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Stimulation of growth factor receptor signal transduction by activation of voltage-sensitive calcium channels

(epidermal growth factor receptor/Ras/mitogen-activated protein kinase)

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ABSTRACT **To understand the mechanisms by which electrical activity may generate long-term responses in the nervous system, we examined how activation of voltage-sensitive calcium channels (VSCCs) can stimulate the Ras/mitogen-activated protein kinase (MAPK) signaling pathway. Calcium influx through L-type VSCCs leads to tyrosine phosphorylation of the adaptor protein Shc and its association with the adaptor protein Grb2, which is bound to the guanine nucleotide exchange factor Sos1. In response to calcium influx, Shc, Grb2, and Sos1 inducibly associate with a 180-kDa tyrosine-phosphorylated protein, which was determined to be the epidermal growth factor receptor (EGFR). Calcium influx induces tyrosine phosphorylation of the EGFR to levels that can activate the MAPK signaling pathway. Thus, ion channel activation stimulates growth factor receptor signal transduction.**

Calcium influx into neurons is the critical transducer of electrical input into biochemical output (1). A wide range of neurotransmitter receptors and second messenger systems have been shown to regulate the influx of extracellular calcium through their effects on voltage-sensitive calcium channels (VSCCs) and other ion channels in a process termed neuromodulation (2–4). However, the mechanisms by which calcium influx elicits long-term neuronal responses are less clear.

Studies of the biochemical responses generated in response to calcium influx have focused on activation of cytoplasmic signaling molecules that directly bind calcium or calcium–calmodulin (CaM) complexes, such as calcium–CaM-dependent adenylate cyclases and protein kinases (5). Calcium influx also leads by indirect mechanisms to activation of the ubiquitous mitogen-activated protein kinase (MAPK) pathway (6, 7), which is a critical intermediate in long-term cellular responses such as proliferation and differentiation (8–10). Recently, we have demonstrated that stimulation of the MAPK pathway in response to calcium influx through L-type VSCCs involves activation of the small guanine nucleotide binding protein Ras (11), a protooncogene product that mediates MAPK activation in response to a wide variety of mitogens, cytokines, and trophic factors such as nerve growth factor (NGF) (8–10). To understand the mechanisms by which electrical activity may initiate long-term responses in the nervous system, we examined how calcium influx leads to activation of Ras.

Growth factors initiate signaling processes that lead to Ras activation by binding to transmembrane receptors that contain intrinsic tyrosine kinase activity or, in the case of cytokine receptors, that are associated through their cytoplasmic domains with nontransmembrane protein tyrosine kinases such as the Src family members (12–14). Ligand binding induces receptor dimerization and autophosphorylation on tyrosine residues. These phosphorylated tyrosines create binding sites for Src homology 2 (SH2) domains, which are present in a number of different signaling molecules that associate with activated growth factor receptors (15, 16). SH2 domains bind to phosphorylated tyrosine residues and adjacent amino acid sequences, which determine the specificity of the interaction.

One class of signaling molecule that inducibly binds to growth factor receptors is the adaptor protein, which lacks catalytic moieties but mediates protein–protein interactions via modular domains such as SH2 domains. One of the adaptor proteins that inducibly associates with tyrosine-phosphorylated growth factor and cytokine receptors is the SH2/collagen protein (Shc) (17). Shc is itself also inducibly phosphorylated on tyrosine in response to growth factor and cytokine stimulation (18–22), which creates a consensus binding site (pYXN) that is recognized by another SH2 domain-containing adaptor protein, growth factor receptor binding protein 2 (Grb2) (23). Grb2 contains, in addition to its SH2 domain, two SH3 domains that mediate its interaction with proline-rich sequences in the Ras guanine nucleotide exchange factor (GEF) termed mSos1 (24–28). Induction of Grb2–Sos1 association with Shc through Y317 is a potential mechanism for Ras activation in response to growth factors and cytokine stimulation (29–35).

