of $G_{\beta\gamma}$-sensitive PI 3-kinase is about 2.5 times higher than that of the p85/p110 isoform of PI 3-kinase. Ten nanomolar wortmannin and 10 μM LY 294002 are close to the ic_{50} values for the inhibition of the p85 isoform of PI 3-kinase but may be lower than the ic_{50} value for the inhibition of the p110γ isoform [34–36]. LPA-induced activation of ERK1 and ERK2 was blocked by lower concentrations of these two inhibitors, but AVP-induced activation of RSK2 was inhibited by a higher concentration of LY 294002. This indicates that the p85 isoform of PI 3-kinase plays a major role in the effect of LPA. However, the p110γ isoform may be more important for AVP-induced activation of the RSK1 and RSK2 pathways.

Many growth factors and mechanical stretch have been shown to stimulate p70 S6K, resulting in cardiac hypertrophy [21]. Activation of p70 S6K has been shown to be involved in the stimulation of mRNA translation. AVP and LPA may stimulate protein synthesis in part via activation of p70 S6K in adult cardiomyocytes.

AII has been shown to stimulate PI 3-kinase in the heart [37]. Wilson *et al.* [38] reported that LPA-induced activation of p70 S6K is blocked by inhibition of PI 3-kinase in clone 1C cells. Another recent report has described LPA activation of PI 3-kinase in murine macrophages [39]. Our data from cardiomyocytes extend these findings. PI 3-kinase was able to activate p70 S6K by PKC-dependent and -independent routes. Since the activation of p70 S6K by LPA was blocked by wortmannin, but not by bisindolymaleimide, LPA-evoked activation of p70 S6K is PI 3-kinase-dependent and PKC-independent. The inhibitory effects of wortmannin on LPA-induced activation of p70 S6K were unlikely to be due to their nonspecific actions, since wortmannin did not reduce the basal levels of ERK1 phosphotransferase activity (data not shown). The stimulating effect of AVP on p70 S6K was abolished by either PI 3-kinase or PKC inhibitors, and the stimulating effect of LPA on p70 S6K was inhibited significantly by PI 3-kinase inhibitors but not by PKC inhibitors. Therefore, PI 3-kinase activation is essential for the activation of p70 S6K. However, the role of PKC in p70 S6K may be stimulator-dependent. Considering the effect of AVP, AII, and ET-1 on the activity of phospholipase D in cardiomyocytes [8, 40] and the time courses of these two agonists, LPA may mediate the effects of AVP partially but not fully. Further studies are needed to examine whether AVP, AII, ET-1, and other agonists are able to increase the cytosolic level of LPA and whether inhibition of the production of phosphatidic acid can block the effect of AVP.

It has been reported that the activation of ERK1 and 2 by LPA and AVP in neonatal rat cardiomyocytes is inhibited by PTX [16]. Our data extend these findings in adult rat cardiomyocytes. In conclusion, AVP and LPA stimulated the ERK1/ERK2→RSK1/RSK2 and PI 3-kinase→p70 S6K pathways via PTX-sensitive G proteins.

We thank R. Wagey for her aid in the PI 3-kinase assays and the reviewers of this manuscript for their helpful comments. Dr. Y-J. Xu was the recipient of a Postdoctoral Fellowship from the Medical Research Council of Canada (MRCC). This research was supported by a grant-in-aid from the Heart and Stroke Foundation of British Columbia and Yukon to Drs. S. L. Pelech and S. Katz.

