Epidermal Growth Factor and Angiotensin II Regulation of Extracellular Signal-Regulated Protein Kinase in Rat Liver Epithelial WB Cells

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ABSTRACT. Activation of extracellular signal-regulated protein kinase (ERK) is considered essential for mitogenesis. In the present study, rat liver epithelial WB cells were used to investigate the relative roles of Ca\(^{2+}\), protein kinase C (PKC), and protein tyrosine phosphorylation in mitogenesis and activation of the ERK pathway stimulated by epidermal growth factor (EGF) and angiotensin II (Ang II). The sensitivity of the ERK pathway to Ca\(^{2+}\) was studied by using 1,2-bis (O-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid (BAPTA) to chelate intracellular Ca\(^{2+}\) and a low extracellular Ca\(^{2+}\) concentration to prevent Ca\(^{2+}\) influx. Agonist-induced PKC activation was diminished by inhibition of PKC by GF-109203X (bisindolylmaleimide) or by down-regulation of PKC by long-term treatment of the cells with phorbol myristate acetate (PMA). Our results show that although activation of PKC was critical for mitogenesis induced by Ang II or EGF, the initial activation of ERK by both agonists in these cells was essentially independent of PKC activation and was insensitive to Ca\(^{2+}\) mobilization. This is in contrast to the findings in some cell types that exhibit a marked dependency on mobilization of Ca\(^{2+}\) and/or PKC activation. On the other hand, an obligatory tyrosine phosphorylation step for activation of ERK was indicated by the use of protein tyrosine kinase inhibitors, which profoundly inhibited the activation of ERK by EGF, Ang II, and PMA. Additional experiments indicated that tyrosine phosphorylation by a cytosolic tyrosine kinase may represent a general mechanism for G-protein coupled receptor mediated ERK activation.

KEY WORDS. rat liver epithelial WB cells; EGF; angiotensin II; MAP kinase; PKC; mitogenesis

MAP\(^{\ddagger}\) kinases are widely expressed protein Ser/Thr kinases [1, 2]. ERK is the best-studied member of the MAP kinase family, and its activation is considered essential for the initiation of mitogenic processes [3, 4]. Upon activation, ERK regulates the activities of a variety of key signal transduction enzymes and transcription factors, which ultimately affect gene expression and mitogenesis [2, 5, 6]. In response to various extracellular stimuli, the ERK signaling pathway can be activated by receptor tyrosine kinases, such as the EGF receptor, or by G-protein coupled receptors, such as the Ang II receptor. Activation of ERK through receptor tyrosine kinases is the best understood of several pathways that link receptor activation to signaling systems that target nuclear proteins. After ligand binding, the growth factor receptor causes activation of its tyrosine kinase and autophosphorylation of tyrosine residues in the cytoplasmic domain of the receptor. Adapter proteins such as GRB2 and SHC bind to specific phosphorytrosine residues of the receptor via their src homology (SH\(_2\)) domains and, through the mediation of Sos, a guanine nucleotide exchange factor, cause activation of Ras, which, in turn, phosphorlylates and activates the Raf-MEK-ERK kinase cascade [2, 6].

In contrast, the pathways for G-protein coupled receptor-mediated activation of ERK are currently less clear [7, 8]. Several studies have shown that receptors coupled to G\(_{\alpha}\) proteins cause an activation of ERK that can be accounted for, to different extents, by activation of phospholipase C\(_{\beta}\) and subsequent activation of PKC and Ca\(^{2+}\) mobilization [9, 10]. Phorbol esters, by directly stimulating PKC, can activate the ERK pathway in many cell types, probably by phosphorylation and activation of Raf-1 [11, 12]. However, the effects of PKC activation are cell type dependent, suggesting different pathways for activation of ERK or the involvement of different PKC isoforms. A similar lack of consistency is also seen in studies on the role of Ca\(^{2+}\) in the regulation of MAP kinase, since both Ca\(^{2+}\)-dependent and
-independent pathways for activation of ERK have been reported [13]. Receptors that are coupled to G\(_i\) also cause a stimulation of ERK activity, and recent evidence indicates that this effect may be mediated by G-protein \(\beta\gamma\) subunits [14–16]. Activation of ERK by the \(\beta\gamma\) subunit pathway requires a tyrosine phosphorylation step since it can be blocked by tyrosine kinase inhibitors such as genistein and herbimycin [17, 18]. However, activation of the ERK signaling pathway by G\(_i\)-coupled receptors may be less dependent upon activation of a tyrosine kinase upstream of Ras [19, 20]. It is apparent that activation of the ERK pathway can be achieved by several mechanisms in an agonist and cell-dependent manner, and its full activation may need input from several different cellular signaling systems.

