Extracellular signal-regulated kinases control expression of G protein-coupled receptor kinase 2 (GRK2)

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Abstract G protein-coupled receptor kinase 2 (GRK2) phosphorylates G protein-coupled receptors resulting in uncoupling from G proteins. Receptors modulate GRK2 expression, however the mechanistic basis for this effect is largely unknown. Here we report a novel mechanism by which receptors use the extracellular signal-regulated kinase (ERK) cascade to regulate GRK2 cellular levels. ERK activation by receptor stimulation elevated endogenous GRK2 while antagonist treatment decreased cellular GRK2. Activating ERK by overexpressing constitutive active MEK-1 or Ras elevated GRK2 protein levels while blocking ERK using PD98059 or dominant negative Ras abolished this effect. These data suggest ERK is a critical regulator of GRK2 levels. © 2002 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.

Key words: G protein-coupled receptor; Receptor desensitisation; Angiotensin II; Mitogen activated kinase; Signal transduction

1. Introduction

G protein-coupled receptors activate multiple molecules including G proteins and the extracellular signal-regulated kinase (ERK)/mitogen-activated protein kinase cascade. A family of G protein receptor kinases (GRKs) rapidly phosphorylate and together with β-arrestin desensitise G protein signalling following agonist occupation [1]. GRK2 is a ubiquitous member of this serine/threonine kinase family [2,3]. The phosphorylated receptors are recognised by cytosolic arrestin proteins that uncouple receptors from G proteins thereby terminating G protein signalling [4]. However, GRK2 and arrestin can also turn on signalling via ERK cascade activation. Arrestin-tagged receptors recruit other molecules including Src kinase to the receptor. This serves to target the receptors for endocytosis (sequestration) through clathrin-coated pits, a process that initiates ERK signalling by several receptors [5]. GRK2-mediated phosphorylation of activated receptors thus plays a critical role in regulating activity and surface expression of G protein-coupled receptors [2,6,7]. In addition, GRK2 regulates the activity of other signalling proteins including Gαq, phosducin and cytoskeletal proteins [3,8,9].

GRK2 activity is regulated by a plethora of signalling molecules such as receptors, Gβγ, lipids, c-Src kinase, protein kinase C and A, calmodulin and ERK [2,7,10]. Within seconds to minutes following receptor activation these proteins regulate GRK2 activity, however, we have little information about the mechanistic basis for regulation of GRK2 cellular levels.

Receptor activity is an important regulator of GRK2 expression [6,11]. In cardiac myocytes, prolonged agonist stimulation (hours) of β-adrenergic receptors induces GRK2 expression while antagonist treatment reduces GRK2 levels [12]. Moreover, recent data suggest that dysregulated GRK2 expression may play a role in the pathophysiology of human diseases such as heart hypertrophy and failure, hypertension and chronic inflammation [13,14]. These results imply that receptors generate a signal that controls GRK2 expression. We herein describe a series of experiments that show ERK activation is required for receptor-mediated changes in GRK2 expression. These data enhance our understanding of the regulatory mechanisms controlling receptor signalling.

2. Materials and methods

2.1. Materials

Cell culture media and additions were from Life Technologies or Sigma. Hormones and other reagents were purchased from Sigma. The secondary antibodies, ECL reagent and hyperfilms were from Amersham. The AT_1 antagonist losartan was a gift from Gaethan Thibault (University of Montreal, QC, Canada). Antibodies against GRK2 were from Upstate (clone C5/1.1) or Santa Cruz Biotechnology (C-15, cat # sc-562). Total ERK-, phospho-ERK-antibodies and PD98059 were obtained from New England Biolabs.

2.2. DNA

Julian Downward (Imperial Cancer Research Fund, London, UK) generously provided plasmid encoding RasV12, Raymond Erickson (Harvard, MA, USA) kindly supplied MEK-DD encoding plasmid, while specific antibodies against Ras was generously provided by Berthe Willumsen (University of Copenhagen).

2.3. Cell culture and transfection

Ventricular myocyte-enriched cultures were prepared from cardiac ventricles of neonatal Wistar rat pups by trypsin digestion and mechanical disruption as described [15]. Cardiomyocytes were dissociated using 1 mg/ml trypsin and 20μg/ml DNase in a 20.1 mM HEPES buffer (pH 7.4). The cells were plated (approx. 5×10^4 cells/cm^2) in minimal essential medium (MEM) supplemented with 5% (v/v) foetal calf serum. On day 3 after preparation, the medium was changed to serum-free MEM, and different compounds were added. On day 4 or 5, cells were processed for immunoprecipitation and Western blotting.

