Research article

Ca²⁺-mediated activation of ERK in hepatocytes by norepinephrine and prostaglandin F₂α: role of calmodulin and src kinases

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Abstract

Background: Previous studies have shown that several agents that stimulate heptahelical G-protein coupled receptors activate the extracellular signal regulated kinases ERK1 (p44mapk) and ERK2 (p42mapk) in hepatocytes. The molecular pathways that convey their signals to ERK1/2 are only partially clarified. In the present study we have explored the role of Ca²⁺ and Ca²⁺-dependent steps leading to ERK1/2 activation induced by norepinephrine and prostaglandin (PG)F₂α.

Results: Pretreatment of the cells with the Ca²⁺ chelators BAPTA-AM or EGTA, as well as the Ca²⁺ influx inhibitor gadolinium, resulted in a partial decrease of the ERK response. Furthermore, the calmodulin antagonists W-7, trifluoperazine, and J-8 markedly decreased ERK activation. Pretreatment with KN-93, an inhibitor of the multifunctional Ca²⁺/calmodulin-dependent protein kinase, had no effect on ERK activation. The Src kinase inhibitors PP1 and PP2 partially diminished the ERK responses elicited by both norepinephrine and PGF₂α.

Conclusion: The present data indicate that Ca²⁺ is involved in ERK activation induced by hormones acting on G protein-coupled receptors in hepatocytes, and suggest that calmodulin and Src kinases might play a role in these signaling pathways.

Background

The extracellular signal regulated kinases ERK1 (p44mapk) and ERK2 (p42mapk) are activated in response to stimulation of receptor tyrosine kinases (RTKs) as well as heptahelical G protein coupled receptors (GPCR) and transmit signals which regulate cell differentiation and growth [1–3]. The molecular steps involved in signaling from GPCRs to ERK are incompletely understood. Data obtained in various cell systems have provided evidence in support of several signaling pathways including protein kinase C (PKC) [4], Ca²⁺-mediated mechanisms [5–12], and trans-activation of receptor tyrosine kinases [13,14]. In hepatocytes several hormones, including vasopressin, angiotensin II, norepinephrine, and PGF₂α, that bind to GPCRs activate ERK [15–17]. The mechanisms mediating the ERK activation by GPCR agonists are not clarified;
there is evidence that protein kinase C is involved [15,18], but a role for Ca\textsuperscript{2+} also appears likely, since all the agents above activate phospholipase C and elevate intracellular Ca\textsuperscript{2+} in hepatocytes [19,20]. Furthermore, agents that elevate intracellular Ca\textsuperscript{2+} through mechanisms bypassing receptors have been found to activate ERK [15,21]. However, agonist-stimulated phospholipase C activity is rapidly down-regulated upon culturing of hepatocytes [22,23], and we recently reported that norepinephrine and PGF\textsubscript{2\alpha} activate ERK under conditions where the level of inositol 1,4,5-trisphosphate (InsP\textsubscript{3}) was only slightly, and transiently elevated [17]. In the present study we have, therefore, examined more closely the role of Ca\textsuperscript{2+} in ERK activation induced by norepinephrine and PGF\textsubscript{2\alpha} and mechanisms downstream of elevated [Ca\textsuperscript{2+}].

Results

Agents that elevate [Ca\textsuperscript{2+}], activate ERK

In agreement with previous observations [15,21] treatment of hepatocytes with thapsigargin, which inhibits Ca\textsuperscript{2+} reuptake to endoplasmatic reticulum [24], and A23187, which induces Ca\textsuperscript{2+} influx, stimulated ERK1/2 activity 2–2.5 fold (Fig. 1A). The elevation of intracellular Ca\textsuperscript{2+} resulting from stimulation with thapsigargin is shown in Fig. 1B. These observations are compatible with a role for Ca\textsuperscript{2+}-elevating mechanisms in the events that trigger ERK1/2 activation in hepatocytes.

