Differential regulation of extracellular signal-regulated protein kinase 1 and Jun N-terminal kinase 1 by Ca^{2+} and protein kinase C in endothelin-stimulated Rat-1 cells

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The extracellular signal-regulated protein kinase (ERK) and Jun N-terminal kinase (JNK) signalling cascades transduce signals from the cell cytoplasm to the nucleus, where they regulate gene expression. The activation of ERK1 by lysophosphatidic acid (LPA) and endothelin 1 (Et-1) was compared in Rat-1 cells. Both stimulated DNA synthesis to a similar degree but, in contrast with LPA, Et-1 did not stimulate sustained ERK1 activation, a signal that is thought to be important for the proliferation of fibroblasts. Et-1, but not LPA, was able to activate JNK1; pharmacological analysis revealed that the same Et_A receptor mediates DNA synthesis, ERK1 and JNK1 activation. However, activation of JNK1 required higher concentrations of Et-1 than was required for stimulation of ERK1 or DNA synthesis. Signalling to ERK1 and JNK1 was partly inhibited by pertussis toxin, suggesting that both pathways are

regulated in part by G_i or G_o proteins. Activation of JNK1 by Et-1 lagged behind ERK1 activation but was not dependent on it because PD98059, an inhibitor of mitogen-activated protein kinase (or ERK) kinase, was without effect on JNK1 activation. In contrast with recent studies, activation of protein kinase C (PKC) or Ca^{2+} fluxes inhibited activation of JNK1 but not ERK1; furthermore inhibition of PKC or sequestration of Ca^{2+} potentiated JNK1 activation by Et-1 but not by anisomycin, and again had little effect on ERK1 activation. These results demonstrate that the same G-protein-coupled receptor can activate both the ERK and JNK signal pathways but the two kinase cascades seem to be separate, parallel pathways that are differentially regulated by PKC and Ca^{2+} . The results are discussed in terms of the role of ERK and JNK in proliferative signalling.

INTRODUCTION

The stimulation of cell proliferation by growth factors involves a co-ordinated series of signalling events that serve to transduce extracellular signals across the plasma membrane and into the nucleus, thereby inducing the expression of a variety of genes that are important for regulating cell cycle progression. Two such genes are the proto-oncogenes *c-fos* and *c-jun*, which are prototypes for a family of transcription factors that dimerize to form the transcription factor complex AP-1 [1]. The expression of *c-Fos* and *c-Jun* is controlled by the activation of specific kinase cascades that are regulated by growth factors.

The extracellular signal-regulated protein kinase (ERK) or mitogen-activated protein kinase (MAPK) cascade is directly implicated in the regulation of *c-Fos* expression. ERKs are serine/threonine kinases that phosphorylate and activate the transcription factor p62TCF/Elk-1, which forms part of the ternary complex that regulates the transcriptional activity of the *c-Fos* promoter serum response element or SRE [2,3]. Activation of ERKs requires a co-ordinated signal transduction cascade that involves growth factor-dependent activation of the Ras proteins, monomeric GTPases encoded by the Ras proto-oncogenes [4]. Activated Ras binds to Raf with high affinity, thereby recruiting it to the plasma membrane where it becomes activated [5,6]. Raf in turn activates mitogen-activated protein kinase (or ERK) kinase (MEK), a dual-specificity kinase which in turn phosphorylates ERK1 and ERK2 at Thr-183 and Tyr-185, thereby activating them [7–9]. Activated ERKs accumulate in the nucleus and phosphorylate, in addition to Elk-1,

several different substrates including, *c-Fos* and p90 ribosomal protein S6 kinase [10,11]. In this way growth factor-dependent signal events at the plasma membrane are transduced into changes in transcriptional machinery and gene expression.

The regulation of *c-Jun* expression and activity is regulated in part by a separate kinase signalling module known as the Jun N-terminal kinase (JNK) cascade. JNK1 and JNK2 are closely related to the ERKs but phosphorylate Ser-63 and Ser-73 of *c-Jun*, thereby activating it [12]. *c-Jun* together with ATF-2 can autoactivate its own promoter via an AP-1 site so that JNK activation leads to expression as well as activation of Jun [13]. JNKs are also named stress-activated protein kinases because they are strongly activated by cellular stresses such as UV radiation, protein synthesis inhibitors and genotoxic agents [12,14]. JNKs are activated by a kinase cascade analogous to that regulating the ERKs. A JNK-activating kinase called SEK phosphorylates JNK at threonine and tyrosine residues similar to those found in ERK; SEK is in turn activated by MEK kinase 1 (MEKK1) [15,16]. In addition to phosphorylating *c-Jun*, according to a recent study JNK might also be able to phosphorylate at least some of the residues on Elk-1 that are normally targeted by ERKs during growth factor stimulation [17]; this might point to a new pathway of cross-talk whereby JNK might regulate both *Fos* and *Jun* expression.

Recent studies in the rat pheochromocytoma cell line PC12 have led to a model proposing that the magnitude and duration of ERK activity are intimately linked to cell fate [18,19]. Specifically, nerve growth factor stimulates a sustained activation

Abbreviations used: BAPTA-AM, bis-(*o*-aminophenoxy)ethane-*N,N,N',N'*-tetra-acetic acid tetra(acetoxymethyl ester); $[\text{Ca}^{2+}]_i$, intracellular Ca^{2+} concentration; ERK, extracellular signal-regulated protein kinase; Et, endothelin; GST, glutathione S-transferase; JNK, Jun N-terminal kinase; LPA, lysophosphatidic acid; MEK, mitogen-activated protein kinase (or ERK) kinase; MEKK, MEK kinase; PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate; PTX, pertussis toxin; SEK, a JNK-activating kinase.

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and nuclear accumulation of ERK and promotes differentiation, whereas epidermal growth factor elicits a transient, non-nuclear activation of ERK and acts as a growth factor. This model raises the question as to what pattern of ERK activation is generated by growth factors in the classical models of cell proliferation such as fibroblasts. A series of studies on the mitogenic effects of thrombin have shown that the protease requires a biphasic, sustained activation of ERK1 in CCL39 fibroblasts to stimulate DNA synthesis [20,21]. In support of this we have demonstrated that in Rat-1 cells lysophosphatidic acid (LPA) stimulates a sustained activation of ERK1 [22], which seems to be required for the mitogenic effects of LPA [23].

