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Requirement of EGF Receptor Kinase for Signaling by Calcium-Induced ERK Activation and Neurite Outgrowth in PC12 Cells

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Membrane depolarization in PC12 cells induces calcium influx via an L-type voltage-sensitive calcium channel (L-VSCC) and increases intracellular free calcium, which leads to tyrosine phosphorylation of epidermal growth factor (EGF) receptor and the associated adaptor protein, Shc. This activated EGF receptor complex then can activate mitogen-activated protein (MAP) kinase, as in nerve growth factor (NGF) receptor activation. In the present study, we investigated the role of EGF receptor in the signaling pathway initiated by membrane depolarization of PC12 cells. Prolonged membrane depolarization induced phosphorylation of extracellular signal-regulated kinase (ERK) within 1 min in undifferentiated PC12 cells. Pretreatment of PC12 cells with the calcium chelator EGTA abolished depolarization-stimulated ERK phosphorylation, but NGF-induced phosphorylation of ERK was not affected. The chronic treatment of phorbol ester, which down-regulated the activity of protein kinase C (PKC), did not affect the phosphorylation of ERK upon depolarization. In the presence of an inhibitor of EGF receptor, neither depolarization nor calcium ionophore increased the level of ERK phosphorylation. These data imply that the EGF receptor is functionally necessary to activate ERK and neurite outgrowth in response to the prolonged depolarization in PC12 cells, and also that PKC is apparently not involved in this signaling pathway.

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Introduction

PC12 cell is widely used in various types of neurochemical studies because of its neuronal property (Green, 1982). This cell-line was established from rat pheochromocytoma and exhibits extended long, branching neuronal-like processes (Kang *et al.*, 1997) in response to nerve growth factor (NGF). Removal of NGF is followed by degeneration of the processes within 24 h (Green and Tischler, 1976).

Many actions of NGF are mediated through a proto-oncoprotein signaling pathway involving NGF receptor/Trk tyrosine kinase, nonreceptor type tyrosine kinase Src, Shc/Grb2 adaptor protein, GTP-binding protein Ras, cytoplasmic serine/threonine Raf kinases, MEK (MAPK or ERK kinase), and mitogen-activated protein kinase (MAPK). Binding of NGF to its receptor induces dimerization of the receptors and thereby autophosphorylation on tyrosine residues. The phosphorylated tyrosine in the receptor molecule generates the binding sites for Shc. Shc itself is also phosphorylated on tyrosine residues in response to NGF. This phosphorylated tyrosine is recognized by growth factor receptor binding protein 2 (Grb2) (Lowenstein *et al.*, 1992). Grb2 contains one SH2 domain and two SH3 domains which mediate the interactions with proline-rich sequences in the Ras guanine nucleotide exchange factor (GEF), termed mSos1 (Koh *et al.*, 1997). These membrane-recruited mSos1 can then activate Ras. Since Ras is localized at the inner surface of the plasma membrane, the interaction between Ras-GTP and Raf within cells will bring Raf protein to the membrane and

then activate Raf (Luo *et al.*, 1996). Activated Raf has been shown to phosphorylate and activate MEK (Dent *et al.*, 1992), which in turn phosphorylates and activates the extracellular signal-regulated kinase (ERK) which is one of the MAPK isotypes (Nakielny *et al.*, 1992). ERKs are translocated into the nucleus upon activation; many transcription factors contain potential ERK phosphorylation sites and, for some transcription factors such as Elk-1, ERKs clearly play a role in activation (Hill *et al.*, 1993). Activation of ERKs may therefore provide the link between cytoplasmic and nuclear signaling events.

The MAP kinase cascade is likely to serve specific functions in different cell types. Since NGF induces neuronal differentiation in PC12 cells (Chung and Hong, 1996), the cascade has been proposed to mediate neuronal phenotype or other neurotrophic responses in this cell-line (Nguyen *et al.*, 1993). In agreement with this model the transfection of PC12 cells with constitutive activity, oncogenic Raf, mimics NGF action by inducing activation of the MAP kinase cascade and neuronal differentiation (Wood *et al.*, 1993). The activated MEK mutant also stimulates neuronal differentiation of PC12 cells (Cowley *et al.*, 1994). In addition to NGF, prolonged depolarization, some neuropeptides, as well as epidermal growth factor (EGF) can also activate the MAPK pathway in PC12 cells.

