ARTICLES AND REVIEWS
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Subcellular Localization of the Type II cAMP-Dependent Protein Kinase

John D. Scott and Daniel W. Carr

Diverse biochemical effects of different neurotransmitters or hormones that stimulate cAMP production may occur through activation of compartmentalized pools of cAMP-dependent protein kinase (PKA). Evidence suggests that compartmentalization of type II PKA is maintained through protein-protein interactions between the regulatory subunit and specific anchoring proteins.

Introduction

Hormone-receptor interactions generate signals that trigger protein kinase-catalyzed phosphorylation events (12). The action of 30 or more catecholamine and peptide hormones, as well as some prostaglandins, proceeds through parallel pathways that elevate intracellular adenosine 3′,5′-cyclic monophosphate (cAMP) and lead to activation of the cAMP-dependent protein kinase (PKA). An apparent paradox, highlighted in Fig. 1, exists in that parallel pathways can all activate the same enzyme, PKA, although it is clear that individual hormones trigger distinct and diverse physiological processes in the same cell. In essence, each hormone elevates cAMP concentrations but promotes PKA-mediated phosphorylation of distinct substrate proteins (12).

One hypothesis to explain these observations is that individual hormones activate specific pools of PKA activity. A potential mechanism to explain this phenomenon is that individual PKA pools are compartmentalized at their site of action, close to their preferred substrates, and only become active when the appropriate hormone elevates cAMP in that particular microenvironment. For this to occur, PKA must be maintained at the correct intracellular loci in close vicinity to its preferred substrates.

Support for this hypothesis has been provided primarily by the work of Rubin, Erlichman, and others (2–4, 7–13) who have shown that the type II PKA holoenzyme ($R_2$2C) is tethered at specific subcellular locations through interaction of its regulatory subunit dimer (R) with specific anchoring proteins. Experimental evidence supporting this hypothesis can be divided into 3 areas.

1) Different hormones activate specific PKA subtypes.

2) cAMP accumulates in different cell compartments in response to different hormones.

3) Type II PKA distribution is dictated by interaction with specific anchoring proteins.

Different hormones activate specific PKA subtypes

Considerable research has focused on elucidating the action of cAMP, since the discovery that it is an intracellular messenger for hormone-mediated events. Individual hormone receptors and adenylate cyclases are coupled physically through interaction with intermediary GTP-binding proteins, such that hormone-receptor binding signals the catalytic unit of adenylate cyclase to increase the synthesis of cAMP. Four molecules of cAMP can bind each dormant PKA holoenzyme complex ($R_2$2C), thereby causing the release of two active catalytic (C) subunits from the R subunit dimer.

Two R subunit classes exist, RI and RII, which contribute to the type I and type II holoenzymes, respectively. Type II PKA is present in all cells, whereas the tissue distribution of type I PKA is more restricted.

Multiple variants of the same holoenzyme type exist in selected tissues arising from the expression of distinct isoforms of RI and RII. RIα and RIIα are expressed in most cell types, whereas RIβ and RIIβ are predominantly expressed in the brain (the molecular characterization of PKA has been recently reviewed in detail; see Refs. 12 and 14).

Using ion-exchange chromatography, several investigators have shown that individual hormones preferentially activate one or the other holoenzyme form in a tissue-specific manner. For example, type I PKA is preferentially activated by glucagon in hepatocytes and by corticotropin-releasing factor, isoproterenol, and forskolin in AtT20 cells. Type II PKA is activated by human gonadotrophic hormone in ovarian follicles, but the same hormone activates type I PKA in the corpus luteum. In osteoblasts, parathyroid hormone activates type I PKA, whereas prostaglandin E_2 (PGE_2) activates type II PKA. These results support the hypothesis that distinct pools of PKA can be differentially activated in the same cell in response to different hormones.