WB cells are epithelial cells that were originally isolated from the livers of adult rats. They show a good responsiveness to Ang II and EGF [20, 21]. The signaling pathways in WB cells initiated by these hormones may play important roles in the development of the liver and its functions. In the present study, rat liver WB cells were used to investigate the roles of Ca\(^{2+}\), PKC, and protein tyrosine kinases in the signaling pathways leading to activation of ERK and mitogenesis after stimulation of cells with EGF and Ang II. The data presented here indicate that although PKC activation induced by EGF and Ang II is essential for the mitogenic effect of these hormones in WB cells, activation of PKC and increased cytosolic free Ca\(^{2+}\) play a minor role in the overall activation of ERK. On the other hand, ERK activation stimulated by EGF and Ang II was inhibited extensively by the tyrosine kinase inhibitors genistein and herbimycin, suggesting that recruitment of a tyrosine kinase exerts a critical role downstream of receptor activation in WB cells. Together with the findings reported by other investigators, it seems that tyrosine phosphorylation by a cytosolic tyrosine kinase may represent a general mechanism for ERK activation stimulated by various agonists.

**MATERIALS AND METHODS**

**Materials**

EGF, anti-ERK2 antibodies, and ERK substrate peptide were purchased from UBI. Fura-2 and BAPTA/AM were obtained from Molecular Probes, and anti-Raf-1 and anti-MEK1/2 antibodies were from Santa Cruz. PMA and Ang II were purchased from Sigma. Genistein, GF-109203X (bisindolylmaleimide), and herbimycin A were obtained from the LC Laboratories.

**Cell Culture**

WB cells (passage 20–30) were plated onto 100-mm plastic culture plates and maintained in Richter’s improved essential medium containing l-glutamine plus 10% fetal bovine serum at 37° in a humidified incubator (5% CO\(_2\), 95% air) until about 80% confluent. Then cells were serum-starved for 24–48 hr in Richter’s medium without fetal bovine serum before treatment with the agonists.

**Measurement of DNA Synthesis**

DNA synthesis was determined by two methods. In the first method, DNA synthesis was measured by \(^{[3]H}\)thymidine incorporation into DNA. Near confluent WB cells were starved for 48 hr and incubated with EGF, Ang II, or PMA for 24 hr. \(^{[3]H}\)Thymidine (25 \(\mu\)Ci/mL) was added 16 hr before the end of the incubation. The cells were quickly washed three times with ice-cold PBS, incubated for 10 min with 2 mL of 10% (v/v) TCA, and washed three times with 2 mL of 95% ethanol. The acid-insoluble pellet was incubated in 800 \(\mu\)L of 0.2 N NaOH, and the solution was neutralized with HCl. The radioactivity was determined by liquid scintillation counting. In the second method, DNA synthesis was measured by an immunocytochemistry method. Briefly, WB cells were plated onto coverslips in 6-well plates. Near confluent WB cells were starved for 48 hr and incubated with agonists in the presence or absence of GF-109203X for 20 hr. BrdU was added to the culture medium, and cells were incubated for a further 4 hr. BrdU incorporation into proliferating cells was determined using the BrdU staining kit obtained from Calbiochem, as described previously [22].

