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Cyclic GMP-dependent protein kinase potentiates serotonin-induced Egr-1 binding activity in PC12 cells

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Abstract

The NO/cyclic GMP (cGMP) signal transduction pathway, which involves the cGMP-dependent protein kinase (PKG), regulates transcription of several genes, including immediate early genes. Using transfection experiments with the PKG-I α cDNA cloned from human aorta, we show here that addition of membrane-permeable cGMP analogues to PC12 cells slightly upregulated ERK MAP (mitogen-activated protein) kinase. Likewise, PKG-I α was found to activate weakly DNA binding activity of the Egr-1 transcription factor. On the other hand, PKG-I α overexpression was shown to tremendously amplify the Egr-1 binding activity induced by the neurotransmitter serotonin, which activates *egr-1* gene expression also via the stimulation of the ERK MAP kinase pathway. Since this potentiation occurred neither at the level of ERK nor at the *egr-1* transcriptional level, the mechanism of amplification probably results from the convergence of ERK and PKG pathways at the level of the transcription factor Egr-1. © 2001 Elsevier Science Inc. All rights reserved.

Keywords: cGMP; cGMP-dependent protein kinase; ERK MAP kinase; Serotonin; *c-fos*; *egr-1*; Egr-1 transcription factor; PC12 cells

1. Introduction

The NO/cyclic GMP (cGMP) signal transduction pathway is involved in a variety of important physiological functions, including neurotransmission, cell differentiation and proliferation, activation of macrophages, vasodilatation and platelet aggregation (reviewed in Refs. [1–3]). NO is also involved in many pathological events, such as neurodegenerative diseases. While most of its direct effects are believed to result from the stimulation of cytosolic guanylyl cyclase, long-term effects of NO are probably mediated by the transcription of a series of genes. In pheochromocytoma PC12 cells, NO-releasing agents and membrane-permeable cGMP analogues increase mRNA expression of the im-

mediate early genes *c-fos* and *junB*, as well as AP-1 DNA binding, via the activation of cGMP-dependent protein kinase (PKG) [4]. Similar results were reported in rat embryo fibroblasts and thyroid epithelial cells [5]. It was subsequently demonstrated, using guanylyl cyclase and PKG transfection experiments, that NO activates the *c-fos* promoter in a strictly cGMP-dependent manner, the effect being mediated by PKG [6]. In PC12 and glioma C6 cells, a series of immediate early genes including *c-fos* and *egr-1* was shown to be induced by natriuretic peptides, the action of which is mediated by membrane-bound guanylyl cyclases [7]. cGMP has also been shown to induce ‘late’ genes, the expression of which is often regulated by transcription factors encoded by immediate early genes. In the brain, NO and cGMP are second messengers of the excitatory neurotransmitter glutamate, and the glutamate/NO/cGMP signal transduction pathway has been shown to control gene expression of proenkephalin, gonadotropin-releasing hormone (GnRH) and Ca²⁺/calmodulin-dependent protein kinase II [8,9].

The mitogen-activated protein (MAP) kinases form another essential link between extracellular stimuli and

Abbreviations: cGMP, cyclic GMP; PKG, cGMP-dependent protein kinase; 8Br-cGMP, 8-bromo-cGMP; 8pCPT-cGMP, 8-*p*-chlorophenylthio-cGMP; MEK, MAP or ERK kinase; 5-HT, 5-hydroxytryptamine or serotonin; MBP, myelin basic protein

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altered patterns of gene expression, since activated MAP kinases translocate to the cell nucleus where they phosphorylate and activate a number of transcription factors, including Elk-1 (reviewed in Ref. [10]). The most extensively characterized MAP kinases are ERK1 and ERK2, also known as the 44 and 42 kDa isoforms, respectively. These Ser/Thr kinases require phosphorylation on both Thr and Tyr residues for activity. This dual phosphorylation is catalyzed by a dual specificity kinase known as MAP kinase kinase or MEK (MAP or ERK kinase), which is itself activated by phosphorylation by enzymes of the Raf family of protein kinases. Although ERKs were first characterized as being activated in response to mitogens such as growth factors, recent evidence suggests that in the central nervous system, the ERK pathway becomes activated in response to stimuli that regulate synaptic function through cytosolic increases in calcium [11,12]. ERK signalling cascade has been shown to contribute to synaptic plasticity and to long-term memory formation (reviewed in Ref. [13]).

We show in this study, using transfection experiments with human PKG-I α , that PKG slightly upregulated ERK activity in cultured PC12 cells. Moreover, PKG expression was found to potentiate transcription factor DNA binding activity induced by the neurotransmitter serotonin (5-hydroxytryptamine, 5-HT). The effect is of particular significance in the case of the transcription factor Egr-1.

