Recently, we have demonstrated that in PC12 cells activation of the Ras/extracellular signal-regulated kinase pathway in response to membrane depolarization or bradykinin is mediated by calcium-dependent transactivation of the epidermal growth factor receptor (EGFR). Here we address the question whether Ca\(^{2+}\)-calmodulin-dependent protein kinase (CaM kinase) has a role in the EGFR transactivation signal. Using compounds that selectively interfere with either CaM kinase activity or calmodulin function, we show that KCl-mediated membrane depolarization-triggered, but not bradykinin-mediated signals involve CaM kinase function upstream of the EGFR. Although both depolarization-induced calcium influx and bradykinin stimulation of PC12 cells were found to induce c-fos transcription through EGFR activation, the former signal is CaM kinase-dependent and the latter was shown to be independent. As PYK2 is also activated upon elevation of intracellular calcium, we investigated the potential involvement of this cytoplasmic tyrosine kinase in EGFR transactivation. Interestingly, we observed that inhibition of CaM kinase activity in PC12 cells abrogated tyrosine phosphorylation of PYK2 upon KCl but not bradykinin treatment. Nevertheless, PYK2 activation in response to both stimuli appeared to be mediated by pathways parallel to EGFR transactivation. Our data demonstrate the existence of two distinct calcium-dependent mechanisms leading either to EGFR-mediated extracellular signal-regulated activation or to PYK2 tyrosine phosphorylation. Both pathways either in concert or independently might contribute to the definition of biological responses in neuronal cell types.

In neurons, transient changes of intracellular calcium levels are critical for a variety of cellular signaling processes. The concentration of intracellular calcium can be increased by influx across the plasma membrane or by release from internal stores (1–3). In neuronal cell cultures, membrane depolarization-induced calcium entry through voltage-gated calcium channels (VGCC)\(^{1}\) can trigger the expression of a number of immediate-early genes such as c-fos, and thereby contributes to physiological responses such as neuronal differentiation and survival (4–6). Elevation of intracellular calcium and its subsequent binding to calmodulin (CaM) has been reported to be an important cellular response upon hormonal stimulation or membrane depolarization (7). Ca\(^{2+}\)-calmodulin is required for the activity of a wide variety of enzymes including protein kinases such as the multifunctional Ca\(^{2+}\)-calmodulin-dependent protein kinases (CaM kinases) (3, 8–11). In PC12 cells, a rat pheochromocytoma-derived cell line that is widely used as a model system for neuronal differentiation, calcium influx through VGCC induces c-fos transcription through a signaling pathway that may include the activation of a CaM kinase (10, 12). Moreover, in hippocampal neurons it has been demonstrated that both membrane depolarization and stimulation of NMDA receptors result in elevation of intracellular calcium concentrations and c-fos induction, but only KCl-induced c-fos expression can be blocked by inhibition of CaM kinases (3). Thus, depending on the mode of calcium entry, different signaling pathways can be activated downstream.

Besides activating Ca\(^{2+}\)-calmodulin-dependent signaling cascades, membrane depolarization-induced calcium influx triggers the ubiquitous Ras/extracellular signal-regulated kinase (ERK) pathway (13, 14). In PC12 cells, 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 tyrosine kinases to Ras (15). This finding, and the earlier report that calcium entry following membrane depolarization triggers tyrosine phosphorylation of the EGFR (13), led to the demonstration that EGFR tyrosine phosphorylation represents an essential signaling event for ERK activation in PC12 cells upon KCl as well as bradykinin stimulation (14). Therefore, in PC12 cells, calcium plays an important role in the EGFR transactivation mechanism, which had previously been demonstrated to be required for G-protein-coupled receptor (GPCR)-mediated ERK activation in Rat-1 fibroblasts and other cell types (16, 17). The critical role of calcium in the EGFR transactivation mechanism is further supported by the recent finding that ERK activation by angiotensin II involves Ca\(^{2+}\)-dependent EGFR stimulation (18).

Cytoplasmic kinases of the Src family have also been reported to be implicated in the activation of the Ras/ERK pathway by various stimuli such as membrane depolarization and GPCR-mediated signals (19–20). Moreover, the cytoplasmic tyrosine kinase PYK2, a relative of the focal adhesion kinase FAK, was reported to link GPCR signaling and the calcium second messenger system influx to the MAPK pathway in PC12 cells (15). These observations raised the possibility that concerted action of receptor tyrosine kinases and cytoplasmic tyrosine kinase activity might result in activation of the Ras/ERK pathway.
Calcium-dependent Transactivation of EGFR and PYK2

Rosine kinases might be necessary to activate distinct signaling cascades in response to GPCR stimulation or membrane depolarization (22).

In the present study, we demonstrate that a calcium-calmodulin-dependent pathway is involved in KCl- but not bradykinin-induced EGFR transactivation. Selective inhibition of CaM kinases strongly attenuates SHC tyrosine phosphorylation and ERK activation in response to membrane depolarization. Furthermore, membrane depolarization and bradykinin stimulation of PC12 cells activate c-fos transcription through distinct calcium-dependent signaling pathways involving the EGFR. Finally, we demonstrate that PYK2 tyrosine phosphorylation upon KCl but not bradykinin stimulation is dependent on CaM kinase function and address the question of PYK2 involvement in calcium-triggered EGFR transactivation.

