

Protein kinase A and human disease

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A symposium on Protein Kinase A and Human Disease was held at the Warren–Magnuson Clinical Center of the NIH, Bethesda, MD, USA, on 10 September 2001.

At a time when the importance of cAMP as a messenger in many hormonal systems was established and the complexity of its functions was becoming apparent, human diseases that were linked to abnormal cAMP responses, such as irregular cell growth and proliferation, were beginning to be elucidated at the molecular level. McCune–Albright syndrome (MAS) and some sporadic growth-hormone (GH)-producing pituitary tumors were found to be caused by mutations in the *gsp* oncogene, the *GNAS1* gene, which codes for the stimulatory α subunit of the G proteins ($G\alpha$). A series of mutations in G-protein coupled receptors (GPCRs) was then identified in tumor-predisposing disorders or sporadic endocrine tumors [1].

More recently, *PRKARIA*, the gene encoding $RI\alpha$, the relatively abundant regulatory subunit type 1A of cAMP-dependent protein kinase A (PKA), the central hub of all cAMP-related signaling, was identified as the gene responsible for the multiple endocrine neoplasia syndrome known as Carney complex (CNC) [2–4]. This is the first time that cAMP-dependent PKA itself was found to be directly involved in oncogenesis in an inherited disease.

This article summarizes several presentations on cAMP and PKA signaling and their role in health and disease given during a recent international meeting held at the NIH that was sponsored by the NICHD; a more comprehensive report and the list of participants are available elsewhere [5].

$G\alpha$ (*GNAS1*): beyond McCune–Albright syndrome

Suzanne Jan de Beur (Johns Hopkins University, Baltimore, MD) and Lee Weinstein (NIDDK, NIH, Bethesda, MD) reviewed the involvement of $G\alpha$ mutations in human disease and

presented new data on imprinting of the *GNAS1* gene from both human and animal studies. The heterotrimeric $G\alpha$ couples transmembrane receptors to adenylyl cyclase and is therefore required for hormone-stimulated cAMP production. Its single-copy gene (*GNAS1*) is located at 20q13; its frequent mutations Arg201 and Gln227 (residues that are required for the GTPase-‘turn-off’ mechanism) lead to constitutive activation and elevated intracellular cAMP. Such somatic mutations are present in a subset of human GH-secreting pituitary tumors and in patients with MAS (a syndrome characterized by acromegaly, ACTH-independent macronodular adrenal hyperplasia, hyperfunctional thyroid nodules and peripheral precocious puberty). Heterozygous inactivating $G\alpha$ mutations produce Albright hereditary osteodystrophy (AHO); maternal inheritance of these mutations leads to resistance to PTH and other hormones (pseudohypoparathyroidism type Ia), whereas paternal inheritance leads only to AHO (pseudopseudohypoparathyroidism). Inactivating $G\alpha$ mutations also lead to progressive osseous heteroplasia (POH). Studies in humans and mice suggest that parental effects in the phenotypes of these disorders are due to tissue-specific imprinting of $G\alpha$, resulting in maternal-specific expression of $G\alpha$ in specific hormone target tissues and biallelic expression of $G\alpha$ in most other tissues. Pseudohypoparathyroidism type Ib (renal PTH resistance without AHO) is associated with a $G\alpha$ imprinting defect with both parental alleles having a ‘paternal-specific’ imprinting pattern. This imprinting defect is predicted to decrease $G\alpha$ expression in tissues where $G\alpha$ is maternally expressed (e.g. proximal renal tubules, resulting in PTH resistance) but has no effect on $G\alpha$ expression in most other tissues. In GH-secreting pituitary tumors, the $G\alpha$ -activating mutations are almost always in the maternal allele, suggesting that parental origin might also affect the phenotypic consequences of activating $G\alpha$ mutations [1].