Compartmentalized cAMP accumulation

PKA activation can only proceed when cAMP is elevated above a threshold concentration. Stimulated adenylate cyclase releases cAMP, which diffuses from discrete points on the membrane and is rapidly me-

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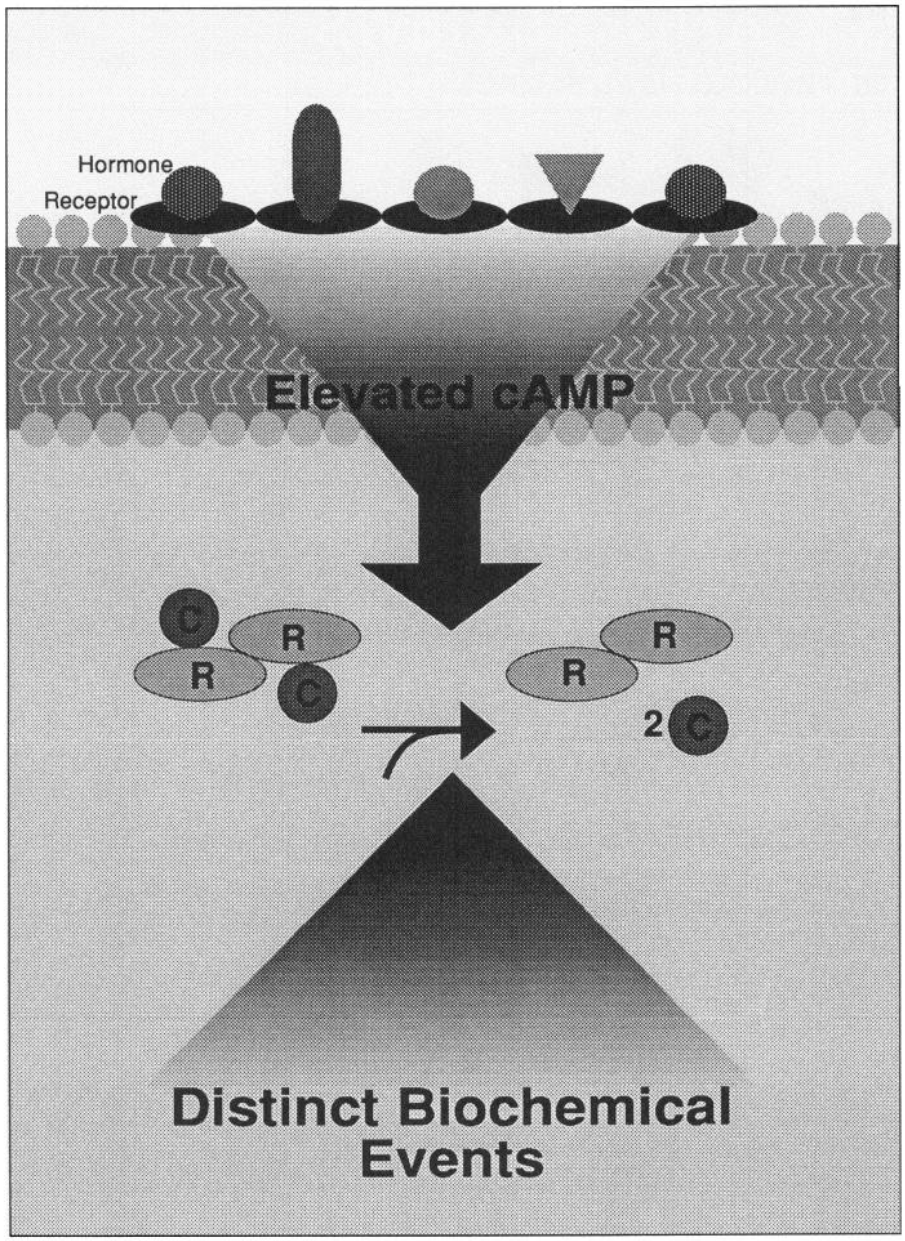

FIGURE 1. Hormone-specific activation of adenosine 3′,5′-cyclic monophosphate (cAMP)-dependent protein kinase. Individual hormones bind their own receptors, which leads to elevation of the second messenger cAMP. Elevated cAMP promotes activation of cAMP-dependent protein kinase and somehow triggers the specific physiological effects associated with the activating hormone. R=regulatory subunit and C=catalytic subunit of cAMP-dependent protein kinase.

tabolized to AMP by phosphodiesterases. Therefore, PKA must be localized either close to the adenylate cyclase complex or in an area that preferentially concentrates cAMP.

A potential mechanism for hormone-specific activation of particular PKA subtypes is the compartmentalized elevation of cAMP. Immunohistochemical analysis of frozen, formaldehyde-fixed cells has shown that localized cAMP concentrations fluctuate in specific subcellular compartments.

