Activation of tyrosine kinases by α_{1A} -adrenergic and growth factor receptors in transfected PC12 cells

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We compared the role of tyrosine kinases in α_{1A} -adrenergic receptor (AR) and growth factor receptor stimulation of mitogen-activated protein kinase pathways in PC12 cells. Norepinephrine (NE) (noradrenaline), epidermal growth factor (EGF) and nerve growth factor (NGF) caused different patterns of tyrosine phosphorylation in PC12 cells stably expressing α_{1A} -ARs. NE increased tyrosine phosphorylation of focal adhesion-related kinase Pyk2 and a 70 kDa protein, probably paxillin, whereas EGF strongly stimulated tyrosine phosphorylation of the EGF receptor and cytokine-activated kinase Jak2. The EGF receptor inhibitor AG1478 inhibited activation of extracellular signal-regulated kinases (ERKs) by EGF but not by NE. EGF and NGF strongly activated tyrosine phosphorylation of Shc and caused association of Src-homology collagen (Shc) with growth-factor-receptor-bound protein 2 (Grb2); however, neither NE

nor UTP caused substantial activation of the Shc/Grb2 pathway. NE, UTP, EGF and NGF all increased tyrosine phosphorylation of Src, and this was inhibited by the Src inhibitor PP2. However, PP2 inhibited ERK activation in response to NE and UTP, but not in response to EGF or NGF. PP2 also completely blocked NE-induced PC12 cell differentiation, but had no measurable effect on NGF-induced differentiation. These studies show that activation of mitogen-activated protein kinase pathways by G-protein-coupled receptors and tyrosine kinase receptors proceed through distinct molecular pathways in PC12 cells, and support an obligatory role for Src activation in mitogenic responses to α_{1A} -ARs in these cells.

Key words: calcium, differentiation, G protein, mitogenic, norepinephrine.

INTRODUCTION

Mitogen-activated protein kinases (MAPKs) are known to play important roles in growth and differentiation. Growth factors acting through receptor tyrosine kinases and many neurotransmitters and hormones acting through G-protein-coupled receptors (GPCRs) activate MAPK pathways and alter cell growth. The signal transduction pathways by which receptor tyrosine kinases activate MAPKs are well established. These receptors dimerize in response to agonist occupation, causing receptor autophosphorylation. A conserved cascade of events is then initiated, including binding of the adaptor proteins Src-homology collagen (Shc) and growth-factor-receptor-bound protein 2 (Grb2), activation of the guanine nucleotide exchange factor Sos, and eventually recruitment and activation of the low-molecular-mass G-protein Ras. Ras then activates Raf, which activates downstream MAPK cascades [1].

GPCRs also activate MAPK pathways, however the mechanisms involved are less clear. In some cases, activation of these pathways is downstream of known second messengers such as cAMP [2], Ca^{2+} [3,4] and/or protein kinase C [5]. In other cells, α and/or $\beta\gamma$ subunits of heterotrimeric G-proteins may directly or indirectly activate the Ras/Raf pathway through adapter proteins, tyrosine kinases and/or phosphoinositide-3-kinase [6]. A number of these studies suggest that GPCRs and growth factors may activate MAPK responses through similar cellular machinery, converging on Ras. We have shown previously that stimulation of α_{1A} -adrenergic receptors (ARs) in stably transfected PC12 cells activates all three arms of the MAPK pathway and causes the cells to differentiate into a neuronal phenotype indistinguishable from that induced by nerve

growth factor (NGF) [7]. This response does not require increased intracellular Ca^{2+} or activation of protein kinase C [8], and different α_1 -AR subtypes activate different mitogenic responses [9]. These experiments suggest that activation of MAPKs by α_1 -ARs may involve tyrosine kinase pathways unrelated to the common second messengers activated by receptors.

To test directly the hypothesis that GPCR and tyrosine kinase receptors may utilize some of the same signalling molecules, we compared tyrosine kinase responses to growth factors and GPCRs in PC12 cells stably expressing α_{1A} -ARs. These cells endogenously express receptors for the epidermal growth factor (EGF) and NGF, as well as P_{2U} purinergic receptors (GPCRs activated by UTP) [10,11]. By directly comparing growth factor- and GPCR-mediated responses in the same cells, we attempted to identify shared elements in their mitogenic pathways and points where the pathways diverge.

