Membrane-bound cAMP-dependent Protein Kinase Controls cAMP-induced Differentiation in PC12 Cells*

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The A126 cell line, a derivative of PC12, is defective in cAMP-induced transcription and does not differentiate in the presence of cAMP. In these cells overexpression of a cAMP-dependent protein kinase (PKA) anchor protein, AKAP75, and of the PKA catalytic subunit substantially increased the fraction of PKAII bound to the membrane, stimulated the transcription of cAMP-induced genes, and induced terminal differentiation. Conversely, wild type PC12 cells expressing a derivative of the AKAP75 protein, AKAP45, which binds the PKA regulatory subunits RII, but fails to locate them to the membranes, induced translocation of PKAII to the cytosol. These cells did not efficiently accumulate PKA catalytic subunit in the nuclei when stimulated with cAMP, did not transcribe cAMP-induced genes, and failed to differentiate when exposed to cAMP. These data indicate that membrane-bound PKA positively controls the transcription of cAMP-induced genes and differentiation in PC12 cells.

The growth and differentiation of several cell types are controlled by cAMP (1–3). In eukaryotes, cAMP binds the regulatory (R) subunit of cAMP-dependent protein kinases (PKA). This binding releases the catalytic subunit (C-PKA) which phosphorylates a wide range of substrate proteins. A fraction of the C-PKA migrates to the nucleus and phosphorylates nuclear proteins and transacting factors (4–6).

The distinctive characteristics of PKA are largely determined by the structure and properties of their R subunits (3, 7, 8). PKAI, consisting of RI type subunits, is typically cytosolic, whereas PKAII, consisting of type II R subunits (α and β), is targeted to certain subcellular locations by specific anchor proteins (AKAPs) (3, 9–11). The localization of PKA may improve the accessibility of the enzyme to upstream and downstream effectors and also to cAMP (10, 11). We have provided evidence linking membrane targeting of PKAII to cAMP-dependent gene transcription in differentiated and non-differentiated cells. In thyroid cells, displacement of immobilized PKAII from perinuclear sites to the cytoplasm impaired cAMP-induced transcription of the thyroglobulin gene (12). Also, in non-neuronal cells positioning of PKAII in the membranes significantly stimulated the rate and the magnitude of transcription of cAMP-induced promoters (13).

We have used PC12 as a model system to study the transmission of cAMP signals under well defined conditions. PC12 cells terminally differentiate in neurons when exposed to cAMP (1, 14). The activation of differentiation program by cAMP depends on the transcription of cAMP-induced genes (4, 6). A PC12 derivative cell line has been isolated, which proliferates and does not differentiate when exposed to cAMP. This cell line, A126, efficiently differentiates when treated with NGF or other agents which stimulate protein kinase C or Ras, indicating that the cAMP and Ras pathways are independently required for the execution of the differentiation program in PC12 cells (14, 15).

The A126 cells fail to transcribe cAMP-induced genes, although they normally grow in presence of the cyclic nucleotide. These cells contain mostly cytosolic PKAII, and we have provided evidence suggesting that the failure to activate cAMP-induced genes is because of the loss of membrane-bound PKAII (16).

Here, we show that A126 cells, overexpressing the anchor protein AKAP75 and the catalytic subunit of PKA, significantly increase the fraction of membrane-bound PKA. These cells transcribe cAMP-induced genes and differentiate when exposed to cAMP, becoming undistinguishable from wild type PC12 cells. These data indicate that membrane-bound PKA positively controls cAMP-dependent transcription and terminal differentiation in PC12 cells.

**MATERIALS AND METHODS**

**Cell Lines—**PC12 and A126 IB2 cells were grown in RPMI medium containing 5% fetal calf serum, 10% horse serum, 100 units/ml penicillin, 100 μg/ml streptomycin, and 2 mM glutamine.

