Cyclic AMP Activates the Mitogen-activated Protein Kinase Cascade in PC12 Cells

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Mitogen-activated protein (MAP) kinases are activated in response to a large variety of extracellular signals, including growth factors, hormones, and neurotransmitters, which activate distinct intracellular signaling pathways. Their activation by the cAMP-dependent pathway, however, has not been reported. In rat pheochromocytoma PC12 cells, we demonstrate here a stimulation of the MAP kinase isozyme extracellular signal-regulated kinase 1 (ERK1) following elevation of intracellular CAMP after exposure of the cells to isobutylmethylxanthine, forskolin, or cAMP-analogues. cAMP acted synergistically with phorbol ester, an activator of protein kinase C, in the stimulation of ERK1. In accordance with this observation, the peptide neurotransmitter pituitary adenylate cyclase-activating polypeptide 38 (PACAP38), which stimulates an immediate upstream activator of ERK1 in the MAP kinase cascade. Supporting this view, forskolin and a CAMP analog were found to increase the activity of MAP kinase in PC12 cells, as well as in combination with phorbol ester. PACAP38 also stimulated in vivo [32P]-labeling of ERK1 and MAP kinase activity. Finally, cAMP or PACAP38 increased by 3-fold nerve growth factor-stimulated neurite formation in PC12 cells, which may be correlated with the potentiating effect of these agents on nerve growth factor-stimulated ERK1 activity.

In mammalian cells the ability to activate the mitogen-activated protein (MAP) kinase cascade is a feature common to many extracellular stimuli including growth factors, hormones, and neurotransmitters. The various stimuli which can activate the MAP kinase cascade employ distinct initial signaling pathways. Some stimuli activate receptor tyrosine kinases (3-5), non-receptor tyrosine kinases (6-8). Other stimuli activate G protein-coupled receptors generating the second messengers diacylglycerol or calcium, or activating ion-channels (9-12). Stimuli generating the prominent second messenger cAMP, however, have not been reported to activate the MAP kinase cascade.

In the case of the receptor tyrosine kinases, a sequence of events leading to MAP kinase activation is emerging. Activated receptor tyrosine kinases increase GTP-binding to c-Ras, through guanine nucleotide exchange factors and adaptor proteins, leading to its activation (13, 14). Activated c-Ras may activate c-Raf kinase by direct binding (15-17). c-Raf can phosphorylate and activate MAP kinase (18-21), which in turn activates MAP kinase (22, 23). How other signaling pathways couple to the cascade is less understood. In some cell types, certain G protein-stimulated pathways and protein kinase C-dependent pathways may also activate the cascade through c-Ras/c-Raf (12, 24, 25), while in other cell types, G protein-stimulated pathways may be predominantly Ras independent and employ MAP kinase kinases distinct from c-Raf (26-29).

The MAP kinase cascade is likely to serve specific functions in different cell types. In rat pheochromocytoma PC12 cells, nerve growth factor (NGF) induces a very robust and sustained activation of the MAP kinase cascade (30-33). Since NGF induces neuronal differentiation in PC12 cells, the cascade has been proposed to mediate neurotrophic signals leading to a neuronal phenotype or other neurotrophic responses in this cell line (30-33). In agreement with this model, the transfection of PC12 cells with constitutively active, oncogenic Ras or Raf mimics NGF action by inducing activation of the MAP kinase cascade and neuronal differentiation of PC12 cells, respectively (24, 25, 34, 35).

Elevation of intracellular CAMP, alone or in combination with NGF, has also been reported to promote neuronal differentiation of PC12 cells (36-39). A recently identified neuropeptide, pituitary adenylate cyclase-activating polypeptide 38 (PACAP38), which generates cAMP and stimulates phosphatidylinositol breakdown, has been shown to promote neurite outgrowth in PC12 cells (40). These observations prompted us to investigate the possibility that cAMP might stimulate the MAP kinase cascade in these cells.

