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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 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 this signaling pathway that may contribute to explain how differential effects of cAMP may be contributed to features of the PKA signaling 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). Cyclic AMP activates a class of cyclic nucleotide gated ion channels (5-7) as well as the guanine exchanging factors Epac1 and Epac2 (exchanging protein directly activated by cAMP) that regulates the activity of the small G-protein Rap1 (8,9). However the principle cAMP receptor in mammalian cells with which the majority of biological effects of cAMP have been associated, is cAMP-dependent protein kinase (PKA; EC 2.7.1.37) (10) (Figure 1). 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 2). Cyclic AMP binds co-operatively to two sites on each R protomer (for review, see (11,12)). 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 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 (13), gene regulation (14), cell growth and division (15), cell differentiation (16,17), and sperm motility (18), as well as ion channel conductivity (19). Therefore, a major question has been to understand how specificity is maintained in this second messenger system.

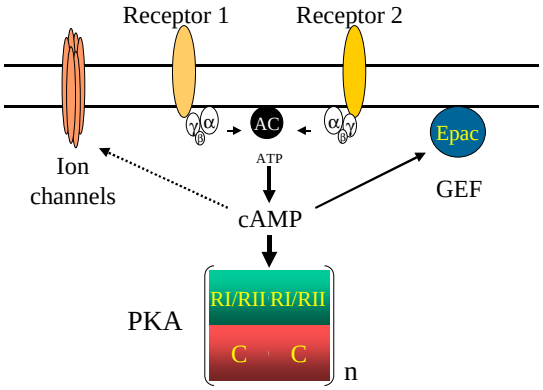

Figure 1. Cyclic adenosine 3',5'-monophosphate (cAMP) is generated from ATP when a ligand binds to a G-protein coupled receptor (Receptor 1 and Receptor 2) that activates adenylyl cyclase (AC). Free cAMP may stimulate and alter the activity of three different cAMP receptor molecules which includes ion channels, Epac which regulates the Rap1 guanine-nucleotide-exhanging factor and various PKA holoenzymes. PKA is considered the major target for cAMP action. RI, RII and C denotes subunits of PKA.

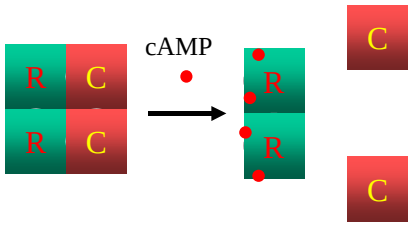

Figure 2. Cyclic AMP-dependent protein kinase (PKA) is a holoenzyme consisting of a regulatory (R) subunit dimer and two catalytic (C) subunits. Activation of PKA occurs when four molecules of cAMP bind to the R subunit dimer, two to each subunit, in a positive cooperative fashion. When both cAMP binding sites (A and B) are occupied the R subunit adopts a confirmation with low affinity for the C subunit and the holoenzyme dissociates. 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 cAMP levels, the relative concentration of PKA subunits, in addition to salt concentration, pH and temperature.

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 DEAE-cellulose columns (20,21). 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 (11). However, molecular cloning techniques have revealed a great heterogeneity in both R and C subunits, which open up for a multiplicity of PKA isozymes.

3.1. Multiple isoforms of regulatory and catalytic subunits of PKA

Cloning of cDNAs for regulatory subunits have identified two RI subunits termed RIα (22,23) and RIβ (24,25) and two RII subunits termed RIIα (26,27) and RIIβ (28,29) 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 have been demonstrated. RIα cDNAs with different leader exons and differentially regulated initiation from two promoters of the RIα gene was shown (30).

Furthermore, two distinct C subunits were initially identified by molecular cloning, and were designated. Cα (31) and Cβ (32,33). The cloning of the Cα and Cβ subunits from human testis by low homology screening also revealed an additional C subunit, designated Cγ? (34-35). Moreover, a novel human X chromosome-encoded protein kinase X (PrKX) was identified (36). This kinase forms a holoenzyme that can be activated by cAMP exclusively with the RI subunit, defining PRKX1 as a novel PKA C subunit isoform. A homologue gene is present at the Y-chromosome, and additional genes encoding proteins highly similar to PRKX1 is present on the X-chromosome, indicating the possibility of additional isoforms of C.

