

# Calcium-Independent Activation of Extracellularly Regulated Kinases 1 and 2 by Angiotensin II in Hepatic C9 Cells: Roles of Protein Kinase C $\delta$ , Src/Proline-Rich Tyrosine Kinase 2, and Epidermal Growth Factor Receptor *trans*-Activation

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## ABSTRACT

Agonist activation of endogenous angiotensin II (Ang II) AT<sub>1</sub> receptors expressed in hepatic C9 cells markedly stimulated inositol phosphate production, phosphorylation of the proline-rich tyrosine kinase PyK-2, and ERK activation. Ang II caused activation of protein kinase C  $\delta$  (PKC $\delta$ ) in C9 cells, and its stimulatory actions on Pyk2 and extracellularly regulated kinase (ERK) phosphorylation were abolished by PKC depletion and selective inhibition of PKC $\delta$  by rottlerin, but not by Ca<sup>2+</sup>-chelators. These effects, and the similar actions of the Src kinase inhibitor PP2 indicate the involvement of PKC $\delta$  and Src kinase in ERK activation. In C9 cells, phorbol-12-myristate-13-acetate (PMA) caused much greater phosphorylation of Pyk2 and ERK than the Ca<sup>2+</sup> ionophore ionomycin, and the effects of PMA and Ang II were abolished in PKC-depleted cells. Ang II in-

creased the association of Pyk2 with Src and with the epidermal growth factor receptor (EGF-R). EGF caused much greater tyrosine phosphorylation of the EGF-R than Ang II and PMA. Ang II-induced activation of ERK, but not Pyk2, was prevented by inhibition of EGF receptor phosphorylation by AG 1478 and of Src kinase by PP1. Ang II also increased the association of the adaptor protein Grb2 with the EGF-R. These findings indicate that Src and Pyk2 act upstream of the EGF-R and that the majority of Ang II-induced ERK phosphorylation is dependent on *trans*-activation of the EGF-R. Ang II-induced ERK activation in C9 cells is initiated by a PKC $\delta$ -dependent but Ca<sup>2+</sup>-independent mechanism and is mediated by the Src/Pyk2 complex through *trans*-activation of the EGF-R.

The rapid intracellular signaling responses of ligand-activated G protein-coupled receptors (GPCRs) are frequently associated with the initiation of phosphorylation cascades that regulate cell growth, differentiation, and function. Many of these GPCR-induced cellular effects are mediated by activation of extracellular signal regulated kinases (ERKs), a subfamily of the MAP kinases. One major route of GPCR-stimulated ERK/MAP kinase activation uses the generic in-

tracellular signaling pathway of receptor tyrosine kinases (RTKs) (Luttrell et al., 1999). This process, which involves RTK *trans*-activation, has been demonstrated for many GPCRs, including receptors for lysophosphatidic acid (LPA), thrombin, bradykinin, endothelin, and angiotensin II (Dikic et al., 1996; Hackel et al., 1999). In many agonist-stimulated cells, GPCR stimulation acts through a variety of effectors to promote activation and tyrosine phosphorylation of the receptors for epidermal growth factor (EGF-R) and platelet-derived growth factor (PDGF-R) (Daub et al., 1997; Hackel et al., 1999; Luttrell et al., 1999). After RTK phosphorylation,

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**ABBREVIATIONS:** GPCR, G protein-coupled receptor; ERK, extracellularly regulated kinase; MAP, mitogen-activated protein; RTK, receptor tyrosine kinase; LPA, lysophosphatidic acid; EGF, epidermal growth factor; PDGF, platelet-derived growth factor; MEK, mitogen-activated protein kinase/extracellularly regulated kinase kinase; Ang II, angiotensin II; AT<sub>1</sub>-R, angiotensin type 1 receptor; PKC, protein kinase C; Pyk-2, proline-rich tyrosine kinase 2; BAPTA, 1,2-bis(2-aminophenoxy)ethane-*N,N,N',N'*-tetraacetic acid; AM, acetoxymethyl ester; AG 1478, 4-(3-chloroanilino)-6,7-dimethoxyquinazoline; AG 1296, 7-dimethoxy-3-phenylquinoxaline; AG 490, *a*-cyano-(3,4-dihydroxy)-*N*-benzylcinnamide; F-12K, Ham's F-12 nutrient mixture, Kaighn's modification; PBS, phosphate-buffered saline; PAGE, polyacrylamide gel electrophoresis; PD123177, 1-[(4-amino-3-methylphenyl)methyl]-5-(dephenylacetyl)-4,5,6,7-tetrahydro-1*H*-imidazol[4,5-*C*]pyridine-6-carboxylic acid; PMA, phorbol-12-myristate-13-acetate; Gö 6983, 2-[1-(3-dimethylaminopropyl)-5-methoxyindol-3-yl]-3-(1*H*-indol-3-yl) maleimide; Ro-31-8220, 3-[1-(amidinothio)propyl]-1*H*-indol-3-yl]-3-(1-methyl-1*H*-indol-3-yl)maleimide; PTX, pertussis toxin; PLC, phospholipase C; PI-3 kinase, phosphatidylinositol 3-kinase; PP2, 4-amino-5-(4-chlorophenyl)-7-(*tert*-butyl)pyrazolo[3,4-*d*]pyrimidine; PD 98059, 2'-amino-3'-methoxyflavone; HEK, human embryonic kidney; VSMC, vascular smooth-muscle cell.

the steps involved in GPCR- and RTK-mediated ERK activation are usually similar and are initiated by tyrosine phosphorylation of adaptor proteins (Shc and Grb2) that recruit guanine nucleotide exchange factors for the small GTPase Ras. The resulting rapid increase in Ras.GTP subsequently activates the ERK-MAPK cascade with sequential phosphorylation of Raf, MAPK/ERK kinase (MEK), and finally ERK. Despite intensive investigation, the natures and roles of the intermediate signaling molecules that link many GPCRs to RTK *trans*-activation are not fully understood.

The type 1 angiotensin II receptor (AT<sub>1</sub>-R) is a typical GPCR and mediates a wide variety of physiological actions in the cardiovascular system, kidney, brain, adrenal glands, and liver (see de Gasparo et al., 2000). In many Ang II target cells, the AT<sub>1</sub>-R interacts primarily with pertussis-toxin insensitive G<sub>q/11</sub> proteins, leading to activation of PLC and generation of diacylglycerol, which activates protein kinase C (PKC), and inositol trisphosphate, which mobilizes Ca<sup>2+</sup> from intracellular stores. However, the AT<sub>1</sub>-R is also coupled to inhibitory G<sub>i</sub> proteins in rat hepatocytes (Pobiner et al., 1991; Tsygankova et al., 1998) and in rat adrenal, pituitary, and renal cells (de Gasparo et al., 2000). The Src family kinases are known to act as convergence points in G<sub>q</sub>- and G<sub>i</sub>-mediated ERK activation (Della Rocca et al., 1997; Luttrell et al., 1997), but the mechanism(s) by which Ang II-induced G<sub>q</sub>- and G<sub>i</sub>-mediated signaling lead to EGF-R *trans*-activation and ERK phosphorylation are less well characterized.