We demonstrate that elevation of the intracellular cAMP stimulates the MAP kinase isozyme, extracellular signal-regulated kinase 1 (ERK1), as well as MAP kinase in PC12 cells. cAMP acts synergistically with phorbol ester, and thus synergizes with PACAP38, 38-amino acid form: PMA, phorbol 12-myristate 13-acetate; PAGE, polyacrylamide gel electrophoresis.
protein kinase C. This interaction may serve as a model for signal integration at the level of the MAP kinase cascade. Such an interaction may be used by different ligands that stimulate cAMP generation or phosphatidylinositol breakdown, respectively, or ligands which stimulate both pathways, like PACAP38. Finally, cAMP increased ERK1 activation as well as neurite outgrowth in response to NGF in PC12 cells.

Most importantly, our observations describe a novel regulation of the MAP kinase cascade, i.e. stimulation by intracellular cAMP. In addition, these data may provide new insight into the neurotrophic effects of cAMP, by trypsinization. Cells were seeded in 12-well dishes at 1 x 10^5 cells/cm^2 and cultured for 1 day before they were shifted to medium containing 0.5% serum and 500 ng/ml MBP or recombinant ERK1 were omitted from the phosphorylation reaction. The phosphorylation of the recombinant ERK1 or [-p32^P]ATP adsorbed to MBP, respectively. More data were not corrected for these minor contributions.

**Materials**—The keyhole limpet hemocyanin-coupled 12-amino-acid carboxyl-terminal of ERK1 (356-367) (TARFQPGAPEAP), synthesized by Neo System (Strasbourg, France) was used to generate rabbit polyclonal antisera (43). For the generation of polyclonal rabbit antisera to MAP kinase kinase, the 17-amino-acid amino-terminal (PKKKPTPLIQL-DW-KY-VWLPAAAR) of CREB was used as an antigen. Sodium orthovanadate, BSA, leupeptin, phenylmethylsulfonyl fluoride, MBP from bovine brain, protein A-Sepharose, Triton X-100, 8-(4-chlorophenylthio)-cAMP, forskolin, cholera toxin, N-(n,N',O'-dibutyryl)-cAMP, NPY, CPT-cAMP, and sodium vanadate were purchased from Sigma. The final experimental concentration of dimethyl sulphoxide, used as a carrier for PM and forskolin, was 0.04% which had no effect on ERK1 activity in itself. Ovine PACAP38 (identical to rat PACAP38) from Peninsula Laboratories, Inc, was in part a generous gift from Prof. J. Fahrenkrug, Bispebjerg Hospital, Copenhagen, Denmark. Mouse 75 NAG was purified and generated as provided to us by Dr P. Kitabgi (Nice-Sophia Antipolis, France).

**RESULTS**

**CAMP Activates the MAP Kinase Cascade in PC12 Cells**

ERK1 activity was then measured using MBP as a substrate. Briefly, pellets with immunoprecipitated MAPKK were washed twice in 50 mM HEPES, pH 7.4, dried, and resuspended in 50 /mu l of 50 mM HEPES, pH 7.4, containing bacterially expressed, recombinant ERK1 and 0.2 mM NaVO_4, 100 units/ml aprotinin, 20 mu g leupeptin, and 0.2 mg/ml phenylmethylsulfonyl fluoride. The phosphorylation cascade was started by the addition of [-p32^P]ATP (50 /mu M, 50 Ci/mmol), 150 /mu M MBP, 15 mM magnesium chloride, 1 mM EDTA added as a 6-fold concentrated mixture to a volume of 10 /mu l. The phosphorylation reaction was allowed to proceed for 10 min at room temperature and was stopped by spotting Whatman P-81 filter papers which were then dropped into 0.1% (v/v) orthophosphoric acid. The papers were washed overnight with several shifts in this solution, rinsed once in ethanol, air dried, and radioactivity was determined by Cerenkov-counting. The reaction blank, which received identical treatment, was a mixture containing all of the reagents except the cell lysate during immunoprecipitation, and was subtracted from all values. Control experiments showed that, when MBP or recombinant ERK1 were omitted from the phosphorylation reaction with immunopurified MAPKK from unstimulated as well as stimulated cells, radioactivity associated with the papers dropped by approximately 90%. The remaining 10% may derive from auto-phosphorylation of the recombinant ERK1 or [-p32^P]ATP adsorbed to MBP, respectively. More data were not corrected for these minor contributions.

