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Accepted Version

Sugden, P. H., Markou, T., Fuller, S. J., Tham, E. L., Molkentin, J. D., Paterson, H. F. and Clerk, A. ORCID: https://orcid.org/0000-0002-5658-0708 (2011) Monophosphothreonyl extracellular signal-regulated kinases 1 and 2 (ERK1/2) are formed endogenously in intact cardiac myocytes and are enzymically active. Cellular Signalling, 23 (2). pp. 468-477. ISSN 0898-6568 doi: 10.1016/j.cellsig.2010.10.024 Available at https://centaur.reading.ac.uk/18087/

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To link to this article DOI: http://dx.doi.org/10.1016/j.cellsig.2010.10.024

Publisher: Elsevier

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Monophosphothreonyl extracellular signal-regulated kinases 1 and 2 (ERK1/2) are formed endogenously in intact cardiac myocytes and are enzymically active

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Running title: Enzymically-active monophosphothreonyl ERK1/2 in cardiac myocytes

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ABSTRACT

ERK1 and ERK2 (ERK1/2) are central to the regulation of cell division, growth and survival. They are activated by phosphorylation of the Thr- and the Tyr- residues in their Thr-Glu-Tyr activation loops. The dogma is that dually-phosphorylated ERK1/2 constitute the principal activities in intact cells. We previously showed that, in neonatal rat cardiac myocytes, endothelin-1 and phorbol 12-myristate 13-acetate (PMA) powerfully and rapidly (maximal at ~5 min) activate ERK1/2. Here, we show that dually-phosphorylated ERK1/2 rapidly (< 2 min) appear in the nucleus following stimulation with endothelin-1. We characterized the active ERK1/2 species in myocytes exposed to endothelin-1 or PMA using MonoQ FPLC. Unexpectedly, two peaks of ERK1 and two peaks of ERK2 activity were resolved using *in vitro* kinase assays. One of each of these represented the dually-phosphorylated species. The other two represented activities for ERK1 or ERK2 which were phosphorylated solely on the Thr- residue. Monophosphothreonyl ERK1/2 represented maximally ~30% of total ERK1/2 activity after stimulation with endothelin-1 or PMA, and their k_{cat} values were estimated to be minimally ~30% of the dually-phosphorylated species. Appearance of monophosphothreonyl ERK1/2 was rapid but delayed in comparison with dually-phosphorylated ERK1/2. Of 10 agonists studied, endothelin-1 and PMA were most effective in terms of ERK1/2 activation and in stimulating the appearance of monophosphothreonyl and dually-phosphorylated ERK1/2. Thus, enzymically-active monophosphothreonyl ERK1/2 are formed endogenously following activation of the ERK1/2 cascade and we suggest that monophosphothreonyl ERK1/2 arise by protein tyrosine phosphatase-mediated dephosphorylation of dually-phosphorylated ERK1/2.

Keywords: ERKs, cardiac myocytes, endothelin, phorbol esters, monophosphorylation.

Abbreviations

AdV, adenovirus or adenoviral; ET-1, endothelin-1; FPLC, fast protein liquid chromatography; MBP, myelin basic protein; MKK, MAPK kinase; PMA, phorbol 12-myristate 13-acetate; PTP, protein Tyr-phosphatase; SDS-PAGE, sodium dodecyl sulphate-polyacrylamide gel electrophoresis.

1. Introduction

The related prototypic MAPKs, ERK1 and ERK2 (ERK1/2), are Pro-directed Ser-/Thr- protein kinases which regulate cell growth, division and survival through their effects on gene expression and other cellular processes [1-3]. In many dividing cells (including cell lines), growth factor-binding to receptor protein Tyr- kinases leads to activation of ERK1/2 [4,5]. This is also the case in primary cultures of (terminally-differentiated) neonatal rat cardiac myocytes [6,7] but here robust ERK1/2 activation is also induced by Gq protein-coupled receptor- /PKC-dependent pathways [8]. Thus, ligands such as endothelin-1 (ET-1) and tumour-promoting phorbol esters (such as phorbol 12-myristate 13-acetate, PMA) essentially stoicheiometrically activate ERK1/2 in these cells [7].

ERK1/2 are the final components of a protein kinase signaling cascade in which members of the Raf MAPK kinase kinase family (Raf-1, A-Raf and B-Raf) phosphorylate and activate MAPK kinases 1 and 2 (MKK1/2), which in turn phosphorylate and activate ERK1/2 [1]. In all 'classical' MAPKs (ERK1/2, JNKs and p38-MAPKα/β) and some 'novel' MAPKs, activation involves the phosphorylation of a Tyr- residue and a Thr- residue in the activation loop Thr-Xaa-Tyr sequence (Thr-Glu-Tyr in ERK1/2) to produce the dually-phosphorylated MAPK species [1]. In mouse or rat, the relevant residues are Thr-203 and Tyr-205 in ERK1, and Thr-183 and Tyr-185 in ERK2. The general belief is that phosphorylation of both Tyr- and Thr- is necessary for activation of ERK1/2.

Activated ERK1 and ERK2 from cardiac myocytes exposed to ET-1 or PMA have been separated into two discrete peaks of activity by MonoQ Fast Protein Liquid Chromatography (FPLC) with ERK2 eluting at a lower NaCl concentration [6,9]. We show here that each of these peaks of ERK1 or ERK2 activity can be resolved further into two

separate peaks. One of these two resolved peaks of ERK1 or ERK2 is dually-phosphorylated [(pT-E-pY)ERK1/2], whereas the second peak is ERK1 or ERK2 monophosphosphorylated on the Thr- residue [(pT-E-Y)ERK1/2]. The important conclusion is that monophosphothreonyl-ERK1/2 can be formed endogenously in cells and contributes significantly to overall ERK1/2 activity.

2. **Materials and methods**

2.1. Antibodies

The antibody against total ERK1/2 was a rabbit polyclonal from Cell Signaling Technology Inc. (cat. no. 9102). (pT-E-pY)ERK1/2 were detected with a Sigma-Aldrich UK mouse monoclonal antibody (cat. no. M9692) or a Cell Signaling Technology Inc. rabbit monoclonal antibody (cat. no. 4377), as specified in the Figure legends. The mouse monoclonal antibodies against (pT-E-Y)ERK1/2 (cat. no. M3557, ERK-YNP, clone 155 in Ref. [10]) and (T-E-pY)ERK1/2 (cat. no. M3682, clone 193 in Ref. [10]) were from Sigma-Aldrich UK. The rabbit anti-FLAG® antibody was from Santa Cruz Biotechnology Inc. (cat. no. sc-7787). Horseradish peroxidase- conjugated anti-mouse (cat. no. P0260) and anti-rabbit (cat. no. P0448) secondary antibodies were from Dako. The Alexa Fluor®488 anti-mouse secondary antibody (cat. no. A11001) and Texas Red-phalloidin (cat. no. T7471) were from Invitrogen.

2.2. Cardiac myocyte isolation and culture

Primary cultures of ventricular cardiac myocytes were prepared from 1- to 2-day-old Sprague-Dawley rats by an adaptation of the method of Iwaki et al. [11] as described elsewhere [12]. Hearts were removed, ventricles were dissected away from atria, and ventricular myocytes were dissociated by serial digestion with 0.4 mg/ml collagenase and 0.6 mg/ml pancreatin in sterile digestion buffer (116 mM NaCl, 20 mM HEPES, 0.8 mM Na_2HPO_4, 5.6 mM glucose, 5.4 mM KCl and 0.8 mM $MgSO_4$, pH 7.35). The first digestion supernatant (5 min, 37ºC, 160 cycles/min in a shaking water bath) was removed and discarded. Cell suspensions from subsequent digestions (20 min, 2 × 25 min, 20 min, 10 min; 37ºC, 136 cycles/min shaking) were recovered by centrifugation (5 min, 60 × g) and the cell pellets resuspended in plating medium [Dulbecco's modified Eagle's medium/medium 199 (4:1 v/v), 15% (v/v) foetal calf serum, 100 units/ml of both penicillin and streptomycin]. The cells were pre-plated on plastic Primaria tissue culture dishes (30 min). The non-cardiomyocytes adhere to the dishes whereas the cardiac myocytes remain unattached. For biochemistry experiments, the non-adherent viable cardiac myocytes from the pre-plates were plated at a density of 4×10^6 cells/dish on 60 mm Primaria dishes pre-coated at room temperature with sterile 1% (w/v) gelatin (Sigma-Aldrich UK). For immunostaining experiments, cardiac myocytes were plated at 2×10^6 cells/dish on 35 mm Primaria dishes containing glass coverslips pre-coated with gelatin as above followed by laminin (Sigma-Aldrich UK, 20 µg/ml in PBS). After 18 h, myocytes were confluent and beating spontaneously. Unless they were to be infected with adenovirus (AdV), serum was then withdrawn and myocytes were incubated in maintenance medium [Dulbecco's modified

Eagle's medium/medium 199 (4:1 (v/v), 100 units/ml of both penicillin and streptomycin] for a further 24 h.

