Cyclic AMP-elevating agents induce the expression of MAP kinase phosphatase-1 in PC12 cells

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Abstract Stimulation of pheochromocytoma PC12 cells by cAMP-elevating agents caused the induction of the immediate early gene *3CH134*, which encodes MAP kinase phosphatase-1 (MKP-1). Forskolin was as potent as serum in stimulating *MKP-1* gene expression, whereas dibutyryl-cAMP and neuropeptide PACAP were less effective. Induction of the *MKP-1* gene was accompanied by neo-synthesis of MKP-1 protein. MAP kinase activation was not involved in the cAMP-induced *MKP-1* gene expression. The MAP kinase inactivation, that would result from MKP-1 induction in response to increased intracellular cAMP level, contributes to explain how hormones or neurotransmitters signaling through cAMP influence cell growth and differentiation.

Key words: Cyclic AMP; Forskolin; Immediate early gene; Mitogen-activated protein kinase phosphatase; Pituitary adenylyl cyclase-activating polypeptide; PC12 cell

1. Introduction

Mitogen-activated protein (MAP) kinases mediate multiple pathways regulating cell growth and differentiation (reviewed in [1,2]). In neuronal cells, MAP kinase activity mediates the actions of growth factors like EGF that stimulate cell proliferation, and factors like NGF that maintain neuronal survival and differentiation. Activation of MAP kinase involves phosphorylation on threonine and tyrosine residues in a TxY motif, that is achieved by a sequential cascade of cytoplasmic protein kinases. Three types of MAP kinases have been described, depending their mode of activation and the nature of the x amino-acid of the TxY motif. The archetypal MAP kinases called extracellular signal-regulated kinases (ERK), ERK1 and ERK2, possess a TEY motif and are activated by the tumor promoter 12-*O*-tetradecanoyl-phorbol-13-acetate (TPA) and by mitogens like EGF. A second MAP kinase family comprises c-Jun N-terminal kinases (JNK) that are activated through phosphorylation on a TPY motif [3]. The third family consists of p38 MAPKs, that are activated through phosphorylation on a TGY motif [4]. JNKs and p38 kinases are activated by cellular stresses such as heat shock or UV irradiation, and by proinflammatory cytokines [2].

Activated MAP kinases of the TEY family have been reported to be specifically dephosphorylated on both T and Y residues by a dual specific protein phosphatase, MAP kinase phosphatase-1 (MKP-1) [5]. This phosphatase was identified as the product of the immediate early gene *3CH134* [6]. The importance of this dual protein phosphatase in cell biology is emphasized by the fact that overexpression of MKP-1 has been shown to block DNA synthesis and to slow cell division [7–9]. The MAP kinase phosphatase family is rapidly expanding (reviewed in [10–12]). It includes hVH-2, also cloned as MKP-2 or TYP-1, as well as hVH-3, hVH-5 and MKP-5, all encoded by immediate early genes. Whereas most of these phosphatases are nuclear proteins, two members of this subclass, MKP-3 (also known as PYST1 or hVH-6) and MKP-4 are localized primarily within the cytosol.

The mechanisms by which *MKP-1* mRNA is synthesized in response to extracellular stimuli is presently a matter of controversy. An early report showed that stimulation of JNK- but not of ERK-induced *MKP-1* gene expression in fibroblasts, suggesting that activation of JNK leads to inactivation of ERK as a result of MKP-1 neo-synthesis [13]. In fact, various reports indicate that transcriptional activation of *MKP-1* takes place through mechanisms involving one or several MAP kinases [10,12,14,15]. Other reports however suggest that this phosphatase can be induced by calcium [16], phosphatidylinositol 3-kinase [17,18], protein kinase C [19] or arachidonic acid [20]. We report here *MKP-1* early gene induction in response to cAMP-elevating agents in PC12 cells, without stimulation of MAP kinases.

