Stimulation of Neurite Outgrowth in PC12 Cells by EGF and KCl Depolarization: A Ca^{2+}-independent Phenomenon

Melanie D. Mark, Yuechueng Liu, Scott T. Wong, Thomas R. Hinds, and Daniel R. Storm

Department of Pharmacology, University of Washington, Seattle, Washington 98195

Abstract. MAP kinase activity is necessary for growth factor induction of neurite outgrowth in PC12 cells. Although NGF and EGF both stimulate MAP kinase activity, EGF does not stimulate neurite extension. We report that EGF, in combination with KCl, stimulates neurite outgrowth in PC12 cells. This phenomenon was independent of intracellular Ca^{2+} increases and not due to enhancement of MAP kinase activity over that seen with EGF alone. However, EGF plus KCl increased intracellular cAMP, and other cAMP elevating agents acted synergistically with EGF to promote neurite outgrowth. Stimulation of neurite outgrowth by cAMP and EGF was blocked by inhibitors of transcription suggesting that synergistic regulation of transcription by the cAMP and MAP kinase pathways may stimulate neurite growth.

R AT pheochromocytoma cell lines (PC12) are a useful model system for examining mechanisms underlying neuronal differentiation and signal transduction. These cells differentiate in response to several growth factors, including NGF and FGF (Greene and Tischler, 1982; Togari et al., 1983). Growth factor induced neurite outgrowth is accompanied by several changes which include increased electrical excitability, induction of the immediate early genes c-*jun*, c-*myc*, and c-*fos*, and enhanced activation of the MAP-kinase cascade (Greene and Tischler, 1976; Greene and Tischler, 1982; Boonstra et al., 1983; Greenberg et al., 1985; Miyasaka et al., 1990; Gotoh et al., 1990). EGF- and NGF-treated PC12 cells share several common properties including rapid membrane ruffling, flattening of cells, and increases in cell adhesion (Connolly et al., 1984). However, NGF but not EGF stimulates neurite outgrowth. EGF functions as a typical mitogen for PC12 cells inducing the same "early" responses as NGF but none of the late differentiation-associated responses (Chao, 1992).

MAP kinase activity is necessary for growth factor induced PC12 cell differentiation (Cowley et al., 1994). The duration of MAP kinase stimulation by NGF and EGF may account for the lack of neurite induction by EGF. NGF and EGF both stimulate rapid increases in MAP kinase activity, however, NGF stimulations are more persistent than EGF (Gotoh et al., 1990; Traverse et al., 1992). Furthermore, NGF treatment of PC12 cells causes a persistent increase in Na^+/K^+ pump activity, whereas the increase caused by EGF decays more rapidly (Boonstra et al., 1983).

Although EGF was originally identified as a potent epidermal proliferative agent (Cohen, 1965), it may also act in combination with other agents to stimulate neuronal differentiation. For example, cultured post-mitotic chick CNS precursor neurons undergo extensive differentiation in the presence of medium supplemented with 15–20% fetal bovine serum (Pettmann et al., 1979). These neurons fail to differentiate in serum-free media not supplemented with growth factors, however, addition of purified EGF induces neuritogenesis. Furthermore, addition of EGF antibodies to serum-supplemented medium blocks neuritogenesis in post-mitotic chick CNS precursor neurons (Rosenberg and Noble, 1989). In another study, EGF was found to promote neuronal differentiation in PC12 cells when cultured on a positively charged modified surface, Primaria, but not on other substrates (Nakafuku and Kaziro, 1993). Collectively, these data suggest that EGF may promote neurite growth in combination with activation of other signal transduction pathways.

In this study, we report that EGF and KCl depolarization act synergistically to promote neurite outgrowth in PC12 cells. An analysis of this phenomenon revealed that EGF and KCl increased intracellular cAMP, and that cAMP and EGF synergistically stimulate neurite extension. These data suggest that neurite outgrowth may be stimulated by sub-threshold activation of the MAP kinase pathway in combination with stimulation of the cAMP signal transduction system.

Materials and Methods

PC12 Cell Culture

PC12 cells were maintained at 37°C in high glucose DME supplemented

Address all correspondence to D. R. Storm, University of Washington School of Medicine, D-429 Health Science Building, Box 357280, Seattle, WA 98195-7280. Tel.: (206) 543-7028. Fax: (206) 685-3822.

with 1% penicillin and streptomycin, 5% bovine calf serum (BCS)[1] and 10% FBS in humidified 95% air/5% CO_2 incubator. Cells were subcultured into 6-well poly-L-lysine–coated (50 μg/ml) tissue culture dishes and treated with various effectors 24–48 h after plating.

Determination of Neurite Outgrowth

When the cultures reached 20–30% confluency, cells were treated with various effectors at the indicated concentrations for 3 d. Cells were photographed and neurite lengths were quantitated from photographs of cells using a ruler. Values for neurite lengths are an average from three independent experiments ±SD ($n = 200$ cells). In some cases, PC12 cells were maintained in Ca^{2+}-free DME media (purchased from GIBCO BRL, Gaithersburg, MD) supplemented with 10% FBS, 5% BCS, and 0.4 mM EGTA (Ca^{2+}-free media) to remove extracellular Ca^{2+} from the serum. Cells were allowed to rest for 24 h and subsequently treated with various agents as indicated in the presence of Ca^{2+}-free media. In other experiments, cells were pretreated with 10 μM nifedipine (Sigma Chemical Co., St. Louis, MO) for 30 min at 37°C before addition of effectors.

Determination of Cyclic AMP Accumulation

Intracellular cAMP levels were measured by determining the ratio of [³H]cAMP to a total ATP, ADP, and AMP pool in [³H]adenine-loaded cells as described previously by Wayman et al. (1994). Briefly, PC12 cells were subcultured into poly-L-lysine–coated 6-well plates and incubated for 1 h at 37°C in serum-free DME containing 1 mM 3-isobutyl-1-methylxanthine (IBMX) and various agents as indicated. Under "Ca^{2+}-free" conditions, serum-free, Ca^{2+}-free high glucose DME (purchased from GIBCO BRL) containing 0.4 mM EGTA was used instead of serum-free DME. In some cases, cells were pretreated with 10 μM nifedipine for 30 min at 37°C before various effectors were added. Reactions were terminated with 1 ml of ice cold 5% trichloroacetic acid containing 1 μM cAMP and separated by ion-exchange chromatography as described by Salomon et al. (1974). Reported data are averages of triplicate determinations.

MAP Kinase Assays

MAP kinase activity was assayed by the general method of Gotoh et al. (1990) using myelin basic protein as a substrate. A mouse anti–MAP kinase monoclonal antibody was purchased from Zymed Laboratories (San Francisco, CA). PC12 cells were incubated with NGF (100 ng/ml) or EGF (100 ng/ml) in the presence or absence of 40 mM KCl for varying periods of time as indicated. Cell extracts were prepared for MAP kinase immunoprecipitation and kinase reactions in SDS polyacrylamide gels were carried out as described in the product information bulletin from Zymed Laboratories. Gels were incubated at room temperature for 1 h in 10 ml of 40 mM Hepes, pH 8.0, 2 mM dithiothreitol, 100 μM EGTA, 5 mM $MgCl_2$, 25 μM ATP with gamma [³²P] ATP (250 μCi; 1 μCi = 37 kBq). The gels were vigorously washed in 5% trichloroacetic acid, 1% NaPPi, dried, and exposed to x-ray film for 10–12 h. MAP kinase activities are expressed as relative activities determined by scanning the exposed film with a Bio-Rad GS-670 densitometer. MAP kinase data obtained with this assay was also confirmed using the method of Ahn et al. (1992).