Novel mechanisms of cAMP signaling through PKA or other pathways

The PKA holoenzyme is composed of two genetically distinct subunits, catalytic (C) and regulatory (R), forming a tetrameric holoenzyme R₂C₂ that dissociates in the presence of cAMP into an R₂(cAMP)₄ dimer and two free catalytically active C subunits (Fig. 1). The only known function for the R subunit is that of inhibiting the C subunit kinase activity. Khew Voon Chin (The Cancer Institute of New Jersey, New Brunswick, NJ) showed previously that genetic mutants of the $RI\alpha$ subunit, but not the C subunit, exhibit resistance to the anticancer agent cisplatin. Transfectants harboring the mutant $RI\alpha$ subunit are also resistant to cisplatin. He found subsequently by yeast two-hybrid screening that the cytochrome c oxidase (Cox) subunit Vb interacts with $RI\alpha$ and the Cox activity is regulated by cAMP. Chin reported that $RI\alpha$ associates with a novel BTB/POZ domain zinc finger transcription factor, RIAZ. Members of the family of BTB/POZ domain zinc finger proteins, including PLZF and BCL6, are associated with oncogenesis. Cotransfecting the fusion of green fluorescence protein to RIAZ (GFP–RIAZ) with $RI\alpha$ results in cytoplasmic localization. The presence of cAMP causes GFP–RIAZ to translocate from the cytoplasm into the nucleus. Deletion of the C-terminus of RIAZ abolishes its interactions with $RI\alpha$ and GFP–RIAZ localizes in the nucleus. RIAZ is aberrantly expressed in a panel of breast cancer cell lines, thus suggesting its role in oncogenesis. These results showed that $RI\alpha$ interacts with and regulates the activity of other proteins in addition to the C subunit kinase [6]. Jacques Dumont (University of Brussels, Belgium) went further and reported that although PKA activation is necessary for all the effects of cAMP, it is clearly not sufficient for all of them [7].

Inherited mutations of $RI\alpha$ cause a multiple tumor syndrome

CNC is an multiple endocrine neoplasia (MEN) syndrome [2]. The complex has

features overlapping those of MAS and other MENs and has been mapped to chromosomes 2 and 17. Because of several similarities to MAS, genes implicated in cyclic nucleotide-dependent signaling had been considered candidates. The gene coding $RI\alpha$, *PRKARIA*, had been mapped to 17q. Constantine Stratakis (NICHD, NIH, Bethesda, MD) reported that loss-of-heterozygosity (LOH) analysis using markers from this region revealed consistent changes in CNC tumors. Lawrence Kirschner from Stratakis' laboratory, cloned the *PRKARIA* genomic structure; sequencing of the gene showed mutations in half of the existing CNC patients [3,4]. Nonsense-mediated mRNA decay was shown in the cells of these patients; the predicted mutant *PRKARIA* protein products were also absent from these cells. In CNC tumors, PKA activity showed increased stimulation by cAMP [3]. Other tumors, including thyroid neoplasms, show mutations of the *PRKARIA* gene. Stratakis concluded from these studies that *PRKARIA* gene behaves, perhaps, as a tumor suppressor in tissues affected by CNC and in some sporadic tumors [2–4].

Non-inherited mutations of $RI\alpha$ are associated with lupus

Systemic lupus erythematosus (SLE) is an idiopathic autoimmune disease characterized by impaired T lymphocyte immune effector functions. Gary Kammer (Wake Forest University School of Medicine, Winston-Salem, NC) reported that cAMP-stimulated, PKA-catalyzed protein phosphorylation is markedly diminished in SLE T cells. Competitive PCR revealed a marked reduction of $RI\alpha$ and $RI\beta$ mRNA and protein levels in these cells. This was associated with a 30% decrease in $RI\alpha$ protein and a 65% reduction in $RI\beta$ protein. T cells from ~25% of SLE subjects had no detectable $RI\beta$ protein. Transient transfection of T cells not expressing $RI\beta$ protein with SLE patient $RI\beta$ cDNA bypassed the block in translation, reconstituting PKA activity and augmenting interleukin-2 production. Of importance was the initial identification of novel *PRKARIA* mRNA mutations, including deletions, transitions and transversions [8]. Kammer concluded that distinct mechanisms account for deficient PKA-I and PKA-II isozyme activities in SLE T cells.