AT<sub>1</sub>-R-mediated ERK activation in Ang II target cells may be dependent on PKC (Arai and Escobedo, 1996; Li et al., 1998), Ca<sup>2+</sup> (Murasawa et al., 1998; Eguchi et al., 1999), or sometimes on both messengers (Sabri et al., 1998). The extent to which PKC participates in Ang II-stimulated ERK activation through EGF-R *trans*-activation varies significantly among individual cell types. In rat liver epithelial (GN4) cells, Ang II has been shown to activate ERK by a PKC-dependent but EGF-R- and Ras-independent pathway in control cells, and by an EGF-R- and Ras-dependent pathway in PKC-depleted cells (Li et al., 1998). In contrast, other evidence indicates that activation of PKC stimulates tyrosine phosphorylation of the EGF-R and Shc and thus causes activation of ERK (Tsai et al., 1997; Prenzel et al., 1999). Furthermore, stimulation of PKC by phorbol esters can also mimic the action of GPCRs (endothelin-1, LPA, and thrombin) by activating the metalloprotease that cleaves heparin-binding EGF-like growth factor to form EGF, which in turn activates the EGF-R and its associated mitogenic cascade (Prenzel et al., 1999).

Recent studies have suggested that the signaling characteristics of endogenously expressed GPCRs not only reflect the molecular mechanisms operating in the specific cell type but also may differ from those observed in cells containing over- or underexpressed ectopic receptors and transducing proteins (Zhang et al., 1996; Tang et al., 2000). Clone 9 cells, which are derived from the normal rat liver and retain an epithelial phenotype, express endogenous AT<sub>1</sub> receptors that are coupled to both G<sub>q</sub> and G<sub>i</sub> proteins and thus represent a useful model in which to study the Ang II-mediated ERK signaling cascade. Although Ang II receptors are abundant in the liver, the actions of Ang II in hepatic cells are less well characterized than those in cardiovascular and renal cell types (de Gasparo et al., 2000). Recently, Ang II was shown to

stimulate AT<sub>1</sub>-R phosphorylation in a PKC- and PI 3-kinase-dependent manner in C9 cells (Garcia-Caballero et al., 2001). In the present study, the mechanism of AT<sub>1</sub> receptor-mediated activation of ERK in C9 cells was found to be mediated by Src/Pyk-2 through EGF-R *trans*-activation, followed by recruitment of Shc and Grb2, in a PKC $\delta$ -dependent manner.

## Experimental Procedures

**Materials.** Ham's F-12 nutrient mixture, Kaighn's modification (F-12K), fetal bovine serum, antibiotic solutions, and EGF were from Invitrogen (Carlsbad, CA). PKC inhibitors, BAPTA-2 AM, PP2, AG 1478, AG 1296, AG 490, wortmannin, and antisera against specific  $\alpha$  subunits and phospho-Pyk2 (402) were purchased from Calbiochem (San Diego, CA). Ang II was from Peninsula Laboratories (Belmont, CA), and pertussis toxin was from LIST Biologicals (Campbell, CA). Anti-phospho-Akt (P-Ser472/473/474) and anti-Akt antibodies were from BD PharMingen (San Diego, CA), antibodies to EGF receptor and Src were from Santa Cruz Biotechnology (Santa Cruz, CA), anti-phospho-EGF receptor (Tyr1173) and anti-phospho-Src (Tyr416) antibodies were from Upstate Biotechnology (Lake Placid, NY). Phosphotyrosine (PY20) and Pyk-2 antibodies were from Transduction Labs (Lexington, KY). Anti-phospho-ERK1/2 (Thr202/Tyr204) and ERK1/2 antibodies were from New England Biolabs (Beverly, MA). Secondary antibodies conjugated to horseradish peroxidase were from Kirkegaard and Perry Laboratories (Gaithersburg, MD). Enhanced chemiluminescence reagents were from Amersham Biosciences (Piscataway, NJ). <sup>125</sup>I-[Sar<sup>1</sup>,Ile<sup>8</sup>]Ang II was from Covance Laboratories (Vienna, VA), and clone 9 rat liver cells were obtained from the American Type Culture Collection (Manassas, VA).

**Cell Culture.** Clone 9 (C 9) rat liver epithelial cells were grown in F-12K nutrient mixture (Kaighn's modification) supplemented with 10% (v/v) fetal calf serum, 100  $\mu$ g/ml streptomycin, 100 IU/ml penicillin, and 250  $\mu$ g/ml Fungizone. For all studies, C9 cells between passages 3 and 12 were used because these cells exhibit maximum expression of their endogenous AT<sub>1</sub> receptors. When [<sup>3</sup>H]inositol labeling was performed, cells were maintained in inositol-free medium for 16 h before experiments.

**Inositol Phosphate Measurements.** [<sup>3</sup>H]Inositol labeling and incubation of C9 cells were performed as described previously (Hunyady et al., 1991). After incubation with [<sup>3</sup>H]inositol in 0.5 ml of inositol-free Dulbecco's modified Eagle's medium for 24 h, cells were washed twice and then incubated for 30 min at 37°C in the same medium containing 10 mM LiCl. After stimulation with 100 nM Ang II for 20 min, reactions were stopped with perchloric acid, the inositol phosphates were extracted, and their radioactivity was measured by liquid scintillation- $\gamma$  spectrometry.

**Preparation of Membranes.** Membranes were prepared from the cells by homogenization with a Teflon-on-glass homogenizer and differential centrifugation as described previously (Shah and Milligan, 1994). Frozen cell pellets were suspended in 5 ml of 10 mM Tris/HCl, 0.1 mM EDTA, pH 7.5, buffer A, and the cells ruptured with 25 strokes of the homogenizer. The resulting suspension was centrifuged at 500g for 10 min in a L5-50B centrifuge (Beckman Coulter, Fullerton, CA) with a Ti 50 rotor to remove unbroken cells and nuclei. The supernatant was further centrifuged at 48,000g for 10 min. The pellet from the second centrifugation was washed with buffer A and recentrifuged at 48,000g for 10 min. The pellet was resuspended in buffer A to give a protein concentration of 1 to 3 mg/ml and stored at -80°C until required.

**Immunoprecipitation.** After treatment with inhibitors and drugs, cells were placed on ice, washed twice with ice-cold PBS, lysed in buffer containing 50 mM Tris, pH 8.0, 100 mM NaCl, 20 mM NaF, 10 mM Na pyrophosphate, 5 mM EDTA, 10  $\mu$ g/ml aprotinin, 10  $\mu$ g/ml leupeptin, 10  $\mu$ g/ml soybean trypsin inhibitor, 10  $\mu$ g/ml pepstatin, and 1 mM 4-(2-aminoethyl)benzenesulfonyl fluoride, and probe-sonicated (Sonifier Cell Disruptor; Branson Ultrasonics, Dan-

bury, CT). Solubilized lysates were clarified by centrifugation at 8000g for 10 min, precleared with agarose, and then incubated with antibodies and protein A or G agarose. The immunoprecipitates were collected, washed four times with lysis buffer, and mixed with Laemmli buffer. After heating at 95°C for 5 min, the samples were centrifuged briefly and the supernatants were analyzed by SDS-PAGE on 8 to 16% gradient gels.