Utilizing microwave-fixing techniques, Barsony and Marx (1) have shown that specific hormones can increase cAMP concentrations in subcellular compartments in epithelial and fibroblast cell lines. For example, isoproterenol and PGE_2 promote cAMP accumulation close to the plasma membrane, whereas calcitonin causes perinuclear accumulation of cAMP. Prolonged treatment with forskolin results in accumulation of cAMP in the nucleus. These authors conclude that the hormone-specific intracellular patterns of cAMP accumulation result from the combined effect of differences in local production rates, local binding to proteins, and intracellular flow.

Site-specific localization of PKA through RII-anchoring proteins

Biochemical and immunocytochemical studies have shown that PKA localization is directed through the R subunit (12). Although both RI isoforms are primarily cytoplasmic, certain tissues contain up to 75% of either RII isoform (RIIα or RIIβ) in particulate form, associated with the plasma membrane, cytoskeletal components, secretory granules, or the nuclear membrane.

The mechanism of type II PKA localization involves protein-protein interactions between RII and specific RII-anchoring proteins. The majority of RII-anchoring activity is detected in high-molecular-weight particulate fractions, implying attachment to membranes and the cytoskeleton. Furthermore, tissue-specific patterns of RII-anchoring proteins have been detected by protein-blotting techniques or fractionation with RII-Sepharose affinity columns.

Using these techniques, we have detected upwards of 30 different RII-anchoring proteins during a survey of protein extracts from 10 different bovine tissues. Presumably, tissue-specific PKA function is directed through unique complements of RII-anchoring proteins, and localization influences which substrates are most accessible to the catalytic subunit on activation. In agreement with this theory, a significant subset of RII-anchoring proteins are PKA substrates themselves that become phosphorylated at multiple sites in response to cAMP.

Recently, we have cloned two novel RII-anchoring proteins that, when expressed in *Escherichia coli*, are PKA substrates in vitro. Both molecules, Ht21 and Ht31, contain clusters of consensus PKA phosphorylation sites (R-R-X-S-X) within a 20-residue region of their sequences. Potentially, phosphorylation may unmask activities in each

anchoring protein and may be a mechanism for triggering individual cAMP-mediated responses.

RII dimerization is required for anchoring-protein interaction

As previously stated, PKA is tethered at precise subcellular sites through interactions between the regulatory subunit (RII) and anchoring proteins (12). In recent months we and others have utilized recombinant DNA and protein chemistry techniques to characterize the site of anchoring protein interaction on RII (9, 13).

To establish the minimum region of RIIα required to bind anchoring proteins, a family of RIIα deletion mutations was constructed, expressed in *E. coli*, and tested for anchoring activity in a solid-phase binding assay using the cytoskeletal component microtubule-associated protein 2 (MAP2) as a substrate. Anchoring-protein interaction occurs through the first 79 amino acids in each RII promoter but, surprisingly, only with the intact RII dimer (13). Residues 1–30 represent an independent RIIα dimerization subdomain, functional when removed from the remainder of the molecule.

Since this region alone is unable to bind MAP2, other regions distal to the dimerization domain must also participate in anchoring-protein interaction. Two distinct peptide subdomains found in each RIIα promoter indeed appear to participate in such interaction. A schematic diagram summarizing these findings is presented in Fig. 2.

Once the components of the MAP2 binding site had been established, other RII-anchoring proteins were screened to see whether they bound the RIIα dimer. In all cases, disruption of dimerization destroyed anchoring-protein binding. Furthermore, competition studies with P75, a bovine brain anchoring protein, suggest that it binds on RII through the same or overlapping sites as MAP2 (13). Therefore, we believe the schematic diagram of the RII-anchoring-protein interaction presented in Fig. 2 represents a general model for how the type II PKA is localized at discrete cellular sites.

This model has implications for hormone-specific control of PKA function. For example, the anchoring-protein receptor site is immediately adjacent to the autoinhibitor domain in RIIα (Fig. 2). The RRVSV sequence motif (residues 92–96 in murine RIIα) is believed to provide a primary contact site with the dormant catalytic subunit. Therefore, the topology of an anchored type II PKA holoenzyme complex (Fig. 2) could place both catalytic subunits in close proximity to the anchoring protein, allowing its rapid and preferential phosphorylation following C subunit activation. In support of this hypothesis, it has been shown that MAP2 is phosphorylated by PKA at multiple sites, incorporating up to 11 moles of phosphate. Separate studies have shown that most RII-anchoring proteins isolated by affinity chromatography on RIIα-Sepharose are also PKA substrates.