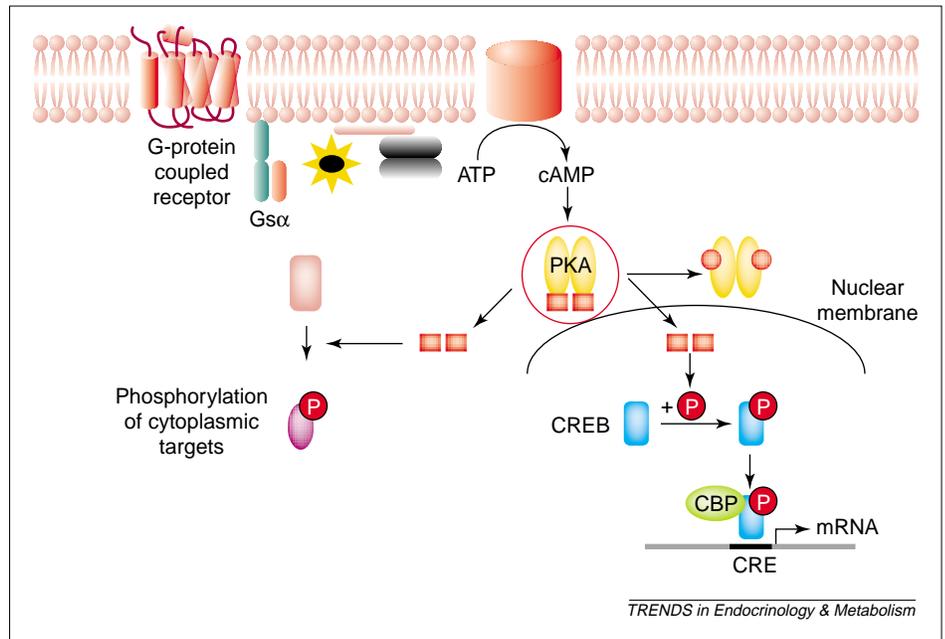


Fig. 1. The cAMP-dependent signaling system starts from the ligand-dependent activation of the G-protein-coupled receptors (GPCRs) and is followed by $G\alpha$ activation, cAMP generation (through adenylyl cyclase) and PKA activation. PKA activation is essentially synonymous with cAMP binding to the regulatory subunits (RIs or RIIs) and release of the catalytic subunits from the PKA tetramer; phosphorylation of targets in the cytoplasm and in the nucleus (mainly CREB but perhaps also other factors) follows.

Complete inactivation of $RI\alpha$ in mouse results in early embryonic lethality

Paul Amieux and Stanley McKnight (University of Washington School of Medicine, Seattle, WA) addressed the function of $RI\alpha$ in mammalian physiology by generating a targeted disruption of the *Prkar1a* gene in mice using homologous recombination in embryonic stem cells. Unlike the three other regulatory subunits for which equivalent targeted disruptions have been made, homozygous *Prkar1a*-knockout embryos were not viable. A closer examination of embryos from *Prkar1a*-heterozygous mating suggested that *Prkar1a*-knockout embryos were already resorbed by day 10.5, owing to some form of cardiovascular failure. Inspection of sections from day 8.5 wild-type and *Prkar1a*-knockout embryos revealed a complete failure in heart tube formation in the mutant embryos. Marker analysis and cell counts at earlier stages of development pointed to a defect in mesoderm production at the primitive streak stage of embryogenesis. Consistent with this observation, the investigators noted later deficiencies in all mesoderm-derived lineages, including axial, paraxial and lateral plate mesoderm. The phenotype of *Prkar1a*-knockout embryos could be rescued by crossing them to mice carrying a targeted disruption of the *Prkaca* gene, supporting the hypothesis that it was

inappropriately regulated catalytic subunit activity that caused the *Prkar1a*-knockout phenotype and not an unknown or unique function of $RI\alpha$. Isolation of fibroblasts from wild-type and *Prkar1a*-knockout embryos revealed a significant alteration in actin-based cytoskeletal morphology. These abnormalities in the actin-based cytoskeleton could account for the failure of mesodermal fibroblastic cells to migrate away from the primitive streak and successfully colonize structures such as the embryonic heart.

cAMP signaling in cancer genesis and treatment

William Miller (University of Edinburgh, Scotland, UK) reviewed evidence that links the expression of PKA signaling with the natural history of breast cancer: (1) breast cancers overexpress PKA in comparison with normal breast; (2) $RI:RII$ ratios are significantly higher in specimens of normal breast that demonstrate increased proliferation; (3) although the $RI:RII$ ratio varies greatly among different breast cancers, those with high $RI:RII$ are associated with poor prognosis in terms of early disease recurrence and death following primary treatment; (4) decrease in *PRKARIA* mRNA following treatment with the antiestrogen, tamoxifen, in responding but not in nonresponding tumors. Thus, it is relevant that increased expression of PKA

could change sensitivity to estrogens and antiestrogens in cancer cells; (5) in model systems of breast cancers (cell lines and xenografts), exposure to antisense R1 α oligonucleotides slows cell proliferation and tumor growth.