EXPERIMENTAL PROCEDURES

Reagents, Antibodies, and Plasmids—Protein A-Sepharose was from Amersham Pharmacia Biotech. KN-62, KN-93, W-7 and ionomicyn were purchased from Calbiochem. Bradykinin and 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. (UBI)), rabbit polyclonal anti-ERK antibody (Santa Cruz), rabbit polyclonal anti-PYK2 antibody (UBI), and mouse monoclonal anti-phosphotyrosine antibody 4G10 (UBI). Rabbit polyclonal anti-Shc antibody has been described before (24). As secondary antibodies, goat anti-mouse and anti-rabbit conjugates (Bio-Rad and Dianova) were used. The ECL system from Amersham Pharmacia Biotech was utilized for immunoblot detection. Stripping and reprobing of blots were performed according to the manufacturer's recommendations.

pUHD15–1neo contains the tetracycline-controlled transactivator coding sequence driven by the human cytomegalovirus promoter/enhancer, pUHD0-3 contains the PhCMV*-1 followed by a multiple cloning sequence (25). These two plasmids were kindly provided by Herbert Bujard and Manfred Gossen. The establishment of the PC12/HER-CD533/Tet cell line has been described before (14). The kinase-negative mutant of PYK2 (PYK2-KM) was constructed by replacing Lys557 with an Ala residue by site-directed mutagenesis (26). The oligonucleotide sequence of the mutant was designed to create a new NruI restriction site. The nucleotide sequence of PYK2-KM was confirmed by DNA sequencing. To generate a PC12/PYK2-KM/Tet cell line the PYK2 cDNA was subcloned into pUHD0-3.

Cell Culture and Generation of PC12 Cell Lines—PC12 cells (kindly provided by Philip Cohen) were cultured in Dulbecco's modified Eagle’s medium, 4500 mg/liter glucose, supplemented with 5% fetal bovine serum and 10% horse serum. PC12 cells are generally grown on plastic dishes coated with collagen (Sigma). The stable transfection of pUHD10-3-HER-CRD533, pUHD-3-PYK2-KM, and pUHD10-3 into tetracycline-controlled transactivator-expressing clone 15-185 was performed with LipofectAMINE. For transfection in 6-cm dishes, cells were incubated for 8–20 h in 2 ml of serum-free medium containing 50 μg/ml hygromycin B (Roche Molecular Biochemicals). The PC12/PYK2-KM/Tet cell line was generated by transfecting PC12 cells with pUHD0-3.

Cell Lysis, Immunoprecipitation, and Western Blotting—Prior to lysis, cells grown to 80% confluence were treated with inhibitors and agonists as indicated and then lysed for 10 min on ice in buffer containing 50 mM HEPES, pH 7.5, 150 mM NaCl, 1% Triton X-100, 1 mM EDTA, 10% glycerol, 10 mM sodium pyrophosphate, 2 mM sodium orthovanadate, 10 mM sodium fluoride, 1 mM phenylmethylsulfonfonyl fluoride, and 10 μg/ml aprotinin. For induction of HER-CRD533 or PYK2-KM expression, cells were cultured for 48 h in the presence or absence of tetracycline, treated with inhibitors and agonists as indicated, and lysed. Lysates were precleared by centrifugation at 13,000 rpm for 10 min at 4 °C. Precleared lysates were immunoprecipitated using the respective antibodies and 20 μl of protein A-Sepharose for 4 h at 4 °C. Precipitates were washed three times with 0.5 ml of HNTG buffer (24), suspended in 2× SDS sample buffer, boiled for 3 min, and subjected to gel electrophoresis. Following SDS-polyacrylamide gel electrophoresis, proteins were transferred to a nitrocellulose membrane and immunoblotted.

MAPK Assay—Endogenous ERK2 was immunoprecipitated from lysates obtained from six-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). 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 per reaction. Reactions were stopped by addition of 30 μl of Laemmli buffer and subjected to gel electrophoresis on 15% gels. Labeled MBP was quantitated using a Phosphoimager (Fuji).

Northern Blot Analysis—Quiescent PC12 cells were stimulated for 30 min with the appropriate agonists prior to treatment with inhibitors or 48 h incubation in the presence or absence of tetracycline. Cells were harvested in 5 ml of ice-cold PBS and total RNA was prepared using the Qiagen Total RNA extraction kit according to manufacturer's recommendations. 10 μg of each RNA preparation were separated on a 1.4% agarose formaldehyde gel, and the 28 S and 18 S RNA were detected by ethidium bromide staining of the gel. The RNA was then transferred to nitrocellulose for hybridization with a 32P-labeled cDNA probe of c-fos full coding sequence.

PC12 Differentiation Assay—PC12/PYK2-KM/Tet cells were seeded into six-well dishes and cultivated for 48 h in medium with or without tetracycline. After 2 days, the medium was replaced by medium containing only 1% horse serum and 50 ng/ml EGF, 50 ng/ml NGF, or no ligand. Photomicrographs of cells were taken 18 h after addition of ligand with a phase-contrast microscope (Nikon) supplemented with a camera.