EXPERIMENTAL

Materials

PC12 cells were obtained from Cindy Miranti and Michael Greenberg (Harvard Medical School, Boston, MA, U.S.A.). The human α_{1A} -AR cDNA [12] was from Gozoh Tsujimoto (National Children's Hospital, Tokyo, Japan). Other materials were obtained from the following sources: Lac-Switch vector system, Stratagene (La Jolla, CA, U.S.A.); AG1478, Calbiochem (La Jolla, CA, U.S.A.); (–)-norepinephrine (NE) (noradrenaline) bitartrate, Dulbecco's modified Eagle's medium (DMEM), penicillin and streptomycin, Sigma Chemical Co. (St. Louis, MO, U.S.A.); lipofectamine (Gibco BRL); phospho-specific extra-

Abbreviations used: AR, adrenergic receptor; DMEM, Dulbecco's modified Eagle's medium; EGF, epidermal growth factor; ERK, extracellular signal-regulated kinase; GPCR, G-protein-coupled receptor; Grb2, growth-factor-receptor-bound protein 2; HRP, horseradish peroxidase; IPTG, isopropyl β -D-thiogalactoside; MAPK, mitogen-activated protein kinase; NE, norepinephrine (noradrenaline); NGF, nerve growth factor; Shc, Src-homology collagen.

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cellular signal-related kinase (ERK) (Thr-202/Tyr-204) antibody, New England Biolabs (Beverly, MA, U.S.A.); phosphotyrosine antibody (P-Y 99), Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.); Pyk2, Src, Shc and Grb2 antibodies, Upstate Biotechnology (Lake Placid, NY, U.S.A.); horseradish peroxidase (HRP)-conjugated anti-mouse IgG and ECL reagent, Amersham (Arlington Heights, IL, U.S.A.); HRP-conjugated anti-rabbit IgG, Bio-Rad (Hercules, CA, U.S.A.).

Cell culture

Transfected PC12 cells were propagated in 75-cm² flasks at 37 °C in a humidified 5% CO₂/95% air incubator in DMEM containing 4.5 g/l glucose, 1.4% (w/v) glutamine, 20 mM Hepes, 100 mg/l streptomycin, 10⁵ units/l penicillin, 10% (v/v) donor horse serum and 5% (v/v) fetal bovine serum [7]. The cells were detached by gentle trituration and subcultured at a ratio of 1:3 upon reaching confluency. Receptor expression was induced by treatment with 1 mM isopropyl β -D-thiogalactoside (IPTG) at least 24 h before each experiment [7]. For measurements of MAPK phosphorylation, 35-mm dishes were seeded at a density of 600 000 cells/2 ml. For studies involving immunoprecipitation, 100-mm dishes were seeded at a density of 6 $\times$ 10⁶ cells/10 ml. Cells were grown to confluency before use.

Transfection

PC12 cells were co-transfected with the LacSwitch repressor (p3'SS) and the pRSVICAT operator vectors containing the human α_{1A} -AR cDNAs [13] by lipofectamine. Cells were propagated for approximately four weeks in the presence of 250 μ g/ml hygromycin and 500 μ g/ml geneticin, and subclones were screened by radioligand binding for low constitutive and high inducible receptor expression.

Immunoblotting

Confluent cells were serum-starved for 2 h before use, and drug treatments were carried out for 15 min at 37 °C. After stimulation, monolayers were lysed with Laemmli sample buffer. Cell lysates were centrifuged and proteins (10 μ g/lane) were resolved by SDS/PAGE and transferred to nitrocellulose. Phosphorylation of ERKs was detected by protein immunoblotting using a 1:1000 dilution of rabbit polyclonal dual phospho-specific antibodies with HRP-conjugated goat anti-rabbit IgG as a secondary antibody [7]. Protein tyrosine phosphorylation was detected by immunoblotting using a 1:2000 dilution of mouse polyclonal phospho-Tyr-specific antibody with HRP-conjugated goat anti-mouse IgG as a secondary antibody. Quantification was performed by densitometry after development of membranes with ECL reagent and exposure to Hyperfilm (Amersham).

