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**The Protein Kinase C Inhibitor, Go6976, Potentiates Agonist-induced MAP
Kinase Activation through Tyrosine phosphorylation of the Epidermal
Growth Factor Receptor**

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Running title: Go6976 stimulates agonist-induced ERK1/2 activation

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Abbreviations: AT₁R, angiotensin type 1 receptor; C9, clone 9 rat liver cells; EGF, epidermal growth factor; ERK1/2, extracellularly regulated kinases 1 and 2; AG1478, 4-(3-chloroanilino)6,7-dimethoxyquinazoline; Go 6983, 2-[1-(3-Dimethylaminopropyl)-5-methoxyindol-3-yl]-3-(1H-indol-3-yl)maleimide; GPCR, G protein-coupled receptor; Go 6976, 12-(2-Cyanoethyl)-6,7,12,13-tetrahydro-13-methyl-5-oxo-5H-indolo(2,3-a)pyrrolo(3,4-c)-carbazole; HEK, human embryonic kidney cells; LPA, lysophosphatidic acid; InsP3, inositol 1,4,5-triphosphates; MAP kinase, mitogen-activated protein kinase; MKP-1, MAP kinase phosphatase-1; PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate; PTP, protein tyrosine phosphatase; Ro-31-8220, 3-[1-[3-(Amidindothio)propyl-1H-indol-3-yl]-3-(1-methyl-1H-indol-3-yl)maleimide; U0126, 1,4-Diamino-2,3-dicyano-1,2-bis(2-aminophenylthio)butadiene; STP, serine-threonine phosphatase; VSMC, vascular smooth muscle cell.

ABSTRACT

Protein kinase C isoforms are important transducers of signals from G protein-coupled receptors (GPCRs) to diverse cellular targets, including extracellularly signal regulated kinases 1 and 2 (ERK1/2). Clone 9 rat hepatocytes (C9 cells) express receptors for angiotensin II (Ang II) type 1 (AT₁-R), lysophosphatidic acid (LPA) and epidermal growth factor (EGF), and their stimulation causes transient ERK1/2 phosphorylation through transactivation of the EGF-R. Inhibition of PKC by Go6983, or PKC depletion by prolonged PMA treatment, attenuated ERK1/2 activation by Ang II and PMA, but not by LPA and EGF. In contrast, another PKC inhibitor, Go6976, enhanced basal and agonist-stimulated phosphorylation of ERK1/2, which was not due to alteration in receptor binding and internalization, stimulation of inositol phosphate production, or activation of Pyk2 and Src tyrosine kinases. However, Go6976 enhanced agonist-induced tyrosine phosphorylation of the EGF-receptor, possibly through inhibition of protein tyrosine phosphatase (PTP), since the PTP inhibitor, sodium orthovanadate, mimicked the effects of Go6976. Selective blockade of EGF-R kinase by AG1478 abolished the ERK1/2 activation induced by Go6976. Similar experiments were conducted in HEK293 cells, which express receptors for LPA and EGF but exhibit no significant cross-communication between them. While Go6976 caused a significant increase in EGF-induced tyrosine phosphorylation of the EGF-R and subsequent ERK1/2 activation, it had no such effects on LPA-induced responses. In CHO cells, which express receptors for LPA but not for EGF, Go6976 also had no significant effect on LPA-induced ERK1/2 activation. These data indicate that Go6976 potentiates agonist-induced ERK1/2 activation through stimulation of tyrosine phosphorylation of the EGF-R.

INTRODUCTION

G protein-coupled receptor (GPCR) signaling is a primary mechanism by which cells transduce changes from the external environment to their interior. GPCRs mediate responses to numerous ligands by binding and activating intracellular heterotrimeric G proteins. Stimulation of the pertussis-toxin insensitive G proteins, $G_{q/11}$, triggers the hydrolysis of membrane inositol phospholipids by phospholipase- $C\beta$ to form two important second messengers, inositol 1,4,5-triphosphate ($InsP_3$) and diacylglycerol (DAG). The binding of $InsP_3$ to its intracellular receptors causes a rise in intracellular Ca^{2+} , and DAG activates protein kinase C (PKC). These second messengers initiate numerous cellular responses, including activation of extracellularly signal regulated protein kinases 1 and 2 (ERK1/2) and regulation of gene expression. Decreases or increases in intracellular cAMP through activation of G_i - or G_s -linked GPCRs are also associated with ERK1/2 activation in several cell types. ERK1/2 are known to be involved in the regulation of several major cellular functions, including survival, growth, secretion, chemotaxis and motility (Luttrell, 2002; Pierce et al., 2002; Shah and Catt, 2002).

Recent studies have shown that GPCR-mediated ERK1/2 activation often occurs through transactivation of receptor tyrosine kinases (RTKs) such as epidermal growth factor receptor (EGF-R) (Fischer et al., 2003; Prenzel et al., 1999). In many cell types, GPCR stimulation leads to ectodomain shedding of heparin-binding EGF (HB-EGF) through activation of matrix metalloproteinases (MMP) or a disintegrin and metalloproteinase (ADAM). The HB-EGF released following GPCR stimulation binds to and activates the EGF-R, which leads to recruitment of specific signaling proteins and adaptor molecules, followed by sequential activation of the Ras/Raf/MEK/ERK cascade (Fischer et al., 2003; Shah and Catt, 2003). In

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many cells, the cross-communication between GPCRs and RTKs is primarily dependent on activation of PKC (Prenzel et al., 1999; Shah et al., 2003; Wetzker and Bohmer, 2003). In some instances, PKC signaling bypasses the EGF-R and causes activation of ERK1/2 through phosphorylation of Raf (Farshori et al., 2003; Kolch et al., 1993). Evidence for the involvement of PKC in GPCR-mediated ERK1/2 activation has been based mainly on the use of PKC inhibitors and the agonist-induced translocation of specific PKC isoforms from the cytosol to the cell membrane, and less often on the use of dominant negative PKC isoforms.

Although recently developed PKC inhibitors have been extensively used in cell signaling studies, some of these compounds exert actions that are unrelated to and independent of PKC inhibition (Davies et al., 2000). For example, the potent PKC inhibitor, Ro31-8220, inhibits MAP kinase phosphatase-1 (MKP-1) and thus causes activation of MAP kinase and Jun kinase in a PKC-independent manner in rat-1 fibroblasts (Beltman et al., 1996). This effect of Ro31-8220 is similar to that of the phosphatase inhibitor, pervanadate (Zhao et al., 1996), and resembles some of the effects of insulin in activating c-Jun N-terminal kinase and glycogen synthase in rat adipocytes and L6 myotubes (Standaert et al., 1999). These studies indicate the need for caution when using PKC inhibitors, and for the confirmation of PKC-dependent signaling mechanisms through multiple approaches. Here we report that Go6976, which preferentially inhibits conventional PKC α (Davies et al., 2000; Greco et al., 2004; Martiny-Baron et al., 1993), also causes sustained potentiation of ERK1/2 activation by Ang II, LPA, EGF and PMA through stimulation of tyrosine phosphorylation of the EGF-R.

Materials and Methods

Materials. F-12K nutrient mixture (Kaighn's modification), DMEM, fetal bovine serum, antibiotic solutions, and EGF were from Invitrogen. PKC inhibitors, LPA, PMA, U0126, sodium orthovanadate and AG1478 were purchased from Calbiochem. Angiotensin II was from Peninsula Laboratories and losartan (DuP 753) and PD123177 were generous gifts from DuPont (Wilmington, DE). Pertussis toxin was from LIST Biologicals. Antibodies against the EGF receptor, Src and phospho-tyrosine EGF-R, PY20 phosphotyrosine and Pyk2 were from Transduction Labs or Santa Cruz Biotechnology Inc. Anti-phospho-ERK1/2 and ERK1/2 antibodies were from Cell Signaling and secondary antibodies conjugated to horse-radish peroxidase were from Kirkegaard and Perry Laboratories (Gaithersburg, MD). ECL reagents were from Pierce Biotchnology. ^{125}I -[Sar¹,Ile⁸]Ang II was from Covance Laboratories (Vienna, VA).

Cell Culture. Clone 9 rat liver epithelial (C9) cells obtained from ATCC were cultured in F-12K nutrient mixture (Kaighn's modification) supplemented with 10% (v/v) fetal calf serum (FCS), 100 $\mu\text{g}/\text{ml}$ streptomycin, 100 IU/ml penicillin and 250 $\mu\text{g}/\text{ml}$ fungizone. For all studies, C9 cells between passages 3 and 12 were used as these exhibit maximum expression of endogenous AT₁ receptors. HEK 293 cells were grown in DMEM containing 10% (v/v) FCS, 100 $\mu\text{g}/\text{ml}$ streptomycin, 100 IU/ml penicillin and genaticin (200 $\mu\text{g}/\text{ml}$). Chinese hamster ovary (CHO) cells were grown in HAM's F12 mdium supplemented with FBS (10%) and L-glutamine. When [^3H]inositol labeling was performed, cells were maintained in inositol-free Medium for 16 h before experiments.

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Receptor internalization. ¹²⁵I-labeled Ang II (0.5-1 μ Ci) was added in HEPES-buffered medium 199 to cells cultured in 24-well plates at 37°C for the indicated times. Incubations were stopped by placing on ice, followed by rapidly washing with two 1 ml aliquots of ice-cold Dulbecco's phosphate-buffered saline. The cells were then treated for 10 min with 0.5 ml of an acid wash solution (150 mM NaCl/50 mM acetic acid) to remove the surface-bound radioligand. The supernatant containing the acid-released radioactivity was collected and the cells were treated with 0.5 M NaOH and 0.05% SDS to solubilize the acid-resistant (internalized) radioactivity. Non-specific binding was measured in parallel experiments. Radioactivity was measured by γ -spectrometry and the percent internalization for each point was calculated, after deducting the respective non-specific values, from the ratio of the acid-resistant binding to the total (acid-resistant plus acid-released) binding.

Inositol phosphate measurements. After incubation with [³H]inositol in 0.5 ml 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 1 μ M Ang II for 20 min, reactions were stopped with perchloric acid and inositol phosphates were extracted and radioactivity was measured by liquid scintillation β -spectrometry.

Immunoprecipitation. After treatment with inhibitors and drugs, cells were placed on ice, washed twice with ice-cold PBS, lysed in the buffer (LB) containing 50 mM Tris, pH 8.0, 100 mM NaCl, 20 mM NaF, 10 mM Na pyrophosphate, 5 mM EDTA, 10 μ g/ml aprotinin, 10 μ g/ml leupeptin, 10 μ g/ml soybean trypsin inhibitor, 10 μ g/ml pepstatin and 1 mM 4-(2-aminoethyl)benzenesulfonyl fluoride, and probe-sonicated (Sonifier Cell Disruptor). Solubilized lysates were clarified by centrifugation at 14,000 rpm for 10 minutes, precleared with agarose,

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and then incubated with antibodies and protein A or G agarose. The immunoprecipitates were collected, washed four times with LB, and mixed with Laemmli buffer. After heating at 95°C for 5 minutes, the samples were centrifuged briefly and the supernatants were analyzed by SDS-PAGE on 8-16% gradient gels.