2.3. Immunofluorescence microscopy

Cardiac myocytes were exposed to ET-1 (100 nM), then washed 3 × with PBS and fixed in 4% (v/v) formaldehyde (10 min, room temperature). Following permeabilisation with 0.1% (v/v) Triton X-100 (10 min, room temperature), non-specific binding was blocked with 2.5% (w/v) bovine serum albumin in 0.1% (v/v) Triton X-100 (10 min, room temperature). Antibodies and fluorophores were diluted in PBS, all incubations were at 37ºC, and coverslips were washed three times in PBS after each stage of the immunostaining procedure. Myocytes were stained with mouse primary monoclonal antibodies to (pT-E-pY)ERKs (Sigma M9692) (1:80, 60 min) and Alexa Fluor®488 anti-mouse secondary (1:200, 60 min). Myofilamentous actin was counterstained with Texas Red-phalloidin (5 U/ml, 20 min) and nuclei were counterstained with Hoechst 33258 (10 µg/ml, 15 min). Fluorescence microscopy was performed using a Zeiss Axio Observer Z1 inverted microscope equipped with a Zeiss LSM710 confocal imaging system, and using a Plan-Apochromat 63×/1.4 oil-immersion objective. Standard excitation and emission presets were chosen for Hoechst 33258, Alexa 488, and Texas Red dyes, which were imaged sequentially to eliminate fluorescence bleed-through. All fields were captured at a resolution of 512 × 512 pixels using a zoom factor of 1.2× with a field dimension of 112 × 112 µm.

2.4. AdV vector for FLAG-ERK2

The AdV-FLAG-ERK2 vector was prepared using the AdEasy™ XL Adenoviral Vector System (Stratagene) using the general methodology described previously [13]. The FLAG sequence (placed 3' to an initiation codon) was introduced into the pShuttle-CMV vector and the mouse ERK2 coding sequence was placed in frame and 3' to the FLAG tag. The FLAG sequence was first introduced between the BglII and KpnI restriction sites in the multiple cloning site of the pShuttle-CMV vector by synthesis of an oligonucleotide cassette containing the FLAG sequence preceded by an ATG start codon. The complementary oligonucleotides used were:

Forward:

5'-GATCTACCATG**GACTACAAAGACGATGACGACAAG**GGTACCGTCGACGC-3';

Reverse:

5'-GGCCGCGTCGACGGTACC*CTTGTCGTCATCGTCTTTGTAGTC*CATGGTA-3' with the FLAG sequence underlined and emboldened, and its antisense complement underlined and italicized. The oligonucleotides were annealed and phosphorylated, resulting in a cassette flanked by 'cut' BglII and NotI sites, which was then inserted into the corresponding restriction sites in the multiple cloning site of pShuttle-CMV. This strategy, which replaces the KpnI-NotI fragment of pShuttle-CMV with an identical sequence in the cassette insert, was used to avoid the potential difficulty of cutting both the BglII and KpnI sites which lie immediately adjacent to each other in pShuttle-CMV. The mouse ERK2 coding sequence was amplified from a glutathione-*S*-transferase-ERK2 fusion construct in pGEX4T-1 (gift of Professor Chris Marshall, Institute of Cancer Research, London) with Pfu polymerase using forward primer: (5'-GACGACAAG**GGTACC**ATGGCGGCCGCAGCAGCGGCCGGC-3')

and reverse primer (5'-CTTATCTAG**AAGCTT**TTAAGATCTGTATCCTGGCTGGAATCTAGCAG-3') to produce ERK2 flanked by KpnI and HindIII sites (underlined and emboldened) which were then used for insertion into the corresponding sites in FLAG-pShuttle-CMV, 3' and in frame with the FLAG sequence. All constructs were sequenced on an ABI3730xl or ABI3130xl automated fluorescent DNA sequencer at the Clinical Sciences Centre Genomics Core Laboratory (Imperial College London). Shuttle plasmids containing verified sequences were then linearised with PmeI and used to transform BJ5183-AD-1 cells which were subsequently screened for homologous recombination. AdV plasmids from positive recombinants were expanded in XL10Gold cells, linearised with PacI and used to transform HEK293 cells, as described in the AdEasy XL Adenoviral Vector System manual. Viruses were amplified through subsequent re-infection of HEK293 cells. Serum was withdrawn from cardiac myocytes 6 h before AdV.FLAG-ERK2 infections. The multiplicity of infection was titrated to give about 10-fold greater expression of FLAG-ERK2 over endogenous ERK2 at 20 h after infection.

2.5. MonoQ FPLC

After the various treatments, cardiac myocytes were washed 3× with ice-cold PBS, scraped into 150 µl Buffer A [20 mM β-glycerophosphate (pH 7.5), 50 mM NaF, 2 mM EDTA, 0.004 mM microcystin LR, 1% (v/v) Triton X-100, 5 mM dithiothreitol, 10 mM benzamidine, 0.2 mM leupeptin, 0.01 mM trans-epoxy succinyl-l-leucylamido-(4-guanidino)butane, 0.3 mM phenylmethylsulphonyl fluoride], and extracts were clarified by centrifugation (10,000 × g, 5 min, 4ºC). Extracts from 4 dishes of uninfected cardiac

myocytes or from 2 dishes of AdV.FLAG-ERK2 infected myocytes were loaded onto a MonoQ HR 5/5 FPLC column and ERK1/2 activities were separated using an Äkta™ FPLC system (GE Healthcare). The column was equilibrated with Buffer B (50 mM Tris/HCl pH 7.3, 2 mM EDTA, 2 mM EGTA, 0.1% (v/v) 2-mercaptoethanol, 5% (v/v) glycerol, 0.03% (v/v) Brij-35, 0.3 mM Na_3VO_4, 1 mM benzamidine and 4 µg/ml leupeptin). Following a 5 ml isocratic wash, discontinuous linear NaCl gradients were formed by mixing Buffer B with Buffer C (Buffer B containing 1 M NaCl) as described in the Figure legends.

2.6. Western blotting

MonoQ FPLC fractions or whole cell supernatant extracts were denatured by heating at 100ºC with 0.33 vol. sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer [300 mM Tris-HCl pH 6.8, 10% SDS (w/v), 13% glycerol (v/v), 130 mM dithiothreitol, 0.2% bromophenol blue (w/v)]. Proteins (25 µl in sample buffer) were separated by SDS-PAGE [10% (w/v) resolving gels with 6% (w/v) stacking gel] and transferred electrophoretically to nitrocellulose. Non-specific binding sites were blocked with 5% (w/v) non-fat milk powder in TBST buffer [20 mM Tris-HCl pH 7.5, 137 mM NaCl, 0.1% (v/v) Tween 20] for 30 min. Blots were incubated with primary antibodies in TBST buffer containing 5% (w/v) bovine serum albumin overnight at 4ºC. Antibody dilutions were: total ERK1/2, 1/1000; pT-E-pY(ERK2) (Cell Signaling Technology antibody), 1/1000; (pT-E-pY)ERK1/2 (Sigma Aldrich UK antibody), 1/30,000; (pT-E-Y)ERK1/2, 1/10,000; (T-E-pY)ERK1/2, 1/4000; FLAG, 1/10,000. The blots were washed with TBST buffer (3 × 5 min, room temperature), incubated with secondary antibodies (1:5000 dilution in TBST buffer containing 1% (w/v) nonfat milk powder, 1 h, room temperature) and then washed

again in TBST buffer (3 × 5 min, room temperature). Bands were detected by enhanced chemiluminescence (Santa Cruz Biotechnology) using Hyperfilm MP and were quantified by scanning densitometry.

2.7. Measurement of ERK1/2 activities.

ERK1/2 activities in FPLC fractions (40 µl sampled) were measured by incorporation of ^{32}P from 0.1 mM [γ-^{32}P]ATP (0.5 µCi per assay) into myelin basic protein (MBP, Upstate, 15 µg per assay) in the presence of 20 mM $MgCl_2$/2 µM protein kinase A inhibitor (Calbiochem). Assays (50 µl final volume) were initiated with 5 µl of 1 mM [γ-^{32}P]ATP/200 mM $MgCl_2$, incubated at 30ºC for 30 min, and terminated by sampling (40 µl) onto P81 paper squares which were then washed in 75 mM H_3PO_4 (4 × 15 min) on a rocking platform. Radioactivity was measured by Čerenkov radiation counting.

In some cases, ERK1/2 activities were examined by 'in-gel' MBP kinase assays. Fractions from FPLC were denatured with SDS-PAGE sample buffer and subjected to SDS-PAGE in 10% polyacrylamide gels containing 0.5 mg/ml MBP incorporated covalently into the gel matrix. After electrophoresis, SDS was removed from the gel by washing (3 × 20 min) with 20% (v/v) 2-propanol in 50 mM Tris/HCl, pH 8.0, then in 5 mM 2-mercaptoethanol in 50 mM Tris/HC1, pH 8.0 (3 × 20 min). Proteins were further denatured by washing the gel in 6 M guanidine HCl in 50 mM Tris/HC1 pH 8.0/5 mM 2-mercaptoethanol (2 × 20 min), then renatured by extensive washing in 50 mM Tris/HCl, pH 8.0, containing 0.04% (v/v) Tween-40 and 5 mM mercaptoethanol at 4ºC. After pre-incubation of the gel at 20ºC for 1 h in 40 mM HEPES, 2 mM dithiothreitol, 10 mM $MgCl_2$, pH 8.0, *in situ* phosphorylation of MBP was performed in 40 mM HEPES, 0.5 mM EGTA,

10 mM $MgCl_2$, 2 μM protein kinase A inhibitor, 0.1 mM [γ-^{32}P]ATP, pH 8.0 (5 ml/gel, containing 12.5 μCi [γ-^{32}P]ATP) at room temperature for 3 h. After extensive washing in 5% (w/v) trichloroacetic acid/1% (w/v) sodium pyrophosphate, gels were dried and autoradiographed.