2. Materials and methods

2.1. PKG-I α cDNA cloning

A polymerase chain reaction (PCR) using a human placenta cDNA library (generously provided by J.M. Garnier, IGBMC, Illkirch, France) as template was used to obtain a DNA fragment of about 1.1 kb corresponding to the N-terminal fragment of PKG-I. The following oligonucleotides were used as primers: 5'-TCTAGGGGATCCGGAGGAGCATGGGCACCTTGCGGGATT-3' which includes a 5' portion of bovine PKG-I α [14] and 5'-TGCTTTAGCTTCTGCATCT-3' corresponding to the 1096–1114 fragment of the human PKG-I β [15]. Using standard techniques, this fragment of DNA was then used as a probe to screen a human aorta cDNA library made in the λ ZAP II system (IGBMC). After the third round of screening, pure plaques were directly recovered as bacterial colonies using the pBluescript/ λ ZAP II in vivo excision system (Stratagene, La Jolla, CA, USA). Nine positive clones were inserted in the Bluescript SK⁺ vector and sequenced. None of the nine clones was found to encode an entire PKG-I isoform. Complete α and β isoforms (identical in their C-terminal 500 amino acids) were obtained by combining two clones, using a single *Nco*I restriction site present in the coding region of both forms. The cDNA encoding PKG-I α isoform was subcloned into the p513 vector, a derivative of the pSG5 mammalian expression vector [16].

2.2. Cell culture and transient transfections

Rat pheochromocytoma PC12 cells, cultured as described before [17], were serum-starved for 15 h before harvesting. Cells were transfected with the PKG expression vector p513 (2 μ g/well) combined with 50 μ l of 45 mg/l cationic polymer polyethylenimine 800 kDa (PEI, Fluka) [18]. The cells were then incubated for 16 h, washed twice and incubated for a further 24 h before experiment.

2.3. PKG assay

PKG activity was measured as described in Ref. [19]. Activity was estimated as the difference between phosphorylation observed in the presence and absence of 10 μ M cGMP of the selective peptide substrate LRKVSQKE. Reaction mixture (80 μ l total volume) contained 20 mM Tris-HCl (pH 7.4), 10 mM MgCl₂, 0.5 mM dithiothreitol, 0.1 mg/ml bovine serum albumin and [γ -³²P]ATP (16 μ M, 100 cpm/pmol). Incubation was carried out for 10 min at 30°C.

For in vitro phosphorylation of MAP kinases, 2000 U of recombinant PKG-I α (Calbiochem, San Diego, CA, USA) was incubated for 30 min in the same medium, except that the peptide substrate was replaced by 400 ng of recombinant ERK2 (Biomol, Plymouth Meeting, PA, USA). Samples were then boiled in SDS sample buffer and ³²P-phosphorylated proteins analysed on sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (10%). For phosphoamino acid analysis, the ERK2 band was cut out from the gel and protein extracted by boiling the gel in 0.05 M NH₄HCO₃ (pH 8.5), 0.1% SDS and 5% β -mercaptoethanol. Protein was hydrolyzed in 6 N HCl at 110°C for 65 min and lyophilized. The residue was resuspended in a phosphoserine, phosphothreonine and phosphotyrosine standard mix (0.7 mg/ml each) and submitted to electrophoresis (1000 V, 50 min) on thin-layer cellulose plastic sheet (MN Polygram CEL 300) in a formic acid/acetic acid/H₂O (25/87/888), pH 1.9 solution. The plate was then dried, sprayed with 0.2% ninhydrin, heated to develop the phosphoamino acid markers and submitted to autoradiography at –70°C for 10 days.

2.4. Measurement of ERK activity

Cells were lysed in 0.5 ml of lysis buffer (10 mM Tris-HCl buffer, pH 7.4, containing 150 mM NaCl, 1% Triton X-100, 0.5% NP-40, 1 mM EDTA, 1 mM EGTA, 0.2 mM sodium vanadate and 0.2 mM phenylmethylsulfonyl fluoride) for 30 min at 4°C. After clarification by centrifugation at 12,000 \times g for 15 min at 4°C, identical amount of protein (200 μ g) from each lysate was incubated for 2 h at 4°C with 1 μ g of anti-ERK2 (Santa Cruz Biotech., Santa Cruz, CA, USA). Immunoprecipitation of the antigen-antibody complex was accomplished by shaking for 2 h at 4°C with 30 μ l of a 75% protein A-Sepharose suspension

(Pharmacia, Uppsala, Sweden), and the Sepharose beads were washed four times with immunoprecipitation buffer. ERK activity was measured by the incubation of 30 μ g Sepharose-bound protein for 30 min at 30°C in a mixture (40 μ l total volume) containing 25 mM Hepes buffer, pH 7.5, 10 mM magnesium acetate, [γ - 32 P]ATP (50 μ M; 2 μ Ci) and 1 μ g of myelin basic protein (MBP; Sigma, St Louis, MO, USA). Reactions were terminated by adding SDS sample buffer. After heating to 95°C for 5 min, the samples were analysed by SDS-PAGE.