Calcium Imaging

PC12 cells were subcultured onto poly-L-lysine–coated, four-chambered NUNC dishes. Within 72 h after subculturing, cells were rinsed once with serum-free, high glucose DME, and loaded with 4 μM Fura-2 at 37°C in the dark. After 40 min of loading with Fura-2, cells were rinsed twice with serum-free DME and allowed to sit for 30 min or pretreated with 10 μM nifedipine for 30 min prior to imaging. Ca^{2+} imaging was carried out in serum-free DME, serum-free DME ± nifedipine, or in Ca^{2+}-free high glucose DME (purchased from GIBCO BRL) containing 0.4 mM EGTA. Calcium imaging was carried out using a Nikon Diaphote inverted microscope. The four-chambered coverglass (Lab-Tek, Nunc Roskilde, Denmark) was epi-illuminated through a 20× objective at 340 and 380 nm using a filter wheel and a 75 W Xenon lamp at 25°C. Emitted fluorescence was collected by the 20× objective and filtered through a 510 nm band pass filter. Fluorescence was subsequently magnified with a 2× lens and

an image was obtained with an intensified CCD camera. The ratios at 340/380 were obtained every 8 s up to 20 min with no observable photobleaching. Control of the camera and filter wheel and the rate of sampling, data collection, data display and analysis was done with the software Image-1/FL (Universal Imaging Corp., West Chester, PA). The system was calibrated using Fura-2–loaded cells at the end of the experiment in order to convert fluorescence ratios into intracellular-free Ca^{2+} (Grynkiewicz et al., 1985). The cells were permeabilized with 10 μM 4-Bromo A23187 in imaging buffer containing 0.4 mM EGTA for determination of R_{min} and R_{max} was obtained using 10 mM Ca^{2+}. The dissociation constant K_d was 224 nM and Sf/b was the fluorescence signal without and with Ca^{2+} at 380 nm.

Quantitation of CRE-mediated Transcription

PC12 cells were stably transfected with the G418 resistant CRE-lacZ reporter construct with Lipofectamine as described in the product information bulletin from (GIBCO BRL). Briefly, cells were plated in 100 mm dishes at ~70% confluency, grown overnight, and transfected with the CRE-lacZ (15 μg DNA/plate) and Lipofectamine (GIBCO BRL) in Opti-MEM. Polyclonal stables were selected with 500 μg/ml G418. PC12 cells stably expressing the CRE-lacZ construct (PC12-CRE-lacZ) were subsequently used to quantitate CRE-mediated transcription as described by the general method of Impey et al. (1994). The cells were treated with various effectors in Ca^{2+}-free, serum-free DME for 1 h, or cells were pretreated with 20 μM H89 for 30 min prior to the addition of effectors. The effectors were removed and replaced with Ca^{2+}-free DME media supplemented with 10% FBS, 5% BCS. Following a 4-h incubation at 37°C, cells were rinsed with PBS plus 1 mM $MgCl_2$, lysed in 1 ml 0.5% NP-40 in PBS plus 1 mM $MgCl_2$ and incubated with 3.5 mM o-nitrophenyl-b-D-galactopyranoside (ONPG) at 37°C for 3 h. Reported data are averages of triplicate determinations.

Expression of Dominant–Negative MAPKK Mutants

PC12 cells were transiently co-transfected with CDM8-lacZ and pc-DNAIII (the vector control) or one of the MAPKK dominant negative mutants (pcDNAIII-MAPKK1aS222A or pcDNAIII-MAPKK1aK97A) (Seger et al., 1994) using Lipofectamine as described in the product information bulletin from GIBCO BRL. Briefly, cells were plated in 100 mm dishes at ~70% confluency, grown overnight, and transfected with pc-DNAIII, pcDNAIII-MAPKK 1a S222A, or pcDNAIII-MAPKK 1a K97A (10 μg DNA/plate) and CDM8-lacZ (5 μg DNA/plate) constructs. After a 7-h incubation at 37°C with 5% CO_2, cells were supplemented and equal volume of DME plus 10% FBS and 5% BCS for 12 h. Transfected PC12 cells were then washed, subcultured into 4 poly-L-lysine–coated 100 mm plates, and incubated for an additional 72 h in Ca^{2+}-free DME. The cells were allowed to rest for 2 h, and then treated with 50 ng/ml EGF and 40 mM KCl. Transfected cells were fixed with −20°C 95% ethanol for 2 min and stained for X-gal (Gold Biotechnology Inc., St. Louis, MO). Positively transfected cells were measured for neurite outgrowth and reported as the average ($n = 175$) neurite length ± SD.

Detection of CREB Phosphorylation

PC12 cells were subcultured into poly-L-lysine–coated 60 mm tissue culture dishes and cell extracts were prepared according to the method of Ginty et al. (1994). Briefly, PC12 cells were treated with various effectors for 10 min unless indicated otherwise, washed twice with PBS lysed in 100 μl boiling SDS sample buffer (40 mM Tris, pH 6.9, 2 mM EGTA, 10% glycerol, 1% dithiothreitol, 1% SDS, .04% bromophenol blue), and boiled for 10 min. Cell extracts were separated by 10% SDS-PAGE and transferred to nitrocellulose membranes. The membrane was blocked with 4% bovine serum albumin in PBS and then incubated with 0.14 μg/ml anti-phospho-CREB (Ginty et al., 1993). Blots were developed using an alkaline phosphatase reaction. The relative density of phospho-CREB bands was quantitated by scanning immunoblots with a densitometer (model GS-670; Bio-Rad Laboratories, Cambridge, MA).

Results

Synergistic Induction of Neurite Outgrowth in PC12 Cells by EGF and KCl Depolarization

To identify signal transduction pathways that might act cooperatively with EGF to stimulate neurite outgrowth, we