In the nervous system, electrical activity may generate long-term responses. Calcium influx can translate electrical input into biochemical output through voltage-sensitive calcium channels (VSCCs). Calcium serves as a second messenger and regulates a number of signaling molecules. It is possible that calcium can mediate the electrical activity-dependent long-term response in the nervous system. In neuronal cell culture, depolarization-induced calcium entry through VSCCs has been shown to mimic and complement the actions of neuronal growth factor, including the neuronal survival and maintenance of neurites after growth factor deprivation (Lampe *et al.*, 1995). In PC12 cells, depolarization-induced calcium entry through VSCCs has been shown to maintain neurites and cell survival after NGF deprivation in serum-free medium (Teng and Greene, 1993). Calcium influx through VSCCs also causes neurite outgrowth in PC12 cells. Ras activity is necessary for neurite outgrowth, which is induced by prolonged depolarization in PC12 cells (Rusanescu *et al.*, 1995).

Prolonged depolarization in PC12 cells phosphorylates and activates EGF receptor in a ligand-independent manner. Activated EGF receptor can recruit Shc/Grb2 and mSos1 and activate the MAPK pathway, like activated NGF receptor/Trk (Rosen and Greenberg, 1996). However, whether the EGF receptor is functionally necessary, as is the exact role of the EGF receptor in this signaling pathway, has not been determined in detail. Also, investigation should identify other signaling pathways which are able to bypass the EGF receptor for activation of ERK upon depolarization. Therefore, in this study, we investigated whether the activation of EGF receptor is functionally necessary for activation of ERK and neurite outgrowth in PC12 cells. Since elevated levels of intracellular calcium could activate protein kinase C (PKC) and then initiate the MAP kinase pathway (Ueda *et al.*, 1996), we also investigated whether the activation of ERK upon depolarization occurred through the activation of PKC in PC12 cells as well.

Materials and Methods

Materials PC12 cells were obtained from the American Type Culture Collection. EGF, NGF, A23187, anti-ERK polycolonal antibody (pAb), anti-rabbit IgG pAb, and rat collagen were from Sigma (St. Louis, USA). Anti-EGF receptor pAb and anti-phosphotyrosine monoclonal antibody (mAb) 4G10 were from Upstate Biotechnology (Lake Placid, USA). Anti-phosphorylated ERK pAb was from New England Biolab. (Beverly, USA). GF109203X, AG1478, and phorbol 12-myristate 13-acetate (PMA) were from Calbiochem (La Jolla, USA). Anti-sheep IgG pAb was from Serotec (Kidlington Oxford, England). Western blotting detection reagent, ECL™, was from Amersham (Buckinghamshire, UK).

Cell culture PC12 cells were cultured on 60-mm tissue culture dishes (Costar, Actton, USA) in RPMI1640 (GIBCO, Gaithersburg, USA) supplemented with 10% heat-inactivated horse serum and 5% fetal bovine serum (GIBCO) in a humidified incubator with 10% CO_2/90% air.

Stimulation Cell membranes were depolarized by addition of an isosmotic solution of KCl (170 mM KCl, 1.8 mM $CaCl_2$, 0.8 mM $MgCl_2$) to a final concentration of 75 mM. Calcium influx was induced by using A23187 with a final concentration of 2.5 μg/ml.

