Critical Role of Calcium-dependent Epidermal Growth Factor Receptor Transactivation in PC12 Cell Membrane Depolarization and Bradykinin Signaling*

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PC12 cells respond to a variety of external stimuli such as growth factors, neurotransmitters, and membrane depolarization by activating the Ras/mitogen-activated protein kinase pathway. Here we demonstrate that both depolarization-induced calcium influx and treatment with bradykinin stimulate tyrosine phosphorylation of the epidermal growth factor receptor (EGFR). Using a tetracycline-controlled expression system in conjunction with a dominant-negative EGFR mutant, we demonstrate that depolarization and bradykinin triggered signals involve EGFR function upstream of SHC and MAP kinase. Furthermore, bradykinin-stimulated EGFR transactivation is critically dependent on the presence of extracellular calcium, and when triggered by ionophore treatment, calcium influx is already sufficient to induce EGFR tyrosine phosphorylation. Taken together, our results establish calcium-dependent EGFR transactivation as a signaling mechanism mediating activation of the Ras/mitogen-activated protein kinase pathway in neuronal cell types.

In neurons, the cytosolic calcium concentration is tightly regulated and represents a critical parameter for a variety of intracellular signaling processes. Intracellular calcium levels are modulated either by release of calcium from internal stores or by calcium entry across the plasma membrane through ligand- or voltage-gated calcium channels (1–3). Stimuli such as growth factors, neurotransmitters, and membrane depolarization by activating the Ras/mitogen-activated protein kinase pathway. Here we demonstrate that both depolarization-induced calcium influx and treatment with bradykinin stimulate tyrosine phosphorylation of the epidermal growth factor receptor (EGFR). Using a tetracycline-controlled expression system in conjunction with a dominant-negative EGFR mutant, we demonstrate that depolarization and bradykinin triggered signals involve EGFR function upstream of SHC and MAP kinase. Furthermore, bradykinin-stimulated EGFR transactivation is critically dependent on the presence of extracellular calcium, and when triggered by ionophore treatment, calcium influx is already sufficient to induce EGFR tyrosine phosphorylation. Taken together, our results establish calcium-dependent EGFR transactivation as a signaling mechanism mediating activation of the Ras/mitogen-activated protein kinase pathway in neuronal cell types.

In neurons, calcium levels can trigger various signaling events, among them the activation of the small G-protein Ras resulting in stimulation of the mitogen-activated protein kinase (MAPK)1 pathway (5). In PC12 cells, a rat pheochromocytoma cell line widely used as a model system for neuronal differentiation, calcium influx rapidly induces tyrosine phosphorylation of the adaptor protein SHC and SHC-Grb2 complex formation, steps known to couple cell surface receptors such as receptor tyrosine kinases to Ras (6). Using a PC12 subline overexpressing a dominant-negative mutant of the cytoplasmatic tyrosine kinase Src, Rusanesca et al. found that inhibition of membrane depolarization induced SHC tyrosine phosphorylation and MAPK activation (7). Moreover, calcium influx following membrane depolarization was recently reported to mediate ligand-independent epidermal growth factor receptor (EGFR) tyrosine phosphorylation in this system (8). Although direct evidence is lacking regarding whether this represents an essential signaling event for activation of the MAPK pathway, this finding raises the possibility that in PC12 cells calcium may play a role in the EGFR transactivation mechanism as previously demonstrated for signaling through G-protein coupled receptor (GPCR) in Rat-1 fibroblasts (9). In addition to membrane depolarization-induced activation of the MAPK pathway (7), GPCR-mediated signaling was also reported to involve Src function (10–13). Moreover, the tyrosine kinase PYK2, a relative of the focal adhesion kinase, was implicated in triggering the MAPK pathway in PC12 cells (6). This raised the possibility that concerted action of receptor tyrosine kinases and cytoplasmatic tyrosine kinases might be necessary to activate certain signaling cascades in response to GPCR stimulation or membrane depolarization (10).

To analyze potential EGFR function in calcium-dependent signaling, we developed a PC12 cell line that expresses the dominant-negative EGFR mutant HER-CD533 under the control of a tetracycline-sensitive promoter system (14). In this system specific EGFR inhibition strongly attenuates SHC tyrosine phosphorylation and MAPK activation in response to both membrane depolarization and bradykinin stimulation. Moreover, we find that calcium ionophore treatment of PC12 cells is sufficient to trigger EGFR tyrosine phosphorylation, whereas EGFR transactivation in response to the GPCR ligand bradykinin is critically dependent on the presence of extracellular calcium. Therefore calcium-dependent EGFR transactivation integrates various extracellular stimuli and provides a link to downstream signal progression.