The parallels we found previously between calcium and growth factor activation of MAPK suggested that tyrosine phosphorylation might be involved in calcium activation of Ras. We report here that calcium influx upon activation of VSCCs leads to tyrosine phosphorylation of Shc and its association with Grb2 and Sos1. In addition, this signaling complex inducibly associates with the epidermal growth factor receptor (EGFR), which is phosphorylated on tyrosine in response to calcium influx to a level that is sufficient to lead to downstream MAPK activation. Our results demonstrate that growth factor receptor signal transduction is activated in response to VSCC stimulation, which may be an important biochemical mechanism by which neuronal activity can generate long-term cellular responses.

MATERIALS AND METHODS

Materials. EGF was from Collaborative Biomedical Products (Bedford, MA), and NGF was purified from mouse salivary glands as described (36). Nifedipine was from Sigma. Anti-Trk antibodies were the generous gift of David Kaplan (67). Other antibodies were obtained from the following vendors: anti-phosphotyrosine [Tyr(*p*)] monoclonal antibody (mAb) 4G10 from Upstate Biotechnology (Lake Placid, NY); anti-Tyr(*p*) mAb PY20 from ICN; anti-Shc polyclonal antibody (pAb) and mAb from Transduction Laboratories (Lexington, KY); anti-Grb2 mAb from Upstate Biotechnology; anti-Grb2

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Abbreviations: VSCC, voltage-sensitive calcium channel; CaM, calmodulin; MAPK, mitogen-activated protein kinase; NGF, nerve growth factor; GEF, guanine nucleotide exchange factor; EGF, epidermal growth factor; EGFR, EGF receptor; mAb, monoclonal antibody; pAb, polyclonal antibody.

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pAb from Santa Cruz Biotechnology (Santa Cruz, CA); anti-Sos1 pAb from Upstate Biotechnology; anti-EGFR pAb from Upstate Biotechnology; anti-ErbB2 from Oncogene Science; goat anti-mouse pAb from Calbiochem; and rabbit anti-sheep pAb from Pierce.

Cell Culture and Stimulation. PC12 cells were obtained from Simon Halegoua (68) and cultured on 100-mm tissue culture dishes (Falcon) in DMEM (GIBCO) supplemented with 10% heat-inactivated horse serum and 5% fetal bovine serum (GIBCO) in a humidified incubator with 10% CO_2/90% air. Cell membranes were depolarized by addition of an isosmotic solution of KCl (or NaCl control) to a final concentration of 50 mM as described (11).

Immunoprecipitation and Immunoblotting. Cells were lysed in HNTG buffer [50 mM Hepes, pH 7.5/50 mM NaCl/1% Triton X-100/10% glycerol (vol/vol)/1.5 mM $MgCl_2$/1 mM EDTA/10 mM sodium pyrophosphate/1 mM Na_3VO_4/100 mM NaF/30 mM 2-(*p*-nitrophenyl) phosphate/1 mM phenylmethylsulfonyl fluoride/10 μg of aprotinin per ml/10 μg of leupeptin per ml] and centrifuged at 10,000 × *g* for 15 min. Supernatants were mixed with primary antibody and rocked at 4°C for 1–4 h. Secondary antibody and protein A-Sepharose (Calbiochem) were added for an additional 1–2 h. Immunoprecipitates were washed three times in HNTG and resuspended in 2× Laemmli sample buffer (80 mM Tris·HCl, pH 6.8/15% glycerol/2% SDS/0.01% bromphenol blue/10% 2-mercaptoethanol). Precipitated proteins were separated by SDS/PAGE, transferred to nitrocellulose, and analyzed by Western blotting as described (11). Antibody binding was detected by enhanced chemiluminescence (ECL; Amersham) with a secondary antibody conjugated to horseradish peroxidase. For analysis of Shc, Grb2, and Sos1 coimmunoprecipitation, 6–12% gradient gels were run, and the nitrocellulose blots were cut horizontally at the 125- and 35-kDa markers for blotting of individual proteins.