Cos-7 cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) foetal calf serum, 10 μg/ml non-essential amino acids and 0.1 μg/ml gentamycin. Transient transfections were

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performed using a DEAE–dextran/adenovirus method as described [16].

2.4. Adenoviral infection

Adenoviral infection was achieved using an E1-deficient type 5 adenoviral containing a GRK2 encoding gene. On day 2, the cells were incubated with virus for 15 min before adding back culture medium. Two adenoviral transgenes were employed; empty vector (Ad5) and a GRK2 containing virus produced in this laboratory using the pAdEasy and pAdTrack from Stratagene.

2.5. Immunoprecipitation and western blot analysis

Cells were lysed in 1.0% Triton X-100, 50 mM Tris–HCl, pH 7.5, containing in addition 100 mM NaCl, 5 mM EDTA, 10 μg/ml Leupeptin, 10 μg/ml Aprotinin, 1 mM PMSF, and 1 mM Na_3VO_4 as described [17]. After centrifugation (12 000 × *g*, 10 min), cellular extracts were immunoprecipitated using GRK2-specific antibodies followed by incubation with protein A–Sepharose for 2 h at 4°C. Immunoprecipitates were washed three times with ice-cold 50 mM Tris–HCl buffer, pH 7.5, containing 100 mM NaCl, 0,1% Triton X-100 and the aforementioned protease inhibitors. Proteins were resolved by SDS–polyacrylamide gel electrophoresis (PAGE) and transferred to polyvinylidene difluoride (PVDF) membranes to be probed with the appropriate antibodies in TBS containing 5% milk, 0,2% Tween 20 (Merck) and 0.02% NaN_3. PVDF membranes were incubated for 2 h at room temperature with the primary antibodies, washed four times in TBS with 0.2% Tween 20, and probed with horseradish peroxidase-conjugated secondary antibodies (Amersham). Blots were developed using the ECL reagent and exposed to Hyperfilm (Amersham). Band density was quantified using laser densitometric analysis.

2.6. Inositol phosphate accumulation

Cells were labelled overnight with 2 μCi [^{3}H]inositol/ml in inositol-free media. Cells were preincubated in PBS with 10 mM LiCl, 1 mM $MgCl_2$ and 90 μM $CaCl_2$, before agonist stimulation for 20 min at 37°C. Inositol phosphates were extracted using 20 mM formic acid and loaded on Evergreen columns, 3 ml, 1 g/ml AG 1×8 resin (Bio-Rad). The columns were washed with 40 mM ammonium hydroxide, pH 9, followed by elution using 2 M ammonium format, 0.1 M formic acid into 4 ml Ultima-Flo scintillation liquid (Packard).

3. Results

3.1. AT_1 activity regulates GRK2 expression

We first validated two GRK2 antibodies for their ability to detect GRK2 in our experimental system. Used directly for immunoblotting both antibodies detected a 80 kDa band in myocyte and Cos-7 cell lysate corresponding to the GRK2 protein standard obtained from Cos-7 cells infected with GRK2-adenovirus (Fig. 1A). Since myocytes contained less GRK2 protein than Cos-7 cells, we immunoprecipitated GRK2 from myocyte lysates before SDS–PAGE. This procedure yielded a stronger GRK2 band (Fig. 1A), and therefore this procedure was employed for further studies. In control experiments, immunoprecipitated GRK2 increased in a linear fashion as a function of increasing amounts of cardiomyocyte lysate (Fig. 1B), and blocking the antibody with peptide antigen abolished the 80 kDa band (not shown). We next examined if AT_1 activation would effect GRK2 expression. Activation of recombinantly expressed AT_1 in Cos-7 increased GRK2 protein after 2 h, and GRK2 remained increased for at least 24 h (Fig. 1C). The AT_1 stimulation caused a long-lasting ERK phosphorylation without changing ERK expression (Fig. 1C). In addition, activation of native AT_1 in cardiomyocytes elevated GRK2 expression (Fig. 1D) with a similar time course (not shown). This effect that was blocked by the AT_1 antagonist losartan suggesting GRK2 expression reflects AT_1 activity (Fig. 1D). These results were confirmed with both GRK2 antibodies.