2.8. Data interpretation

Graphs were constructed using GraphPad Prism 4.0 software and results are presented as means ± S.E.M. Areas under the curves were calculated using Origin Pro 8 software.

3. RESULTS

3.1. Immunofluorescence microscopy

Immunofluorescence microscopy showed that (pT-E-pY)ERK1/2 appeared transiently in cardiac myocytes exposed to 100 nM ET-1 (Fig. 1). At zero time, some background staining of (pT-E-pY)ERK1/2 was detectable (Fig. 1). By 2 min, staining had increased dramatically in the nucleus and, to a lesser extent, in the cytoplasm. After 5 min, staining was maximal in both compartments but, by 10 min, staining was declining in both. This transience is consistent with enzyme activity measurements [6].

3.2. MonoQ FPLC of extracts of cardiac myocytes exposed to ET-1 or PMA

By reducing the flow rate and using a shallow NaCl gradient, we separated five peaks of MBP kinase activity (peak A and peaks I-IV) in extracts of cardiac myocytes exposed for 5 min to 100 nM ET-1 (Fig. 2A) or 1 μM PMA (Fig. 2B), conditions that maximally activate ERK1/2 in these cells [7,9]. Activities in Peaks I-IV with ET-1 or PMA were about 6-fold greater than in the controls. PD184352 is an allosteric inhibitor of MKK1/2 [14]. Exposure of cardiac myocytes to PD184352 (2 μM, 15 min) reduced MBP kinase activities to below control values and prevented subsequent activation of MBP kinase peaks I-IV by ET-1 (Fig. 2C). These results, together with previous findings [6,9], suggest that the MBP kinase activities in Peaks I-IV represent either ERK1 and ERK2 themselves, or that one or more of the activities represents downstream effector protein kinases activated by ERK1/2.

The MonoQ fractions in Fig. 2A-B, were immunoblotted for total (i.e. phosphorylated + non-phosphorylated) ERK1/2 (Fig. 2D). In extracts from control cells, 42 kDa ERK2 eluted over fractions 16-22 (maximal in fractions 18-19) and 44 kDa ERK1 eluted over fractions 24-29 (maximal in fractions 26-27) (Fig. 2D). In extracts of cells exposed to ET-1 or PMA, ERK1 and ERK2 eluted at slightly higher NaCl concentrations because of the negative charges introduced by phosphorylation. MBP kinases (Fig. 2A-B) in Peak I (peak fractions 19-20 eluting at ~0.14 M NaCl) and Peak II (peak fractions 23-24 eluting at ~0.16 M NaCl) contained ERK2 (Fig. 2D). Peak III (peak fractions 29-30 eluting at ~0.17 M NaCl, and overlapping with Peak II) contained ERK1 as did Peak IV (peak fractions 40-41 eluting at ~0.20 M NaCl) (Fig. 2A,B,D).

Selected fractions were immunoblotted with antibodies recognising various phosphorylated forms of ERK1/2. In extracts of myocytes exposed to ET-1 or PMA,

antibodies recognising (pT-E-pY)ERK1/2 showed that Peak II (fractions 23 and 25) contained (pT-E-pY)ERK2 and Peak IV (fraction 41) contained (pT-E-pY)ERK1 (Fig. 2E). Monoclonal antibodies selective for (pT-E-Y)ERK1/2 [10] showed that Peak I (fractions 18 and 20) contained (pT-E-Y)ERK2 and Peak III (fractions 28 and 30) contained (pT-E-Y)ERK1 (Fig. 2E). The (T-E-pY)ERK1/2-selective antibody [10] did not detect any significant immunoreactivity in Peaks I-IV, though it may have detected some immunoreactivity in Peak A (fraction 7) (Fig. 2E). We cannot definitely exclude the possibility that there is some formation of (T-E-pY)ERK1/2 because the antibody may simply be unable to detect it, but we were unable to detect any signal using a variety of dilutions. Our interpretation of these experiments is that, in addition to (pT-E-pY)ERK1/2, (pT-E-Y)ERK1/2 can be readily detected in cardiac myocytes exposed to ET-1 or PMA in intact cells and these species are enzymically active.

In addition to ERK1/2, a 46 kDa 'ERK' protein was detected in fractions 6-7 and which corresponded to the MBP kinase activity in Peak A eluting at ~0.11 M NaCl (Fig. 2A-B). This may be ERK1b, identified previously in Rat1 cells, which is expressed in highest abundance in heart [15]. ERK1b is an alternatively-spliced 406 residue protein containing a 26-residue insert between residues 340 and 341 in rat ERK1 [15]. In comparison with peaks I-IV, Peak A MBP kinase was relatively poorly activated (~2-fold) by ET-1 (Fig. 2A) or PMA (Fig. 2B).

We also performed 'in-gel' MBP kinase assays [7] on selected fractions. Such assays require the renaturation of denatured phosphorylated ERK1/2. Interestingly, only the dually-phosphorylated forms (fractions 21, 22, 38 and 39) of ERK1 (migrating at 44 kDa) and ERK2 (migrating at 42 kDa) were able to renature (Fig. 2F) and phosphorylate MBP.

3.3. Relative contributions and specific activities of phospho-ERK1/2 species

Integration of the curves in Fig. 2A-B, showed that the enzymic activity attributable to (pT-E-Y)ERK1/2 is considerable and represents ~30% of the total MBP kinase activity in cardiac myocytes exposed to 100 nM ET-1 (9 separate preparations of cardiac myocytes) or 1 μM PMA for 5 min (5 separate preparations of myocytes).

Approximate relative specific activities (turnover numbers or k_{cat}) of (pT-E-Y)ERK1/2 and (pT-E-pY)ERK1/2 were estimated from data in Fig. 2A,B,E on the following basis. In extracts of control cardiac myocytes, antibodies against total ERK1/2 or any of the phospho-ERK1/2 species did not detect significant immunoreactivity in fractions 23 and 41. On activation of ERK1/2 by ET-1 or PMA, immunoreactivity became detectable with antibodies against total ERK1/2 or (pT-E-pY)ERK1/2, but not (pT-E-Y)ERK1/2, in fractions 23 (ERK2) and 41 (ERK1). Thus, essentially all enzymic activity and total immunoreactivity in these fractions was attributable to (pT-E-pY)ERK1/2. The ratio of measured enzymic activity to total ERK1/2 in fractions 23 and 41 thus provided a measure of the specific activity for (pT-E-pY)ERK1/2

For fractions 20 and 30, antibodies against total ERK1/2 detected immunoreactivity but negligible enzymic activity in control incubations of cardiac myocytes. However, both immunoreactivity and significant enzymic activity was detected in cardiac myocytes exposed to ET-1 or PMA but (pT-E-Y)ERK1/2 were the only phosphorylated species detectable. The ratio of measured enzymic activity to total ERK1/2 in fractions 20 and 30 thus provided a ***minimum*** measure of the specific activity for (pT-E-Y)ERK1/2 because the contribution of inactive ERK1/2 to the denominator cannot be assessed. From the data shown, the minimum specific activities (mean ± S.E., n = 5) of (pT-E-Y)ERK1 and (pT-E-Y)ERK2 were

calculated to be 29 ± 1% and 30 ± 2% of those of (pT-E-pY)ERK1 and (pT-E-pY)ERK2, respectively.

3.4. Infection of cardiac myocytes with AdV.FLAG-ERK2.

In order to ensure that activities in Peaks I and III (Fig. 2A-B) were not MBP kinases activated downstream from ERK1/2, we infected cardiac myocytes with AdV.FLAG-ERK2. Infected and uninfected myocytes were exposed to 100 nM ET-1 (5 min) and MBP kinase activities measured following MonoQ FPLC. The additional negative charge of the FLAG epitope shifted the elution of FLAG-ERK2 activity to a position between Peaks III and IV (Fig. 3A). Treatment of AdV.FLAG-ERK2-infected myocytes with ET-1 resulted in a large increase in MBP kinase activity in fractions 46 - 62 (note that fraction numbers in Fig. 3 are not comparable to those in Fig. 2 because fraction size was reduced). Fractions 48 (minor peak of FLAG-ERK2 activity) and 54 (major peak of FLAG-ERK2 activity) were characterised further (Fig. 3B). As expected, total ERK2 or FLAG immunoreactivity was present in much greater abundance in fractions 48 and 54 from AdV.FLAG-ERK2-infected myocytes (control or ET-1-treated) than in uninfected myocytes (Fig. 3B). (pT-E-pY)ERK1/2 were not detected in uninfected or AdV.FLAG-ERK2-infected control myocytes, though there was some (pT-E-Y)ERK1/2 immunoreactivity detectable in fraction 48 of the AdV.FLAG-ERK2-infected controls (Fig. 3B). We do not understand the reasons for this. In AdV.FLAG-ERK2-infected myocytes exposed to ET-1, a strong (pT-E-pY)ERK2 signal was detected in fraction 54 with a weaker signal in fraction 48 (Fig. 3B). Conversely, (pT-E-Y)ERK2 was readily detected in fraction 48 with a weaker signal in fraction 54 (Fig. 3B). These data confirm that all the MBP kinase activities in peaks I-IV (Fig. 2A-B) are

monophosphorylated or dually-phosphorylated ERK1 or ERK2, rather than MBP kinases downstream from ERK1/2.