![Figure 1](http://www.biomedcentral.com/1471-2121/3/5)

**Figure 1**

ERK1/2 activation and Ca\textsuperscript{2+} response in hepatocytes. A: At 3 h after the time of seeding hepatocytes were preincubated with timolol (10 \(\mu\)M) for 30 min prior to stimulation with thapsigargin (1 \(\mu\)M), A23187 (10 \(\mu\)M) or norepinephrine (10 \(\mu\)M) for 5 min before they were harvested and ERK 1/2 activity assessed. Results represent mean ± S.E.M. of five different experiments. B: Single cell measurement of [Ca\textsuperscript{2+}]\textsubscript{i} as described in Materials and Methods. Results given as ratio (345/385 fluorescence) represent a typical single cell response after stimulation with thapsigargin (10 \(\mu\)M) in a fura-2 AM loaded hepatocyte.

Activation of ERK by norepinephrine and PGF\textsubscript{2\alpha} involves Ca\textsuperscript{2+}

We then examined the role of Ca\textsuperscript{2+} in activation of ERK1/2 induced by stimulation of \(\alpha\textsubscript{1}\)-adrenoceptors (with norepinephrine in the presence of timolol) and prostaglandin receptors (using PGF\textsubscript{2\alpha}) [21,25,26]. The hepatocytes were pretreated with BAPTA-AM, which is activated intracellularly to bind Ca\textsuperscript{2+}, EGTA, which binds extracellular Ca\textsuperscript{2+} and eventually may deplete intracellular Ca\textsuperscript{2+} [27,28], or gadolinium, a competitive inhibitor of Ca\textsuperscript{2+} influx [29–31]. BAPTA-AM completely attenuated the norepinephrine-induced rise of [Ca\textsuperscript{2+}]\textsubscript{i} (Fig. 2A), while the ERK1/2 activity in response to norepinephrine was partially decreased (Fig. 2B,2C). ERK1/2 activity induced by PGF\textsubscript{2\alpha} and the Ca\textsuperscript{2+} ionophore A23187 was also inhibited by BAPTA-AM, while the TPA response was unaffected (Fig. 2B,2C,2D). When the cells were pretreated with EGTA, the initial peak of the Ca\textsuperscript{2+} elevation was only slightly affected, while the prolonged phase of the Ca\textsuperscript{2+}-response was abolished (Fig. 3A). The activation of ERK1/2 by norepinephrine or PGF\textsubscript{2\alpha} was partly decreased by EGTA (Fig. 3B,3C,3D). EGTA also markedly decreased the ERK1/2 response induced by A23187 and thapsigargin, while the TPA-induced ERK1/2 activation was unaffected (Fig. 3B,3C). Pretreatment with gadolinium decreased the adrenergic activation almost to the level obtained by EGTA (Fig. 4A). Gadolinium also decreased the A23187-induced activation of ERK1/2 (Fig. 4B). Taken together, the results suggest a role for Ca\textsuperscript{2+} in the activation of ERK by norepinephrine and PGF\textsubscript{2\alpha} and that this involves Ca\textsuperscript{2+} influx as well as release from internal pools.

Effect of antagonists of calmodulin and the multifunctional Ca\textsuperscript{2+}/calmodulin-dependent protein kinase in ERK activation in hepatocytes

A major mechanism for Ca\textsuperscript{2+}-induced signaling is through formation of a complex with calmodulin [32,33]. Calmodulin has been found to stimulate as well as inhibit ERK1/2 activity [12,34,35]. We therefore examined the role of calmodulin in these pathways. Pretreatment of hepatocytes with the calmodulin inhibitors trifluoperazine, I-8, and W-7 markedly inhibited the ERK1/2 activation after stimulation with norepinephrine and PGF\textsubscript{2\alpha} (Fig. 5). The results were confirmed with immunoblots (Fig. 6). Activation of ERK1/2 by A23187 was also markedly sensitive to pretreatment with W-7 (Fig. 5, 6).

