The Type and the Localization of cAMP-dependent Protein Kinase Regulate Transmission of cAMP Signals to the Nucleus in Cortical and Cerebellar Granule Cells*

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CAMP signals are received and transmitted by multiple isoforms of cAMP-dependent protein kinases, typically determined by their specific regulatory subunits. In the brain the major regulatory isoform RIIβ and the RII-anchor protein, AKAP150 (rat) or 75 (bovine), are differentially expressed. Cortical neurons express RIIβ and AKAP75; conversely, granule cerebellar cells express predominantly RIα and RIα. Cortical neurons accumulate PKA catalytic subunit and phosphorylated cAMP responsive element binding protein very efficiently into nuclei upon cAMP induction, whereas granule cerebellar cells fail to do so. Down-regulation of RIIβ synthesis by antisense oligonucleotides inhibited cAMP-induced nuclear signaling in cortical neurons. Expression in cerebellar granule cells of RIIβ and AKAP75 genes by microinjection of specific expression vectors, markedly stimulated cAMP-induced transcription of the lacZ gene driven by a cAMP-responsive element promoter.

These data indicate that the composition of PKA in cortical and granule cells underlies the differential ability of these cells to transmit cAMP signals to the nucleus.

CAMP formed by adenyl cyclases after stimulation of G-protein-coupled receptors binds the regulatory subunits (R) of the tetrameric PKA1 holoenzyme and promotes dissociation of the catalytic subunits (C-PKA). A fraction of C-PKA translates to the nucleus and stimulates CAMP-dependent gene expression (1–3). Multiple isoforms of PKA are determined by their specific regulatory subunits. Four regulatory subunits (RIα, RIIα, RIβ, and RIIβ) have been cloned. PKA containing RIIβ is the predominant PKA isoform in the brain and is expressed in the cortex, whereas in the brainstem and cerebellum (except Purkinje cells) RIIβ has not been found (4–6). In mammalian brain, signals triggered by cAMP are targeted to specific effector sites by the tethering of cAMP-dependent protein kinases to intracellular compartments (4, 7, 8). PRAI1 is bound to membranes via specific anchor proteins (AKAPs), which bind R subunits. Bovine brain AKAP75 has been studied as prototype of kinase A anchor protein and shares high homology with human AKAP79 and rat AKAP150 (9, 10). These proteins have similar properties, related sequences, and are recognized by the same antibodies (9–11). AKAP150/75 and RIIβ are co-localized in the dendritic cytoskeleton and perikarya of forebrain neurons; both proteins have not been found in cerebellar granule cells (6).

Although the structure and expression pattern of the PKA regulatory subunits and AKAPs are well documented, the functional role of these proteins in the transduction of cAMP signals is still poorly understood. It is not known how the different PKA isoforms in different districts of the central nervous system receive and transmit cAMP signals.

We have chosen primary cortical and granule cerebellar neurons as prototype cells with different PKA composition and localization. PKA in cortical neurons is mainly of IIβ type and is membrane-associated by AKAP150/75. Conversely, granule cerebellar cells do not express AKAP75/150 and RIIβ. The R subunits expressed by these cells are RIα and RIα (6, 12).

We have studied the activation by CAMP of these enzymes and the transmission of the signals to the nucleus by measuring the accumulation of C-PKA in the nucleus, CREB phosphorylation, and the transcription of a CAMP-induced promoter following CAMP stimulation. Also, we have manipulated the composition of PKA in granule and cortical cells by down-regulating RIIβ in cortical cells or by expressing AKAP75 and RIIβ in granule cells, respectively.

The results presented here indicate that RIIβ and the PKA-anchor protein, AKAP75, amplify the transmission of CAMP signals to the nucleus and suggest that the composition of PKA might influence the ability of the cell to receive and transmit CAMP signals to the nucleus.