RESULTS

To examine whether tyrosine phosphorylation might be involved in calcium activation of Ras, we determined the effect of membrane depolarization on tyrosine phosphorylation of Shc and its association with Grb2 in the pheochromocytoma cell line PC12. Membrane depolarization of both NGF-differentiated and undifferentiated PC12 cells has been demonstrated to activate signaling pathways that are also activated by direct electrical stimulation of primary neurons in culture as well as by neuronal excitation *in vivo* (37). Undifferentiated PC12 cells were membrane depolarized by exposure to elevated levels of extracellular KCl to induce calcium influx through VSCCs, or cells were treated with NGF or EGF. Shc proteins were immunoprecipitated and analyzed by protein immunoblotting with antibodies to phosphotyrosine. Equal amounts of Shc were immunoprecipitated as shown by immunoblotting for Shc proteins (Fig. 1*A Bottom*). KCl induced tyrosine phosphorylation of the 48- and 56-kDa Shc isoforms, as did NGF and EGF treatment (Fig. 1*A Top*). The 65-kDa Shc isoform was inducibly tyrosine phosphorylated to a lesser extent by NGF and EGF but not by KCl. Although this may simply be due to a detection limit in the assay, the differential phosphorylation of the three Shc isoforms in response to the different stimuli may reflect specificity in the signaling pathways. These results demonstrate that membrane depolarization leads to inducible tyrosine phosphorylation of Shc, an adaptor protein that is involved in Ras activation in response to growth factor stimulation.

To determine whether KCl-induced Shc phosphorylation led to its functional association with Grb2, Shc immunoprecipitates (Fig. 1*A*) from PC12 cells treated with KCl, NGF, or EGF were analyzed for coprecipitation of Grb2 by immunoblotting with anti-Grb2 antibody. Membrane depolarization

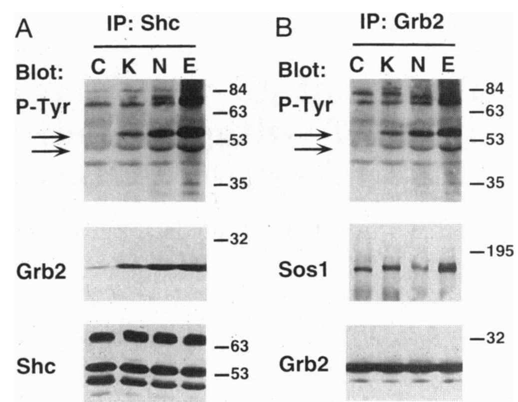

FIG. 1. Calcium induction of Shc tyrosine phosphorylation and Grb2 association. (*A*) PC12 cells were incubated with 50 mM NaCl control solution (lane C), 50 mM KCl (lane K), 100 ng of NGF per ml (lane N), or 10 ng of EGF per ml (lane E) for 5 min. Lysates were immunoprecipitated with anti-Shc pAb. Washed immunoprecipitates (IP) were separated by PAGE and transferred to nitrocellulose for immunoblotting with a mixture of anti-Tyr(*p*) antibodies 4G10 and PY20, anti-Grb2 mAb (*Middle*), or anti-Shc mAb (*Bottom*). Positions of migration of prestained molecular size markers (kDa) (Sigma) are shown. Arrows indicate phosphorylated Shc isoforms of 48 and 56 kDa. (*B*) PC12 cells were treated as in *A*. Lysates were immunoprecipitated with a pAb against Grb2, and precipitated proteins were analyzed by immunoblotting with anti-Tyr(*p*) antibodies (*Top*), anti-Sos1 pAb (Upstate Biotechnology; *Middle*), or anti-Grb2 mAb (*Bottom*) to confirm that equal levels of Grb2 were immunoprecipitated. Positions of migration of prestained molecular size markers are shown.