Immunoblot analysis. Cells cultured to 60-70% confluence were serum-deprived for 24 h before treatment with the indicated reagents and drugs. After aspiration of the media, cells were washed twice with ice-cold PBS and lysed in 100 μ l of Laemmli sample buffer. The samples were briefly sonicated, heated at 95 °C for 5 minutes, and centrifuged for 5 min. The supernatants were electrophoresed on SDS-PAGE (8-16%) gradient gels and transferred to PDVF nylon membranes. Phosphorylated and non-phosphorylated ERK1/2 antibodies were detected with ERK1/2-specific antibody. Blots were probed with horseradish peroxidase-conjugated secondary antibody, then visualized with ECL (enhanced chemiluminescence reagent; Amersham Pharmacia or Pierce Biotechnology) and quantitated by laser scanning densitometry. In some cases, blots were stripped and reprobed with other antibodies as described above.

Results

Ang II (100 nM) caused a marked increase in ERK1/2 activation in C9 cells as measured by immunoblotting the phosphorylated ERK1/2. Ang II-induced ERK1/2 activation was blocked by the AT₁ receptor antagonist, DuP753 but not by the AT₂ receptor antagonist, PD123177, indicating that the Ang II effect is mediated through AT₁-R activation in C9 cells (Fig. 1A). Inhibition of PKC by Go6983 attenuated Ang II-induced ERK1/2 activation, whereas the PKC α inhibitor, Go6976, stimulated Ang II-induced responses (Figure 1B-C). Ang II-induced ERK1/2

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activation was transient in C9 cells, but was potentiated and sustained in the presence of Go6976 (Fig. 1D).

Ang II is known to stimulate inositol phosphates (InsP) production through activation of phospholipase-C β 1 (PLC- β 1). In C9 cells, Ang II caused a 3-fold increase in InsP production that was not affected by Go6976, suggesting that the PKC inhibitor exerts its stimulatory effects downstream of PLC β 1 activation. Also, Go6976 had no effect on Ang II binding to its receptor or AT $_1$ -R internalization in C9 cells (data not shown). Ang II-induced ERK1/2 activation in C9 cells is primarily dependent on PKC. Consistent with the predominant role of PKC in Ang II action, the PKC activator phorbol myristate acetate (PMA) caused a marked but transient increase in stimulation of ERK1/2 activation that was prevented by the PKC inhibitor, Go6983 (Figure 2A). In contrast, pretreatment of cells with Go6976 led to a sustained increase in ERK1/2 activation by PMA (Figure 2B).

Since C9 cells express receptors for LPA that are primarily coupled to a pertussis toxin sensitive G protein (G $_i$), we examined the effect of Go6976 on the G $_i$ -linked ERK1/2 cascade. As shown in Fig. 3A, LPA-induced ERK1/2 activation was not affected by PKC depletion but was sensitive to pertussis-toxin. Moreover, three PKC inhibitors had no effect on LPA responses (Fig. 3B), indicating that LPA exerts its effects through G $_i$ in a PKC-independent manner. Time-course studies revealed that LPA stimulation caused transient activation of ERK1/2, reaching a peak at 5 min and declining rapidly thereafter. In contrast, pretreatment of cells with Go6976 potentiated LPA-induced ERK1/2 responses that were maintained up to 60 min (Fig. 3C).

The role of growth factor-linked tyrosine kinase signaling pathways in ERK1/2 activation is well documented. C9 cells express endogenous EGF receptors and stimulation by EGF caused

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marked ERK1/2 activation that was not affected by PKC inhibitors (Fig. 4A) or PKC depletion (data not shown). The EGF-induced ERK1/2 activation was transient, with a maximum response at 5 min and a rapid decline thereafter. However, pretreatment of cells with Go6976 led to marked and sustained ERK1/2 activation by EGF (Fig. 4B). These data indicate that Go6976 potentiates ERK1/2 activation that is induced by activation of G_q/PKC (Ang II), G_i (LPA) and PKC (PMA).

Several GPCR agonists are known to cause ERK1/2 activation through transactivation of the EGF-R (Wetzker and Bohmer, 2003). To determine whether signals originating from Ang II, LPA and PMA cause ERK1/2 activation through the EGF-R, experiments were performed with the selective EGF-R kinase antagonist, AG1478. As shown in Fig. 5A, pretreatment of C9 cells with AG1478 abolished ERK1/2 activation induced by EGF and markedly diminished that caused by Ang II, PMA and LPA. These results show that Go6976 causes potentiation of ERK1/2 activation following stimulation of GPCRs coupled to G_q and G_i, and RTKs. Moreover, the signals originating from GPCR stimulation converge on the EGF-R and appear to follow the signaling characteristics of EGF stimulation in terms of the onset and duration of ERK1/2 activation. Since tyrosine phosphorylation of the EGF-R is the central step for ERK1/2 signaling by all agonists used in this study, we next determined whether Go6976 potentiates agonist-induced ERK1/2 activation through an effect on EGF-R phosphorylation. EGF caused transient phosphorylation of its receptor as indicated by a phospho-specific antibody against tyrosine 1068. However, in the presence of Go6976, the agonist-stimulated EGF-R phosphorylation was significantly increased (Fig. 5B-D).

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Time-course studies showed that Go6976 caused marked activation of ERK1/2 in C9 cells as early as 10 min, and the maximum effects were observed at 30-60 min. Go6976 also increased phosphorylation of the EGF-R and ERK1/2 in a concentration-dependent manner (Figs. 6A, B). The mechanism of Go6976-induced ERK1/2 activation was examined by using selective inhibitors of signaling pathways. While inhibition of EGF-R with its selective antagonist, AG1478, and of MEK1/2 with U0126, abolished ERK1/2 activation, inhibition of Src by PP2 and of metalloproteinases by GM6001 had no effect (Fig. 6C,D), further indicating the role of EGF-R in mediating the stimulatory effects of Go6976. In C9 cells, Ang II causes activation of Pyk2 activation in a Src-dependent manner (Shah and Catt, 2002). However, Go6976 had no effect on Pyk2 phosphorylation (data not shown). The time-dependent decrease in agonist-stimulated ERK1/2 activation is caused by the dephosphorylation of protein kinase substrates by cellular phosphatases that include protein tyrosine phosphatases (PTPs), serine/threonine phosphatases (STPs) and dual specificity MAP kinase phosphatases (MKPs) (Chernoff, 1999; Tiganis, 2002). Inhibition of PTP by sodium orthovanadate (vanadate) increased basal ERK1/2 activation and also prolonged the actions of Ang II, EGF and LPA (Fig. 7A,B,D,E). However, the STP inhibitor, okadaic acid, had no such effects (Fig. 7A,C).

Since Go6976 is known to inhibit agonist-mediated ERK1/2 activation in other cell types (Matrougui et al., 2000; Sellers et al., 2001), we also examined the cell-specificity of potentiation of ERK1/2 activation by Go6976. For this purpose, similar experiments were performed in human embryonic kidney (HEK 293) cells, which also express endogenous receptors for EGF and LPA. Time-course studies revealed that LPA caused transient activation of ERK1/2, and pretreatment of HEK 293 cells with Go6976 enhanced ERK1/2 activation at early time points (5-

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15 min). However, it did not elicit the prolonged responses that were observed in C9 cells (Fig. 8A). To determine if this is due to the absence of EGF-R involvement, we examined the effect of EGF-R blockade by AG1478 on LPA responses in HEK293 cells. As shown in Fig. 8B, AG1478 completely abolished the effect of EGF but had only a minor inhibitory effect on LPA-induced responses. In contrast, the transient phosphorylation of EGF-R and ERK1/2 during EGF stimulation was replaced by sustained signals in the presence of Go6976 (Fig. 9A, B). These data indicate that the stimulatory effects of Go6976 on GPCR-mediated ERK1/2 activation are dependent on the involvement of EGF-R transactivation, and hence appear to be cell-type specific. To further verify the critical role of the EGF-R as a target of Go6976 action, similar experiments were performed in CHO cells, which lack EGF-R but express endogenous receptors for LPA. In this case, Go6976 caused only a slight and transient increase in ERK1/2 activation at early time points (5, 10 min) (Fig. 10).

Discussion

The PKC family of serine/threonine kinases is comprised of 12 distinct mammalian protein kinases, which are subdivided into three main subfamilies according to their activation profiles; conventional PKCs (cPKCs; α 1, β 1, β II and γ), novel PKCs (nPKC; δ , ϵ , η , θ), atypical PKCs (aPKC; ζ and λ) and the more distantly related PKC μ /PKD and PKC ν . PMA and other phorbol esters have a diacylglycerol-like structure and can activate both c- and n-PKC isoforms (Violin and Newton, 2003). Although PKC can cause direct activation of the Raf-1/MEK/ERK cascade (Farshori et al., 2003; Kolch et al., 1993), GPCR-mediated PKC activation can transduce signals through RTKs in various cell types (Prenzel et al., 1999; Shah and Catt, 2002; Shah et al., 2003). Hepatic C9 cells are derived from normal rat liver and express endogenous receptors for

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Ang II, LPA and EGF. In these cells, agonist-induced ERK1/2 activation is primarily dependent on PKC and occurs through transactivation of the EGF-R. Our data show that stimulation of C9 cells with Ang II, LPA, EGF and PMA cause highly transient phosphorylation of ERK1/2. However, pretreatment of cells with the PKC α inhibitor, Go6976, leads to sustained potentiation of agonist-induced ERK1/2 activation. The stimulatory effect of Go6976 on ERK1/2 activation appears to be mediated at the level of the EGF-R, since upstream signaling molecules such as PLC β 1 and tyrosine kinases such as Src are not affected. However, Go6976-induced activation of ERK1/2 was blocked by a selective antagonist of EGF-R, AG1478 (Fig. 6).