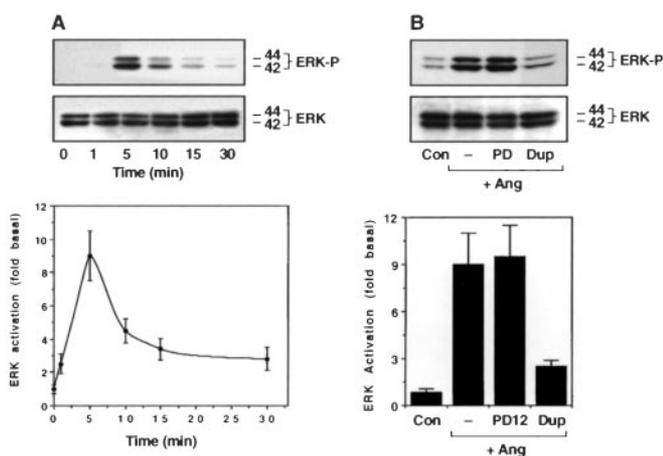
**Immunoblot Analysis of ERK1/2.** C9 cells were grown in six-well plates in F-12K nutrient mixture (Kaighn's modification) supplemented with 10% fetal calf serum. At 60 to 70% confluence, cells were serum-starved for 24 h before treatment with the indicated reagents. After treatment at 37°C, media were aspirated and cells were washed twice with ice-cold PBS, then lysed in 100 μl of Laemmli sample buffer. The samples were briefly sonicated, heated at 95°C for 5 min, and centrifuged for 5 min. The supernatants were electrophoresed on SDS-PAGE (8 to 16%) gradient gels and transferred to polyvinylidene difluoride nylon membranes. Immunoblotting was done using horseradish peroxidase-conjugated secondary antibody, followed by visualization with enhanced chemiluminescence reagent (Amersham Biosciences) and quantification with a scanning laser densitometer. In some cases, blots were stripped and reprobbed with other antibodies as described above.

### Results

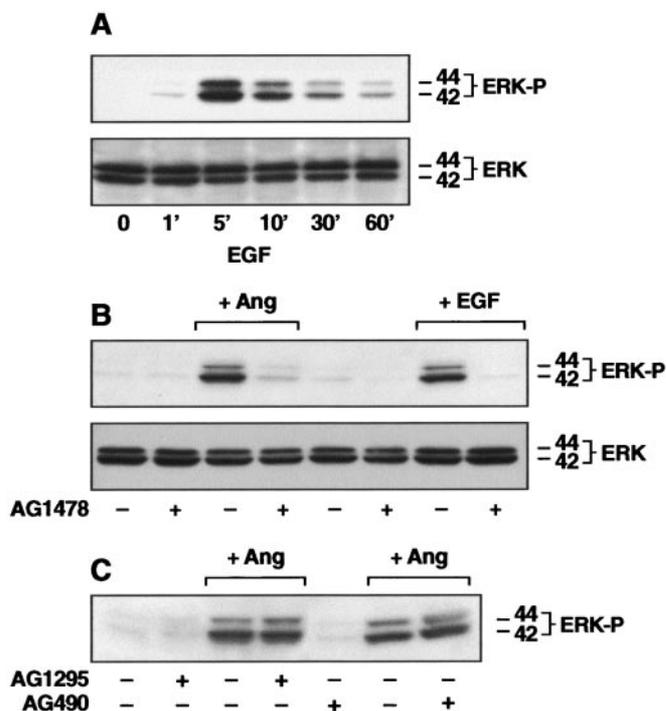
Stimulation of C9 cells with 100 nM Ang II caused a rapid and transient increase of ERK activity that reached a maximum at 5 min and then declined over the next 30 min (Fig. 1A). Agonist-induced ERK activation was almost abolished by the AT<sub>1</sub> receptor antagonist, losartan (DuP753), but was unaffected by the AT<sub>2</sub> receptor antagonist PD123177 (Fig. 1B). C9 cells also express receptors for tyrosine kinase-linked growth factors, and EGF stimulation (50 ng/ml) caused a marked increase in ERK phosphorylation to a peak at 5 min and a subsequent decline (Fig. 2A). Treatment with the EGF-R kinase inhibitor AG 1478 almost abolished ERK activation induced by both Ang II and EGF (Fig. 2B). However, there was no such effect of the PDGF-R kinase inhibitor AG 1295 or the Janus kinase inhibitor AG 490 (Fig. 2C). These

data illustrate the dominant contribution of EGF-R *trans*-activation to Ang II-mediated ERK phosphorylation in C9 cells.

Ang II causes a rapid increase in intracellular Ca<sup>2+</sup> in C9 cells (Garcia-Caballero et al., 2001), but Ca<sup>2+</sup>-chelators (BAPTA and EGTA) did not impair ERK activation by Ang II or EGF (Fig. 3A). The Ca<sup>2+</sup>-calmodulin kinase inhibitor KN93 also had no effect on ERK activation (Fig. 3B). These results clearly demonstrate that ERK activation by Ang II and EGF is not dependent on Ca<sup>2+</sup> signaling in C9 cells. Ang II and EGF activate PKCδ and -ε isoforms in rat liver epithelial WB cells (Maloney et al., 1999), and Ang II causes selective activation of PKCδ in C9 hepatic cells (Garcia-Caballero et al., 2001). Both depletion of PKC by preincubation of cells with PMA (2 μM for 20 h) and PKC blockade by Gö 6983 (which inhibits PKC α, β, γ, and δ) attenuated ERK activation by Ang II and PMA. In contrast, Ro-31-8220 (which inhibits PKC α, β, γ and ε) inhibited PMA- but not Ang II-induced ERK activation (Fig. 4, A–C). These data provide further evidence for a role of PKCδ in Ang II action in C9 cells. Consistent with this, PKCδ was translocated from cytosol to membranes by Ang II and PMA and was down-regulated by prolonged PMA treatment (Fig. 4, D–F). Furthermore, treatment with the selective PKCδ inhibitor rottlerin [see Watters and Parsons (1999) and references therein] completely abolished ERK activation by Ang II and PMA (Fig. 5, A and B). This action of rottlerin was specific for its effect on PKCδ, because it did not block the effect of anisomycin (Fig. 5C), which activates MAP kinase independently of PKC (Yu et al., 1996). These data indicate that Ang II-induced ERK activation in



**Fig. 1.** A, time course of Ang II-stimulated ERK1/2 activation in C9 cells. Cells were grown to 70% confluence and serum-starved for 20 to 24 h before treatment with 100 nM Ang II for the indicated times. The cells were then washed twice with ice-cold PBS and lysed in Laemmli sample buffer, sonicated, heated to 95°C for 5 min, and centrifuged before SDS-PAGE analysis on 8 to 16% gradient gels. B, blockade of Ang II-stimulated ERK activation by the AT<sub>1</sub>-R receptor antagonist, DuP753 (Dup, 10 μM), but not by the AT<sub>2</sub>-R antagonist, PD123177 (PD, 10 μM). Antagonists were added 20 min before the addition of Ang II (100 nM) for 5 min. The lower shows the total ERK1/2. Blots were stripped and reprobbed with ERK1/2 antibody



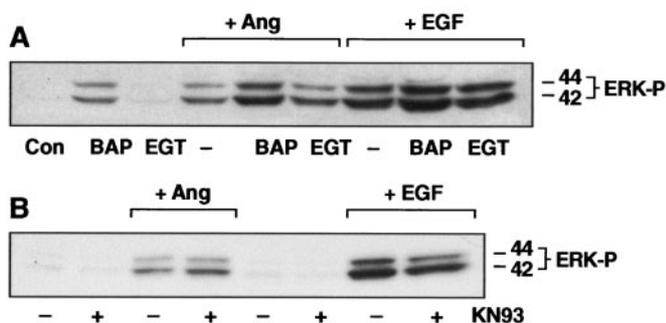
**Fig. 2.** A, time course of EGF-induced activation of ERK1/2 in C9 cells. B, effects of EGF receptor kinase inhibition by AG 1478 (100 nM) on ERK activation by Ang II and EGF. C, lack of effects of the PDGF-receptor kinase inhibitor AG 1295 (1 μM) and the Janus kinase inhibitor AG 490 (10 μM) on Ang II-induced ERK activation. Cells were treated with inhibitors for 20 min before stimulation with Ang II (100 nM) or EGF (50 ng/ml) for 5 min.