Although anchoring-protein contact occurs at sites between residues 30 and 79 on each RIIα protomer, the orientation of these contact sites may be dictated by dimerization, which occurs between residues 1 and 30. We predict that if RIIα protomers dimerize in a parallel alignment, the topology of the anchored holoenzyme complex places both C subunits on the same face of the holoenzyme as the anchoring protein. In this configuration, RII-anchoring proteins would be ideal substrates for the kinase and could be instantly phosphorylated on PKA activation.

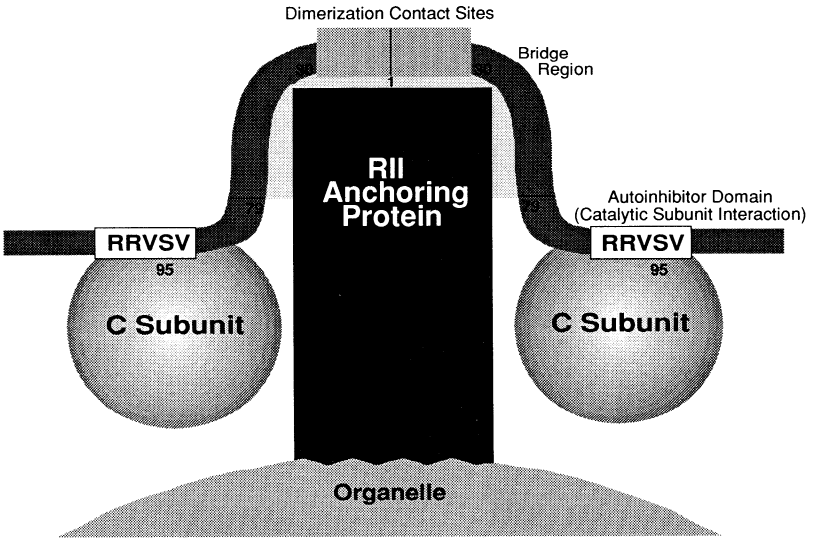

FIGURE 2. Model for RII-anchoring protein receptor site. Schematic summarizes our findings and shows how the first 79 residues of murine RIIα could interact with an RII-anchoring protein and thereby target type II protein kinase (PKA) holoenzyme to specific subcellular locations.

Alternatively, if RII protomers dimerize in an antiparallel alignment, one C subunit may face into the anchoring protein, whereas the other will face away from it. In this confirmation, the C subunit would be more able to diffuse and phosphorylate other proteins, within its microenvironment. Current work in our laboratory is focusing on elucidating the orientation of RIIα dimerization and the availability of the anchoring protein to the C subunit.

RII-anchoring proteins

Although RII-anchoring proteins may play a central role in cAMP-mediated signal transduction, little is known about their structure, subcellular location, or tissue distribution. So far, only four RII-anchoring proteins have been characterized in any detail. Cytoskeletal attachment of type II PKA occurs through interactions between RII and MAP2. RII also associated with a bovine brain calmodulin-binding protein designated P75 (2).

Several P75 analogues, ranging in size from M_r 60,000 to 150,000, have been reported in different species and may represent members of a family of structurally related RII-anchoring proteins (2). It has also been demonstrated that the P75/150 family of proteins binds to calmodulin. Because proteins in the P75/150 family associate with both PKA and calmodulin, these RII-binding proteins appear to function in both

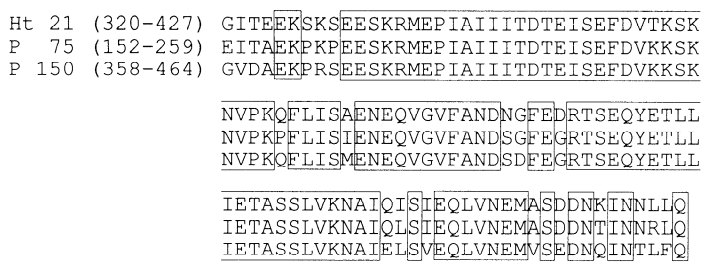

FIGURE 3. RII-binding domains of the P75/150 family of RII-anchoring proteins. Sequences of COOH-terminal RII-binding domains of Ht21 (residues 320–327), P75 (residues 152–259), and P150 (residues 358–464). Boxed areas represent sequence identity.

cAMP and Ca^{2+}-mediated transduction pathways.