RESULTS

Membrane Depolarization but Not Bradykinin-mediated EGFR Transactivation Is Blocked by CaM Kinase and Calmodulin Inhibitors—KCl-induced calcium influx or bradykinin stimulation of PC12 cells results in the rapid induction of EGFR tyrosine phosphorylation (13, 14). Moreover, our recent work demonstrated that such EGFR transactivation is critically dependent on the presence of extracellular calcium (14). These results strongly indicated an involvement of calcium-dependent proteins in EGFR transactivation. In an initial attempt to identify such transactivation signal elements, we employed the compounds W-7 and KN-62, which have been reported to inhibit the actions of calmodulin and CaM kinase, respectively (27, 28). As shown in Fig. 1A, pretreatment of PC12 cells with 5 μM KN-62 strongly interfered with KCl-mediated EGFR transactivation. In contrast, KN-62 pretreatment had only minor effect on bradykinin-induced EGFR tyrosine phosphorylation and, as expected, EGF-mediated receptor tyrosine phosphorylation was unaffected by preincubation with KN-62. As previous work demonstrated that KN-62 might also directly interfere with Ca2+ channel function independently of CaM kinase inhibition (29, 30), we further examined this question utilizing the structurally and functionally unrelated compound W-7. Pretreatment of PC12 cells with W-7 (5 μM) also blocked KCl-induced EGFR tyrosine phosphorylation, whereas this reagent had only marginal effects on bradykinin- or EGF-triggered EGFR activation. Similar results were obtained using KN-93, another CaM kinase inhibitor (31) (data not shown). Taken together, these observations support the conclusion that, in PC12 cells, EGFR transactivation is mediated through calcium-dependent signaling mechanisms, either involving CaM kinases in response to membrane depolarization, or operating without them as shown for bradykinin stimulation.

Tyrosine phosphorylation of the adaptor protein SHC represents a prominent receptor-proximal signaling step following EGF activation (32). The effects of CaM kinase inhibition on EGFR transactivation suggested that SHC tyrosine phosphorylation might be regulated in a similar manner. In order to verify this prediction, we investigated the role of CaM kinase in KCl- and bradykinin-induced SHC tyrosine phosphorylation. Therefore, we pretreated PC12 cells with W-7, stimulated the cells, and immunoprecipitated SHC using a specific antibody. The Western blot analysis indicated that inhibition of CaM kinase activity achieved by pretreatment of PC12 cells with the
inhibitor W-7 specifically abolished membrane depolarization-stimulated SHC tyrosine phosphorylation, whereas the bradykinin- and EGF-induced response remained unaltered (Fig. 1C, upper panel). Similar results on SHC tyrosine phosphorylation were obtained using the CaM kinase inhibitors KN-62 or KN-93 (data not shown), further supporting an essential role of CaM kinase function in the membrane depolarization-triggered cell response.

Previously, we demonstrated that ionomycin-induced calcium influx into PC12 cells is sufficient to induce EGF tyrosine phosphorylation (14). We next addressed the question whether ionomycin-induced ERK2 transactivation is dependent on CaM kinase function. To answer this question, PC12 cells were either control-incubated or pretreated with W-7 or KN-62, and then subjected to calcium ionophore stimulation. As shown in Fig. 1D, both inhibitors of CaM kinase activity did not affect EGF tyrosine phosphorylation in response to ionomycin. Moreover, SHC tyrosine phosphorylation following calcium ionophore stimulation was not influenced by pretreatment of PC12 cells with either inhibitor (data not shown). This shows that CaM kinase activity is not involved in ionomycin-triggered EGF transactivation, indicating that, depending on the mode of stimulation, elevation of intracellular calcium can lead to the activation of alternative, CaM kinase-independent transactivation mechanisms sufficient to trigger EGF tyrosine phosphorylation.

**The Role of CaM Kinase in Membrane Depolarization- and Bradykinin-induced ERK2 Activation**—We next investigated the role of CaM kinase function in the activation of the ERK MAPK pathway in PC12 cells. To address this question, we examined the effect of CaM kinase inhibition on activation of ERK2 following KCl or bradykinin treatment of PC12 cells. ERK2 activity was measured with an immunocomplex assay using myelin basic protein (MBP) as an exogenous substrate. As shown in Fig. 2, pretreatment of PC12 cells with KN-62 or W-7 strongly attenuated ERK2 activation upon KCl treatment, whereas bradykinin-mediated ERK-2 activity was not inhibited, but even slightly enhanced in the case of KN-62 preincubation. As expected, EGF-mediated ERK2 activation was unaffected by pretreatment of cells with both inhibitors. Thus, in extension of our previous work, these results establish a depolarization-triggered signaling cascade involving downstream function of CaM kinase and EGF prior to activation of the ERK MAPK pathway.