C9 cells is not Ca<sup>2+</sup>-dependent but is highly dependent on PKCδ.

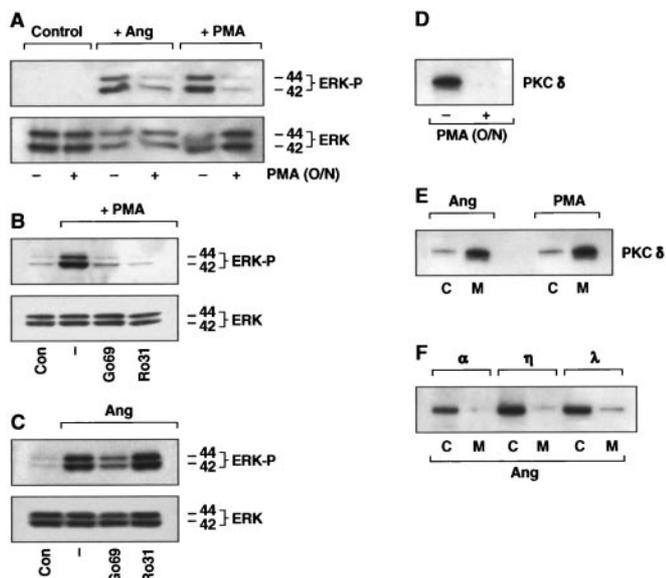
Coupling of the AT<sub>1</sub>-R to G<sub>i</sub> proteins has been reported in several rodent cell types, including rat hepatocytes and liver cell lines. In C9 cells, Ang II-induced AT<sub>1</sub>-R phosphorylation is partially pertussis toxin (PTX)-sensitive (Garcia-Caballero et al., 2001). In the present study, exposure of C9 cells to PTX (50 ng/ml for 16 h) caused a significant reduction in Ang II-stimulated ERK activation (Fig. 6A). Because agonist-induced receptor activation causes changes in membrane levels of the interacting G protein(s) (Shah and Milligan, 1994), we measured membrane-associated G<sub>i</sub> α-subunit levels in agonist-treated C9 cells. As shown in Fig. 6B, Ang II caused a significant loss of the Gα<sub>3</sub> subunit from the membranes within 5 min. There was no significant change in Gα<sub>1</sub> or

Gα<sub>2</sub> proteins (data not shown). These data are consistent with the observations of AT<sub>1</sub>-R coupling to both G<sub>q</sub> and G<sub>i</sub> proteins in hepatocytes (Pobiner et al., 1991; Tsygankova et al., 1998; Garcia-Caballero et al., 2001). To compare the effects of Ang II with those of activation via a typical G<sub>i</sub>-coupled receptor, we analyzed the effects mediated by endogenous LPA receptors, which are primarily coupled to G<sub>i</sub> (Della Rocca et al., 1997). Treatment of C9 cells with LPA (1 μM) caused marked but transient ERK activation that peaked at 5 min and rapidly declined to basal (Fig. 6C). As expected, PTX inhibited LPA-induced ERK activation, consistent with the predominant signaling of LPA through G<sub>i</sub>-linked pathways (Fig. 6D). The LPA-induced activation of ERK was also significantly reduced by rottlerin and AG 1478 but was not affected by Ca<sup>2+</sup> chelation with BAPTA-2 AM (Figs. 6, E and F), again indicating a role of PKCδ and EGF-R activation, but not [Ca<sup>2+</sup>]<sub>i</sub>, in GPCR-induced ERK activation in C9 cells

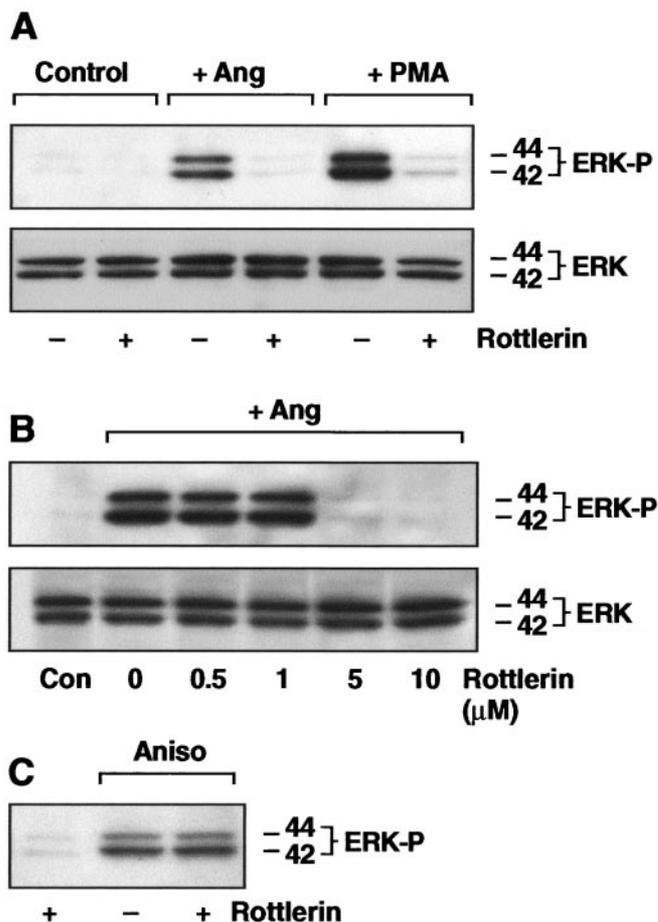
Stimulation of G<sub>q</sub> and G<sub>i</sub>-linked GPCRs has been shown to activate the MAP kinase pathway through multiple mechanisms, including the interaction of G<sub>i</sub> βγ-subunits with PLC-β, phosphatidylinositol 3-kinase (PI 3-kinase) (Hawes et



**Fig. 3.** A, effects of Ca<sup>2+</sup> chelation on ERK activation by Ang II and EGF. Cells were pretreated with BAPTA-AM (10 μM) or EGTA (0.5 mM) for 15 min before stimulation with Ang II (100 nM) or EGF (50 ng/ml) for 5 min. B, lack of effect of the Ca<sup>2+</sup>-calmodulin kinase inhibitor KN-93 (1 μM) on ERK activation induced by Ang II and EGF.



**Fig. 4.** A, effect of PKC depletion by PMA pretreatment (2 μM for 16 h, overnight) on ERK activation by Ang II and PMA. B and C, effects of PKC inhibitors Gö 6983 (Go69, 1 μM) and Ro-31-8220 (Ro31, 1 μM) on ERK activation by PMA (B) and Ang II (C). Cells were pretreated with inhibitors for 20 min followed by stimulation with PMA (100 nM) or Ang II (100 nM) for 5 min. D, down-regulation of PKCδ by PMA treatment. Cells were treated with PMA (2 μM) for 16 h (overnight) and cell lysates were analyzed and immunoblotted for PKCδ. E, translocation of PKCδ by Ang II and PMA. C9 cells were treated with Ang II and PMA (100 nM) for 5 min, cytosol (C) and membranes (M) were collected, analyzed by SDS-PAGE, and immunoblotted with PKCδ antibody. F, lack of effect of Ang II on translocation of PKC isoforms α, η, and λ from cytosol to membranes.