EXPERIMENTAL PROCEDURES

Reagents, Antibodies, and Plasminoids—Protein A-Sepharose was purchased from Pharmacia Biotech Inc. Fura-2 was from Molecular Probes. Bradykinin and ionomycin were purchased from Calbiochem. All other reagents were obtained from Sigma. Antibodies used were rabbit polyclonal anti-EGFR antibody (Santa Cruz), sheep polyclonal anti-EGFR antibody (Upstate Biotechnology Inc.), rabbit polyclonal anti-ERK2 antibody (Santa Cruz), and mouse monoclonal anti-phosphotyrosine antibody 4G10 (Upstate Biotechnology Inc.). Rabbit polyclonal anti-Shc antibody has been described (21). As secondary antibodies, goat anti-mouse and anti-rabbit conjugates (Bio-Rad and Dianova) were used. For immunoblot detection, the ECL system from Amersham Corp. was

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1 The abbreviations used are: MAPK, mitogen-activated protein kinase; GPCR, G-protein-coupled receptor; EGFR, epidermal growth factor; EGFR, EGF receptor; MBF, myelin basic protein; tTA, tetracycline-controlled transactivator; NGF, nerve growth factor.
Utilizing. Stripping and reprobing of blots were performed according to the manufacturers’ recommendations.

pUHD15-1neo contains the tetracycline-controlled transactivator (tTA) coding sequence driven by the human cytomegalovirus promoter/enhancer. pUHD10-3 contains the PhCMV*-1 followed by a multiple cloning sequence (14). These two plasmids were kindly provided by Hermann Bujard and Manfred Gossen. The establishment of the PC12 cell clone 15–1/5, which stably expresses tTA, will be described elsewhere.2 To generate a PC12/HER-CD533 construct, the HER-CD533 cDNA was subcloned as an EcoRI fragment into pUHD10-3.

Cell Culture and Generation of PC12 Cell Lines—PC12 cells (kindly provided by Iro’s modified Eagle’s medium, 4500 gliter glucose, supplemented with 5% fetal bovine serum and 10% horse serum. PC12 cells were generally grown on collagen (Sigma)-coated plastic dishes. The stable transfection of pUHD10-3-HER-CD533 or pUHD10-3 into tTA-expressing clone 15–1/5 was performed with Lipofectamine. For transfection in 6-cm dishes, cells were incubated for 8–20 h in 2.0 ml of serum-free medium containing 5 µl of LipofectAMINE, 2.0 µl of pUHD-3-HER-CD533 or pUHD10-3, and 0.2 µg of PSV2-hph, which contains the hygromycin-resistant gene. The PSV2-hph was a generous gift from Marianne Dieckmann and Paul Berg from Stanford University. Cells were selected with 200 µg/ml hygromycin B (Boehringer Mannheim). Colonies were cloned, expanded, and further analyzed.

Cell Lysis, Immunoprecipitation, and Western Blotting—Prior to experiments, cells were cultured for 48 h in the presence or the absence of tetracycline for induction of HER-CD533 expression and treated with inhibitors and agonists as indicated. Cells were then lysed as previously (21). Precleared lysates were immunoprecipitated, and subjected to gel electrophoresis, and immunoblotted as described (9, 21).

MAPK Assay—Endogenous ERK2 was immunoprecipitated from lysates obtained from 6-well dishes using 0.4 µg of anti-ERK2 antibody, washed three times with HNTG buffer, and washed once with kinase buffer (20 mM HEPES, pH 7.5, 10 mM MgCl₂, 1 mM dithiothreitol, 200 µM sodium orthovanadate). The kinase reactions were performed in 30 µl of kinase buffer supplemented with 0.5 mg/ml myelin basic protein, 50 µM ATP, and 1 µCi of [γ-32P]ATP for 10 min at room temperature. Reactions were stopped by the addition of 30 µl of Laemmli buffer and subjected to gel electrophoresis on 15% gels.