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1. *Abbreviations used in this paper:* BCS, bovine calf serum; CaM, calmodulin; IBMX, 3-isobutyl-1-methylxanthine; ONPG, o-nitrophenyl-b-D-galactopyranoside.
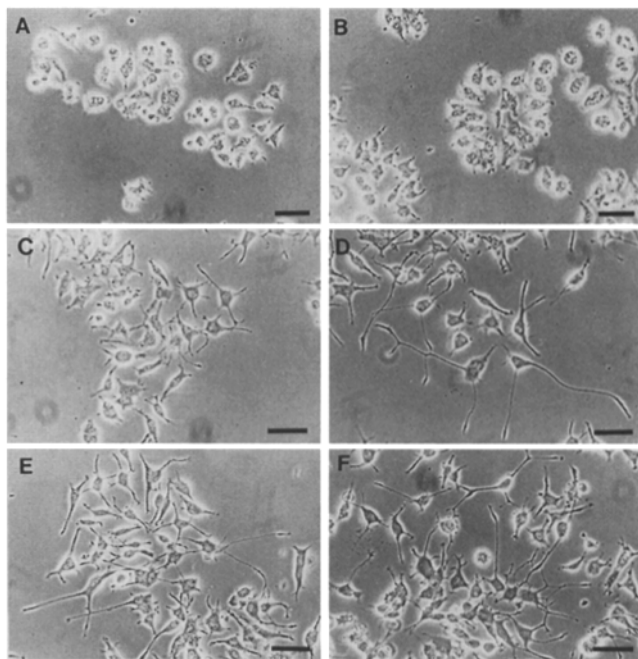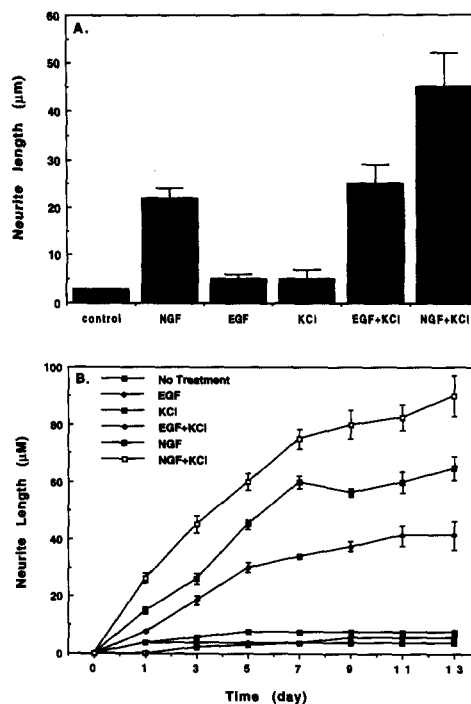
Figure 1. Stimulation of PC12 cell neurite outgrowth by combinations of EGF and KCl. (*A*) Untreated PC12 cells; (*B*) PC12 cells treated with 50 ng/ml EGF; (*C*) PC12 cells treated with 20 ng/ml NGF; (*D*) cells treated with 20 ng/ml NGF and 50 mM KCl; (*E*) cells treated with 50 ng/ml EGF and 50 mM KCl; (*F*) cells treated with 100 ng/ml EGF and 50 mM KCl. PC12 cells were grown as described in Materials and Methods. Phase–contrast photographs of treated and untreated PC12 cells were taken three days after treatment. Bars, 30 μm.

Figure 2. Average PC12 cell neurite length stimulated by EGF and KCl. (*A*) Average neurite lengths produced after three days in culture with no treatment (*control*), 20 ng/ml NGF, 50 ng/ml EGF, 50 mM KCl were quantitated as described in Materials and Methods. (*B*) PC12 cells were treated with 50 ng/ml EGF, 40 mM KCl, 50 ng/ml NGF, 50 ng/ml EGF and 40 mM KCl, 50 ng/ml NGF and 40 mM KCl, or no treatment (*control*). Average neurite lengths were quantitated as described in Materials and Methods. Reported neurite lengths are the average ± SD (*n* = 200 cells).

examined the effect of EGF on neurite extension under conditions which activate other signal transduction pathways. Depolarization by KCl induces neurite outgrowth in PC12 cells grown on a monolayer of 3T3 fibroblast cells (Saffell, 1992), suggesting that combinations of depolarization and factors released from fibroblasts may stimulate neurite outgrowth. Therefore, we examined the effect of EGF and combinations of EGF and KCl on neurite extension in PC12 cells. Untreated PC12 cells and EGF treated cells showed no significant induction of neurite outgrowth (Fig. 1, *A* and *B*). NGF treatment promoted extensive neurite outgrowth and the length of these neurites was enhanced by the addition of KCl (Fig. 1, *C* and *D*). Although EGF alone did not stimulate neurite outgrowth, the combination of EGF and KCl did (Fig. 1, *E* and *F*).

KCl or EGF alone induced only minor increases in average process length from 3.0 ± 0.6 μm to 5.0 ± 2.0 μm (Fig. 2). The average neurite length produced by EGF plus KCl (25.6 ± 4.0 μm) was comparable to NGF alone (23.2 ± 2.0 μm). Neurite lengths stimulated by NGF plus KCl were approximately twofold greater than NGF alone. Neurite outgrowth stimulated by EGF and KCl or NGF alone persisted for at least 13 d indicating that neurite formation is not transient (Fig. 2 *B*). Three other PC12 cell lines demonstrated stimulation of neurite outgrowth by combinations of EGF and KCl, illustrating that this response is not a unique property of one cell line.

The phenomenon described above was dependent upon the concentration of EGF and KCl (data not shown). In

the presence of 50 ng/ml EGF, half-maximal stimulation of neurite outgrowth was observed at 25 mM KCl with a maximum effect at 40 to 50 mM KCl (data not shown). In the presence of 50 mM KCl, half maximal stimulation of neurite length was at 1 ng/ml EGF. These data demonstrated that EGF can promote neurite extension in PC12 cells when paired with another signal transduction pathway stimulated by KCl depolarization.

EGF/KCl-induced Neurite Outgrowth is Ca^{2+} Independent

Treatment of PC12 cells with 50 mM KCl causes membrane depolarization, activation of voltage-sensitive Ca^{2+} channels, and significant increases in intracellular Ca^{2+} (Stallcup, 1979; Ritchie, 1979). In order to evaluate the contribution of Ca^{2+} signal transduction pathways for neurite outgrowth, neurite extension was examined in the presence of agents that prevent increases in intracellular Ca^{2+} or antagonize calmodulin (CaM). Neither calmidazolium, a CaM antagonist, nor KN-62, an inhibitor of CaM kinases, affected neurite outgrowth stimulated by EGF and KCl (Fig. 3). Nifedipine, an L-type voltage-sensitive Ca^{2+} channel blocker, did not block EGF and KCl stimulated neurite outgrowth. Treatment of cells with EGTA in Ca^{2+}-free media also did not affect neurite extension caused by EGF and KCl.

To ensure that intracellular Ca^{2+} levels did not increase
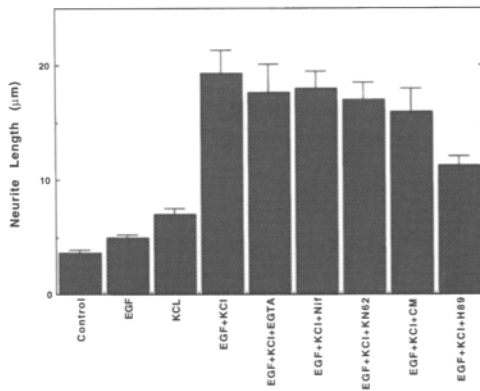
*Figure 3.* EGF and KCl depolarization-induced neurite outgrowth is independent of increases in intracellular Ca^{2+}. Average neurite lengths produced after three days in culture with 50 ng/ml EGF, 40 mM KCl, 50 ng/ml EGF and 40 mM KCl, 50 ng/ml EGF and 40 mM KCl and 0.4 mM EGTA, 50 ng/ml EGF and 40 mM KCl and 10 μM nifedipine (*Nif*), 50 ng/ml EGF and 40 mM KCl and 1 μM KN-62, 50 ng/ml EGF and 40 mM KCl and 1 μM calmidazolium (*CM*), or 50 ng/ml EGF and 40 mM KCl and 10 μM H89. Cells treated with EGTA were in "Ca^{2+}-free" media as described in the Materials and Methods. Neurite lengths are the average ± SD (n = 200 cells).

in the presence of nifedipine or EGTA, intracellular Ca^{2+} was monitored using Fura-2. In the presence of Ca^{2+}, addition of EGF plus KCl increased cytosolic free Ca^{2+} fivefold relative to untreated control cells (Fig. 4 *A*). However, no increases in intracellular Ca^{2+} were detectable when cells were treated with EGF and KCl in the presence of 10 μM nifedipine or 0.4 mM EGTA in Ca^{2+} free buffer (Fig. 4, *B* and *C*). Therefore, neurite outgrowth stimulated by EGF and KCl was not dependent upon increases in intracellular Ca^{2+}.