We are particularly interested in the pathways by which 'serpentine' or G-protein-coupled receptors regulate cell proliferation. Thrombin, LPA and endothelin (Et) play major roles in orchestrating proliferative responses to injury. They are growth factors for vascular and airway smooth-muscle cells [24–26], and Et-1 is a growth factor for mesangial cells [27]. Studies involving targeted disruption of the Et-1 gene indicate that Et-1 plays a major role in cardiovascular homeostasis and that it is vital for normal mouse development [28]. In addition, Et-1 and LPA are implicated as autocrine factors in the genesis and maintenance of malignant tumours. Et-1 levels are significantly elevated in the plasma and ejaculate of men with metastatic prostate cancer, and Et-1 can act as a growth factor for a variety of prostate cancer cell lines [29]. In addition, LPA is the major autocrine growth factor in the ascites cells of a wide variety of ovarian tumours [30].

In this study we show that whereas Et-1 and LPA are equally good growth factors in Rat-1 cells, Et-1 elicits a more transient activation of ERK1, in contrast with the effects of LPA in Rat-1 cells and thrombin in CCL39 cells [21–23]. We reasoned that Et-1 must be activating some additional signal pathway that might be able to substitute for the sustained phase of ERK activity in regulating gene expression. We were prompted to examine JNK activation for two reasons. First, although JNKs are predominantly activated by stresses, they have also been shown to be activated by certain G-protein-coupled receptors in a Ca^{2+} -dependent manner [32,33]. Secondly, the ability of JNK to mediate the activation of Elk-1 by anisomycin [17] provided a plausible link between the JNK pathway and a transcription factor normally regulated by the ERK cascade. Here we show that Et-1, but not LPA, does indeed activate both ERK1 and JNK1 and present evidence that the two kinase cascades are regulated independently. In particular JNK1 activation, but not ERK1 activation, is inhibited by protein kinase C (PKC) or Ca^{2+} , in contrast with recent reports [32,33]. The results are discussed in terms of the pathways regulating ERK1 and JNK1 and the role of these kinases in mitogenic signalling.

MATERIALS AND METHODS

Materials

BQ-123 [cyclo-(D-Trp-D-Asp-Pro-D-Val-Leu)] and BQ-788 [*N*-cis-2,6-dimethylpiperidinocarbonyl-L- γ -methyl-leucyl-D-1-methoxycarbonyltryptophanyl-D-norleucine] were from Research Biochemicals International. Cell culture reagents were from Gibco BRL and pre-poured SDS/PAGE materials were from Novex Gel Systems. 1-Oleoyl-LPA was obtained from Avanti Polar lipids. Et-1 and Et-3 were from Sigma or Calbiochem. Pertussis toxin (PTX) was from List Biologicals or Calbiochem. GF109203X was from Calbiochem. Bis-(*o*-aminophenoxy)ethane-*N,N,N',N'*-tetra-acetic acid tetra(acetoxymethyl ester) (BAPTA-AM) was from LC Laboratories.

JNK1 C-terminal antisera was from Santa Cruz Biotechnology. [γ - ^{32}P]ATP and [^3H]thymidine were from NEN–DuPont. All other reagents, including Protein A–Sepharose beads, phorbol esters and myelin basic protein, were from Sigma.

Cells and cell culture

Rat-1 cells were maintained in Dulbecco's modified Eagle's medium containing glutamine, penicillin/streptomycin and 10% fetal bovine serum. Cells were washed once in serum-free medium and placed in fresh serum-free medium 24 h before starting the experiments described. A Rat-1 cell line that expressed physiological levels of an epitope-tagged ERK2 construct (mycERK2) was isolated and grown under identical conditions but with the addition of G418 to the media.

Cell stimulation

For ERK1 and ERK2 assays, experiments were performed on 6-well plates of confluent, quiescent cells that had been serum-starved for 24–36 h. Medium was replaced with 1 ml of serum-free Dulbecco's modified Eagle's medium containing the desired growth factor, and stimulations were performed at 37 °C for the required time. Incubations were terminated by aspiration and addition of ice-cold TG lysis buffer [20 mM Tris/HCl (pH 8.0)/1% (w/v) Triton X-100/10% (v/v) glycerol/137 mM NaCl/1.5 mM MgCl_2 /1 mM EGTA/50 mM NaF/1 mM Na_3VO_4 /1 mM Pefabloc/20 mM leupeptin/10 $\mu\text{g}/\text{ml}$ aprotinin]. All manipulations of cell lysates were performed at 4 °C. The plates were rocked for 20 min, and lysates were collected into Eppendorf tubes and centrifuged for 10 min at 14000 rev./min to remove nuclei and detergent-insoluble material. A Bio-Rad microprotein assay was used to determine protein content and values were routinely found to vary by no more than 10%.

Immune-complex kinase assays for ERK1/ERK2

Anti-peptide antibodies directed against the extreme C-termini of ERK1 (E1.2) were as described previously [22]; this serum exclusively immunoprecipitates ERK1. Cell lysates, typically 100 μg of protein, were immunoprecipitated with 3 μl of crude antiserum and Protein A–Sepharose beads at 4 °C for 2–3 h. ERK2 was assayed from mycERK2-expressing cells by immunoprecipitation with an anti-myc antibody (9E10) prebound to Protein G–Sepharose beads. Immunoprecipitates were collected by centrifuging for 10 s at 14000 rev./min and washed twice with 1 ml of TG lysis buffer. Immune complexes were washed in kinase buffer [30 mM Tris (pH 8.0)/20 mM MgCl_2 /2 mM MnCl_2] before being resuspended in 30 μl of a mix of kinase buffer, 6 μg of myelin basic protein, 10 μM unlabelled ATP and 2.5 μCi of [γ - ^{32}P]ATP per sample. Incubations were conducted for 30 min at 30 °C and were terminated by the addition of hot 4 $\times$ SDS/PAGE sample buffer, followed by boiling for 5 min at 95 °C. Samples were resolved by SDS/PAGE [14% (w/v) gel] with the Novex gel system. The gel was stained with Coomassie Brilliant Blue, dried and autoradiographed. The Coomassie Blue-stainable MBP band was excised from the dried gel and the incorporated radioactivity determined by scintillation counting.

Immune-complex kinase assays for JNK1

Confluent Rat-1 cells were serum-starved overnight before stimulation with various agents. Cells were lysed in ice-cold lysis buffer and cell lysates collected as above.