2. Materials and methods

2.1. Measurement of DNA synthesis

Rat pheochromocytoma PC12 cells were cultured as described [21]. They were serum-starved for 15 h and then treated with the various agents together with 10 μM bromodeoxyuridine (BrdU). After 20 h of BrdU incorporation, cells were fixed with 4% paraformaldehyde and permeabilized with 0.2% Triton X-100. Cells were incubated for 2 h with a monoclonal anti-BrdU antibody (Janssen Biochimica) at a 1:50 dilution in PBS, 3% bovine serum albumin and after extensive washes in PBS, they were further incubated for 1 h with peroxidase-coupled anti-mouse IgG (Dako) at a 1:100 dilution in PBS/bovine serum albumin. Peroxidase activity was revealed with chloronaphtol.

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Abbreviations: MAP, mitogen-activated protein; ERK, extracellular signal-regulated kinase; TPA, 12-*O*-tetradecanoyl-phorbol-13-acetate; JNK, c-Jun N-terminal kinase; MKP-1, MAP kinase phosphatase-1; PKA, cAMP-dependent protein kinase; Bt_2cAMP, dibutyryl cAMP; Rp-8-Br-cAMPS, 8-bromoadenosine 3′,5′-monophosphorothioate; PACAP, pituitary adenylyl cyclase-activating polypeptide; BrdU, bromodeoxyuridine

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2.2. Northern blot and Western blot analysis

Total RNA was isolated from PC12 cells by the guanidinium isothiocyanate method [22], separated by electrophoresis and blotted onto Hybond N^+ membranes (Amersham). Specific *MKP-1* mRNA was detected as described previously [21] using a ^{32}P-labeled cDNA probe [5]. For quantitative analysis, densitometry was performed on autoradiograms with an image analyzer. MKP-1 protein was detected by Western blots performed as described [21], using anti-MKP-1 antibody (Santa Cruz Biotech., Santa Cruz, CA, USA).

2.3. JNK assay

Serum-starved PC12 cells were stimulated with various agents and lysed in 0.5 ml of lysis buffer (10 mM Tris–HCl buffer, pH 7.4, containing 150 mM NaCl, 1% Triton X-100, 1 mM EDTA, 1 mM EGTA, 0.2 mM sodium vanadate, 0.2 mM phenylmethylsulfonyl fluoride and 0.5% NP-40) for 30 min at 4°C. The lysates were incubated for 2 h at 4°C with 1 μg of anti-JNK2 (Santa Cruz Biotech., USA) and immunoprecipitation was accomplished by shaking for 1 h at 4°C with 50 μl of a 10% protein A suspension (Pansorbin). After extensive washing, JNK activity was assayed in the resuspended immunoprecipitation pellet. 30 μg protein were incubated at 30°C for 30 min in 40 μl total volume of 25 mM HEPES buffer, pH 7.5, containing 10 mM magnesium acetate, [γ-^{32}P]ATP (50 μM; 2 μCi), and 1 μg of glutathione *S*-transferase–c-Jun(1-79) (GST–c-Jun) fusion protein (Stratagene, USA). After heating to 95°C for 5 min, the samples were analyzed by SDS(10%)–PAGE.

2.4. In vivo MAP kinase activation

MAP kinase activation was assayed in vivo by following Elk-1 phosphorylation using the PathDetect reporting system (Stratagene). Cells were transfected with the several plasmids using the polyethylenimine technique [23]. The cells were then incubated for 16 h, washed and after another 24 h, they were treated with the various agents for 6 h. Luciferase was then extracted and its activity assayed by mixing 20 μl of cell extract with 100 μl of a reaction mixture containing 40 mM tricine (pH 7.8), 0.5 mM ATP, 0.5 mM EDTA, 10 mM $MgSO_4$, 10 mM dithiothreitol, 0.5 mM coenzyme A and 0.5 mM luciferin, and the emitted light was measured.