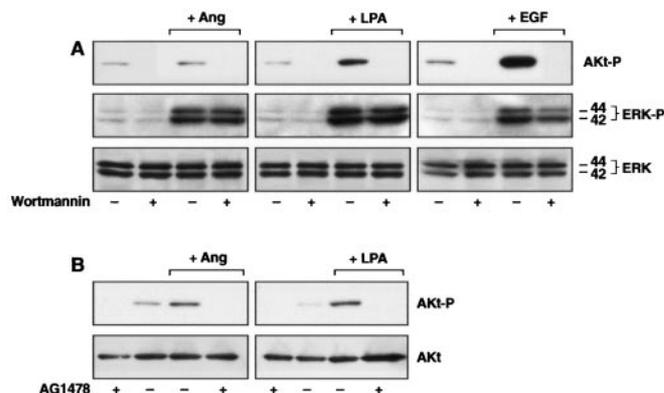
**Fig. 5.** A, effect of PKCδ inhibitor rottlerin (10 μM) on ERK activation by Ang II or PMA. B, dose-dependent inhibition of Ang II-induced ERK activation by rottlerin. C, lack of effect of rottlerin on ERK activation by anisomycin (which causes PKC-independent ERK activation). Serum-starved C9 cells were incubated with inhibitors for 20 min and then treated with Ang II (100 nM), PMA (100 nM), or anisomycin (10 μM) for 5 min. After washing twice with ice-cold PBS, cells were collected in Laemmli sample buffer, analyzed, and immunoblotted for phosphorylated ERK1/2.

al., 1996), and Src (Luttrell et al., 1997). Recently, Ang II was found to stimulate PKB/Akt phosphorylation as well as wortmannin-sensitive AT<sub>1</sub>-R phosphorylation in C9 cells, suggesting the involvement of PI 3-kinase in these responses (Garcia-Caballero et al., 2001). An analysis of the role of lipid kinases in agonist-stimulated ERK activation revealed that whereas wortmannin blocked the phosphorylation of PKB/Akt by Ang II, LPA, and EGF, it did not cause a corresponding decrease in agonist-induced ERK activation (Fig. 7A). Moreover, AG 1478 completely inhibited phosphorylation of PKB/Akt by both Ang II and LPA (Fig. 7B). These results indicate that GPCR-mediated activation of PI 3-kinase-linked PKB/Akt in C9 cells occurs through EGF-R activation but has no role in ERK activation by GPCRs.

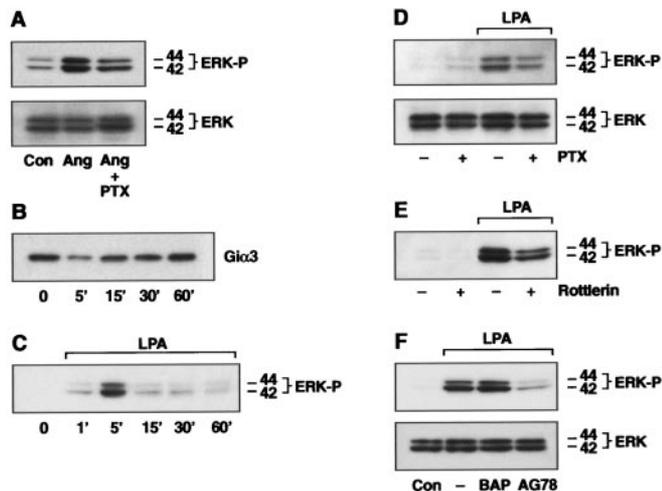
From the above data, it is clear that GPCR-mediated ERK activation in C9 cells is largely attributable to EGF-R trans-activation. The roles of Ca<sup>2+</sup> and PKC in this process were determined by analyzing tyrosine phosphorylation of the EGF-R by immunoprecipitation with a specific antibody and immunoblotting with phosphotyrosine antibody (PY20) and also by direct immunoblotting with a site-specific (Tyr1173) EGF-R phosphotyrosine antibody. This revealed that both Ang II and PMA, but not ionomycin, stimulated tyrosine phosphorylation of the EGF-R that was relatively minor compared with that elicited by EGF (Fig. 8A). EGF-induced tyrosine phosphorylation of EGF-R was abolished by AG 1478 but not by BAPTA or PP2 (Fig. 8B). Pretreatment of cells with rottlerin reduced the tyrosine phosphorylation of EGF-R induced by Ang II (Fig. 8C), but Ca<sup>2+</sup>-chelation by BAPTA had no such effect (data not shown).

To identify the intermediate signaling molecules linking PKCδ to EGF-R activation, we examined the roles of Src kinase and Pyk2 in this cascade. Time course studies revealed that Ang II caused rapid phosphorylation of c-Src that

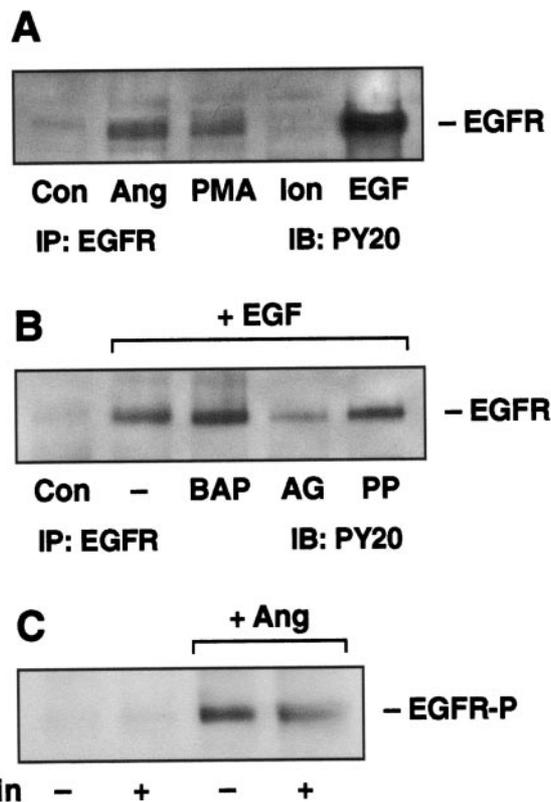
was detectable as early as 30 s (Fig. 9A). Consistent with this, the Src kinase inhibitor PP2 reduced ERK activation by Ang II (Fig. 9B). As expected, Ang II-induced ERK phosphorylation was abolished by the MEK inhibitor PD 98059. Moreover, c-Src activation by Ang II was markedly inhibited by



**Fig. 7.** A, effects of the PI 3-kinase kinase inhibitor, wortmannin, on phosphorylation of Akt/PKB and ERK induced by Ang II (100 nM), LPA (1 μM), and EGF (50 ng/ml). Wortmannin (100 nM) was added 20 min before agonist treatments for 5 min. B, inhibitory effects of AG 1478 (100 nM) on phosphorylation of PKB/Akt induced by Ang II and LPA. The lower shows total Akt protein detected by stripping and reprobing the blots with Akt antibody.



**Fig. 6.** A, effect of pertussis toxin (PTX, 50 ng/ml for 24 h) on ERK activation by Ang II (100 nM for 5 min). B, time course of changes in membrane G<sub>α3</sub> levels during Ang II stimulation. Cells were treated with Ang II (10 nM) for the times indicated, and membranes were solubilized, analyzed by SDS-PAGE, and immunoblotted with Gα3-subunit antisera. C, time course of effects of LPA (1 μM) on ERK activation. D, treatment with pertussis-toxin (50 ng/ml for 16 h) inhibits ERK activation by LPA (1 μM for 5 min). E, effect of the PKCδ inhibitor rottlerin (10 μM) on ERK activation by LPA. F, effects of BAPTA (BAP; 10 μM) and the EGF-R kinase inhibitor AG 1478 (AG78; 100 nM) on ERK activation by LPA (1 μM). Cells were treated with inhibitors for 20 min before stimulation with Ang II or LPA.