Intracellular Calcium Measurements—For dye loading, the cells were incubated with Fura-2-acetoxymethylster (5 µmol/liter, 30 min) supplemented with 250 µmol/liter sulfinpyrazone (22) to prevent dye leakage. Fluorescence measurements were made as described before (23). The intracellular free Ca²⁺ concentration was calculated according to the method described by Grynkiewicz et al. (24).

RESULTS AND DISCUSSION

To analyze EGFR function in response to stimuli such as membrane depolarization or GPCR activation in PC12 cells, we employed the tetracycline-controlled gene expression system for expression of the dominant-negative EGFR mutant HER-CD533 (14, 15). This mutant lacks the cytoplasmic domain and disrupts EGFR downstream signaling by forcing endogenous wild-type receptors into signaling-incompetent heterodimers. In the PC12/HER-CD533/Tet cell line, expression of HER-CD533 is suppressed in the presence of tetracycline, whereas removal of tetracycline results in an approximately 20-fold induction within 48 h, as determined by metabolic labeling and subsequent quantification of immunoprecipitated mutant reporters.

Fig. 1. Inducible expression of HER-CD533 and the effect of HER-CD533 on EGFR tyrosine phosphorylation and calcium mobilization in PC12 cells. The PC12 cell line PC12/HER-CD533/Tet and the control cell line were cultivated for 48 h with or without 1 µg/ml tetracycline. A, cells were labeled with [35S]methionine, and serum-starved in Dulbecco’s modified Eagle’s medium, 4500 gliter glucose, supplemented with 5% fetal bovine serum and 10% horse serum. PC12 cells were generally grown on collagen (Sigma)-coated plastic dishes. The stable transfection of pUHD-3-HER-CD533 or pUHD10-3 into tTA-expressing clone 15–1/5 was performed with Lipofectamine. For transfection in 6-cm dishes, cells were incubated for 8–20 h in 2.0 ml of serum-free medium containing 5 µl of LipofectAMINE, 2.0 µl of pUHD-3-HER-CD533 or pUHD10-3, and 0.2 µg of PSV2-hph, which contains the hygromycin-resistant gene. The PSV2-hph was a generous gift from Marianne Dieckmann and Paul Berg from Stanford University. Cells were selected with 200 µg/ml hygromycin B (Boehringer Mannheim). Colonies were cloned, expanded, and further analyzed.

The PC12 cell line PC12/HER-CD533/Tet was loaded with the dye Fura-2-acetoxymethylster and treated with bradykinin (1 µM) or KCl (75 mM) in a calcium-free medium. Calcium release from internal stores was measured upon bradykinin or KCl treatment. Fluorescence was measured as described under “Experimental Procedures.” White bars correspond to cells treated with tetracycline, and filled bars correspond to cells treated without tetracycline for 48 h. Data represent the means of three independent experiments (± S.D.). Ab, antibody.
When these cells were subjected to 75 mM extracellular KCl to trigger membrane depolarization in the presence of tetracycline, this treatment stimulated EGFR tyrosine phosphorylation in agreement with previous observations (8) (Fig. 1A). The same effect was achieved by treatment of undifferentiated PC12/HER-CD533/Tet cultures with bradykinin in analogy to previously reported findings for the GPCR ligands endothelin-1, lysophosphatidic acid, and thrombin in Rat-1 fibroblasts (9). In PC12/HER-CD533/Tet, expression of HER-CD533 upon removal of tetracycline strongly interfered with EGFR tyrosine phosphorylation upon treatment with KCl, bradykinin, and EGF, whereas in control cells these stimuli resulted in increased EGFR tyrosine phosphorylation unaffected by the removal of tetracycline (Fig. 1B). Reprobing with anti-EGFR antibody revealed that comparable amounts of protein were present, which supported the conclusion that EGFR inhibition was due to dominant-negative HER-CD533 action. Importantly, in PC12/HER-CD533/Tet cells elevation of cytoplasmatic calcium upon bradykinin incubation or membrane depolarization was similar when HER-CD533 expression was either induced or repressed (Fig. 1C), indicating that functional coupling to calcium mobilization was unaffected by EGFR inhibition.