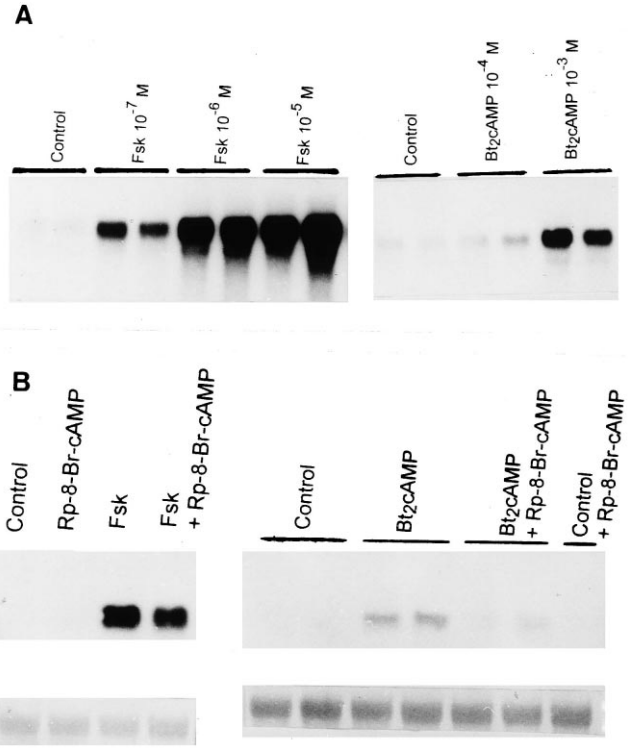

Fig. 2. Effect of various concentrations of forskolin and Bt_2cAMP (A), and of PKA inhibitor Rp-8-Br-cAMPS (B) on *MKP-1* mRNA expression. A: PC12 cells were treated with the indicated concentration of the agents for 30 min. B: Rp-8-Br-cAMPS (0.2 mM) was added 4 h before 1 μM forskolin (Fsk) or 1 mM Bt_2cAMP. Total RNA was analyzed as described in Section 2 and Fig. 1.

3. Results

3.1. Induction of the MKP-1 gene by cAMP elevating agents

The effect of various agents on *MKP-1* gene transcription was examined in serum-starved PC12 pheochromocytoma cells. Fig. 1A shows that addition of 20% serum highly induced gene induction, whereas NGF produced only a modest induction. Quantitative analysis indicated that NGF produced approximately 15% of the effect achieved by serum. Surprisingly, 10 μM of the adenylyl cyclase activator forskolin was found as potent as serum in inducing *MKP-1* gene transcription. *MKP-1* mRNA expression was already induced after 15 min incubation, and was highly enhanced at 30 min, in agreement with kinetics described for the expression of an immediate early gene. Increasing intracellular Ca^{2+} concentration by adding the Ca^{2+} ionophore A23187 (Fig. 1B) resulted in an induction of the *MKP-1* gene transcription to a level corresponding to 15% of the induction achieved by serum. An even weaker induction was caused by TPA (4% of the serum effect).

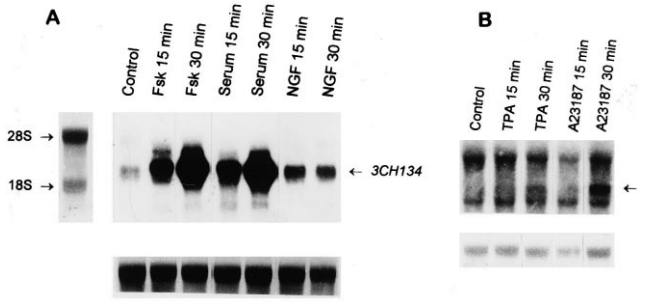

Fig. 1. Northern blot analysis of immediate early gene *MKP-1* induction by various agents in PC12 cells. Total RNA (20 μg/lane) was extracted from serum-starved PC12 cells incubated with (A) 10 μM forskolin (Fsk), 20% fetal calf serum, or 50 ng/ml NGF, and (B) 100 ng/ml TPA, or 10 μM of the Ca^{2+} ionophore A23187. The right side arrow indicates hybridization signal of *MKP-1*. The lower panel presents the 28S rRNA revealed by methylene-blue staining of the blot, showing equal loading of RNA. The figures are representative of three experiments.