with KCl led to the inducible association of Shc with Grb2, as did NGF and EGF treatment (Fig. 1*A Middle*). This KCl-induced association of Shc with Grb2 was also demonstrated by first immunoprecipitating Grb2 and then immunoblotting with antibodies to phosphotyrosine to detect coprecipitated Shc proteins (Fig. 1*B Top*). In addition, by immunoblotting Grb2 immunoprecipitates with antibodies to Sos1, we found that Grb2 is constitutively bound to Sos1 in PC12 cells, as others have previously shown (Fig. 1*B Middle*) (38). Thus, KCl-stimulated tyrosine phosphorylation of Shc can induce its association with the Grb2–Sos1 complex.

Evidence suggests that the Ras GEF must be targeted to the plasma membrane in order to activate Ras (39). In the case of growth factor stimulation, this can be accomplished by association of the adaptor protein–GEF complexes with the receptor tyrosine kinase itself. This can occur by receptor binding directly to Grb2–Sos1 complexes through the Grb2 SH2 domain (24–28) or through binding Shc–Grb2–Sos1 complexes through the Shc SH2 domain (33–35, 40–42) or a phosphotyrosine-binding domain at the Shc N terminus (43, 44). To determine how calcium influx might target the Shc–Grb2–Sos1 signaling complex to the plasma membrane, we examined whether KCl treatment led to association of Shc, Grb2, or Sos1 with a tyrosine-phosphorylated protein that could act as a membrane anchor. Shc, Grb2, and Sos1 proteins were immunoprecipitated and analyzed for coprecipitating proteins containing phosphotyrosine by immunoblotting with anti-phosphotyrosine antibodies. KCl induced the association of an ≈180-kDa tyrosine-phosphorylated protein with Shc, Grb2, and Sos1 (Fig. 2). Surprisingly, this protein comigrated with a tyrosine-phosphorylated protein that was coprecipitated in response to EGF stimulation. A low or undetectable level of pp180 was detected in Shc, Grb2, and Sos1 immunoprecipitates from NGF-treated cells. We failed to detect coprecipitation of the NGF receptor $p140^{trk}$ in Shc immunoprecipitates in response to NGF, possibly because of lower endogenous levels of Trk than EGFR in these cells.

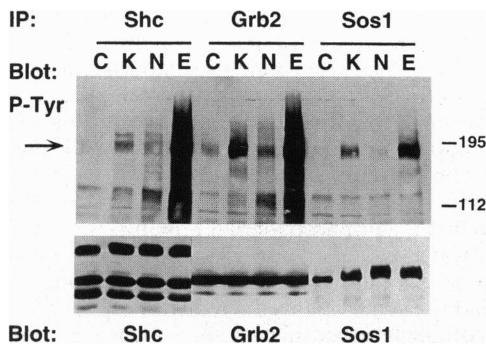

FIG. 2. Calcium-induced association of Shc, Grb2, and Sos1 with pp180. PC12 cells were treated with 50 mM NaCl control solution (lanes C), 50 mM KCl (lanes K), 100 ng of NGF per ml (lanes N), or 10 ng of EGF per ml (lanes E) for 5 min. Lysates were immunoprecipitated with pAb against Shc, Grb2, or Sos1, and precipitated proteins (IP) were analyzed by immunoblotting with anti-Tyr(p) antibodies (*Top*) or with antibodies to the precipitated proteins to confirm equal recovery (*Bottom*). Positions of migration of prestained molecular size markers (kDa) are shown. Arrow indicates pp180.