Tyrosine phosphorylation of the adaptor protein SHC represents a prominent receptor-proximal signaling step upon EGFR activation (16). Analysis of crude cell lysates indicated that the HER-CD533 mutant specifically abolished EGF-stimulated SHC tyrosine phosphorylation, whereas the NGF-induced response remained unaltered (Fig. 2A). The identity of the 52-kDa phosphotyrosine-containing protein as the major SHC isoform was confirmed by reprobing immunoblots with a specific antibody. To answer the question of whether the previously reported membrane depolarization- or bradykinin-induced SHC tyrosine phosphorylation (6) is mediated through transactivation of the EGFR, we immunoprecipitated SHC after stimulation of PC12/HER-CD533/Tet cells in the presence or the absence of tetracycline. As shown in Fig. 2B, HER-CD533 induction suppressed both KCl- and bradykinin-stimulated SHC tyrosine phosphorylation, demonstrating an essential role of the EGFR in both signals.

We next analyzed the role of EGFR function in the activation of the MAPK pathway in PC12 cells. To address this question, we examined the effect of EGFR inhibition on activation of ERK-2 following KCl or bradykinin treatment. ERK-2 activity was measured with an immunocomplex kinase assay using myelin basic protein as an exogenous substrate. As shown in Fig. 3 (upper panel), expression of HER-CD533 strongly and reproducibly attenuated ERK-2 activation upon KCl or bradykinin treatment by approximately 80%. For reasons currently unknown, the stimulation of MAPK activity following bradykinin treatment was reproducibly weaker compared with stimulation with KCl. As expected, EGF-induced MAPK stimulation was completely suppressed, whereas the NGF-induced response, included as a control, was not significantly affected. Interestingly, despite weaker stimulation of Shc tyrosine phosphorylation (Fig. 2A), NGF induced MAPK activity as potently as 1 ng/ml EGF. This suggested that upon NGF stimulation additional signal transducers such as the recently described Grb2-binding protein FRS2 may contribute to the activation of the Ras/MAPK pathway (17).

Furthermore, any influence of tetracycline on MAPK activation was excluded using control cells (Fig. 3, lower panel). Similar effects on MAPK activation upon these treatments were obtained with nanomolar concentrations of the EGFR-specific inhibitor AG1478 (data not shown).

The recently reported finding of ligand-independent EGFR tyrosine phosphorylation upon membrane depolarization-mediated calcium influx (8) raised the question of whether calcium may be critical for EGFR transactivation in PC12 cells. As shown in Fig. 4A, elimination of extracellular calcium with 3 mM EGTA for 5 min completely abolished the increase of EGFR tyrosine phosphorylation upon bradykinin stimulation. More-
over, when we used the calcium ionophore ionomycin to directly elevate intracellular calcium levels, enhanced phosphorylation of the EGFR on tyrosine was readily detected. Tetracycline-controlled expression of HER-CD533 strongly interfered with EGFR function in PC12 cells (18). Interestingly, elevated intracellular calcium levels was also reported to trigger tyrosine phosphorylation and activation of the cytoplasmatic tyrosine kinase PYK2 in PC12 cells (6). Moreover, this tyrosine kinase had been suggested to link stimuli such as membrane depolarization and bradykinin to MAPK activation. Because PYK2 was reported to interact with Src upon bradykinin stimulation (10), this association might be required for Src to efficiently phosphorylate its cellular sub-

strates on tyrosine residues. Moreover, because overexpression of a dominant-negative Src mutant had been reported to interfere with depolarization-induced SHC tyrosine phosphorylation (7), our findings raise the question how the EGFR and the cytoplasmatic tyrosine kinase complex Src/PYK2 are functionally linked in PC12 cells. Because oncogenic Src appears to activate the EGFR by tyrosine phosphorylation at nonregular sites (19), Src and PYK2 could act upstream and utilize EGFR for further signal transmission. Alternatively, to reconcile the results presented here and those reported earlier (6, 10), EGFR transactivation might occur independently and parallel to Src/PYK2 with SHC binding to phosphorylated EGFR and subsequent SHC phosphorylation by activated Src.

The tetracycline-controlled inducible expression system used in this study to analyze EGFR function in PC12 cells may be instrumental for finding answers to these questions. Because, at the concentrations used, tetracycline is without any detectable effect and the temporal and quantitative parameters of mutant EGFR expression can be tightly controlled, this system provides an excellent tool for the examination of multiple elements in the cellular signal transmission network of the PC12 model system. In the context of the nervous system where the EGFR is found in various areas (20), our findings provide a new basis for the investigation of GPCR mediated signals and their significance for biological phenomena such as neuronal cell survival and neurodegenerative disease.

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