Stimulation of Neurite Extension by EGF and KCl Is Not Mediated by an Enhancement in MAP Kinase Activity

Since NGF stimulates neurite outgrowth and induces a prolonged increase in MAP kinase activity, we examined the effects of EGF and combinations of EGF and KCl on MAP kinase activity in the presence and absence of Ca^{2+} (Fig. 5). NGF and EGF both induced a rapid increase in MAP kinase activity but the duration and magnitude of this response was greater with NGF. In the presence of Ca^{2+}, KCl alone stimulated MAP kinase activity (Fig. 5 *B*) and enhanced stimulations by either NGF (Fig. 5 *A*) or EGF (Fig. 5 *B*), consistent with the report that depolarization of PC12 cells enhances MAP kinase activity through a Ca^{2+}-dependent process (Rosen et al., 1994). In the presence of nifedipine, however, stimulation of MAP kinase activity by EGF and KCl was no greater than EGF alone. The data reported in Fig. 5 was confirmed using both MAP kinase assays described in Materials and Methods. Since synergistic stimulation of neurite growth by EGF and KCl was Ca^{2+} independent, stimulation of neurite extension by combinations of EGF and KCl was not attributable to increases in MAP kinase activity above that seen with EGF alone. However, activation of the MAP kinase pathway was required for this phenomenon because
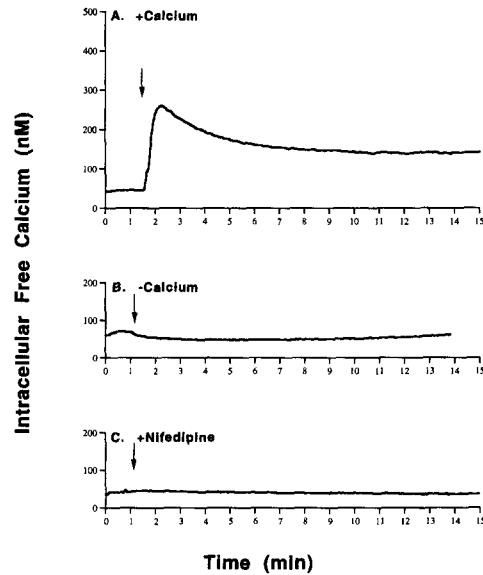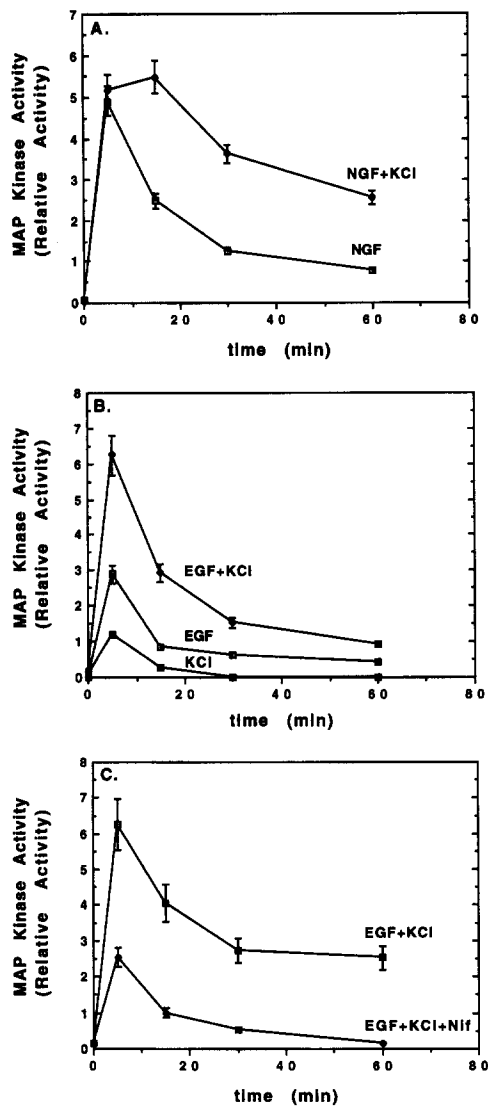

Figure 4. Increases in intracellular Ca^{2+} stimulated by KCl are blocked by Nifedipine and EGTA. Intracellular Ca^{2+} levels were determined in PC12 cells using the Ca^{2+}-sensitive dye Fura-2 and recorded as the ratio of 340/380 as described in the Materials and Methods. Measurements were recorded following the addition of 50 ng/ml EGF and 40 mM KCl (as indicated by the *arrow*) in the presence of (*A*) 2.3 mM Ca^{2+}; (*B*) 0.4 mM EGTA in Ca^{2+}-free media; or (*C*) 2.3 mM Ca^{2+} and 10 μM nifedipine.

expression of dominant–negative MAP kinase kinase (MAPKK) mutants (Seger et al., 1994) in PC12 cells blocked neurite outgrowth stimulated by combinations of EGF and KCl (Fig. 6).

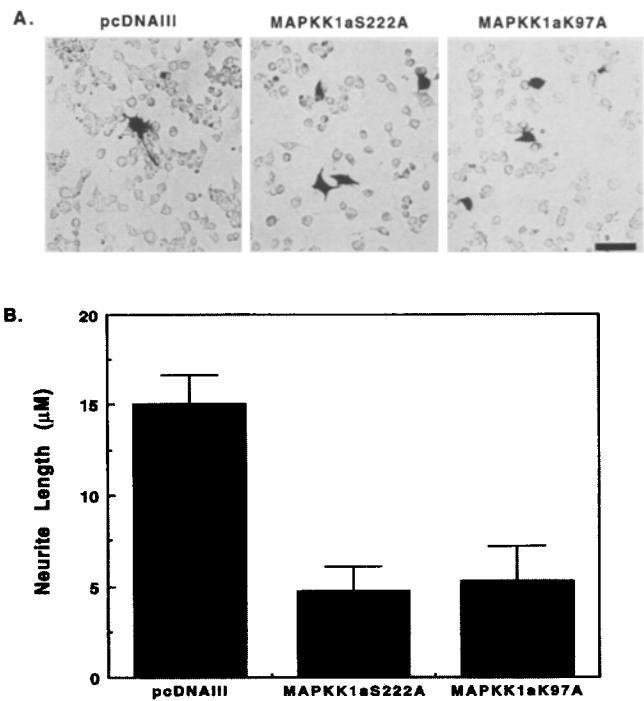### Contribution of the cAMP Pathway to EGF- and KCl-induced Neurite Outgrowth

An analysis of the effect of various protein kinase inhibitors on neurite extension revealed that H89, an inhibitor of cAMP-dependent protein kinase partially inhibited neurite extension promoted by EGF and KCl (Fig. 3). We could not achieve a complete inhibition of neurite outgrowth because higher concentrations of H89 were cytotoxic to the cells after 72 h of treatment. Since EGF increases intracellular cAMP in luteal cells (Budnik and Mukhopadhyay, 1991, 1993) and EGF receptors have been demonstrated to directly interact with G-proteins (Sun et al., 1995), we measured intracellular cAMP levels in PC12 cells treated with EGF and KCl. PC12 cells were treated with EGF, KCl, or combinations of EGF and KCl in Ca^{2+}-containing or Ca^{2+}-free media with 0.4 mM EGTA (Fig. 7). Intracellular cAMP levels were consistently lower in Ca^{2+}-containing buffer than in Ca^{2+}-free buffer, possible because of the presence of Ca^{2+} inhibitable adenylyl cyclase activity. Cells treated with EGF alone demonstrated no significant elevation in intracellular cAMP. On the other hand, treatment with KCl and EGF caused a consistent increase in cAMP. In the presence or absence of Ca^{2+}, the combination of EGF and KCl increased intracellular cAMP by 50 and 80%, respectively. NGF ± KCl had no measurable effect on intracellular cAMP in the presence or absence of Ca^{2+} (data not