Calmodulin may act on several regulatory enzymes [32,36–40], including the Ca\textsuperscript{2+}/calmodulin-dependent protein kinases, which have been implicated in the activation of ERK1/2 [7–9]. We explored a possible role for the multifunctional Ca\textsuperscript{2+}/calmodulin-dependent protein kinase in ERK1/2 activation in hepatocytes stimulated by norepinephrine and PGF\textsubscript{2\alpha}. Pretreatment of the cells with KN-93, an inhibitor of the multifunctional Ca\textsuperscript{2+}/calmod-
ulin-dependent protein kinase [41], did not decrease the activation of ERK1/2 either by norepinephrine, PGF$_{2\alpha}$, or A23187 (Fig. 5, 6). In supplementary experiments we examined the effects of higher concentrations of KN-93 (up to 100 µM) or prolonged exposure times (up to 24 hours) which in none of the cases resulted in a decreased ERK1/2 activation (data not shown). These results suggest that calmodulin might be involved in hormone-induced activation of ERK1/2 in hepatocytes, however the data do not support a role for the multifunctional Ca$^{2+}$/calmodulin-dependent protein kinase in Ca$^{2+}$/calmodulin-mediated activation of ERK1/2 in hepatocytes.

**Inhibitors of src kinases attenuate ERK activation in hepatocytes**

Src kinases [42] have been implicated in the mechanisms resulting in ERK1/2 activation in response to stimulation of both $G_\text{q}$ and $G_\text{i}$-coupled heptahelic receptors [43–45], and several observations suggest that activation of Src in these pathways involves Ca$^{2+}$[46,47]. Data obtained in this study showed that the Src inhibitors PP1 and PP2, which are reported to primarily inhibit the Lck, Fyn, and Hck subtypes of Src kinases [48], markedly decreased the PGF$_{2\alpha}$-induced ERK1/2 activation and led to partial inhibition of the effect of norepinephrine, while the EGF induced ERK1/2 response was not reduced (Fig. 7). Furthermore, ERK1/2 activation induced by A23187 and
thapsigargin was also decreased after Src inhibition (Fig. 7). The results suggest a role for Src kinases in the mechanisms leading to ERK1/2 activation both by PGF 2α and norepinephrine, and that this step at least in part may be located distal to increases in the intracellular level of Ca2+.

Discussion
The present findings confirm previous reports of a role for Ca2+ in ERK1/2 activation in hepatocytes [15,21] and suggest that release of Ca2+ from intracellular stores as well as influx of extracellular Ca2+ is of importance for the hormone-induced activation of ERK1/2. Furthermore, the results suggest that calmodulin and Src kinases might be involved in the Ca2+-dependent activation of ERK1/ERK2.

Evidence from several experimental models suggest that activation of ERK1/2 may occur through Ca2+-dependent as well as Ca2+-independent mechanisms [5,28,49–54]. The present data suggest that Ca2+ is involved in activation of ERK1/2 in hepatocytes in response to norepinephrine and PGF2α. The ERK1/2 response was decreased by chelation of intracellular and extracellular Ca2+ with BAPTA-AM and EGTA, respectively, as well as by gadolinium, which competitively inhibits Ca2+ influx. It may appear that extracellular and intracellular Ca2+ act in a concerted, possibly sequential manner in the mechanisms involved in activation of ERK1/2 by norepinephrine and PGF2α. An integration of Ca2+ signals from the extracellular and intracellular environment is presumably due to store-operated Ca2+ influx [31,55,56]. The mechanisms that initiate Ca2+ influx subsequent to depletion of intracellular stores are incompletely understood, but recent studies have suggested that direct interaction between InsP3 receptors and calcium channels in the plasma membrane may lead to activation of the calcium channels [57]. A diffusible Ca2+ influx factor may also be involved [58]. Previous studies have suggested that hormone-induced Ca2+ influx involves heterotrimeric Gt proteins in hepatocytes [59,60]. It is notable that norepinephrine and PGF2α activate ERK1/2 in the presence of a barely detectable increase in intracellular InsP3[17]. This may suggest either the occurrence of local elevations of InsP3 which do not affect global InsP3, or that Ca2+ pools are regulated by other mechanisms such as generation of sphingosine-1-phos-

![Figure 3](image-url)
phate [61]. A role for ryanodine-sensitive Ca\(^{2+}\) stores in the endoplasmatic reticulum has also been proposed in hepatocytes [62] and EGTA-sensitive pools that are located in plasma membrane micro villar structures have been described [63].