3.5. Time courses of ERK1/2 phosphorylation

Cardiac myocytes were exposed to 100 nM ET-1 for various times and cell extracts were immunoblotted directly with (pT-E-pY)ERK1/2 (Fig. 4A) or (pT-E-Y)ERK1/2 antibodies (Fig. 4B). (pT-E-pY)ERK1/2 were detectable within 30 *s* and maximal at 1 - 2 min (Fig. 4A), whereas (pT-E-Y)ERK1/2 were not detected before 1 min but was maximal at ~ 5 min (Fig. 4B). These findings suggest (but do not prove) a precursor-product relationship with (pT-E-Y)ERK1/2 resulting from dephosphorylation of (pT-E-pY)ERK1/2. The time course of activation of ERK1/2 by ET-1 was also examined by MonoQ FPLC (Fig. 5A). Areas under individual peaks were integrated and plotted against time (Fig. 5B). Peak II [(pT-E-pY)ERK2] and Peak IV [(pT-E-pY)ERK2] were detectable within 30 *s*, rose to a maximum at 1 - 2 min, then gradually declined over the subsequent 40 min (Fig. 5A-B). Peak I [(pT-E-Y)ERK2] and Peak III [(pT-E-Y)ERK1] were not detected until 1 min, then increased to a maximum at 2 - 5 min (Peak I) or 1 - 2 min (Peak III) before declining subsequently. These findings were confirmed by immunoblotting fractions containing the highest activities of Peaks I-IV (fractions 19, 23, 29 and 40, respectively) with antibodies against (pT-E-pY)ERK1/2 and (pT-E-Y)ERK1/2 (Fig. 5C). As an aside, there were two bands of total ERK2 in the 0.5 - 2 min samples of fraction 19 [which contains only (T-E-Y)ERK2 and (pT-E-Y)ERK2]. The more slowly migrating band is presumably (pT-E-Y)ERK2. Migration of ERK2 is reduced by phosphorylation and the result here showed that phosphorylation of the Thr- residue alone may be adequate to cause the shift.

3.6. Comparison of agonist efficacy in (pT-E-Y)ERK1/2 and (pT-E-pY)ERK1/2 formation in cardiac myocytes

Numerous agonists stimulate ERK1/2 activities and phosphorylation in cardiac myocytes. We compared the MonoQ FPLC activity profiles for 10 agonists (Fig. 6A) and derived the relative contribution of each peak of activity to the total activity (i.e. the sum of activities in all four peaks) using activation by ET-1 (100 nM, 5 min) as an internal standard and taking it as 100%. The time of sampling was when the response to each agonist was maximal and maximally-effective concentrations were chosen. In terms of total activity, ET-1 and PMA caused the greatest activation and H_2O_2 the least (Fig. 6A). Essentially the same spectrum of activation was seen for Peak II [(pT-E-pY)ERK2, Fig. 6C] and Peak IV [(pT-E-pY)ERK1, Fig. 6E], and plots of activities in these peaks against total activity relative to ET-1 were linear ($p < 0.0001$, data not shown). However, for Peak I [(pT-E-Y)ERK2], there appeared to be a 'threshold' effect with disproportionately large effects of ET-1 and PMA relative to total activity (Fig. 6B,F). This contrasted with the other monophosphorylated species in Peak III [(pT-E-Y)ERK1, Fig. 6D] where the abundance of (pT-E-Y)ERK1 was proportional to the total ERK1/2 activities (Fig. 6G). Thus the relative abundance of (pT-E-Y)ERK reflects the abundance of total (pT-E-pY)ERK only in the case of ERK1.

4. Discussion

Classically, activation of ERK1/2 involves phosphorylation of the Thr- and Tyr-residues in the activation loop Thr-Glu-Tyr by the dual-specificity kinases, MKK1/2 in an

ordered, 'distributive' manner [1,22-26]. In non-phosphorylated ERK2 (and presumably ERK1), the activation loop Tyr- lies in a hydrophobic pocket with its -OH group buried, whilst the Thr- is on the surface. First of all, Tyr-185 is phosphorylated by MKK1/2 causing a conformational change, with the phospho-Tyr- side chain moving to the surface of the protein making four interactions with two Arg- residues and thus presumably stabilising the (T-E-pY)ERK2 conformation of the enzyme. The MKK1/2 then dissociates and second MKK1/2 binds to (T-E-pY)ERK2 and phosphorylates the Thr- residue, thereby forming a further 8 ionic contacts. The Tyr- phosphorylation is important in protein substrate recognition whereas the Thr- phosphorylation improves the geometry of the active site. This model is supported by demonstration of distinct structures for the non-phosphorylated, Tyr-phosphorylated, and dually-phosphorylated forms of ERK1 [27]. The ordered mechanism has led to the conclusion that phosphorylation of both Thr- and Tyr- residues is essential for full activity of ERK1/2. It became generally assumed that monophosphorylated forms are, at best, low k_{cat} species and, if they occur endogenously in cells *in vivo*, it is only in low abundances. However, an alternative interpretation is that phosphorylation of the Tyr- is an obligatory step for stabilisation of the 'transition state', and that it precedes and is required for the Thr- phosphorylation. However, once the activation loop Thr- is phosphorylated, the phosphate of phospho-Tyr could be dispensable. This may explain why, although (pT-E-pY)ERK1/2 activities renatured in 'in-gel' kinase assays (Fig. 2F), (pT-E-Y)ERK1/2 activities did not because the Tyr-phosphorylation is necessary for renaturation.

Many agonists activate ERK1/2 the ERK1/2 cascade in cardiac myocytes to varying degrees (Fig. 6) with ET-1 and PMA being most potent (as we have shown previously [7,9]). (pT-E-pY)ERK1/2 appeared rapidly (within 2 min) in both the nuclei and cytoplasm of cardiac myocytes exposed to ET-1 before declining (Fig. 1), a similar response to that seen

with PMA [28]. Thus, (pT-E-pY)ERK1/2 are correctly localised to regulate ET-1-mediated transcriptional events, the majority of which are dependent on activation of the ERK1/2 cascade in cardiac myocytes [29,30]. However, we were unconvinced that we could detect any production of (pT-E-Y)ERK1/2 by immunostaining (results not shown) and suggest that this is simply a problem with immunostaining with the (pT-E-Y)ERK1/2 antibody.

Here, we show that (pT-E-Y)ERK1/2 can be responsible for up to ~30% of total ERK1/2 activity in mammalian cells (Fig. 2). To our knowledge, this is the first direct demonstration of significant **endogenous** (pT-E-Y)ERK1/2 activity *in vivo*. The general assumption has been that (pT-E-Y)ERK1/2 or (T-E-pY)ERK1/2 are essentially enzymically-inactive and that, if they do occur *in vivo*, they only occur in low abundances. However, *in vitro*, recombinant (pT-E-F)ERK2 was about 7- to 8-fold more active than (T-E-F)ERK2, though the resulting activity was still <0.5% of that of (pT-E-pY)ERK2 [26,31]. Similarly, (A-E-pY)ERK2 was about 100-fold more active than (A-E-Y)ERK2 but this was only about 2% of the activity of (pT-E-pY)ERK2. However, activation loop mutants of ERK2 was used in these experiments and these may not be of strict relevance to (T-E-Y)ERK2. Zhou and Zhang [32] generated (pT-E-Y)ERK2 and (T-E-pY)ERK2) *in vitro* by treating recombinant (pT-E-pY)ERK2 with the protein Tyr-phosphatase (PTP) PTPN7 (HePTP) or the protein Ser-/Thr- phosphatase PP2C until activities had decreased to a minimum and (pT-E-pY)ERK2 was not detectable immunologically. The k_{cat} values of (T-E-Y)ERK2, (pT-E-Y)ERK2, (T-E-pY)ERK2 and (pT-E-pY)ERK2 were 1, 227, 620 and 8940 s^{-1}, respectively, with MBP as substrate with corresponding '*K*m' values of 22.9. 15.5, 15.2, and 10.0 µM. A similar rank order was obtained with, the transactivation domain of the Elk-1 transcription factor, a more 'physiological' substrate. Our estimates of the relative specific activities of (pT-E-Y)ERK1/2 to (pT-E-pY)ERK1/2 of ~30% are somewhat higher that the 7% of Zhou & Zhang [32].