**Fig. 8.** A, agonist-induced phosphorylation of the EGF-R. After treatment with Ang II (200 nM, 1 min), ionomycin (Ion; 10 μM), EGF (10 ng/ml for 1 min) or PMA (100 nM for 2 min), serum-starved cells were collected in radioimmunoprecipitation assay lysis buffer followed by immunoprecipitation with EGF-R antibody, SDS-PAGE analysis, and immunoblotting with anti-phosphotyrosine antibody (PY20). B, effects of BAPTA (BAP; 10 μM), AG 1478 (AG; 100 nM) and the Src kinase inhibitor PP2 (100 nM) on EGF-R phosphorylation. C, effect of rottlerin (10 μM) on EGF-R phosphorylation induced by Ang II (200 nM for 2 min). Cells were treated with inhibitors for 20 min before agonist stimulation.

rottlerin but not by BAPTA and AG 1478 (Fig. 9C). PKC depletion also caused a reduction in Src activation in response to stimulation by Ang II and PMA (Fig. 9D). These data suggest the involvement of c-Src in a PKC-dependent manner during agonist-stimulated ERK activation and also show that c-Src must act upstream of the EGF-R in C9 cells.

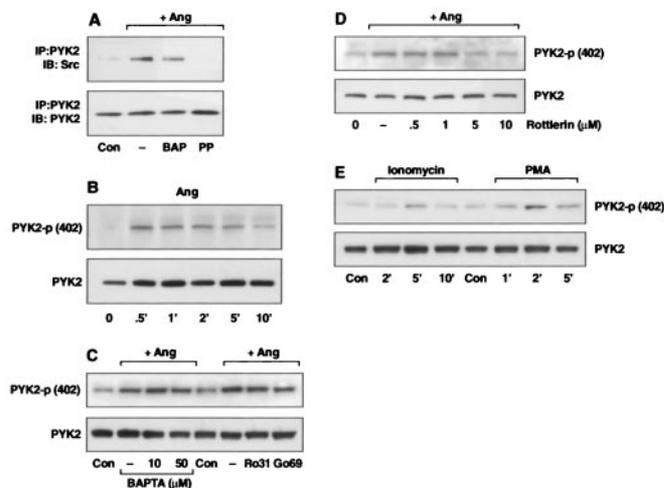
Src activation by GPCRs often leads to phosphorylation of the proline-rich tyrosine kinase, Pyk2 (Dikic et al., 1996). In C9 cells, Ang II stimulation enhanced the association of Src with Pyk2, as determined by immunoprecipitation of Pyk2 and immunoblotting with Src antibody (Fig. 10A). Moreover, Ang II caused rapid phosphorylation of Pyk2 as measured by phosphorylation of the tyrosine residue 402, which is the target site of activation by Src (Figs. 10B). Pyk2 activation was not affected by BAPTA-2 AM (10 or 50  $\mu$ M) but was inhibited by rottlerin (Fig. 10, C and D). Consistent with this finding, PMA caused greater stimulation of Pyk2 than the  $Ca^{2+}$ -ionophore ionomycin (Fig. 10E), suggesting that Pyk2 is the potential target of PKC stimulation during GPCR-induced ERK activation in C9 cells.

Because ERK activation by the  $AT_1$ -R and other GPCRs involves *trans*-activation and phosphorylation of the EGF-R, we determined whether the Src/Pyk2 complex interacts with the latter receptor. Stimulation of C9 cells by Ang II, but not EGF, increased the association of Pyk2 with the EGF-R (Fig. 11A). Activation of the EGF-R is known to cause ERK phosphorylation through recruitment of adaptor proteins such as Shc, Grb2, and Sos (see Hackel et al., 1999). Like several other GPCRs,  $AT_1$ -R activation increased the association of Grb2 with the EGF-R (Fig. 11B). These findings indicate that Ang II-induced ERK activation in C9 cells involves Src/Pyk2 and EGF-R activation, followed by recruitment of adaptor proteins to form the Shc/Grb2/Sos complex.

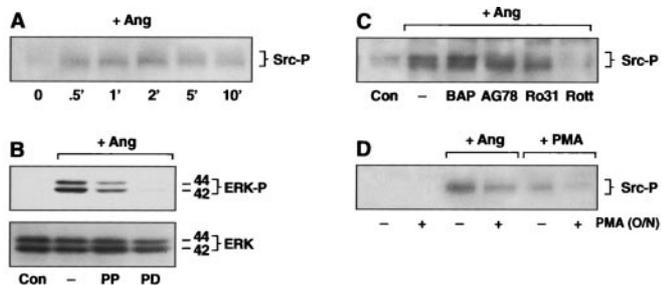
### Discussion

ERKs 1 and 2 are 44- and 42-kDa members of the MAP kinase family and are involved in the regulation of gene expression, protein synthesis, cell growth and proliferation, and, in some cases, cell differentiation and secretion (Gutkind, 1998). ERK phosphorylation was initially observed after ligand activation of such RTKs as the EGF receptor, but many  $G_q$ -,  $G_i$ -, and  $G_s$ -coupled receptors also initiate the ERK cascade through *trans*-activation of the EGF-R (Gutkind,

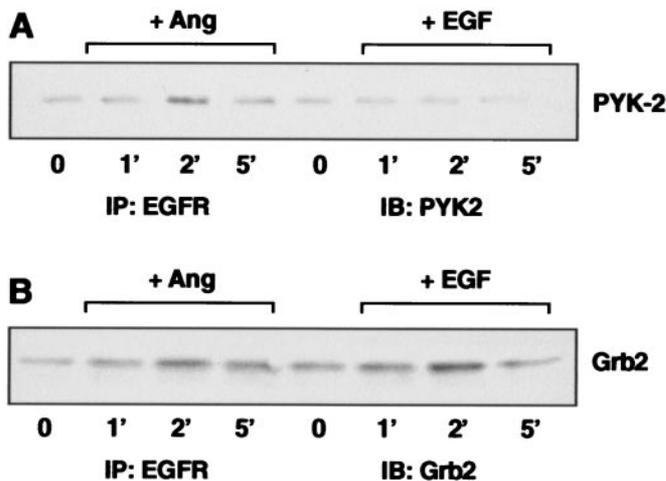
1998; Hackel et al., 1999; Luttrell et al., 1999). This mode of ERK activation often involves sequential steps that include *trans*-activation of the RTKs (EGF-R) in a Src kinase and Pyk-2 dependent manner (Daub et al., 1996; Luttrell et al., 1997; Eguchi et al., 1999; Tang et al., 2000). However, Pyk2 and EGF-R can also independently cause ERK activation in PC12 cells (Zwick et al., 1999). The  $AT_1$ -R is coupled to both  $G_q$  and  $G_i$  proteins in C9 cells, in which we found  $Ca^{2+}$  to have little or no direct role in the activation of Pyk2 or ERK. However, PKC $\delta$  proved to be a critical effector in Ang II-



**Fig. 10.** A, Ang II-induced association of Src with Pyk2. Serum-starved C9 cells were treated with Ang II (100 nM for 1 min), washed with ice-cold PBS, and lysed in radioimmunoprecipitation assay lysis buffer containing protease inhibitors. Cell lysates were immunoprecipitated with Pyk-2 antibody before SDS-PAGE analysis and immunoblotted with anti-Src or anti-Pyk2 antibodies. The blots were stripped and reprobed with Pyk-2 antibody to detect total Pyk2 protein (bottom). B, time course of Ang II-induced stimulation of Pyk-2 phosphorylation detected by a Tyr402-specific phosphotyrosine antibody. C, effects of BAPTA, Ro-31-8220 (Ro31; 1  $\mu$ M), and Gö 6983 (Go69, 1  $\mu$ M) on Pyk2 (Tyr402) phosphorylation by Ang II. D, concentration-dependent inhibition of Ang II-stimulated Pyk2 (Tyr402) phosphorylation by rottlerin. E, effects of PMA (100 nM) and ionomycin (10  $\mu$ M) on Pyk2 (Tyr 402) phosphorylation. Agents and inhibitors were added 20 min before stimulation with 100 nM Ang II for 1 min. Bottom, total Pyk2 protein in blots that were stripped and reprobed with Pyk2 antibody.