Figure 6. Inhibition of EGF- and KCl-stimulated neurite outgrowth by transient expression of MAPKK dominant–negative mutants. (*A*) Phase contrast photographs of PC12 cells transiently co-transfected with CDM8-lacZ and pcDNAIII (vector control) or one of the MAPKK dominant–negative mutants, pcDNAIII-MAPKK1aS222A or pcDNAIII-MAPKK1aK97A. Bar, 30 μM. Transfected cells were treated with 50 ng/ml EGF and 40 mM KCl for 72 h under calcium free conditions, stained for X-gal to identify transfected cells, and phase contrast photographs were taken as described in the Materials and Methods. (*B*) Average neurite lengths of transfected cells were measured and reported as average ± SD as described in the Materials and Methods. *n* = 175 for each set of cells scored.

Figure 5. KCl stimulation of MAP kinase activity is Ca^{2+} dependent. Kinetics for stimulation of MAP kinase activity by (*A*) 100 ng/ml NGF ± 40 mM KCl; (*B*) 100 ng/ml EGF ± 40 mM KCl or 40 mM KCl alone; (*C*) 100 ng/ml EGF and 40 mM KCl ± 10 μM nifedipine. Relative MAP kinase activity was determined as described in the Materials and Methods. The various effectors were added at time zero and the data points are the average of triplicate determinations ± SD.

shown). These data illustrate that EGF receptors in PC12 cells are coupled to stimulation of cAMP levels when the cells are depolarized with KCl. The mechanism for synergistic stimulation of adenylyl cyclase activity by EGF and membrane depolarization has not been elucidated. However, adenylyl cyclase activity in cultured neurons is regulated by the membrane potential in a Ca^{2+}-independent manner (Reddy, R., and Storm, D. R., unpublished observations) suggesting that some cells may contain voltage sensitive adenylyl cyclase activity.

The data described above raised the interesting possibility that cAMP may act synergistically with EGF to stimulate neurite outgrowth. Consequently, we examined the effect of dibutyryl-cAMP and EGF on neurite extension.

High concentrations of cAMP elevating agents including forskolin and dibutyryl-cAMP stimulate neurite outgrowth (Gunning et al., 1981; Heidemann et al., 1985). We were interested in determining if low levels of cAMP would synergistically stimulate neurite extension in combination with EGF. PC12 cells were treated with various concentrations of dibutyryl-cAMP in the presence or absence of EGF and neurite lengths were monitored (Fig. 8 *A*). Dibutyryl-cAMP alone stimulated neurite outgrowth only at concentrations of 300 μM or higher. However, low levels of dibutyryl-cAMP (5–100 μM) synergistically stimulated neurite outgrowth with EGF. In the presence of 50 μM dibutyryl cAMP, half-maximal stimulation of neurite growth occurred at 30 ng/ml EGF (Fig. 8 *B*).

The general phenomenon described above was confirmed using two other agents that elevate intracellular cAMP, pertussis toxin and forskolin. Pertussis toxin causes small increases in cAMP by catalyzing the ADP-ribosylation of G_i (Katada and Ui, 1982; Bokoch et al., 1983). Pertussis toxin alone, at concentrations as high as 200 ng/ml, had no effect on neurite extension (data not shown). However, the toxin synergistically stimulated neurite extension with EGF. The neurites stimulated by pertussis toxin and EGF were shorter than that produced by EGF and KCl, or
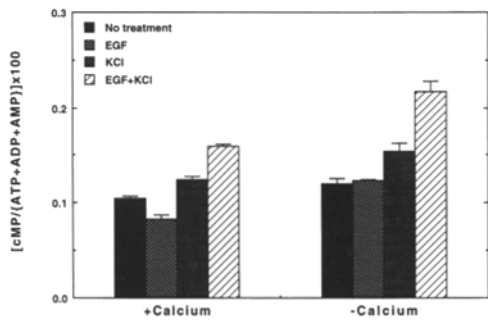
Figure 7. Synergistic increases in intracellular cAMP by EGF and KCl. PC12 cells were treated with 100 ng/ml EGF, 40 mM KCl, or 100 ng/ml EGF and 40 mM KCl in the presence or absence of calcium and intracellular cAMP levels were quantitated as described in the Materials and Methods. Calcium-free conditions were generated using 0.4 mM EGTA in calcium-free media as described in the Materials and Methods.

EGF and dibutyryl-cAMP, probably because the increase in intracellular cAMP stimulated by pertussis toxin was lower.

Forskolin at concentrations greater than 1.0 μM stimulated neurite outgrowth, but lower concentrations (0.01–0.1 μM) did not (Fig. 8 C). Combinations of 0.1 μM forskolin and EGF synergistically stimulated neurite outgrowth. Neurite extensions produced by combinations of EGF and forskolin or EGF and dibutyryl-cAMP were comparable in the presence or absence of Ca^{2+}.

In order to determine if the levels of intracellular cAMP produced by EGF and KCl were sufficient to account for

neurite outgrowth, we compared the forskolin dose response curves for stimulation of intracellular cAMP and neurite outgrowth with EGF (Fig. 8 D). Forskolin at 0.3 μM increased intracellular cAMP to levels comparable to that produced by 100 ng/ml EGF plus 40 mM KCl (cAMP ratio = 0.2 to 0.3). This forskolin concentration also gave maximum stimulation of neurite growth when paired with EGF. We conclude that low levels of cAMP produced by KCl and EGF, which were 80% over basal, were sufficient to explain the synergistic stimulation of neurite extension caused by KCl and EGF.

In the presence of Ca^{2+}, KCl alone activated MAP kinase activity but the stimulation was only 20–30% of that caused by EGF or NGF (Fig. 5). It was of interest to determine if low levels of MAP kinase stimulation would also synergize with cAMP. Combinations of 40 mM KCl and 50 μM dibutyryl-cAMP also synergistically stimulated neurite outgrowth suggesting that differentiation may be promoted by pairing of relatively low levels of MAP kinase activation with cAMP (Fig. 8 E). Neurite outgrowth stimulated by KCl and cAMP was Ca^{2+} dependent, consistent with previous data reporting that KCl stimulation of MAP kinase activity is Ca^{2+} dependent (Rosen et al., 1994).