Both (pT-E-Y)ERK1/2 and (T-E-pY)ERK1/2)] have been detected by immunofluorescence or immunoblotting in intact ErbB1-overexpressing CHO cells following short-term (up to 15 min) exposure to EGF [10]. They were thought to arise from selective dephosphorylation of (pT-E-pY)ERK1/2 by either PTPs or protein Ser-/Thr- phosphatases, but were assumed to be enzymically-inactive. (pT-E-Y)ERK1/2 were predominantly cytoplasmic and (T-E-pY)ERK1/2 were predominantly nuclear. Additionally, (T-E-pY)ERK1/2 formed by the action of PP2A phosphatase have been detected in the nuclei of cell lines but also co-localised with the Golgi apparatus during the G_2/M phase of the cell cycle, when they may be involved in Golgi disassembly [33,34]. Their role in the Golgi seemed to be dependent on the monophosphorylation itself rather than ERK1/2 activity.

Other studies using a Tyr- to Phe mutation in the T-G-Y phosphorylation motif of p38-MAPKα indicated that it can be enzymically-activated [to (pT-G-F)p38-MAPKα, etc.] by its MAPK kinase *in vitro*, but its activity is only ~5% that of (pT-G-pY)p38-MAPKα [35]. *In vivo*, constitutively-active MKK6 transfected into HEK293T cells activated transfected (T-G-F)p38-MAPKα, but this did not lead to activation of an AP-1-luciferase reporter above that seen in cells transfected with constitutively-active MKK6 alone [35]. Largely-analogous results were obtained with mutants of the yeast p38-MAPKα orthologue HOG-1 where phosphorylation of Thr-, but not Tyr-, was essential for both catalytic and biological activity [36].

Our data do not conflict with the accepted ordered phosphorylation mechanism. The appearance of (pT-E-Y)ERK1/2 is delayed relative to (pT-E-pY)ERK1/2 (Fig. 3), consistent with obligate initial phosphorylation of the Tyr-residue. As suggested [10], it seems likely that (pT-E-Y)ERK1/2 are generated through dephosphorylation of (pT-E-pY)ERK1/2 by one or more PTPs. On the assumption that a 'classical' Cys-dependent PTP dephosphorylates

(pT-E-pY)ERK1/2, ~15 candidates have been identified as being expressed in cardiac myocytes by our transcriptomics data (A. Clerk, unpublished data). A final question relates to the potential role of (pT-E-Y)ERK1/2 *in vivo*. The biological role(s) of (pT-E-Y)ERK1/2 is unclear. Given that pY-185 in ERK2 forms part of the substrate recognition pocket, (pT-E-Y)ERK1/2 may differ from (pT-E-pY)ERK1/2 in terms of substrate specificity.

ACKNOWLEDGEMENTS

We thank primarily the Fondation Leducq and secondarily the British Heart Foundation for support, and Professors Rony Seger (Weizmann Institute, Rehovoth, Israel) and Chris Marshall (Institute of Cancer Research, London, UK) for helpful discussions.

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FIGURE LEGENDS

Fig. 1. Immunofluorescence microscopy of (pT-E-pY) ERK1/2 in cardiac myocytes exposed to endothelin-1 (ET-1). Following exposure to ET-1 (100 nM) for the times indicated, myocytes were stained for nuclei (blue), (pT-E-pY)ERK1/2 (green, Sigma-Aldrich antibody), or actin filaments (red). The combined overlay images are also shown. Representative fields

(out of 3) from an individual experiment are shown and the experiment was repeated 3 times on separate preparations of myocytes. Scale bar = 50 µm.

Fig. 2. MonoQ FPLC of ERK1/2 in extracts of cardiac myocytes. Myocytes were exposed to 100 nM ET-1 for 5 min, 1 µM PMA for 5 min, or to 2 µM PD184352 for 15 min followed by 100 nM ET-1 for 5 min, and extracts were applied to MonoQ columns. Following a 5 ml isocratic wash, the NaCl concentration was increased in a stepwise fashion to 0.1 M (2 ml). ERK1/2 activities were eluted with a linear NaCl gradient (0.1 M-0.22 M NaCl, 12 ml). Flow rate was 0.5 ml/min, 0.25 ml fractions were collected and MBP kinase activities measured. When shown, the broken line denotes the NaCl concentration. For the MonoQ FPLC profiles (A-C): ▲, control (A-C); ●, ET-1 (A,C); or PMA (B); ■, PD184352 pre-exposure followed by ET-1 (C). Experiments where responses to both ET-1 or PMA were examined contemporaneously (A and B) was repeated on 4 different preparations of myocytes with similar results. For the effects of PD184352 (C), the experiment was repeated once with similar results. D, E. Immunoblotting of MonoQ FPLC fractions (A and B). D, total (i.e. phosphorylated + non-phosphorylated) ERK1/2 was immunoblotted across the MonoQ FPLC fractions. E. Selected fractions of peak MBP kinase activity following MonoQ FPLC separations (A,B) were immunoblotted for (pT-E-pY)ERK1/2 (Cell Signaling Technology Inc. antibody), (pT-E-Y)ERK1/2, (T-E-pY)ERK1/2 or total ERK1/2. F, 'in gel' MBP kinase assays. Selected fractions containing peak MBP kinase activity following MonoQ FPLC of myocytes exposed to 100 nM ET-1 for 5 min (A) were examined by 'in-gel' MBP kinase assays. The experiment was repeated once with similar results.

Fig. 3. MonoQ FPLC of ERK1/2 in extracts of AdV.FLAG-ERK2-infected cardiac myocytes following exposure to ET-1. Myocytes were uninfected, or were infected with AdV.FLAG-ERK2 and exposed to 100 nM ET-1 for 5 min as required. A, ERK1/2 activities were separated by MonoQ FPLC (see Legend to Fig. 1), except that flow rate and fraction size were reduced to 0.25 ml/min and 0.15 ml, respectively, and MBP kinase activities measured. Control values (no infection or exposure to ET-1) were essentially at baseline values on this scale. ▲, myocytes infected with AdV.FLAG-ERK2, no ET-1 exposure; ● myocytes infected with AdV.FLAG-ERK2 and exposed to ET-1; ■, uninfected myocytes exposed to ET-1. The broken line denotes the NaCl concentration. B, fractions 48 and 54 (5 µl/lane) were immunoblotted. For (pT-E-pY)ERK2 detection, the Sigma-Aldrich antibody was used. The experiment was repeated with similar results.

Fig. 4. Phosphorylation of ERK1/2 in fresh extracts of cardiac myocytes exposed to ET-1. Cardiac myocytes were exposed to 100 nM ET-1 for the times indicated, extracts were prepared immediately and were examined by immunoblotting with antibodies to (A, B) total ERK1/2 (centre panels); A, antibodies to (pT-E-pY)ERK1/2 (Sigma-Aldrich antibody) (upper panel), or B, (pT-E-Y)ERK1/2 (upper panel). A, B, The lower panels show quantification of immunoblots for A, (pT-E-pY)ERK1 or (pT-E-pY)ERK2, or B, (pT-E-Y)ERK1 or (pT-E-pY)ERK2. ●, solid line, ERK1; ■, broken line, ERK2. Results are means ± S.E.M. for 4 different preparations of myocytes.

Fig. 5. Time courses of activation and phosphorylation of ERK1/2 by ET-1 in extracts of cardiac myocytes examined by MonoQ FPLC. Experiments were carried out as described in the Legend to Fig. 2 with the exception that the length of exposure of myocytes to 100 nM