**Fig. 9.** A, time course of Ang II-induced Src phosphorylation by Ang II (100 nM). B, effects of the Src kinase inhibitor, PP2 (100 nM) and the MEK inhibitor, PD98059 (PD, 10  $\mu$ M) on ERK1/2 activation by Ang II. Serum-starved C9 cells were incubated with inhibitors for 20 min and then stimulated with Ang II (100 nM). C, effects of BAPTA (BAP; 10  $\mu$ M), AG 1478 (AG78; 100 nM), Ro-31-8220 (Ro31; 1  $\mu$ M), and rottlerin (Rott; 10  $\mu$ M) on Src activation by Ang II. D, effects of PKC depletion by PMA pretreatment (2  $\mu$ M for 16 h) on stimulation of Src phosphorylation by Ang II (100 nM; 1 min) and PMA (100 nM for 3 min).



**Fig. 11.** A, time course of the association of Grb2 and Pyk2 with the EGF-R during treatment of C9 cells with Ang II (100 nM) or EGF (50 ng/ml) for the times indicated. Cell lysates were sonicated and analyzed by 10% SDS-PAGE after immunoprecipitation with EGF-R antibody, followed by immunoblotting with Grb2 or Pyk2 antibodies.

mediated ERK phosphorylation through activation of Src and Pyk2, and subsequent *trans*-activation of the EGF-R. Consistent with this, PMA-induced phosphorylation of Pyk2 and ERK was much greater than that elicited by the Ca<sup>2+</sup> ionophore ionomycin.

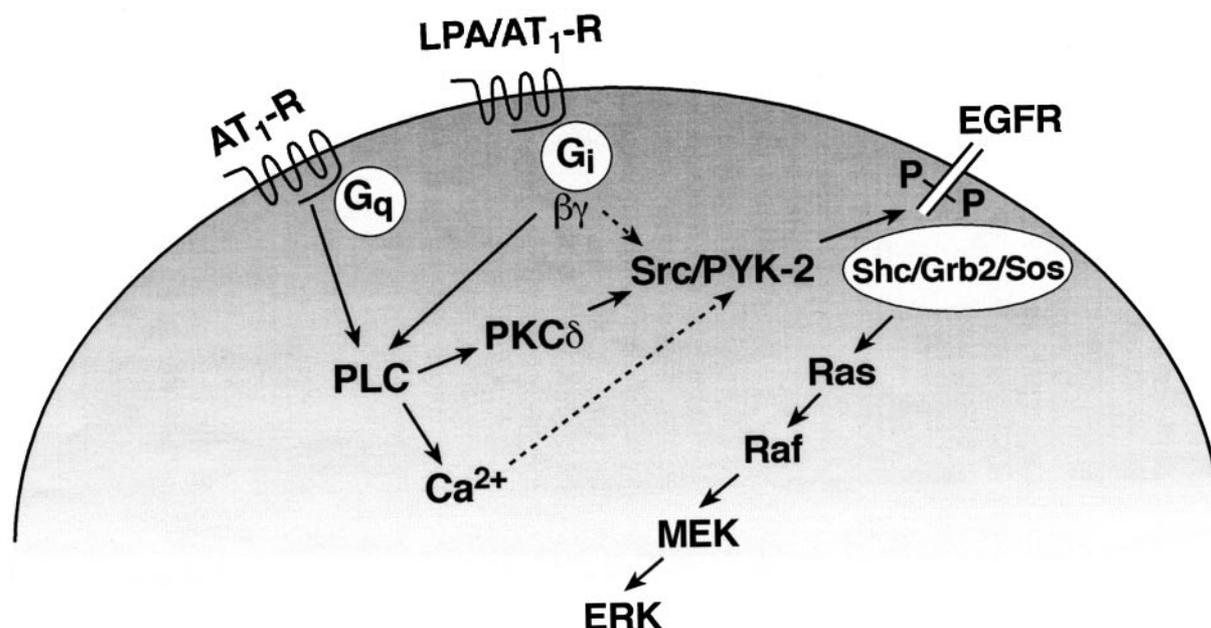
Although there is no binding site for Ca<sup>2+</sup> on Pyk2 (Dikic et al., 1996; Li et al., 1998), there is substantial evidence for a role for Ca<sup>2+</sup> in GPCR-mediated phosphorylation of Pyk2, EGF-R, and ERK in several cell types. These include  $\beta_2$ -adrenergic receptors in HEK-293 cells (Della Rocca et al., 1997), muscarinic receptors in intestinal epithelial cells (Keely et al., 2000), and AT<sub>1</sub> receptors in VSMCs (Sabri et al., 1998; Eguchi et al., 1999) and cardiac fibroblasts (Murasawa et al., 1998). In contrast, the present findings in C9 cells showed that Ca<sup>2+</sup> chelation by BAPTA-2 AM and EGTA had no inhibitory effect on Pyk2 or EGF-R activation but instead increased the ERK activation induced by Ang II and EGF (Fig. 3). However, Ang II- and LPA-induced Pyk2 and ERK activation were consistently inhibited by the selective PKC $\delta$  inhibitor, rottlerin, and by PKC depletion (Figs. 4–6). The lack of involvement of Ca<sup>2+</sup> in mitogenic responses to Ang II and LPA may be a characteristic of hepatocytes, because similar observations have been reported in WB and GN4 hepatic cell lines (Li et al., 1998; Maloney et al., 1999; Yang et al., 1999). Interestingly, Ca<sup>2+</sup>-chelation by BAPTA inhibits cellular phosphatases and augments the Ang II-mediated activation of Src kinase (Fyn), Raf-1, and ERK in rat liver WB cells (Maloney et al., 1999). Thus, signaling through PKC seems to be a predominant pathway used by Ang II in hepatic cells.

Ang II is known to activate PKC $\delta$  and - $\epsilon$  in rat liver WB cells (Maloney et al., 1999), PKC $\alpha$ , - $\beta_2$ , - $\epsilon$ , - $\gamma$ , and - $\zeta$  in heart cells (Takeishi et al., 1999), PKC $\zeta$  in VSMCs (Liao et al., 1997), and PKC $\delta$  in C9 cells (Garcia-Caballero et al., 2001). Consistent with the latter finding, our results show that Ang II-, LPA- and PMA-induced phosphorylation of Src, Pyk2, and ERK, respectively, was inhibited by rottlerin, a selective

inhibitor of PKC $\delta$  (Watters and Parsons, 1999), and by PKC depletion (Figs. 4, 9, and 10). PKC-dependent activation of Pyk2 and/or ERK has been demonstrated in several other cell types, including Chinese hamster ovary cells (Arai and Escobedo, 1996), adrenal glomerulosa cells (Tian et al., 1998), VSMCs (Sabri et al., 1998), platelets (Ohmori et al., 1999), and COS-7 and HEK-293 cells (Prenzel et al., 1999).