To further implicate the cAMP signal transduction pathway in EGF and KCl stimulated neurite outgrowth, we examined neurite extension in PKA-deficient PC12 cells (Ginty et al., 1991). As previously reported by Ginty et al. (1991) NGF stimulated neurite outgrowth in PKA-deficient PC12 cells was comparable to that obtained with the parental PKA$^+$ cell line (Fig. 9 A). Neurite extension caused by EGF plus KCl was inhibited in PKA-deficient PC12 cells compared to the parental cell line which dis-
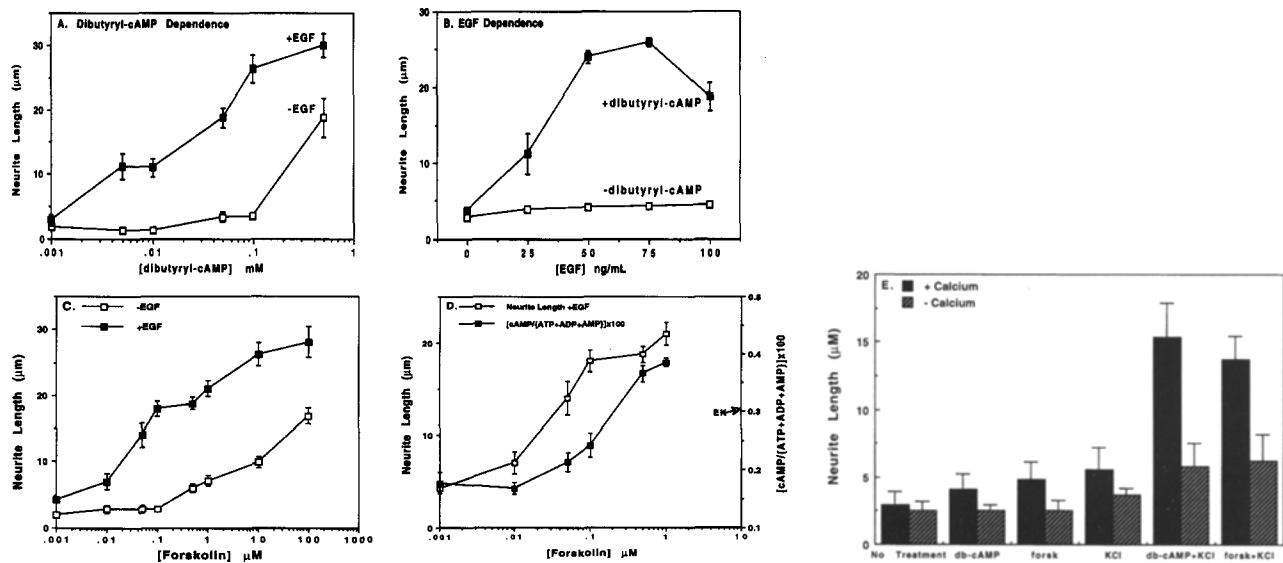
Figure 8. EGF and cAMP synergistically stimulate neurite outgrowth in PC12 cells. (A) PC12 cells were grown in the presence or absence of 50 ng/ml EGF with increasing concentrations of dibutyryl-cAMP. (B) PC12 cells were grown in the presence or absence of 0.05 mM dibutyryl-cAMP with increasing concentrations of EGF. (C) PC12 cells were grown in the presence (+EGF) or absence (−EGF) of 50 ng/ml EGF with increasing concentrations of forskolin. (D) Neurite length and intracellular cAMP produced by 100 nM EGF and increasing concentrations of forskolin were measured in parallel sets of cells. The amount of intracellular cAMP produced by 100 ng/ml EGF and 40 mM KCl (EK) is indicated with an arrow. In A–D, PC12 cells were treated in Ca^{2+}-free media and 0.4 mM EGTA. (E) PC12 cells were treated with 0.05 mM dibutyryl-cAMP, .5 μM forskolin, 40 mM KCl, 0.05 mM dibutyryl-cAMP and 40 mM KCl, .5 μM forskolin and 40 mM KCl, or no treatment (control) in Ca^{2+}-free media and 0.4 mM EGTA or Ca^{2+}-containing media. Neurite lengths were recorded after three days as described in the Materials and Methods and are the average ± SD (n = 200 cells).
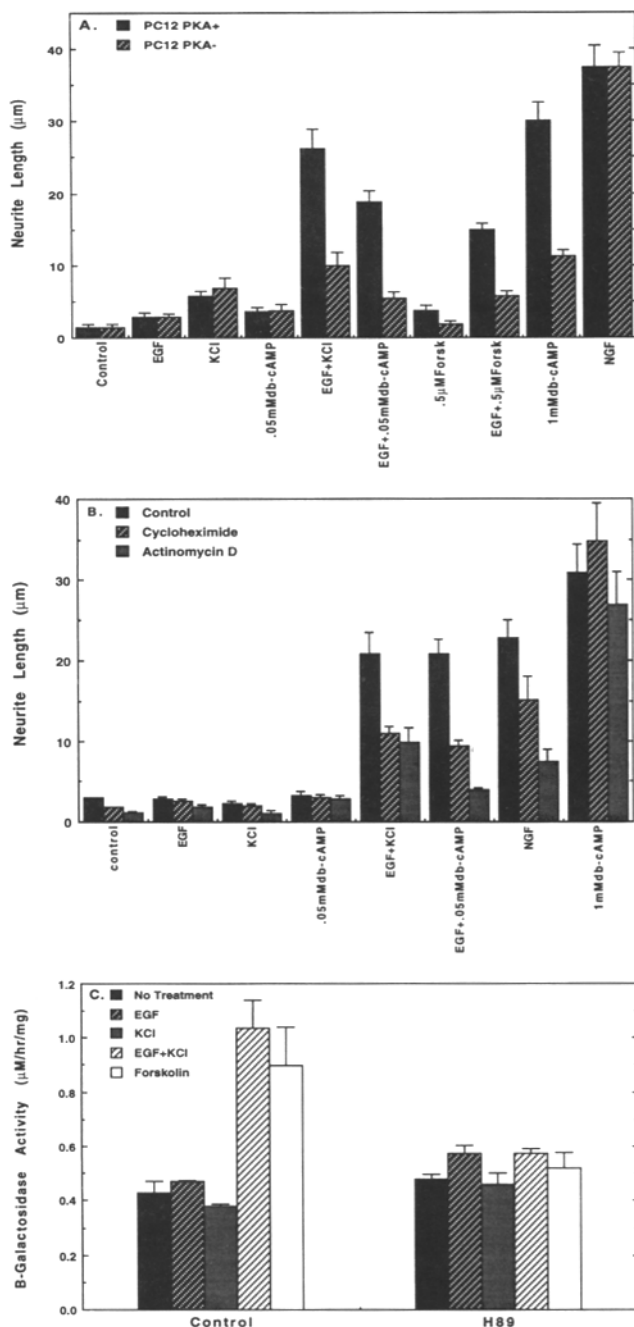
Figure 9. EGF- and KCl-stimulated neurite outgrowth is depressed in PC12 PKA⁻ cells and is dependent upon transcription. (*A*) PC12 cells deficient in PKA (*PC12 PKA⁻*) or the parental cell line (*PC12 PKA⁺*) were treated with 50 ng/ml EGF, 40 mM KCl, 0.05 mM dibutyryl-cAMP, 50 ng/ml EGF and 40 mM KCl, 50 ng/ml EGF and 0.05 mM dibutyryl-cAMP, 0.5 µM forskolin, 50 ng/ml EGF and 0.5 µM forskolin, 1 mM dibutyryl-cAMP, or 50 ng/ml NGF. (*B*) PC12 cells were pretreated with 0.05 µg/ml actinomycin D or 1 µg/ml cycloheximide. Following 1 h of pretreatment, cells were treated with 50 ng/ml EGF, 40 mM KCl, 0.05 mM dibutyryl-cAMP, 50 ng/ml EGF and 40 mM KCl (*EK*), 50 ng/ml EGF and 0.05 mM dibutyryl-cAMP, 50 ng/ml NGF or 1 mM dibutyryl-cAMP. Neurite lengths were quantitated after three days and are the average ± SD (*n* = 200 cells). (*C*) PC12 cells stably expressing a CRE-lacZ construct were treated with 50 ng/ml EGF, 40 mM KCl, 50 ng/ml EGF and 40 mM KCl, or 10 µM forskolin for 1 h under Ca²⁺-free conditions then assayed for

played significant neurite outgrowth. Stimulation of neurite outgrowth by EGF and dibutyryl-cAMP or EGF and forskolin were both depressed in the PKA-deficient PC12 cells.

