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SPECIFICITY IN THE cAMP/PKA SIGNALING PATHWAY. DIFFERENTIAL EXPRESSION, REGULATION, AND SUBCELLULAR LOCALIZATION OF SUBUNITS OF PKA.

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1. ABSTRACT

A large number of hormones, neurotransmitters and other signal substances utilize cyclic adenosine 3'5' cyclic monophosphate (cAMP) as an intracellular second messenger. Cyclic AMP regulates a number of different cellular processes such as cell growth and differentiation, ion channel conductivity, synaptic release of neurotransmitters, and gene transcription The principle intracellular target for cAMP in mammalian cells is the cAMP-dependent protein kinase (PKA). The fact that this broad specificity protein kinase mediates a number of discrete physiological responses following cAMP engagement, has raised the question of how specificity is maintained in the cAMP/PKA system. Here, we will describe features of PKA signaling pathway that may contribute to explain how differential effects of cAMP may be maintained in this pathway.

2. CYCLIC AMP AND THE cAMP-DEPENDENT PROTEIN KINASE (PKA) SIGNALING SYSTEM

Reversible protein phosphorylation is a key regulatory mechanism in eukaryotic cells. Protein phosphorylation was first demonstrated to regulate the activity of glycogen phosphorylase in response to glucagon (1,2). A heat-stable factor mediating the effect of glucagon on the phosphorylation status of glycogen phosphorylase was next identified as 3',5'-cyclic adenosine monophosphate (cAMP) (3), and the concept of cAMP as an intracellular second messenger to a wide range of hormones, neurotransmitters, and other signaling substances was developed (4). The target for cAMP was purified and identified as a cAMP regulated protein kinase (5), termed cAMP-dependent protein kinase (PKA; EC 2.7.1.37). In the absence of cAMP, PKA is an enzymatically inactive tetrameric holoenzyme consisting of two catalytic subunits (C) bound to a regulatory subunit (R) dimer (Figure 1). Cyclic AMP binds co-operatively to two sites on each R protomer [for review, see (6,7)]. Upon binding of four molecules of cAMP, the enzyme dissociates into an R subunit dimer with four molecules of cAMP bound and two free, active C subunits that phosphorylate serine and threonine residues on specific substrate proteins.

At present, the cAMP/PKA signaling pathway is known to be activated by a number of different receptors that upon binding of their respective ligands, transduce their signals over the cell membrane by coupling to G-proteins. These G-proteins interact with adenylyl cyclase on the inner membrane surface either to activate or to inhibit the production of cAMP. Receptors that activates PKA through generation of cAMP, regulates a vast number of cellular processes such as metabolism (8), gene regulation (9), cell growth and division (10), cell differentiation (11,12), and sperm motility (13), as well as ion channel conductivity (14). Therefore, a major question for scientists working in the field of the cAMP/PKA signaling system has been to understand how specificity is maintained in this second messenger system.

3. ISOZYMES OF PKA

Initially, two different isozymes of PKA, termed type I and II (PKAI and PKAII, respectively), were identified based on their pattern of elution from

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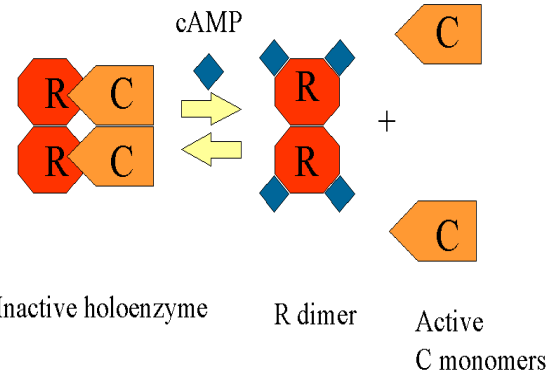

Figure 1. Cyclic AMP-dependent protein kinase (PKA) is a holoenzyme consisting of a regulatory (R) subunit dimer and two catalytic (C) subunits. Activation of PKA occurs when four molecules of cAMP bind to the R subunit dimer, two to each subunit, in a positive cooperative fashion. When both cAMP binding sites (A and B) are occupied the R subunit adopt a confirmation with low affinity for the C subunit and the holoenzyme dissociate. The relation between free C subunits, the R subunit dimer and the intact holoenzyme is an equilibrium which is determined by several factors, that include the relative concentration of PKA subunits, cAMP in addition to salt concentration, pH and temperature.

DEAE-cellulose columns (15,16). The PKAI and PKAII, eluting at salt concentrations between 25 and 50 mM and 150 and 200 mM NaCl, respectively, were shown to contain C subunits associated with two different R subunits, termed RI and RII (6). However, over the last 10 years molecular cloning techniques have revealed a great heterogeneity in both R and C subunits which reveal the potential of multiple isozymes of PKA.