**Effect of CaM Kinase and EGF Inhibition on Membrane Depolarization- and Bradykinin-induced c-fos Transcription**—

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**Fig. 1. Ca^2+-calmodulin-dependent kinase-inhibition of KCl-, bradykinin-, and ionomycin-mediated EGFR and SHC tyrosine phosphorylation.** A, PC12 cells were serum-starved for 20 h and pretreated with 5 μM KN-62 or an equal volume of Me2SO for 20 min before stimulation with 75 mM KCl, 1 μM bradykinin, or 1 ng/ml EGF for 90 s. After lysis, the EGFR was immunoprecipitated (IP) using polyclonal anti-EGFR antibody. Tyrosine-phosphorylated EGFR was detected by immunoblotting with monoclonal anti-phosphotyrosine (αPY) antibody (upper panel), followed by reprobing of the same filter with anti-EGFR antibody (lower panel). B, quiescent PC12 cells were preincubated with 5 μM W-7 or an equal volume of Me2SO for 20 min and stimulated with KCl (75 mM), bradykinin (1 μM), or EGF (1 ng/ml). After cell lysis, EGFR tyrosine phosphorylation (upper panel) was detected as described under A, followed by reprobing of the same filter with anti-EGFR antibody (lower panel). C, quiescent PC12 cells were pretreated with 5 μM W-7 (20 min) or an equal volume of Me2SO and stimulated with KCl (75 mM), bradykinin (1 μM), or EGF (1 ng/ml). After cell lysis, SHC was immunoprecipitated using polyclonal anti-SHC antibody. Tyrosine-phosphorylated SHC was detected by immunoblotting with αPY antibody (upper panel). The amount of SHC was further analyzed by reprobing of the same filter with anti-SHC antibody (lower panels). D, quiescent PC12 cells were preincubated with KN-62 (5 μM), W-7 (5 μM), or an equal volume of SO for 20 min prior to stimulation with 1.8 μM ionomycin. After lysis, the EGFR was immunoprecipitated (IP) using polyclonal anti-EGFR antibody. Tyrosine-phosphorylated EGFR was detected by immunoblotting with αPY antibody (upper panel), followed by reprobing of the same filter with anti-EGFR antibody (lower panel).
Early work in PC12 cells indicated that membrane depolarization leads to c-fos expression by activation of L-type VGCC (5). Moreover, results published by Greenberg and colleagues demonstrated that expression of the c-fos gene following calcium influx through VGCC involves CaM kinase II function (4, 6). To assess the role of CaM kinase in bradykinin-induced c-fos gene expression, PC12 cells were incubated with KN-62 prior to stimulation with KCl, bradykinin or EGF and induction of c-fos transcription was determined by Northern blot analysis. As shown in Fig. 3A (upper panel), inhibition of CaM kinase function by pretreatment of PC12 cells with KN-62 strongly interfered with KCl-induced c-fos gene transcription, confirming earlier results, which suggested an important function of CaM kinase in the transmission of the L-type Ca^{2+} channel signal to the nucleus (3). In contrast, expression of the c-fos gene after bradykinin stimulation is not inhibited, but appears to be enhanced in the presence of KN-62. As expected, EGF-mediated c-fos expression is not affected by CaM kinase inhibition. Similar results were obtained using additional CaM kinase (KN-93) or calmodulin (W7) inhibitors (data not shown), indicating that enhancement of c-fos transcription upon CaM kinase inhibition prior to bradykinin stimulation is not due to a nonspecific side effect of the inhibitor. This experiment strongly suggests that, in comparison to membrane depolarization, bradykinin induces c-fos transcription via a CaM kinase-independent signaling pathway. A similar CaM kinase-independent mechanism for c-fos induction is used by the NMDA receptor and involves the function of a serum response element (SRE) (6). This pathway may include activation of CaM kinases or Ras/ERK-mediated phosphorylation of SRE-binding proteins, or both. (3, 33). As it is known that CaM kinases can induce CREB/Ca^{2+} response element-dependent transcription of the c-fos gene (3), we wanted to know whether KCl-mediated ERK2 activation is essential to induce c-fos transcription or acts in parallel to CaM kinase-induced promoter activation. To investigate the impact of the EGFR/ERK MAPK pathway on c-fos gene expression, we used the recently described PC12/HER-CDS33/Tet cell line (14), which expresses the dominant-negative EGFR mutant HER-CDS33 under the control of a tetracycline-sensitive promoter system. This mutant lacks the cytoplasmic domain and disrupts EGFR downstream signaling by engaging endogenous wild-type receptors to form signaling-competent heterodimers (34). As we have previously demonstrated, expression of HER-CDS33 in this cell line is suppressed in the presence of tetracycline, whereas removal of tetracycline results in an approximately 20-fold induction within 48 h (14). As shown in Fig. 3B (upper panel), expression of HER-CDS33 strongly attenuated expression of c-fos mRNA upon KCl or bradykinin stimulation, demonstrating that EGFR tyrosine phosphorylation is an important step in KCl- and bradykinin-mediated signaling to the nucleus. As expected, EGF-induced c-fos transcription was completely suppressed, whereas the NGF-mediated response, included as a control, was not affected. These experiments demonstrate that the EGFR/ERK MAPK pathway is necessary to mediate activation of the c-fos promoter upon membrane depolarization and bradykinin stimulation of PC12 cells. Moreover, these findings show that CaM kinase-induced CREB phosphorylation is not sufficient to induce c-fos transcription following KCl treatment of PC12 cells. Effect of CaM Kinase Inhibition on Membrane Depolarization-induced PYK2 Tyrosine Phosphorylation—Not only the EGFR, but also the cytoplasmic tyrosine kinase PYK2 has been implicated between stimuli which trigger calcium elevation and ERK activation (15). Although protein kinase C has been suggested to play a role in the regulation of PYK2 (35, 36), the mechanism by which calcium activates PYK2 is not known. Here, we tried to analyze whether PYK2 tyrosine phosphorylation is regulated in a common or distinct calcium-dependent
mode relative to EGFR activation. Therefore, we examined the influence of CaM kinase inhibition on PYK2 tyrosine phosphorylation in PC12 cells. As shown in Fig. 4, pretreatment of PC12 cells with 5 μM KN-62 strongly attenuated PYK2 tyrosine phosphorylation upon membrane depolarization, whereas the bradykinin-mediated response was not affected by CaM kinase inhibition. This result indicated that PYK2 tyrosine phosphorylation and EGFR transactivation are regulated by similar or identical calcium-dependent pathways. In contrast, pretreatment of PC12 cells with the protein kinase C inhibitor GF109203X had nearly no effect on membrane depolarization- and bradykinin-mediated PYK2 tyrosine phosphorylation (data not shown).