PKC inhibitors have been extensively used to define the role of PKC and its isoforms in signaling studies (Goekjian and Jirousek, 1999). However, the ability of some of these inhibitors to exert effects that are independent of PKC inhibition raises the question of their specificity and selectivity in GPCR signaling studies. In particular, Go6976 has also been widely used as an inhibitor of PKC α (Davies et al., 2000; Hou et al., 2000; Iankov et al., 2002; Lang et al., 2004; Martiny-Baron et al., 1993; Matrougui et al., 2000) and has caused variable effects in different cell types. It inhibits neurotrophin-intrinsic tyrosine kinase activity (Behrens et al., 1999) and reduces PMA-induced protein phosphorylation in neutrophils (Djafarzadeh and Niggli, 1997) and PMA-induced JNK activation in human lung cancer cells (Lang et al., 2004). Moreover, it decreases Ang II-induced ERK activation in rat mesenteric resistance arteries (Matrougui et al., 2000), protein synthesis in human cardiac fibroblasts (Hou et al., 2000) and vasoconstrictor responses in the pulmonary vascular bed (De Witt et al., 2001). It also protects microglial and mesencephalic neurons from LPS-induced death by inhibiting TNF- α release through suppression of p38MAPK (Jeohn et al., 2000). In contrast, Go6976 exerts stimulatory effects on

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MAPK phosphorylation that cannot be explained by its PKC α inhibitory properties. For example, it increases basal ERK1/2 activity in keratinocytes (Praskova et al., 2002), and basal and IL-6-induced ERK1/2 activation in plasmacytoma cells (Iankov et al., 2002). More recent studies have shown that arginine vasopressin causes ERK1/2 activation through EGF-R transactivation in rat mesangial cells, and that Go6976 stimulates basal as well as agonist-mediated ERK1/2 responses in these cells (Ghosh et al., 2004). Our data indicate that Go6976 stimulates basal and agonist-mediated ERK1/2 activation in C9 cells by amplifying and prolonging tyrosine phosphorylation of the EGF-R. The stimulatory effect of Go6976 appears to be mediated through inhibition of tyrosine phosphatases, since it markedly potentiates agonist-induced EGF-R tyrosine phosphorylation and mimics the effects of the PTP inhibitor, vanadate (Fig. 7). Furthermore, another PKC inhibitor, Ro318220, is known to inhibit MKP-1 and thus to increase ERK1/2 phosphorylation (Beltman et al., 1996). Interestingly, Ro318220 also potentiates the effects of Ang II in C9 cells (data not shown). These and other observations indicate that the extent and nature of PKC involvement in signaling pathways cannot necessarily be revealed simply by inhibitor-based studies.

GPCR-mediated transactivation of RTKs has a key role in the regulation of essential physiological processes and in the pathophysiology of hyperproliferative diseases. Binding of a ligand to its RTK results in receptor dimerization and autophosphorylation of multiple tyrosine residues in the cytoplasmic domain that provide assembly points for various signaling proteins. Consistent with their functional importance, RTKs are subject to multiple levels of control and their activities are continuously monitored and tightly regulated (Schlessinger, 2000). This is essential since aberrant expression of RTKs has been implicated in several disease processes,

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including cancer (Fischer et al., 2003). The several mechanisms that control the attenuation and termination of RTK activity include negative-feedback regulation and activation of cellular phosphatases (Schlessinger, 2000; Schlessinger, 2003; Tiganis, 2002). In this regard, PTPs have a critical role in maintaining EGF-R in their dephosphorylated state. Accordingly, inhibition of PTP activity causes enhancement of basal tyrosine phosphorylation (Tiganis, 2002). All three major classes of phosphatases, PTPs, STPs and MKPs, have been reported to regulate MAP kinase signaling cascades (Camps et al., 2000; Saxena and Mustelin, 2000). Thus, inhibition of phosphatases leads to enhancement of basal as well as agonist-stimulated RTK activity and ERK1/2 activation (Camps et al., 2000; Chernoff, 1999; Schlessinger, 2000). In general, phosphatase interactions with specific protein kinases culminate in the termination of signaling process, and steady-state phosphorylation is governed by the coordinated actions of protein kinases and phosphatases that maintain phosphorylation and dephosphorylation in a dynamic equilibrium. Our data in C9 cells show that PTP inhibition by sodium orthovanadate, but not by the STP inhibitor, okadaic acid, increases basal as well as agonist-stimulated responses which mimic the effects of Go6976 (Fig. 7).

In C9 cells, Ang II-induced ERK1/2 phosphorylation is primarily mediated by PKC activation (Shah and Catt, 2002). Previous studies have shown that activation of PKC by GPCRs, PDGF or PMA results in EGF-R phosphorylation at multiple Ser and Thr residues, which leads to inhibition of EGF-R kinase activity (Schlessinger, 2000). However, in C9 cells, PKC causes tyrosine phosphorylation of the EGF-R with subsequent activation of ERK1/2 (Shah and Catt, 2002). Moreover, blockade of EGF-R kinase activity attenuates PMA-induced ERK1/2 activation (Fig. 5). Our data show that Go6976 not only increases GPCR-mediated ERK1/2

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activation, but also potentiates the effects of PKC activation by PMA. To examine whether the stimulatory effects of Go6976 are PKC-dependent, we performed similar experiments using LPA, a G_i -coupled agonist, and EGF, an RTK agonist. Both LPA and EGF caused transient ERK1/2 activation in a PKC-independent manner (Fig. 5). However, Go6976 potentiated the effects of LPA and EGF, as observed for Ang II and PMA in C9 cells. In these cells, Ang II, PMA and LPA cause ERK1/2 activation through transactivation of the EGF-R. Since our results exclude the role of signaling molecules upstream of the EGF-R in Go6976 action, and EGF-R is the convergence point for downstream signaling of these agonists, we examined the effects of Go6976 on tyrosine phosphorylation of the EGF-R. This revealed that Go6976 markedly potentiates and prolongs the phosphorylation of the EGF-R at Tyr1068, possibly via inhibition of EGF-R dephosphorylation, leading to sustained activation of downstream molecules such as ERK1/2. This is further supported by our results showing that selective inhibition of EGF-R kinase by AG1478 abolishes the effects of Go6976 on ERK1/2 activation (Fig. 6C).

To further evaluate the central role of the EGF-R in mediating enhanced and sustained ERK1/2 activation by during Go6976 treatment, we studied HEK293 cells, which show minimal cross-communication of LPA with the EGF-R. In these cells, LPA elicited transient ERK responses and Go6976 caused much less potentiation of ERK1/2 activation by LPA than that observed in C9 cells (Fig. 8). However, Go6976 markedly stimulated tyrosine phosphorylation of the EGF-R and ERK1/2 activation following EGF stimulation of HEK 293 cells, indicating an effect at the level of the EGF-R (Fig. 9). To further assess the role of the EGF-R in this cascade we used CHO cells, which lack EGF-R but express endogenous receptors for LPA. In these cells, LPA caused transient ERK1/2 activation that was not affected by the presence of Go6976. Thus,

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Go6976 appears to selectively potentiate the effects of EGF and GPCR agonists that cause ERK1/2 activation through transactivation of the EGF-R, probably through inhibition of tyrosine phosphatase(s). The nature of the phosphatase(s) that are inhibited by Go6976 remains to be identified. However, mice lacking PTP1B exhibit increased and sustained phosphorylation of the EGF-R (Haj et al., 2003). Moreover, the dual specificity protein phosphatase, cdc25A, interacts both physically and functionally with the EGF-R in Hep3B human hepatoma cells and its inhibition by Cpd5 caused prolonged EGF-R tyrosine phosphorylation (Wang et al., 2002).

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Legends for Figures

Fig. 1. A, Ang II causes ERK1/2 activation in C9 cells through the AT₁-R. C9 cells were grown to 70% confluence and serum-deprived for 20-24 h. AT₁- and AT₂-receptor antagonists, Dup753 (Dup; 10 μM) and PD123177 (PD; 10 μM) were added 20 minutes before the addition of Ang II (100 nM) for 5 minutes. ERK1/2 activation was measured with phospho-specific ERK1/2 antibodies. B, Effects of PKC inhibitors on Ang II-induced ERK1/2 activation. The PKC inhibitors, Chel (chelerythrine), Go6983, and Go6976 (1 μM) were added 20 min before addition of Ang II for 5 min. C, Time-course of the effects of Ang II on ERK1/2 activation in the absence (top panel) and presence of Go6976. Cells were treated with Go6976 (1 μM) for 20 min and then stimulated with Ang II (100 nM) for the indicated time periods. Immunoblot data are representative of 3 similar experiments.

Fig. 2. A, Effects of PKC inhibitors on PMA-induced ERK1/2 activation in C9 cells. The PKC inhibitors, Go 6983, Go6976, and R0318220 (1 μM) were added 20 min before addition of Ang II (100 nM) for 5 min. B, Time-course of the effects of PMA on ERK1/2 activation. Pretreatment of C9 cells with Go6976 causes sustained ERK1/2 activation induced by PMA (100 nM). Cells were treated with Go6976 (1 μM) for 20 min followed by stimulation with PMA for the indicated time periods.

Fig. 3. A, Effects of PKC depletion and pertussis toxin (PTX) on LPA-induced ERK1/2 activation in C9 cells. Cells were treated with PMA overnight (PMA O/N) and PTX (50 ng/ml for 16 h) and stimulated with LPA (1 μM) for 5 min. B, Effects of PKC inhibitors on LPA-induced ERK1/2 activation. PKC inhibitor treatments were as described in Fig 1B. C, Effects of

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Go6976 on LPA-induced ERK1/2 activation. LPA-induced ERK1/2 activation was measured in the absence and presence of Go6976 (1 μ M; 20 min).

Fig. 4. A, Effects of PKC inhibition by chelerythrine (Chel; 1 μ M), Go6983 and Ro318220 (1 μ M) on EGF-induced ERK1/2 activation in C9 cells. Inhibitors were added 20 min before stimulation with EGF (10 ng/ml) for 5 min. B, Time-course of the effects of EGF alone and in the presence of Go6976 (1 μ M; 20 min).

Fig. 5. A, Role of EGF-R transactivation in agonist-stimulated ERK1/2 activation. Serum-deprived C9 cells were treated with the selective EGF receptor kinase inhibitor, AG 1478 (100 nM), and then stimulated with Ang II (100 nM), EGF (10 ng/ml), PMA (100 nM) and LPA (1 μ M) for 5 min. B-D, Effects of Go6976 on agonist-induced tyrosine phosphorylation of the EGF-R. C9 cells were treated for increasing time periods with EGF (10 ng/ml), Ang II (100 nM) and LPA (1 μ M) in the absence and presence of Go6976 (1 μ M). Immunoblots were performed with phospho-specific antibody against tyrosine 1068 of the EGF-R.

Fig. 6. A, Time-course of the effects of Go6976 on ERK1/2 phosphorylation in C9 cells. Cells were treated with Go6976 (1 μ M) for the indicated time periods. B, Concentration-dependent effects of Go6976 (30 min) on phosphorylation of the EGF-R (Y1068) and ERK1/2 in C9 cells. C, Cells pretreated with the EGF-R antagonist, AG1478 (200 nM), MEK inhibitor, U0126 (10 μ M), and Src inhibitor, PP2 (1 μ M) for 20 min were stimulated with Go6976 (1 μ M) for 30 min. D, Lack of an effect of metalloproteinase inhibitor, GM6001, on Go6976-induced ERK1/2 activation. C9 cells were treated with varying concentrations of GM6001 for 20 min and stimulated with Go6976 for 30 min. Cell lysates were analyzed for phosphorylation of ERK1/2.

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Fig. 7. A, Time-course of the effects of PTP inhibition by sodium orthovanadate (vanadate; 100 μ M), and STP inhibition by okadaic acid (200 nM), on ERK1/2 activation in C9 cells. B-C, Effects of vanadate (100 μ M) and okadaic acid (200 nM) on ERK1/2 activation by Ang II. C9 cells were treated with vanadate and okadaic acid for 15 min and stimulated with Ang II for the indicated time periods. D-E, Effects of vanadate (100 μ M) and okadaic acid (200 nM) on ERK1/2 activation by LPA and EGF. C9 cells were pretreated with inhibitors for 15 min and stimulated with EGF (10 ng/ml) or LPA (1 μ M) for the indicated time periods.