Immunoprecipitation

Confluent cells were serum-starved for 2 h before further treatment. Cells were washed twice with ice-cold PBS containing 1 mM sodium orthovanadate and lysed on ice with RIPA lysis buffer (1% Nonidet P40, 25 mM Hepes, 50 mM NaCl, 50 mM NaF, 5 mM EDTA, 1 mM PMSF, 1 mM sodium vanadate, 10 μ g/ml aprotinin and 10 μ g/ml leupeptin). Cell lysate was centrifuged at approx. 10 000 g for 15 min at 4 °C in a micro-centrifuge. The supernatant containing 1 mg of protein was incubated with 10 μ g of anti-phosphotyrosine (P-Y-99) antibody at 4 °C for 2 h followed by addition of 20 μ l of Protein A-agarose. After overnight incubation at 4 °C, the sample was centrifuged

and the immunoprecipitates were washed three times with lysis buffer. For Shc or Src immunoprecipitation, 40 μ g of antibody was added to 1 mg of total cellular protein lysate and incubated at 4 °C overnight. The mixture was incubated with 40 μ l of Protein A/G-agarose for an additional 2 h. The precipitates were then washed three times with lysis buffer. After boiling in 30 μ l of 2 $\times$ SDS sample buffer [100 mM Tris/HCl, pH 6.8, 200 mM dithiothreitol, 4% (w/v) SDS, and 20% (w/v) glycerol], 15 μ l of supernatant was subjected to SDS/PAGE and transferred to a nitrocellulose membrane. Protein bands were detected by probing sequentially with the primary antibody, HRP-conjugated secondary antibody (1:5000) and ECL reagent.

Differentiation

PC12 cells were grown in collagen-coated 6-well plates at a starting density of 60 000 cells/ml. After induction with 1 mM IPTG for 2 days, agonists were added to the medium and pictures were taken 36–48 h after agonist addition.

RESULTS

Protein tyrosine phosphorylation activated by α_{1A} -ARs and growth factor receptors in stably transfected PC12 cells

We compared the patterns of protein tyrosine phosphorylation stimulated by agonists in PC12 cells stably expressing α_{1A} -ARs. PC12 subclone α_{1A} -3 was pretreated with IPTG for 48 h to induce receptor expression, expressing α_{1A} -ARs at approx. 293 $\pm$ 65 fmol/mg of protein [7]. Figure 1 shows that treatment with NE, EGF or NGF for 15 min caused different patterns of tyrosine phosphorylation. Exposure to NE specifically increased phosphorylation of a $\sim$ 70 kDa protein, whereas exposure to EGF increased phosphorylation of a high-molecular-mass protein identified as the 170 kDa EGF receptor by immunoprecipitation (results not shown), as well as several smaller

Figure 1 Differential activation of tyrosine kinases by α_{1A} -adrenergic and tyrosine kinase receptors

Tyrosine phosphorylation stimulated by 100 μ M NE, 50 ng/ml EGF or 50 ng/ml NGF. Cells were treated with the indicated agonists for 15 min and lysed. Cell lysates were immunoprecipitated with an anti-phosphotyrosine antibody, eluted and then immunoblotted with anti-phosphotyrosine (top), anti-Pyk2 (middle), or anti-Jak2 antibody (bottom). In this and subsequent figures lanes marked 'B' show basal levels.

Figure 2 Tyrosine phosphorylation of paxillin activated by NE and NGF

Cells were treated with agonists for 15 min and lysed. Cell lysates were immunoprecipitated with an anti-paxillin antibody, eluted and then immunoblotted with an anti-phosphotyrosine (P-Y) antibody.

(20–50 kDa) proteins. Phosphorylation of specific proteins was identified by immunoprecipitation with an anti-phosphotyrosine antibody, elution and Western blotting with antibodies to known proteins. NE, but not EGF or NGF, increased tyrosine phosphorylation of the focal adhesion-related kinase Pyk2, whereas EGF, but not NE or NGF, stimulated tyrosine phosphorylation of the cytokine-activated kinase Jak2. To examine whether the 70 kDa protein tyrosine phosphorylated in response to NE was paxillin [14], cell lysates were immunoprecipitated with an anti-paxillin antibody and blotted with an anti-phosphotyrosine antibody. Figure 2 shows that NE, and to a lesser extent NGF,

Figure 3 Effect of the EGF receptor tyrosine kinase inhibitor AG1478 on tyrosine phosphorylation (top) and ERK activation (bottom) stimulated by 100 μ M NE or 50 ng/ml EGF

Cells were pre-treated with or without 500 nM AG1478 for 30 min before addition of NE or EGF for a further 15 min. Total protein (10 μ g) was loaded in each lane and blots were probed with an anti-phosphotyrosine antibody (top) or dual phospho-specific ERK antibody (bottom).