2.5. Immunoblotting

Rabbit polyclonal anti-PKG antibody was kindly provided by Prof. U. Walter (University of Würzburg, Germany). For detection of PKG, PC12 cell lysates were separated by SDS-PAGE (10%) and the separated proteins transferred onto nitrocellulose membrane. The resulting blots were blocked in PBS containing 0.2% casein and 0.1% (v/v) Tween-20, followed by incubation for 2 h shaking at room temperature with primary anti-PKG antibody diluted 1:2000. Blots were washed in 0.1% (v/v) Tween 20 in PBS and then incubated in a 1:20,000 dilution of the biotinylated secondary goat anti-rabbit antibody for 30 min shaking at room temperature, followed by streptavidin–alkaline phosphatase conjugation. Antibody binding was detected by a chemiluminescent technique (Tropix, USA) and exposed to Kodak Biomax autoradiographic film for 10–30 min.

2.6. RNA isolation and Northern blot analysis

Total RNA was isolated from PC12 cells by the guanidium isothiocyanate method, separated by electrophoresis through 1% agarose formaldehyde gels and blotted onto nylon membranes [17]. Briefly, membranes were prehybridized for 2 h at 42°C in a mixture containing 50% formamide, 0.9 M NaCl, 50 mM NaH₂PO₄, 5 mM EDTA, 0.1% Ficoll, 0.1% polyvinylpyrrolidone and 0.5% SDS, pH 7.7. Specific mRNAs were detected using DNA probes [32 P]-labelled and added to the mixture which was then incubated for a further 24 h at 42°C. Membranes were washed at high stringency and exposed to X-ray films at –80°C for a period of 2–5 days.

2.7. Nuclear extracts and electrophoretic mobility shift assay

Nuclear protein extraction from PC12 cells and DNA binding activity monitored by the electrophoretic mobility shift assay were performed as described earlier [17]. The probe used was a 21-bp fragment containing the Egr-1 consensus binding site (italic) from the 5' upstream sequence of the murine *egr-1* gene itself: 5'-CCCGGC-GCGGGGCGAGGGCG-3' and complementary strand. Nuclear extract (10 μ g) was incubated with 2 μ g of

poly(dI–dC) and the [32 P]-labelled oligonucleotides for 20 min at room temperature in a 10 mM Tris–HCl buffer, pH 7.5, containing 50 mM NaCl, 10 μ M ZnCl₂, 1 mM EDTA, 1 mM dithiothreitol and 5% glycerol. DNA–protein complexes were resolved on a 4% polyacrylamide gel (38:2 acrylamide to bisacrylamide) in 0.25 \times TBE (1 \times TBE: 50 mM Tris–Borate, pH 8.3, 1 mM EDTA). The gels were then dried and exposed to X-ray film for 24 h.

3. Results

3.1. ERK activation is required for immediate early gene induction by 5-HT

We previously reported the induction of *c-fos* and *egr-1* immediate early genes by the neurotransmitter 5-HT in PC12 cells [17]. Gene induction was accompanied by increased AP-1 and Egr-1 protein binding to their respective

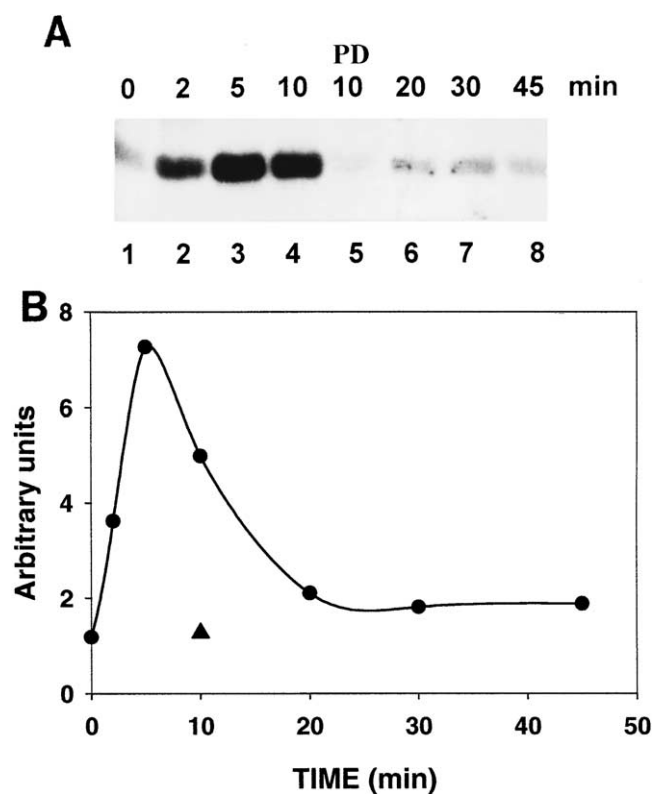


Fig. 1. Time course of the stimulation of ERK activity by 5-HT. PC12 cells were treated with 1 μ M 5-HT for the indicated time, in minutes. In lane 5, cells were incubated together with 5-HT and 20 μ M PD98059 for 10 min. Cells were then lysed and identical amount of protein from each lysate was immunoprecipitated with anti-ERK2 antibodies, as described in Materials and Methods. (A) shows ERK activity assayed in the immunoprecipitate as the phosphorylation of myelin basic protein (MBP) in an assay medium containing [γ - 32 P]ATP, followed by SDS-PAGE analysis and autoradiography. (B) Densitometric analysis of data obtained in (A) after scanning the film. (●) 1 μ M 5-HT. (▲) 1 μ M 5-HT plus 20 μ M PD98059. The figure is representative of two experiments.