Fig. 2A presents the dose-dependency of forskolin and dibutyryl cAMP (Bt_2cAMP) in inducing *MKP-1* gene transcription. Forskolin provoked gene induction at a concentration as low as 0.1 μM. Treatment of PC12 cells with 1 mM of the membrane permeant cAMP analogue Bt_2cAMP for 30 min resulted also in a significant increase in *MKP-1* mRNA, reaching 34% of the induction achieved by 1 μM forskolin (Fig. 2A). The addition of the selective cAMP-dependent protein kinase (PKA) inhibitor 8-bromoadenosine 3′,5′-monophosphorothioate (Rp-8-Br-cAMPS, Biolog, Germany) at 0.2 mM partially abolished the *MKP-1* gene transcription induced by the cAMP-elevating agents (Fig. 2B). It inhibited the effect of forskolin by 54% and that of Bt_2cAMP by 80%. Similar results were obtained with the less selective PKA inhibitor H89 (data not shown). These results show that forskolin and Bt_2cAMP produced their effect on gene transcription probably through the activation of PKA.

We next verified that the *MKP-1* gene could also be induced by hormones known to use cAMP as a second messen-

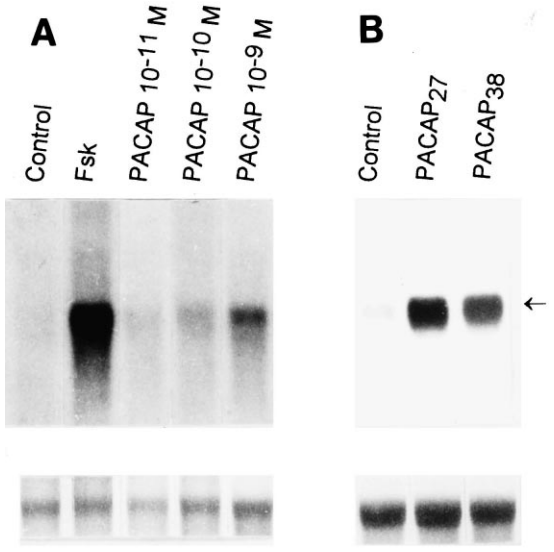

Fig. 3. Effect of PACAP peptides on *MKP-1* gene induction. Cells were treated for 30 min (A) with 10 μM forskolin (Fsk), or with the indicated concentration of PACAP27, and (B) with 1 nM of PACAP27 or PACAP38. Total RNA was analyzed as described in Section 2 and Fig. 1.

ger. Addition of the neuropeptide pituitary adenylyl cyclase-activating polypeptide (PACAP27) to PC12 cells resulted in an increase in *MKP-1* mRNA expression. However, 1 nM PACAP27 produced an induction that corresponded only to 18% of that produced by 1 μM forskolin (Fig. 3A). Fig. 3B shows that PACAP27 and PACAP38 produced a very similar induction of the *MKP-1* gene.

3.2. Synthesis of MKP-1 protein by forskolin

In order to verify that *MKP-1* gene induction resulted in the neo-synthesis of the corresponding protein, we measured the induction of the MKP-1 protein. Fig. 4 shows that both

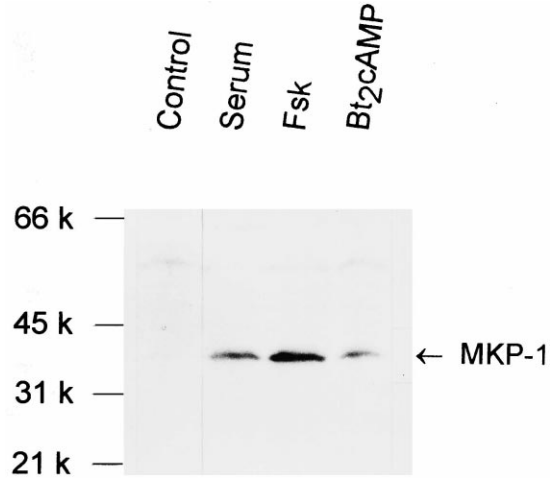

Fig. 4. Western blot analysis showing MKP-1 protein synthesis by cAMP-elevating agents. PC12 cells were incubated for 1 h with 20% fetal calf serum, 10 μM forskolin (Fsk), or 1 mM Bt_2cAMP. The amount of protein loaded in each lane was normalized, using the Bradford reagent technique. Immunoreactive signals were visualized by chemiluminescence. Apparent molecular weights of marker proteins are indicated at the left. The figure is representative of two experiments. Arrow indicates MKP-1 protein.