Immunoprecipitations were performed on equivalent amounts of clarified lysate. Protein A–Sepharose (40 μl) and 4 μl of anti-JNK1 antiserum were added to each lysate. Samples were rotated

for 2 h at 4 °C. Immunoprecipitates were collected by brief centrifugation and washed once with lysis buffer and once with 'JNK' kinase buffer [20 mM Hepes (pH 7.5)/20 mM β -glycerophosphate/10 mM $MgCl_2$ /1 mM dithiothreitol/50 μ M sodium orthovanadate] before pellets were drained completely with a fine-gauge Hamilton syringe. The immune complexes were then resuspended in 30 μ l of a mix of 'JNK' kinase buffer, 1 μ M unlabelled ATP, 100 mM *p*-nitrophenyl phosphate, glutathione S-transferase (GST)-conjugated c-Jun and 2.5 μ Ci of [γ - 32 P]ATP. Incubations were conducted for 30 min at 30 °C and terminated by the addition of 10 μ l of 4 $\times$ SDS/PAGE sample buffer. After being boiled for 5 min, samples were resolved by SDS/PAGE [12% (w/v) gel], stained, dried and autoradiographed. The Coomassie Blue-stained GST-c-Jun bands were excised from the dried gels and the radioactivity incorporated was determined by scintillation counting.

Assay of DNA synthesis by [3 H]thymidine incorporation

Matched confluent cultures of Rat-1 cells were washed once in serum-free medium and then incubated in fresh serum-free medium for 24 h before being stimulated with the appropriate growth factors for a further 24 h in the presence of 5 mM unlabelled thymidine. Reinitiation of DNA synthesis was assayed by the incorporation of a pulse of [3 H]thymidine (1 μ Ci/ml) during the last 5 h of stimulation. The radioactivity incorporated into trichloroacetic acid-precipitable material at the end of the stimulation was determined after solubilization in 0.1 M NaOH and liquid-scintillation counting.

Reproducibility and analysis of results

ERK1 and JNK1 kinase assays were performed on duplicate samples except for time-course studies, where single points were used. We and others in the field [20,21] have found these time courses to be highly reproducible. Assays of [3 H]thymidine incorporation were performed in duplicate. In all cases a representative experiment is shown and is accompanied by pooled data from between three and nine experiments. The pooled data were used for statistical analysis by Student's *t* test with the Statview program for Macintosh; *P* values are quoted in the text or indicated by * (*P* < 0.05) or ** (*P* < 0.01).

RESULTS

LPA and Et-1 stimulate DNA synthesis to the same extent in Rat-1 cells

Rat-1 cells were treated with serum, LPA or Et-1 and assayed for their ability to promote DNA synthesis in quiescent Rat-1 cells. The results showed that at maximal doses, Et-1 exhibited a mitogenic efficacy similar to that of LPA. Both agonists stimulated DNA synthesis, which amounted to 50–60% of that observed with 10% fetal bovine serum. Results from a single representative experiment, expressed as [3 H]thymidine incorporated into trichloroacetic acid-precipitable DNA, were 1508 ± 492 c.p.m. for basal, 24160 ± 407 c.p.m. for 10% serum, 15730 ± 2536 c.p.m. for 100 μ M LPA and 14900 ± 1581 c.p.m. for 100 nM Et-1 (means $\pm$ S.D. for duplicate stimulations). In addition, maximal doses of Et-3 were also able to elicit a robust response, similar in magnitude to that seen with Et-1 (results not shown).

Et-1 and Et-3 activate ERK1 in a transient manner

In Rat-1 fibroblasts mitogenic doses of LPA activated ERK1 in a co-ordinated biphasic manner consisting of an early peak

Figure 1 A comparison of the kinetics of LPA- and endothelin-stimulated ERK1 activity in Rat-1 cells

Quiescent, serum-starved Rat-1 cells were stimulated with Et-1 (□), Et-3 (○), LPA (■) or vehicle (▲) for various times up to 3 h. Detergent lysates were immunoprecipitated with E1.2 antisera and ERK1 activity was determined by immune-complex kinase assays with MBP as a substrate. Results are expressed as fold increase in ERK1 MBP kinase activity and were pooled from *n* = 3–8 experiments. ERK1 activity was statistically elevated over vehicle control for all agonists between 5 and 30 min but only for LPA after 2 or 3 h (*P* < 0.05).

followed by a smaller sustained phase (Figure 1). The response to LPA remained significantly elevated above basal (*P* < 0.05) for the full 3 h of the time course (Figure 1) and was still significantly elevated after 7 h [23]. In contrast, Et-1 and Et-3 activated ERK1 in a transient manner (Figures 1 and 2B), with a smaller peak of activity at 5–10 min than that observed with LPA. In some experiments Et-1-stimulated ERK1 activity declined rapidly from 10 to 30 min (Figure 2B), whereas in others the peak response was broader so that the 30 min value was close to that seen at 10 min (Figure 1). However, in all cases ERK1 activity was not significantly elevated above basal from 1 h onwards (*P* > 0.05) in contrast with the effects of LPA at these times. ERK2 activation, measured with an epitope-tagged ERK2 construct stably expressed at physiological levels in a clonal Rat-1 cell line (R1MycERK2), showed a similar pattern with a sustained phase of activation only being seen with LPA (results not shown). Thus in Rat-1 cells, Et-1 stimulated DNA synthesis despite only activating ERK1 and ERK2 in a transient manner, contrasting with the idea that sustained ERK activation is required to promote DNA synthesis [18,20,21].

Endothelin-1 activates JNK1 in Rat-1 cells

Because Et-1 stimulated DNA synthesis but gave only transient ERK activation, we reasoned that it must be activating an additional signalling pathway that could regulate gene expression during the *G*₁ phase of the cell cycle. JNK phosphorylates and activates c-Jun but has recently also been shown to activate Elk-1, a transcription factor that regulates c-Fos expression in response to activation of the ERK cascade [17]. In addition, some G-protein-coupled receptors have been shown to activate JNK [32,33].

Figure 2A shows that Et-1 did indeed activate JNK1. In these experiments we stimulated cells, prepared lysates and divided them into equal portions that were assayed in parallel for either

Figure 2 Kinetics of JNK1 (A) and ERK1 (B) activation by Et-1

(A) Serum-starved Rat-1 cells were treated with vehicle (□) or 100 nM Et-1 (■) for various times up to 3 h. Cells were lysed and equal aliquots of lysate were assayed for JNK1 activity by immunoprecipitation of endogenous JNK1, followed by a kinase assay that measured phosphorylation of GST-c-Jun. Results are expressed as fold increase in JNK1 activity. (B) Equal portions of the same cell lysates as were used in (A) were assayed for ERK1 activation after immune precipitation of endogenous ERK1. Results are expressed as fold increase in ERK1 MBP kinase activity. The results shown were pooled from $n = 3$ experiments and are representative of three to five experiments in which both ERK1 and JNK1 were assayed and gave similar results.