Connection between EGFR Transactivation and PYK2 Function—Recent findings implicating PYK2 in GPCR-induced ERK activation (20), together with our finding of similar Ca2+-dependent regulation of PYK2 and EGFR tyrosine phosphorylation, raise the question of a functional connection between the two kinases in relation to ERK activation. One must consider the possibilities of PYK2 action upstream, downstream or even parallel to EGFR signaling. To assess the role of PYK2 function in membrane depolarization- and bradykinin-mediated EGFR tyrosine phosphorylation, we developed a PC12 cell line that expresses a kinase-inactive PYK2 mutant (PYK2-KM) under the control of a tetracycline-sensitive promoter system (25). In the PC12/PYK2-KM/Tet cell line, expression of PYK2-KM is suppressed in the presence of tetracycline, whereas removal of tetracycline results in an induction of PYK2 expression within 48 h, as determined by immunoblot analysis of crude cell lysates (Fig. 5). As shown in Fig. 5 (upper panel), expression of the PYK2-KM mutant did not affect EGFR tyrosine phosphorylation in response to either bradykinin or KCl treatment. Moreover, analysis of crude cell lysates (lower panel) revealed that the 52-kDa SHC isoform was tyrosine-phosphorylated in response to both stimuli both in the presence and absence of tetracycline, indicating that SHC tyrosine phosphorylation following membrane depolarization and bradykinin stimulation occurs independently of PYK2 function. Thus PYK2 does not seem to be involved in the cross-talk between membrane depolarization- or GPCR signals and the EGFR in PC12 cells.

As PYK2 was implicated in GPCR-induced ERK/MAPK activation (20), we investigated the effect of PYK2-KM expression on bradykinin-mediated ERK2 activation by measuring the ERK2 activity with an immunocomplex assay using MBP as an exogenous substrate. As shown in Fig. 6, expression of the kinase-negative PYK2 mutant (PYK2-KM) reproducibly attenuated ERK2 activation upon bradykinin treatment, but also following EGF stimulation (0.3 and 1.0 ng/ml) by approximately 40%. This result confirmed our conclusion that PYK2 is not involved in membrane depolarization- and bradykinin-mediated EGFR transactivation, but rather seems to act downstream of EGFR signaling.

However, the interpretation of these experiments is critically limited, as the expression level of PYK2-KM might not be high enough to suppress PYK2-mediated signaling or the mutant might not be properly localized to be effective. Nevertheless, the expression level of the kinase-inactive PYK2 mutant was high enough to observe dramatic effects on cell-substrate and cell-cell interactions of the PC12/PYK2-KM/Tet cell line. As expected, treatment of PC12/PYK2-KM/Tet cells with NGF (50 ng/ml) in the presence of tetracycline results in the induction of neurite outgrowth, whereas EGF treatment (50 ng/ml) in the presence of tetracycline stimulates cell proliferation (Fig. 7, a–c). Interestingly, expression of the kinase-inactive PYK2 mutant upon removal of tetracycline resulted in a loss of cell-to-substrate adhesion and formation of cell aggregates within 6 h following stimulation with both NGF or EGF (Fig. 7, e and f), whereas in unstimulated cells expression of PYK2-KM had no effect on their adhesion properties (Fig. 7d). Similarly, recent findings demonstrated that stable expression of an ERK inhibitory mutant in PC12 cells resulted in the formation of calcium-dependent cell aggregates, which were less adherent to the substrat (37). Together with our observations, these findings suggest a role of PYK2-mediated ERK activation in the regulation of PC12 cell-cell interactions.

Membrane Depolarization- and Bradykinin-mediated PYK2 Tyrosine Phosphorylation Is Not Dependent on EGFR Func-
Calcium-dependent Transactivation of EGFR and PYK2