3.1. Multiple isoforms of regulatory and catalytic subunits of PKA

Cloning of cDNAs for regulatory subunits have identified two RI subunits termed RIα (17,18) and RIβ (19,20) and two RII subunits termed RIIα (21,22) and RIIβ (23,24) as separate gene products. The RIα and RIβ subunits are dissimilar, but reveal high homology (81 % identity at the amino acid level) as do the RIIα and RIIβ subunits (68 % identity at the amino acid level). Recently, alternative splice variants of the RIα subunit has been demonstrated. RIα cDNAs with different leader exons and differentially regulated initiation from two promoters of the RIα gene was shown (25).

Furthermore, two distinct C subunits were initially identified by molecular cloning, and were designated Cα (26) and Cβ (27,28). The cloning of the Cα and Cβ subunits from human testis by low homology screening also revealed an additional C subunit, designated Cγ (29). Recent work has also revealed the existence of splice variants of the human form of Cα (Cα2), which is catalytically inactive due to truncation of the C-terminal region resulting in a 224 amino acid protein (30). Furthermore, a splice variant of the bovine form of Cβ (Cβ2) where the mRNA encodes a protein with an additional amino terminal 47 amino acids (31) is identified. Recently, three brain specific splice variants of the mouse Cβ form (Cβ-1, Cβ-2 and Cβ-3) have also been cloned (32). Cβ-1 correspond to the previously described Cβ (27), whereas Cβ-2 and Cβ-3 represent N-terminal truncated splice variants, expected to be catalytically fully active.

3.2. Features of the regulatory and the catalytic subunits of PKA

3.2.1. Structure of the regulatory subunits

The RI and RII subunits contain an amino terminal dimerization domain, a region responsible for interaction with the C subunit, and in the carboxy terminus, two tandem cAMP binding sites, termed sites A and B (33,34). Dimerization was initially discovered by the fact that proteolytic cleavage in the hinge region of the molecule would produce a monomeric R subunit with cAMP binding activity (35). For the RI subunits, dimerization involves two disulfide bridges (Cys16 and Cys37) (36). Dimerization of the RII subunit does not involve cysteines, but the domain responsible for dimerization resides in the amino terminal part of the protein (amino acids 1-30) (37). Despite that residues in the amino terminus (amino acids 1-5) of the RII dimer interact with anchoring proteins, it is assumed that additional contact points within the region 1-82 may exsist (37). The hinge region of the molecule, that has a site sensitive to proteolysis, is involved in binding to the substrate binding site of the C subunit. The RII subunits serve as true substrates and are autophosphorylated by the C subunit. In contrast, the RI subunits are not phosphorylated and bind as pseudosubstrates. Of the two tandem cAMP binding sites, only site B is exposed in the inactive tetrameric PKA complex [reviewed in (7)]. Binding of cAMP to this site enhances binding of cAMP to the A site in a positively co-operative fashion, as a result of a conformational change in the molecule. The characteristics of the two cAMP binding sites have been described in detail elsewhere [reviewed in (6,7,38)] as have the relative affinities and site selectivities of a wide array of chemically modified cAMP analogs (39). The crystal structure of a monomeric RI deletion mutant (1-91) has been reported (40), and provide a model for cAMP- binding

3.2.2. Structure of the catalytic subunits

All the C subunits (Cα, Cβ, Cγ) have catalytic core motifs that are common to all protein kinases (41,42) and involve a MgATP binding site as well as a peptide binding site. The crystal structure of the murine Cα subunit was the first protein kinase crystal structure available (43) and has served as a template for modeling of all the other kinases. The catalytic subunit is a nearly globular protein with two lobes. The small, amino terminal lobe is involved in MgATP-binding, whereas the larger carboxy terminal lobe is involved in peptide binding and catalysis. Both MgATP and the peptide come together for catalysis in the cleft between the two lobes. The C subunits (except the inactive Cα2) contain a domain that involves additional sites apart from the peptide binding site (44). This site is capable of binding the heat stable inhibitor of PKA, PKI. PKI, which contains a NES (nuclear export signal), has the ability of transporting the C subunit from the nucleus to the cytosol

and serves as a major regulator of C subunit activity (45). Interestingly, the Cγ subunit is mutated at amino acid 133 and does not bind PKI and may thus not be exported from the nucleus (45b,46). Furthermore, all the C subunits except bovine Cβ2 and mouse Cβ-2, have the potential of being myristylated at the N-terminus, a modification that may serve to stabilize the C subunit secondary structure (44,47). Despite that the bovine Cβ2 lacks a myristylation site, the N-terminal extension which is hydrophobic, may serve the same function (15).