The EGFR can associate with Grb2 directly through binding of the Grb2 SH2 domain to phosphorylated Y1068 or Y1086 in the receptor, as well as indirectly via binding of Shc to phosphorylated Y1148 or Y1173 (41, 42, 45). The observation that a 180-kDa protein inducibly associated with Shc, Grb2, and Sos1 in response to KCl as well as EGF raised the possibility that both proteins might be the EGFR. This possibility was examined directly by immunoprecipitating Grb2 and immunoblotting with antibodies to the EGFR. Membrane depolarization with KCl induced Grb2 association with the EGFR, although to a lesser extent than treatment with 10 ng of EGF per ml (Fig. 3, lanes 1–3). This result suggests that the 180-kDa protein that is coprecipitated with Shc, Grb2, and Sos1 in response to KCl stimulation is the EGFR. Thus, calcium influx induces the association of a growth factor receptor with downstream signaling proteins that can trigger Ras activation.

To determine whether KCl-induced EGFR association with Shc, Grb2, and Sos1 was due to calcium influx through VSCCs, the effect of a specific channel antagonist on the interaction was examined. Pretreatment of PC12 cells with the L-type VSCC antagonist nifedipine or with the calcium chelator EGTA for 15 or 5 min, respectively, completely blocked the ability of KCl to induce the association of Grb2 with the EGFR, whereas they had no effect on EGF-induced association (Fig. 3, lanes 4–9). These results demonstrate that KCl-induced association of the EGFR with Grb2 is dependent on the influx of extracellular calcium through L-type VSCCs and is not a nonspecific effect of membrane depolarization. This finding is consistent with previous observations that nifedipine and nimodipine, another dihydropyridine antagonist of L-type VSCCs, block KCl activation of Ras and that KCl activation of MAPK is blocked by nifedipine but not by ω-conotoxin, an inhibitor of N-type VSCCs (ref. 11; unpublished data). The importance of L-type VSCCs in mediating the signaling effects of membrane depolarization we observe reflects the fact that they are the primary carrier of voltage-sensitive calcium current in undifferentiated PC12 cells (46).

Since Shc and Grb2 specifically recognize tyrosine-phosphorylated proteins, the finding that these adaptor molecules inducibly associate with the EGFR upon VSCC activation suggested that calcium influx was leading to tyrosine phosphorylation of the receptor. To examine the effect of calcium influx on the phosphotyrosine content of the EGFR, the EGFR was immunoprecipitated after KCl or EGF treatment and analyzed by immunoblotting with antibodies to phosphotyrosine. Calcium influx led to inducible tyrosine phosphorylation of the EGFR within 20 sec of membrane depolarization (Fig. 4 *Top*). This calcium-induced phosphorylation of the EGFR can therefore account for the inducible association of the receptor with Shc–Grb2–Sos1 in response to membrane depolarization.

To determine whether calcium-induced EGFR tyrosine phosphorylation was likely to lead to physiologically meaningful receptor responses, we titrated down the dose of EGF to a level (1 ng/ml) that produced an induction of EGFR tyrosine phosphorylation comparable to that induced by KCl (Fig. 4 *Top*). We then examined whether this level of EGFR phosphorylation was sufficient to produce downstream responses to EGF. MAPK activation was monitored as an indicator of physiologically important signal transduction, since MAPK activation has been demonstrated to be critical for a variety of long-term cellular responses to extracellular stimuli, including cell proliferation and differentiation (8–10). Both membrane depolarization and treatment with EGF (1 ng/ml) led to inducible tyrosine phosphorylation of the 42- and 44-kDa isoforms of MAPK. The identification of these tyrosine phosphorylated bands as activated MAPK was confirmed by immunoblotting with antibodies that specifically recognize the