**Cell Lysate Preparation**

WB cells were treated with or without agonists for the indicated times, then washed twice with ice-cold PBS, and scraped into cell lysis buffer containing 50 mM HEPES (pH 7.5), 150 mM NaCl, 1 mM Na\(_2\)VO\(_4\), 50 mM pyrophosphate, 100 mM NaF, 1 mM EGTA, 1.5 mM MgCl\(_2\), 1% (v/v) Triton X-100, 10% (v/v) glycerol, 10 \(\mu\)g/mL of apritinin, 10 \(\mu\)g/mL of leupeptin, and 1 mM phenylmethylsulfonyl fluoride. The cells were incubated in the lysis buffer for 1 hr at 4°, and then were centrifuged at 15,000 g at 4° for 20 min. The insoluble material was discarded, and the supernatant was designated as whole cell lysate. The protein concentration was determined by the method of Bradford [23], using BSA as the standard.

**Protein Kinase Assays**

ERK activation was determined either by the gel shift assay using anti-ERK2 antibodies by western-blot analysis, or by a kinase assay using myelin basic protein (MBP) peptide as substrate. In the gel shift assay, the appearance of a slower migrating band on the gel, due to phosphorylation of threonine and tyrosine residues of ERK, was used as an indication of its activation [22]. The kinase activity assay was performed as described [24] with some modifications. The kinase assay buffer contained 20 mM HEPES (pH 7.4), 10 mM MgCl\(_2\), 20 mM glycerophosphate, 1 mM dithiothreitol (DTT), 2 mM EGTA, 0.1 mM sodium orthovanadate, 20 \(\mu\)M \([\gamma\text{-}^{32}\text{P}]\)ATP (10 Ci/mmol), 10 \(\mu\)g/mL of
leupeptin, 10 μg/mL of aprotinin, 1 mg/mL of MBP peptide substrate, and 0.1 g/mL of BSA. The reaction mixtures were incubated for 20 min at 30°C, and the reaction was terminated by the addition of 15 μL of 40% TCA (w/v). After centrifugation at 12,000 g for 5 min, 20 μL of supernatant was spotted onto p81 phosphocellulose paper filters, which were washed five times with 1% (v/v) phosphoric acid. After the final wash, the filters were dried and transferred to scintillation vials. The radioactivity was determined in triplicate assays by liquid scintillation counting.

MEK and Raf-1 activities were assayed by an immunocomplex assay method. MEK and Raf-1 were immunoprecipitated with their specific antibodies. The kinase activities were determined using GST-MEK for Raf-1 and GST-ERK2 for MEK as substrates, respectively, as described previously [25].

PKC activities in the cytosol and particulate fractions were determined by measuring the incorporation of 32P from [γ-32P]ATP into histone H1 in the presence of 40 μg/mL of phosphatidyserine and 4 μg/mL of 1,2-dioleoyl-sn-glycerol in a final volume of 50 μL kinase buffer as used for the ERK kinase assay. The reaction was started by adding cell lysate (5 μg of protein). After incubation for 5 min at 30°C, the reaction was stopped by the addition of 10 μL of 25% (w/v) TCA; the remaining procedures were the same as described for the ERK activity assay.

**Immunoblotting**

The protein samples were subjected to SDS–PAGE and transferred onto nitrocellulose membrane. The membrane was first washed for 15 min with PBS containing 0.1% (v/v) Tween 20 (PBST). Nonspecific binding of proteins was blocked with 3% BSA in PBST for 1 hr, followed by washing twice with PBST. Then the membrane was incubated with primary antibodies for 1 hr at room temperature, followed by three washes with PBST. The membrane was incubated with secondary antibodies for 30 min. After washing with PBST, the immunoblots were visualized by the ECL (enhanced chemiluminescence) system.

**Measurement of Cytosolic Free Ca\(^{2+}\)**

Intracellular Ca\(^{2+}\) was measured in single cells using fluorescence microscopy, as described previously [26].