Our data further suggest that calmodulin, which has previously been implicated in growth regulation in liver [64], is involved in activation of ERK1/2. The ERK1/2 responses induced by norepinephrine and PGF\(_{2\alpha}\) were markedly decreased after pretreatment with the calmodulin inhibitors trifluoperazine, J-8, or W-7. Besides calmodulin, it is conceivable that the effect of Ca\(^{2+}\) is mediated through other Ca\(^{2+}\)-binding proteins [39,65]. Calmodulin may also act in a Ca\(^{2+}\)-independent manner [38,66], which might account for the more pronounced inhibition of hormone-stimulated ERK activity by calmodulin antagonists than by agents inhibiting the Ca\(^{2+}\) signal. Alternatively, non-specific effects produced by calmodulin antagonists in higher doses might explain their relatively stronger inhibition. Among the downstream targets of calmodulin, the Ca\(^{2+}\)/calmodulin-dependent protein kinases have been implicated in ERK1/2 activation in smooth muscle cells [8,9], but not in other cells [67,68]. Furthermore, the multifunctional Ca\(^{2+}\)/calmodulin-dependent protein kinase was located downstream of ERK1/2 activation by platelet-derived growth factor (PDGF) in vascular smooth muscle cells [69]. Pretreatment of hepatocytes with KN-93 did not decrease ERK1/2 activation induced by hormones or the Ca\(^{2+}\) ionophore A23187. Thus, while the multifunctional Ca\(^{2+}\)/calmodulin-dependent protein kinase exerts several effects in hepatocytes, including growth inhibition under certain conditions [70–72], it does not appear to be involved in ERK activation. It is of interest that the \(\alpha_1\)-adrenoceptor-induced c-fos expression in fibroblasts was also observed to involve calmodulin, but not the multifunctional Ca\(^{2+}\)/calmodulin-dependent protein kinase [73].

Increasing evidence suggest a role of Src kinases downstream of Ca\(^{2+}\)/calmodulin in ERK1/2 signaling [10,14,46]. The present results suggest that Src kinases may be involved in ERK1/2 activation induced by PGF\(_{2\alpha}\) and norepinephrine, while the EGF induced ERK1/2 response appears to be independent of these Src kinases. Furthermore, the ERK1/2 activation induced by the Ca\(^{2+}\) ionophore A23187 or by thapsigargin was partially decreased by Src inhibition suggesting a role of Src distal to increases in intracellular Ca\(^{2+}\). Of the possible downstream targets for Ca\(^{2+}\)/calmodulin in ERK signaling in hepatocytes our findings thus lend support to a role of Src kinases, although the results do not exclude the possibility that Src kinases and calmodulin act in parallel pathways leading to ERK activation.

While the present results show a role for Ca\(^{2+}\) in ERK1/2 activation by norepinephrine and PGF\(_{2\alpha}\), it is notable that even complete inhibition of Ca\(^{2+}\) signaling only partially inhibited ERK1/2 activity. Taken together with previous observations that inhibition of PKC almost completely inhibited ERK1/2 activation by norepinephrine, vasoressin, and angiotensin II [18], the results suggest that several mechanisms contribute to and may act in concert in the hormonal stimulation of ERK1/2 in hepatocytes.

**Conclusion**

Our present data indicate that both extracellular and intracellular Ca\(^{2+}\) is involved in hormone-induced ERK1/2 activation in cultured hepatocytes, and suggest that calmodulin and Src kinases might play a role in these signaling pathways, while the multifunctional Ca\(^{2+}\)/calmodulin-dependent protein kinase does not appear to be involved.
Figure 5
Effect of inhibitors of calmodulin (Trifluoperazine, J-8, W-7) and the multifunctional Ca\(^2+\)/calmodulin dependent protein kinase (KN-93). Hepatocytes were cultured for 3 h before preincubation with timolol (10 \(\mu\)M) for 30 min in the presence or absence of indicated inhibitors prior to stimulation for 5 min with norepinephrine (10 \(\mu\)M), PGF\(_{2\alpha}\) (10 \(\mu\)M) or A23817 (10 \(\mu\)M), before cells were harvested and ERK1/2 activation assessed. A: Pretreatment with trifluoperazine (50 \(\mu\)M) in 0.5 % DMSO. The results represent one typical experiment out of three and are expressed as percent of untreated control. B: Pretreatment with J-8 (10, 25, and 50 \(\mu\)M) in 0.5 % DMSO. The results represent mean ± S.E.M of three experiments and are expressed as percent of corresponding control values. C: Pretreatment with W-7 (100 \(\mu\)M) or KN-93 (20 \(\mu\)M) in 0.5 % DMSO, while the final DMSO concentration during incubation with agonist was 1 %. Results represent mean ± S.E.M. of five experiments and are expressed as percent of untreated control.
**Isolation and culture of hepatocytes**