Many studies have shown that the P75/150 family is expressed specifically in neural and endocrine tissues and preferentially associates with the RIIβ isoform. Ht21, a human thyroid RII-anchoring protein we have recently cloned and characterized, is a member of the P75/150 family. Sequence comparison of P75, P150, and Ht21 reveals strong homology among all three molecules in the carboxyl-terminal 107 amino acids (Fig. 3). Two lines of evidence suggest that this is a conserved RII-binding domain: deletion of the last 26 residues in P75 or the last 15 in P150 destroys interaction with RIIβ, whereas expression of the last 107 amino acids of Ht21 alone produces a functional RII-anchoring fragment.

While the carboxyl-terminals of all three proteins share a highly conserved RII-binding domain, the remainder of each molecule is structurally distinct. This lack of homology could be explained by species drift, since P150 was cloned from mouse, P75 from bovine, and Ht21 from humans, although this is unlikely because of the degree of divergence.

An alternative and more attractive hypothesis is that the amino-terminal portion of each P75/150 family member is a distinct domain that targets the RII-anchoring protein to specific cellular compartments. For example, the amino-terminal portion of P150 could promote membrane association, whereas the amino-terminal portion of Ht21 promotes interaction with a different cellular organelle. Furthermore, since the amino-terminal region of Ht21 is a substrate for PKA, it is possible that phosphorylation may alter its subcellular anchoring or its calmodulin-binding properties. The characterization of the amino-terminal regions of the P75/150 family of RII-anchoring proteins is an important topic for future research.

Amphipathic helices form the RII-binding sites on anchoring proteins

Since several anchoring proteins apparently bind to the same or overlapping sites on RIIα, it seemed likely that these molecules share a common RII-binding domain. The site on MAP2 that contacts RIIβ has been identified as a 31-residue peptide in the amino-terminal region of the molecule (10, 11), whereas the RII-binding domains in P75 and P150 are believed to include the last 26 and 15 amino acids of the proteins, respectively (2, 3). Comparison of these sequences revealed no striking homology (Fig. 4*A*), leading us to examine the RII-binding site in each anchoring protein for a conserved secondary structure binding motif.

Computer-aided secondary structure predictions of each putative RII-binding site showed a high probability of amphipathic helix formation. The distinction between the hydrophobic and hydrophilic faces can be clearly seen when the sequences are drawn in a helical-wheel configuration (Fig. 4*B*). In each RII-anchoring