ERK1 or JNK1 activation. As a result all experiments had internal controls and this approach allowed us to compare the regulation of the two enzymes in Rat-1 cells under identical cellular conditions. Activation of ERK1 by Et-1 was again transient, peaking at 10 min and declining to basal by 60 min (Figure 2B). In the same samples JNK1 activation lagged behind that of ERK1 but rose to a peak after 30 min of stimulation with Et-1, at which time JNK1 was activated 8 ± 2.5 -fold over the control (mean $\pm$ S.D. of 11 experiments). Activation of JNK1 by Et-1 was also transient, returning to basal levels by 1–2 h. In the same assays no activation of JNK1 by LPA was observed (results not shown).

Activation of ERK1, JNK1 and DNA synthesis by Et-1 is mediated by the Et_A receptor in Rat-1 cells

The ability of Et-1 to activate both the ERK1 and JNK1 cascades raised the question of whether the same or different receptors were mediating activation of these pathways. There are two well-characterized endothelin receptors, which are widely distributed in vascular and muscular tissue types [34,35] and can be distinguished by their affinities for the three Et isopeptides. The Et_A receptor is Et-1 selective showing a distinct order of

affinities (Et-1 > Et-2 $\gg$ Et-3), whereas the Et_B receptor has similar affinities for all three isopeptides.

The endothelins stimulated DNA synthesis in a dose-dependent manner in Rat-1 cells (Figure 3A) with EC₅₀ values of 0.65 ± 0.3 nM for Et-1 (mean $\pm$ S.D., $n = 6$ experiments) and 150 ± 17.3 nM for Et-3 (mean $\pm$ S.D., $n = 3$; results not shown). The EC₅₀ for Et-1 in the presence of BQ-123 (an Et_A receptor antagonist [36]) was shifted to the right by approximately one order of magnitude to 6.5 ± 3.5 nM (significantly different from Et-1 alone, $P < 0.05$), whereas BQ-788 (a potent and selective Et_B receptor antagonist [37]) gave a small and non-significant shift (1.2 ± 0.7 nM) (Figure 3A). BQ-123 abolished DNA synthesis in response to Et-3, whereas BQ-788 had only a small effect on the EC₅₀ for Et-3 (287 ± 99 nM) (results not shown). These results are consistent with the stimulation of DNA synthesis by Et-1 and Et-3 in Rat-1 cells proceeding via an Et_A receptor.

We next assayed ERK1 and JNK1 activation at different doses of Et-1 by using the time points of maximum response for ERK1 (10 min) or JNK1 (30 min). Activation of ERK1 by Et-1 exhibited an EC₅₀ of 0.3 ± 0.25 nM (mean $\pm$ S.D., $n = 3$ experiments) (Figure 3B), which agreed well with that for stimulated DNA synthesis and was consistent with the affinity of Et-1 for the Et_A receptor [38]. The EC₅₀ for activation of ERK1 by Et-3 was 85 ± 49 nM (results not shown) and agreed well with the K_d for the dissociation of Et-1 from the Et_A receptor by Et-3 [38]. Activation of JNK1 by Et-1 exhibited an EC₅₀ of 17 ± 11 nM, at least one order of magnitude to the right of that for ERK1 activation and DNA synthesis; a disparity was also noted for the EC₅₀ for Et-3-stimulated activation of JNK1, which was 350 ± 132 nM. This disparity in EC₅₀ values for ERK1 and JNK1 was not due to degradation of Et-1 during the stimulation time required for maximal JNK1 activation, which was 20 min longer, because we have found that the reconstitution of Et-1 in media for up to 3 h before addition to cells has no apparent effect on the efficacy or potency of Et-1 responses (results not shown).

The relative EC₅₀ values for Et-1- and Et-3-stimulated ERK1 and JNK1 activity were consistent with an Et_A receptor mediating both responses. This was confirmed by the demonstration that the Et_A antagonist BQ-123 strongly and significantly inhibited activation of both ERK1 and JNK1 by Et-1, whereas BQ-788 had little reproducible effect (Table 1). Taken together, these results suggest that Et-1 activates both ERK1 and JNK1 via the same Et_A receptor but that JNK1 is activated only at doses of Et-1 well in excess of those needed for ERK1 activation or DNA synthesis.

Et-1-stimulated ERK1 and JNK1 activation proceeds via PTX-sensitive and PTX-insensitive pathways

Because Et-1 seemed to activate both JNK1 and ERK1 via the same receptor in Rat-1 cells, experiments were performed to determine at which point the signal paths diverge. PTX is known to block interactions between receptor and G_i or G_o proteins by ADP-ribosylating the C-terminus of G_q or G_o. In Rat-1 cells it is known that Et-1 activates inositol lipid hydrolysis via a PTX-insensitive pathway that probably reflects activation of PLC β by a member of the G_q family of GTPases [39].

Rat-1 cells pretreated with or without PTX were stimulated with Et-1 and assayed for JNK1 and ERK1 as described in the Materials and methods section. Under these conditions PTX completely inhibits LPA-mediated inhibition of adenylate cyclase in intact Rat-1 cells [40] and blocks LPA-stimulated high-affinity GTPase activity in Rat-1 cell membranes [41]. Table 2 shows that the activation of both ERK1 and JNK1 by Et-1 were inhibited

Figure 3 Dose-response curves for Et-1-stimulated DNA synthesis (A) and ERK1 or JNK1 activation (B) in Rat-1 cells

(A) Quiescent, serum-starved Rat-1 cells were stimulated with increasing concentrations of Et-1 alone (■) or with BQ-123 (▲) or BQ-788 (□) at 1 μ M for 24 h. [3 H]Thymidine was added for the last 5 h and trichloroacetic acid-precipitable radioactivity (mean $\pm$ S.D. of duplicate stimulations) was measured by liquid-scintillation counting. Results are from a single experiment representative of four giving similar results. (B) Quiescent, serum-starved Rat-1 cells were stimulated with increasing concentrations of Et-1 for 10 min (ERK1) or 30 min (JNK1). Detergent lysates were used to immunoprecipitate ERK1 (■) or JNK1 (□) and enzyme activities in the immune complex were assayed as described in the text. Results are expressed as a percentage of the maximum response and are pooled from $n = 3$ (ERK1) or $n = 4$ (JNK1) experiments.

Table 1 The effect of BQ123, BQ788 and PD98059 on Et-1-stimulated ERK1 and JNK1 activity in Rat-1 cells

Quiescent, serum-starved Rat-1 cells were pretreated for 20 min with BQ-123 (1 μ M), BQ-788 (1 μ M) or PD98059 (50 μ M) before stimulation with Et-1 for 10 min (ERK1) or 30 min (JNK1). Detergent lysates were prepared and assayed for ERK1 or JNK1 activity in immune complexes as described in the text. ERK1 activity is expressed as 32 P incorporated into MBP; JNK1 as 32 P incorporated into GST-c-Jun. Results from a single representative experiment performed in duplicate are shown, and also as pooled data (percentage of maximum) from $n = 4$ experiments (in duplicate) expressed as a percentage of the maximum response. The asterisks indicate the statistical significance with respect to the corresponding control value: ** $P < 0.01$; * $P < 0.05$.