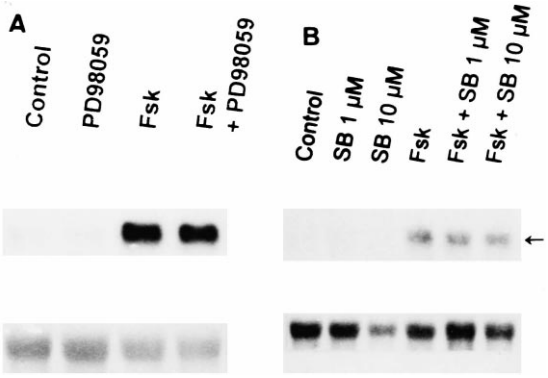

Fig. 5. Effect of MAP kinase inhibitors on forskolin-induced *MKP-1* gene expression. Cells were treated for 30 min (A) with 1 μM forskolin (Fsk) and 20 μM PD98059, or (B) with 1 μM Fsk and 1 or 10 μM SB203580. PD98059 and SB203580 were added 30 and 60 min before forskolin, respectively. Total RNA was analyzed as described in Section 2 and Fig. 1. The figure is representative of three experiments.

forskolin and Bt_2cAMP induced MKP-1 synthesis, as detected by the band appearing at M_r 40 000, in agreement with the apparent M_r reported in [7]. The intensity of the band induced by forskolin was higher than that induced by serum.

3.3. Cyclic AMP-elevating agents failed to activate MAP kinases

We investigated whether cAMP-elevating agents were promoting *MKP-1* gene expression through the activation of MAP kinases. Fig. 5A shows that PD98059, a specific inhibitor of the kinase upstream to ERK1 and ERK2, did not inhibit the forskolin-induced *MKP-1* induction. Also, the p38 kinase inhibitor SB203580 failed to abolish the forskolin-induced *MKP-1* induction (Fig. 5B). Moreover, neither forskolin nor Bt_2cAMP were found to stimulate JNK activity in whole cell extracts, whereas JNK enzymatic activity was

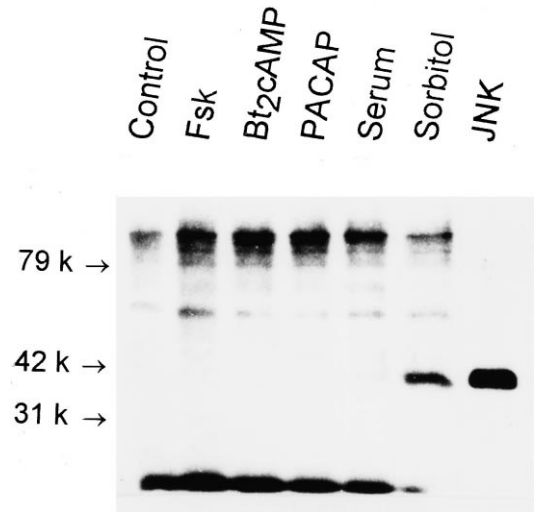

Fig. 6. Effect of serum and cAMP-elevating agents on JNK activity. PC12 cells were incubated for 15 min with 10 μM forskolin (Fsk), 1 mM Bt_2cAMP, 1 nM PACAP27, 20% fetal calf serum, or 0.5 M sorbitol. Whole cell extracts were prepared as described and JNK assayed for its ability to phosphorylate GST-c-Jun. The right lane (JNK) shows the phosphorylation of GST-c-Jun by purified recombinant JNK. The figure is representative of three separate observations.