References

1. Walker BR, Childs ME and Adams EM, Direct cardiac effects of vasopressin: Roles of V1- and V2-vasopressinergic receptors. *Am J Physiol* **255:** H261–H265, 1988.
2. Xu YJ and Gopalakrishnan V, Vasopressin increases cytosolic free [Ca^{2+}] in the neonatal rat cardiomyocyte: Evidence for V_1 subtype receptors. *Circ Res* **69:** 239–245, 1991.
3. Van Der Bent V, Church DJ, Valloton MB, Meda P, Kem DC, Capone AM and Lang U, $[Ca^{2+}]_i$ and protein kinase C in vasopressin-induced prostacyclin and ANP release in rat cardiac myocytes. *Am J Physiol* **266:** H597–H605, 1994.
4. Xu YJ, McNeill JR, Wilson TW and Gopalakrishnan V, Vasopressin accelerates protein synthesis in neonatal rat cardiomyocytes (RC). *Can J Cardiol* **10:** 107A, 1994.
5. Tahara A, Tomura Y, Wada K-i, Kusayama T, Tsukada J, Ishii N, Yatsu T, Uchida W and Tanaka A, Effect of YM087, a potent nonpeptide vasopressin antagonist, on vasopressin-induced protein synthesis in neonatal rat cardiomyocyte. *Cardiovasc Res* **38:** 198–205, 1998.
6. McNeill JR, Role of vasopressin in the control of arterial pressure. *Can J Cardiol* **61:** 1226–1235, 1983.
7. Uretsky BF, Verbalis JG, Generalovich T, Valdes A and Reddy PS, Plasma vasopressin response to osmotic and hemodynamic stimuli in heart failure. *Am J Physiol* **248:** H396–H402, 1985.
8. Tran K, Zha X and Choy PC, Enhancement of phospholipid hydrolysis in vasopressin-stimulated BHK-21 and H9C2 cells. *Mol Cell Biochem* **151:** 69–76, 1995.