Immunoblotting PC12 cells were incubated for various times for specific stimulus. The medium was aspirated and the cell monolayers were solubilized in SDS sample buffer [62.5 mM Tris-HCl (pH 6.8), 2% w/v SDS, 10% glycerol, 50 mM DTT, 0.1% w/v bromphenol blue]. The solubilized samples were sonicated for 10–15 s to shear DNA and centrifuged at 10,000 × *g* for 15 min. Proteins in the supernatant were separated by SDS/PAGE, transferred to nitrocellulose, and analyzed by Western blotting as described previously (Ausubel *et al.*, 1995). The antibody binding was detected by enhanced chemiluminescence (ECL; Amersham, Buckinghamshire, UK) with a secondary antibody conjugated with horseradish peroxidase as described by the manufacturer.

Neurite assay Prior to plating, culture dishes were treated with 1 mg/ml collagen and then rinsed with the medium. Cells were plated in the concentrations of about 5000 cells/cm^2. Differentiation of PC12 cells was determined by scoring the number and length of the neurite. After 4 d of growing in collagen-coated dishes, cells were washed with phosphate buffered saline (PBS, pH 7.3) and fixed with fixing solution (4% formaldehyde in PBS). Fixed cells were randomly photographed. Well-isolated single cells which possess more than one neurite

with a length longer than a diameter of the cell body were investigated.

Results

To approach the involvement of PKC and EGF receptors in depolarization-induced activation of the MAP kinase pathway and neurite outgrowth in PC12 cells, we tested for the phosphorylation of ERK, one isotype of MAPK, and neurite outgrowth in the presence of an inhibitor of PKC and EGF receptors.

An elevated level of K^+ ions (75 mM) in the media induces depolarization of plasma membrane and phosphorylates ERK within 1 min in undifferentiated PC12 cells (Fig. 1). It was suggested that the depolarization of the plasma membrane induces Ca^{2+} influx via an L-type voltage sensitive calcium channel (L-VSCC) (Rosen *et al.*, 1994). To confirm that the calcium influx is the primal effector for ERK phosphorylation upon depolarization of the plasma membrane, we examined whether the elimination of extracellular calcium could abolish depolarization-stimulated phosphorylation of ERK. Pretreatment of PC12 cells with the calcium chelator EGTA for 3 min abolished depolarization-stimulated ERK phosphorylation (Fig. 2, lanes 2 and 5), but NGF-induced phosphorylation of ERK was not affected (Fig. 2, lanes 1 and 4). In addition, Ca^{2+} ionophore A23187 induced phosphorylation of ERK. Induction of ERK phosphorylation with addition of A23187 in the medium was abolished by elimination of extracellular Ca^{2+} using EGTA (Fig. 2, lanes 3 and 6). Based on these results and other investigation (Rosen *et al.*, 1994), it seems that depolarization-induced phosphorylation of ERK depends on calcium influx, and that this influx is sufficient for phosphorylation of ERK in PC12 cells.

Various extracellular stimuli could activate the MAP kinase pathway. Most act through direct phosphorylation of Raf by protein kinase C (PKC) (Terada *et al.*, 1995). Because elevated levels of intracellular free calcium can activate PKC, we investigated whether depolarization-induced phosphorylation of ERK is mediated through PKC activation. As expected, stimulation of PKC with a PKC agonist, phorbol 12-myristate 13-acetate (PMA), could phosphorylate ERK; in the presence of PKC inhibitor GF109203X, phosphorylation of ERK did not increase (Fig. 3, lanes 2 and 6). To investigate the possibility that a Ca^{2+} influx could stimulate PKC and in turn phosphorylate Raf to activate the MAP kinase pathway, we down-regulated PKC activity using GF109203X. In the presence of GF109203X, NGF-induced ERK phosphorylation was not affected, indicating that the MAP kinase pathway is not affected by GF109203X (Fig. 3, lanes 1 and 5). Depolarization- and A23187-induced phosphorylation of ERK was also not affected by GF109203X (Fig. 3, lanes 3, 4, 7, and 8). This result suggests that Ca^{2+} influx-mediated activation of MAP kinase rarely depends on the activity of

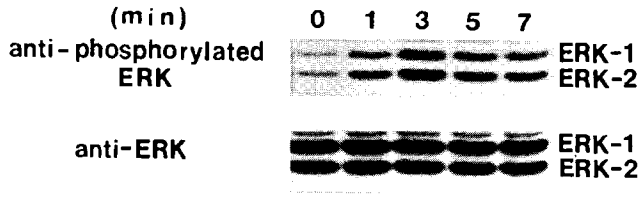

Fig. 1. Time-dependent phosphorylation of ERK upon depolarization. K^+ ion in the medium was elevated to 75 mM with an isosmotic KCl solution (170 mM). The total amount of ERK in each lane was the same, as shown in the lower panel.