Fig. 8. A, Time-course of the effects of LPA in HEK293 cells in the absence and presence of Go6976 in HEK293 cells. B, Effects of the EGF-R antagonist, AG1478, on agonist-induced ERK1/2 activation. HEK293 cells were treated with AG1478 (200 nM) for 20 min and stimulated with EGF (10 ng/ml) and LPA (1 μ M).

Fig. 9. A, Time-course of the effects of EGF on tyrosine phosphorylation of the EGF-R at Tyr 1068 in the absence and presence of Go6976 in HEK293 cells. B, Time-course of effects of EGF on ERK1/2 activation in the absence and presence of Go6976 in HEK293 cells.

Fig. 10. Time-course of the effects of LPA on ERK1/2 activation in the absence and presence of Go6976 in Chinese hamster ovary (CHO) cells.

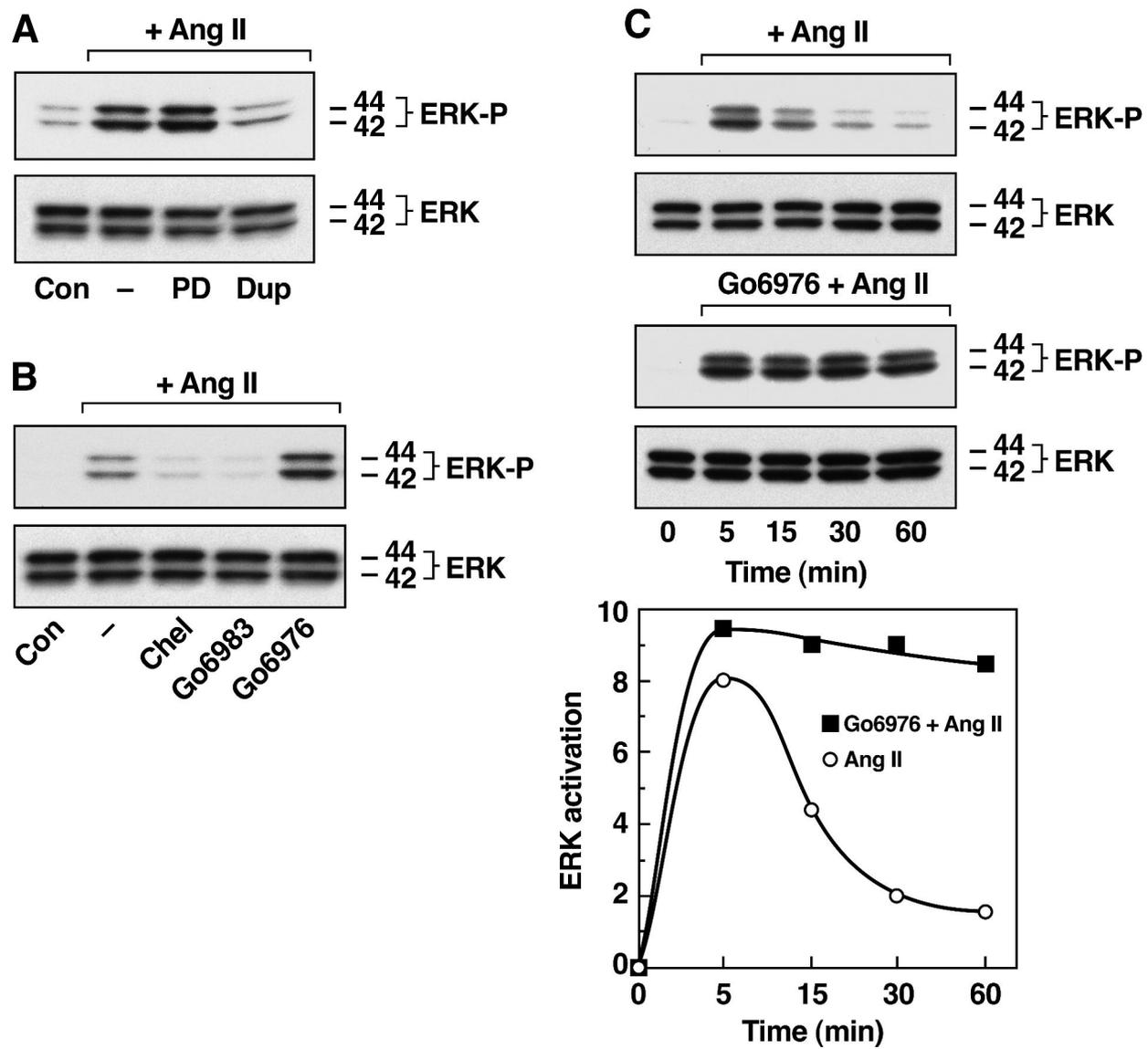


Figure 1

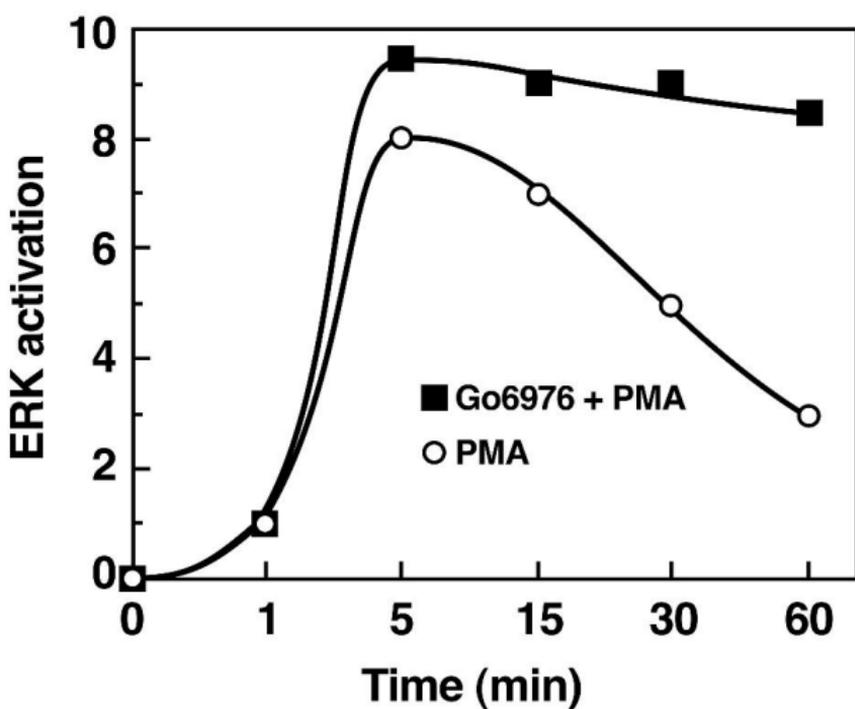
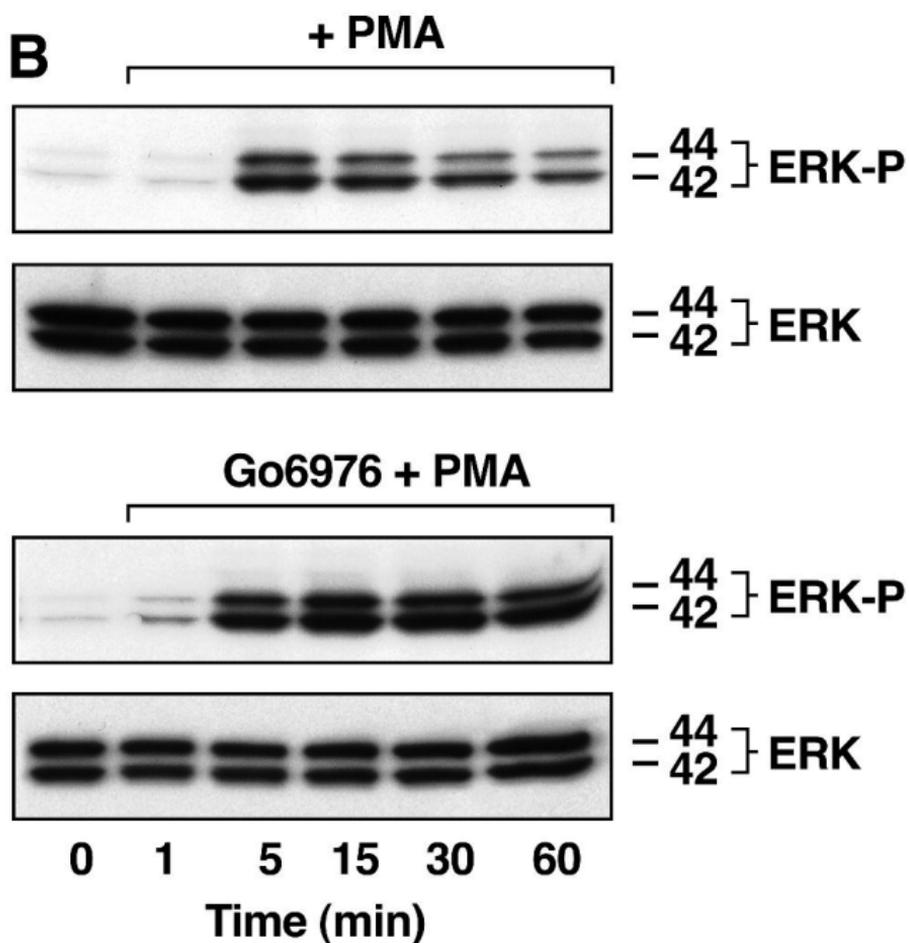
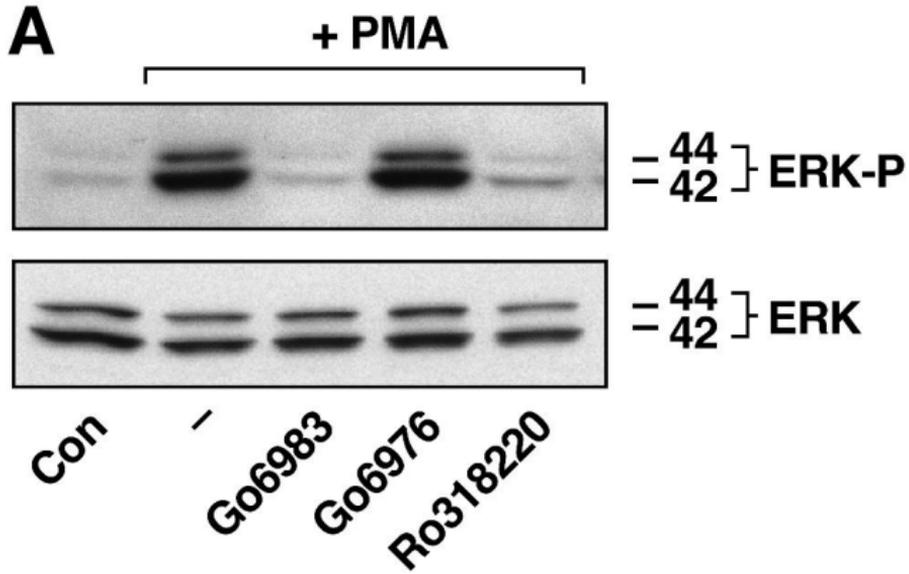