Splice variants of both Cα and Cβ have been reported. Three splice variants designated Cα1, Cα2 and Cα-s have been identified (37,38). Cα2 was cloned from interferon-treated cells and was shown to be catalytically inactive due to truncation of the C-terminal region resulting in a 224 amino acid protein and may thus represent a pseudogene or translocation of little significance. In contrast, the Cα-s subunit isolated from ovine sperm flagellum was shown to be catalytically active (38). Cα-s, which has been identified and cloned from human sperm (39), is an N-terminally truncated form of Cα with an apparent molecular mass of 39-kDa. The Cα and Cα-s are different in the N-terminal most probably due to alternative use of two different forms of exon 1 of the Cα gene (39). In the case of the Cβisoform, several splice variants have been identified in different species. In the bovine, the isoform bCβ was first identified and is homologous to the human, rat and mouse Cβ variant. In addition to bCβ, a bCβ2 variant has been identified and cloned (20). Bovine Cβ and bCβ2 are dissimilar in the N-terminal end presumably due to alternative use of two different forms of exon 1, as is the case for the Cα and Cα-s isoforms. From the cDNA sequence of bCβ and bCβ2 one would expect the presence of proteins of approximately 40 kDa (bCβ) and 47 kDa (bCβ2), respectively. In the mouse, one splice variant (mCβ1) that is ubiquitously expressed and the brain specific splice variants of Cβ (mCβ2 and mCβ3) were identified and cloned (40). Mouse Cβ1 is homologous to the previously described Cβ isoform from human, rat and bovine (32). Mouse Cβ2 and mCβ3 are truncated in the N-terminal end when compared to the mCβ1 isoform. The

differences are due to alternative use of exon 1 in the mCβ gene (40). Both mCβ2 and mCβ3 have been demonstrated as proteins of relative molecular mass of 38-kDa.

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 (41,42). 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 (43). For the RI subunits, dimerization further involves stable α helix configuration by amino acids 12 through 61. As evident from *in vitro* studies, disulfide bridges between Cys16 and Cys37 on opposite strands indicate an anti-parallel orientation of the dimer, whether such disulfide bonds are present in the intracellular environment remain elusive (44). Dimerization of the RII subunit is antiparallel, but does not involve cysteine bridges. A recent study shows that the N-terminal amino acids 1 through 44 of RIIα encompassed both the dimerization interface as well as the interaction with A-kinase anchoring proteins (AKAPs, see below). By solution NMR it was demonstrated that amino acids 1-44 of RIIα form an X-type four-helix bundle dimerization motif with an extended hydrophobic face at the N-teminal end where the hydrophobic face of the AKAP amphipatic helix docks in (45).

Association of the R and the C subunit involves two different mechanisms of interaction. One mechanism depends on acidic residues between amino acids 15 and 258 in the R subunit which make electrostatic interactions with specific domains in the C subunit (46). In addition, the hinge region of both the RI and RII molecules is involved in binding to the substrate binding site of the C subunit. Interestingly, RII but not RI is autophosphorylated by the C subunit.

Of the two tandem cAMP binding sites that are located in the C-terminal domain, only site B is exposed in the inactive tetrameric PKA complex (reviewed in (12)). 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 (11,12)) as have the relative affinities and site selectivities of a wide array of chemically modified cAMP analogs (47). The crystal structure of a monomeric RI deletion mutant (Δ1-91) that was refined to 2.8 Å, has been reported (48,49), and provides a model to study cAMP- binding.

3.2.2. Structure of the catalytic subunits

With the exception of Cα2, all the C subunits retain the catalytic core motif common to all protein kinases (50,51). The crystal structure of the murine Cα subunit was the first protein kinase crystal structure available (52) and has served as a template for modeling of several other kinases. The crystal structure of Cα demonstrates this protein as a nearly globular protein with two lobes in addition to a free rotating domain consisting of the N-terminal 50 amino acids encoded by exon 1 and some of exon 2 (52). The small, amino terminal lobe of the C subunit 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.