MATERIALS AND METHODS

Primary Cultures of Cortical and Cerebellar Granule Cells—Primary cultures were obtained as described previously for striatal neurons with...
some modifications (13). Briefly, cortices of 16-day-old rat embryos were dissected and incubated with papain. Tissue fragments were mechanically dissociated and the cells plated in polylysine-coated dishes in 1:1 minimum Eagle’s medium/F12 medium containing 2 mM glutamine and 10% fetal calf serum. Cerebellar granule cells were obtained from 7-day-old rats by trypsinization. Tissue fragments were mechanically dissociated. The cells were plated in polylysine-coated dishes in 25 cm² BME containing 10% fetal calf serum. 24 h after plating, 10 μM Cytosine C Arabinoside was added to the cultures to prevent the growth of non-neuronal cells. Under these conditions the contamination of glial cells, measured by staining with glial fibrillar proteins, was less than 10%. For each experiment, 7-day-old rat embryos were used (21, 22). AKAP plasmids contained the sequence of AKAP75 bound to a specific DNA sequence motif (CRE) and, upon phosphorylation of CREB peptide (peptide 31–53 of CREB), it binds to a region of the rat sequence of RIIα (peptide 200–230). The residues 53–73 from the start codon of the rat protein (cross-linked to soybean trypsin inhibitor). The total IgGs were purified, and the specificity of each preparation was tested by immunoprecipitation, immunofluorescence, and immunoblot by preadsorbing the antibodies to the specific peptides or control peptides (12, 18).

Microinjection of DNA Expression Vectors—Cerebellar granule cells were grown for 7 days and injected with the DNA solutions in phosphate buffer (25 ng/ml of each plasmid). The plasmid vectors used were: CMV-GFP (CLONTECH); RSV-lacZ; CRE-lacZ containing five CRE elements and the vasoactive intestinal peptide promoter driving lacZ gene (19, 20). In some experiments plasmid vectors expressing C-PKA were used (21, 22). AKAP plasmids contained the sequence of AKAP75 or AKAP45 driven by the cytomegalovirus promoter (20). All plasmids were prepared in the rat thyroid cell line, FRTL-5, and in the PC12 cell line by stable transfection. The expression of the specific proteins was measured by immunonanlyses (immunoblot, immunofluorescence, and immunoprecipitation) and Northern blot (18, 24).

The injection apparatus consisted of a phase contrast microscope connected to a computer-aided image analyzer (AIS automatic image system, Gyrus, Germany). A computer-operated microinjector (Ep- pendorf, Germany). In each experiment 150 cells/dish and 2 dishes/ DNA were injected. 18 h after the injection the cells were stimulated with 0.1 mM forskolin in the presence of 100 mM IBMX for 4 h, washed in phosphate-buffered saline solution and fixed with 4% paraformaldehyde for 30 min. Cells were permeabilized with 0.1% Triton X-100 and after extensive washes were incubated first with the monoclonal anti-β-galactosidase antibody (Sigma) and then with fluorescein-tagged goat anti-mouse IgG antibody (Sigma) in phosphate-buffered saline containing 0.2% skin porcine gelatin for 30 min at room temperature. In all experiments, the fluorescein-tagged anti-mouse IgG antibodies were injected alone as control of the microinjection procedures. The specificity of the signal was tested by omitting the first antibody. Under our conditions the efficiency of injection was about 20%.

**RESULTS**

**Differential Response to cAMP of PKA in Granule Cells and Cortical Neurons**—The binding of cAMP to the inactive PKA tetrameric holoenzyme induces its dissociation, thereby releasing active catalytic subunits in the cytoplasm. We have studied cAMP signaling in primary cultures of cortical neurons and cerebellar granule cells by measuring the cytoplasmic and nuclear PKA catalytic activity following cAMP stimulation for various periods of time. We have used forskolin, a stimulator of adenyl cyclase, and IBMX, the inhibitor of cAMP phosphodiesterases, to maintain constant cAMP levels. Fig. 1A shows the time course of cytoplasmic PKA activity in cortical and granule cells stimulated by 10 μM forskolin in the presence of 0.5 mM IBMX. In granule cells, cAMP-induced PKA activity was marked and persistent. In cortical cells PKA activation was lower, peaking at 15 min of cAMP stimulation and returning to the basal value within 20 min. The activation of PKA in granule cerebellar cells reached the plateau between 1 and 5 min and remained constant up to 30 min after the initial cAMP stimulation. The cAMP dose-response curve indicated that the cerebellar granule cells PKA was activated at lower cAMP concentration relative to PKA in cortical neurons (Fig. 1B).