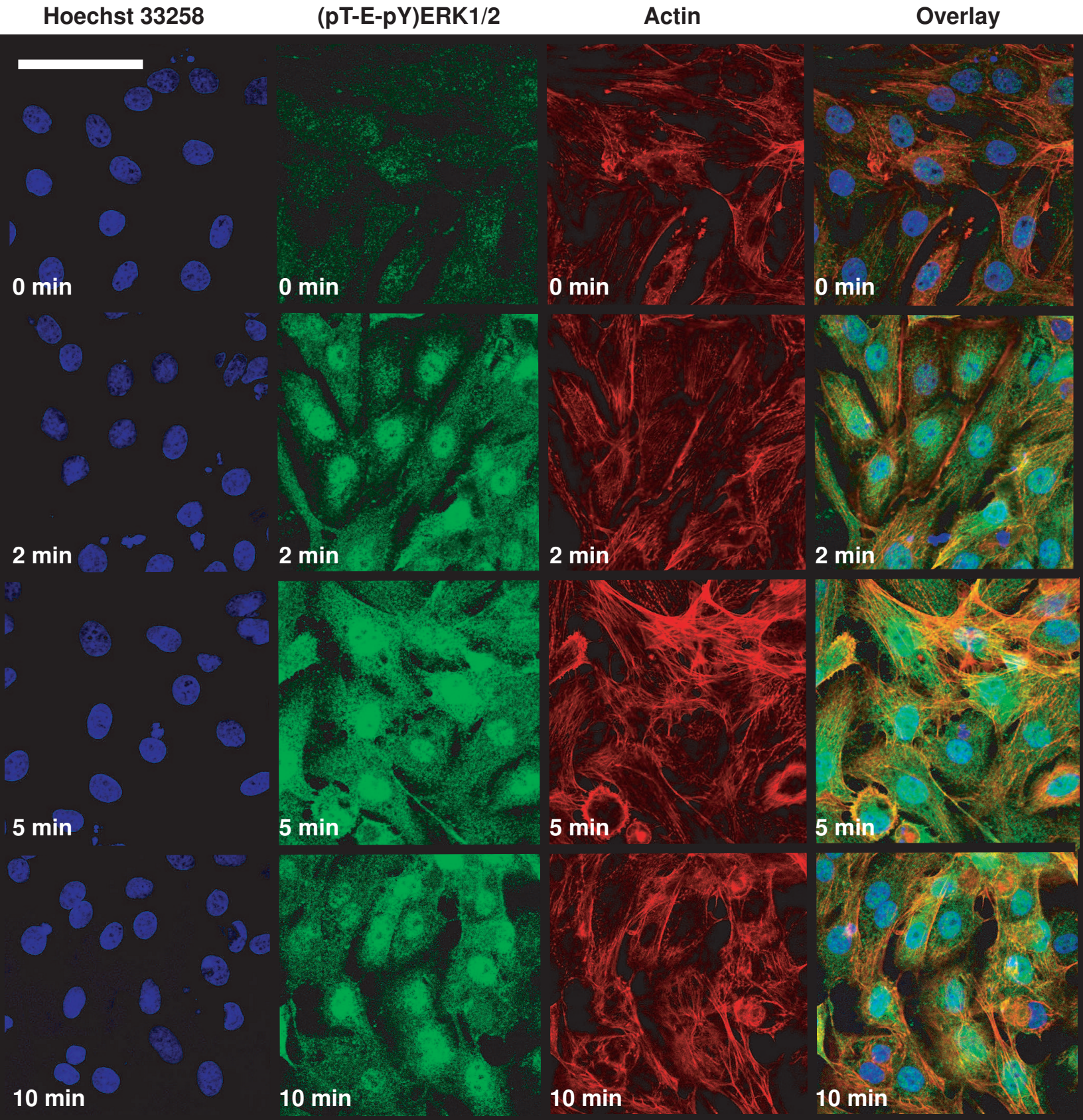
ET-1 was varied. A, MonoQ FPLC. MBP kinase activities were expressed as a percentage of total MBP kinase activity following the standard exposure of myocytes to (maximally-activating) 100 nM ET-1 for 5 min. Elution profiles are offset to prevent overlapping, but the baseline values are identical all each cases. Results are representative of 3 different preparations of myocytes. B, MBP kinase activities in peaks I-IV (as defined in Fig. 1). Activities in the individual peaks I-IV from MonoQ FPLC were calculated by curve fitting and are shown as means ± S.E.M. for 3 independent observations on separate preparations of myocytes. ●, solid line, Peak I (pT-E-Y)ERK2; ○, solid line, Peak II (pT-E-pY)ERK2; ■, broken line, Peak III (pT-E-Y)ERK1; □, broken line, Peak IV (pT-E-pY)ERK1. C, fractions 19, 23, 29 and 40 (peaks I-IV, respectively, Fig. 5A) were immunoblotted with antibodies against (pT-E-pY)ERK1/2 *(*top panels), (pT-E-Y)ERK1/2 (centre panels), or total ERK1/2 (bottom panels). Results are representative of 3 different preparations of myocytes.

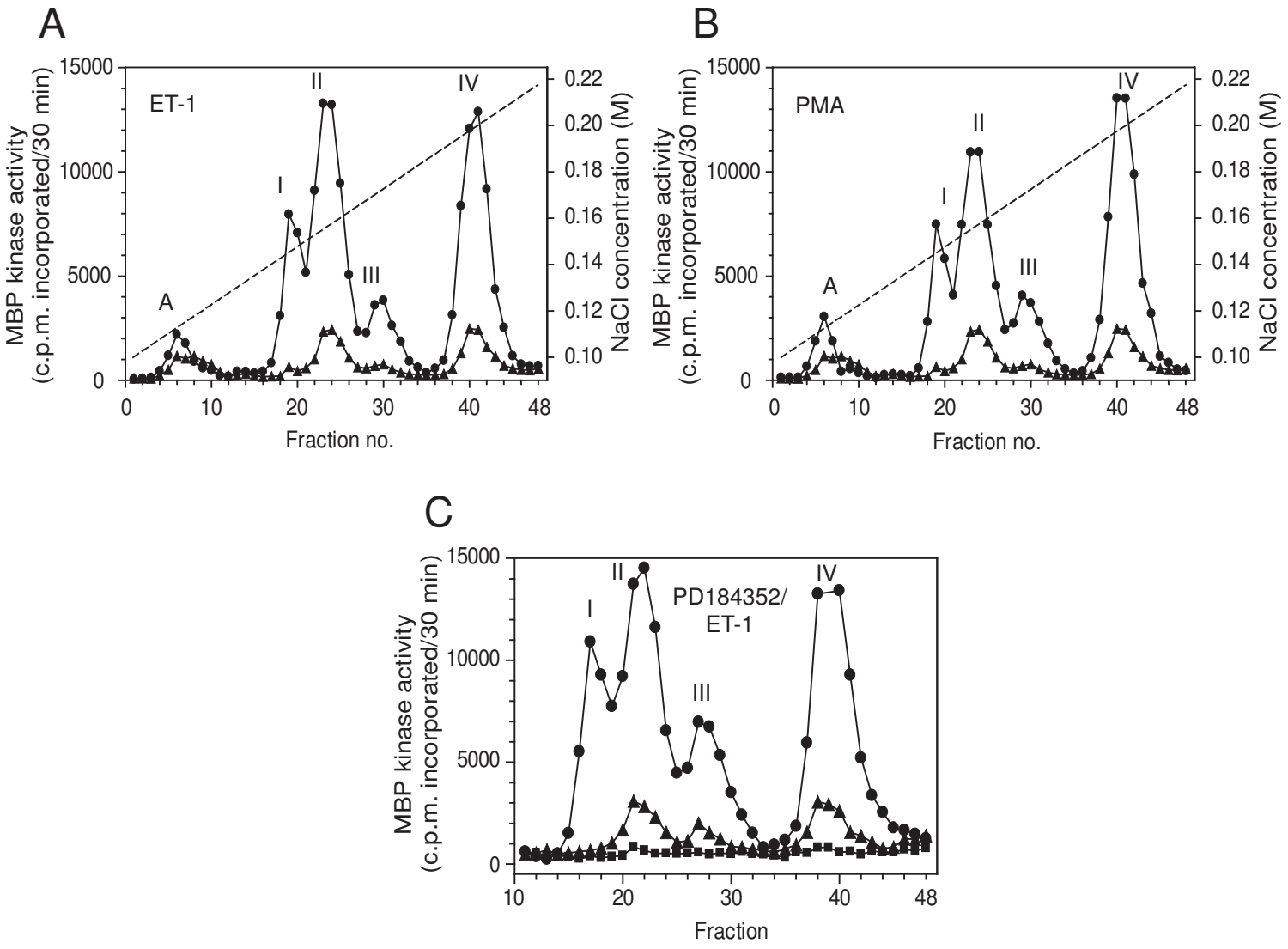
Fig. 6. Comparison of activation of ERK1 and ERK2 in cardiac myocytes by various agonists as determined by MonoQ FPLC. Myocytes were exposed to agonists at their maximally effective concentrations and times, and ERK1/2 activities in the extracts were examined using MonoQ FPLC. Agonist concentrations and lengths of exposure were: ET-1, 100 nM for 5 min (n = 8); PMA 1 μM for 5 min (n = 5); bradykinin (BK) 10 μM for 5 min (n = 3); EGF, 20 ng/ml for 5 min (n = 3); PDGF, 20 ng/ml for 5 min (n = 3); phenylephrine (PE), 100 μM for 5 min (n = 5); hyperosmotic shock (HOS) with 0.5 M sorbitol (n = 3) for 30 min; H_2O_2 0.1 mM for 15 min (n = 3); IL-1β, 10 ng/ml for 15 min (n = 3); isoprenaline (ISO), 50 μM for 5 min (n = 3). MBP kinase activities in fractions were measured and peaks of activity were integrated. In all cases, ET-1 standards (myocytes exposed to 100 nM ET-1 for 5 min) were included, and the total activity or the activity in each peak was expressed as a

percentage relative to the total activity induced by ET-1. A, total activity; B, Peak I activity [(pT-E-Y)ERK2]; C, Peak II activity [(pT-E-pY)ERK2]; D, Peak III activity, [(pT-E-Y)ERK1]; E, Peak IV activity [(pT-E-pY)ERK1]. F, G, activities in Peak I, Fig. 6B [(pT-E-Y)ERK2 relative to the ET-1 total activity standard] and Peak III, Fig. 6D [(pT-E-Y)ERK1 relative to the ET-1 total activity standard] were plotted against total activity relative to the ET-1 standard (Fig. 6A) for each agonist. All results are means ± S.E.M. F, points were fitted to a fourth-order polynomial non-linear regression equation ($r^2 = 0.98$). G, points were fitted to a linear regression. In both cases, the lines were forced through the origin.