Stimulus	ERK1 activity		JNK1 activity	
	(c.p.m.)	Pooled data (% of maximum)	(c.p.m.)	Pooled data (% of maximum)
Vehicle	39585 $\pm$ 2198		796 $\pm$ 201	
Et-1	111449 $\pm$ 11572	100	8011 $\pm$ 2237	100
Et-1 + BQ123	51816 $\pm$ 3763	26 $\pm$ 11*	3588 $\pm$ 566	27 $\pm$ 20*
Et-1 + BQ788	106736 $\pm$ 7421	104 $\pm$ 16	9591 $\pm$ 1363	106 $\pm$ 13
Et-1 + PD98059	28614 $\pm$ 3593	2 $\pm$ 3**	13372 $\pm$ 1658	119 $\pm$ 40

Table 2 Effect of pertussis toxin on Et-1-stimulated ERK1 and JNK1 activity

Quiescent, serum-starved Rat-1 cells were pretreated with vehicle or 100 ng/ml pertussis toxin for 18 h before stimulation with 100 nM Et-1 for 10 min (ERK1) or 30 min (JNK1). ERK1 and JNK1 activities were assayed as described in the text. ERK1 activity is expressed as 32 P incorporated into MBP; JNK1 as 32 P incorporated into GST-c-Jun. Results from a single representative experiment performed in duplicate are shown, and also as pooled data (percentage of maximum) from $n = 4$ experiments (in duplicate) expressed as a percentage of the maximum response. The asterisks indicate the statistical significance with respect to the corresponding control value: * $P < 0.05$.

Stimulus	ERK1 activity		JNK1 activity	
	(c.p.m.)	Pooled data (% of maximum)	(c.p.m.)	Pooled data (% of maximum)
Vehicle	25469 $\pm$ 251		924 $\pm$ 48	
Et-1	67526 $\pm$ 7641	100	10614 $\pm$ 1403	100
Et-1 + PTX	47561 $\pm$ 1025	47 $\pm$ 10*	4589 $\pm$ 258	43 $\pm$ 11*

by 40–50% in PTX-treated cells, implying that the activation of both kinases proceeds, at least partly, through a G_i or G_o -regulated pathway. In the same series of experiments, LPA-stimulated ERK activation was strongly inhibited by PTX (72 $\pm$ 13%; mean $\pm$ S.D., $n = 6$ determinations; results not

shown); however, LPA did not activate JNK1 so the effect of PTX could not be determined. The simplest interpretation of these differential sensitivities to PTX is that they represent the relative use of G_i - or G_o -regulated pathways by LPA or ET-1 to activate ERK1 and JNK1.

Figure 4 Et-1-stimulated JNK1 activation is negatively regulated by Ca^{2+}

(A, C) Serum-starved Rat-1 cells were treated with vehicle (■, △) or with 3 mM EGTA and 20 μM BAPTA-AM (□) for 20 min before a 10, 30 or 60 min stimulation with Et-1 (■, □). A time course of treatment with 5 μM ionomycin (▲) or vehicle (△) was also performed. Cells were lysed and equal aliquots used to assay JNK1 activity (A) or ERK1 activity (C). Results are expressed as fold increase in JNK1 or ERK1 activity (means $\pm$ S.D.) and are pooled from $n = 4$ (ERK1) or $n = 6$ (JNK1) experiments. (B, D) Serum-starved Rat-1 cells were treated with 5 μM ionomycin (□) or vehicle (■) for 20 min before a 10, 30 or 60 min stimulation with Et-1 (■, □) or vehicle (△). Cells were lysed and equal aliquots used to assay JNK1 activity (B) or ERK1 activity (D). Results are expressed as fold increase in JNK1 (B) or ERK1 (D) activity over the control (means $\pm$ S.D.) and are pooled from $n = 4$ (ERK1) or $n = 3$ (JNK1) experiments. The asterisks indicate statistical significance with respect to the control value: * $P < 0.05$.

The ability of PTX to inhibit both ERK1 and JNK1 activation by Et-1 was not due to a toxic effect of PTX because epidermal growth factor, a non-G-protein-coupled receptor agonist, was not impaired in its ability to stimulate DNA synthesis or ERK1 in PTX-treated Rat-1 cells [23,40] or in CCL39 cells ([42], and S. J. Cook, unpublished work). In addition, PTX-treated cells were still responsive to classical stress stimuli such as anisomycin or UV. For example, JNK1 activity (in c.p.m. of ^{32}P incorporated into GST-c-Jun) was 1357 ± 160 in untreated cells, 28453 ± 33 in response to anisomycin (25 $\mu\text{g}/\text{ml}$; 1 h) and 27902 ± 3473 in response to UV (800 mJ followed by a 1 h recovery). In PTX-treated cells the basal value was 2006 ± 215 , that with anisomycin was 26576 ± 2629 and that with UV was 31102 ± 225 . In no case was there a statistically significant difference between the control and PTX-treated data sets. Thus PTX does not compromise the ability of Rat-1 cells to respond to either tyrosine kinase growth factors (the ERK1 pathway) or cellular stresses (the JNK1 pathway) but rather acts as a selective inhibitor of G_i - or G_o -dependent signalling pathways to these kinases.

The slower activation of JNK1 by Et-1 is not dependent on prior activation of ERK

The fact that the peak of ERK1 activation preceded the peak of JNK1 activation (Figure 2) raised the possibility that activation

of JNK1 was dependent on prior activation of the ERK cascade. To address this we used the MEK1 inhibitor PD98059 [43]. Rat-1 cells were treated with PD98059 for 20 min before stimulation with Et-1. ERK1 activation was severely inhibited (more than 95% inhibition) but PD98059 had no significant effect on the activation of JNK1 by Et-1 (Table 1). This suggests that activation of JNK1 does not require a functional MEK-ERK signal cascade, so if there is any cross-talk between the two pathways it does not occur at the level of MEK1. LPA-stimulated ERK1 activation was inhibited by PD98059 (results not shown) but again LPA alone was unable to activate JNK1.

Et-1-stimulated JNK1 activation is negatively regulated by Ca^{2+}

Et-1 elevates intracellular Ca^{2+} levels in Rat-1 cells [44–46], and activation of JNK by muscarinic acetylcholine receptors and angiotensin II receptors has been shown to be Ca^{2+} -dependent [32,33] so we studied the effects of Ca^{2+} on Et-1-stimulated JNK1 and ERK1 activation.