Nuclear C-PKA accumulation, a sensitive marker of cAMP stimulation, was very efficient in cortical cells compared with granule cerebellar cells (Fig. 1C), although the cytoplasmic PKA activity was similar in the two cell types (Fig. 1D). The cortical enzyme, albeit dissociated poorly, contributed significantly to the total mass of nuclear PKA because the absolute amount of cytoplasmic PKA was higher in cortical than in granule cerebellar cells (see the legend of Fig. 1).

These data indicate that the granule cells PKA, stimulated by cAMP, drains effiently, but does not transmit C-PKA to the nucleus. In contrast, cortical cells respond very efficiently to cAMP with a significant increase of nuclear C-PKA activity.

We suggest that the composition of PKA in the two cell types might be the cause of the different responses to cAMP. Indeed, the regulatory RII type is abundantly expressed in cortical cells, is membrane-bound, binds cAMP with lower affinity and has a longer half-life compared with RIs and RIIα (8, 12, 15, 18, 25). These features suggest that PKAIIR might be the sensor of continuous and persistent cAMP signals.

**CREB Phosphorylation in Granule and Cortical Cells**—The biologically relevant effect of PKA translocation to the nucleus is the regulation of gene expression mediated by the phosphorylation of the nuclear transcription factor CREB. CREB is bound to a specific DNA sequence motif (CRE) and, upon phosphorylation by PKA catalytic subunit (26) or Ca³⁺/calmodulin-dependent protein kinase IV (27), binds the adaptor proteins (CBP and p300). The association of CREB with these proteins facilitates the assembly of the transcriptional machinery and leads to the activation of cAMP-induced genes (28). We assayed CREB phosphorylation in cortical and granule cells stimulated with forskolin or CPM and immunoblot of nuclear proteins with antibodies that recognize the phosphorylated form of CREB (17). Fig. 2 shows that the phosphorylation of CREB (PCR) was strongly induced by forskolin (10 μM, 10 min) in cortical neurons. In granule cells the PCR signal was detectable in basal conditions but was only slightly induced by forskolin.
did not induce the de novo synthesis of CREB, because the total amount of the protein was not modified by forskolin treatment (Fig. 2). The phosphorylation of CREB was induced by a cAMP-dependent pathway, because it was mimicked by the cAMP analogue, 8-bromo-cAMP and was inhibited by 10 μM of the cell-permeable PKA inhibitor, H89 (14) (data not shown).

Because the PCREB signal detected by Western blot might originate from contaminating glial cells present in our cultures, we have investigated the effect of cAMP-elevating agents in pure type I astrocyte cultures. Under the conditions described in Fig. 2, stimuli-dependent cAMP accumulation did not induce CREB phosphorylation (data not shown).

Inhibition of RII β Expression Impairs Nuclear Response to cAMP in Cortical Neurons—To test whether the expression of PKAII β affects CREB phosphorylation in cortical cells, we treated cortical neurons with specific anti-RII β antisense oligonucleotides for 72 h (see “Materials and Methods”). This treatment specifically reduced RII β protein levels (over 50% reduction, Fig. 3 lane 2), but did not affect the other membrane-bound PKA regulatory subunit RII α, as shown by the immunoblot with specific anti-RIIβ or RIIα antibodies (Fig. 3). The reduction of RII β protein was also detected by the ligand binding assay or overlay (23, 29) in RIIβ antisense-treated cell extracts. Also, total PKA activity was not affected by anti-RIIβ antisense treatment (data not shown). RII β levels did not change in cells treated with mismatched oligonucleotides (Fig. 3, lane 1 and legend).

Cortical cells treated with the RII β antisense oligonucleotide were stimulated with forskolin and CREB phosphorylation as-
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**Fig. 3.** Treatment with RIIβ antisense oligonucleotides reduces RIIβ content in cortical neurons. Representative immunoblots of total cellular extracts from cortical neurons treated with mismatched (1) or anti-RIIβ antisense oligonucleotides (2) using specific anti-RIIα (upper panel) or anti-RIIβ (lower panel) specific antibodies (see “Materials and Methods”). The lower graph shows the relative amount of RIIα and RIIβ derived from the densitometric analysis of the immunoblots in three experiments. The treatments with specific oligonucleotides are described under “Materials and Methods.” RIIβ and RIIα content was not influenced by treatment with mismatched oligonucleotides. Some experiments we noticed a 25% reduction of both RII proteins in cells treated with nonspecific oligonucleotides.