It is well established that the EGFR plays a key role in the transduction of mitogenic signals in response to membrane depolarization (18) or activation of G-protein-coupled receptors (16–18, 38). In PC12 cells, the KCl- or bradykinin-triggered EGFR transactivation pathway requires the presence of extracellular calcium (14). We demonstrate here that membrane depolarization—but not bradykinin-mediated EGFR tyrosine phosphorylation—is dependent on Ca^{2+}-calmodulin-dependent kinase function (Fig. 1, A and B). This conclusion is derived from experimental results that involve inhibitors, which, although established in the literature, may interfere with the function of molecules other than CaM kinase or calmodulin. KN-62 was shown to reduce Ca^{2+} channel activity in the pancreatic β-cell line HIT-T15 (29) and adrenal chromaffin cells (30), whereas in hippocampal CA1 neurons KN-62 and KN-93 do not affect Ca^{2+} currents (39). As we were able to verify the presented data using the structurally and functionally unrelated compound W-7 (27), our data strongly indicate that in PC12 cells membrane depolarization-induced EGFR tyrosine phosphorylation requires CaM kinase function in PC12 cells. A number of studies have shown that CaM kinases, among them CaM kinase II and CaM kinase IV, are activated by an increase in intracellular calcium level mediated either by calcium influx through VGCC (3, 6) or by Ca^{2+} release from intracellular stores (40). Although treatment of PC12 cells with the calcium ionophore ionomycin results in calcium influx into the cells, under these conditions inhibition of CaM kinase activity does not affect EGFR tyrosine phosphorylation (Fig. 1D). This suggests that only calcium entry through L-type VGCC requires a CaM kinase-dependent pathway for EGFR transactivation. In contrast, bradykinin and ionomycin-induced Ca^{2+} influx, which is due to a distinct temporal and spatial mode of Ca^{2+} entry, seem to utilize additional Ca^{2+}-dependent mediators, whose function is sufficient to trigger EGFR tyrosine phosphorylation in PC12 cells.

Focusing on the tyrosine kinase substrate SHC and the MAPK ERK2 for further analysis, we found that inhibition of CaM kinases abrogates KCl-mediated SHC tyrosine phosphorylation as well as membrane depolarization-induced ERK activation (Figs. 1C and 2). Earlier findings, such as those of Muthalif et al. (30), have suggested that stimulation of vascular smooth muscle cells with norepinephrine, a G_{i}\text{-coupled receptor agonist that activates Ca^{2+} influx primarily through L-type VGCC, induces ERK activation by activating CaM kinase II (41). Nevertheless, there is no report showing that norepinephrine-mediated ERK activation involves EGFR transactivation, although GPCR-mediated EGFR tyrosine phosphorylation was recently reported in this cell system in response to angiotensin II (18).

When we investigated the role of CaM kinase in membrane depolarization-and bradykinin-induced c-fos gene expression, we found that inhibition of CaM kinase activity suppressed c-fos gene expression only upon KCl treatment, but not following bradykinin stimulation (Fig. 3A). This confirms earlier results of Greenberg and colleagues, which support the involvement of CaM kinase II in c-fos transcription following VGCC activation, but not upon NMDA receptor-stimulated Ca^{2+} influx (4, 6). Moreover, previous reports have shown that elevation of intracellular calcium activates Ras (6, 13, 14) and downstream the MAPKs ERK1 and ERK2 resulting in the promotion of gene transcription. The transcription factor Elk-1 is activated via this signaling cascade (42) and induces c-fos gene expression through binding to the CREB, which binds to the CREL response element (36). Phosphorylation of Ser^{135} of CREB, which acts as a critical positive regulatory site (41), has been reported to be directly mediated by certain CaM kinase isofoms in the nucleus (10, 12, 44). Furthermore, cAMP-activated protein ki-
nase A (45), as well as another NGF-induced CREB kinase (46), which is activated by a Ras-dependent mechanism in PC12 cells, is able to phosphorylate CREB at Ser 133. The phosphorylation of CREB at another site, Ser 142, appears to inhibit CREB's transcriptional activation potential, as it has been shown that CaM kinase II, which phosphorylates Ser 133 and Ser 142, is much less effective in inducing CREB-dependent transcription than CaM kinase IV, which only phosphorylates Ser 133 (47, 48). This negative regulatory function of CaM kinase II on CREB-induced transcription may be the reason for the increase of bradykinin-mediated c-fos gene expression upon preincubation with CaM kinase inhibitors (Fig. 3A). Taken together, these experiments demonstrate that the EGFR/ERK MAPK pathway is necessary to mediate activation of the c-fos promotor upon membrane depolarization and bradykinin stimulation of PC12 cells. Furthermore, in contrast to bradykinin, KCl-induced c-fos transcription is not only dependent on EGFR signaling, but also requires CaM kinase function.

Another tyrosine kinase that has been implicated in ERK/MAPK activation involving calcium signals is PYK2 (15). Investigating the role of PYK2 in the membrane depolarization- and bradykinin-mediated EGFR transactivation pathway, we demonstrated that preincubation of PC12 cells with KN-62 abrogates PYK2 tyrosine phosphorylation upon KCl, but not upon bradykinin stimulation (Fig. 4). As PYK2 was reported to interact with Src upon bradykinin stimulation (20), this association may be required for Src to efficiently phosphorylate its cellular substrates. This interpretation is supported by the observation that overexpression of a dominant-negative Src mutant interferes with depolarization-induced SHC tyrosine phosphorylation (19). Taken together with our finding that PYK2 and EGFR tyrosine phosphorylation are regulated by similar calcium-dependent pathways, we raised the question of how the EGFR and the cytoplasmic tyrosine kinase complex PYK2/Src are functionally linked in PC12 cells. Interestingly, experiments with the PC12/PYK2-KM/Tet cell line (Fig. 5) suggest that dominant-negative PYK2 does not interfere with KCl- or bradykinin-induced EGFR tyrosine phosphorylation in PC12 cells. Nevertheless, we were able to demonstrate that expression of PYK2-KM reduced bradykinin-mediated ERK activation approximately by 30–40% (Fig. 6). On the other hand, we observed that ERK2 was inhibited to the same extent upon stimulation with low doses of EGF (0.3 and 1.0 ng/ml) when PYK2-KM was expressed (Fig. 6). Furthermore, we show that expression of the dominant-negative EGFR mutant HER-CD533 had no influence on PYK2 tyrosine phosphorylation upon KCl or bradykinin treatment (Fig. 8, upper panel), whereas in the same experiment inhibition of EGFR function completely abrogated tyrosine phosphorylation of downstream signaling molecules (Fig. 8, lower panel). Thus, these experiments suggest that PYK2 acts in parallel to the EGFR signaling pathway and might play an important role in the regulation of cell adhesion properties as indicated by our findings (Fig. 7).