**Plasmids and DNA Transfections—**The plasmids used in this study were the following: CRE-CAT (17); C-PKA (2); AKAP75 and AKAP45 (18, 19, 21); RSV-lacZ (20); and RSV-Neo (21). Transfections were performed by calcium phosphate (21). Stable transfected cells were generated as follows. 10<sup>6</sup> cells/100-mm dish were transfected with a total amount of 20 μg of DNA: 5 μg of RSV-Neo, 5 μg of AKAP75, 5 μg of C-PKA, and salmon sperm to 20 μg. 72 h later, the cells were split and fresh medium was added. Selection was carried in G418 for 3 weeks. Single colonies (~50) were pooled and expanded for further studies. Transient transfections were carried with a total amount of 10 μg of DNA: 5 μg of CRE-CAT and 5 μg of RSV-lacZ. 44 h later, the cells were stimulated with 0.5 mM 8-Br-cAMP for 4 h. CAT activity was assayed in extracts containing equivalent units of β-galactosidase (0.1, 0.2 unit), (1 unit is the absorbance at 420 nm of the extracts incubated with 1 mg/ml 2-nitrophenol-D-galactopyranoside transferase at 37 °C for 1 h). Experiments showing differences of lacZ expression more than 2-fold have been eliminated.

**Fractionation of Cell Extracts—**The cells were washed with phosphate-buffered saline and lysed mechanically as described previously.
Fig. 1. Membrane/cytosol partition of RII subunits in A126 cells expressing AKAP75. A, ligand blotting analysis of membrane and cytosolic fractions with labeled RIIβ subunit. Cytosolic (C) and membrane (P) fractions were prepared as described previously (16). Purified RIIβ subunit was labeled with C-PKA as described under "Materials and Methods." The specific activity was 3.5 × 10^6 cpm/µg. The lane labeled RIIβ corresponds to 1 ng of purified protein. Panels B and C are immunoblots of the same cellular fractions probed with antibodies to RIIβ (B) and RIIα (C), respectively. The histograms shown in the lower part of the figure indicate the relative amount of RII present in the pellet (black) or cytosol (white) fraction of each cell line indicated by the numbers. The values on the ordinates, expressed in arbitrary units, were generated by densitometric scanning of the blots.

(1). The pellet was treated with AT buffer (15 mM NaCl, 60 mM KCl, 15 mM Hepes (pH 7.8), 2 mM EDTA, 0.3 M sucrose, 14 mM Kemptide (Sigma). Kemptide phosphorylation was monitored by spotting 20 µl of the incubation mixture on phosphocellulose filters (Whatman, P81) and washing with 75 mM phosphoric acid, as described previously (12). The radioactivity retained on the filters was determined by scintillation counting in 4 ml of scintillation liquid (EcoLite, ICI). Free C-PKA activity was evaluated by subtracting cpm obtained in the absence of cAMP from the values obtained in the presence of PKA inhibitor peptide. Data were expressed as picomoles of [32P]phosphate transferred to the peptide substrate during a 10-min incubation in the presence (PKA holoenzyme) or absence (free C-PKA) of 10 µM cAMP. At the concentrations used, PKA inhibitor peptide did not inhibit the binding of phosphorylated Kemptide to phosphocellulose filters (12, 13, 16).

Neurile Outgrowth Assay—Cells (2–4 × 10^4) grown in RPMI medium were induced with 1 mM 8-Br-cAMP for 12 days and 100 ng/ml NGF (Upstate Biotechnology Inc.) for 5 days. The cells received fresh medium supplemented with 8-Br-cAMP or NGF at 2-day intervals. A neurite was identified as a process whose length was 1.5 times the cell body. 200 cells were counted for each plate, and the percentage of cells with neurites was calculated as described (15).

RNA Analysis—Total RNA (30 µg) was size-fractionated in 2.2 M formaldehyde:1% agarose gel and transferred to N-Hybond filters (Amersham Pharmacia Biotech). Hybridization was performed at 42 °C for 15 h in 50% formamide, 0.1% SDS, 5× SSC, 5× Denhardt’s solution. The filters were washed at 42 °C in 0.2× SSC (22).