Male Wistar rats (170–220 g) fed ad libitum were used. Parenchymal liver cells were isolated by in vitro collagengase perfusion and low-speed centrifugation [74] with modifications as previously described [75]. Cell viability was at least 95 %, measured as the ability to exclude trypan blue. The cells were suspended in medium and plated in Costar wells at 20,000 cells/cm², unless otherwise specified. The culture medium (0.2 ml/cm²) was a 1:1 mixture of Dulbecco’s modified Eagle’s medium and Waymouth’s medium MAB 87/3 containing 16.8 mM glucose [76], supplemented with penicillin (100 U/ml), streptomycin (0.1 mg/ml), dexamethasone (25 nM) and insulin (100 nM). The cultures were gassed with 95 % air/5 % CO₂ and kept at 37°C.

**Measurement of ERK activity**

The measurement of ERK1/2 activity was performed as previously described [17,77]. In brief, the hepatocyte cultures were exposed to agonists for 5 minutes before rinsing and scraping the cells into a 10 % ethylene glycol buffer. The lysate was centrifuged (15,800 x g) for 10 min, and the supernatant was mixed with phenyl-Sepharose which was washed twice in a 10 %, twice in a 35 % ethylene glycol buffer, and finally ERK1/2 was eluted with a 60 % ethylene glycol buffer [78]. The eluate was assayed for ERK1/2 activity with MBP as substrate, thereafter spotted onto P81 paper (Whatman, Maidstone, UK), which was washed, dried and counted in a liquid scintillation counter. Protein content was determined with the BCA Protein Assay (Pierce, Rockford, IL, U.S.A.).

**Immunoblotting**

Aliquots with 20 µg cell protein (total cell lysate prepared in Laemmli buffer) were electrophoresed on 10 % polyacrylamide gels (acylamide:N’N’-bis-methylene acrylamide 30:0.8) followed by protein electrotransfer to nitrocellulose membranes and immunoblotting with a polyclonal antibody against the dually threonine- and tyrosine phosphorylated forms of ERK1 and ERK2 (Promega Corporation, Madison, WI). Assessment of the multifunctional Ca²⁺/calmodulin dependent protein kinase was performed by immunoblotting using an antibody against the phosphorylated form of the enzyme. Immunoreactive bands were visualised with ECL Western blotting detection reagents (Amersham International).

**Measurement of cytosolic Ca²⁺ in single hepatocytes**

The calcium measurements were done as described previously [20,79]. Freshly isolated hepatocytes (50,000/cm²) were plated onto glass coverslips coated with collagen and kept in the culture medium for 30 minutes in an atmosphere of 95 % air/5 % CO₂ at 37°C. The cells were loaded in Krebs-Ringer-Hepes buffer (KRH) with 1 % albumin and 16.8 mM glucose, supplemented with 5 µM fura-2 and

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**Materials and Methods**

**Materials**

Dulbecco’s modified Eagle’s medium, Waymouth’s medium MAB 87/3, penicillin and streptomycin were from Gibco, Grand Island, NY, U.S.A. Adenosine 5’-triphosphate, collagen, collagengase, EGTA, phenylmethylsulfonyl fluoride, benzamidine, leupeptin, pepstatin A, myelin basic protein (MBP), norepinephrine, prostaglandin F₂α, epidermal growth factor, insulin, timolol, gadolinium nitrocellulose membranes and immunoblotting with a polyclonal antibody against the dually threonine- and tyrosine phosphorylated forms of ERK1 and ERK2 (Promega Corporation, Madison, WI). Assessment of the multifunctional Ca²⁺/calmodulin dependent protein kinase was performed by immunoblotting using an antibody against the phosphorylated form of the enzyme. Immunoreactive bands were visualised with ECL Western blotting detection reagents (Amersham International).