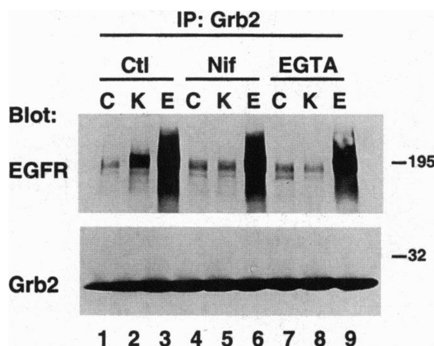

FIG. 3. Calcium induction of Grb2 association with the EGFR. PC12 cells were treated with 50 mM NaCl control solution (lanes C), 50 mM KCl (lanes K), or 10 ng of EGF per ml (lanes E) for 2 min. Lysates were immunoprecipitated with anti-Grb2 pAb, and precipitated proteins (IP) were analyzed by immunoblotting with anti-EGFR pAb (*Top*) or anti-Grb2 mAb (*Bottom*). Cells were pretreated as follows: lanes 1–3, vehicle control for 15 min (Ctl); lanes 4–6, 5 μM nifedipine for 15 min (Nif); lanes 7–9, 3 mM EGTA for 5 min (EGTA). Positions of migration of prestained molecular size markers (kDa) are shown.

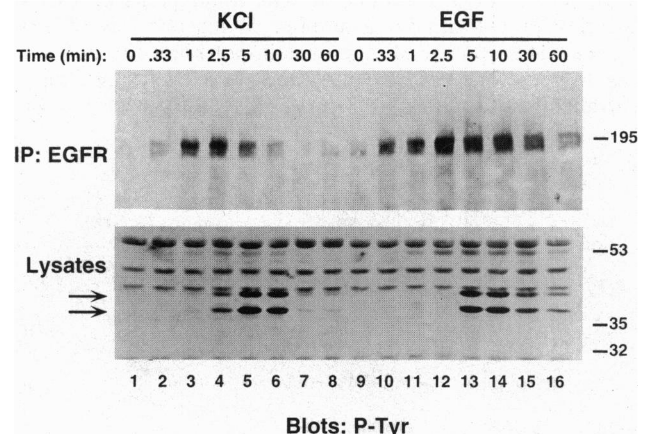

FIG. 4. Calcium induction of EGFR tyrosine phosphorylation and downstream signaling. PC12 cells were treated with 50 mM KCl or 1 ng of EGF per ml for the indicated times. Lysates were immunoprecipitated with anti-EGFR pAb, and immunoprecipitated proteins (IP) (*Top*) or samples of lysates (*Bottom*) were analyzed by immunoblotting with anti-Tyr(p) antibodies. Positions of migration of prestained molecular size markers (kDa) are shown. Arrows indicate positions of the 42- and 44-kDa MAPK isoforms.

phosphorylated and activated form of MAPK (L.B.R., David D. Ginty, and M.E.G., unpublished observations). Thus, calcium influx in response to L-type VSCC activation leads to tyrosine phosphorylation of the EGFR to an extent that is sufficient to induce downstream signaling to MAPK. Notably, the dose of EGF used in this experiment is 10-fold higher than doses reported to generate physiological responses in neurons (47, 48), suggesting that the comparable level of EGFR tyrosine phosphorylation produced by calcium influx is likely to be a physiologically important mechanism of signal transduction in response to neuronal activity.

Finally, we addressed the generality and specificity of the calcium signaling response by examining whether KCl induced tyrosine phosphorylation of other growth factor receptors. KCl treatment led to tyrosine phosphorylation of the EGFR family member ErbB2 (Fig. 5*A*) as well as a 100-kDa protein that is recognized by an antibody generated against a consensus tyrosine kinase domain (unpublished data). In contrast, KCl did not induce tyrosine phosphorylation of the insulin receptor (unpublished data) or the NGF receptor Trk, which was robustly phosphorylated in response to NGF (Fig. 5*B*). These results suggest that calcium influx may activate the signal transduction pathways of other receptor tyrosine kinases in addition to the EGFR but that there is specificity in the signaling responses generated. Thus, stimulation of growth factor receptor signaling may be a general mechanism by which calcium influx generates long-term responses in cells.