DNA consensus sequences. We now show that 5-HT signal transduction comprises the activation of ERK. Fig. 1A shows the time course of 5-HT-induced ERK activation by following the phosphorylation of MBP. Corresponding densitometric analysis shows that ERK activation was maximal 5 min after 5-HT addition to PC12 cells and decreased thereafter (Fig. 1B). Inhibition of the kinase upstream to ERK1/2 by PD98059 abolished the 5-HT-induced ERK activation.

Similarly, addition of PD98059 abolished the *c-fos* mRNA synthesis observed in response to 5-HT (Fig. 2), as well as the 5-HT-induced Egr-1 binding activity (Fig. 3). Characterization of the Egr-1 binding complex in PC12 cells was established in Ref. [17]. Similar results were obtained when investigating *egr-1* gene induction or AP-1 binding activation by 5-HT (data not shown). Taken together, these results show that ERK activity is necessary for the early gene expression induced by 5-HT.

3.2. ERK activity in PKG-1 transfected cells

Several laboratories, including ours, have shown that activation of the NO/cGMP pathway induces immediate early genes through PKG activation. We therefore checked whether PKG could actually activate early gene expression by modulating ERK activity. Fig. 4 shows that adding recombinant ERK2 to a standard in vitro PKG assay medium containing recombinant PKG-I α resulted in a clear

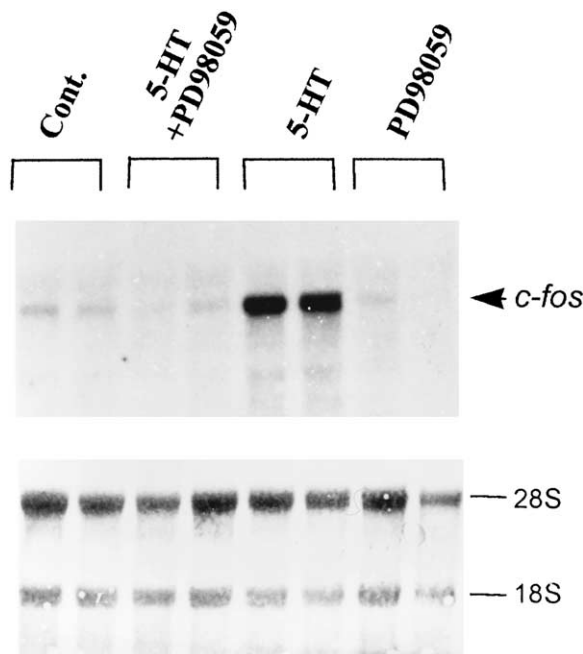


Fig. 2. Northern blot analysis of *c-fos* induction by 5-HT. Serum-starved PC12 cells were incubated for 40 min with 1 μ M 5-HT, 20 μ M PD98059 or with both compounds, as indicated. Twenty μ g of extracted total RNA was used for each lane. The lower panel presents the rRNA revealed by methylene blue staining of the blot, showing equal loading of RNA. This figure is representative of three experiments.

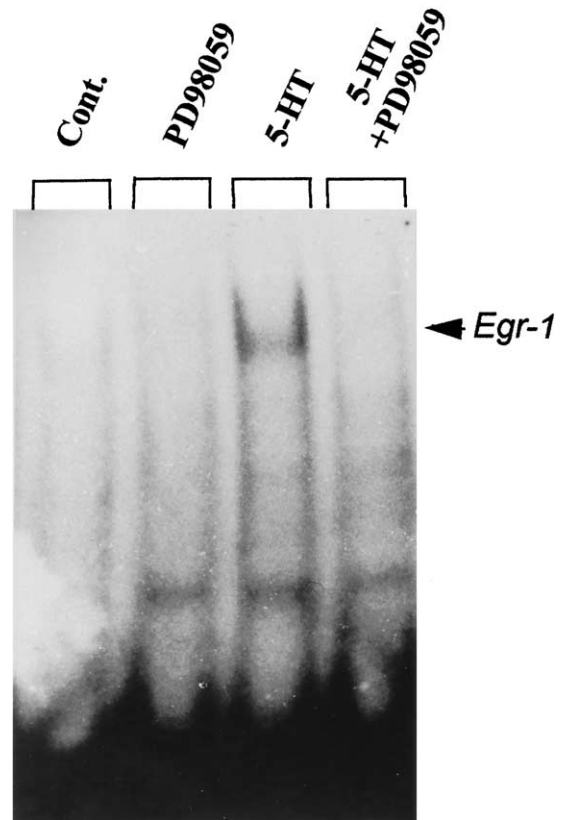


Fig. 3. Mobility shift assay of nuclear extracts from PC12 cells treated with 5-HT. Nuclear extracts were prepared 1 h after stimulation of serum-starved cells with 1 μ M 5-HT, 20 μ M PD98059 or with both compounds, as indicated. Ten μ g of protein sample was incubated with a [32]P-labelled synthetic DNA probe containing the Egr-1 consensus site. The figure is representative of four experiments.