4. REGULATION OF LEVELS AND EXPRESSION OF THE REGULATORY AND CATALYTIC SUBUNITS

In several cells and tissues extensive studies have been performed in order to demonstrate differential expression of R and C subunits. Levels of expression of the different PKA subunits are subject to regulation by hormones acting through G-protein coupled receptors (48-50), mitogen signals through receptors associated with protein tyrosine kinases (PTK) (51) as well as by steroid hormones (52). Regulation of PKA by hormones acting through cAMP may serve as an autologous sensitization/-desensitization mechanism of the cAMP effector system.

Cyclic AMP mediated regulation of PKA subunits acts through gene transcription (53,54) and mRNA stability (55), as well as altered stability of the R and C proteins after dissociation of the holoenzyme by cAMP (54,56). Protein kinase C represents another major signaling pathway in cells and crosstalk between these two signaling systems is seen beyond cAMP at the level of PKA (57,58).

4.1. Developmental expression of regulatory and catalytic subunits

Gonadal tissues have a high level of α subunits as well as β subunits of PKA, and the rat testis has proved to be a good model system for studies on differential regulation of the various PKA subunits during development. Age studies of whole rat testes revealed distinct developmental changes in the expression of PKA subunits (49,59,60). At prepubertal stages, 10-15 days of age, the presence of RIα (2.8 and 3.2 kb), RIIα (6.0 kb), RIIβ (3.2 kb), and Cα (2.4 kb) mRNAs was detected. These are the mRNA species seen in somatic cells. During early puberty, 15-25 days of age, germ cells increase exponentially and the first haploid cells are observed between 21 and 24 days of age. At later stages the large number of germ cells dominate the testis and dilute signals from somatic cells in whole testis studies. During this time period, the large RIα, RIIα, and RIIβ mRNAs declined concomitantly with the appearance of low molecular weight mRNAs of RIα (1.7 kb), RIIα (2.2 kb), and RIIβ (1.6 kb). These shorter messages result from germ cell specific use of alternative polyadenylation site signals (60). Small molecular weight mRNAs for RIα and RIIα were observed between 20 and 30 days of age and after day 40, respectively. Together with the appearance of the short RIα mRNA, expression of RIβ was also detected and the levels of Cα mRNA (2.4 kb) increased. Differential expression of PKA subunits in various germ cell fractions has been demonstrated in that the short message of RIα (1.7 kb) as well as RIβ (2.4 kb) are present in pachytene spermatocytes (PS) and round spermatids (RST) both at 32 and 44 days of age. A lower level of expression that can be accounted for by contamination from the RST fraction is observed in elongating spermatids (ES). In contrast, mRNAs for the RII subunits are not detected at 32 days whereas a high level expression of RIIα mRNA (2.2 kb) can be detected at 44 days when the ES fraction can be purified. RIIβ (1.6 kb) is also detected at 44 days, but appears stronger in the RST fraction. The Cα message (2.4 kb) is observed at high levels both in the PS and RST fractions. Studies of mRNA expression in isolated seminiferous tubules at different stages and *in situ* hybridization revealed a similar pattern of expression (61). Similar regulation of RIα, RIIα, RIIβ, and Cα protein has been reported recently (49). Taken together, the RIα, RIβ, and Cα subunits in germ cells are induced at premeiotic and meiotic stages, whereas the RII subunits are induced only during spermatid elongation. The Cβ mRNA was detected in peritubular cells and Leydig tumor cells but not in Sertoli cells or germ cells (60).

4.2. Hormonal regulation of PKA subunits in rat Sertoli cells

Rat Sertoli cells serves as a good model system for studies of hormone responsiveness in general and of PKA regulation in particular. FSH and cAMP induce messenger RNA for RIα, RIIα, RIIβ, and Cα with similar kinetics. However, the responses differ greatly in magnitude. Whereas cAMP-dependent stimulation of RIα, RIIα, and Cα mRNAs are 2 to 4-fold, the increase in RIIβ mRNA is approximately 50-fold (50,53). The up-regulation of RIα, RIIβ, and Cα mRNAs after treatment by cAMP is, at least partly, due to an increased transcriptional activity (53), and in the case of RIIβ also involves increased stability of the mRNA (55). In Sertoli cells, similar regulatory changes are observed in RIα, RIIα and RIIβ protein (62).