**RESULTS**

**Stimulation of DNA Synthesis and Activation of ERK by Ang II, EGF, and PMA**

To compare the mitogenic effects of Ang II and EGF, WB cells were incubated in serum-free medium to arrest them at the G0 stage of the cell cycle and subsequently were treated with the different agonists. As shown in Fig. 1A, Ang II (1 μM) and EGF (100 ng/mL) both stimulated DNA synthesis, as determined by [3H]thymidine incorporation into DNA. Separate experiments showed that the maximal effects of [3H]thymidine incorporation were obtained with these concentrations of agonists (data not shown). Interestingly, PMA, a well-characterized pharmaceutical activator of PKC, also showed a mitogenic effect (Fig. 1A), indicating that PKC may be involved in mediating the mitogenic effects of Ang II and EGF. To test this possibility, the PKC pathway was blocked by two methods. It is known that long-term pretreatment of cells with PMA results in PKC degradation and a loss in total PKC activity, a phenomenon known as PKC down-regulation [9, 10]. As shown in Fig. 1A, pretreatment of the cells for 48 hr with PMA prior to acute stimulation totally abolished the mitogenic effect of PMA, indicating the effectiveness of PKC down-regulation. Under these experimental conditions, Ang II-stimulated DNA synthesis was blocked completely, while EGF-stimulated DNA synthesis was attenuated markedly (Fig. 1A). It is possible that the long-term incubation of cells with PMA may have an inhibitory effect on mitogenesis by a mechanism other than down-regula-
kinase activity method. The basal kinase activity (0 min) was determined by the gel shift assay. The lower band indicates the inactive form of ERK2. The bar graph represents the total ERK activity obtained with the kinase assay method. The basal kinase activity (0 min) was 1420 cpm/1.5 μg protein. A similar result was obtained in three separate experiments.

To ascertain that PKC was indeed activated over the same time periods that ERK activity increased after stimulation with EGF or Ang II, both PKC translocation and activity were examined. It is known that upon activation by various stimuli, PKC translocates from the cytosol to the cell membrane. Therefore, redistribution of PKC has been regarded as an indication of its activation [28]. Stimulation of WB cells with Ang II (5 min), EGF (5 min), or PMA (10 min) caused a decrease of total PKC activity in the particulate fraction and a corresponding increase of PKC activity in the cytosolic fraction (data not shown). These results demonstrate that a redistribution of the PKC isoforms took place within 5 min of stimulation by EGF or Ang II. PKC down-regulation by prolonged treatment of the cells with PMA, or acute inhibition of PKC by GF-109203X, essentially abolished the effects of Ang II and EGF on PKC activation in both the soluble and particulate fractions (data not shown). PKC is encoded by a multiple gene family, and eleven members have been described to date [28]. Immunoblotting using isoform-specific antibodies detected four PKC isoforms (α, δ, ε, and ζ) in the whole cell lysate of WB cells [29]. In resting cells, PKC-α, -δ, and -ε were present mainly in the cytosol, while PKC-ζ was distributed approximately equally in the cytosol and the particulate fractions. EGF and Ang II treatment increased the amount of PKC-δ and -ε in the particulate fraction, but the distributions of -α and -ζ were not affected, as determined by western-blot analysis (data not shown). These results indicate that the EGF- and Ang II-stimulated activation of total PKC may be accounted for by the δ and ε isoforms of PKC.

To investigate the role of PKC in Ang II- and EGF-induced activation of ERK, WB cells were incubated with GF-109203X prior to the addition of EGF and Ang II. As shown in Fig. 3, the most pronounced effect of GF-109203X was to inhibit almost entirely the acute activation of ERK by PMA, thereby confirming the effectiveness of GF-109203X in inhibiting PKC under the conditions of the experiments. In contrast, neither EGF- nor Ang II-mediated activation of ERK was affected appreciably by this PKC inhibitor. It is evident, therefore, that during the first hour of ERK activation by EGF and Ang II, PKC plays a minor role in the overall effects of the hormones on ERK activation.

Role of Ca²⁺ in the Activation of ERK by EGF and Ang II

Since Ca²⁺ has been implicated as a potential regulator of the ERK pathway in many cell types, two approaches were used to investigate the Ca²⁺ sensitivity of ERK activation by Ang II and EGF in WB cells. The first method employed BAPTA/AM to increase the Ca²⁺ buffering capacity of the cytosol. As shown in Fig. 4, preincubation of fura-2-loaded WB cells with 5 μM BAPTA/AM for 30 min did not affect the resting cytosolic free Ca²⁺ but was sufficient to abolish the Ca²⁺ transient induced by Ang II. The EGF-induced Ca²⁺ transient was similarly inhibited by BAPTA/AM pretreatment (data not shown). The second approach was to lower the extracellular free Ca²⁺ concentration to 1–10
by the addition of a 0.1 mM excess of EGTA over Ca\(^{2+}\) to the incubation medium immediately prior to hormone addition. This treatment has little effect on the Ang II- or EGF-induced mobilization of intracellular Ca\(^{2+}\), but prevents Ca\(^{2+}\) entry and refilling of the internal Ca\(^{2+}\) pools.