Myristylation of the C subunit was initially thought to be important for stabilization of the C subunit by embedding of the myristyl group in a hydrophobic cleft in the globular protein (53,54). An amino terminal Gly serves as a site for myristylation in Cα1 and Cβ1, but not in other splice variants as, e.g. mCβ3 (40). Mouse Cβ3 is not myristylated most probably due to the fact that the most amino terminal sequence is (H_2N- Gly-Leu-X-) and not (H_2N-Gly-Asn-X-) as is the case for Cα and Cβ1 (55). Thus, the importance of myristylation for structural stability and activity *in vivo* may be questioned since several splice variants do not have motifs allowing N-terminal myristylation, yet they are fully catalytically active. It may be speculated that the myristyl group serves to increase the lipofilic properties of the C subunit when binding the RII- but not the RI subunit, by altering the conformation and exposing the myristyl group (56).

A conserved autophosphorylation motif (-Lys-Lys-Gly-Ser^{10}-) is encoded by exon 1 in both Cα1 and Cβ1 (57) and at Thr 9 in Cγ (34). Interestingly, site directed mutatation of Cα1 in Ser10 resulted in decreased activity as well as reduced solubility of the protein, implying an important role for Ser10 phosphorylation (57). Despite this, it may be questioned to what extent Ser10 phosphorylation is required for *in vivo* activity of all C subunits since it is not present in Cβ2 and Cβ3, which are both enzymatically active (40). All the C subunits except Cγ contain a domain that is capable of binding PKI through interaction with several amino acids including Arg133 (58). 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 (59). Interestingly, Cγ has a Gln in position 133 instead of Arg, and it has been shown that Cγ does not bind PKI and may thus not be exported from the nucleus (60).

Although the Cα1 and the Cβ1 isoforms are 91 % identical in amino acid sequence, Cα1 exhibits a 3-5 fold lower K_m for certain peptide substrates and a 3 fold lower IC_{50} for inhibition by the protein kinase inhibitor PKI and RIIα than does the Cβ1 (61). This suggests unique features associated with the various C subunits, which may imply that they may exhibit different functions *in vivo*.

4. LEVELS AND EXPRESSION OF THE REGULATORY AND CATALYTIC SUBUNITS

In several cells and tissues at various stages of development and differentiation extensive studies have been performed in order to demonstrate differential expression of R and C subunits. In an early study by Cadd (62) it was demonstrated that in mice RIα is expressed in

the heart and central nervous system (CNS), whereas RIβ expression is more restricted to nervous tissues such as the spinal cord and the brain. Furthermore, RIIα and RIIβ are both expressed in the brain, and show distinct patterns of expression with RIIα predominantly expressed in the heart and RIIβ expressed in the liver and fat tissue (63). During male germ cell differentiation a distinct pattern of expression of PKA subunits is demonstrated. The C subunit isoforms Cα-s and Cγ are expressed exclusively in male germ cells primarily in late pachytene spermatocytes and haploid cells (35). RIα is expressed in early haploid cells and RIIα is expressed later in spermatogenesis during spermatid elongation (64).

Levels of expression of the different PKA subunits are subject to regulation by hormones acting through G-protein coupled receptors (65-67), mitogenic signals through receptors associated with protein tyrosine kinases (PTK) (68) as well as by steroid hormones (69). Regulation of PKA by hormones acting through cAMP may serve as an autologous sensitization/-desensitization mechanism of the cAMP effector system. Interestingly it has been shown that cAMP mediated regulation of PKA subunits acts through gene transcription (70,71) and mRNA stability (72), as well as altered stability of the R and C proteins after dissociation of the holoenzyme by cAMP (71,73). Protein kinase C represents another major signaling pathway in cells and cross talk between these two signaling systems is seen beyond cAMP at the level of PKA (74,75).

Upstream regulatory sequences have been reported for the genes encoding RIα (30,76), RIβ (77), RIIα (78), RIIβ (79,80), Cα (81), Cβ (81), and Cγ (35). All these genes except Cγ 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 (30,82).

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 (76) and man (30). Furthermore, cloning of an alternatively spliced mRNA with a different leader exon leads to the identification of two alternatively initiated promoters in the RIα gene that are differentially regulated (30). 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 (83-85). Thus, regulation of the RIIβ gene by FSH and cAMP have been subject to extensive studies in granulosa- and Sertoli cells where a 10 to 50-fold induction of its mRNA is seen (28,70,86). Studies of the transcriptional regulation of the RIIβ gene revealed that the cAMP-responsiveness resides within a distinct region upstream of the translation initiation codon (79). In fact it was discovered that a novel mechanism was operative in regulation of RIIβ responsiveness by which FSH regulates response genes through immediate early upregulation of C/EBP-β (87).