Figure 4 Left panels: stimulation of Shc tyrosine phosphorylation (top panel) and Shc/Grb2 association (bottom panel) by 100 μ M NE, 100 μ M UTP, 50 ng/ml EGF or 50 ng/ml NGF; right panels: time-course of Shc tyrosine-phosphorylation and Shc/Grb2-association stimulated by 100 μ M NE or 50 ng/ml NGF

Left panels: cells were treated with the indicated agonists for 5 min and lysed. Cell lysates were immunoprecipitated with anti-Shc antibody and blots were probed with either an anti-phosphotyrosine (P-Y; top) or an anti-Grb2 (bottom) antibody. Right panels: NE and NGF were incubated with the cells for the indicated times and immunoprecipitated Shc was probed with anti-Grb2 antibody (top) or anti-phosphotyrosine antibody (bottom).

Figure 5 Src tyrosine phosphorylation stimulated by 100 μ M NE, 100 μ M UTP, 50 ng/ml EGF or 50 ng/ml NGF

Cells were treated with indicated agonists for 5 min before whole cell lysates were obtained. Immunoprecipitation was carried out using either anti-Src (right panel) or anti-phosphotyrosine (left panel) antibodies and the blots were probed with anti-phosphotyrosine and anti-Src antibody respectively.

Figure 6 Effect of PP2 on Src tyrosine phosphorylation stimulated by NE and NGF

Cells were treated with 100 μ M NE or 50 ng/ml NGF for 15 min after 30 min pretreatment with 10 μ M PP2. Src was immunoprecipitated and probed with anti-phosphotyrosine antibody.

strongly activates tyrosine phosphorylation of paxillin in α_{1A} -transfected PC12 cells.

EGF receptor transactivation is not involved in NE-stimulated ERK activity

In some cell lines, activation of ERKs by GPCRs requires transactivation of EGF receptors [15,16]. To determine whether this was true in our cells, we examined the effect of the EGF receptor tyrosine kinase inhibitor AG1478 [17] on ERK activation by NE and EGF. Figure 3 shows that NE-stimulated ERK phosphorylation is not inhibited by 500 nM AG1478, a

Figure 7 Effect of PP2 on ERK activation stimulated by 100 μ M NE, 100 μ M UTP, 50 ng/ml EGF or 50 ng/ml NGF

Cells were treated with 10 μ M PP2 for 30 min before addition of various agonists for a further 5 min. Cell lysates were immunoblotted with anti-ERK antibody that specifically recognizes dual-phosphorylated ERKs.

concentration which completely inhibits EGF activation of ERKs and tyrosine phosphorylation of the EGF receptor (Figure 3).

Activation of the Shc/Grb2 pathway

We examined whether the adaptor proteins Shc and Grb2, which are essential for growth factor-mediated ERK activation, are involved in responses to α_{1A} -ARs in PC12 cells. Tyrosine phosphorylation of Shc and the association of Shc and Grb2 was monitored after agonist activation. Figure 4 shows that 5 min exposure to EGF or NGF strongly activates tyrosine phosphorylation of Shc and causes association with Grb2. However, 5 min exposure to NE or UTP caused no significant increase in Shc tyrosine phosphorylation or Grb2 association (Figure 4). The time course of responses to NE and NGF are also shown in Figure 4. The response to NGF peaked at 2 min after agonist addition. Conversely, NE showed little activation of Shc phosphorylation or association with Grb2 during the 15 min period studied (Figure 4).

Activation of Src

We also examined activation of the tyrosine kinase Src by the various agonists in transfected PC12 cells. Figure 5 shows that treatment with NE, EGF or NGF for 5 min caused a marked activation of Src tyrosine phosphorylation. Treatment with UTP for 5 min gave a smaller increase in Src tyrosine phosphorylation.