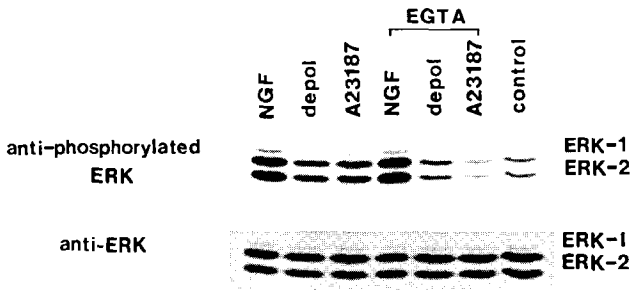

Fig. 2. Calcium influx mediates membrane depolarization-induced phosphorylation of ERK. Prolonged depolarization (75 mM KCl, 3 min) and calcium ionophore A23187 (2.5 μg/ml, 5 min) induced phosphorylation of ERK. Pretreatment with EGTA (1 mM, 3 min) abolished the phosphorylation of ERK induced by prolonged depolarization or A23187. NGF (20 ng/ml, 5 min)-induced phosphorylation of ERK was not affected. Elevated Na^+ ion (75 mM, 3 min) was used as a control.

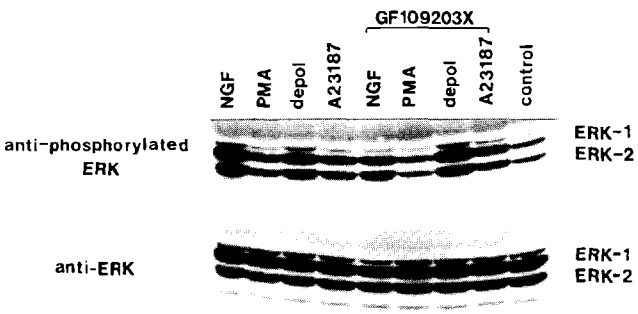

Fig. 3. PKC inhibitor, GF109203X, did not affect phosphorylation of ERK upon depolarization. Pre-incubation of GF109203X (1 mM, 5 min) did not affect phosphorylation of ERK upon depolarization and calcium influx. Phosphorylation of ERK by PMA (1.25 μg/ml, 5 min) was reduced but phosphorylation of ERK by NGF was not affected by GF109203X.

GF109203X-sensitive PKC. To further investigate the role of PKC in the phosphorylation of ERK upon depolarization, we depleted PMA-sensitive PKC by chronic treatment with PMA. Phosphorylation of ERK by PMA was greatly reduced by chronic treatment with PMA (Fig. 4, lanes 2 and 6). Consistent with the previous result,

the phosphorylation of ERK upon depolarization was not affected by chronic treatment with PMA (Fig. 4, lanes 3 and 7). Taken together, it seemed that at least GF109203X and PMA-sensitive PKCs are not involved in the phosphorylation of ERK induced by depolarization in PC12 cells.