Different mechanisms are involved in the regulation of the RIIβ and RIα genes. Whereas transcriptional activity of the RIα gene is induced with similar kinetics as that of the *c-fos* gene, the induction of the RIIβ gene is increasing throughout the observation period. Furthermore, the RIα gene is superinduced by combined treatment with cAMP and a protein synthesis inhibitor (cycloheximide). In contrast, inhibition of protein synthesis almost completely blocks the cAMP-mediated induction of the RIIβ gene (53). Regulation of the RIIα gene appears to be qualitatively similar to that of RIIβ, but is quantitatively less pronounced.

The RIα and RIIβ genes are also subject to regulation by PKC (58). Again the mechanisms of regulation appear to be different. PKC-dependent activation of RIα is unaffected by cycloheximide whereas induction of RIIβ is dependent of on-going protein synthesis (58). Cyclic AMP and TPA have additive effects on the regulation of the RIα message, whereas TPA inhibits the cAMP-mediated induction of the RIIβ gene.

Thus, there is extensive evidence showing differential mechanisms of regulation of the R subunit

genes. The RIα gene seems to be regulated by cAMP with similar characteristics as the cAMP response element (CRE) regulated *c-fos* gene. The 5'-flanking sequence of the RIα gene also contains a consensus CRE that is conserved between pig (63) and man (25). Furthermore, cloning of an alternatively spliced mRNA with a different leader exon led to the identification of two alternatively initiated promoters in the RIα gene that are differentially regulated (25). In contrast, the RIIβ gene has a regulation by cAMP distinct from that of RIα and *c-fos*, and belongs to a group of genes which respond to cAMP with slower kinetics and have cAMP-responsive regions distinct from the classical CRE, TRE, and AP-2 elements (64-66). It has therefore been of great interest to study the transcriptional regulation of the RIIβ gene.

4.3. Mitogen regulation of PKA subunits in lymphoid cells

Lymphoid cells have proved to serve as good model systems to study how exogenous signals regulate the levels of PKA subunits. T lymphocytes are activated to proliferation, differentiation and effector function through the T cell antigen receptor CD3 (TCR/CD3) complex (51). These cells were shown to express both PKA I and II consisting of $RI\alpha_2C\beta_2$ and $RII\alpha_2C\beta_2$, respectively (67). During antigen stimulation, PKA specific phosphotransferase activity decreased up to 40-45% within 3 hours of stimulation. This coincided with a decrease in the levels of immunoreactive C and a marked decrease (50-80%) in Cβ but not Cα mRNA levels. Furthermore, R subunit activity measured as specific [^{3}H]-cAMP-binding was only marginally influenced by TCR/CD3 stimulation, as was the case with the level of immunoreactive RIα and RIIα protein. In contrast, the mRNA level for RIα but not RIIα revealed a transient increase (3-5 fold) between 3 and 24 hours upon stimulation. The regulatory effects of TCR/CD3 stimulation on RIα and Cβ mRNAs was blocked by pre-treatment with the PTK inhibitor herbimycin A (Herb A), and enhanced by cross-linking the membrane associated protein tyrosine phosphatase CD45. Furthermore, during TCR/CD3 stimulation a significant induction of cAMP and activation of PKA was observed. Moreover, since PKC activation is also associated with TCR/CD3 triggering (51), it was interesting to observe that stimulation with 8-CPT-cAMP or phorbol 12-myristate 13-acetate (PMA), respectively, could mimic the TCR/CD3-mediated effects on the RIα mRNA. This indicates that the observed regulatory effects on PKA subunits by TCR/CD3 stimulation may involve PKA and/or PKC activity.

Upon T cell receptor triggering, an initial peak of cAMP and PKA activity (51,68) is observed that may serve as an acute negative modulator and a negative feedback of signaling through TCR. This is followed by regulatory changes of R and C subunit levels within hours of stimulation. The biological implication of this regulation may be that the R/C ratio is transiently increased, leading to a down-regulation of PKAI activity, which may be important for the G/S transition of the cell cycle, following TCR-induced mitogen stimulation (67) . Similar reciprocal regulation of levels of RIα mRNA and protein was observed in a panel of lymphoid cell lines investigated for PKA regulation, levels of cAMP and cell growth rate (69).

4.4. Transcriptional regulation of the genes for PKA subunits.

Upstream regulatory sequences have been reported for the genes encoding RIα (25,63), RIβ (70), RIIα (71), RIIβ (72,73), Cα (74), and Cβ (74). All these genes have GC-rich and TATA-less promoters which are characteristics of highly regulated genes expressed at a low level. Furthermore, the human gene for RIα has two promoters directing expression of two alternate initiated RIα mRNAs with different 5' non-translated regions. The two different promoters provide a more complex regulation of the RIα mRNA and proteins (25,75).