EGF-and KCl-induced Neurite Outgrowth Is Dependent on Transcription and Translation

The initiation of neurite outgrowth by intracellular cAMP, which may involve rapid reorganization of the cytoskeleton (Puck, 1977; Sloboda et al., 1975), is independent of transcription (Gunning et al., 1981). In contrast, NGF induced neuronal differentiation in PC12 cells is dependent on transcription (Burstein and Greene, 1978). Therefore, we examined the effect of inhibitors of transcription and translation on neurite extension stimulated by EGF and cAMP. Because neurite outgrowth was measured three days after treatment with EGF and other agents, the concentrations of cycloheximide and actinomycin D used were limited to 1.0 and 0.05 µg/ml, respectively. Higher concentrations of these drugs were toxic to the cells during three days of treatment. In agreement with previous studies, neurite outgrowth stimulated by 1 mM dibutyryl-cAMP was not affected by cycloheximide or actinomycin D (Fig. 9 *B*). Stimulation of neurite extension by NGF, combinations of EGF and KCl, or EGF and dibutyryl cAMP were sensitive to cycloheximide or actinomycin D (Fig. 9 *B*). Although complete inhibition of neurite outgrowth by cycloheximide or actinomycin D was not obtained, this may be due to the relatively low concentrations of these agents required for the experiments.
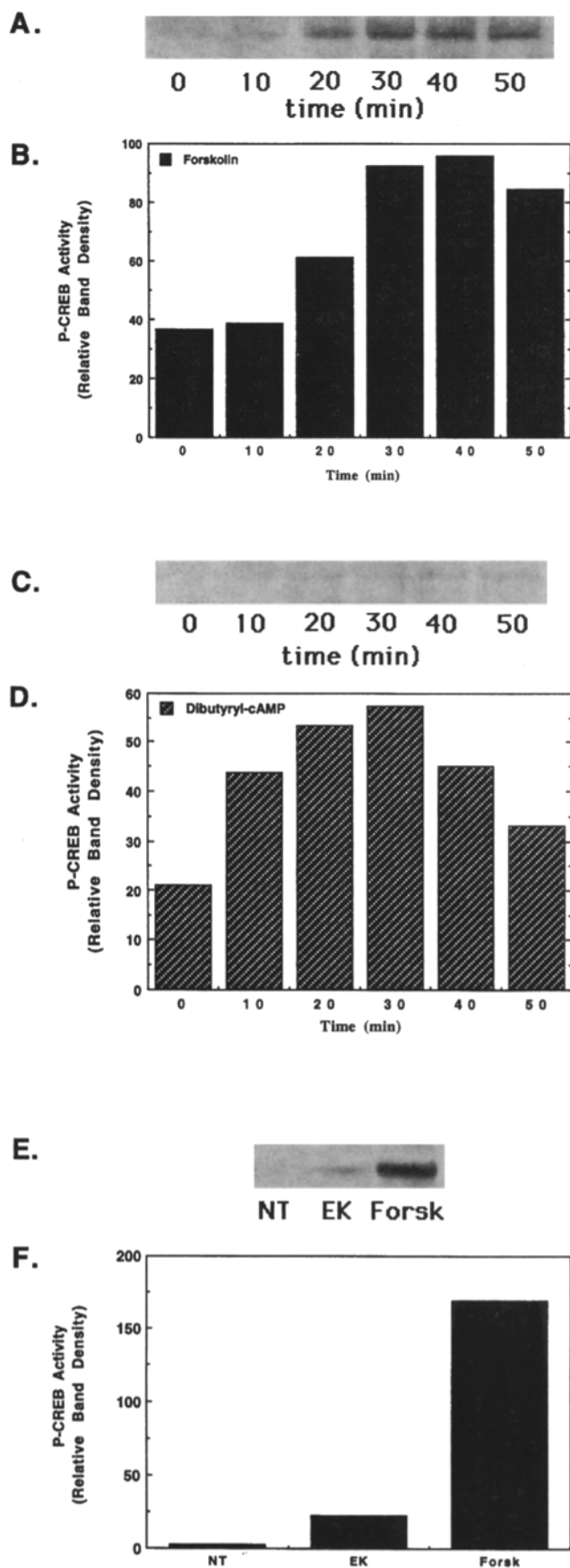
Given the small increases in cAMP stimulated by EGF and KCl it was also interesting to determine if CREB was phosphorylated under these conditions (Fig. 10). Using an antibody specific for CREB phosphorylated on Ser-133 (Ginty et al., 1993), Western analysis indicated that 50 µM dibutyryl-cAMP or 0.5 µM forskolin stimulated phosphorylation of CREB 2.7 fold and 2.5 fold after 30 min of exposure. Furthermore, EGF and KCl increased phosphorylation of CREB 2.5-fold after 10 min of exposure. Most importantly, EGF and KCl stimulated CRE-mediated transcription 2.5 ± 0.2-fold and this response was completely inhibited by H89 (Fig. 9 *C*). These data indicate that the low levels of cAMP generated by KCl and EGF were sufficient to activate CRE-mediated transcription mediated by PKA phosphorylation of CREB.

Discussion

Growth factor induced activation of MAP kinase is necessary for PC12 cell differentiation (Cowley et al., 1994), and transfection with constitutively activated ras or raf stimulates neurite outgrowth (Thomas et al., 1992; Wood et al., 1992, 1993; Noda et al., 1985). Although EGF and NGF both cause a rapid increase in MAP kinase activity in PC12 cells, NGF but not EGF stimulates neurite extension. This difference may be due to additional pathways stimulated by NGF (Chao et al., 1992) or to a more pro-

CRE-mediated transcription as described in the Materials and Methods. In some cases, cells were pretreated with 20 µM H89 for 30 min prior to treatments.

A.

0   10   20   30   40   50
time (min)

B.

■ Forskolin

P-CREB Activity
(Relative Band Density)

100
80
60
40
20
0

0   10   20   30   40   50
Time (min)

C.

0   10   20   30   40   50
time (min)

D.

▨ Dibutyryl-cAMP

P-CREB Activity
(Relative Band Density)

60
50
40
30
20
10
0

0   10   20   30   40   50
Time (min)

E.

NT   EK   Forsk

F.

P-CREB Activity
(Relative Band Density)

200
150
100
50
0

NT   EK   Forsk

longed activation of MAP kinase activity by NGF (Gotoh et al., 1990; Traverse et al., 1992). It was our goal to determine if EGF will stimulate neurite outgrowth when paired with additional signal transduction pathways. The data in this study indicate that EGF and KCl depolarization synergistically stimulate neurite outgrowth as a result of simultaneous activation of the cAMP and MAP kinase signal transduction pathways.