phosphorylation of ERK2 (Fig. 4A) as revealed by the labelled band of relative molecular mass (M_r) 42 k. This

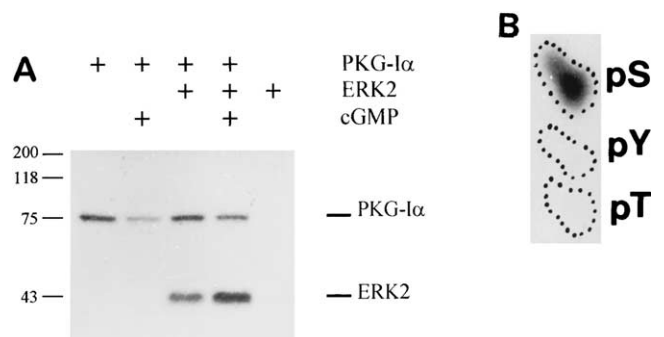


Fig. 4. PKG phosphorylates ERK2 in vitro on serine residue(s). (A) Purified recombinant PKG-I α (2000 U/assay) was incubated with 400 ng of ERK2 for 30 min at 30°C in a standard PKG assay medium containing [γ - 32]ATP, in the presence or absence of 10 μ M cGMP. Proteins were then separated on SDS-PAGE (10%) and submitted to autoradiography. (B) [32]P-labelled ERK2 was cut out from the gel shown in (A) and hydrolyzed. Resulting amino acid residues were separated by electrophoresis on thin-layer cellulose and submitted to autoradiography. Positions of standard phospho-Ser (pS), phospho-Tyr (pY) and phospho-Thr (pT) are indicated by dotted lines.

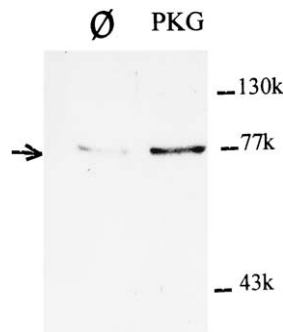


Fig. 5. Western blot analysis showing that PKG-I α is expressed in PC12 cells as a 77-k protein. Immunoblot using polyclonal anti-PKG antibody on extracts from cells transfected with 2 μ g p513 vector containing the PKG-I α cDNA (PKG) or empty vector (\emptyset). Immunoreactive signals were visualized by chemiluminescence. Apparent molecular weights of marker protein are indicated at the right. Arrow indicates PKG protein.

result cannot be explained in terms of MAP kinase autophosphorylation, since this was very low under the conditions we used. At the PKG concentration of 2000 U/assay, MAP kinase phosphorylation was found to be cGMP-dependent, as opposed to PKG autophosphorylation. After cutting the phosphorylated ERK2 band out from the gel and the recovered protein analysed for phosphoamino acid content, we found that PKG phosphorylated ERK2 on Ser residue(s) only (Fig. 4B). Obviously, it did not phosphorylate ERK2 on its activation site since MAP kinases are known to require phosphorylation on both Thr and Tyr residues for activity.

To test the hypothesis of ERK regulation by PKG, we cloned human PKG-I α by screening a human aorta cDNA

library with a DNA fragment obtained by PCR using primers complementary to bovine PKG-I. The human α isoform was found to be 96% identical with regard to nucleotide and 99.7% for amino acids by comparison with the bovine isoform [14]. When PKG-I α was expressed in PC12 cells from the cDNA subcloned in the p513 mammalian expression vector, significant PKG activity could be measured as evaluated by phosphorylation of the selective peptide substrate LRKVSQEQ (data not shown). We also verified that the expressed protein reacted with an antibody directed against bovine PKG-I. The Western blot analysis presented in Fig. 5 shows that cells transfected with PKG-I α exhibited a 77-k band corresponding roughly to the expected PKG-I α Mr. A faint signal was detected in control cells transfected with the corresponding empty vector.

We then measured ERK activity in PC12 cells transfected with PKG-I α by following the phosphorylation of MBP. Fig. 6A shows that basal non-stimulated MAP kinase activity in control cells was increased on addition of cell-permeable PKG activators to the culture medium. Quantitative densitometric analysis shows that ERK activity was increased by about 30%, with a similar effect being noted when 8-bromo-cGMP (8Br-cGMP) or the more selective PKG activator 8-*p*-chlorophenylthio-cGMP (8pCPT-cGMP) was added to the cells (Fig. 6B). In cells overexpressing PKG, basal activity of ERK was increased. This activation probably reflects that of transfected PKG, which was found to be partially active even without the addition of cGMP analogues. When these analogues were added to the cells, thus fully activating PKG, a further increase in ERK activity (by about 75%) was observed, so that the overall stimulation