Regulation of the RIIβ gene have been subject to extensive studies. RIIβ was first isolated and cloned from rat granulosa cells (23) where a 6 to 10-fold induction of its mRNA by cAMP is seen (76). Studies of the 5'-flanking region of the rat RIIβ gene in ovarian granulosa cells revealed that the cAMP-responsiveness resided within a distinct region (-395 to -293) upstream of the translation initiation codon (72).

For transfection in Sertoli cells, 5'-deletions of the RIIβ flanking region were inserted in front of a CAT reporter gene. Basal CAT activity directed from the different constructs was reduced to approximately 50 % when the region -723 to -395 was included. The same region conferred a 4-fold cAMP responsiveness to the CAT reporter gene. In contrast, transfections of the same constructs into rat testis peritubular cells revealed that the cAMP-responsiveness as well as the inhibition of basal activity that resided within the region -723 to -395 was specific to Sertoli cells. Mapping of the cAMP-responsive region by gel retardation and DNAse I footprinting experiments identified several protected regions that are candidates for novel cAMP responsive elements (77).

5. PKA ISOZYME COMPOSITION AND CHARACTERISTICS

It is generally assumed that the catalytic subunits associate freely with homodimers of all the R subunits. However, PKAI holoenzymes are more readily dissociated by cAMP *in vitro* than PKAII holoenzymes (6,78). Furthermore, when RII is overexpressed in 3T3 cells, the C subunit will preferably be bound to RII, whereas RI will be present as free dimer (79). This indicates that PKAII holoenzyme forms preferentially compared to PKAI under physiological circumstances either due to lower sensitivity to cAMP or due to kinetics of association/dissociation influenced by salt and MgATP [reviewed in (7)]. This observation is confirmed in mice that are genetically null mutant for the RIIβ subunit where RIα is induced and PKAI is formed, not as result of increased transcription of the RIα gene, but rather due to an increased half life (up to 5 fold) of the RIα protein when associated with C (80). Furthermore, the PKAI ($RI\alpha_2C_2$ and $RI\beta_2C_2$) and PKAII ($RII\alpha_2C_2$ and $RII\beta_2C_2$) holoenzymes have been reported to have distinct biochemical properties. RIβ holoenzymes are 2 to 7-fold more sensitive to cyclic nucleotides than RIα holoenzymes (81-83). RIIα and RIIβ holoenzymes elute from DEAE-cellulose columns at different positions in the PKAII area, and RIIα expressed at high levels will compete with RIIβ in binding the C subunit, indicating

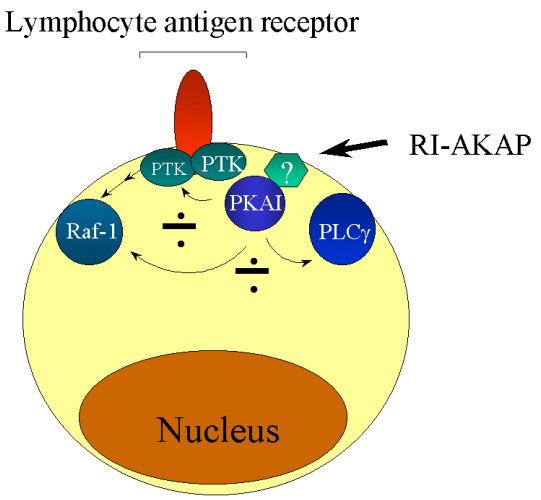

Figure 2. PKAI is redistributed from an apparently cytoplasmic distribution to localize with the antigen receptor in lymphoid cells upon lymphocyte activation and capping. PKAI associated with components of the antigen receptor or an associated A kinase anchoring protein (AKAP) for PKAI (RI) serves as a negative modulator of signals through these receptors. Anchored PKAI is located in close proximity to relevant substrates for PKAI phosphorylation, such as protein tyrosine kinases, phospholipaseCγ-1 (PLCγ-1) and the serine/threonine protein kinase Raf-1, all of which are important mediators of signals associated with a complete activation of lymphocytes leading to effector function and clonal expansion.

either a higher affinity for the C subunit or a higher threshold for cAMP induced dissociation (84).

Characterization of a cell line almost completely devoid of PKAII, revealed the presence of an isozyme consisting of an RIα-RIβ heterodimer with associated phosphotransferase activity. This isozyme elutes in the position of PKAII by DEAE-cellulose chromatography (85). Formation of RIα-RIβ heterodimeric complexes was also demonstrated *in vitro* by coimmunoprecipitation, using recombinant proteins (85). Furthermore, some experimental evidence support the possible existence of RIIα-RIIβ heterodimeric complexes that can be formed *in vitro* from recombinant proteins, and such complexes may also be detected in extracts from human testicular tissue (Taskén, unpublished results).