Lymphoid cells have proved to serve as good model systems to study how mitogenic 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 (68). These cells were shown to express both PKA I and II, consisting of $RI\alpha_2C_2$ and $RII\alpha_2C_2$, respectively (88). Upon T cell receptor triggering, an initial peak of cAMP and PKA activity is observed that may serve as an acute negative modulator and a negative feedback of signaling through the TCR/CD3 complex (68,89). This is followed by regulatory changes of R and C subunit levels revealed as a decrease (40-45%) in PKA specific phosphotransferase activity, which is coincided with a decrease in the levels of immunoreactive C and a marked decrease (50-80%) in the Cβ but not Cα mRNA levels within 3 hours of stimulation (68). Similar reciprocal regulation of level 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 (90).

5. PKA ISOZYME COMPOSITION AND CHARACTERISTICS

It is generally assumed that the C subunits associate freely with dimers of all the R subunits. However, PKAI holoenzymes are more readily dissociated by cAMP *in vitro* than PKAII holoenzymes (11,91). Furthermore, when RII is over-expressed in NIH 3T3 cells, the C subunit will preferably bind to RII, whereas RI will be present as free dimers (92). The mechanism for this observation may involve several features such as lower sensitivity of PKAII to cAMP compared to PKAI and differential kinetics of association/dissociation influenced by salt and MgATP between the two holoenzyme types (reviewed in (12)). This indicates that PKAII holoenzymes are assembled preferentially over PKAI under physiological conditions. Despite this, it was recently shown that ablation of the RIIβ and RIβ subunits by gene targeting (knockout, KO), did not result in quantitative compensation by RIIα in the RIIβ KO or by RII in the RIβ KO as would be expected. Instead, Amieux et al. (93) could demonstrate induction of RIα and PKAI assembly in both the RIβ and the RIIβ KO as a result of a 4-5-fold increase in the half-life of RIα protein when binding to the C subunit. Together, this demonstrates that complex mechanisms influenced by multiple factors are governing to what extent PKAI and PKAII assembly is preferred *in vivo*.

It has been reported that PKAI ($RI\alpha_2C_2$ and $RI\beta_2C_2$) and PKAII ($RII\alpha_2C_2$ and $RII\beta_2C_2$) holoenzymes have distinct biochemical properties. RIβ containing holoenzymes are 2 to 7-fold more sensitive to cyclic nucleotides than are RIα containing holoenzymes (94-96). In addition, 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

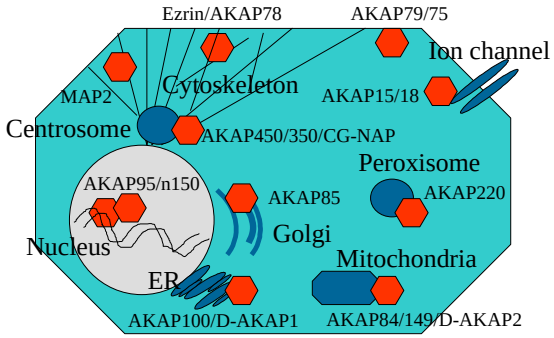

Figure 3. Cyclic AMP-dependent protein kinase II (PKAII) is targeted to different subcellular compartments through binding to A kinase anchoring proteins (AKAPs). At present more than 20 AKAPs have been cloned and it has been suggested that some cells may express as many as 10 to 15 different AKAPs located to different compartments. These compartments may include the nucleus (AKAP95/n150), cytoskeleton (AKAP78, ezrin, MAP2), centrosome (AKAP450/350/CG-NAP), ion channels (AKAP15/18), peroxisomes (AKAP220), the Golgi (AKAP85), mitochondria (AKAP84/149), endoplasmatic reticulum (ER, AKAP100) and membranes (AKAP79/75).

in binding the C subunit, indicating either a higher affinity for the C subunit or a higher threshold for cAMP induced dissociation (97).

Finally, the presence of an isozyme consisting of an RIα-RIβ heterodimer with associated phosphotransferase activity has been reported (98). Interestingly, this isozyme elutes at the position of PKAII by DEAE-cellulose chromatography, implying different biochemical properties of holoenzymes containing R-subunit heterodimers compared to R-subunit homodimers.