Moreover, contradictory results concerning PYK2-dependent ERK activation in PC12 cells could be due to the fact that ERK

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**Fig. 7. Effect of the kinase-inactive PYK2 mutant on cell adhesion properties in NGF- and EGF-stimulated PC12 cells.** PC12/PYK2-KM/Tet cells were seeded into six-well dishes and cultivated for 48 h in medium with tetracycline (a–c) or without tetracycline (d–f). Then no ligand (a, d), EGF (b, d), or NGF (c, f) were added to final concentrations of 50 ng/ml, respectively. Photographs of the cells were taken 18 h after addition of ligands.

**Fig. 8. HER-CD533 expression has no effect on tyrosine phosphorylation of PYK2.** PC12/HER-CD533/Tet cells were pretreated as indicated with or without tetracycline for 48 h, serum-starved for 20 h, and stimulated with bradykinin (1 μM) or KCl (75 mM). After lysis, PYK2 was immunoprecipitated (IP) using polyclonal anti-PYK2 antibody. Tyrosine-phosphorylated PYK2 was detected by immunoblotting with monoclonal anti-phosphotyrosine (αPY) antibody (upper panel), followed by reprobing of the same filter with anti-PYK2 antibody (upper middle panel) in order to detect the tyrosine-phosphorylated SHC, ERK1, and ERK2 isoforms. The amount of SHC in crude lysates was further analyzed by reprobing of the same filter with anti-SHC antibody (upper panel).
activation upon KCl and bradykinin stimulation is not exclusively mediated by an EGFR-dependent pathway. Incomplete inhibition (80%) of KCl- and bradykinin-mediated ERK activation by expression of HER-CD533 (14) indicates that other upstream transducers such as PYK2 (15, 20), Src family kinases (21, 22, 38), or protein kinase C (51–53) are involved in KCl- or GPCR-mediated induction of ERK. With respect to these results, it seems likely that the impact of the EGFR transactivation pathway depends on cell type and the specific physiological state of the cell.

Interestingly, in the present study, we found differences between membrane depolarization- and GPCR-mediated EGFR transactivation. Although we were able to demonstrate that a Ca\(^{2+}\)-calmodulin-dependent pathway is involved in KCl-, but not in bradykinin-triggered EGFR transactivation, the mechanism of CaM kinase-dependent EGFR transactivation is still unknown. It was reported that CaM kinase II is able to phosphorylate the EGFR at serines 1046/1047 based on a consensus sequence (54); this process, however, seems to be important for the desensitization and down-regulation of the EGFR. Nevertheless, it might be possible that direct phosphorylation of the EGFR or interaction with intermediate signaling factors which have been modified by CaM kinase action might lead to KCl-induced EGFR transactivation.

Our data establish the existence of two distinct calcium-dependent mechanisms that converge on the EGFR as a central signal relay element, which then activates the Ras/ERK pathway resulting in the initiation or modulation of gene transcription. These pathways and those involving PYK2 are parts of a complex signaling network that controls and defines diverse responses of neuronal cell types to physiological stimuli.