Figure 5 shows that BAPTA/AM or EGTA treatment of the cells had no inhibitory effect on either the Ang II- or EGF-induced activation of ERK. Thus, the ERK signaling pathway initiated by stimulation of either Ang II or the EGF receptors is Ca\(^{2+}\) independent in WB cells.

\[\text{Effect of Protein Tyrosine Kinase Inhibitors on EGF- and Ang II-induced Activation of ERK}\]

Since the maximal ERK activation induced by EGF or Ang II could not be accounted for by PKC activation or Ca\(^{2+}\) mobilization, another pathway(s) must be involved in mediating the effects of these hormones on the activation of ERK in WB cells. Tyrosine phosphorylation of components of the Ras-activated protein kinase cascade is thought to be responsible for activation of ERK by growth factors. In the case of EGF-induced activation of ERK, evidence points to the kinase domain of the receptor itself being responsible for the first committed tyrosine phosphorylation step. The identity of a participating tyrosine kinase in the Ang II-induced pathway for activation of ERK is less well established. To determine whether a tyrosine kinase step was involved in Ang II-mediated activation of ERK, WB cells were pretreated with genistein in order to inhibit tyrosine kinase activity prior to the addition of the stimulating agents. Genistein is a widely used protein tyrosine kinase inhibitor [18, 21]. It profoundly inhibited the EGF-induced activation of ERK, as expected, but it also inhibited almost entirely the activation of ERK induced by Ang II (Fig. 6A). These results suggest that a cytosolic tyrosine kinase is activated as a consequence of binding of Ang II to its G-protein coupled receptor. Further experiments showed that genistein did not affect the activities of either immunoprecipitated MEK or ERK in vitro (data not shown). To further confirm the involvement of a tyrosine kinase step in Ang II signaling, the cells were stimulated with Ang II in the presence of another protein tyrosine kinase inhibitor, herbimycin A [18]. ERK and its upstream kinases MEK and Raf-1 were immunoprecipitated with their specific antibodies. As shown in Figure 6B, the...
activities of all three kinases stimulated by Ang II were attenuated markedly by herbimycin A. This result is consistent with that obtained with the genistein experiment and supports the notion that a tyrosine kinase is involved in mediating the Ang II-stimulated ERK signaling pathway. In addition, it also indicates that the tyrosine kinase acts upstream of the Raf-MEK-ERK cascade.

**Effect of Genistein on the Activation of ERK Induced by Other Agonists**

To test whether tyrosine phosphorylation by a cytosolic tyrosine kinase may represent a general mechanism for ERK activation, the cells were pretreated with genistein, and stimulated with PMA, which activates ERK in a PKC-dependent manner, or with phenylephrine, vasopressin, or thrombin, which are known to exert their effects through G-protein coupled receptors. In all cases, the activation of ERK by these agonists was inhibited by at least 50% after treatment of the cells with genistein (Fig. 7). Thus, a tyrosine phosphorylation step seems to be involved in the activation of the ERK pathway mediated by both G-protein coupled receptors and by the PKC signaling pathway.

**DISCUSSION**

The ERK signaling cascade is a prominent cellular pathway used by many growth factors and hormones to mediate mitogenesis. One of the earliest responses of cells to EGF and Ang II is an activation of PKC and mobilization of intracellular Ca^{2+} as a consequence of activation of PLC-γ and PLC-β [30]. Thus, activation of PKC and increased Ca^{2+} mobilization are thought to initiate important signaling events that contribute to the final physiological effects of EGF and Ang II in different cells. Although it is well established that EGF can activate the ERK signaling pathway through the Ras-Raf-MEK-ERK cascade by its receptor tyrosine kinase activity, it is evident that ERK activity may also be regulated in many other ways. Ras, Raf, PKC, and Ca^{2+}-dependent and -independent activation of ERK has been reported in a variety of cell types [7, 8, 10, 14, 17, 20, 31–39].