9. Eichholtz N, Jalink K, Fahrefort I and Moolenaar WH, The bioactive phospholipid lysophosphatidic acid is released from activated platelets. *Biochem J* **291:** 677–689, 1993.
10. Toker A and Cantley L, Signalling through the lipid products of phosphoinositide-3-OH kinase. *Nature* **387:** 673–676, 1997.
11. Yokumura A, Iimori M, Nishioka Y, Kitahara M, Sakashita M and Tanaka S, Lysophosphatidic acids induce proliferation of vascular smooth muscle cells from rat aorta. *Am J Physiol* **267:** C204–C210, 1994.
12. Brindley DN and Waggoner DW, Phosphatidate phosphohydrolase and signal transduction. *Chem Phys Lipids* **80:** 45–47, 1996.
13. An S, Dickens MA, Bleu T, Hallmark OG and Gootzl E, Molecular cloning of the human Edg2 protein and its identification as a functional cellular receptor for lysophosphatidic acid. *Biochem Biophys Res Commun* **231:** 619–622, 1996.
14. Pelech SL, Charest DL, Mordret GP, Siow YL, Palaty C, Campbell D, Charlton L, Samiei M and Sanghera JS, Networking with mitogen-activated protein kinases. *Mol Cell Biochem* **127/128:** 157–169, 1993.
15. Aharonovitz O, Aboulafia-Etzion S, Leor J, Battler A and Granot Y, Stimulation of 42/44 kDa mitogen-activated protein kinases by arginine vasopressin in rat cardiomyocytes. *Biochim Biophys Acta* **1401:** 105–111, 1998.
16. Bogoyevitch MA, Clerk A and Sugden PH, Activation of the mitogen-activated protein kinase cascade by pertussis toxin-sensitive and -insensitive pathways in cultured ventricular cardiomyocytes. *Biochem J* **309:** 437–443, 1995.
17. Xu YJ, Yau L, Yu L, Elimban V, Zahradka P and Dhalla NS, Stimulation of protein synthesis by phosphatidic acid in cardiomyocytes. *Biochem Pharmacol* **52:** 1735–1740, 1996.
18. Tudan C, Jackson JK, Charlton L, Pelech SL, Salh B and Burt HM, Activation of S6 kinase in human neutrophils by calcium pyrophosphate dihydrate crystals: Protein kinase C-dependent and phosphatidylinositol-3-kinase-independent pathways. *Biochem J* **331:** 531–537, 1998.
19. Wagey R, Pelech SL, Duronio V and Krieger C, Phosphatidylinositol 3-kinase: Increased activity and protein level in amyotrophic lateral sclerosis. *J Neurochem* **71:** 716–722, 1998.
20. Kim SO, Irwin P, Katz S and Pelech SL, Expression of mitogen-activated protein kinase pathways during postnatal development of rat heart. *J Cell Biochem* **71:** 286–301, 1998.
21. Laser M, Kasi VS, Hamawaki M, Cooper GIR, Kerr CM and Kuppuswamy D, Differential activation of p70 and p85 S6 kinase isoforms during cardiac hypertrophy in the adult mammal. *J Biol Chem* **273:** 24610–24619, 1998.
22. Levine JS, Koh JS, Triaca V and Lieberthal W, Lysophosphatidic acid: A novel growth and survival factor for renal proximal tubular cells. *Am J Physiol* **273:** F575–F585, 1997.
23. Baldo A, Sniderman AD, St. Luce S, Zhang X-J and Cianflone K, Signal transduction pathway of acylation stimulating protein: Involvement of protein kinase C. *J Lipid Res* **36:** 1415–1426, 1995.
24. Ui M, Okada T, Hazeki K and Hazeki O, Wortmannin as a unique probe for an intracellular signalling protein, phosphoinositide 3-kinase. *Trends Biochem Sci* **20:** 303–307, 1995.
25. Gopalakrishnan V, Xu YJ, Sulakhe PV, Triggle CR and McNeill JR, Vasopressin (V1) receptor characteristics in rat aortic smooth muscle cells. *Am J Physiol* **261:** H1927–H1936, 1991.
26. Ohta Y and Hartwig JH, Phosphorylation of actin-binding protein 280 by growth factors is mediated by p90 ribosomal protein S6 kinase. *J Biol Chem* **271:** 11858–11864, 1996.
27. Sadoshima J, Qiu Z, Morgan JP and Izumo S, Angiotensin II and other hypertrophic stimuli mediated by G protein-coupled receptors activate tyrosine kinase, mitogen-activated protein kinase, and 90-kD S6 kinase in cardiac myocytes. The critical role of Ca^{2+}-dependent signaling. *Circ Res* **76:** 1–15, 1995.
28. Sando J and Chertihin OI, Activation of protein kinase C by lysophosphatidic acid: Dependence on composition of phospholipid vesicles. *Biochem J* **317:** 583–588, 1996.
29. Pelech SL and Sanghera JS, MAP kinases: Charting regulatory pathways. *Science* **257:** 1355–1356, 1992.
30. McLees A, Graham A, Malarkey K, Gould GW and Plevin R, Regulation of lysophosphatidic acid-stimulated tyrosine phosphorylation of mitogen-activated protein kinase by protein kinase C- and pertussis toxin-dependent pathways in the endothelial cell line EAhy 926. *Biochem J* **307:** 743–748, 1995.
31. Ferrell JE Jr, MAP kinases in mitogenesis and development. *Curr Top Dev Biol* **33:** 1–60, 1996.
32. Daub H, Wallasch C, Lankenan A and Ullrich A, Signal characteristics of G protein-transactivated EGF receptor. *EMBO J* **16:** 7032–7044, 1997.
33. Ferby IM, Waga I, Hoshino M, Kume K and Shimizu T, Wortmannin inhibits mitogen-activated protein kinase activation by platelet-activating factor through a mechanism independent of p85/p110-type phosphatidylinositol 3-kinase. *J Biol Chem* **271:** 11684–11688, 1996.
34. Stephens L, Smrcka A, Cooke FT, Jackson TR, Sternweis PC and Hawkins PT, A novel phosphoinositide 3 kinase activity in myeloid-derived cells is activated by G protein $\beta\gamma$ subunits. *Cell* **77:** 83–93, 1994.
35. Imai Y and Clemmons DR, Roles of phosphatidylinositol 3-kinase and mitogen-activated protein kinase pathways in stimulation of vascular smooth muscle cell migration and deoxyribonucleic acid synthesis by insulin-like growth factor-I. *Endocrinology* **140:** 4228–4235, 1999.
36. Somwar R, Sumitani S, Taha C, Sweeney G and Klip A, Temporal activation of p70 S6 kinase and Akt1 by insulin: PI 3-kinase-dependent and -independent mechanisms. *Am J Physiol* **275:** E618–E625, 1998.
37. Saad MJ, Velloso LA and Carvalho CR, Angiotensin II induces tyrosine phosphorylation of insulin receptor substrate 1 and its association with phosphatidylinositol 3-kinase in rat heart. *Biochem J* **310:** 741–744, 1995.
38. Wilson M, Burt AR, Milligan G and Anderson NG, Wortmannin-sensitive activation of $p70^{s6k}$ by endogenous and heterologously expressed G_i-coupled receptors. *J Biol Chem* **271:** 8537–8540, 1996.
39. Koh JS, Lieberthal W, Heydrick S and Levine JS, Lysophosphatidic acid is a major serum noncytokine survival factor for murine macrophages which acts via the phosphatidylinositol 3-kinase signaling pathway. *J Clin Invest* **102:** 716–727, 1998.
40. Eskildsen-Helmond YE, Van Heugten HA and Lamers JM, Regulation and functional significance of phospholipase D in myocardium. *Mol Cell Biochem* **157:** 39–48, 1996.