There is now abundant evidence for the frequent involvement of EGF-R *trans*-activation in GPCR-mediated ERK activation (Daub et al., 1997; Hackel et al., 1999; Luttrell et al., 1999; Saito and Berk, 2001). Whereas Ang II-induced EGF-R phosphorylation is dependent on Ca<sup>2+</sup> in VSMCs (Murasawa et al., 1998; Eguchi et al., 1999), it is Ca<sup>2+</sup>-independent in cardiac fibroblasts (Wang et al., 2000). Moreover, PKC-dependent phosphorylation of the EGF-R has been reported for the muscarinic acetylcholine receptor in HEK-293 cells (Tsai et al., 1997), thyrotropin-releasing hormone in GH3 cells (Wang et al., 2000), muscarinic acetylcholine receptor in Rat-1 cells, and thrombin and PMA in HEK-293 cells (Prenzel et al., 1999). We observed that both Ang II and PMA stimulated EGF-R phosphorylation in C9 cells, albeit of lesser magnitude than that evoked by EGF. Both rottlerin treatment and PKC depletion inhibited Ang II-induced Pyk2 and ERK responses (Figs. 4 and 10). This indicates that PKC plays a major role in transducing Ang II signals to ERK activation, and acts upstream of EGF-R *trans*-activation.

In contrast to this, Li et al. (1998) found that Ang II caused ERK activation by an EGF-R- and Ras-independent but PKC-dependent pathway in rat liver epithelial (GN4) cells, but by an EGF-R- and Ras-dependent pathway in PKC-depleted GN4 cells. The negative regulatory effect of PKC on EGF-R and ERK pathway was attributed to an as-yet-undefined PKC isoform present only in GN4 cells, but the possibility of cell-type specific effects was also raised. This discrepancy could be related to the higher concentrations of PMA (5  $\mu$ M), Ang II (1  $\mu$ M) and PKC inhibitor, GF-109203X (3–10  $\mu$ M) used in that study. Moreover, GN4 cells were



**Fig. 12.** Schematic representation of the signaling pathways involved in Ang II action in C9 cells. Ang II stimulates PKC $\delta$  and Src/Pyk2 association through activation of G<sub>q</sub> and/or G<sub>i</sub> proteins. The Src/Pyk2 complex associates with and activates the EGF-R, leading to recruitment of adaptor proteins including Shc/Grb2/Sos and subsequent activation of the ERK phosphorylation cascade.

derived by chemical transformation of a normal rat liver epithelial cell line WB (see Yu et al., 1996), and Ang II caused 5-fold higher activation of both Pyk2 and c-Jun NH<sub>2</sub>-terminal kinase in these cells compared with parent WB cells (Yu et al., 1996). We used C9 hepatocytes that have lower AT<sub>1</sub>-R density (Kozlowski et al., 1993) and exhibit signaling characteristics closer to those of WB cells (Tsygankova et al., 1998; Maloney et al., 1999; Yang et al., 1999; Melien et al., 2000; Garcia-Caballero et al., 2001). Therefore, AT<sub>1</sub>-R density in specific cell types, and variations in the signaling characteristics caused by variable amounts of signaling proteins, such as Pyk2, may contribute to the differential responses in these hepatic cell lines.

Although earlier studies suggested that Pyk2 had no link with MAP kinase activation (Yu et al., 1996), ERK activation by G<sub>q/11</sub>- and G<sub>i</sub>-coupled receptors, including the AT<sub>1</sub>-R, was shown to be dependent on Pyk2 activation (Eguchi et al., 1999; Avraham et al., 2000; Rocic and Lucchesi, 2001). The overexpression of a phosphorylation-deficient Pyk2 mutant attenuated agonist-stimulated ERK activation (Dikic et al., 1996; Murasawa et al., 1998; Eguchi et al., 1999), and an antisense Pyk2 oligonucleotide abolished the activation of Pyk2 and ERK in response to Ang II stimulation (Rocic and Lucchesi, 2001). According to the cell type, Pyk2 activation may be dependent on both Ca<sup>2+</sup> (Dikic et al., 1996; Eguchi et al., 1999; Keely et al., 2000) and PKC (Sabri et al., 1998; Avraham et al., 2000). However, our data clearly indicate the dependence on PKC $\delta$ , but not Ca<sup>2+</sup>, of Ang II-induced Pyk2 activation, because the latter was prevented by rottlerin and PKC inhibition. Also, PMA caused much greater Pyk2 activation than ionomycin, as shown in Fig. 10.

The ability of the Src kinase inhibitor PP2, but not AG 1478, to inhibit Src and Pyk2 activation indicates that Ang II-induced Src/Pyk-2 activation precedes EGF-R *trans*-activation. Furthermore, Ang II increased the association of Pyk2 and Grb2 with the EGF-R (Fig. 11). In these regards, the AT<sub>1</sub>-R seems to follow the same pattern as GPCRs for LPA, bradykinin, and endothelin (Dikic et al., 1996), which promote the association of Src with Pyk2 and its phosphorylation at Tyr402 by Src kinase. Subsequently, the Src/Pyk2 complex activates the EGF-R (Andreev et al., 2001), leading to tyrosine phosphorylation of Shc and Grb2 adaptor proteins that recruit guanine nucleotide exchange factors for Ras. Activated Ras subsequently engages the ERK-MAPK cascade involving Raf, MEK, and finally ERKs (Dikic et al., 1996; Luttrell et al., 1997; Hackel et al., 1999). A diagram to indicate the signaling pathways activated by Ang II in C9 cells is shown in Fig. 12.

Recent studies in cultured fibroblasts derived from Src- and Pyk2-deficient mouse embryos have shown that both Src and Pyk2 are essential for GPCR-mediated activation of MAP kinase (Andreev et al., 2001). Furthermore, studies in fibroblasts from EGF-R-deficient mice revealed that GPCR-induced MAP kinase activation is not dependent on EGF-R phosphorylation. Our findings in C9 cells are consistent with the necessity of Src kinase and Pyk2 in *trans*-activation of the EGF-R but differ in that the latter is the predominant route of signaling to ERK activation. This implies that alternative pathways present in embryonic fibroblasts do not operate in C9 cells, rendering them dependent on *trans*-activation of the EGF-R for signaling via Grb2 and Ras to the MAP kinase pathway.

In C9 cells, Ang II-induced phosphorylation of the AT<sub>1</sub> receptor, but not inositol phosphate production and elevation of [Ca<sup>2+</sup>]<sub>i</sub>, is partially inhibited by PTX (Garcia-Caballero et al., 2001). Similar studies point to a significant role of G<sub>i</sub> proteins in Ang II-mediated ERK activation in liver epithelial WB cells (Tsygankova et al., 1998; Melien et al., 2000; Garcia-Caballero et al., 2001). The likely targets of G<sub>i</sub>  $\beta\gamma$ -subunits in agonist-stimulated ERK activation are PI-3 kinase and PLC (Hawes et al., 1996), as well as Src (Luttrell et al., 1997). Our studies indicate that the involvement of PI 3-kinase in ERK activation by Ang II and LPA can be excluded because wortmannin, which inhibits phosphorylation of Akt/PKB, had no effect on Pyk2 and ERK activation (Figs. 7). In C9 cells, it seems that PI 3-kinase affects upstream targets, such as AT<sub>1</sub>-R phosphorylation, but not the downstream events of GPCR-induced ERK activation. In this hepatic cell line, Ang II-induced ERK activation is dependent on both G<sub>q</sub> and G<sub>i</sub> proteins and is predominantly mediated by PKC $\delta$ -dependent activation of Src and Pyk2 followed by *trans*-activation of the EGF receptor.

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