RESULTS

Relocation of PKAII to the Membranes Amplifies cAMP-induced Transcription and Reverses the Differentiation Block in A126 Cells—A126 cells do not differentiate when exposed to cAMP because cAMP-stimulated transcription is defective. We previously found that these cells contain mostly cytosolic PKAII, and we have provided evidence suggesting that the failure to activate cAMP-sensitive genes was dependent on the absence of membrane-bound PKAII (16). To prove that when PKAII is localized to the membranes it can reactivate cAMP-induced transcription, we transfected A126 cells with plasmid constructs expressing the RII-binding protein AKAP75, or the catalytic subunit of PKA, or both genes. Stable transfecants were selected, pooled (−50 for each DNA construct), analyzed for the expression of the plasmid vectors, and further characterized. Cells transfected with C-PKA contained two-fold higher PKA activity compared with NEO or AKAP75 transfecants (data not shown). We first determined the cytosolic/membrane partition of endogenous RII subunits in transfected cells. Fig. 1 shows RIIα and RIIβ present in the cytosolic (C) and membrane (P) fractions derived from transfected cells. The...
absence or presence of 1 mM 8-Br-cAMP, respectively; limiting in these cells, as demonstrated by the residual RII expressing C-PKA and AKAP75 in the absence or presence of 1 mM 8-Br-cAMP, respectively. RII to the membranes (see the histogram below the blot). As to preferentially cytosolic/membrane partition of RII levels of AKAP75, and under these conditions AKAP75 affects the cytosol (Fig. 1, panel B). The levels of these RII binding proteins are high in PC12 and reduced in A126 cells. A minor band of 52 kDa is detected in the lower part of the blot. This band corresponds to RII subunits, which are better defined in the B (RIIβ) and C (RIIα) panels of Fig. 1. RIIβ is found exclusively in the cytosol of A126 cells (Fig. 1, A and B, lanes 1, and Ref. 16). Expression of AKAP75 in combination with C-PKA subunit significantly increases RIIβ in the membrane fraction (Fig. 1B, lane 6). In these transfectants, a significant fraction of RIIβ translocates to the membranes (see the histogram below the blot). As to RIIα, we did not detect significant variations relative to control cells (Fig. 1, panel C), indicating that AKAP75 expression influences predominantly the membrane-cytosplasmic partition of RIIβ. This finding might be explained by the higher binding affinity of AKAP75 to RIIβ relative to RIIα (23). AKAP75 is limiting in these cells, as demonstrated by the residual RIIβ in the cytosol (Fig. 1B). These clones do not express very high levels of AKAP75, and under these conditions AKAP75 affects preferentially cytosolic/membrane partition of RIIβ but not RIIα. In transfectants expressing high levels of AKAP75, both RIIα and RIIβ are targeted to the membranes (data not shown, and Ref. 13).

To determine the biological effects of membrane-bound PKA, we tested cAMP-induced transcription in the cells mentioned above by assaying the transient expression of CAT driven by a cAMP-induced promoter (CRE). Fig. 2 shows that cAMP-induced transcription was markedly stimulated in the cells transfected with C-PKA and AKAP75. The effects of AKAP75 and C-PKA were synergistic, indicating that the increase in total PKA by C-PKA and membrane anchoring by AKAP75 expression independently contributed to the stimulation of cAMP-induced transcription, defective in A126 cells (Fig. 2). A126 cells were originally isolated as cells resistant to cAMP-induced differentiation, we therefore tested whether the expression of AKAP75 and C-PKA could rescue this phenotype. Fig. 3A shows that A126 cells expressing exogenous AKAP75 and C-PKA (A75+C-PKA) differentiated efficiently when stimulated with cAMP (75% of the cells showed neurite outgrowth versus 10% in control cells). This effect was cAMP-dependent since these cells did not differentiate in the absence of cAMP (Fig. 3A, A75+C-PKA).

We also measured the growth profile of these cells in the presence of cAMP (Fig. 3B). The growth of cells expressing AKAP75 and C-PKA was markedly inhibited by cAMP, whereas control or mock-transfected cells proliferated in the presence of cAMP for 12 days. Cells expressing AKAP75 or C-PKA alone proliferated more slowly than control A126 cells in the presence of cAMP. These data indicate that relocation of PKA to the membrane reverses the block in cAMP-induced
immunoblotting with specific antibodies to RII AKAP45 vector were isolated and analyzed by ligand blotting and cells expressing AKAP45. Pools of PC12 cells transfected with antibodies. Fig. 4 panel A blots of the same cellular fractions as in the membrane (black generated by densitometric scanning of the blots. The values on the ordinates expressed in arbitrary units were lower part of the figure indicate the relative amount of RII present in and analyzed by immunoblot with anti-RII tant, AKAP45, translocates RII and PKAII to the cytosol (24).