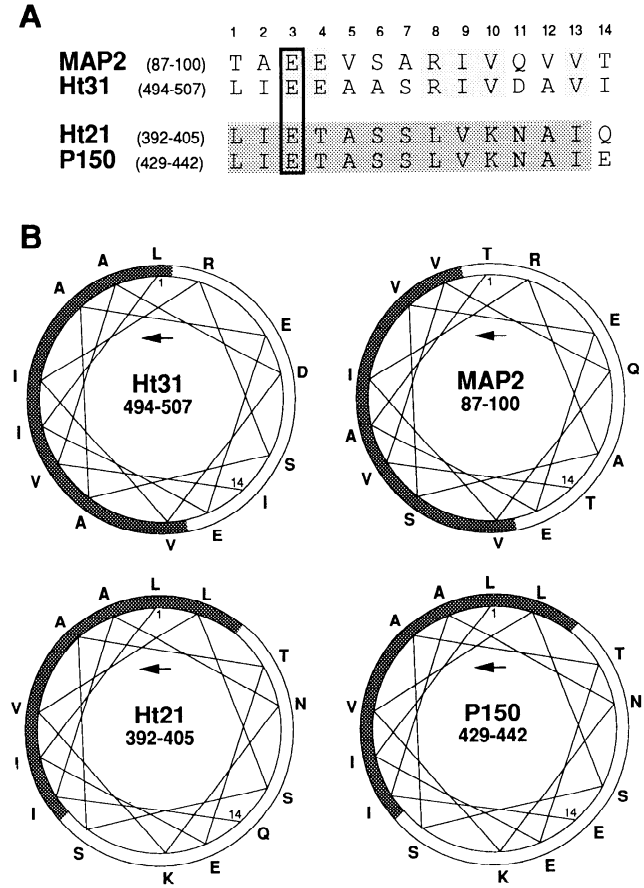

FIGURE 4. Sequence comparison and helical-wheel analysis of RII-anchoring proteins. *A*: sequences of 4 RII-anchoring proteins, MAP2 (residues 87–100), Ht31 (residues 494–507), Ht21 (residues 392–405), and P150 (residues 429–442), are aligned. The invariant glutamic acid residue present in all 4 proteins is boxed; light-shaded areas indicate homology between MAP2 and Ht31, and dark-shaded areas indicate homology between Ht21 and P150. *B*: helical wheel representation of RII-anchoring protein sequences. Each sequence was drawn as an α-helix with 3.6 amino acid residues/turn. Shaded areas identify hydrophobic face of each amphipathic helix. Amino acid residues are represented by single-letter codes.

protein there was a similar alignment of acidic residues throughout the hydrophilic face of each putative helix.

Analysis of Ht31, a novel human thyroid RII-anchoring protein of 1,035 amino acids we recently cloned, identified a potential amphipathic helix between residues 494 to 509. This sequence (Leu-Ile-Glu-Glu-Ala-Ala-Ser-Arg-Ile-Val-Asp-Ala-Val-Ile-Glu-Gln) was 43% identical to region within the RII-binding site of MAP2 (Fig. 4*A*). To determine whether residues 494–509 of Ht31 were involved in RII binding, a 318-amino acid fragment representing residues 418–736 of Ht31 was expressed in *E. coli*. Ht31Δ 418–736 bound RIIα as assessed by solid-phase binding and gel-shift assays (4).

To determine whether an intact amphipathic helix was required for RII binding, a family of Ht31 point mutants was produced in the Ht31Δ 418–736 fragment. The introduction of proline into an α-helix conformation disrupts the secondary structure of the region and causes a 20° bend in the peptide backbone. The introduction of proline into the amphipathic helix region of Ht31 diminished or abolished RIIα binding (4). Weak RIIα binding was observed with mutant Ht31 Pro/Ala 498, which contained a mutation at position 5 in the putative helix region. No RIIα binding was detected with either Ht31 Pro/Ile 502 or Ht31 Pro/Ile 507.

In contrast, proline substitution of Ala 522, which lies 12 residues downstream of the amphipathic helix region, had no apparent effect on RII binding.

These results suggest that disruption of protein secondary structure between residues 498 and 507 of Ht31 diminishes or abolishes RII binding. Recently, we have synthesized a peptide that spans the putative amphipathic helix region of Ht31. This peptide binds RIIα with an affinity of approximately 1 nM (unpublished observation). Circular dichroism analysis of this peptide suggests it can adopt an α-helical conformation.

One characteristic of the amphipathic helix motif is the ordered placement of alternating pairs of hydrophobic and hydrophilic amino acids within the linear sequence of a protein. In addition to this criterion, each RII-anchoring protein contains acidic amino acids distributed over the hydrophilic face of the helix (4). In particular, an invariant glutamic acid at position 3 is located within the first turn of the amphipathic helix (Fig. 4*B*). Therefore, the overall sequence characteristics of an RII-anchoring site appear to be an approximately 14-residue region rich in acidic and hydrophobic amino acids with a high probability for amphipathic helix formation.

The lack of a consensus RII binding sequence is consistent with the hypothesis that each molecule contains a common secondary structure. Another example of conserved secondary structure is the family of 20 or so calmodulin-binding peptides, which exhibit little or no conservation in primary structure other than an abundance of basic and hydrophobic amino acids (6). The basic face of these amphipathic peptides forms ionic interactions with the acidic central helix of calmodulin. In fact, synthetic peptides consisting of alternating pairs of leucine and lysine residues have nanomolar binding affinities for calmodulin.

Because RII dimerization is required for binding, it is likely that the amphipathic helix of the RII-anchoring protein interacts with sites on both RII subunits. The nature of these interactions is unclear but may involve ionic interactions between the acidic face of the amphipathic helix and basic residues located on both RII protomers. Whereas the amphipathic helix region is responsible for RII association, it is clear that other regions in each anchoring protein are responsible for specific interaction with membranes, organelles, or the cytoskeleton. This two-fold binding could potentially determine the subcellular location of the PKA and, presumably, anchor the kinase in close proximity to its preferred substrates.