Links of PKA with cancer make this holoenzyme a clear target for novel cancer therapies; Giampaolo Tortora (University of Napoli, Italy) and his coworkers have contributed to the identification and development of a variety of selective inhibitors of PKA-type I, including a hybrid DNA–RNA mixed backbone oligonucleotide. They have demonstrated that the PKA-type I inhibitors have a cooperative antitumor effect with several cytotoxic drugs *in vitro* and *in vivo*, especially taxanes and epidermal growth factor receptor inhibitors.

These and other studies [9] provide the molecular proof of the initial hypothesis: two isoforms of the regulatory subunits (RI and RII) of cAMP-dependent protein kinase that bind cAMP are inversely expressed during ontogeny and cell differentiation. Together, these cAMP-binding receptor proteins might regulate the growth of normal cells and their differentiation into nondividing states. Cancer cells can also be made to differentiate and stop growing when the functional balance of these receptor proteins is restored by treatment with site-selective cAMP analogs or with an antisense oligodeoxynucleotide, suggesting new approaches to cancer treatment [10]. Yoon Cho-Chung (NCI, NIH, Bethesda) showed how cDNA microarrays paint the molecular portrait of the reverted tumor phenotype that is inducible with antisense-suppression of R1 α , restoring the normal balance of RI and RII in tumors that undergo regression [11].

'Downstream' effects and possible 'parallel' interactions of PKA activation

Jérôme Bertherat (University of Paris, Cochin Hospital, Paris, France) reported his work on CREB, the ubiquitous ultimate effector of most PKA actions in the nucleus (Fig. 1). CREB binds to the cAMP response element (CRE) after phosphorylation on Ser133 by PKA; it then stimulates transcription. Targeted expression of dominant negative mutants of CREB in transgenic mice lead to somatotrophs or thyroid hypoplasia. In GH-producing adenomas, CREB is always expressed and is highly phosphorylated by PKA. After transfection in pituitary GH-cells, the mutant *gsp*, as well as

overexpression of wild-type G α , stimulates transcription of various CRE-dependent promoters via CREB in a specific Ser133-dependent manner. Activation of the cAMP pathway by ACTH is required for adrenal cortex activity. Alterations of CRE binding proteins are observed in the human adrenocortical cancer cell line H295R; CREB expression is greatly reduced or completely lost in the majority of adrenocortical cancers and a subset of benign adrenocortical adenomas [12].

CREB represents a 'vertical' interaction of the PKA system; however, several important interactions in signaling pathways occur in parallel circuits. Such novel interactions were either documented by Michael Beaven (NHLBI, NIH, Bethesda, MD), who presented evidence that PKA, PKC and CAM kinase II synergistically regulate phospholipase D and secretion in mast cells, or hypothesized (on the basis of clinical similarities with CNC) by Charis Eng (Ohio State University, Columbus, OH), who presented her work on Cowden syndrome (a disease with similarities to CNC) and the phosphatase PTEN that is responsible for it.

cAMP and PKA – directors of a celestial orchestra

In 1986, Michael Gottesman (NCI, NIH, Bethesda, MD) predicted that '...sustained expression of the [PKA] catalytic subunit, either because of high cAMP levels from an altered adenylate cyclase or phosphodiesterase system, or because of mutations in R or C, could act like an oncogene product in certain cell types'. He had also said that cAMP had reappeared in the study of tumorigenesis 'like a Halley's comet' [13]. This is certainly true today, and to conclude with another space analogy, it is important to remember in future studies of cAMP and PKA, that like 'in 2001: A Space Odyssey, it takes many disconnections to neutralize the computer Hal' [14], cAMP and PKA stand in the middle of several signal transduction cascades that interact in all directions and coordinate a complex concerto.

Acknowledgements

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