Taken together this demonstrates the existence of multiple R and C subunits harboring different biochemical features and activities. When assembled, they may give rise to a number of PKA holoenzymes with different biological characteristics and activities. A number of different PKA holoenzyme may certainly account for some of the specificity seen in the cAMP/PKA signaling pathway.

6 SPECIFIC EFFECTS OF CAMP ARE MEDIATED THROUGH SUBCELLULARLY ANCHORED PKA ISOZYMES

Although an increasing number of reports demonstrate specific effects mediated by a particular PKA isozyme, most of these reports imply that such effects are associated with differential expression and subcellular localization of various PKA isozymes (Figure 3). We will briefly discuss to what extent regulatory effects of cAMP depends on specific PKA isozymes that requires subcellular anchoring.

6.1. PKAI mediates specific effects of cAMP at distinct subcellular sites

It is generally assumed that compartmentalization of PKA is mediated through binding of the R subunit to subcellular components (99). Furthermore, it is also thought that PKAI ($RI\alpha_2C_2$, $RI\beta_2C_2$) is soluble and preferentially located to the cytosol (100). Lymphoid cells have proved to be a good model system to study the specificity in cAMP signaling mediated by PKAI. Cell growth of normal lymphoid cells and a number of lymphoid cell lines including the B lymphoid cell line Reh are inhibited by cAMP (90,98). In Reh cells proliferation was inhibited by stable transfection with Cα, an effect that could be counteracted by cotransfection of a dominant negative mutant of RIα, that does not bind cAMP (101). Experiments in normal T and B-lymphocytes further showed that cAMP-dependent activation of PKAI, but not PKAII, was necessary and sufficient to inhibit proliferation induced through the antigen receptor complex on both T cells (TCR/CD3) and B cells (BCR/Ig). In addition to this, it has been demonstrated that dysfunction of T cells isolated from patients with HIV (human immune deficiency virus) and CVI (common variable immunodeficiency) could be reversed by addition of PKAI antagonists (102,103). Specifically, combination of PKAI selective antagonist and IL-2 normalize immune function of T cells from all patients examined (104). This implies an important role of cAMP in regulating antigen receptor induced proliferation and clonal expansion of lymphoid cells and testifies to the role of PKAI in mediating these effects. In support of the role of PKAI in mediating specific effects of cAMP in leukocytes it has been demonstrated that cAMP-dependent inhibition of natural killer (NK) cell cytotoxicity is mediated by PKAI (105), and that PKAI mediates cAMP-induced apoptosis of a myeloid leukemia cell line (IPC-81) (106). Further evidence for specific roles of PKAI *in vivo* were obtained when mice null mutated for the RIβ subunit was 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 of the hippocampus. RIα, RIIα and RIIβ are also expressed in the hippocampus (62) but appears unable to compensate functionally for the loss of RIβ (107). Also, when comparing synaptic plasticity in the developing visual cortex in normal and RIβ null mutated mice, it was observed abnormalities in extracellularly recorded LTP, LTD and pair-pulse facilitation (108). Finally, in RIβ null mutant mice it was shown that this subunit was necessary to produce the full response to tissue injury-evoked pain in contrast to nerve injury-evoked pain, suggesting a distinct role of RIβ containing PKA holoenzyme in sensory nerves (109). In summary, these studies imply that holoenzymes containing RIα or RIβ appears to differ functionally from other isozymes of PKA, further providing evidence for cAMP effects mediated through specific isozymes of PKAI *in vivo*.