It was reported that depolarization of plasma membrane induced phosphorylation of the EGF receptor (Rosen and Greenberg, 1996). To examine whether this EGFR activation is functionally necessary to transmit the Ca^{2+} signal for the activation of ERK, we used AG1478, the specific inhibitor of the EGF receptor. AG1478 has high specificity for the EGF receptor in the nanomolar range (Levitzki and Gazit, 1995). As expected, AG1478 did not affect NGF-induced ERK phosphorylation (Fig. 5, lanes 1 and 5). Since NGF transmits the signal to ERK through a proto-oncogenic signaling pathway, including the NGF receptor, Shc/Grb2, mSos1, and Ras, we concluded that AG1478 does not affect this signaling pathway, but specifically inhibits the kinase activity of EGF receptor. Pretreatment of PC12 cells with AG1478 inhibited phosphorylation of ERK which was induced by EGF (Fig. 5, lanes 2 and 6). Both depolarization- and A23187-induced phosphorylation of ERK was also greatly reduced in the presence of AG1478 (Fig. 5, lanes 3, 4, 7, and 8). Based on these results, the kinase activity of EGF receptor largely contributes to the phosphorylation of ERK upon calcium influx.

To determine whether the kinase activity of EGF receptor is necessary for depolarization-induced neurite outgrowth of PC12 cells, cells were depolarized in the presence or absence of AG1478. To enhance neurite outgrowth, cells were cultured with a low concentration of NGF. To confirm that cells were potentially able to extend neurites, the number of neurites was determined with the cells extending more than one neurite per cell. Depolarization by the elevated level of K^+ was shown to enhance neurite outgrowth in undifferentiated PC12 cells (Fig. 6). The average number of neurites was significantly reduced to the control level ($p < 0.01$) when depolarized PC12 cells were cultured in the presence of AG1478 (Fig. 6B). As NGF-induced phosphorylation of ERK was

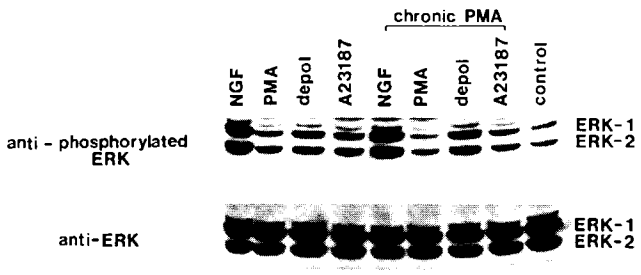

Fig. 4. Depletion of PKC did not affect phosphorylation of ERK upon depolarization. Chronic treatment of PMA (1.25 μg/ml, 1 d) did not affect phosphorylation of ERK upon depolarization. Phosphorylation of ERK induced by NGF (20 ng/ml, 5 min) was not affected by chronic treatment of PMA. Phosphorylation of ERK by PMA was reduced by chronic treatment of PMA.

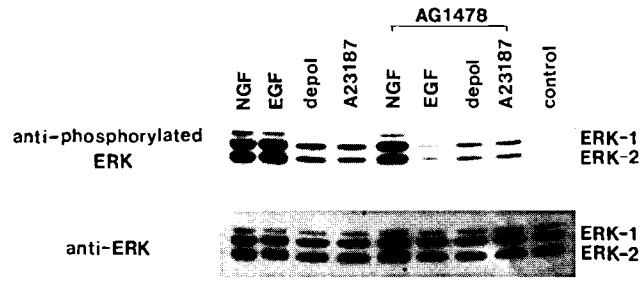

Fig. 5. Kinase activity of EGF receptor is functionally necessary for phosphorylation of ERK upon calcium influx. Phosphorylation of ERK by EGF (20 ng/ml, 5 min) was completely abolished in the presence of AG1478 (500 nM) but phosphorylation of ERK by NGF (20 ng/ml, 5 min) was not affected. Phosphorylation of ERK by depolarization and A23187 (2.5 μg/ml, 5 min) was greatly reduced by AG1478.