Figure 2

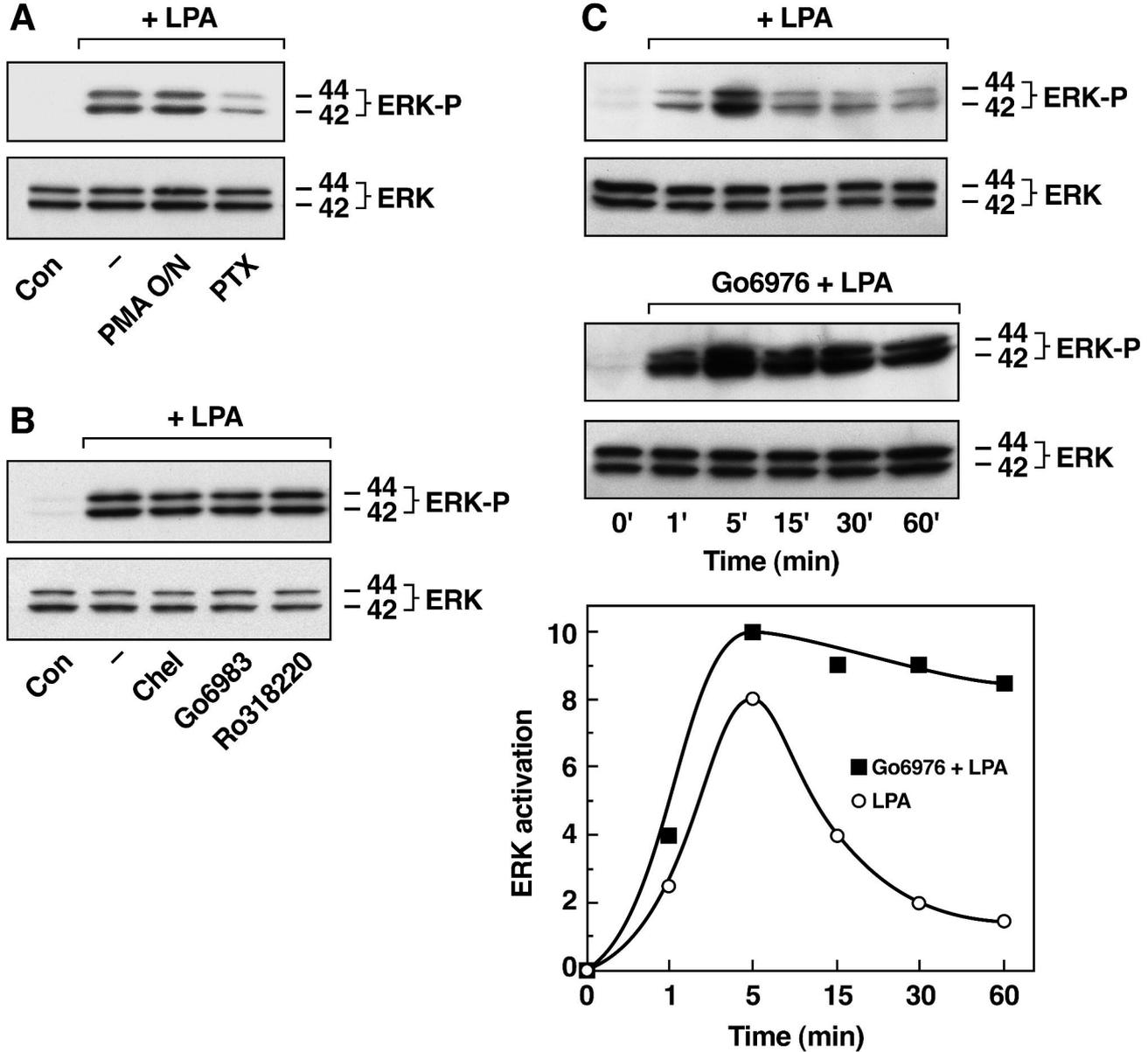


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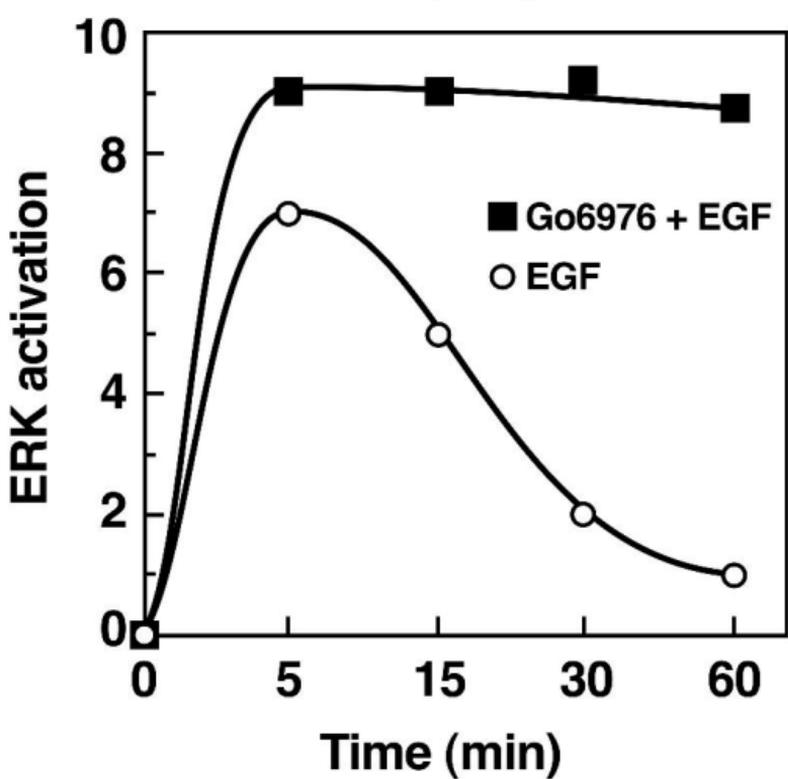
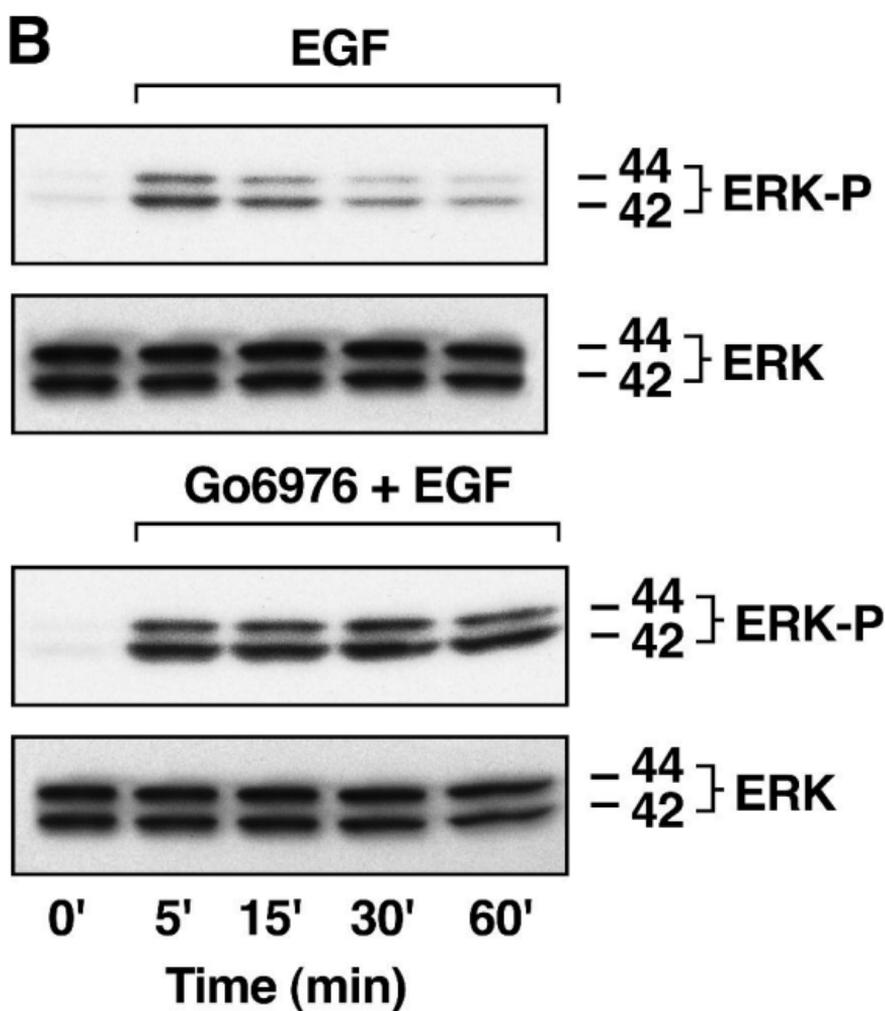
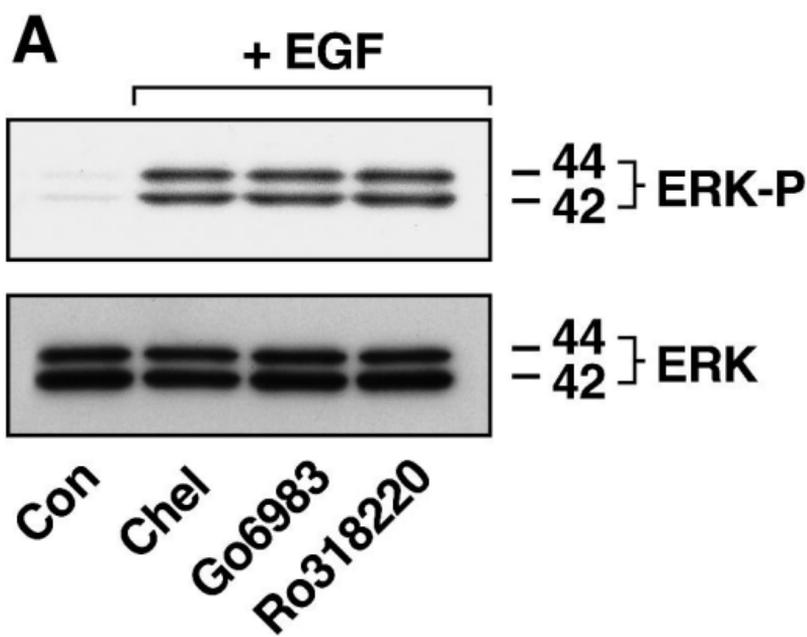