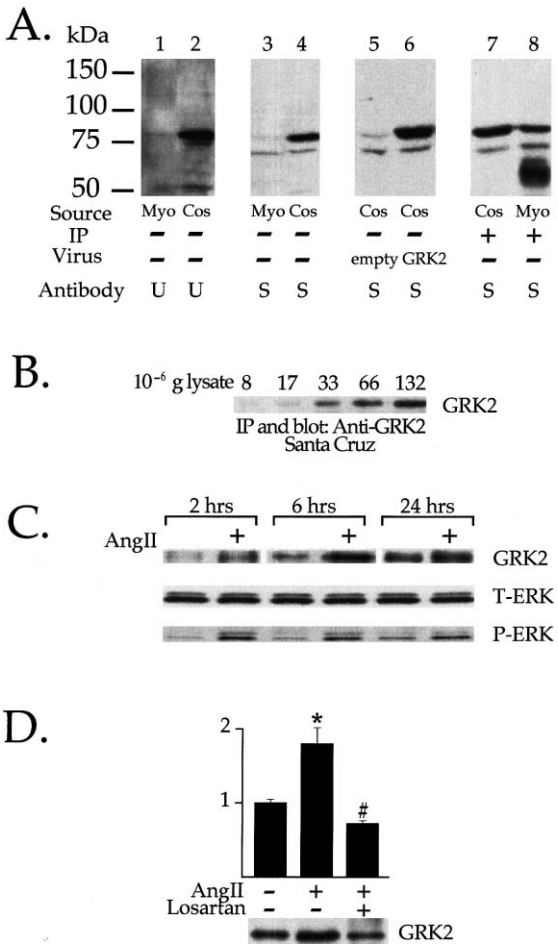

Fig. 1. GRK2 expression in cardiomyocytes and Cos-7 cells. A: (lanes 1–4) GRK2 Western blot obtained using anti-GRK2 antibodies from Upstate (U) or Santa Cruz (S). Equal amounts of cellular protein (70 μg) were subjected to SDS–PAGE. A: (lanes 5 and 6) GRK2 Western blot of lysate (20 μg protein) from Cos-7 cells infected with empty vector adenovirus (lane 5) or GRK2 containing virus (lane 6). A: (lane 7 and 8) Cell lysates were immunoprecipitated (IP) and electrophoresed before GRK2 Western blotting. B: GRK2 levels increase as a function of the amounts of cellular protein. Data are representative of three experiments. C: IP and Western blot of GRK2 protein from Cos-7 cells as a function of time in the presence and absence of 100 nM AngII. Samples were normalised for protein content. Western blots for total ERK (T-ERK) serve as loading controls while blots for phospho-ERK (P-ERK) indicate ERK activation. Data are representative of four experiments. D: GRK2 IP and Western blot in the presence or absence of 100 nM AngII or losartan for 24 h in serum-free medium. Histograms are densitometric quantification of scanned GRK2 immunoblots of lysates treated as indicated. Results are the means ± S.E.M. of double determinations from four experiments and were analysed by Student's *t*-test. $^*P < 0.01$ for AngII versus control, $^{\#}P < 0.01$ for losartan+AngII versus AngII.

3.2. ERK1 and ERK2 inhibition down-regulate GRK2

Since ERK phosphorylates GRK2 [18], we speculated the ERK cascade could play a role in regulation of GRK2 expression. Two different experiments showed ERK inhibition decreased GRK2 levels. First, PD98059, a pharmacological MEK-1 inhibitor prominently decreased GRK2 levels, suggesting ERK activation is required for GRK2 expression in both cardiomyocytes and Cos-7 cells (Fig. 2A,B). Interestingly, the most prominent PD98059 effect was a reduction in GRK expression in an agonist-independent manner. In