Treatment with EGTA (to chelate extracellular Ca^{2+}) together with BAPTA-AM [to buffer intracellular Ca^{2+} ($[\text{Ca}^{2+}]_i$)] effectively prevents increases in $[\text{Ca}^{2+}]_i$ in many cell types including Rat-1 cells [45,46]. In contrast with the study of Mitchell et al. [32], 20 μM BAPTA-AM/3 mM EGTA potentiated the ability of Et-1 to activate JNK1 (Figure 4A) so that the onset of JNK1

Figure 5 Et-1-stimulated JNK1 activation is negatively regulated by PKC

(A) Serum-starved Rat-1 cells were treated with vehicle (△, ■), 1 μ M PMA for 36 h (▲) or 5 μ M GF109203X (□) for 20 mins before a 10, 30 or 60 min stimulation with Et-1 (■, ▲, □) or vehicle as a control (△). Cell lysates were prepared and assayed for JNK1 activity. Results are expressed as fold increase in JNK1 activity over control and are pooled from $n = 3$ (PMA, 36 h) or $n = 6$ (GF109203X) experiments. (B) Serum-starved Rat-1 cells were treated with Et-1 alone (■) or 100 nM PMA for 20 min before a 10, 30 or 60 min treatment with Et-1 (□), or vehicle (○). Cells were lysed and JNK1 activity was assayed as previously described. Results are expressed as fold increase in JNK1 activity over control and are pooled from $n = 3$ –5 experiments. The asterisks indicate statistical significance with respect to the control value: ** $P < 0.01$; * $P < 0.05$.

activation was accelerated and the response was more sustained. For example, JNK1 activation after a 10 min Et-1 stimulation in the presence of BAPTA-AM/EGTA (7.6 ± 2.1 -fold, $n = 5$) was greatly potentiated compared with the normal response after 10 min of Et-1 (1.6 ± 0.6 -fold; values are significantly different, $P < 0.01$). In addition, the Et-1 response was clearly sustained at 60 min in the presence of BAPTA-AM/EGTA (11.6 ± 3.8 -fold) in contrast with the normal Et-1 response after 60 min (2.3 ± 1.0 -fold; significantly different, $P < 0.005$). However, the magnitude of the maximum response observed after 30 min was not significantly potentiated. Treatment with ionomycin alone to elevate $[Ca^{2+}]_i$ was unable to activate JNK1 (Figure 4A). Thus increased $[Ca^{2+}]_i$ is not sufficient to activate JNK1 but seems rather to inhibit the pathway.

To confirm this observation we pretreated Rat-1 cells with ionomycin to increase $[Ca^{2+}]_i$ and assayed the effect on the

subsequent activation of ERK1 or JNK1 by Et-1. Activation of JNK1 by Et-1 was completely abolished by pretreatment with ionomycin, indicating that pretreating to elevate $[Ca^{2+}]_i$ blocks activation of JNK1 (Figure 4B). For example, the activation of JNK1 observed after 30 min of stimulation with Et-1 (5.8 ± 2.1 -fold) was significantly decreased by pretreating with ionomycin (0.7 ± 0.5 -fold, statistically significant difference, $P < 0.001$). This again showed that increasing Ca^{2+} levels inhibits JNK1.

Ionomycin or A23187 alone were able to stimulate ERK1, so $[Ca^{2+}]_i$ increases are sufficient to activate ERK1 (Figure 4C). However, EGTA/BAPTA-AM treatment had no statistically significant effect on Et-1-stimulated ERK1 activation assayed after 10, 30 or 60 min (Figure 4C), showing that Et-1 can activate ERK1 just as efficiently in the absence of Ca^{2+} . The lack of effect of BAPTA-AM/EGTA treatment was not due to lack of efficacy of these agents because the ability of the Ca^{2+} ionophore A23187 to activate ERK1 was completely inhibited by this treatment. For example, ERK1 activity was elevated 8.6 ± 2.7 -fold after 10 min with A23187, whereas in BAPTA-AM/EGTA-treated cells the response was increased 1.9 ± 1.2 -fold and in EGTA-treated cells 1.7 ± 0.7 -fold (both treated data sets significantly different from the control A23187 response, $P < 0.05$). Similar results were obtained with ionomycin (results not shown). It is therefore clear that the BAPTA-AM/EGTA treatment indeed inhibited Ca^{2+} -dependent signalling.

In the same experiments, where pretreatment with ionomycin abolished JNK1 activation by Et-1, there was little effect on Et-1-stimulated ERK1 activation (Figure 4D). Thus the effects of Ca^{2+} seemed for the most part to be selective for the JNK1 cascade.

Et-1-stimulated JNK1 activation, but not ERK1 activation, is negatively regulated by PKC

The role of PKC in ERK1 and JNK1 activation by Et-1 was investigated by treating cells with the selective PKC inhibitor GF109203X [47]. When GF109203X-treated cells were stimulated with Et-1 there was a substantial potentiation of the ability of Et-1 to activate JNK1 (Figure 5A). As with BAPTA-AM/EGTA treatment, the onset of JNK1 activation was accelerated so that significant activation could be seen at earlier times such as 10 min. In addition, GF109203X also potentiated the maximum response observed after 30 min of treatment with Et-1; for example JNK1 activity was increased 7.3 $\pm$ 2.3-fold by Et-1 in control cells ($n = 5$) but in the presence of GF109203X this response was increased to 29.4 ± 6.4 -fold ($n = 5$); this was a statistically significant increase ($P < 0.005$). Similar, though less marked, results were observed when cells were pretreated with phorbol 12-myristate 13-acetate (PMA) for 48 h to down-regulate PKC (Figure 5A). Such a treatment depletes Rat-1 cells of PKC- α , PKC- δ and PKC- ϵ but not PKC- ζ [48]. Under these conditions JNK1 activation was 14.6 ± 0.6 -fold over the control compared with the response to Et-1 alone (7.3 ± 2.3 -fold); these values were significantly different ($P < 0.05$).

A 20 min pretreatment with PMA to activate PKC abolished subsequent JNK1 activation by Et-1, supporting the conclusion that activation of PKC inhibits activation of JNK1 by Et-1 (Figure 5B). For example, JNK1 activation in response to Et-1 after 30 min was 5.8 ± 2.6 -fold over the control, whereas after acute PMA pretreatment this response was decreased to 1.5 ± 0.5 -fold (mean $\pm$ S.D., $n = 5$); these values were significantly different at $P = 0.03$. Under similar conditions pretreatment with PMA had no effect on ERK1 activation by Et-1 (results not shown), indicating that, as with Ca^{2+} , PKC regulates the pathways leading to activation of JNK1 and ERK1 separately.