Figure(s)

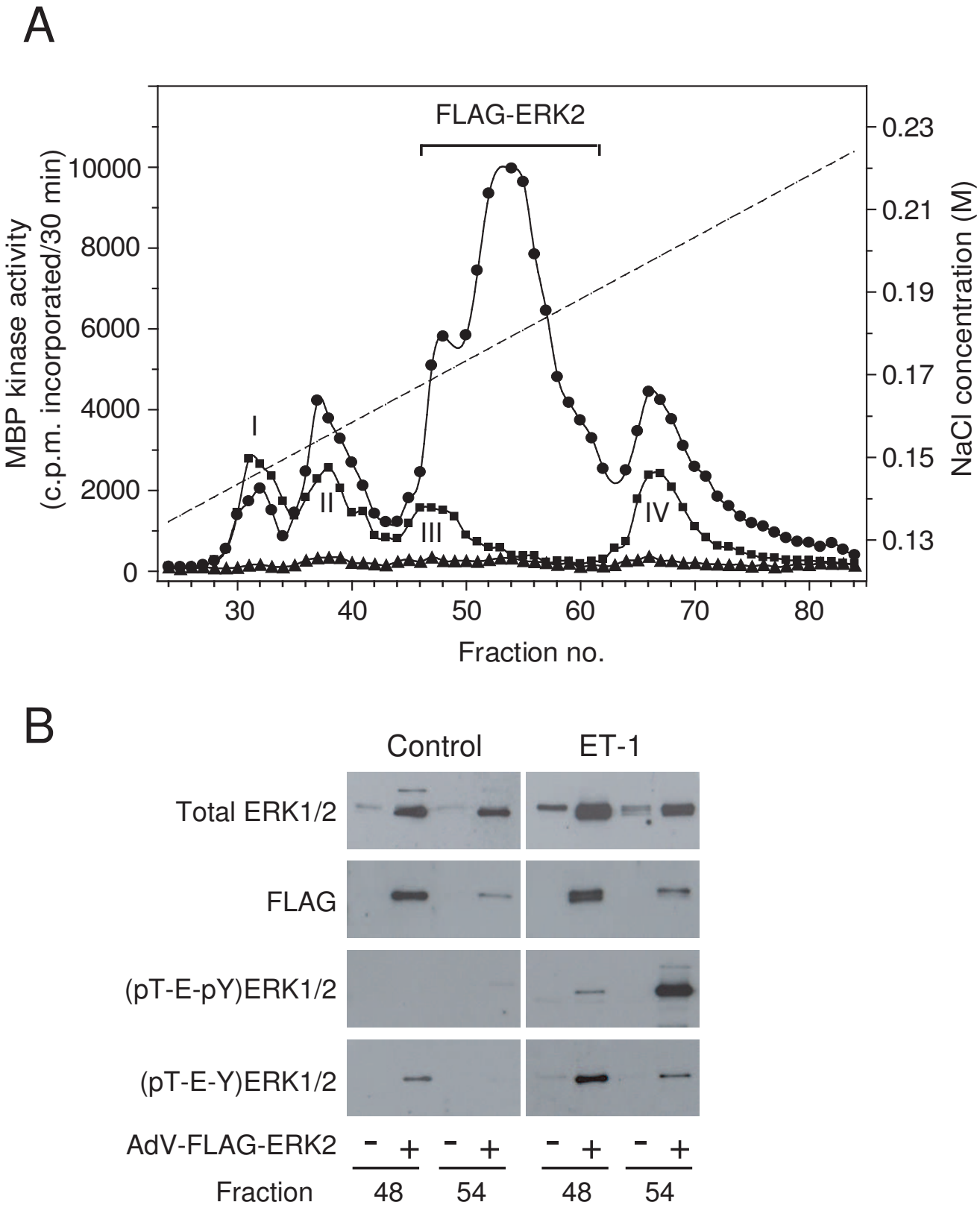
Sugden et al. Figure 1.