**CAMP Stimulates ERK1 in PC12 Cells**—We first measured ERK1 activity in PC12 cells treated with a variety of agents which share the ability to raise the intracellular level of cAMP or being themselves a CAMP analogue. After stimulation of ERK1 in PC12 cells, the cell monolayers were solubilized. ERK1 was immunopurified from the cell lysates, and its activity was measured in vitro using myelin basic protein as a substrate. The various agents, shown in Fig. 1, were found to increase the activity of ERK1 from 2- to 12-fold above the level seen in unstimulated cells (Fig. 1). Maximal activation occurred rapidly, within 5-25 min depending on the agent, except for cholera toxin, which may be due to the fact that it raises intracellular CAMP only slowly. The agents increase CAMP in widely differing ways. IBMX increases CAMP levels by inhibiting its degradation by CAMP phosphodiesterase (46). Cholera toxin activates the G protein Gs through ADP-ribosylation of its a subunit, which subsequently stimulates adenylyl cyclase (47). Forskolin increases intracellular CAMP by direct binding and activation of adenylyl cyclase (46). Dibutyryl-cAMP (dbcAMP) permeates the cell membrane and is metabolized in the cell to generate the active CAMP analogue (49). Cyclic AMP (CPT-CAMP) is a membrane-permeant CAMP analogue (50). The second messenger CGMP is structurally related.
to cAMP and elicits some cellular responses equipotently to cAMP (51). However, cGMP, when added as diisopropyl-cGMP, had no effect on ERK1 activity, indicating that the cAMP effect is not due to cross-reaction with a cGMP-dependent signaling pathway (data not shown). The stimulatory effect of CAMP on ERK1 is not limited to the PC12 cell subclone used in this study, since we observed similar effects of cAMP in two other PC12 subclones (data not shown).

Importantly, the five agents used in Fig. 1 increase intracellular cAMP each in their own distinct way. Their sole common denominator is the ability to elevate intracellular cAMP. We therefore conclude that cAMP can activate ERK1 in PC12 cells.

CAMP Acts Synergistically with Phorbol Ester to Stimulate ERK1 in PC12 Cells—Although CAMP stimulates ERK1, it has a rather modest effect compared with certain other activators in PC12 cells, e.g. phorbol ester (PMA), an activator of protein kinase C (52). CAMP, however, was found to greatly amplify the stimulatory effect of PMA on ERK1 in PC12 cells. Fig. 2A shows the time course of ERK1 activation by CPT-cAMP and PMA, both used at maximally stimulating concentrations. At each time point tested, the effect of PMA was potentiated by a factor of 3 to 4 by the presence of CPT-cAMP.

IBMX, cholera toxin, forskolin, and dibutyryl-cAMP (but not diisopropyl-GMP, not shown) were also found to potentiate the effect of PMA on ERK1, indicating that the potentiation was indeed due to an increase in intracellular cAMP (Fig. 2B).

Fig. 1. Stimulating effect of agents raising intracellular cAMP on the activity of ERK1 in PC12 cells. Serum-starved PC12 cells were incubated with the various agents at the following concentrations and for the following periods of time: IBMX: 1 mM, 10 min; cholera toxin: 500 ng/ml, 30 min; forskolin (FOR): 10 μM, 15 min; dib-cAMP: 1 mM, 20 min; CPT-cAMP: 1 μM, 20 min. At the end of the incubation period, cells were solubilized. ERK1 was immunoprecipitated from cell extracts, and its kinase activity was measured using exogenous MBP as substrate as described under "Experimental Procedures." ERK1 activity is expressed as fold stimulation of the basal level of unstimulated PC12 cells (buffer condition). Data are means ± S.D. of three to six experiments performed in triplicate.