Src is required for NE- and UTP-mediated ERK activation

Since all the agonists tested activated Src, we examined whether this activation was required for ERK activation using the Src family tyrosine kinase inhibitor PP2 [18]. Figure 6 shows that treatment with 10 μ M PP2 effectively inhibited tyrosine phosphorylation of Src stimulated by NE and NGF. PP2 treatment also inhibited the basal level of Src phosphorylation. Interestingly, as shown in Figure 7, the same PP2 treatment blocked NE- and UTP-stimulated ERK activation, but had no effect on NGF- or EGF-stimulated ERK activation.

Src is required for NE-induced cell differentiation

To study further the role of Src in the α_{1A} -AR-mediated MAPK pathway, we examined the effect of PP2 on PC12 cell differentiation induced by NE and NGF. As shown in Figure 8, treatment of PC12 cells with 10 μ M PP2 completely inhibited differentiation induced by NE, but had no effect on differentiation induced by NGF.

DISCUSSION

The different patterns of tyrosine phosphorylation caused by activation of α_{1A} -ARs and tyrosine kinase receptors in PC12 cells suggest that the signals generated by these receptors do not converge at a step proximal to receptor activation. Neither EGF receptor transactivation nor activation of the Shc/Grb2 pathway was found after activation of α_{1A} -ARs. In addition, Src appears to be required for α_{1A} -AR, but not growth factor receptor-induced activation of ERKs and differentiation. These observations suggest that there is little or no overlap in the signalling pathways activated by α_{1A} -ARs and growth factor receptors in these cells.

Few similarities were observed in the proteins tyrosine phosphorylated in response to α_{1A} -ARs and growth factor receptors. Activation of α_{1A} -ARs was found specifically to increase tyrosine phosphorylation of a 70 kDa protein, probably paxillin, which has been reported to be tyrosine phosphorylated following activation of other GPCRs [14,19]. NE also activated the focal adhesion-related kinase, Pyk2, which has previously been implicated in mitogenic responses to other GPCRs [3,4]. EGF specifically increased tyrosine phosphorylation of the EGF receptor, as expected, and also activated the cytokine-activated tyrosine kinase Jak2, as previously reported [20].

Studies with other GPCRs have shown that ERK activation may sometimes involve transactivation of receptor tyrosine kinases such as the EGF receptor [15,16]. Activation of angiotensin II receptors in vascular smooth muscle cells was found to cause a calcium-dependent activation of the EGF receptor,

Figure 8 Effect of PP2 on PC12 cell differentiation in response to NE or NGF

PC12 cells were pre-incubated with or without 10 μ M PP2 for 30 min before addition of 100 μ M NE or 50 ng/ml NGF. Photographs were taken 48 h after agonist addition.

thereby recruiting Shc and Grb2 and activating ERKs [15]. However, EGF receptor transactivation does not appear to be involved in responses to α_{1A} -ARs in PC12 cells. There was no detectable tyrosine phosphorylation of the EGF receptor after exposure to NE, and the specific inhibitor of EGF receptor tyrosine kinase activity AG1478 had no effect on NE-stimulated ERK activity, although it completely blocked both EGF-induced receptor phosphorylation and ERK activation. Similarly, release of calcium is not required for this response [8], suggesting that a calcium-dependent EGF receptor activation could not play a role. Although the pathways involved depend on the type of GPCR and cell phenotype [21], transactivation of EGF receptors is not always required for GPCR-mediated ERK activation.