**Fig. 4.** Down-regulation of RIIβ reduces phosphorylated CREB and C-PKA in the nuclei of cortical neurons. Top panels, immunoblot of phosphorylated CREB in nuclear extracts of cortical neurons treated with mismatched oligonucleotides (1) or RIIβ specific antisense (2) and stimulated with 10 μM forskolin (FSK) as described in Fig. 2. Total CREB is shown in the lower blot obtained from the same cell extracts. Lower panels, nuclear C-PKA accumulation in cortical cells treated with mismatched oligonucleotides (1) or RIIβ specific antisense (2) and stimulated with (+) or without (−) 10 μM forskolin (FSK) as described in Fig. 2. The basal nuclear C-PKA in mismatched or RIIβ oligonucleotide-treated cells was 2.8 ± 0.5 pmol/min/μg in cortical cells and 2.7 ± 0.4 pmol/min/μg, respectively.

To test the effects of the expression of RIIβ and AKAP75 on cAMP-induced transcription, a reporter gene (lacZ) driven by five tandemly linked cAMP-responsive elements, (5× CRE-lacZ), was co-injected with the plasmids indicated above. Cells were stimulated for 4 h with 1 μM forskolin, and the expression of the lacZ gene under control of the CRE promoter was monitored by immunofluorescence with anti-β-galactosidase antibodies. Fig. 6 shows a representative picture of granule cells microinjected with the combination AKAP75-RIIβ and CRE-lacZ genes. Treatment with forskolin for 4 h significantly increased the number of lacZ positive cells.

**Table I** shows that cells microinjected with a control plasmid carrying the lacZ fused to a constitutive non-cAMP-dependent promoter (the long terminal repeats of Rous sarcoma virus, RSV-lacZ) efficiently synthesized β-galactosidase. Cells microinjected with the CRE-lacZ construct did not show β-galactosidase signal following forskolin stimulation. Injection of RIIβ or AKAP75 expression vectors stimulated CRE-lacZ expression (3 to 6 and 2.8 to 5 positive cells). Co-injection of RIIβ and AKAP75 expression vectors resulted in a marked increase in the number of lacZ-expressing cells (4 to 14 positive cells, − or + forskolin, respectively). The number of positive cells was dramatically reduced if a mutant version of AKAP75 was co-injected with RIIβ, AKAP45, which binds RII but fails to localize it to the membranes (18, 32, 33) or with a vector expressing PKI, the specific PKA inhibitor (34). Moreover pretreatment of microinjected cells with 10 μM H89, a PKA inhibitor (14), resulted in a significant reduction of β-galactosidase-expressing cells (data not shown). The data, derived from six independent
periments, indicate that the combination of RIIβ-AKAP expression vectors significantly increased the number of β-galactosidase positive cells in the presence of cAMP.

DISCUSSION

Targeting protein kinases and phosphatases in proximity of their substrates represents an important mechanism to convey intracellular signals to specific cellular sites (4, 7). Two different protein families bind PKA to the cell compartments: the Akinase anchor proteins AKAPs and the microtubule-associated proteins MAP2. AKAPs bind the regulatory subunit RIIβ with nanomolar affinity and localize PKA to the dendritic cytoskeleton and other cellular compartments (Golgi apparatus, primary branches of dendrites and perikarya), whereas MAP2 predominantly binds the RIIα isoform (5). AKAP 150/75, the rat or the bovine PKA anchor protein, can also bind RIIα but with lower affinity (35–37). The levels of RIIβ and AKAP 75 differ in cell populations of brain areas and their expression pattern is strictly correlated. Accordingly, in cortical neurons, which express AKAP 75, RIIβ is the most abundant PKA regulatory subunit; conversely, in cerebellar granule cells AKAP 75 and RIIβ has not been found (5, 8, 12). Because the amounts of RIIα and MAP2 are comparable in both cell populations (8, 12), we decided to test if the expression of RIIβ-AKAP might interfere with the cytosolic and nuclear responses to cAMP in cortical neurons and cerebellar granule cells. PKA activity and CREB phosphorylation were measured as functional correlates of cAMP stimulation. Although it has been shown that CREB is also a substrate for other protein kinases (27, 38), we have analyzed only cAMP-induced events.