6. SUBCELLULAR LOCALIZATION OF PKA

Compartmentalization of PKA is mediated through binding of the R subunit to subcellular components (86). In general, PKAI ($RI\alpha_2C_2$, $RI\beta_2C_2$) is soluble and is preferentially located to the cytosol. However, there are an increasing number of reports of RIα association with subcellular components of the cell. In T cells, RIα associates with the TCR/CD3 complex under T cell activation and capping (87). Moreover, it was recently demonstrated that RIα binds to the adapter protein Grb2 an association which allow PKAI to interact with the epidermal growth factor receptor in epithelial MCF-10A cells (88). Furthermore, a recent report demonstrated a dual-specificity A kinase anchoring protein (AKAP) for both RIα and RIIα. This AKAP is designated D-AKAP1 (89). In contrast to PKAI, PKAII isozymes ($RII\alpha_2C_2$, $RII\beta_2C_2$) are generally associated with the particulate fraction of the cell through the hydrophobic interaction of AKAPs with the hinge region of the RII (90). A number of different anchoring proteins have been identified and serve to sequester PKAII with the cytoskeletal elements such as microtubules (MAP2), postsynaptic densities and cortical actin (AKAP79/75), filopodia (Gravin/AKAP250), actin-binding proteins (ezrin/AKAP78) and centrosomes (AKAP350) (91-95). Also organelle associated AKAPs have been identified, such as AKAP100 of the smooth sarcoplasmatic reticulum, AKAP220 on peroxisomes, AKAP85 bound to the Golgi and AKAP84/149 in mitochondria (96-101). Furthermore, despite the absence of PKA R subunits from the nucleus, nuclear AKAPs (AKAP95, hAKAP150) have been identified, the biological significance of these AKAP are still elusive as PKAII holoenzyme complex is excluded from the nuclei in interphase (102). As a further refinement of specificity in binding of PKAII to AKAPs, it has been demonstrated preferential association of AKAP95 with RIIα and not RIIβ (102), and that RIIα but not RIIβ associate with the Golgi apparatus where as RIIβ preferentially associate with centrosomes (103). Interestingly, it has recently been reported that some AKAPs (AKAP79, Gravin) function as signaling scaffold proteins by binding and assembly of different signaling proteins such as phosphatase 2B (Calsineurin) and PKC in addition to PKAII (104).

7. EFFECTS OF CAMP MEDIATED BY SPECIFIC ISOZYMES OF PKA

Since the unrevealing of a multitude of PKA isozymes, a key question has been to what extent different effects of cAMP may be mediated by specific isozymes. Approaches such as selective activation of one PKA isozyme by the use of combinations of cAMP analogs to complement each other in the preferential activation of PKAI or PKAII has demonstrated isozyme-specific effects of cAMP in cells. However, a major break through in understanding the role of various isozymes of PKA *in vivo*, was first made by creating mice that are null mutant for PKA subunits.

7.1. Cyclic AMP effects mediated by PKAI

It is generally assumed that specific isozymes of PKA localized to subcellular structures, mediates distinct effects of cAMP. The PKAI isozymes ($RI\alpha_2C_2$, $RI\beta_2C_2$) appears generally soluble and freely distributed in the cytoplasm (105). Thus, it may appear that PKAI is promiscuous in its phosphorylation of proteins and regulates all activities that are triggered by cAMP. However, lymphoid cells have proved to be good model systems to demonstrate the specificity in cAMP signaling. Cell growth of Reh cells which are practically devoid of PKAII (85) are inhibited by cAMP. In Reh cells, stable transfection with Cα proliferation was specifically inhibited, an effect that could be counteracted by cotransfection of a dominant negative mutant of RIα, that does not bind cAMP (106). These results testify to the role of the C subunit in mediating cAMP-dependent inhibition of cell proliferation in

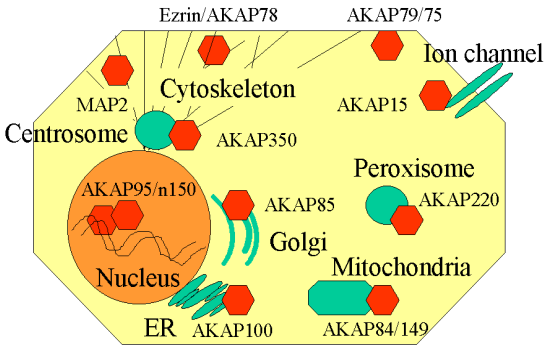

Figure 3. Cyclic AMP-dependent protein kinase II (PKAII) is targeted to different subcellular compartments through binding to A kinase anchoring proteins (AKAPs). At present more than 20 AKAPs have been cloned and it has been suggested that some cells may express as many as 10 to 15 different AKAPs located to different compartments. These compartments may include the nucleus (AKAP95/n150), cytoskeleton (AKAP78, ezrin, MAP2), centrosome (AKAP350), ion channels (AKAP15), peroxisomes (AKAP220), the Golgi (AKAP85), mitochondria (AKAP84/149), endoplasmatic reticulum (ER, AKAP100) and membranes (AKAP79/75).