The potential convergence of signals initiated by GPCR and tyrosine kinase receptors was examined by studying activation of the adaptor protein Shc and its association with Grb2. Tyrosine phosphorylation of Shc is important in linking receptor tyrosine kinase activation to Ras activation in growth factor signalling [22–25]. Activation of several GPCRs has also been shown to increase Shc tyrosine phosphorylation, and this has been suggested to play an important role in MAPK activation. Stimulation of the G_i -coupled lysophosphatidic acid receptor, α_{2A} -ARs, or overexpression of $G\beta_1\gamma_2$ subunits has been shown to increase tyrosine phosphorylation of Shc [26]. This activation also appears to be downstream of EGF receptor transactivation. Soltoff [10] obtained similar results studying ERK activation by the endogenous P_{2Y2} receptor in PC12 cells. In that study, purine nucleotides increased Shc activation, apparently after EGF receptor transactivation caused by increases in intracellular Ca^{2+} and activation of protein kinase C. However, in our cells we observed little or no increase in Shc tyrosine phosphorylation in response to either NE or UTP, consistent with the lack of EGF receptor transactivation discussed above. We also found little or no association of Grb2 with Shc after NE or UTP stimulation. In contrast, EGF and NGF caused the expected large increases in Shc tyrosine phosphorylation and a strong association with Grb2. These results suggest that NE-stimulated ERK activation does not involve activation of the Shc/Grb2 signalling pathway. The difference between our results and those of Soltoff [10] are probably due to the phenotypic variation often observed with PC12 subclones [11].

Activation of the tyrosine kinase Src is involved in mitogenic responses in many cells. Src activation has been implicated in many GPCR pathways, including those for both G_i - and G_q -coupled receptors [27]. Since little or no Shc activation was observed after α_{1A} -AR stimulation, we tested whether Src might be involved. Tyrosine phosphorylation of Src was strongly increased following treatment with NE, EGF or NGF. As expected, the selective inhibitor of Src family protein kinases, PP2, blocked Src activation by all agonists. PP2 pretreatment also blocked ERK activation in response to NE, but not in response to EGF or NGF. This suggests that Src is activated by both GPCRs and receptor tyrosine kinases in PC12 cells, but this activation plays a role in mitogenic responses to GPCRs but not tyrosine kinase receptors.

Src activation has also been shown to be associated with lysophosphatidic acid and α_2 -adrenergic receptor mediated ERK activation [21]. Overexpression of Src caused tyrosine phosphorylation of Shc and association of Shc and Grb2. Della Rocca et al. [5] also studied the role of Src in ERK activation stimulated by α_{1B} - and α_{2A} -ARs after overexpression in HEK293 cells. They found that overexpression of a dominant-negative mutant of c-Src or of the negative regulator of c-Src function, Csk, reduced α_{1B} - and α_{2A} -AR mediated ERK activation. The role of Src in activation of c-Jun N-terminal kinase and p38

MAPK was examined by Nagao et al. [28], after overexpression of $G\alpha_q/11$ in HEK 293 cells. Overexpression of the constitutively activated $G\alpha_{11}$ mutant stimulated Src kinase activity, and this response was blocked by PP2 and overexpression of Csk. These results are in agreement with our results, and support a critical role for Src in mediating G_q -coupled receptor-stimulated ERK activity in PC12 cells. In support of these results, we found that PP2 inhibited PC12 cell differentiation induced by NE, but not by NGF. This is consistent with the PP2-mediated inhibition of ERK activation by NE but not NGF, and the known role of ERK activation in PC12 cell differentiation.

Many studies have shown that NGF activates Src, but the role of this response in ERK activation and differentiation is not clear. Our results suggest that inhibition of Src activation does not affect ERK activation or PC12 differentiation stimulated by NGF. Activation of Src by NGF may be involved in cellular responses other than activation of ERKs and differentiation. Sato et al. [29] studied NGF-dependent tyrosine phosphorylation of Shc in PC12 cells and found that inhibition of Src by PP1 blocked this response. However, they did not examine the effect of PP1 on ERK activation. In our cells, PP1 does not inhibit NGF-stimulated ERK activation (results not shown). However, NGF may use other members of the Src protein tyrosine kinase family to mediate these pathways. Qian et al. [30] in their studies with developing neurons showed that different substrates containing SH2 domains are involved in NGF function, depending on the types of neurons. Thus, different Src family members with differential PP2 sensitivity may be involved in NGF-mediated ERK activation and differentiation in PC12 cells.

In summary, we have identified different signalling molecules involved in ERK activation by GPCRs and tyrosine kinase receptors in PC12 cells. Our results suggest a differential involvement of Shc and Grb2 in these two pathways, as well as differential involvement of Pyk2 and Src tyrosine kinases. Src appears to be required for GPCR-mediated ERK activation and differentiation in these cells, although it does not appear to play a major role in response to NGF.

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