![Image](image_url)

**Fig. 5. Expression of exogenous RIIβ and AKAP75 genes in cerebellar granule cells.** Cerebellar granule cells were microinjected with DNA vectors expressing RIIβ, AKAP75, and green fluorescent protein genes as described under "Materials and Methods." 24 h later the cells were fixed and with specific antibodies to AKAP75 or RIIβ. Column A shows the phase-contrast microphotograph of microinjected cells. Column B shows the staining of GFP-positive cells, indicated by arrows, with antibodies to AKAP75 (1) or to RIIβ (2 and 3). The efficiency of the microinjection was ca. 20%. The expression of the microinjected gene was maximal at 24–36 h after the injection. In 2B and 3B the faint signal visible in noninjected is presumably originated by the RIIβ endogenous gene or by cross-reactivity with RIIα.

**Table I** shows the number of positive cells (see "Materials and Methods").

| DNAa | CRE-LacZ | RSV-LacZ |
|------|----------|----------|
|      | −FSK     | +FSK     |
| RSI-LacZ | 16.0 ± 1.0 | 15.0 ± 1.0 |
| CRE-LacZ | 0.5 ± 0.2 | 0.8 ± 0.5 |
| +RIIβ | 3.0 ± 0.3 | 6.0 ± 0.6 |
| +A75 | 2.8 ± 0.5 | 5.1 ± 0.8 |
| +RIIβ+A75 | 4.1 ± 1.2 | 14.0 ± 1.2 |
| +RIIβ+A45 | 3.5 ± 0.5 | 4.9 ± 0.6 |
| +RIIβ+A75+PKI | 3.5 ± 0.4 | 4.5 ± 1.2 |

“Indicates the DNA expression vectors used for microinjection (see "Materials and Methods").

The number of positive cells is relative to 100 cells, and it is the mean of six experiments.

The response to cAMP signals was different in granule cerebellar and cortical cells. In granule cerebellar cells, cytoplasmic PKA dissociated very efficiently, but nuclear C-PKA accumulation induced by cAMP was rather weak. Cortical cells, on the other hand, did not activate efficiently cytoplasmic PKA but accumulated PKA in the nuclei in response to cAMP. The different PKA composition and localization in the two cell types might account for these different responses. Granule cells contain mainly type I and type IIα PKA, which bind cAMP with higher affinity compared with type PKAIIβ (15, 18, 25). These subunits are soluble (RIα) or partly soluble (RIIα) in the cytosol (10, 12). PKAI and PKAIIS dissociate efficiently at low cAMP levels (Fig. 1B). PKA present in cortical cells, composed mainly of type IIβ isoenzyme, dissociates at high cAMP levels and rapidly reassociates (Fig. 1, A and B). Membrane-bound PKAII might be the sensor of persistent cAMP signals and the preferential source of nuclear C-PKA in cortical cells. This interpretation is strengthened by the observation that the CAMP-dependent nuclear signaling is inhibited in cortical cells with lower RIIβ levels (Fig. 4) or in the RIIβ-defective mouse (30, 31).
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Increasing evidence indicate that CREB is a multifunctional transcription factor that can be activated by cAMP and Ca^{2+}-dependent transduction pathways (41, 42), and its activation is critical for long-term memory formation in different biological systems (43–46). Stimuli that generate long-lasting long-term potentiation have been shown to induce CRE-mediated gene expression that was reduced by L-type Ca^{2+} channel blockers (14), but the cAMP pathway appears to be necessary for the cellular processes related to long-term memory (20, 47).

As to the biological correlates of our findings, we suggest that the differential composition and localization of PKA in discrete populations of neurons might explain the different activation of cAMP-induced transcription in these cells by the same type of signal. Consistent with our data, it has been found that in RIIB defective mouse gene induction by cAMP in striatal neurons is significantly inhibited (31).

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![Fig. 7. Coexpression of RIIB and AKAP75 genes in cerebellar granule cells stimulates cAMP-induced transcription. Cells were microinjected with GFP, RIIB, AKAP75, and CRE-lacZ as described under “Materials and Methods.” 15 h after the microinjection, the cells were treated with 50 μM forskolin and 0.5 mM IBMX for 5 h (indicated in the top panels, +). Phase-contrast microphotographs and staining with anti-β-galactosidase antibody are shown on the left and right, respectively.](image-url)
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