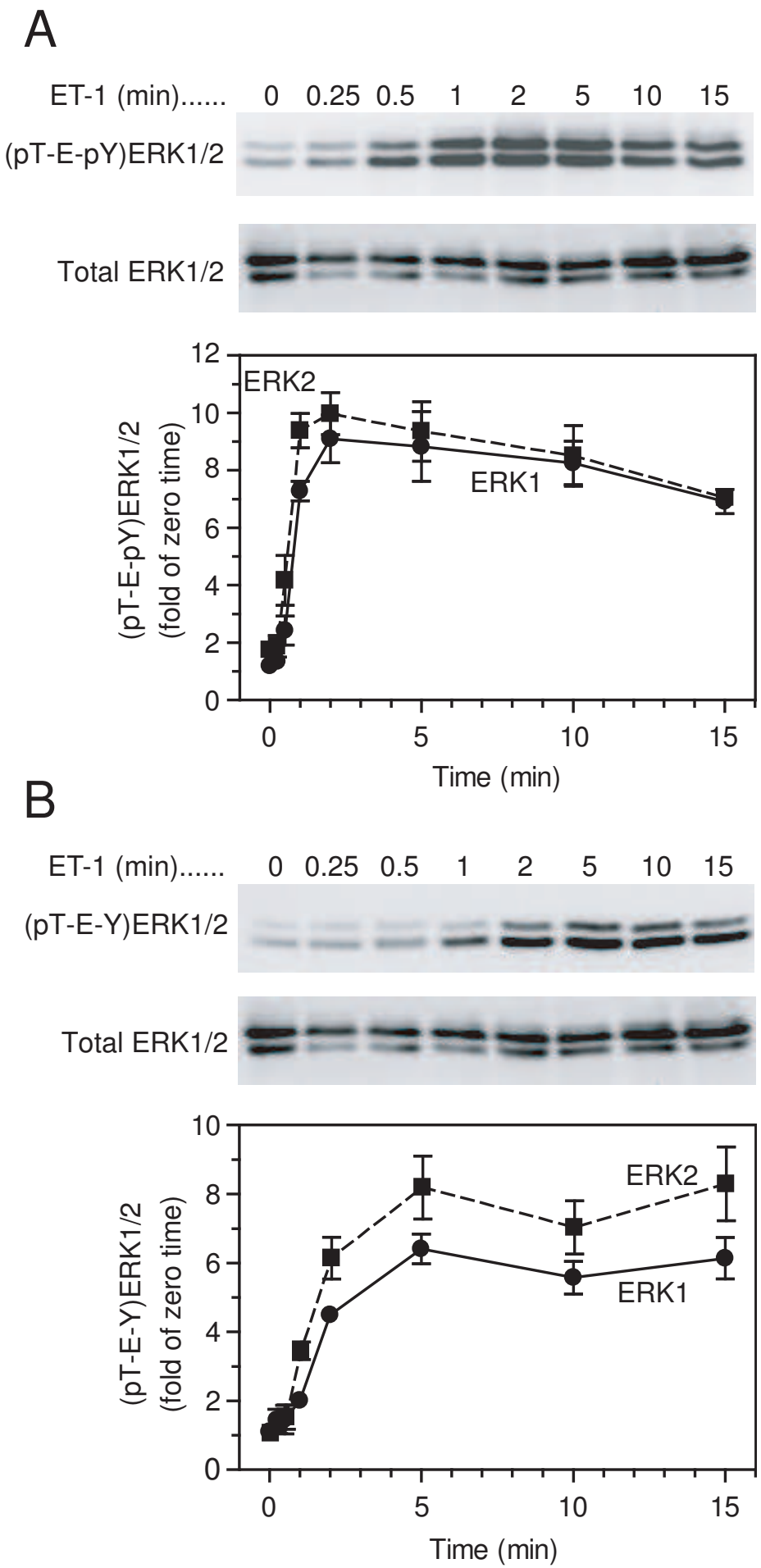


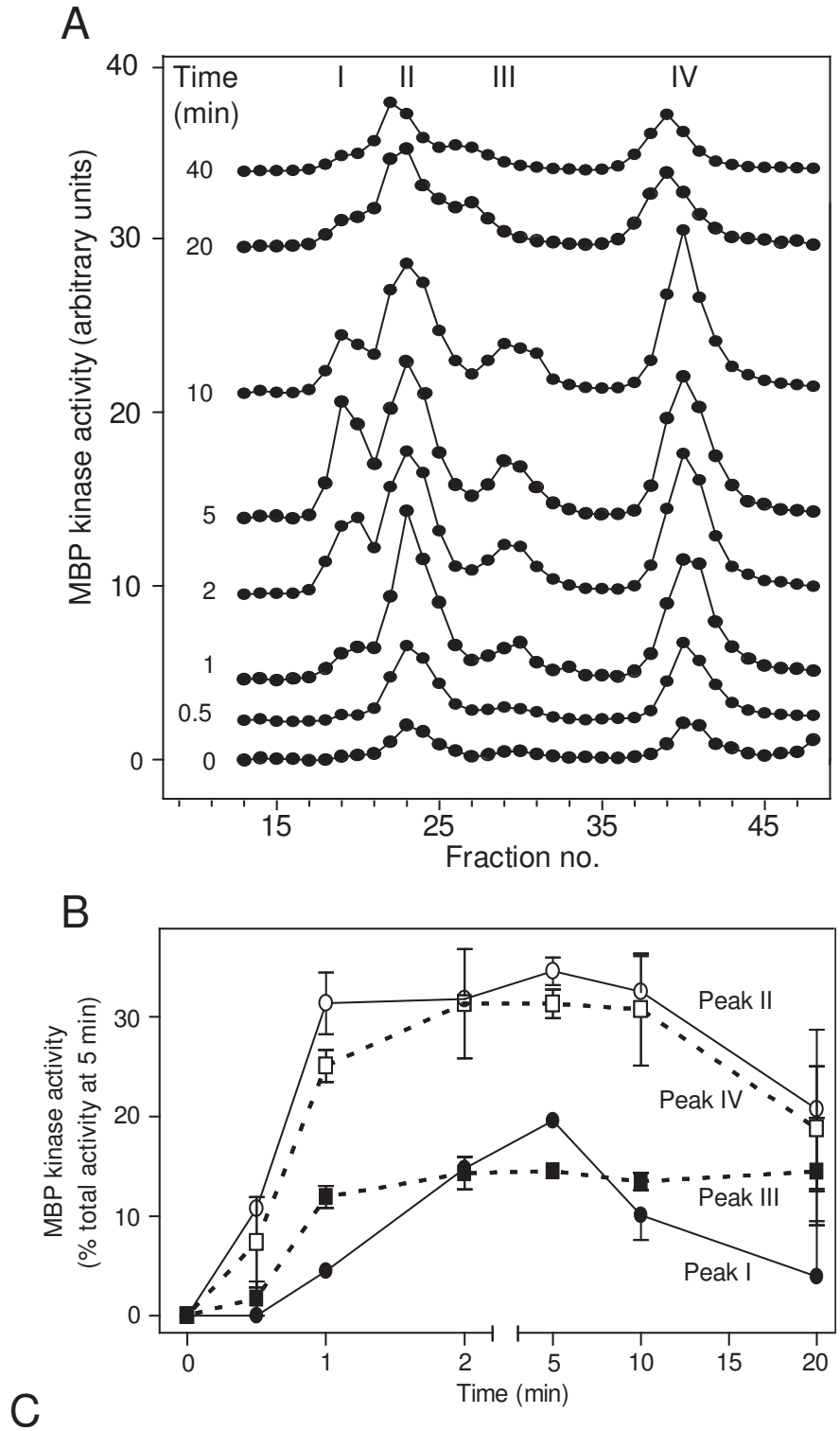

A
ET-1
I
II
III
IV
A
MBP kinase activity (c.p.m. incorporated/30 min)
NaCl concentration (M)
Fraction no.
B
PMA
C
PD184352/ ET-1
Fraction
D
Control
ET-1
PMA
Fraction
Total ERK1/2
E
Control
ET-1
PMA
(pT-E-pY)ERK1/2
(pT-E-Y)ERK1/2
(T-E-pY)ERK1/2
Total ERK1/2
Fraction
F
52
38
Fraction

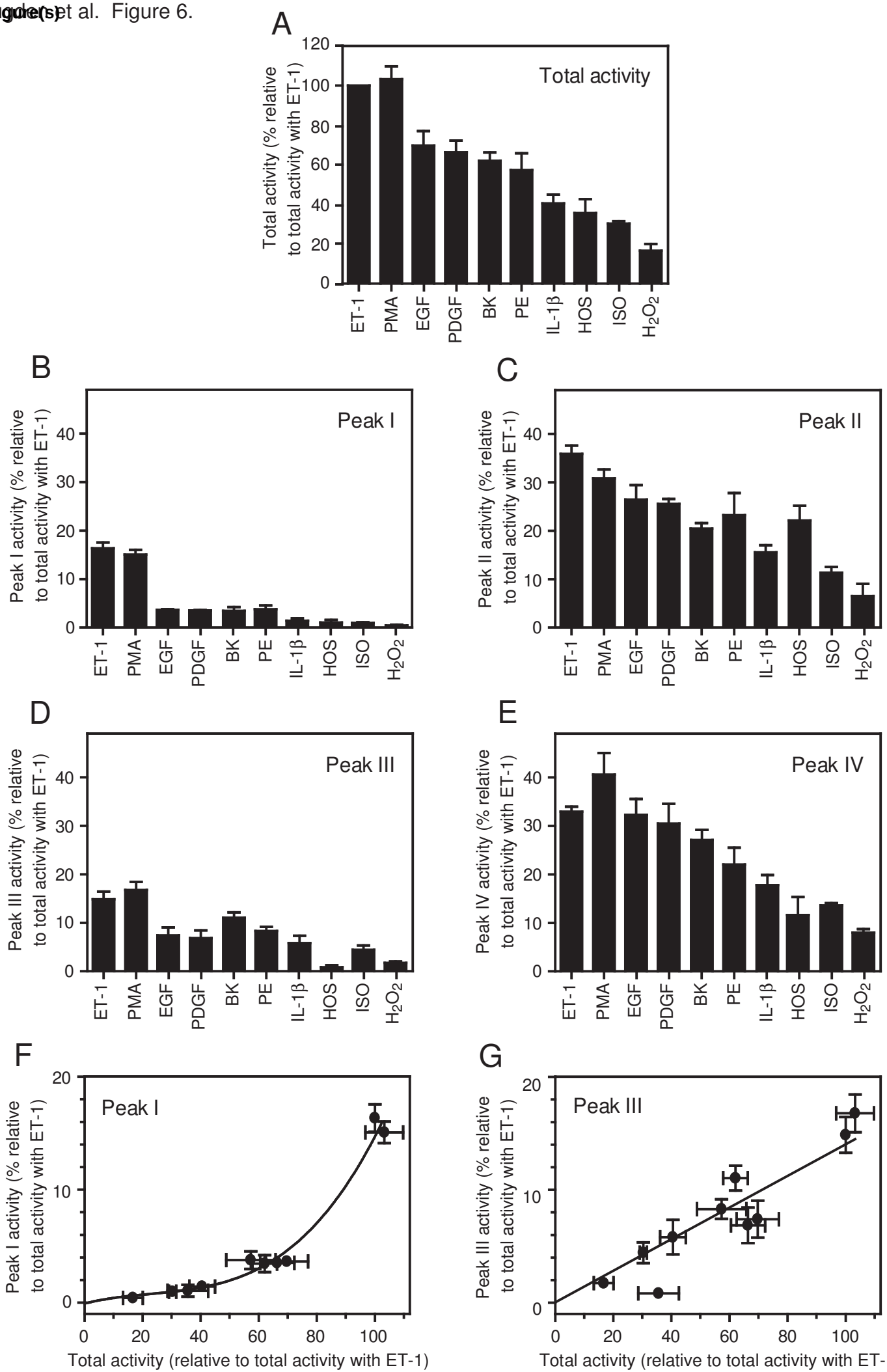
Figure(s)

Sugden et al. Figure 3.




A
ET-1 (min).....
0
0.25
0.5
1
2
5
10
15
(pT-E-pY)ERK1/2
Total ERK1/2
ERK2
ERK1
(pT-E-pY)ERK1/2
(fold of zero time)
Time (min)
B
ET-1 (min).....
0
0.25
0.5
1
2
5
10
15
(pT-E-Y)ERK1/2
Total ERK1/2
ERK2
ERK1
(pT-E-Y)ERK1/2
(fold of zero time)
Time (min)


A
Time (min)
I
II
III
IV
40
20
10
5
2
1
0.5
0
MBP kinase activity (arbitrary units)
Fraction no.
B
MBP kinase activity (% total activity at 5 min)
Peak II
Peak IV
Peak III
Peak I
Time (min)
C
Time (min) 0 0.25 0.5 1 2 5 10
(pT-E-pY)ERK1/2
(pT-E-Y)ERK1/2
Total ERK1/2
Fraction 19
Fraction 23
Fraction 29
Fraction 40


A
Total activity
Total activity (% relative to total activity with ET-1)
120
100
80
60
40
20
0
ET-1
PMA
EGF
PDGF
BK
PE
IL-1β
HOS
ISO
H2O2
B
Peak I
Peak I activity (% relative to total activity with ET-1)
40
30
20
10
0
C
Peak II
Peak II activity (% relative to total activity with ET-1)
D
Peak III
Peak III activity (% relative to total activity with ET-1)
E
Peak IV
Peak IV activity (% relative to total activity with ET-1)
F
Peak I
Peak I activity (% relative to total activity with ET-1)
20
10
0
0
20
40
60
80
100
Total activity (relative to total activity with ET-1)
G
Peak III
Peak III activity (% relative to total activity with ET-1)
Total activity (relative to total activity with ET-1)