ERK2, another MAP kinase isoenzyme expressed in PC12 cells (5), appeared to be regulated by cAMP in much the same way as ERK1. In experiments performed like the ones shown in Fig. 2A, but instead using antibodies directed against the COOH terminus of ERK2, we found that CAMP by itself stimulated ERK2 activity only slightly but potentiated severalfold the effect of PMA (data not shown).

Finally, we tested a neuropeptide, PACAP38, which stimulates cAMP synthesis as well as phosphatidylinositol breakdown in PC12 cells (40, 53). PACAP38 was found to be an efficient activator of ERK1, showing a rapid, but transient, activity peak, reached within 5 min (lower curves in Fig. 8). Half-maximal and maximal stimulation of ERK1 by PACAP38 were observed at approximately 25 and 200 nM, respectively (data not shown). These values are consistent with the binding affinity of the PACAP type 1 receptor and the biological potency of PACAP38 in PC12 cells and chromaffin cells (40, 53–55) suggesting receptor specificity and physiological relevance of ERK1 stimulation by PACAP38. PACAP38 stimulation of ERK1 was inhibited by 40% following protein kinase C downregulation, through overnight preincubation with PMA (data not shown), suggesting protein kinase C involvement in the PACAP38 response. Forskolin acted non-additively with PACAP38 on ERK1 activation (data not shown), indicating cAMP involvement in the PACAP38 response. Taken together, the observations with PACAP38 suggest that the synergistic stimulation of ERK1 by cAMP and activators of protein kinase C, shown in Fig. 2, may occur in response to a physiological stimulus in PC12 cells.

CAMP Acts Synergistically with Receptor Tyrosine Kinases on
ERK1 Activation in PC12 Cells—In PC12 cells, peptide growth factors like NGF or epidermal growth factor (EGF) are among the strongest activators of ERK1 (30–33) capable of increasing ERK1 activity more than 100-fold in our assay system. In contrast, we found that insulin-like growth factor I (IGF-I) by itself has a weak effect in PC12 cells. However, a strong synergistic activation of ERK1 was observed by CPT-cAMP or forskolin in the presence of a maximal concentration of IGF-I (Fig. 3). In combination with the strongly stimulating growth factors, CPT-cAMP or forskolin were also found to potentiate ERK1 activation in a more additive manner, as shown with NGF, used at a maximally stimulating concentration (see Fig. 9). In combination with EGF, however, a potentiation of EPK was most clearly observed, when EGF was used at a half-maximal concentration (data not shown).

These observations indicate that in PC12 cells cAMP also potentiates activation of ERK1 by growth factors, which initiate their signaling through tyrosine kinase receptors.

cAMP Increases ERK1 Phosphorylation in Intact PC12 Cells—The activity of ERK1 is known to be stimulated upon phosphorylation. We therefore investigated whether elevation of intracellular cAMP increased the level of ERK1 phosphorylation. Cells were metabolically labeled with [32P]orthophosphate and exposed to various agonists. Thereafter, cells were solubilized, and cell extracts were subjected to immunoprecipitation with antibodies to ERK1. The immunoprecipitates were analyzed by SDS-PAGE followed by autoradiography.

We found that both CPT-cAMP and forskolin increased [32P]phosphate incorporation in a 44-kDa protein (Fig. 4, left and right panels, respectively). This protein has previously been identified as ERK1 (33, 43, 56). PMA also increased ERK1 phosphorylation. However, when PMA was added in combination with CPT-cAMP or forskolin, the level of ERK1 phosphorylation was markedly higher than with either agent alone. Phosphorylation of ERK1 in response to NGF was also increased by CPT-cAMP. Finally, PACAP38 increased ERK1 phosphorylation to a high level after 5 min of stimulation, which had returned to near-basal levels within 45 min.