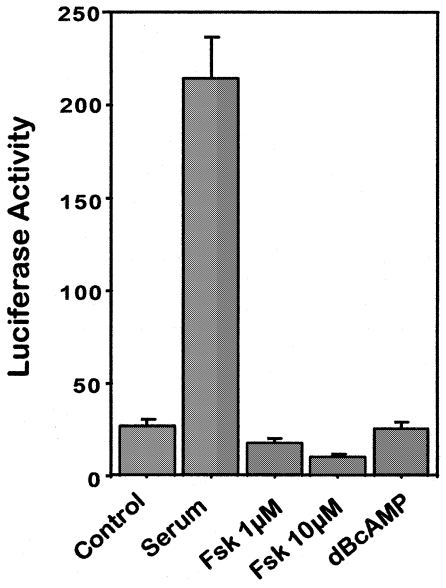

Fig. 7. In vivo Elk-1 phosphorylation in response to serum and cAMP-elevating agents. PC12 cells were transfected with 1 μg pFR-Luc reporter plasmid and 50 ng pFA-Elk fusion trans-activator plasmid encoding the activation domain of Elk. At 40 h post-transfection, cells were treated for 6 h with 20% fetal calf serum, 1 or 10 μM forskolin (Fsk) or 1 mM Bt_2cAMP, and then collected for luciferase assay. Bars represent the mean (arbitrary units) ± S.D. of three experiments. Transfection with a plasmid constitutively expressing an upstream activator of JNK produced a luciferase activity of 470 arbitrary units.

strongly activated by osmotic stress induced by sorbitol (Fig. 6). Very similar results were obtained when JNK activity was measured in the complex obtained after immunoprecipitation of cell lysate by an antibody reacting with both JNK1 and JNK2 (data not shown). To further investigate whether MAP kinases participated in the induction of *MKP-1* by cAMP, we examined the in vivo Elk-1 phosphorylation in response to cAMP-elevating agents, since Elk-1 is known to be a common substrate of the three MAP kinase families. Fig. 7 shows Elk-1 phosphorylation revealed by the luciferase activity expressed from a reporter vector under the control of a fusion activator protein containing the GAL4 DNA binding domain and the Elk-1 activation domain. Serum growth factors produced a tremendous phosphorylation of Elk-1, whereas no such increase was observed in response to forskolin or Bt_2cAMP, confirming that none of the MAP kinase pathways was stimulated in response to cAMP-elevating agents. The participation of phosphatidyl inositol 3-kinase activation can also be ruled out since its inhibitor wortmannin did not significantly modify the cAMP-induced *MKP-1* gene transcription (data not shown).

3.4. *MKP-1 induction correlates with the reduction in the number of cells entering S phase*

PKA-mediated inhibition of cell division is commonly revealed by blockade of the passage of growth factor-stimulated cells into S phase of the cell cycle. To determine whether this blockade correlates with cAMP-dependent MKP-1 induction, we examined the kinetics of cell cycle re-entry (Table 1). Stimulation of quiescent cells with serum resulted in the re-entry of 97% of the cell population into S phase of the cell cycle. Forskolin, which failed to stimulate entry into S phase on its own, attenuated serum-mediated increases in BrdU incorporation by approximately 44%. Forskolin added 2 h before serum, thus inducing expression of MKP-1 protein, inhibited the serum-mediated BrdU incorporation to about 90%.

4. Discussion

We report here the induction of the early gene *3CH134*, encoding the MAP kinase phosphatase MKP-1, in response to various extracellular stimuli in serum-starved PC12 cells. Highest *MKP-1* mRNA induction was obtained with serum, in agreement with an early study in which the gene was identified [6]. We also found that MKP-1 gene and protein were very strongly induced by the cAMP-elevating agents forskolin and Bt_2cAMP. This finding was confirmed by using the neuropeptides PACAP. Receptors for PACAP27 and PACAP38 have been shown to be present on PC12 cells, coupled to adenylyl cyclase, and to both adenylyl cyclase and phospholipase C, respectively [24]. Both peptides produced a very similar effect on *MKP-1* gene induction, strongly suggesting that it is actually the cAMP increase that mediates their effects on *MKP-1* gene transcription.