The mechanism for specific effects of cAMP

mediated by PKAI has been suggested to involve subcellular localization of this enzyme. Quiescent T- and B cells contain soluble PKAI ($RI\alpha_2C\beta_2/C\alpha_2$) and particulate PKAII ($RII\alpha_2C\beta_2/C\alpha_2$) in a proportion of 3:1 (47,88,110). When activated through the antigen receptor, RIα translocates from the cytosol and associates with the antigen complex of both T and B cells (110,111). Despite that this implies PKAI-specific AKAPs in lymphoid cells, no such proteins have yet been identified in these cells. However, PKAI-specific AKAP binding site has recently been reported in the sperm AKAP FSC1 (112) and a PKAI-specific AKAP was reported from C. elegans designated AKAP-CE (113). Furthermore, dual-specific AKAPs, which bind both RI and RII, have been identified. These includes the fibrous sheath polypeptide of mouse sperm (FSC1) also designated AKAP82. FSC1/AKAP82 binds RII with high affinity but in addition contains an RI-binding site as discussed above (114-116). Furthermore, Huang and coworkers (117,118) have by using two-hybrid screening and RIα as bait cloned and identified two dual-specific AKAPs designated D-AKAP1 and D-AKAP2. They could demonstrate that these AKAPs associate with both RI and RII subunits and that binding requires an R subunit dimer with an intact N-terminal domain (119). D-AKAP1 has identity to the previously cloned S-AKAP84/AKAP121/AKAP149 shown to bind RII (120-122), which all are splice variants of the same gene. These AKAPs targets PKA to the mitochondria and endoplasmic reticulum (120,121). Interestingly, a previous report describes PKAI-specific acute regulation of Leydig cell steroidogenesis, which includes regulation of cholesterol transport across mitochondrial membranes and regulation of the rate-limiting p450ssc enzyme on the inner mitochondrial membane (123). Furthermore, it was recently demonstrated that phosphorylation and inactivation of the proapoptotic molecule BAD requires mitochondria-anchored PKA (124) making it interesting to speculate if D-AKAP1 or other PKAI-AKAPs mediates targeted PKA (type I?)-specific mitochondrial effects. In addition, D-AKAP2 is assumed to be a member of a new protein family ubiquitously expressed at all embryonic stages as well as in all tissues of the adult. D-AKAP2 contains an R subunit interaction domain as well as a RGS (regulator of G protein signaling) domain. The latter may imply D-AKAP2 as a site for coupling G protein-dependent cAMP formation and specific effects of PKA in different cells.

Apart from the studies on lymphoid cells and Leydig cells indicating that specific effects of cAMP are mediated by anchored PKAI, no direct evidence has been provided which demonstrate that cellular effects of cAMP associated with anchored PKAI. This may be explained by studies done on characterization of the interaction domain of D-AKAP1 with the various R subunits (125). This demonstrated that the affinity of RI for D-AKAP1 is 2 orders of magnitude lower than that of RII. Thus, it is likely that RI association with D-AKAP1 only occurs in the absence of RII *in vivo*, but that other PKAI-specific AKAPs may exist.

6.2. PKAII is targeted to subcellular structures via A kinase anchoring proteins (AKAPs) and mediates discrete cAMP responses

PKAII has been demonstrated to mediate specific effects of cAMP on distinct cellular functions by the use of cAMP analogs that acts synergistically to activate either PKAI or PKAII. The first report documented that cAMP-mediated regulation of lipolysis in adipocytes was mediated by synergistic activation PKAII and not PKAI (126). Interestingly, similar effects have been shown *in vivo* in adipose tissue of mice lacking the RIIβ subunit (63,127). These studies points to PKAIIβ as the holoenzyme mediating cAMP effects at the level of cultured cells as well as in intact animals. In other systems, such as sperm cells, is has proved difficult to dissect which PKA isozyme is mediating the various effects of cAMP on function and motility. This may be due to the fact that RII specific- and dual-specific AKAPs such as AKAP82 (115), AKAP121, AKAP149 (D-AKAP1), FSC1, AKAP110 (128) and AKAP220 (129) are differentially located in various compartments of the cell. In addition, results have demonstrated overlapping expression patterns between the RI and RII isofoms in sperm (128,130). Despite this, a previous study demonstrated that incubating normal sperm with the synthetic peptide S-Ht31 that is able to penetrate the cell membrane, completely impair motility (131). This suggests that sperm motility require anchored PKA.

The role and specificity of cAMP/PKA signaling in neuronal tissues have been under thorough investigation in order to understand behavior and learning. To solve these very complicated questions models employing learning deficient Drosophila mutants were initially used. Amongst several, two mutants were identified that had defects in the phosphodiesterase encoded by the dunce+ gene and the Ca2+ sensitive adenylyl cyclase encoded by the rutabaga gene (132,133). Since then, a number of studies have been performed in the snail Aplysia demonstrating that cAMP/PKA activity is required for establishing learning and memory. In particular, these studies have demonstrated the important role of the cAMP/PKA signaling pathway as a mediator of short-term modifications by phosphorylation of ion channels and long-term modifications requiring protein synthesis and synaptic remodeling. In more complex systems such as mammals, the role of cAMP/PKA has been monitored in cultured neurons and in neurons of discrete sections of the brain. In most areas of the brain RIIβ is expressed at different levels. In the motor neurons of the striatum which requires cAMP for optimal synaptic response to dopaminergic drugs, the RIIβ containing PKAII holoenzyme is expressed at high levels. Studies on RIIβ KO mice demonstrated that motor learning and the regulation of neuronal gene expression require RIIβ containing PKA holoenzymes, whereas the acute locomotor effects of dopaminergic drugs were relatively unaffected by this PKA deficiency (134). Moreover, when treated with haloperidol, RIIβ ablated mice were unable to induce the acute cataleptic response normally observed in rodents and which is seen as an adverse effect in human. This occurred through interference with synthesis and release, and indicates a direct role for RIIβ containing PKAII as a mediator of haloperidol-