Table 3 Activation of JNK1 by anisomycin is not potentiated by GF109203X

Serum-starved Rat-1 cells were pretreated with 5 μ M GF109203X for 20 min and then stimulated with 50 μ g/ml anisomycin for 30 min or 100 nM Et-1 for 10 or 30 min. Detergent lysates were assayed for JNK1 activity as described in the text. Results are expressed as radioactivity incorporated into GST-c-Jun from two experiments performed under identical conditions. Abbreviation: n.d., not determined.

Stimulus	JNK1 activity (c.p.m)	
	Expt. 1	Expt. 2
Control	640	354
Et-1 (10 min)	1444	n.d.
Et-1 (10 min) + GF109203X	5011	n.d.
Et-1 (30 min)	n.d.	2151
Et-1 (30 min) + GF109203X	n.d.	7362
Anisomycin	15687	6154
Anisomycin + GF109203X	14628	5091

Pretreatment with GF109203X did inhibit PMA-stimulated ERK1 activation, indicating its efficacy as a PKC inhibitor, but Et-1-stimulated ERK activation was largely unaffected by GF109203X treatment, suggesting that the Et-1-stimulated ERK1 activation is largely independent of PKC. For example, Et-1-stimulated ERK activation was 7.2 ± 2.2 -fold over the control after 10 min of treatment with Et-1 alone. In the presence of GF109203X this response was 12.6 ± 5.5 -fold but although several experiments showed a small increase this was not significantly different by *t* test ($P < 0.05$). Results from a representative experiment were: basal, 11020 ± 223 c.p.m.; PMA (10 min), 53044 ± 7764 c.p.m.; PMA + GF109203X, 10731 ± 533 c.p.m.; Et-1 (10 min), 48803 ± 6113 c.p.m.; Et-1 + GF109203X, 46746 ± 6154 c.p.m. (mean $\pm$ S.D., duplicate determinations).

JNK1 activation by anisomycin is insensitive to GF109203X

To try to determine the site of action of GF109203X we compared its effects on JNK1 activation by a classical stress stimulus, anisomycin [49], with that of Et-1. In side-by-side analysis the activation of JNK1 observed with anisomycin was unaffected by GF109203X, whereas the response to Et-1 was again potentiated (Table 3). In separate experiments we determined that the EC_{50} for JNK1 activation by Et-1 was not altered by pretreatment with GF109203X (Et-1, 17 ± 3 nM; Et-1 + GF109203X, 18.5 ± 1 nM; results not shown), suggesting that receptor affinity was unchanged by this treatment. These results suggest that GF109203X is unlikely to be inhibiting a shared component such as MEKK, SEK or JNK1 itself but instead might affect one of the elements coupling the Et-1 receptor to the JNK1 cascade.

DISCUSSION

The ERK and JNK cascades are agonist-stimulated protein kinase cascades that transduce signals into the nucleus to modulate the activity of Elk-1, and therefore the expression of c-Fos (ERK), and c-Jun (JNK). The ERK cascade is strongly activated by growth and differentiation factors, and sustained activation is thought to be an important signal for promoting cell proliferation and differentiation [18–22]. The JNK cascade is strongly activated by cellular stresses but its role in proliferative signalling is unclear [14,50]. In the course of comparing the effects of LPA and Et-1 on DNA synthesis and ERK activation we discovered that Et-1 is a growth factor for Rat-1 cells but elicits only transient ERK activation. We observed that Et-1 is,

in addition, able to activate the JNK1 pathway, and biochemical analysis revealed that the ERK1 and JNK1 pathways are regulated quite differently; in particular, the activation of JNK1 by Et-1 is subject to a strong negative regulation by Ca^{2+} and PKC.

Et-1 is a growth factor but does not stimulate sustained ERK activity

We found that Et-1 stimulated both ERK activity and DNA synthesis with essentially identical EC_{50} values via the same Et_A receptor, indicating a strong pharmacological correlation between these two events. This is consistent with previous reports [51–54]. For LPA and epidermal growth factor in Rat-1 cells this correlation is strengthened by the observation that they both elicit sustained ERK1 activity [22,23]. The sustained activation of ERK1 and 2 is associated with their nuclear accumulation, providing a link to transcriptional regulators such as p62TCF/Elk-1. PC12 cells require a sustained activation of ERK to differentiate in response to nerve growth factor [18,19], whereas in model proliferative systems such as CCL39 cells there is a strong correlation between sustained ERK activity and DNA synthesis for α -thrombin [20,21]. The observation that Et-1 exhibits similar proliferative efficacy as LPA but elicits only a transient ERK activation clearly questions the necessity for sustained ERK activity in mitogenic signalling and raises the question of why LPA, but not Et-1, stimulates sustained ERK activity. On the latter point we note that up to 70% of LPA-stimulated ERK activity proceeds through a G_i -regulated pathway, whereas PTX inhibits only 40% of the response to Et-1. This suggests that the degree of coupling to a G_i -regulated pathway might correlate positively with sustained ERK activation, as seems to occur in CCL39 cells [20].

The transient ERK1 response seen here with Et-1, which is reminiscent of the hypertrophic response to Et-1 seen in cardiac myocytes [54], suggests that the notion of sustained ERK activation's being important for proliferative signalling is an oversimplification. It is now apparent that the ERK cascade is just one of several kinase cascades (such as JNK and p38) that can converge to regulate gene expression in the nucleus [55] in response to a variety of growth factors, trophic agents and stresses. Recent studies suggest that the JNK pathway might also be able to regulate c-Fos expression [17], whereas some G-protein-coupled receptors stimulate a novel pathway converging on the serum response factor of the c-Fos promoter, which may be regulated by the small GTPase Rho [56]. The duration of ERK signalling and translocation to the nucleus will depend on the agonist, receptor and tissues involved as well as other factors such as duration of receptor signalling [11,18,19,21,57], qualitative differences in signal pathway [20,23] and the expression of MKP-1, the nuclear, dual-specificity phosphatase that inactivates ERK1 and ERK2 in the nucleus [58]. Therefore, although ERK activation might be an absolute requirement for the G_1 to S transition, different growth factors might make different use of combinations of sustained or transient ERK together with activation of additional signal pathways to execute the changes in gene expression required for G_1 progression.