Since several studies show that *MKP-1* gene transcription is induced by MAP kinases [11,12], our finding raises the question whether its induction by cAMP involved the activation of one (or more) MAP kinase pathway(s). In fact, only ERK1 and ERK2 were shown to be activated by cAMP in PC12 cells [25,26]. But we found that ERKs were not involved in the mechanisms of *MKP-1* induction since agents such as TPA or NGF, which are well characterized for activating ERKs, were very weak *MKP-1* activators. Moreover, inhibition of the ERK pathway by PD98059 did not reduce the forskolin-stimulated *MKP-1* expression. We also found no evidence in the present study for JNK being activated by cAMP-elevating agents, despite the fact that *MKP-1* was reported to be strongly induced by JNK activation [13]. Moreover, the osmotic stress inducer sorbitol, which strongly stimulates JNK enzymatic activity, was not found to induce *MKP-1* gene transcription (data not shown). Also, *MKP-1* induction was not abolished by the p38 kinase inhibitor SB203580. Finally, cAMP-elevating agents were not found to phosphorylate and activate in vivo Elk-1, a common substrate of the three MAP kinase families. Together, our results indicate that in PC12 cells none of the MAP kinase pathways was involved in *MKP-1* gene induction by cAMP. This was also the case when *MKP-1* was induced by arachidonic acid in vascular smooth muscle cells [20], or by PKC activators in CCL39

Table 1
cAMP-elevating agents reduce the number of cells entering S phase

Treatment	Cells incorporating BrdU (%)
Control	6.5
Serum	97.4
Forskolin	7.3
Forskolin+serum	54.3
Forskolin+serum 2 h later	10.2

PC12 cells (24-well plates) were rendered quiescent after 5 days in culture by overnight serum removal. Following a 20-h stimulatory period with 20% fetal calf serum or 10 μM forskolin in the presence of 10 μM BrdU, the percentage of cells which had incorporated BrdU were counted (at least 150 cells for each treatment). Data are from a single experiment representative of three performed.

fibroblasts [27]. Alternatively, PKA might induce *MKP-1* gene expression by activating a transcription factor through the inhibition of its dephosphorylation, since PKA is known for phosphorylating proteins of the I-1 family which, once phosphorylated, become strong inhibitors of Ser/Thr protein phosphatase type-1 (reviewed in [28]). Interestingly, the protein phosphatase inhibitor okadaic acid was found to induce *MKP-1* gene transcription in PC12 cells [29].

Although in most cells cAMP is without effect or inhibits the growth factor-activated ERK pathway [30], there are conflicting reports on whether this is the case in PC12 cells [31,32]. Stimulation of MAP kinase activity by cAMP was actually reported in PC12, Swiss 3T3 and COS-7 cells. In PC12 cells, a positive cAMP effect on MAP kinase pathway is accompanied by a marked increase in the number of neurite-bearing cells [25,26]. It is difficult to conciliate the present data on cAMP-mediated MKP-1 induction with a role in neurite outgrowth since (i) NGF was not found to activate substantially MKP-1 gene transcription, (ii) MAP kinase activation was not involved in the events leading to MKP-1 induction, and (iii) MKP-1 activation would result in even lower MAP kinase activity. The MKP-1 activation in response to the increased intracellular cAMP level reported here is probably involved in the inhibition of PC12 cell proliferation, since the effect of cAMP in attenuating serum-mediated increases in the number of cells that re-enter S phase was correlated with MKP-1 expression. On the other hand, MKP-1 overexpression has already been shown to block DNA synthesis and to inhibit cell division [7–9]. It appears that the regulation of MKP-1 expression is achieved by complex mechanisms that are specific to the cell type considered. These mechanisms have to be established in order to fully comprehend the role of MKP-1 in cell biology.

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