induced gene expression and cataleptic behavior (135). In these studies, the effect of gene targeting reflects the requirement for RIIβ in order to mediate specific effects of cAMP in nervous tissue, but does not explore to what extent anchoring is required. This is in contrast to an earlier study by Rosenmund et al. (136) who demonstrated that PKAII anchoring is necessary for cAMP-mediated regulation of AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazole-propionic acid)/kainate Ca^{2+}-channels in cultured hippocampal neurons. This study was the first demonstration that anchoring of PKAII is crucial for the regulation of cellular events.

In neuronal tissue of the CNS as well as the peripheral nervous system (PNS) several AKAPs have been identified. Microtubule-associated protein 2 (MAP2) was the first protein identified as an AKAP in brain and was shown to anchor PKAIIβ (137). Furthermore, the human AKAP79 (and ortologs AKAP75 /AKAP150 in bovine and mouse, respectively) (138-141) has been identified in brain where it predominantly is expressed in cerebral cortex. AKAP79 was first identified located to the post-synaptic densities (PSD) that are structures on the internal surface of excitatory synapses beneath the post-synaptic membrane where it has been implicated in regulation of various ion channels (AMPA/kainate receptors, L-type Ca^{2+} channels) as has several anchoring proteins such as AKAP18 (L-type Ca^{2+} channels), Yotiao (NMDA-receptor NR1 subunit) and ezrin (CFTR) (142). Thus, several AKAPs may be located post-synaptically in a number of different cells that are innervated, including muscle fibres (neuromuscular junction) and cardiomyocytes (143). AKAP79 is expressed in a number of non-neuronal tissues, suggesting that it participates also in functions other than those of the nervous system (144,145). AKAP79 is located to the cell membrane in different cell types through interaction with phosphatidylinositol-4, 5-bisphosphate (146) and to cortical actin (144). Furthermore, AKAP150/AKAP75 by localization of PKAII, transmits cAMP signals to the nucleus (147). In addition, the ROMK1 channel in the kidney, which is believed to be a native K^+ secretory channel, is also associated with AKAP79 (148). Finally, AKAP79 also tethers PKAII to β-adrenergic receptors (149). Anchoring as a requirement for PKAI to mediate specific effects of cAMP has in most cases been demonstrated with studies on ion channels. However, PKAII and not PKAI have been shown to localize with the Golgi-centrosomal area in most cells implying that PKAII is associated with mediating effects of cAMP on cell metabolism and cellular trafficking and microtubule dynamics (150-152). Interestingly, we have recently revealed a differential distribution of RIIα and RIIβ in the Golgi-centrosomal area (153), demonstrating that RIIβ is located to centrosomes in differentiated but not in undifferentiated cells, whereas RIIα is associated with centrosomes as well as to the trans-Golgi network in both differentiated and undifferentiated cells. In the latter case RIIα was localized with microtubule associated vesicles. Together, this may imply that PKAII isozymes containing either RIIα or RIIβ may be associated with different functions with respect to vesicle transport and cell cycle control. The lipid anchored AKAP15/18 has three splice variants designated α, β and γ which show differential localization to the apical and basolateal membrane compartments (154-158) which implicates sorting of AKAP18 through the Golgi and into targeted vesicles. Also, Golgi fractionation studies led to characterization of a yet unidentified 85-kDa AKAP that may be responsible for the abundance of PKA in this area (159).