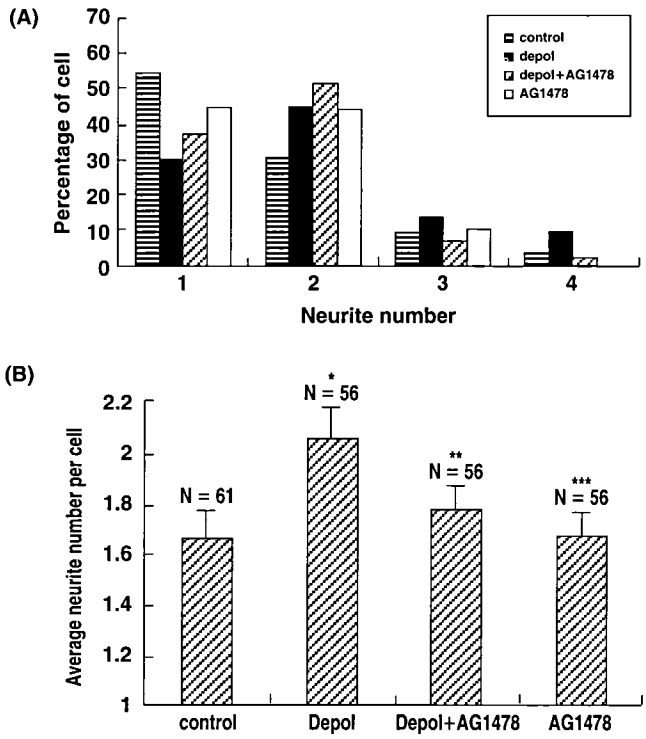

Fig. 6. Neurite outgrowth of PC12 cells induced by depolarization. Cells were cultured in the presence of NGF (5 ng/ml) to enhance neurite outgrowth. Dishes were coated with rat collagen. PC12 cells were treated with elevated Na^+ (control), elevated K^+ (depol), elevated K^+ and AG1478 (500 nM) (depol+AG1478), and AG1478 alone (AG1478). (A) Distribution of cells according to their neurite number in each group. (B) Neurite numbers per cell were determined. N represents the number of cells investigated per each group. The cells extending more than one neurite were investigated. Error bars indicate SEM (standard error of mean). * $p < 0.01$, ** $p < 0.01$, *** $p < 0.01$.

not affected by AG1478 as shown in Fig. 5, nor did it affect NGF-induced neurite outgrowth (Fig. 6B). The portion of cells which extended 3 or 4 neurites per cell was increased about two fold in depolarized PC12 cells compared to the control cells. In the presence of AG1478, this portion of cells was greatly reduced (Fig. 6B). This result indicates that activation of EGF receptor is also needed in prolonged depolarization-induced neurite outgrowth in PC12 cells.

Discussion

Intracellular free calcium acts as a messenger in various signaling pathways. In particular, influx of Ca^{2+} in neurons acts as a translator of electrical activity into biochemical activity. Calcium acts as a survival factor and is also involved in neurotransmitter release in neurons. In the present study, we showed that the kinase activity of the EGF receptor was necessary to transmit the Ca^{2+} signal to ERK activation and then induce neurite outgrowth upon membrane depolarization in the neuronal-like PC12 cell-line. Previous studies showed that membrane depolarization in PC12 cells induced phosphorylation of the EGF receptor which then could recruit the adaptor protein Shc/Grb2 (Rosen and Greenberg, 1996). In order to test whether the kinase activity of the EGF receptor is functionally necessary to transmit a Ca^{2+} signal for ERK activation, AG1478 was used to specifically inhibit EGF receptor kinase.

We first attempted to investigate whether the calcium influx which was induced by membrane depolarization could activate ERK. Depolarization with elevated extracellular K^+ levels in the medium induced phosphorylation of ERK, which occurred within 1 min in undifferentiated PC12 cells. Without depolarization, calcium influx by a calcium ionophore could also induce the phosphorylation of ERK. Furthermore, elimination of extracellular calcium using EGTA could dramatically abolish the phosphorylation of ERK induced by both depolarization and calcium ionophore. These results and previous studies clearly showed that depolarization-induced activation of ERK is mediated by Ca^{2+} influx, and that Ca^{2+} influx without depolarization is also sufficient for phosphorylation of ERK in PC12 cells.