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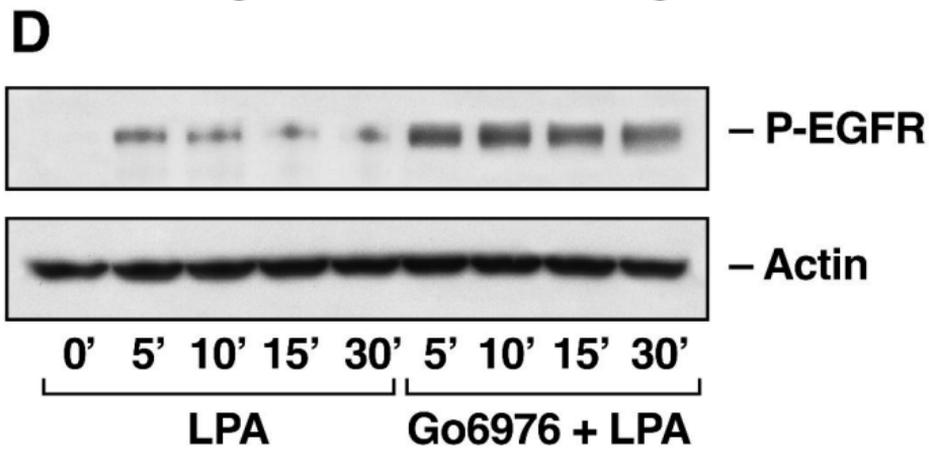
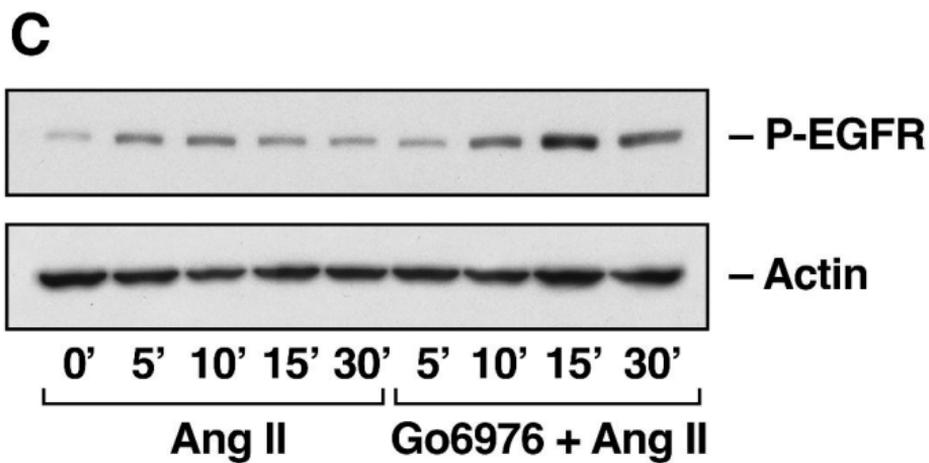
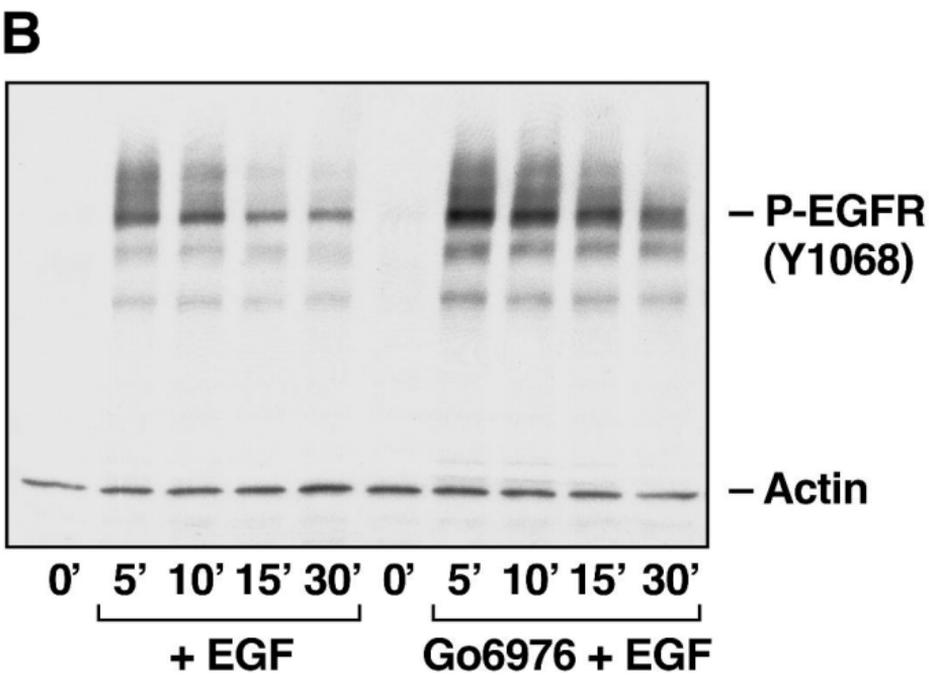
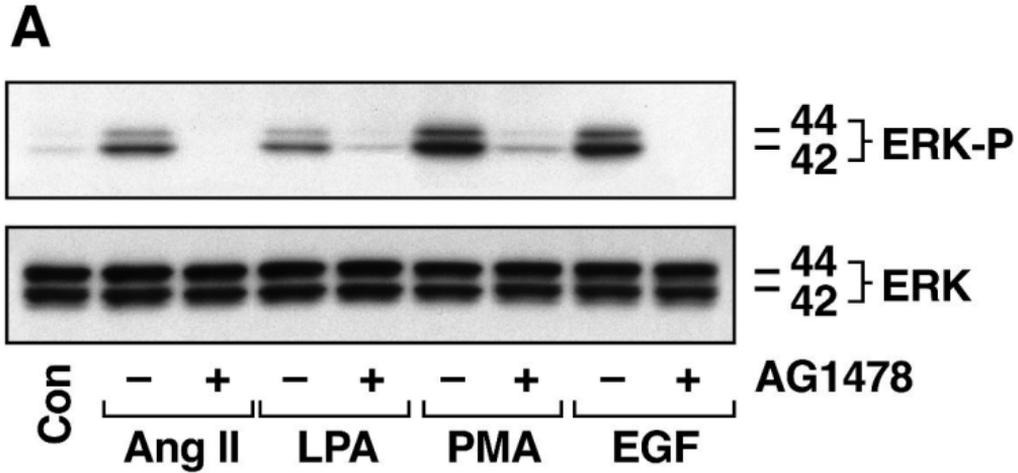


Figure 5

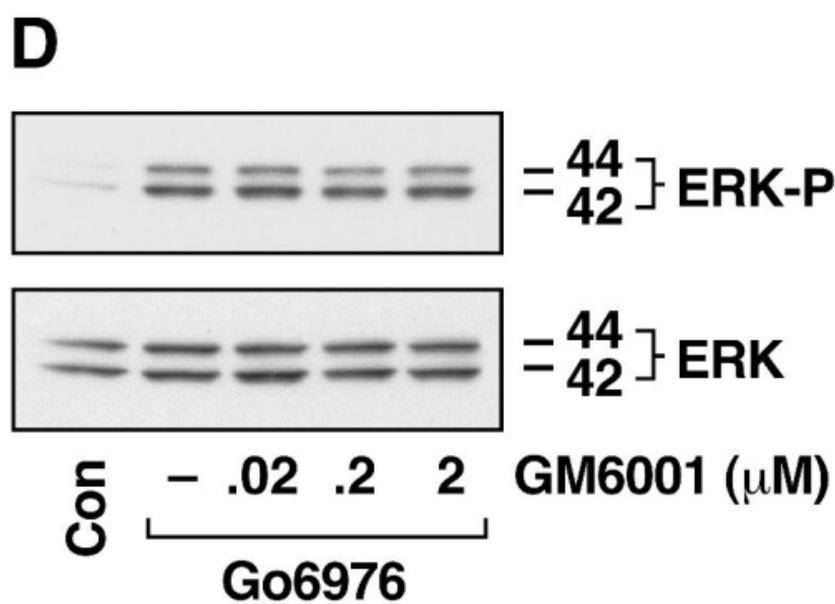
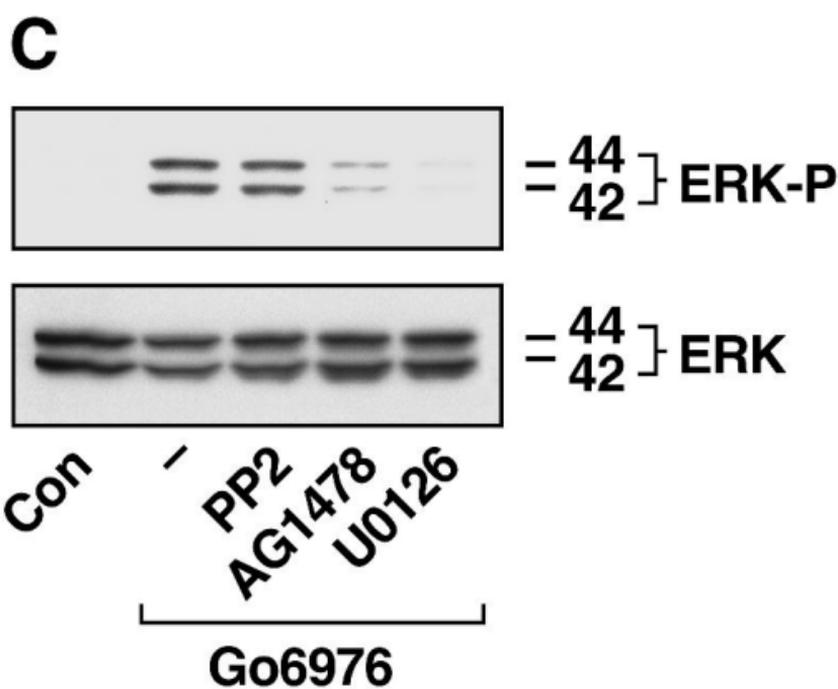
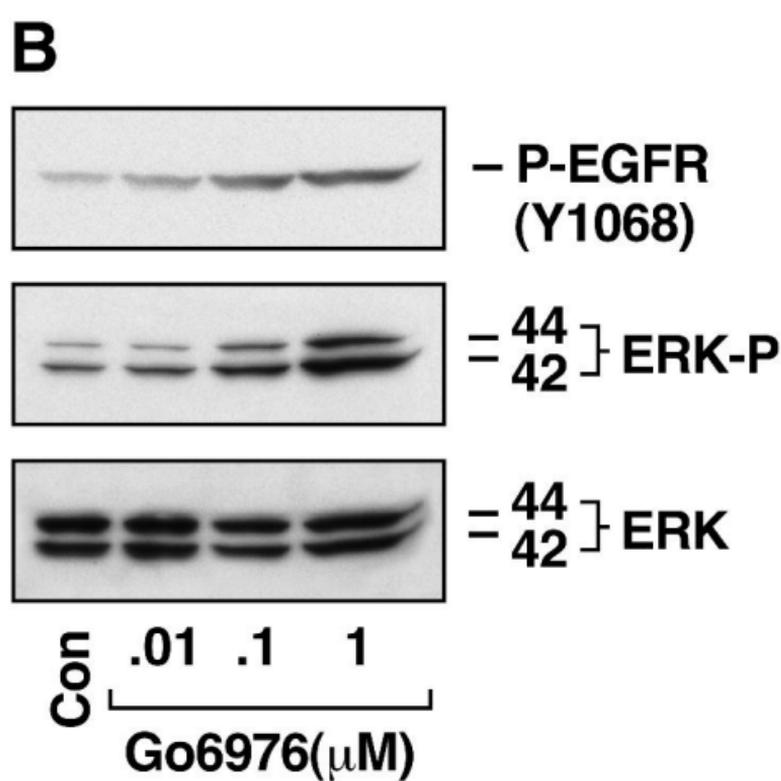
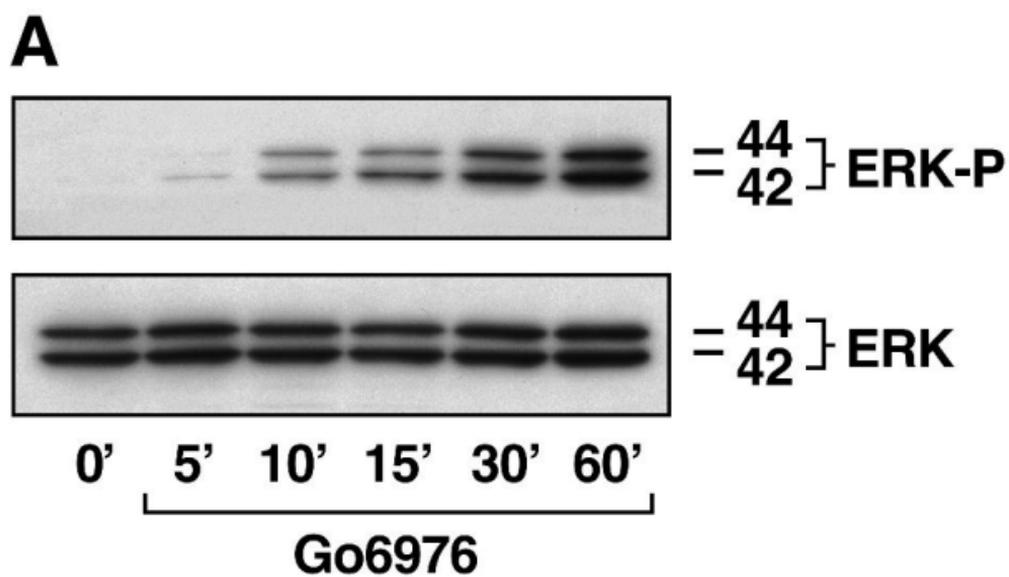