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REFERENCES

1. Boettman, M. D., and Berridge, M. J. (1995) Cell 83, 675–678
2. Gallin, W. J., and Greenberg, M. E. (1995) Curr. Opin. Neurobiol. 5, 367–374
3. Ghosh, A., and Greenberg, M. E. (1995) Science 269, 239–247
4. Greenberg, M. E., Ziff, E. B., and Greene, L. A. (1986) Science 234, 80–83
5. Morgan, J. I., and Curran, T. (1986) Nature 322, 552–555
6. Badig, H., Ginty, D. D., and Greenberg, M. E. (1993) Science 260, 181–186
7. Rasmussen, H., and Rasmussen, J. E. (1990) Curr. Top. Cell Regul. 31, 1–109
8. Hanso, P. L., and Schuman, H. (1992) Annu. Rev. Biochem 61, 559–582
9. Colbran, R., Schwerer, C. M., Hashimoto, Y., Fong, Y.-L., Rich, D. P., Smith, M. K., and Sonderling, T. R. (1989) Biochem. J. 258, 313–325
10. Sheng, M., Thompson, M. A., and Greenberg, M. E. (1991) Science 252, 1427–1431
11. Enslen, H., Sun, P., Brickley, D., Sonderling, S., Klar, E., and Sonderling, T. R. (1994) J. Biol. Chem. 269, 15520–15527
12. Dash, P. K., Karl, K. A., Colcos, M. A., Prywe, R., and Kandel, E. R. (1991) Proc. Natl. Acad. Sci. U. S. A. 88, 4756–4762
13. Riesen, L. B., Ginty, D. D., Weber, M. J., and Greenberg, M. E. (1994) Neuron 12, 1207–1221
14. Zwick, E., Daub, H., Aoki, N., Yamaguchi-Aoki, Y., Tinhofer, I., Maly, K., and Ulrich, A. (1997) J. Biol. Chem. 272, 24767–24770
15. Lev, S., Moreno, H., Martínez, R., Canoll, P., Peles, E., Musacchio, J. M., Plowman, G. D., Rudy, B., and Schlessinger, J. (1995) Nature 376, 737–745
16. Daub, H., Weiss, F. U., Wallasch, C., and Ulrich, A. (1996) Nature 379, 557–560
17. Daub, H., Wallasch, C., Lankena, A., Herrlich, A., and Ulrich, A. (1997) EMBO J. 16, 101–112
18. Eguchi, S., Namaguchi, K., Iwashaki, H., Matsumoto, T., Yamakawa, T., Tsunomiyi, H., Motley, E. D., Kawakatsu, H., Owada, K. M., Hirata, Y., Marumo, F., and Inagami, T. (1998) J. Biol. Chem. 273, 8890–8896
19. Rusanescu, G., Qi, H., Thomas, S. M., Brugge, J. S., and Halesgou, S. (1995) Neuron 15, 1415–1425
20. Dik, L., Tokwa, G., Lev, S., Courtegrade, A. S., and Schlessinger, J. (1996) Nature 383, 547–550
21. Luttrell, L. M., Hawes, B. E., van Biesen, T., Luttrell, D. K. Lansing, T. J., and Lefkowitz, R. J. (1996) J. Biol. Chem. 271, 19443–19450
22. Sadoshima, J., and Izumo, S. (1996) EMBO J. 15, 775–787
23. Schieffer, B., Paxton, W. G., Chai, Q., Marrero, M. B., and Bernstein K. E. (1996) J. Biol. Chem. 271, 10329–10333
24. Seedorf, K., Kostka, G., Lammers, R., Bashkin, P., Daly, R., Burgess, W. H., van der Bliek, A. M., Schlessinger, J., and Ulrich, A. (1994) J. Biol. Chem. 269, 16009–16014
25. Gossen, M., and Bujard, H. (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 5547–5551
26. Bonfini, L., Migliaccio, E., Pelici, G., Lanfranchese, L., and Pelici, P. G. (1996) Trends Biochem. Sci. 21, 257–261
27. Ely, C. M.; Oddie, K. M., Litz, J. S.; Rossomando, A. J.; Kanner, S. B.; Sturgill, T. W., and Parson S. J. (1990) J. Cell Biol. 110, 731–742
28. Redemann, N., Holzmann, B., van Ruden, T., Wagner, E. F., Schlessinger, J., and Ullrich, A. (1992) Mol. Cell. Biol. 12, 491–498
29. Schlaepfer, D. D., Testart, M., Derkinderen, P., Sasaki, T., and Girault, J.-A. (1996) J. Biol. Chem. 271, 28542–28546
30. Maurer, J. A., Winter, B. W., and McKay, D. B. (1996) J. Neurochem. 66, 105–113
31. Mamiya, N., Goldenring, J. R., Tsunoda, Y., Modlin, I. M., Yasui, K., Usuda, N., Ishikawa, T., Natesuma, A., and Hida, K. (1993) Biochem. Biophys. Res. Commun. 195, 608–615
32. Metthews, R. P. (1994) Science 267, 10329–10333
33. Metthews, R. P., Guthrie, C. R., Wailes, L. M., Zhoa, X., Means, A. R., and McIntyre, G. S. (1984) Mol. Cell. Biol. 14, 6107–6116
34. Sheng, M., McFadden, G. J., and Greenberg, M. E. (1991) Neuron 4, 571–582
35. Gonzalez, A. G., and Montminy, M. R. (1989) Cell 59, 675–680
36. Ginty, D. D., Bonni, A., and Greenberg, M. E. (1994) Cell 77, 713–725
37. Sun, P., Enslen, H., Myung, P. S., and Maurer, R. A. (1994) Genes Dev. 8, 2527–2539
38. Tan, S. E., Wenthold, R. J., and Sonderling, T. R. (1994) J. Neurosci. 14, 1123–1129
39. Metthews, R. P. (1994) Mol. Cell. Biol. 14, 6104–6112
40. Wang, Y., and Simonson, M. S. (1996) Mol. Cell. Biol. 16, 5915–5923
41. Zou, Y., Konuro, I., Yamazaki, T., Aikawa, R., Kudoh, S., Shiogina, I., Hiroi, Y., Mizuno, T., and Yazaki, Y. (1996) J. Biol. Chem. 271, 33592–33597
42. Li, X., Lee, J. W., Graves, L. M., and Earp, H. S. (1998) EMBO J. 17, 2574–2583
43. Tsig, W., Morelli, A. D., and Peralta, E. G. (1997) EMBO J. 16, 4597–4605
44. Countaway, J. L., Nairn, A. C., and Davis, R. J. (1997) J. Biol. Chem. 272, 1129–1140