The labeling of an additional phosphoprotein, having a Mr of approximately 90,000, was increased by stimulation with CPTcAMP and PMA, NGF, or PACAP38 at 5 min. We have previously shown that this protein corresponds to the pp90rsk protein kinase, which immunoprecipitates with ERK1 (33, 56) and which is a presumed physiological substrate of ERK1 (4, 57).

In summary, the increase in the phosphorylation of ERK1 in response to cAMP (alone or in combination with other stimuli) paralleled the stimulation of its kinase activity observed in
Fig. 4. *In vivo*[^32]P phosphorylation of ERK1. PC12 cells, metabolically labeled with[^32]Porthophosphate, were incubated with agonists (200 nM PACAP38, 1 mM CPT-cAMP (CPT), 10 μM forskolin (For), 2 μM PMA, 50 ng/ml NGF) for the period of time and in combination as indicated. Following solubilization ERK1 was immunoprecipitated from the cell extracts, dissolved in Laemmli buffer, and subjected to SDS-PAGE. We show an autoradiograph of the dried gel from two representative experiments (left and right panels, respectively) out of several experiments with similar results.

played a similar, dense network of neurites. The effect of cAMP or PACAP38 may thus be characterized as an acceleration of the NGF-induced neuronal differentiation process. As PACAP38 has the potential to stimulate also protein kinase C, we tested the effect of PMA in combination with forskolin or NGF (Fig. 7). PMA led to only a small potentiation of NGF-induced neurite outgrowth during the first 24 h, but thereafter did not potentiate the neuronal differentiation of PC12 cells. Prolonged exposure of cells to PMA, however, is known to downregulate protein kinase C, which may explain the lack of any detectable effects in this long term experiment.

NGF is known to induce a robust and prolonged activation of ERK1 in PC12 cells as previously described (30–33) and shown in Fig. 8. Interestingly, PACAP38, while having a transient effect itself, increased the sustained activation of ERK1 by NGF in a more than additive manner. This potentiation was seen with a half-maximally as well as a maximally stimulating concentration of NGF (Fig. 8, A and B, respectively). Forskolin, CPT-cAMP, and PMA also increased NGF-stimulated ERK1 activity during the sustained secondary phase of the NGF-response as measured after 1 h of stimulation (Fig. 9). At this time point, all of these agents had very little stimulatory effect on ERK1 activity in the absence of NGF.

In summary, these data suggest that there may be a causal relationship between the potentiating effect of cAMP (and PACAP38) on NGF-induced ERK1 activation and NGF-induced neurite outgrowth.

**DISCUSSION**

In the present study we describe the activation and phosphorylation of ERK1 in response to several agents known to increase the intracellular level of cAMP. Since these agents act at distinct levels in the pathway of cAMP generation or degradation we conclude that the second messenger cAMP can stimulate ERK1 in PC12 cells. In mammalian cells, the vast majority of cAMP effects can be attributed to activation of the cAMP-dependent protein kinase. This may also be the case for cAMP stimulation of ERK1 in PC12 cells. However, protein kinase A-independent cAMP responses have been described. For instance, the inhibitory effect of cAMP on glucose transport is thought to be mediated by direct binding of the nucleotide to the glut 4 transporter molecule (58). Further, cardiac pace-
cAMP Activates the MAP Kinase Cascade in PC12 Cells

FIG. 6. PACAP38 activates MAP kinase kinase in PC12 cells. Serum-starved PC12 cells were incubated for 5 or 45 min with PACAP38 (200 nM). At the end of the incubation period, cells were solubilized, MAP kinase kinase was immunopurified from the cell extracts, and its activity was measured as described in Fig. 5 and under "Experimental procedures." Phosphorylation of MBP by ERK1, activated by MAP kinase kinase immunopurified from stimulated PC12 cells, is expressed as percent over MBP phosphorylation by ERK1 incubated with MAP kinase kinase immunopurified from unstimulated PC12 cells (basal). Data are means ± S.D. of three experiments performed in triplicate.