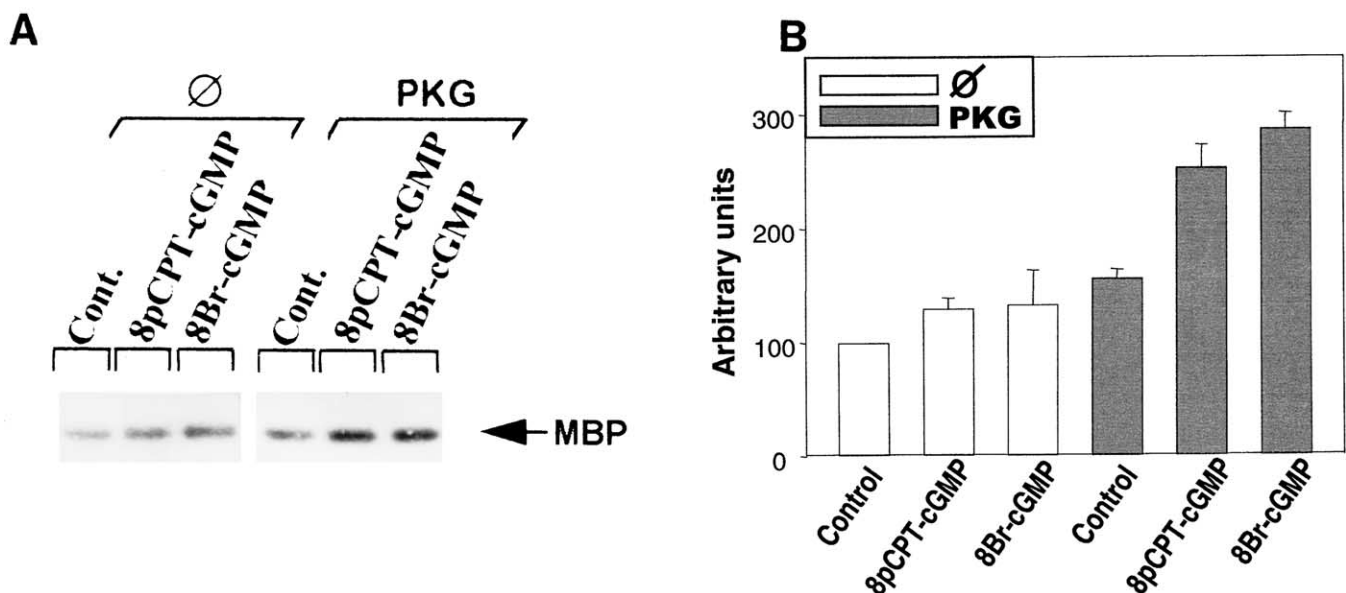


Fig. 6. ERK activity in PC12 cells transfected with PKG-I α . Cells were transfected with 2 μ g p513 vector containing the PKG-I α cDNA (PKG) or vector lacking a cDNA insert (\emptyset), and treated for 20 min with 0.5 mM 8pCPT-cGMP or 8Br-cGMP. Cells were then lysed and identical amount of protein from each lysate was immunoprecipitated with anti-ERK2 antibodies. (A) ERK activity assayed in the immunoprecipitate as the phosphorylation of MBP in an assay medium containing [γ - 32 P]ATP, as described in Materials and Methods and legend to Fig. 1. (B) Densitometric analysis of data obtained from three experiments similar to that shown in (A). Each bar represents the average \pm S.D., expressed in arbitrary units.

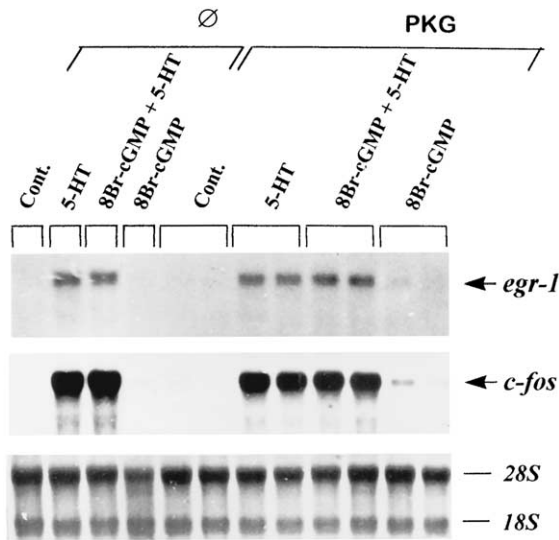


Fig. 7. Northern blot analysis of *egr-1* and *c-fos* induction in PKG-I α overexpressing cells. Total RNA was extracted from PC12 cells transfected with 2 μ g p513 vector containing the PKG-I α cDNA (PKG) or vector lacking a cDNA insert (\emptyset), and treated for 40 min with 1 μ M 5-HT or 0.5 mM 8Br-cGMP, as indicated. Twenty μ g of total RNA was used for each lane. The lower panel presents the rRNA revealed by methylene blue staining of the blot, showing equal loading of RNA. This figure is representative of three experiments.

(from basal activity in control cells to maximal activity in transfected cells) was almost threefold.