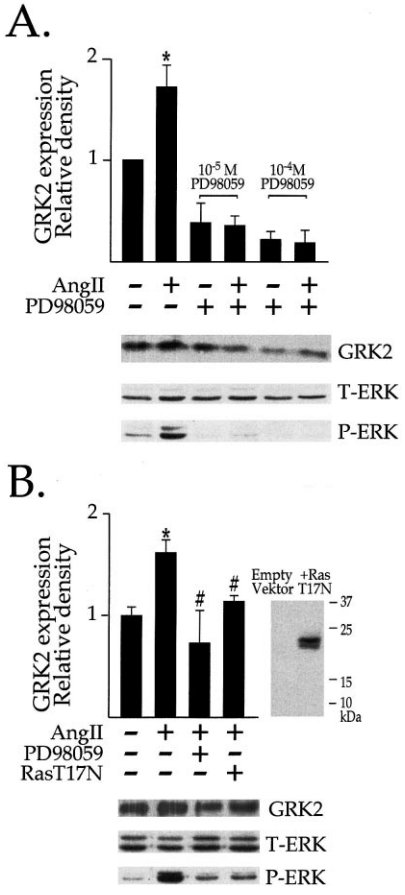

Fig. 2. ERK/mitogen-activated protein kinase blockade reduces GRK2 expression. A: Cardiac myocytes were treated with the MEK-1 inhibitor PD98059 with and without 100 nM AngII for 24 h in serum-free medium. Cell lysates were immunoprecipitated with antibodies against GRK2, ERK or phospho-ERK and Western blots prepared. Histograms are densitometric quantification of scanned immunoblots representative of four independent experiments. $^{*}P < 0.01$. B: The AT_1 was recombinantly expressed with or without co-expression of RasT17N in Cos-7 cells. Histograms are densitometric quantification of scanned GRK2 immunoblots. Expression of RasT17 was confirmed by immunoblotting using an antibody against Ras. Results are the means ± S.E.M. of double determinations from three to six experiments. $^{*}P < 0.01$ for AngII versus control, $^{\#}P < 0.05$ for # versus AngII.

control experiments PD98059 blocked ERK activation without changing ERK expression (Fig. 2A,B). Secondly, transient overexpression of dominant negative RasT17N (verified by Western blotting) prevented the angiotensin II (AngII) effect on GRK2 expression (Fig. 2B). We infer from these data that ERK activation is required for control of GRK2 expression in cultured cardiomyocytes.

3.3. Other receptors up-regulate GRK2 through ERK activation

To examine whether ERK activity serves as a general signal for regulation of GRK2 expression in cardiomyocytes, we analysed the effects of activating two other receptors; α_1-adrenergic receptors (a G protein-coupled receptor), and EGF receptors that belong to the tyrosine kinase family of receptors. Agonist stimulation of both receptors elevated GRK2 expression by a mechanism that required ERK activation, since their GRK2 response was blocked by PD98059 (Fig. 3A).

3.4. Constitutively active Ras and MEK-1 up-regulate GRK2

We examined if ERK activation by overexpression of constitutively active upstream activators in the ERK cascade would affect GRK2 expression. Indeed, overexpression (verified by Western blotting) of RasV12 and MEK-DD protein elevated GRK2 levels while ERK expression remained unchanged in Cos-7 cells (Fig. 3B). The RasV12 response was specific for ERK activation since PD98059 completely prevented the GRK2 induction by RasV12. Taken together these experiments show that ERK activation is both necessary and sufficient for elevation of GRK2 in cardiomyocytes and Cos-7 cells.

3.5. GRK2 down-regulation enhanced AT_1 signalling

To begin to analyse if GRK2 down-regulation affects AT_1 signalling, we measured the accumulation of inositol phosphates after GRK2 down-regulation by PD98059. In both

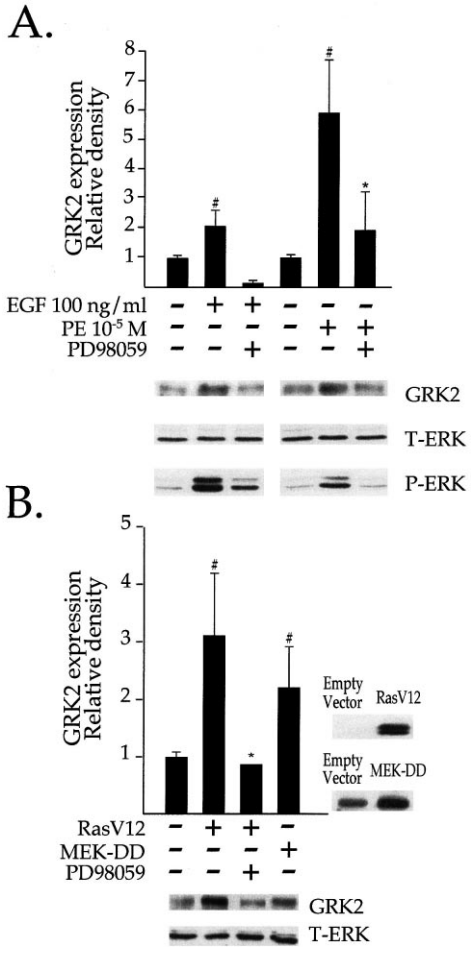

Fig. 3. ERK activation controls GRK2 expression. A: GRK2, total ERK and phospho-ERK immunoblots of lysates from cardiomyocytes treated with epidermal growth factor (EGF) and phenylephrine (PE) for 24 h. The GRK2 expression was quantified by densitometric analysis of Western blots using anti-GRK2 antibodies. Data are the means ± S.E.M. of double determinations from four to five experiments. $^{*}P < 0.1$, $^{\#}P < 0.01$. B: Quantification of scanned GRK2 immunoblots from transiently transfected Cos-7 cells in the presence and absence of constitutively activated up-stream MAP kinase partners RasV12 and MEK-DD and PD98059. T-ERK, MEK-DD and RasV12 immunoblots are shown. Results are the means ± S.E.M. of double determinations from three experiments. $^{*}P < 0.1$, $^{\#}P < 0.01$ for RasV12+PD98059 versus RasV12.