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AM, 0.25 % (v/v) DMSO, 0.025 % Pluronic F-127 and 250 µM sulfinpyrazone for 90 minutes at 37°C. After loading, the cells were washed once and incubated with 400 µl KRH buffer with 16.8 mM glucose. Cells were preincubated with timolol (10 µM) in the presence or absence of EGTA (5 mM) for 15 minutes after loading and the experiments were performed in the same buffer. Preincubation with timolol (10 µM) and DMSO or BAPTA-AM (40 µM) was performed within the last 25 minutes of the loading period. 100 µl norepinephrine was injected after 60 seconds of registration to a final concentration of 10 µM. The experiments were carried out at 37°C. Single cell Ca²⁺ measurements were based on the ratios of the fluorescence with excitation at 345 and 385 nm. Because fura-2 AM is partly compartmentalized in hepatocytes after loading, we did not calculate apparent values for [Ca²⁺]ᵢ from the ratios. The equipment consisted of a PTI-Δ-scan excitation device, a Nikon inverted microscope, a Hamamatsu CCD video camera and a Sony video recorder.

**List of abbreviations**

- BAPTA-AM, 1,2-bis(o-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid tetra (acetoxymethyl)ester; EGF, epidermal growth factor; EGTA, ethyleneglycol-bis(β-aminoethyl)-N,N,N',N'-tetraacetic acid; ERK1/2, extracellular signal-regulated kinase 1 and 2; Gd, gadolinium chloride (hexahydrate); GPCR, G protein-coupled receptor; J-8; (N-8-Aminoocetyl)-5-iodo-1-naphthalenesulfonamide; InsP₃, inositol (1,4,5)-trisphosphate; KN-93, 2-[N-(2-hydroxyethyl)-N-(4-methoxybenzenesulfonyl)] amino-N-(4-chlorocinnamyl)-N-methylbenzylamine; MBP, myelin

**Figure 7**

Effect of inhibition of Src kinases on ERK1/2 activation. A-D: Hepatocytes were cultured for 3 h prior to preincubation for 30 min with timolol (10 µM) and the Src kinase inhibitors PP1 or PP2 at indicated concentrations in 0.5 % DMSO before stimulation for 5 min with norepinephrine (10 µM), PGF₂α (10 µM), EGF (10 nM), thapsigargin (1 µM), or A23187 (10 µM). A: ERK1/2 activity, given as percent of untreated control, induced by hormonal agents expressed as the mean ± S.E.M. of four experiments after treatment with PP1 (10 µM). The inset shows an immunoblot of the effect of PP1 (20 µM) on thapsigargin-induced ERK1/2 response. B: A23187-induced ERK1/2 response after PP1 (10 µM) treatment. Results represent mean ± S.E.M of three experiments. C: Dose-response curve for the effect of PP2 on ERK1/2 activity induced by A23187. Results represent mean ± S.E.M of three experiments. All activity measurements (A-C) are expressed as percent of untreated control. D: Immunoblots showing the effect of PP1 (10 µM) or PP2 (10 µM) on ERK1/2 responses. Antibody against dually phosphorylated ERK1/2 was used.
basic protein; NE, norepinephrine; PGF$_{2\alpha}$, prostanadlin F$_{2\alpha}$; PP1, 4-aminophosphoryl-7-(t-butyl)pyrazolo [3,4-d]pyrimidine; PP2, 4-aminophosphoryl-7-(t-butyl)pyrazolo [3,4-d]pyrimidine; RTK, receptor tyrosine kinase. TFP, trifluoperazine dimaleate; Thaps, thapsigargin; TPA, 12-O-tetradecanoyl phorbol-13-acetate; W-7, N-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide.

Acknowledgements
This work was supported by The Norwegian Cancer Society, The Research Council of Norway, and the Novo Nordisk Foundation. We thank Eva Østby and Ellen Johanne Johansen for technical help and John-Arne Røttingen for helpful discussions.

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