3.3. PKG-I potentiates the 5-HT-induced *Egr-1* DNA binding activity

We next measured the ability of 5-HT to induce immediate early genes in PKG overexpressing PC12 cells. Fig. 7 shows that *c-fos* and *egr-1* mRNA synthesis provoked by 5-HT was not significantly increased by the PKG activator 8Br-cGMP either in control cells or in cells transfected with the PKG-I α expressing vector. Also, the transcriptional level of both genes reached upon 5-HT treatment was not different in PKG overexpressing cells as compared to control cells.

Transcription factor AP-1 and *Egr-1* binding activities were measured in conditions similar to those used for determining early gene induction. AP-1 binding activity in control cells was strongly stimulated by 5-HT, whereas 8Br-cGMP caused a weaker AP-1 activation (data not shown). Basal AP-1 binding activity was slightly enhanced in cells transfected with the PKG-I α expression vector. The effect of

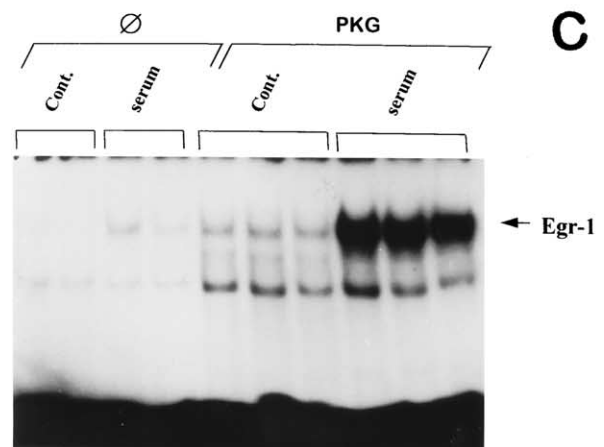
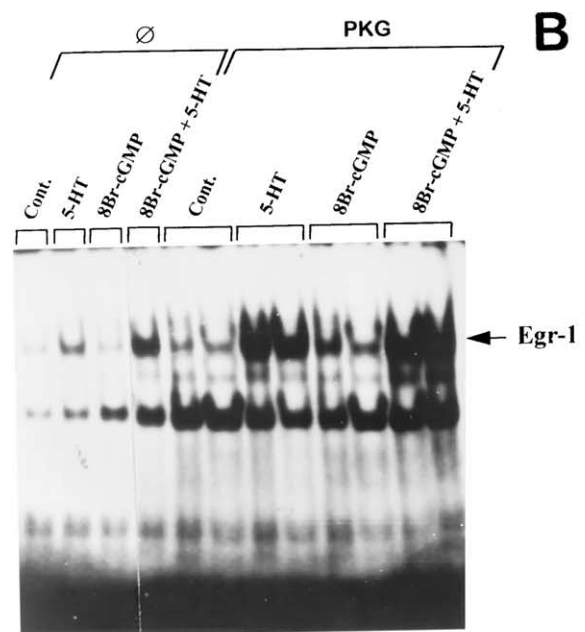
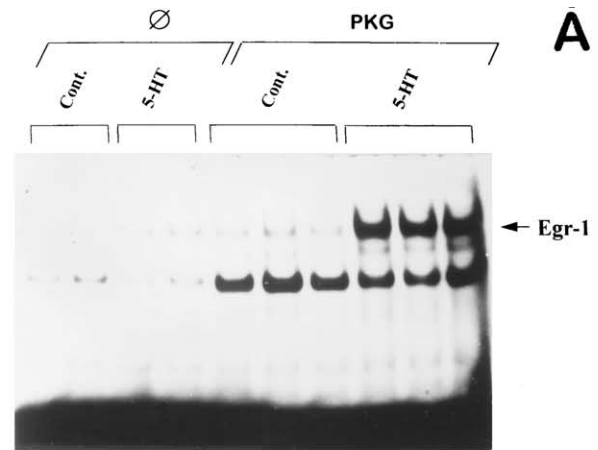


Fig. 8. PKG-I α overexpression potentiates 5-HT-induced (A, B) and serum-induced (C) *Egr-1* binding activation in PC12 cells. Electrophoretic mobility shift assay was carried out with nuclear extracts from cells transfected with 2 μ g p513 vector containing the PKG-I α cDNA (PKG) or vector lacking a cDNA insert (\emptyset). Nuclear extracts were prepared 1 h after stimulation of cells with 1 μ M 5-HT, 0.5 mM 8Br-cGMP or 20% fetal calf serum, as indicated. Ten μ g of protein sample was incubated with [32]P-labelled synthetic DNA probe containing the *Egr-1* consensus site. Arrow indicates the *Egr-1* complex.

5-HT and 8Br-cGMP added together was only about 20% above the sum of the effects of each compound added separately, an increase that did not reach statistical significance (data not shown).