PKC is positively regulated by Ca^{2+} and the activation of PKC initiates the MAP kinase pathway leading to ERK activation. Therefore, we tested whether PKC is involved in Ca^{2+} influx-induced ERK activation. As shown in Fig. 3, GF109203X-sensitive PKC is apparently not involved in depolarization-induced ERK activation in PC12 cells. This result still did not exclude the possibility that GF109203X-insensitive PKC might be involved in ERK activation induced by depolarization of plasma membrane. However, a similar result was obtained when we used chronic treatment of PMA which depletes PMA-sensitive PKC (Fig. 4). These two results strongly suggest that PKC is not involved in the phosphorylation of ERK upon calcium influx although PKC may be activated by, or involved in, elevation of Ca^{2+} levels by some other manner in the cytoplasm of the cell (Messing *et al.*, 1989).

Specific inhibition of EGF receptor activity with tyrphostin, AG1478, abolished depolarization-induced phosphorylation of ERK. Previous studies have indicated that the activation of the ERK upon depolarization is through the Ras-dependent signaling pathway and Ras is positively regulated by activation of EGF receptor (Rusanescu *et al.*, 1995; Rosen and Greenberg, 1996). This observation indicates that the Ca^{2+} signaling pathway leading to activation of the ERK shares a proto-oncogenic signaling pathway which is activated by a growth factor like EGF. Thus, the EGF receptor provides the point of cross-talk between Ca^{2+} and the EGF-induced signaling pathway.

How can the EGF receptor activate ERK upon depolarization? A possible answer based on our results as well as others is that the elevated level of intracellular free calcium may activate tyrosine kinases such as PYK2 and Src (Lev *et al.*, 1995; Rusanescu *et al.*, 1995; Rosen and Greenberg, 1996). One or a group of these Ca^{2+}-activated tyrosine kinases, which were activated by an elevated level of calcium, could phosphorylate the EGF receptor to enhance its kinase activity. The activated EGF receptor could possibly autophosphorylate and then create a binding site for the adaptor protein. Therefore, the EGF receptor may not merely act as a docking site for the adaptor protein when the Ca^{2+} influx induced the activation of ERK. Based on our results, however, the kinase activity of the EGF receptor may create a phosphorylation site for the adaptor protein; this initial activation of the EGF receptor is followed by activation of the ERK downstream pathway.

Ca^{2+} acts as a translator of electrical activity into biochemical activity in the nervous system. Recent reports indicate that the activation of MAPK is important for learning and memory in *Aplysia* (Bailey *et al.*, 1997; Martin *et al.*, 1997). They also reported that MAPK activation was induced by the activation of protein kinase A (PKA) both in *Aplysia* and in PC12 cells. We tested whether the EGF receptor is also involved in activation of ERK through activation of PKA. The level of cAMP was increased by forskolin and this treatment induced phosphorylation of ERK in PC12 cells (data not shown). In the presence of AG1478, however, the phosphorylation of ERK was not affected (data not shown). This result indicated that the EGF receptor is apparently not involved in phosphorylation of ERK by activation of PKA in PC12 cells.

Continued electrical activity induces long-term memory and this is accompanied with new synaptic growth in the nervous system. To approach the possibility that activity of the EGF receptor is necessary for neurite outgrowth upon

depolarization in PC12 cells, we investigated the effect of AG1478 on the neurite outgrowth of PC12 cells which was induced by prolonged membrane depolarization. The number of neurites per cell was significantly increased by prolonged depolarization and this increase was greatly inhibited by the presence of AG1478. This implies that the activity of EGF receptor is involved in the neurite outgrowth which was induced by depolarization through the action of ERK. A length assay of neurites was also performed in this study. There was no significant difference between AG1478 treated cells and nontreated cells (data not shown). One possible explanation for this incongruous result is that the basal level of NGF in all the experimental groups was sufficient for continuing the neurite outgrowth once it had been initiated.

Our results provide a clue to explain neuronal plasticity induced by continual electrical activity. Further research will be necessary to identify the exact nature of the tyrosine kinase molecule which activates the EGF receptor, and to investigate whether the ERK activation through the activation of the EGF receptor is sufficient for neurite outgrowth upon depolarization or whether some other signaling pathway is also activated by calcium influx.

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