DISCUSSION

Our results demonstrate that activation of VSCCs can lead to tyrosine phosphorylation of the EGFR and its association with the adaptor proteins Shc and Grb2 and the guanine nucleotide exchange factor Sos1. A number of studies have demonstrated that Shc, Grb2, and Sos1 act as signaling mediators in growth factor receptor activation of Ras. Taken together, these findings provide a mechanism by which calcium influx could activate the Ras/MAPK pathway and demonstrate that growth factor receptors can be functionally coupled to their downstream signaling pathways in response to ion-channel activation.

The mechanism by which calcium influx induces EGFR tyrosine phosphorylation is not yet clear. Although it is possible that L-type VSCC activation could lead to the calcium-induced release of EGF and autocrine stimulation of the receptor, we favor a model of ligand-independent EGFR activation by calcium for a number of reasons. The EGFR precursor is a transmembrane protein not known to be packaged in secretory vesicles (49), and we have found that a neutralizing antibody to EGF prevents tyrosine phosphorylation of the EGFR in response to EGF but not in response to L-type VSCC activation (unpublished data). Thus, calcium does not lead to EGFR tyrosine phosphorylation by autocrine release of EGF itself. We think a more likely mechanism could involve calcium activation of a cytoplasmic tyrosine kinase that could phosphorylate the receptor C-terminal tail (Fig. 6). This trans-phosphorylation of the EGFR by a calcium-responsive cytoplasmic tyrosine kinase may then activate the EGFR kinase so that it autophosphorylates on the same sites that are phosphorylated in response to EGF binding. Members of the Src family of cytoplasmic tyrosine kinases are good candidates for mediating calcium-induced tyrosine phosphorylation of the EGFR. Although we are unaware of evidence that Src directly binds the EGFR, Src transformation of fibroblasts leads to tyrosine phosphorylation of the EGFR and likely to activation of its kinase activity as well (50). In addition, Src is activated in response to ionomycin treatment of keratinocytes (51), and targeted gene disruption of the Src family member *fyn* suggests that the Fyn protein may play a role in calcium-dependent responses in the nervous system, such as synaptic potentiation and memory formation (52). Alternatively, calcium influx could increase EGFR tyrosine phosphorylation by activating an as yet uncharacterized tyrosine kinase or by inhibiting a protein tyrosine phosphatase. Whether or not calcium activates the EGFR kinase activity *per se*, it does activate EGFR signal transduction by inducing association of the receptor with the downstream signaling proteins Shc, Grb2, and Sos1.

Although calcium activation of growth factor receptor signaling is likely to involve other receptor tyrosine kinases, we have also found evidence for its specificity. For example, KCl treatment of PC12 cells does not induce tyrosine phosphorylation of the insulin receptor (unpublished data) or the NGF receptor Trk (Fig. 5*B*). In addition, the observed effects of calcium appear to be specific to certain cell types. We have not detected inducible tyrosine phosphorylation of the EGFR or Shc in response to KCl treatment of cortical neurons, where calcium influx may activate Ras through a specific CaM-binding GEF that is not detectable in PC12 cells (69). In other studies, ionomyucin treatment of A431 cells and extracellular calcium addition to keratinocytes inhibited EGFR tyrosine phosphorylation (53, 54). These inhibitory effects may involve calcium activation of serine/threonine kinases, since phosphorylation of the EGFR on serine and threonine residues can down-regulate both EGF binding and receptor tyrosine kinase activity (55). The cell specificity of calcium effects on EGFR tyrosine phosphorylation suggests that the stimulation we observe is not simply due to a conformational change in the EGFR induced by calcium ions, as has been described *in vitro*