Sequence comparison of the four amphipathic helix regions (Fig. 4*A*) suggests that there may be two classes of RII-anchoring proteins. Ht31 and MAP2 are 43% similar over the 14-residue amphipathic helix region, whereas Ht21 and P150 have 93% sequence similarity (4). It is of interest to note that MAP2 has a sevenfold higher affinity for the RIIα isoform than for RIIβ (7).

It will be of interest to establish whether Ht21 and Ht31 preferentially bind to a particular RII isoform. The differential subcellular distribution of RIIα and RIIβ in neurons, demonstrated by electron microscope immunocytochemistry, may reflect, in part, a preferential interaction with distinct RII-anchoring proteins (8). Structural and functional analysis of additional RII-anchoring proteins is required to establish if, in fact, there are two classes of RII-anchoring sites and if they preferentially associate with a specific RII isoform. Nevertheless, the preferential interaction of RII isoforms with specific classes of RII-anchoring proteins is an attractive mechanism to explain the subcellular localization of different PKA isoforms.

The C subunit may also be adapted for anchoring, since it is myristylated at the amino-terminus (12). Myristylation of several molecules involved in signal transduction such as other protein kinases, phosphoprotein phosphatases, and GTP-binding proteins is commonly thought to promote targeting to sites on or near membranes. However, a mutant C subunit that cannot become myristylated is fully functional (5). It is quite possible that myristylation of the C subunit, in conjunction with anchoring by the regulatory subunits, preferentially maintains the kinase in hydrophobic environments.

Recently, a splice variant of the bovine PKA catalytic subunit, Cβ2, has been identified. It contains a putative amphipathic helix at the amino-terminus, replacing the normal myristylation signal (15). The role of this amphipathic helix is unknown, but the authors suggest it may function to tether PKA catalytic subunit to membranes. Potentially, amphipathic helices could be responsible for anchoring both RII and Cβ2 subunits, such that the active catalytic subunit is immobilized and remains close to its regulatory subunit on cAMP activation. Under these conditions, the accessibility of PKA substrates would be tightly controlled and kinase activity would be highly regulated, since rapid reassociation of the holoenzyme complex

would occur after cAMP concentrations returned to basal levels.

Conclusions

The type II PKA is dispersed at specific sites throughout the cell because of interaction of the regulatory subunit of PKA with anchoring proteins. We believe that PKA anchoring is important in controlling which substrate proteins become phosphorylated by individual kinase pools. Colocalization of the kinase with its physiological substrates is important as it ensures their rapid phosphorylation in response to elevated cAMP. To tightly control which pools of anchored kinase become active, compartmentalized changes in cAMP must occur.

The recent studies of Barsony and Marx (1) agree with this hypothesis and support the notion that hormone action promotes accumulation of cAMP in different cellular compartments. Therefore, it is conceivable that individual hormones will activate different pools of kinase localized in particular cellular compartments, thereby triggering phosphorylation of specific substrate proteins. Changes in the activity of the newly phosphorylated proteins will potentiate the hormonal response by altering the physiology of the cell.

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Regulation by Nitroxidergic Nerve of Arterial Tone

Noboru Toda and Tomio Okamura

Nitric oxide plays a crucial role in transmitting information from nonadrenergic, noncholinergic vasodilator nerve to cerebroarterial smooth muscle. Since nitric oxide acts as transmitter, the nerve is termed "nitroxidergic." Mesenteric and temporal arterial tone appears to be regulated by reciprocal nitroxidergic and noradrenergic innervation.

Cerebral artery responsiveness to neural, chemical, and physical stimuli differs qualitatively and quantitatively from that of peripheral arteries. Responses to efferent autonomic nerve stimulation of dog cerebral arteries contrast with those of mesenteric, renal, carotid, and femoral arteries that are innervated dominantly by vasoconstrictor noradrenergic nerves (3).

The addition of nicotine produces relaxations of cerebral artery strips that are abolished by hexamethonium but not influenced by treatment with atropine, β-adrenoceptor antagonists, histaminergic H_1 and H_2 receptor antagonists, aminophylline, cyclooxygenase inhibitors, and oua-

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