FIG. 7. Effect of cAMP, PACAP38, or PMA on NGF-stimulated neurite formation in PC12 cells. PC12 cells were cultured in 0.5% serum in the presence of NGF (50 ng/ml), forskolin (10 μM), CPT-CAMP (1 mM), PACAP38 (200 nM), or PMA (2 μM) in combination as indicated. After 1, 2, and 3 days, the fraction of neurite bearing PC12 cells was determined and expressed as a percentage of total cells. Data are mean ± S.D. of three to six experiments performed in duplicate.

 maker ion channels (59), ion channels in olfactory receptor cilia (51), and potassium/calcium-specific Drosophila eog channels (60) have been reported to be activated directly by cAMP. PC12 cells are neuroendocrine cells in which several biological responses to extracellular stimuli are elicited via ion channel activation. A mechanism for ERK1 regulation involving the activation of ion channels by cAMP may therefore be a possibility.

cAMP increased the in vivo phosphorylation of ERK1 as well as the activity of MAPKK in PC12 cells. It therefore appears that cAMP stimulates ERK1 through activation of the MAP kinase cascade operating at the level of MAPKK or upstream of it. The synergistic interaction observed between cAMP and PMA on ERK1 was reflected in MAPKK activation, suggesting that this interaction also occurred upstream in the MAP kinase cascade, again being at least at the level of MAPKK. The MAP kinase cascade seems to utilize several activators upstream of MAPKK including Raf-1 kinase, MAPKK kinases, and possibly other functional equivalents, perhaps in a cell type-specific or pathway-specific manner (27, 29). We found a very pronounced electrophoretic mobility shift of Raf-1 kinase in response to NGF, which is indicative of its activation, as described previously in PC12 cells (25, 61). We observed no such mobility shift of Raf-1 in response to CPT-cAMP or forskolin (data not shown). While we cannot exclude that cAMP is activating Raf-1 without causing a concomitant mobility shift, our data would indicate that cAMP activates MAPKK through a MAPKK kinase, or a functional equivalent, which is distinct from Raf-1 kinase. We are currently investigating whether the other members of the Raf-family, A-Raf and B-Raf, could be involved.

Our observations may have several biological implications. An interesting feature of the cAMP stimulation of ERK1 is its
synergistic interaction with PMA, which presumably reflects synergy with the protein kinase C-dependent pathway. This finding opens the possibility that the MAP kinase cascade may integrate and amplify signals originating from receptors employing cAMP and receptors using phosphatidyl inositol breakdown products as second messengers, respectively. By extension, receptors activating both pathways, may equally well employ this mechanism. With PACAP38 we found a time course and efficiency of activation/phosphorylation of ERK1, MAPKK, and pp90RSK, which were overall similar to that observed with cAMP in combination with PMA. It is reasonable to believe that PACAP38 employs the synergistic interaction between cAMP and phosphatidyl inositol-derived second messengers to activate the MAP kinase cascade in PC12 cells. With the exception of the early events, which are cAMP synthesis, phosphatidylinositol breakdown, and increases in the intracellular calcium, nothing is known about PACAP38 signal generation. The present findings, showing the activation/phosphorylation of MAPKK, ERK1 as well as pp90RSK, make these kinases putative transducers of PACAP38-stimulated signaling. These observations may be relevant for the understanding of the endocrine function of chromaffin cells, as recent evidence strongly suggests that in the adrenal medulla PACAP38 functions as a peptide neurotransmitter to induce catecholamine secretion from the chromaffin cells (55, 62). Rapid and transient activations may be relevant for the understanding of the endocrine peptide neurotransmitter to induce catecholamine secretion of the early events, which are CAMP synthesis, phosphatidylinositol-derived second messengers to activate the kinase cascade in PC12 cells. With the exception

**Addendum**—During the reviewing process of our manuscript, three papers were published demonstrating that, in contrast to the stimulatory action of cAMP in PC12 cells shown in this work, increasing intracellular calcium concentrations can antagonize the stimulatory action of CAMP in PC12 cells shown in this work, increasing intra-cellular calcium.

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cAMP Activates the MAP Kinase Cascade in PC12 Cells

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