Since KCl depolarization elevates intracellular Ca^{2+} and increases MAP kinase activity (Rosen et al., 1994), neurite outgrowth stimulated by EGF and KCl might be due to enhancement of MAP kinase over that seen with EGF. However, enhancement of EGF-stimulated MAP kinase activity by KCl was blocked by nifedipine whereas neurite outgrowth stimulated by EGF and KCl was not inhibited by nifedipine or excess EGTA. Consequently, increases in intracellular Ca^{2+}, or stimulation of MAP kinase activity over that normally produced by EGF cannot explain neurite outgrowth generated by KCl and EGF.

There is increasing evidence that EGF receptors may couple to activation of adenylyl cyclases via G_s-protein as well as the MAP kinase signal transduction cascade (Knecht and Catt, 1983; Ran et al., 1986; Bravo et al., 1987; Ball et al., 1990; Budnik and Mukhopadhyay, 1991, 1993). Although EGF alone did not increase intracellular cAMP in PC12 cells, it did raise intracellular cAMP when paired with KCl depolarization and this increase was Ca^{2+} independent. Other agents which elevate intracellular cAMP including forskolin, pertussis toxin, and dibutyryl cAMP also synergistically stimulated neurite outgrowth with EGF. Furthermore, EGF and KCl stimulation of neurite outgrowth was depressed in PC12 cells deficient in cAMP-dependent protein kinase.

A quantitative analysis of the cAMP concentrations required for this phenomenon indicated that relatively small cAMP increases (80% over basal) synergistically stimulated neurite outgrowth with EGF. The levels of cAMP generated by EGF and KCl were, by themselves, not suffi-

Figure 10. EGF and KCl-induced phosphorylation of CREB at Ser-133 in PC12 cells. (*A*) Immunoblot analysis of CREB phosphorylation at Ser-133 following .5 μM forskolin treatment for 0–50 min. (*B*) The relative band densities of phospho-CREB following .5 μM forskolin treatment were measured by densitometry. (*C*) Immunoblot analysis of CREB phosphorylation at Ser-133 following .05 mM dibutyryl-cAMP treatment for 0–50 min. (*D*) The relative band densities of phospho-CREB following .05 mM dibutyryl-cAMP treatment were measured by densitometry. (*E*) Immunoblot analysis of EGF- and KCl-induced CREB phosphorylation at Ser-133 in PC12 cells. PC12 cells were left untreated (*NT*) or treated with 50 ng/ml EGF and 40 mM KCl (*EK*) or 100 μM forskolin (*Forsk*) for 10 min. (*F*) The relative band densities of phospho-CREB following 50 ng/ml EGF and 40 mM KCl (*EK*), 100 μM forskolin (*Forsk*), or untreated (*NT*) treatments were measured by densitometry. PC12 cells were treated with various effectors, lysed in SDS sample buffer and boiled. Cell extracts were separated by SDS-PAGE and transferred to nitrocellulose membrane, and blots were incubated with anti-phospho-CREB antibodies as described in the Materials and Methods. Immunoblots were developed with a secondary antibody conjugated to alkaline phosphatase. The relative density of phospho-CREB bands were quantitated with a Bio-Rad GS-670 densitometer.

cient to promote neurite outgrowth and only stimulated neurite outgrowth with EGF. Synergistic increases in MAP kinase kinase activity by cAMP and NGF have been directly correlated to enhanced neurite outgrowth produced by NGF and cAMP in PC12 cells (Frodin et al., 1994). However, the phenomenon described in our study was not due to cAMP enhancement of MAP kinase activity. The small increases in cAMP caused by EGF and KCl were not sufficient to enhance MAP kinase activity (data not shown).

There are several mechanisms that might account for synergistic stimulation of neurite outgrowth by cAMP and EGF. Over-expression of EGF receptors in PC12 cells leads to prolonged MAP kinase activity and allows them to respond to EGF by producing neurites (Traverse et al., 1994). One possible explanation for EGF and KCl stimulated neurite outgrowth is an increase in EGF receptor number. However, under Ca^{2+}-free conditions, KCl did not stimulate an enhancement in MAP kinase activity over that seen with EGF alone (Fig. 5 C). Therefore, it seems unlikely that the phenomenon described in this study was due to changes in EGF receptor number.

High levels of cAMP alone will stimulate neurite outgrowth, possibly by phosphorylation of cytoskeletal proteins and rearrangement of the cytoskeleton (Sloboda, 1975; Puck, 1977; Gunning et al., 1981). Neurites stimulated by EGF and cAMP persisted for at least 13 d whereas processes initiated by cAMP alone were transient and retract after several days. In contrast to process outgrowth stimulated by cAMP alone, EGF and cAMP stimulation of neurite outgrowth was sensitive to inhibitors of transcription and protein synthesis, as is NGF-stimulated neurite outgrowth. Therefore, the transcription of one or more genes required for neurite outgrowth may be synergistically regulated by the cAMP and MAP kinase pathways.

EGF receptors also activate Jak1 and at least two stat proteins, Stat1 and Stat3, which mediate transcriptional responses through interactions with the SIE element (Fu and Zhang, 1993). However, our data with dominant–negative MAP kinase mutants indicated that MAP kinase activity is required for the cooperative phenomenon described in this study. Rapamycin, an inhibitor of p70 S6 kinase (Kuo et al., 1992) did not inhibit neurite outgrowth stimulated by EGF and KCl indicating that this pathway did not contribute to this phenomenon (data not shown). Our data is most consistent with mechanisms involving cross-talk between the MAP kinase and cAMP pathways. For example, the c-fos promoter has both cAMP (Verma and Sassone-Corsi, 1987) as well as serum response elements (Treisman, 1986; Greenberg et al., 1987) and c-fos expression in fibroblasts is synergistically regulated by EGF and cAMP (Ran et al., 1986). This mechanism is not limited to immediate early genes and expression of late response genes crucial for neurite growth may also be subject to dual regulation by the cAMP and MAP kinase pathways. For example, NGF stimulated expression of transin, a late gene product whose expression correlates with neurite outgrowth, is enhanced by cAMP (Machida et al., 1991). Alternatively, cAMP may be synergistic with EGF because of its anti-proliferative activity. When PC12 cells are treated with NGF they stop dividing and differen-

tiate with process outgrowth. The correlation between cessation of proliferation and morphological differentiation suggests that the antimitotic activity of NGF may be required for neurite outgrowth (Burstein and Greene, 1978). In fact, NGF may function as a progression factor with both mitogenic and antimitogenic activity that allows cells to reach G1 before they differentiate (Rudkin et al., 1989). EGF is a mitogen for PC12 cells (Huff et al., 1981) but cAMP is antimitogenic (Huffaker et al., 1984) and inhibits DNA synthesis (Bothwell et al., 1980). Therefore, cAMP may synergize with EGF by inhibiting or retarding cell proliferation and allowing differentiation to occur. Regardless of the mechanism, the phenomenon described in this study is a novel method for regulation of neurite outgrowth which is distinguishable from other mechanisms documented in the literature.

In summary, our data indicate that cross-talk between the MAP kinase and cAMP signal transduction system may be sufficient to stimulate neurite outgrowth. To the extent that PC12 cells are a reasonable model system for neurons, these observations have important implications for control of neurite outgrowth in neurons. Suboptimal levels of MAP kinase stimulation by various effectors including activity-dependent depolarization may synergistically stimulate neurite outgrowth when paired with low level cAMP signals generated by neurotransmitter activation of adenylyl cyclases.

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