This effect is seen more dramatically in the overlay blot shown in Fig. 4A. The same cell extracts were also analyzed by immunoblotting with specific anti-RIIβ and anti-RIIα antibodies. The 52-kDa band corresponds to the RII subunits, RIIα and RIIβ-specific antibodies. Fig. 4A shows an overlay experiment using labeled RIIα after SDS denaturation. Panel B shows an immunoblot of the same extracts probed with specific antibodies to AKAP75. The arrows indicate the endogenous AKAP150 and the exogenous AKAP45. Panels C and D show representative immunoblots of the same cellular fractions as in panel A probed with anti-RIIα and anti-RIIβ antibodies, respectively. The histograms shown in the lower part of the figure indicate the relative amount of RII present in the membrane (black) or supernatant (white) fraction of the transfectants. The values on the ordinates expressed in arbitrary units were generated by densitometric scanning of the blots.

Transcription and restores cAMP-induced differentiation in A126 cells.

Cytosolic Translocation of PKAII Inhibits cAMP-induced Transcription and Blocks the Differentiation in PC12 Cells—The experiments shown above indicate that membrane-bound PKA stimulates the transcription of cAMP-induced genes and the differentiation of A126 cells. To conclusively determine the role of membrane-bound PKA in the amplification of cAMP signals, we performed the complementary experiment. We expressed in the wild type PC12 cells a derivative of AKAP75 protein, which contains a deletion at the NH terminus. Since this segment of the protein contains the protein domain which is necessary for membrane binding, overexpression of this mutant, AKAP45, translocates RII and PKAII to the cytosol (24).

PC12 stable transfectants expressing AKAP45 were isolated and analyzed by immunoblot with anti-RIIα- and -RIIβ-specific antibodies. Fig. 4A shows an overlay experiment using labeled RIIβ as probe. In control cells (NEO), we detected two major RII-binding proteins of size ~110–150 and 85 kDa. These proteins were not detected in AKAP45-transfected cells. Fig. 4B shows a Western blot of the same extracts with specific anti-bodies to bovine the AKAP75 (18). The two arrows indicate the endogenous AKAP150 (homologous to the bovine AKAP75) and the exogenous AKAP45 (18). Note that the endogenous AKAP150 was down-regulated in cells expressing AKAP45. This effect is seen more dramatically in the overlay blot shown in Fig. 4A. The same cell extracts were also analyzed by immunoblotting with specific anti-RIIβ and anti-RIIα antibodies. The 52-kDa band corresponds to the RII subunits, RIIα and RIIβ (Fig. 4D) and RIIα (Fig. 4D). Expression of AKAP45 significantly increased RII subunits in the cytosol.

PKA activation by cAMP was measured in these cells. Fig. 5 shows that cAMP-induced accumulation of nuclear C-PKA was substantially reduced in AKAP45-expressing cells compared with control cells (Fig. 5, upper panel). Also, cytoplasmic PKA was not efficiently activated by cAMP (Fig. 5, lower panel) or forskolin (data not shown), when compared with control cells. It is of interest to note that inefficient PKA activation was found in A126 cells, which are refractory to cAMP-induced differentiation (14, 16). The ratio PKAII/PKAI in PC12 expressing AKAP45 was 14.4 ± 0.2 and 11.4 ± 0.5 pmols/µg/min, respectively. Nuclear PKA activity (upper panel) is shown as picomoles of [32P]ATP incorporated/10 min/µg of nuclear proteins.

Nuclear cAMP signaling in PC12 cells expressing A45 was significantly down-regulated as shown by inefficient accumulation of nuclear C-PKA following cAMP stimulation (Fig. 5,
upper panel). Activation of transcription of a CRE promoter following cAMP stimulation was reduced in terms of magnitude and kinetics (Fig. 6). PC12 cells exposed to cAMP stop growing and differentiate (14, 15).

We have determined the growth rate of PC12 expressing AKAP45 in the presence or absence of cAMP. The left panel in Fig. 7 shows that the growth of these cells is not inhibited by cAMP as control cells. Furthermore, the induction of differentiation genes (c-Fos, H-Ferritin, and vgf) by cAMP was significantly depressed in A45-expressing cells (Fig. 7, right panel). The induction of the same genes by NGF was unchanged in AKAP45-expressing cells. Neurite outgrowth, a very sensitive marker of cAMP biological response in PC12 cells, was significantly depressed in PC12-A45 cells (Fig. 8B).