Colocalization and coimmunoprecipitation of RIIα with CDK1 (the mitotic kinase $p34^{cdc2}$) has been reported (160). Both RIIα and RIIβ have recently been demonstrated as substrates for cdc2 kinase *in vitro*(161,162). Moreover, in the case of RIIα phosphorylation, Keryer et al. (153) could demonstrate that this R subunit is hyperphosphorylated on Thr54 by CDK1 at metaphase and that this occurs concomitantly with dissociation of RIIα with the centrosome. Taken together with other reports, these studies suggest that the level of PKAII and its localization during the cell cycle is pivotal. Initially an RII anchoring protein of approximately 350 kDa was identified at the protein level and found to locate PKAII to centrosomes (163). This AKAP has been shown to be identical to a 453-kDa protein which was recently cloned and characterized by several groups and was designated AKAP450/AKAP350/CG-NAP (164-166). The gene encoding AKAP450 was shown to harbor coding sequence for the previously published shorter splice variant Yotiao which is located to the neuromuscular junction and synapses of neurons (167) where it mediates cAMP effects on the NMDA receptor (168). AKAP450 was shown to be associated with the centrosome and Golgi structures and may through anchoring of various PKAII isoenzymes, be important for the regulation of microtubule stability and Golgi function. A very recent study reports that another protein in the pericentriolar matrix, pericentrin, is implicated in centrosomal targeting of PKA via a novel PKA binding domain that involve several Leu residue clusters spaced over approximately 100 amino acids instead of an amphipatic helix region (169).

Recent studies show that the nuclear AKAP95 (170) is redistributed from nuclear matrix and associates with the condensing chromatin upon mitosis entry and before nuclear envelope breakdown (171). Use of immuno-blocking antibodies demonstrated that AKAP95 but not PKA was required for chromatin condensation and that AKAP95 associated with condensins. Furthermore, AKAP95 recruited PKAIIα onto condensed chromatin after nuclear envelope breakdown, and PKA was required for maintenance of condensed chromatin throughout mitosis.

7. AKAPS ASSEMBLE SIGNAL COMPLEXES IMPORTANT FOR INTRACELLULAR SIGNALING

It has been established that AKAPs also targets other molecules important for intracellular signaling to subcellular domains. In the case of AKAP79, this protein has been identified as a molecule able to target the phosphatase calcineurin (PP2B) (172) and the protein kinase, PKC (164), to cellular membrane structures. In this way AKAP79 has been shown to serve as a regulator of PP2B-dependent NFAT activity (173) and Ca^{2+}/

Calmodulin-dependent PKC activity (174). Moreover, recently it was shown that AKAP79/150 anchors PKAII, PKC and PP2B to the β2-adrenoreceptor, facilitating receptor phosphorylation and down stream signaling (149). AKAP75, AKAP220 and AKAP250 (Gravin) are also shown to target signaling molecules to cellular structures. AKAP75 was found to colocalize with adenylyl cyclase (145), whereas AKAP250 have high sequence homology to proteins that bind PKC (175). Interestingly, AKAP250 also associated with β-adrenoreceptor and mediates regulation of protein kinase and phosphatase activity associated with G-protein coupled receptors (57,175). Finally, AKAP220 has been shown to associate with type 1 protein phosphatase (PP1) in the rat (176) revealing this AKAP as a protein locating phosphatase activity presumably to peroxysomes (177).

Together this demonstrate that AKAPs may orchestrate tight regulation of several proteins that may serve as substrates for PKA and enzymes that are important for signal transduction in various cells.

8. CONCLUDING REMARKS

In summary, this brief review describes by the use of a few examples how multiple PKA isozymes withdifferent biochemical properties and targeted in the cell by association with various AKAPs may convey specificity in the cAMP signaling pathway. It should, however, be mentioned that there are many more examples of how PKA by association with other AKAPs may mediate specific effects of cAMP. In the future, it will be important to understand molecular determinants for preferential association between the various PKA isozymes and the different AKAPs. Furthermore, specificity may not only be mediated by R-anchoring to AKAPs. It was recently reported that ablation of Cβ in mouse produced animals with altered LTD and LTP in the hippocampus (178). This together with the observation that Cα-s is only expressed in sperm and is targeted to the sperm flagellum (38) may imply specific functions associated with features of the C subunit as well. Finally, the recent report demonstrating that cAMP can induce events that are independent of PKA, such as regulation of ion channel activity and GEF activity (7-9) and that the PKA C subunit can be activated independently of cAMP (179) indicate complex pathways mediating effects of cAMP.

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