Et-1 activates ERK1 and JNK1 via the same Et_A receptor

At least one additional pathway that Et-1 activates is the JNK cascade, which is also activated by other G-protein-coupled receptors [32,33,59]. Et-1 stimulated a pronounced increase in JNK1 activity that in kinetic terms lagged somewhat behind the activation of ERK, confirming the recent reports by Bogoyevitch et al. [25] and Shapiro et al. [60], which were published during the

completion of this work. We have extended these observations by showing that both ERK1 and JNK1 are activated via an E_{A} receptor but JNK1 is significantly activated only at high receptor occupancy, whereas the activation of ERK is correlated more closely in dose terms with the K_{d} for the E_{A} receptor [38] and for DNA synthesis (Figure 3A). A similar disparity in EC_{50} values was recently reported for the ability of cholecystokinin to activate ERK and JNK in rat pancreatic acini [61]; the reason for this disparity is currently unclear and experiments are in progress to investigate this issue.

Activation of ERK1 and JNK1 by Et-1 were both partly inhibited by PTX, suggesting that a G_i or G_o protein is partly involved in the activation of both cascades. The PTX-insensitive component is unlikely to be a G_q -PLC β pathway because ERK activation is not inhibited by Ca^{2+} chelators or PKC inhibition, and JNK1 activation is greatly potentiated by these agents. Activated mutants of $G\alpha_{13}$ are able to activate JNK [62] and these G-proteins are perhaps interesting candidates to mediate the PTX-insensitive effects of Et-1.

These results are consistent with Et-1 acting through a single receptor class (E_{A}) to activate at least two independent kinase cascades, ERK1 and JNK1. Both ERK1 and JNK1 activation proceed via G_i -regulated and non- G_i -regulated pathways, and the non- G_i -regulated pathway is probably not via activation of Ca^{2+} fluxes or PKC. LPA activates ERK via a Ras-dependent pathway [20,22,63] regulated by a PTX-sensitive GTPase; whether the ' G_i -regulated pathway' to JNK1 is related to that regulating the ERK cascade remains to be seen. Despite these similarities the pathways seem to be parallel and independent of each other, at least at the level of MEK, because the ERK cascade can be completely inhibited by PD98059 without any impact on JNK1 activation.

JNK1, but not ERK1, activation is inhibited by Ca^{2+} and PKC

JNK activation by G-protein-coupled receptors has been reported to be Ca^{2+} -dependent [32] but PKC-independent [59] by using ectopically expressed M_1 and M_2 acetylcholine receptors. Ca^{2+} -dependent JNK activation was also described in epithelial cells stimulated via endogenous angiotensin II receptors [33]. Since Et-1 gives a strong increase in $[\text{Ca}^{2+}]_i$ in Rat-1 cells [45,46] it seemed important to address the role of Ca^{2+} and PKC in regulating the response because these issues had not been previously addressed [25,60]. The ability of ionomycin or PMA alone to activate ERK1 but not JNK1 indicates that increases in $[\text{Ca}^{2+}]_i$ or PKC activity alone are not sufficient to activate JNK1, contrasting the study in epithelial cells [33]. In contrast with all previous reports, we found that buffering increases in $[\text{Ca}^{2+}]_i$ or inhibiting PKC potentiated and accelerated the onset of JNK1 activation in response to a subsequent Et-1 stimulus. The complementary observations, that PMA or ionomycin pretreatment inhibited subsequent JNK1 activation by Et-1, suggest that Et-1 stimulated $[\text{Ca}^{2+}]_i$ elevation or PKC activation might normally inhibit JNK1 activation. In such a model the slower onset of JNK1 activation might coincide with the time at which $[\text{Ca}^{2+}]_i$ declines or PKC is inactivated and relocates back to the soluble fraction [64]. This negative regulation was quite selective for JNK1 because ERK1 activity in the same samples was relatively unaffected by manipulating PKC or $[\text{Ca}^{2+}]_i$. Thus in addition to the studies with PD98059 these results provide further evidence that ERK1 and JNK1 are regulated independently in Et-1-stimulated Rat-1 cells.

The effects of Ca^{2+} and PKC described here are in stark contrast with their effects in some other systems and this might be a reason for the debate over whether or not growth factors

activate the JNK1 pathway [14,50]. Contrasting results might be obtained in cell lines that differ in the profiles of PKC or PLC isoenzymes expressed; hence JNK activation by Ca^{2+} -mobilizing agonists might be seen in some cell types but not in others. One major avenue for future work is to define the site at which Ca^{2+} and PKC intercede or cross-talk to inhibit the JNK1 pathway. Our analysis suggests that the effect is not on receptor affinity or the canonical MEKK-SEK-JNK cascade itself but rather on the specific signalling components between the E_{A} receptor, G-protein and the upstream regulators of the MEKK-SEK-JNK1 pathway. Significantly, the effects of GF109203X and Ca^{2+} chelation are not specific for Et-1 or for Rat-1 cells. We have recently found that LPA, which normally does not activate JNK1 in Rat-1 cells, can do so if cells are pretreated with GF109203X or BAPTA-AM/EGTA (J. Beltman and S. Cook, unpublished work). This suggests that the effects described for GF109203X are not likely to be due to the effects of PKC on Et-1 receptor function [39]. Furthermore, Zohn et al. [33] have recently shown that PKC down-regulation potentiates angiotensin II-stimulated JNK activation in rat liver epithelial cells. The complexity of JNK signalling pathways and their interactions with other signal pathways (PKC, Ca^{2+} and ERK) will only increase as it is now known that there are at least 10 different variants of the three JNK genes [31]. Future experiments will aim at addressing the activation and regulation of these different isoforms by the generation of splice-specific reagents.

Finally, the role of JNK1 activation in mitogenic signalling remains an outstanding issue. Et-1 is the only growth factor we have found that significantly activates JNK1 in Rat-1 cells, but maximal stimulation of DNA synthesis and ERK activation are observed at doses of Et-1 (1–3 nM) at which JNK1 is not activated. This result implies that JNK1 is not required for the proliferative effects of Et-1, although we cannot rule out the possibility that a small amount of active JNK1 at low Et-1 concentrations could synergize with other signal pathways in promoting DNA synthesis. Because JNK1 is activated only at high or maximal receptor occupancy it might serve a protective function in regulating the expression of a distinct set of AP-1-regulated genes in response to high agonist concentrations that might be associated with tissue damage or pathogenic states [61]. Indeed, the stimuli that strongly activate JNK (UV and genotoxic stresses) promote growth arrest or apoptosis rather than proliferation. If JNK1 activation is not the other pathway that acts in concert with transient ERK activation to regulate gene expression, then what is? One candidate is the putative pathway regulated by the small GTPase Rho, which is thought to activate a signal pathway regulating the serum response factor, which acts at the serum response element of the c-Fos promoter [56]. Experiments are in progress to investigate these issues by using LPA and Et-1 signalling in Rat-1 cells as a model system.

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