The situation was much less ambiguous concerning the transcription factor Egr-1 (Fig. 8). PKG-induced potentiation of the Egr-1 binding activation produced by 5-HT was very substantial, making it observable without prior activation of the expressed PKG by 8Br-cGMP. Fig. 8A shows that cells transfected with the PKG-I α expression vector exhibited enhanced basal Egr-1 binding activity. Upon 1 μ M 5-HT addition to these cells, Egr-1 activity was very much above the activity elicited by 5-HT in control cells added to that observed in non-stimulated transfected cells. The effect was even more enhanced when the expressed PKG was fully activated by 8Br-cGMP (Fig. 8B). The potentiation was clearly apparent in control cells, and a dramatic enhancement of Egr-1 activity was observed in PC12 cells overexpressing PKG-I α and treated with both 8Br-cGMP and 5-HT when compared to cells treated with each compound alone or treated with both compounds in control cells (Fig. 8B). This amplification phenomenon is not restricted to 5-HT and can be generalized. Fig. 8C shows that in cells treated with 20% serum, the effect of growth factors on Egr-1 binding activity was also considerably potentiated in PKG overexpressing cells.

4. Discussion

NO-releasing agents and cGMP analogues have been shown to increase the mRNA levels of a series of immediate early genes, including *c-fos* and *junB* [4–7]. Stimulation of the NO/cGMP signal transduction pathway triggers early gene transcription through the activation of PKG, independently of changes in calcium or cAMP [5,6]. Using transfection experiments with the PKG-I α cDNA we cloned from human aorta, it is reported here that in addition to AP-1, PKG also activates DNA binding activity of the Egr-1 transcription factor that contains three repeated zinc finger consensus sequences [20]. Furthermore, PKG-I α overexpression was shown to amplify tremendously the 5-HT-induced Egr-1 binding activity. The signal transduction pathway followed by 5-HT includes stimulation of a 5-HT₂ receptor subtype and activation of a member of the focal adhesion kinase (FAK) family of M_r 115 kDa [17], leading ultimately to gene transcription. Here we show that activation of ERK is a necessary event for 5-HT to induce early gene expression.

Overexpression of PKG-I in PC12 cells was found to enhance only weakly ERK basal activity. This was expected when considering that PKG-I phosphorylated ERK in vitro on Ser residue(s) only, while phosphorylation on Thr and Tyr residues was known to be required for MAP kinase activation. Hence, PKG may only upregulate through Ser phosphorylation the ERK enzyme that is already activated

by the MEKs. Stimulation of ERK via activation of the NO/cGMP transduction pathway has been reported in several other systems. In T-cells, ERK pathway was activated by NO, but the target was identified as being Ras [21,22]. ERK MAP kinases were also found to be activated by NO in hippocampus [23] and by the cGMP/PKG signalling pathway in pinealocytes [24] and in vascular smooth muscle cells [25].

Overexpression alone of PKG-I was also found to slightly enhance the basal activity of both AP-1 and Egr-1, confirming that transfected PKG-I α was partially active in the absence of permeant cGMP analogues or without stimulation of the NO/cGMP pathway. This was already observed when the activity of PKG itself or that of ERK was measured in cells transfected with the PKG expression vector. It is not clear if the spontaneous activity of the transfected kinase was the consequence of a high endogenous cGMP level. Alternatively, the effect might result from elevated PKG concentrations, since this kinase was also activated in the absence of added cGMP when high amounts of the enzyme were present in in vitro assays. Stimulation of the transfected kinase by cell-permeant cGMP analogues further increased binding activities of the transcription factors AP-1 and Egr-1. It should, however, be emphasized that for the effect we describe here to take place under physiological conditions, the availability of sufficient PKG to be activated by the NO/cGMP pathway is required. The paucity of PKG expression in some tissues may explain why in some cells NO-regulated gene transcription cannot be detected, or requires the presence of calcium ionophores [9,26]. The PC12 cells used in this study have a very low, but detectable endogenous PKG level which could produce in control cells effects similar to those described in transfected cells, although to a corresponding lesser extent.

MAP kinase signalling pathways represent appropriate targets for gene regulation, since activated MAP kinases are known to migrate into the nucleus where they phosphorylate and activate an array of transcription factors [10]. In our experiments, the stimulation of PKG might have upregulated ERK, which in turn phosphorylated Elk-1, producing the accumulation of Egr-1 and of the AP-1 component c-Fos, through the activation of the serum response element present in their gene promoter [27]. It is noteworthy that a potentiation of calcium-induced ERK activation by NO was recently reported in PC12 cells [28]. But it appears that the mechanism of amplification of 5-HT-induced Egr-1 binding activity by PKG which we report here probably did not result from a convergence occurring at the level of ERK, since the potentiation occurred neither at the level of ERK nor at the *egr-1* transcriptional level. The mechanism of amplification rather represents the convergence of ERK and PKG pathways at the level of the transcription factor Egr-1. Phosphorylation of Egr-1 was indeed shown to increase its DNA binding efficiency [29,30], but unfortunately, the kinase(s) responsible for this mechanism is (are) not known. Recently, however, PKG itself has been shown to trans-

locate into the nucleus by an active transport mechanism which requires a nuclear localization signal and is regulated by cGMP [31], which makes PKG a likely candidate for phosphorylating directly Egr-1, hence producing the observed amplification.

Independent of the mechanisms involved, the convergence of 5-HT-induced ERK-mediated signal transduction cascade with the activation of PKG pathway may act as a coincidence detector [32]. When active together, these two signal transduction pathways assure that the response to 5-HT neurotransmission will be maximal.

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