lymphoid cells, but do not define the PKA holoenzyme responsible for mediating the cAMP effect. However, since Reh contains almost exclusively PKAI, this result strongly indicate that the inhibitory effect of cAMP on lymphoid cell proliferation can be mediated via this isozyme. The inhibitory effect of cAMP through PKAI on cell proliferation of lymphoid cells have further been verified in T and B cells. Both these cells contain PKAI ($RI\alpha_2C\beta_2$) and PKAII ($RII\alpha_2C\beta_2$) in a proportion of 3:1 (67,107). In resting T and B cells the PKAI is 75 % soluble whereas 75-90 % of the PKAII is particulate. Quiescent T and B cells can be activated to proliferate by cross-linking antigen receptor complexes (TCR/CD3 and BCR/Ig -complex, respectively). To test whether PKAI or PKAII mediates the inhibitory effect on proliferation of lymphoid cells, chemically modified cAMP analogs selective for either site A or site B of PKAI and PKAII (39,108) were used. The combination of 8-piperidino-cAMP (8-pip) and 8-aminohexylamino-cAMP (8-AHA) synergized in inhibiting incorporation of [^{3}H]-thymidine in proliferating T and B cells when compared to the effect of 8-AHA alone. No such synergism was observed when inhibition by 8-(4-chlorophenylthio) cAMP (8-CPT) was examined in the absence and presence of a small priming dose of N^6-benzoyl-cAMP (N^6-Bnz) that by itself had no effect on T and B cell proliferation. The combination 8-pip/8-AHA synergies strongly for T cells and more slightly for B cells in the activation of PKAI since 8-pip reveals high affinity for the A site of RI whereas 8-AHA binds to the B site of both RI and RII with equal affinity. This is contrary to activation of PKAII where both 8-pip and 8-AHA compete for binding to the B site. In contrast, the combination of N^6-Bnz and 8-CPT tends primarily to activate PKAII. This is because 8-CPT binds to the B site of RII with much higher affinity than to the PKAI B site and N^6-Bnz binds to the A site of both RI and RII. Thus, inhibition of T and B cell proliferation by cAMP appears to be a PKAI-mediated effect. Furthermore, using the same approach on natural killer (NK) cells, it has been demonstrated that cAMP-dependent inhibition of NK cell cytotoxizity is mediated by PKAI (109). In conclusion, PKAI is necessary and sufficient to mediate specific effects of cAMP on antigen receptor signaling and has established PKAI colocalized with the antigen receptor on lymphoid cells as a key negative modulator of immune responses that may be of clinical importance, e.g. in lupus patients (110) (Figure 2). Furthermore, isozyme-specific effects of PKAI has been demonstrated in that cAMP-induced apoptosis of a myeloid leukemia cell line (IPC-81) is mediated by PKAI (111).

Further evidence for specific roles of PKA *in vivo* was first obtained when mice null mutant for the RIβ subunit were generated. These animals appeared healthy and fertile, but examination of brain slices revealed that they had lost the ability to undergo long term depression (LTD) in the Schaffer Collateral pathway. RIα, RIIα and RIIβ are also expressed in the hippocampus (112) but appears unable to compensate functionally for the loss of RIβ (113). Thus, holoenzymes containing RIβ appears to differ functionally from both other isozymes of PKAI and PKAII, strongly suggesting specific roles of PKAI holoenzymes *in vivo*.