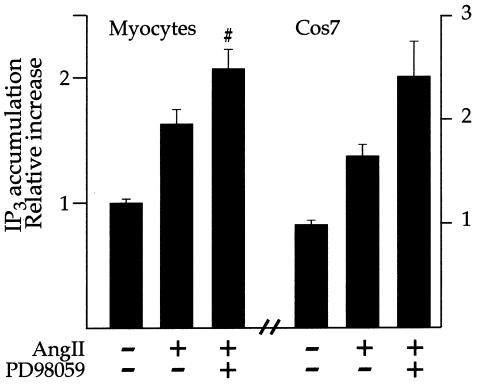

Fig. 4. GRK2 down-regulation enhanced AT_1-induced inositol phosphate accumulation. GRK2 expression was reduced using PD98059 as indicated. AT_1 natively expressed in cardiomyocytes (left panel) or transiently in Cos-7 cells (right panel) were stimulated with 10^{-7} M AngII for 20 min and the inositol phosphate accumulation measured. Results are the means ± S.E.M. of triple determinations from three (left panel) or two (right panel) experiments. $^{\#}P < 0.01$.

cell systems, this treatment increased inositol phosphate accumulation in response to AT_1 activation, suggesting the decreased GRK2 levels reduce receptor desensitisation, Fig. 4.

4. Discussion

Cells receive inputs from many different surface receptors at the same time and must interpret and integrate them in the context of each other. GRK2 plays a key role in this process; it has multiple different receptor substrates and regulation of cellular GRK2 levels represents a way to control signalling through G protein-coupled receptors [6,7]. However, we have little knowledge of the molecular mechanisms involved in such a regulation. We provide evidence that prolonged AT_1 activation in cardiomyocytes increase the cellular complement of GRK2 while the AT_1 antagonist losartan reduces this parameter. These data are in line with previous reports. Thus, increased GRK2 expression was observed in mitogen-activated lymphocytes, in mouse hearts following treatment with β-adrenergic agonists or pressure-induced hypertrophy, and in transgenic mice with cardiac overexpression of β-adrenergic receptors [12,19,20].

We hypothesised receptors generate specific signals that increase GRK2 expression, and present a series of experiments showing ERK activation may represent such a signal. This inference is based on four different experiments. First, the ERK pathway blocker PD98059 abolished the GRK2 induction by AT_1 stimulation in cultured cardiomyocytes. Interestingly, the tyrosine kinase c-Src also regulates GRK2 expression. Accordingly, Src-mediated phosphorylation of GRK2 increases its degradation through the proteasome pathway [7,11]. Taken together with our observations, we speculate that c-Src and ERK exert a differential regulation on GRK2 expression. Thus, c-Src decreases GRK2 expression while ERK activation increases GRK2 levels.

Secondly, the ERK-dependent regulation of GRK2 expression could be extended to other cell lines, since blocking ERK activation down-regulated GRK2 in Cos-7 cells expressing recombinant AT_1 receptors. In addition, overexpression of dominant negative RasT17N, an alternative way of preventing ERK activation, blocked the AngII effect on GRK2 expression, confirming the notion that ERK activation is required for regulation of GRK2 expression. This suggests the result may represent a biologically significant and general mechanism.

Thirdly, the link between ERK activation and GRK2 expression is shared by other endogenous receptors in cardiomyocytes. The α_1-adrenergic and the EGF receptors also use ERK to regulate GRK2 expression, since PD98059 abolished the elevation in GRK2 expression produced by these receptors. If other RTKs also regulate GRK2 expression through ERK activation, this might represent a novel type of desensitisation involving receptor cross-talk by which excessive RTK activity elevates GRK2 expression leading to decreased G protein-coupled receptor signalling.

Fourthly, if the inference that ERK regulates cellular GRK2 levels is correct, any way of increasing ERK activity should elicit the effect. This prediction was fulfiled since activating ERK using constitutively active Ras and MEK-1 elevated GRK2 expression in Cos-7 cells. It is striking that the ERK cascade regulates GRK2 expression, since ERK is activated by very diverse stimuli including G protein-coupled receptors, receptor tyrosine kinases, integrins, ion channels and mechanical stretch receptors [21,22]. It is tempting to speculate that ERK activation by these molecules is linked to GRK2 expression suggesting cells have evolved a way of adjusting receptor signalling to the general level of cell signalling by multiple proteins.

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