Figure 6

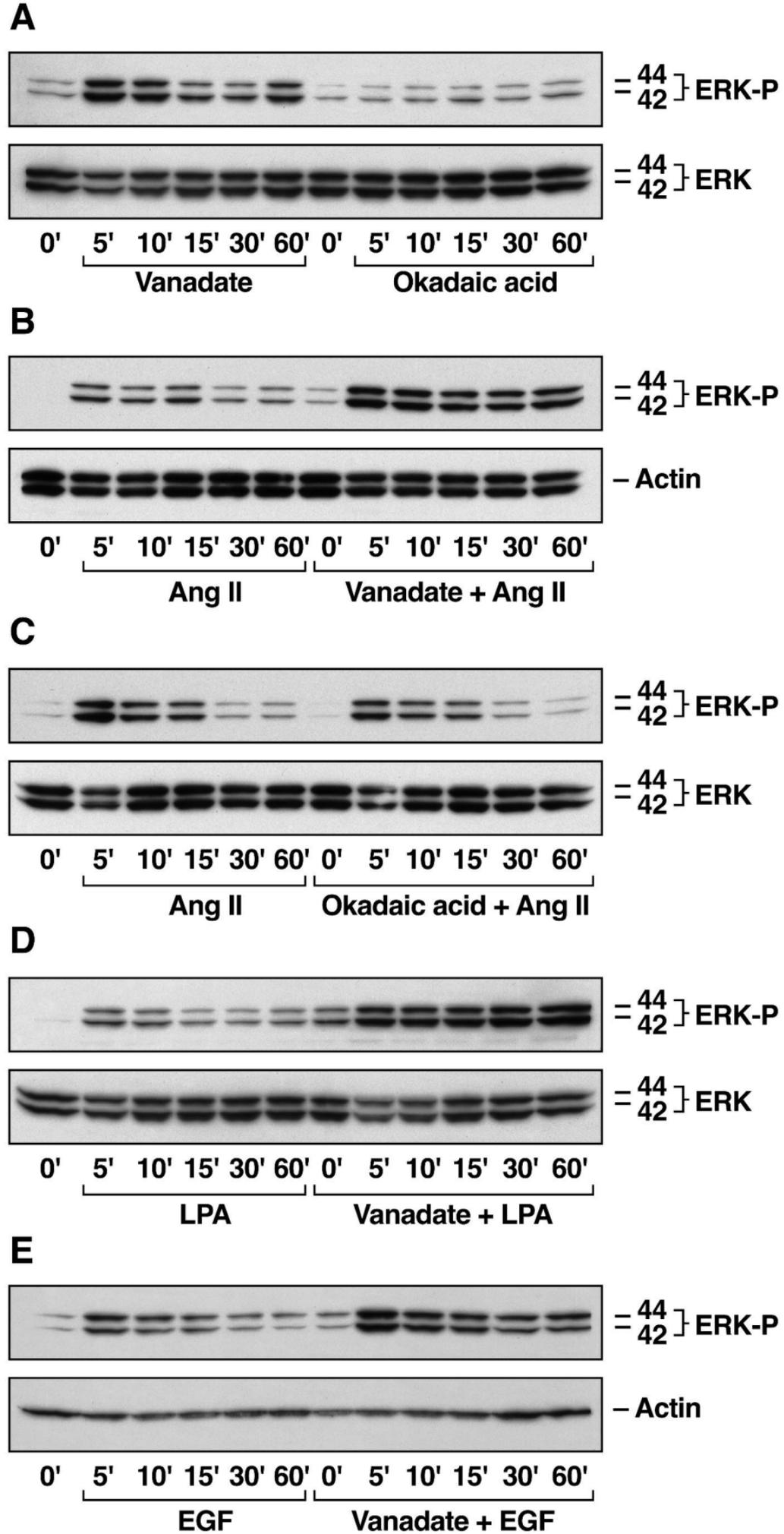


Figure 7

HEK 293 Cells

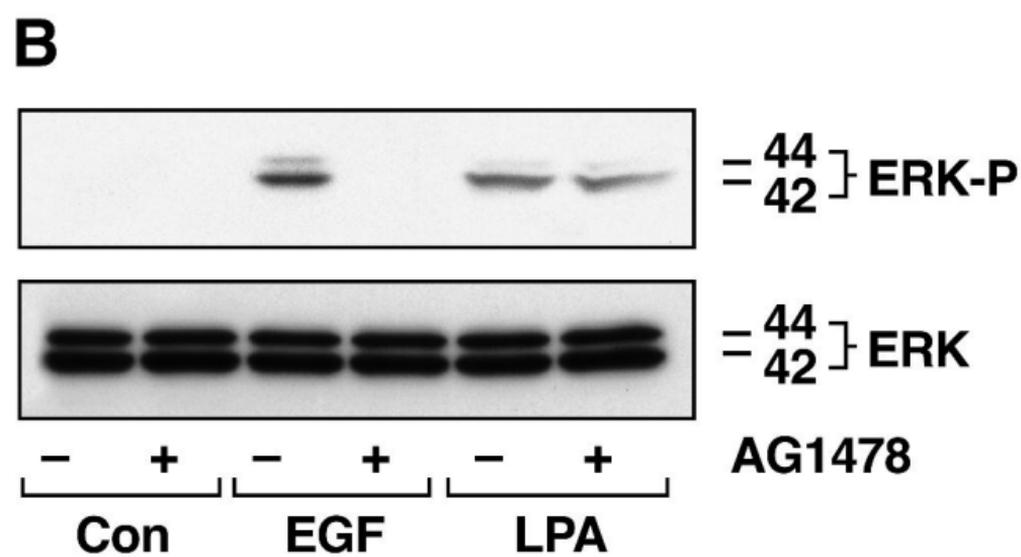
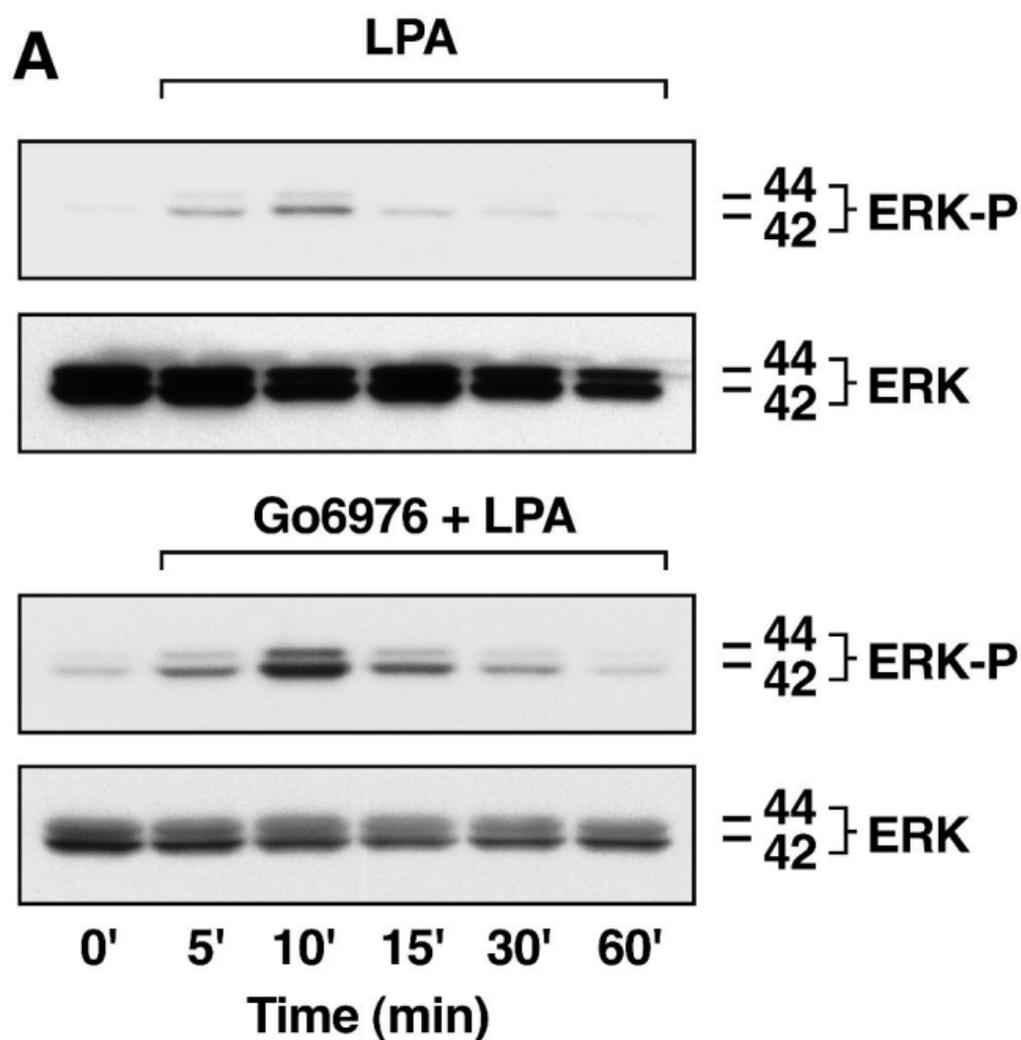
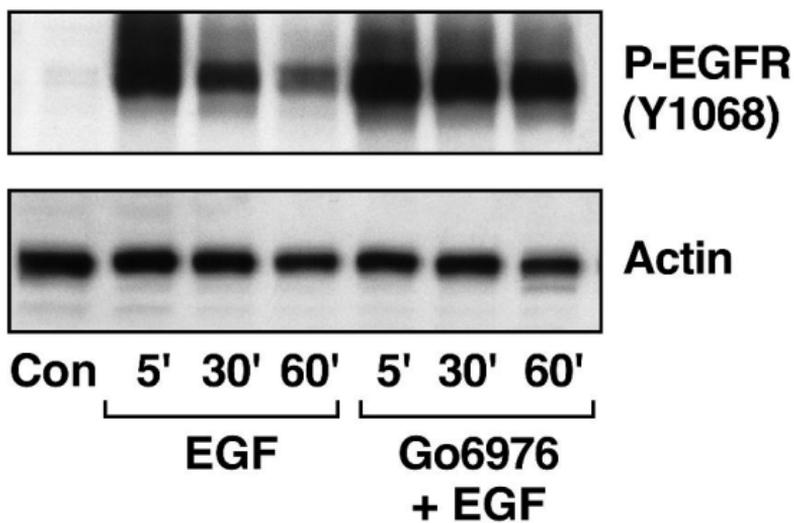