7.2. Cyclic AMP effects mediated by PKAII

Both RIIα and RIIβ have been reported to localize to the Golgi-centrosomal area of different cell types (114). Centrosomal localization is in agreement with the observations in T cells and suggests involvement of PKAII in cell cycle control and formation of the spindle apparatus. Colocalization and coimmunoprecipitation of RIIα of PKAII with $p34^{cdc2}$ kinase has also been reported (115), whereas RIIβ has recently been shown to serve as a substrate for cdc2 kinase *in vitro* (116). However, a specific function of PKAII from these studies that can be ascribed to this localization remains to be shown. Furthermore, a study by Rosenmund *et al.* (1994) showed that PKAII activity was associated with regulation of AMPA (alpha-amino-3-hydroxy-5-methyl-4-isoxazole-propionic acid)/kainate Ca^{2+}-channels. Disruption of PKAII (RII) binding to the AKAP associated with the AMPA receptor impairs the PKAII-dependent regulatory effect on the Ca^{2+} flux in cultured hippocampal neurons. Similarly, specific anchoring of PKAII was necessary for cAMP-mediated modulation of the L-type calcium channel in heart skeletal muscle (117). Also, PKAII ($RII\beta_2C_2$) has been shown to mediate cAMP-dependent activation of lipolysis and glycerol release from adipocytes i*n vitro* (118). Interestingly, similar effects have been shown *in vivo* in adipocytes of mice lacking the RIIβ subunit (119). Disruption of the mouse RIIβ gene leads to a profound change in PKA composition in both white and brown adipose tissue (WAT, BAT), where RIIβ normally is the principal R subunit. WAT was significantly diminished in these animals despite normal food uptake and the animals were protected against diet-induced obesity and fatty liver. In mutant BAT, levels of RIα were induced generating an isozyme switch from PKAII to PKAI. Moreover, these studies also showed that the RIα containing holoenzyme is more readily activated by cAMP and causes an induction of uncoupling protein

(UCP), increased metabolic rate and elevated body temperature, which together contribute to a chronically lean phenotype of RIIβ null mutant mice. These results are the first to demonstrate a specific effect of PKAII ($RII\beta_2C_2$) *in vivo* which was not compensated for by upregulation of PKAII holoenzymes.

8. SUMMARY AND PERSPECTIVES

A large number of hormones, neurotransmitters, and other signaling substances that bind to G-protein coupled cell-surface receptors, converge their signals at one sole second messenger, cAMP. The question of how specificity can be maintained in a signal transduction system where many extracellular signals that lead to a vast array of intracellular responses, all are mediated through one second messenger system, has been subject to thorough investigation and a great deal of speculation. An increasing number of PKA isozymes consisting of homo- or heterodimers of R subunits (RIα, RIβ, RIIα, RIIβ) with associated catalytic subunits (Cα, Cβ, Cγ) may contribute to the answer to this problem. Furthermore, the various PKA isozymes display distinct biochemical properties and the heterolous subunits of PKA reveal cell-specific expression and differential regulation at the level of gene transcription, mRNA stability and protein stability in response to a wide range of hormones and other signaling substances. Moreover, the existence of a number of anchoring proteins specific to either RI or RII subunits that localizes either PKAI or PKAII to distinct subcellular loci, strongly supports the idea that specific functions can be assigned to the various PKA isozymes. This is further strengthened by the demonstration that selective activation of PKAI is necessary and sufficient for cAMP-mediated inhibition of T and B cell proliferation and NK cell function which is compatible with the notion of isozyme-specific effects of PKAI. The observation that T and B cell activation is also associated with redistribution and colocalization of PKAI with the antigen receptor, strongly support the idea of anchoring as a way of maintaining specificity of cAMP effects mediated by PKAI (Figure 2). Thus, it is intriguing to investigate if RI holoenzymes colocalize with other receptors as well, such as activating NK cell receptors. The molecular mechanisms by which PKAI eliminate the signaling through antigen receptors is elusive. Also, AKAPs specific for RI remains to be demonstrated.

In the case of RII, a large number of AKAPs have been demonstrated that localize RII to different subcellular compartments (Figure 3). However, with the exception of cAMP-mediated modulation of AMPA/kainate channels in neurons and the L-type calcium channel in heart skeletal muscle no exact functions of PKAII specifically localized to distinct AKAPs have yet been demonstrated. The fact the PKAI ($RI\alpha_2C_2$) can not compensate for the loss of PKAII ($RII\beta_2C_2$) in WAT in mice that are null mutant for the RIIβ subunit, indicate that a number of different cAMP effects yet to be characterized, are specifically mediated through soluble and not anchored PKA isozymes and *vice versa*.

Finally, a recent report demonstrate defects in synaptic plasticity in neurons of mice that are null mutant for the Cβ subunit. Interestingly, these effects could not be compensated for by the Cα subunit which quantitatively is expressed at a much higher level in the same cells (120). Furthermore, Cγ does not bind PKI and may not be exported from the nucleus via PKI containing NES (46,121). In addition, a very recent report demonstrate that Cα but not Cβ, Cγ nor any R subunit bind specifically to the cytosolic NFκB inhibitor IκB, which binds and sequester the transcription factor NFκB to the cytosol. In this study it was also demonstrated that Cα is activated through an cAMP-independent way through degradation of IκB (122). Together these results demonstrate distinct effects of a particular C subunits that may be induced in an either cAMP-dependent or -independent way. However, most importantly, these results gives us further insight in to the complex way of how specificity may be maintained in the cAMP and PKA signaling pathway.

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