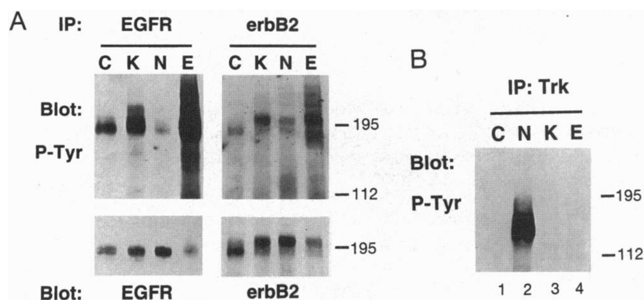

FIG. 5. Calcium induction of ErbB2 but not Trk tyrosine phosphorylation. PC12 cells were treated with 50 mM KCl (lanes K), 100 ng of NGF per ml (lanes N), or 10 ng of EGF per ml (lanes E) for 3 min (lanes C, controls). Lysates were immunoprecipitated with anti-EGFR pAb or anti-ErbB2 (*A*) or anti-Trk (*B*) antibody, and precipitated proteins (IP) were analyzed by immunoblotting with anti-Tyr(*p*) antibodies. Positions of migration of prestained molecular size markers (kDa) are shown.

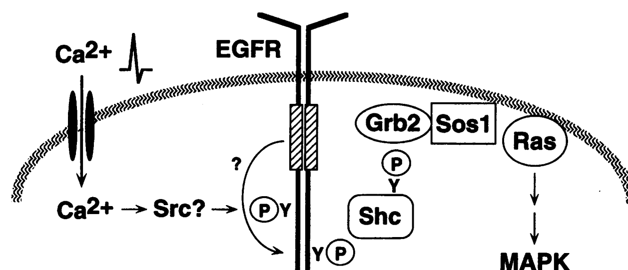

FIG. 6. Calcium induction of EGFR signal transduction. Membrane depolarization leads to calcium influx through VSCCs. This leads to tyrosine phosphorylation of the EGFR, possibly by activating a cytoplasmic tyrosine kinase such as Src. Trans-phosphorylation of the EGFR by the cytoplasmic tyrosine kinase may activate the receptor to autophosphorylate on the same sites that would be phosphorylated in response to EGF binding. Calcium-induced tyrosine phosphorylation of the EGFR leads to its association with the downstream adaptor proteins Shc and Grb2, which allows localization of the guanine nucleotide exchange factor Sos1 to the plasma membrane, where it can activate Ras and initiate signaling to MAPK.

in the presence of millimolar concentrations of Mg^{2+} or Mn^{2+} (56). The distinct effects of increased cytosolic calcium on EGFR tyrosine phosphorylation in different cell types may reflect the differential expression of calcium-responsive signaling intermediates or distinct modes of calcium entry into the cells, which can generate different signaling responses (5).

Stimulation of growth factor receptor signaling pathways in response to VSCC activation has general implications for how calcium signals may be transduced into biochemical responses in neurons. EGF and its receptor are expressed in a number of areas in the nervous system (48, 57), and expression of the EGFR family members ErbB2 (c-neu), ErbB3, and ErbB4 has also been detected in brain (58–60). In addition, a family of ligands that bind the ErbB proteins, the neuregulins, has recently been discovered, which can act as trophic factors in the nervous system (61). This widespread expression of EGFR family signaling machinery in the nervous system suggests that it could be used in response to activity-dependent calcium influx via the mechanism described here. For example, one long-term response to membrane depolarization-induced calcium influx is enhanced survival of certain neuronal populations, such as cerebellar granule cells (62, 63). EGF itself has been shown to be a survival factor for certain types of neurons as well, including cerebellar granule neurons (47, 64, 65). Thus, one mechanism by which neuronal activity may enhance neuronal survival may be through calcium activation of EGFR signaling pathways. Calcium stimulation of growth factor signaling pathways may be a general mechanism for activity-dependent regulation of survival and trophic responses in the nervous system (62, 66).

Note Added in Proof. While this manuscript was in press, others also observed that calcium influx leads to Shc phosphorylation in PC12 cells. The Shc phosphorylation event was shown to be mediated by Src (70) and/or a novel 112-kDa calcium-responsive tyrosine kinase, PYK2 (71).

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