Figure 8

HEK 293 Cells

A



B

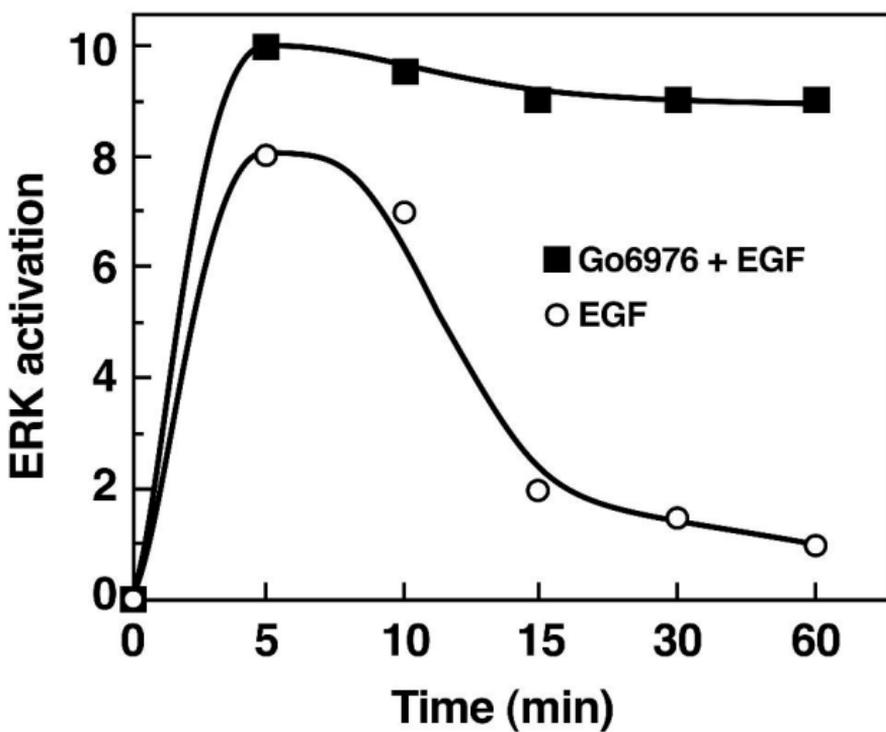
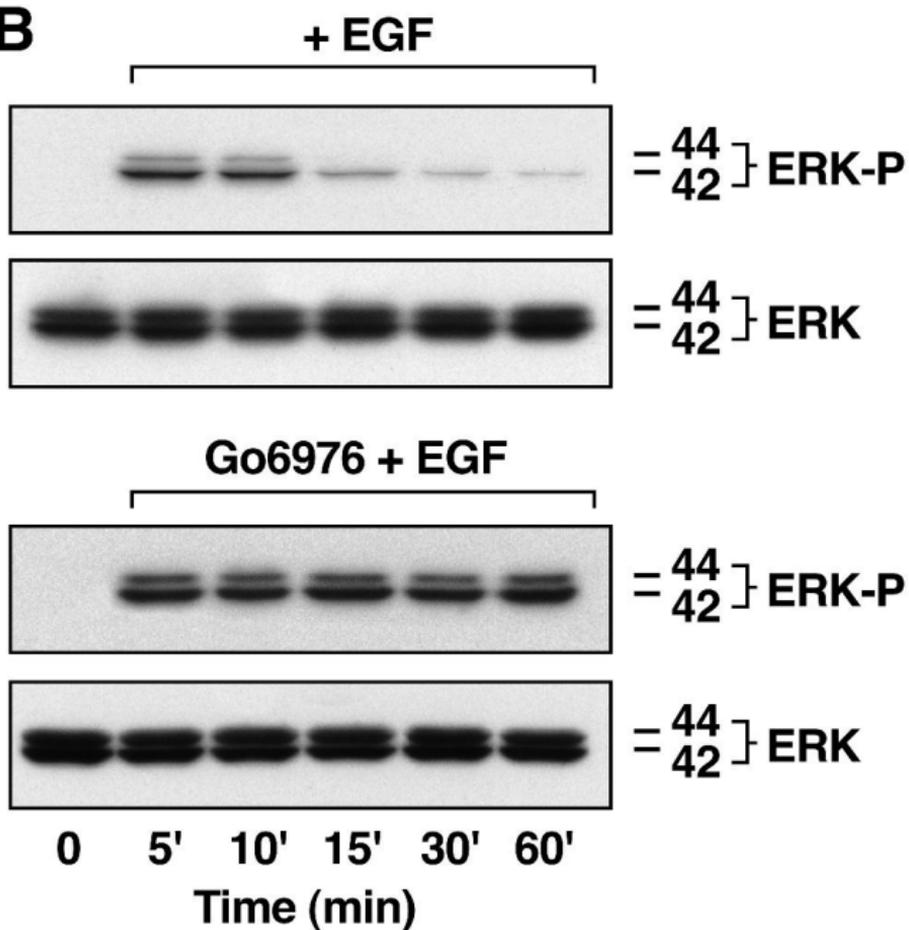
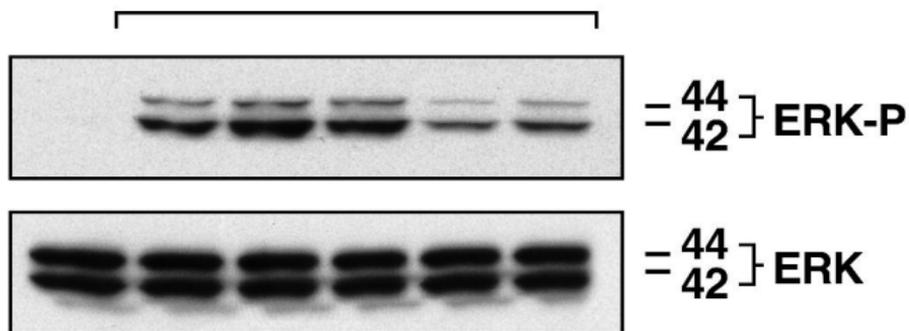


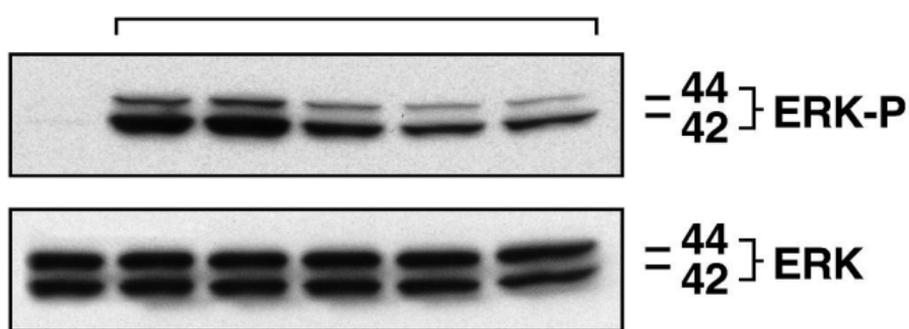
Figure 9

CHO Cells

LPA



Go6976 + LPA



0 5' 10' 15' 30' 60'

Time (min)

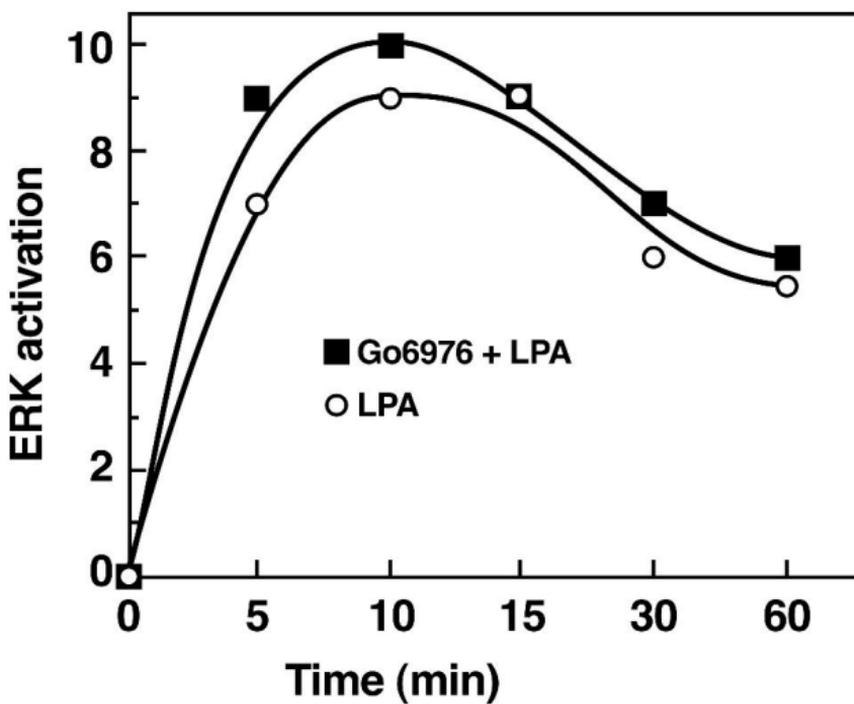


Figure 10