In the present study with WB cells, both EGF-mediated and Ang II-mediated activation of ERK were essentially independent of PKC activation. Therefore, the role of PKC activation seems to be insignificant in the early phase of ERK activation in these cells. However, PKC activation is critical for the mitogenic effect of both EGF and Ang II. It is likely, therefore, that PKC exerts its effect on mitogenesis through other components in the signaling pathway rather than by activation of ERK in WB cells. There appears to be a similar lack of consistency for the role of Ca^{2+} in ERK activation. For example, in foreskin fibroblasts and carci-
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Ca²⁺ play a critical role in the activation of ERK in a direct manner [41]. In rat liver WB cells and GN4 cells, Ang II activates at least two tyrosine kinases, which are thought to be recruited and activated by G-protein receptors. JAK, and some members of c-Src tyrosine kinases, are involved in, but also play a major role in the activation of ERK [13]. In cardiac myocytes and vascular smooth muscle cells, Ang II-induced ERK activation was Ca²⁺ dependent, since chelation of intracellular Ca²⁺ by BAPTA completely inhibited the activation of ERK induced by Ang II [39, 40]. In contrast, the present study demonstrates that in WB cells, short-term activation of ERK by Ang II or EGF was essentially Ca²⁺ independent. This was shown under the two conditions that were minimally invasive to the cell. The first was to incubate the cells with a low concentration of BAPTA/AM, so that the intracellular Ca²⁺ buffering was increased just sufficiently to prevent the Ang II- or EGF-induced Ca²⁺ transient, while the second was to add a 0.1 mM excess of EGTA over Ca²⁺ to the cell medium just prior to the addition of Ang II or EGF. This latter condition allowed an essentially normal Ca²⁺ transient, but no Ca²⁺ entry. Since neither treatment affected the Ang II- or EGF-induced activation of ERK, we can conclude that the transient Ca²⁺ increase was not obligatory for the early activation of ERK.

The fact that neither PKC activation nor Ca²⁺ mobilization could account for the observed ERK activation induced by Ang II and EGF in WB cells indicates that other pathways predominate. Recently, accumulating evidence has shown that activation of ERK mediated by G-protein coupled receptors requires tyrosine phosphorylation events. Cytosolic tyrosine kinases, such as Hck, Lyn, JAK, and some members of c-Src tyrosine kinases, are thought to be recruited and activated by G-protein receptors directly or indirectly upon their activation by ligand binding [41]. In rat liver WB cells and GN4 cells, Ang II activates at least two tyrosine kinases, which are thought to play a critical role in the activation of ERK in a direct Ca²⁺-independent manner [20]. In agreement with these findings, our results show that tyrosine kinase(s) is not only involved in, but also plays a major role in the activation of ERK induced by Ang II. Using a genetic approach, Wan and coworkers [42] recently found that a non-receptor tyrosine kinase cascade is located upstream of the ERK pathway activated by G-protein coupled receptors. Among these tyrosine kinases, Syc in conjunction with Fyn and Yes can recruit and phosphorylate the adapter molecule SHC, which subsequently assembles the signal complex of SHC/Grb2/Sos and initiates the Raf-MEK-ERK pathway [42]. This model provides a possible explanation for our results in WB cells, which demonstrate that the ERK pathway, when activated by EGF or Ang II, showed a similar sensitivity to inhibition by genistein. It can be concluded that a Ca²⁺-independent tyrosine phosphorylation step is critical for upstream activation of the Ras-Raf-MEK-ERK cascade initiated by both EGF and Ang II. Tyrosine phosphorylation, through the intrinsic tyrosine kinase activity of the EGF receptor, is required for EGF to activate ERK, while tyrosine phosphorylation through cytosolic tyrosine kinases seems to play a critical role in the activation of ERK induced by G-protein coupled receptors.

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