Table I summarizes the data and illustrates that the expression of A45 in PC12 cells, which results in translocation of PKAII to the cytosol, down-regulates the cAMP biological response.

**DISCUSSION**

cAMP induces terminal differentiation of PC12 cells. This effect is mediated by the stimulation of transcription of cAMP-responsive genes (16, 25). The mutant cell line A126 is defective in cAMP-induced transcription and does not differentiate when exposed to cAMP (14, 26). PKA is present and active, albeit reduced, but is localized exclusively in the cytosol (16). Expression of a PKA anchor protein, AKAP75, relocates PKAII to the membranes and activates cAMP-induced transcription. Co-expression of C-PKA and AKAP75 completely reverses the block of cAMP-induced transcription and differentiation. Note that C-PKA or AKAP75 alone did not restore cAMP-induced transcription in A126 cells (Fig. 2 and Ref. 16). On the other hand, C-PKA is also required for membrane localization of RIIβ because the expression of RIIβ binding proteins is stimulated by PKA (Fig. 1, panel A and Ref. 27).

A126 cells were selected for their ability to grow in the presence of high cAMP concentrations (14). These cells became resistant to cAMP inhibition of growth probably by reducing the pool of membrane-bound PKAII (Fig. 1). However, we cannot exclude that the A126 phenotype might result from other yet unknown mutations in pathways directly or indirectly affecting cAMP signaling. One way to demonstrate the cause-effect relationship between membrane-bound PKA and cAMP-induced differentiation was to generate the A126 defective phenotype in wild type PC12 by manipulating only the localization of endogenous PKA. The experiments shown here indicate that the expression of a mutated variant of AKAP75, AKAP45, which binds RII but fails to localize it to the membranes, alters the membrane- cytosol partition of RII and significantly increases the fraction of cytosolic RII. cAMP-induced nuclear accumulation of C-PKA and cAMP-induced transcription are significantly down-regulated in these transfectants,
indicating that membrane-bound PKA positively regulates cAMP-induced transcription (Fig. 6). The effects elicited by AKAP45 are solely dependent on RII binding because this mutant does not bind membrane, PKC, or calcineurin (19, 21). Recently, we have shown that a version of AKAP45, which does not bind RII, does not bind membrane, PKC, or calcineurin (19, 21).

PC12 cells expressing AKAP45 mimic A126 mutant cells and become refractory to cAMP signaling. Thus, these cells proliferate and fail to differentiate when exposed to cAMP. Biochemical comparative analysis of PC12 cells expressing AKAP45 indicate that they are very similar to A126 because 1) endogenous RII levels are not altered (Ref. 16, and data not shown), 2) cytoplasmic PKA is not efficiently activated by cAMP in PC12-A45 and A126 cells (Ref. 16, and Fig. 5), and 3) the expression of RII-binding proteins is down-regulated (Figs. 1 and 4). We have previously shown that A126 cells contain an excess of R subunits in the cytosol that efficiently buffers exogenous C-PKA, added to the extracts. Thus, PKA in these cells is efficiently activated in vitro but not in vivo (16). We have reproduced this phenotype in PC12 that express AKAP45, which do not respond to cAMP (Figs. 5 and 8). We suggest that excess of R subunits in the cytosol inhibits PKA dissociation and reduces cAMP-dependent activation of the enzyme. PC12 cells expressing AKAP45 show the same PKA phenotype as A126 cells.

As to the endogenous PKA anchor proteins in PC12, a comparative analysis of the overlay blots of the AKAP45 and AKAP75 transfectants indicates that 1) two major protein species binding RII are present in the membranes of PC12 cells, one ~150 kDa and the other ~85 kDa of molecular mass (Fig. 4, panel A); 2) the expression of both proteins is reduced in A126 and PC12-A45 cells; and 3) the levels of expression of each of the endogenous RII-binding proteins is modulated differentially in response to cAMP signaling (Fig. 1, panel A, see also Ref. 27 for cAMP-induced AKAP). The reduction of the AKAP proteins in A126 might be a consequence of the down-regulation of the expression of cAMP-induced genes (27). This further down-regulated cAMP-induced transcription and differentiation. The definition of the biological roles of the PC12 AKAPs requires the molecular cloning of the species indicated above. In conclusion, we have demonstrated that membrane-bound PKA amplifies cAMP signals to the nucleus, and it is essential in cAMP-induced differentiation of PC12 cells.

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