

Using InCELLect™ Cell-Permeable, Stearated Peptides to Probe cAMP-Dependent Protein Kinase-Mediated Cellular Signaling Reactions In Vivo



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Protein kinase- and phosphatase-mediated phosphorylation and dephosphorylation are key mechanisms used in cellular regulation. In addition to activation, precise subcellular localization is required for signaling specificity in response to a stimulus. Protein-protein interaction facilitates appropriate associations among members of a signaling cascade, which can be in the form of a scaffold or an anchor. Peptides derived from anchoring proteins can disrupt the interaction between enzymes and their anchoring proteins. A peptide derived from the A Kinase-Anchoring Protein (AKAP) Ht-31 (human thyroid) disrupts A kinase anchoring and thus A kinase-mediated responses in several model systems.

We have generated stearated forms of the peptide, InCELLect™ AKAP St-Ht31^(a) (Cat.# V8211), and its corresponding control peptide, InCELLect™ St-Ht31P^(a) (Cat.# V8221), which are cell permeable and facilitate the study of AKAP in mediating PKA responses. This article describes recent research using Promega's stearated peptides—InCELLect™ AKAP St-Ht31 Inhibitor Peptide and InCELLect™ St-Ht31P Control Peptide—on PKA anchoring in the cell.

INTRODUCTION

Protein kinases and phosphatases control a myriad of cellular signal transduction processes such as cell growth, differentiation, development, metabolic pathways, gene expression and cell death. It has been estimated that one-third of the proteins in a typical mammalian cell are phosphorylated, and about 200 protein kinases and 100 protein phosphatases have been identified. In fact, it is predicted that approximately 2–3% of the genes in the entire genome of an eukaryotic cell code for protein kinases (1). The specificity of some kinases mediating phosphorylation reactions is attained by a strict substrate specificity that limits the action of these kinases to a single or limited number of potential targets. However, most protein kinases and phosphatases have multiple substrates in vivo, which may explain their diverse physiological functions. Hence, the specificity of ligand-mediated response is not only dependent on the activation of the protein kinase/phosphatase but also on the proximity of these enzymes to their physiological targets. Therefore, molecular recognition of signaling molecules is critical in relaying specific signals in the cell (2). Several protein modules have been shown to confer the recognition required for protein-protein interactions and coordinate signaling among the appropriate members of a signaling cascade. The association of proteins in a cascade can be in the form of a scaffold where each member of a cascade interacts with a specific motif on the scaffolding protein platform (3,4) or in the form of an anchor that brings two or more members together via separate binding domains (5–8). Anchoring proteins are multivalent molecules and thus bring together two or more molecules to a subcellular locus such as the cytoskeletal apparatus or nuclear compartment. Thus, these proteins selectively alter the signaling pathways by binding to specific domains in their target protein.

ROLE OF AKAPs IN CELL SIGNALING

The discovery of PKA (9), as well as its role in phosphorylating many cellular proteins and enzymes, is generating increasing interest. Studies of the heterogeneity, function and regulation of PKA provide insight into the enzymology of diverse protein kinases and into the role of these kinases in cellular processes (10). Furthermore, the subcellular localization of A kinase, as a result of its binding to various anchoring proteins, adds an additional element to A kinase signaling specificity. The anchoring proteins for A kinase are called AKAPs, for A Kinase-Anchoring Proteins. AKAP binds to the regulatory (R) subunits of PKA. They have been implicated in many PKA-mediated cellular processes (11). Such processes involve altering the activity of AMPA/kainate channels, regulation of a specific glutamate receptor-gated ion channel, modulation of the L-type Ca²⁺ channels in skeletal muscle (11), hormone-mediated insulin secretion in clonal beta cells (12), vasopressin-mediated translocation of aquaporin-2 into cell membrane of renal principal cells (13), motility of mammalian sperm (14,15) and acrosome reaction (AR) of mammalian sperm (16).

The AKAPs range between 15 and 300kDa and have been detected in a variety of tissues and in several subcellular compartments (11). The affinity constant for RII-AKAP interaction ranges between 1 and 11nM, within the intracellular concentration of RII and most AKAPs, suggesting that this complex can be formed in vivo at basal cAMP levels (6). Each protein contains two classes of binding sites, a conserved “anchoring motif” that binds the regulatory subunit (R) of PKA and a “targeting domain” that directs the subcellular localization of the PKA-AKAP complex through association with subcellular organelles, membranes or proteins. The R binding sequence of a human thyroid AKAP (Ht31) was shown to form an amphipathic α -helical structure with acidic residues at the hydrophilic face. (Amphipathic proteins contain both polar and nonpolar moieties.) This helix is required for recognition of the regulatory subunit of RII α , and mutations that disrupt this secondary helical structure reduce binding to RII in vitro (17,18). Most recently, it was reported that the size of the aliphatic side chain at the middle position determines the specificity of binding AKAP to either RI or RII, where small side chains favors RI and long aliphatic side chains are necessary for RII (19).

IN VIVO STUDIES OF AKAPs IN CELL SIGNALING

The discovery of AKAPs and AKAP-derived peptides, with their high affinity to form complexes with PKA, has stimulated interest in exploring the role of these proteins in vivo using several models of cell signaling (reviewed in references 6,7,11). Although transfection of cells with expression vectors containing genes for AKAPs might be useful, several drawbacks make the results difficult to interpret. These include whether expression is at a physiological level and what is the subcellular location of the expressed protein. Alternatively, others have either microinjected the AKAP-derived peptide (Ht-31), as well as its corresponding control peptide (Ht-31P), into cells or used artificially permeabilized cells to

enhance the uptake of the peptides. The peptide Ht-31 has an amphipathic helical structure and was shown to be effective in interrupting PKA binding to Ht-31 in vitro and in vivo when microinjected into cells (11), and the control peptide Ht-31P was ineffective, confirming the specificity of the active peptide in disrupting PKA-mediated responses. Although these techniques have been successfully used to explore the role of AKAP-derived peptides in vivo, they require sophisticated equipment and training of personnel on their use (e.g., microinjection) or raise concern over cellular damage caused by permeabilizing agents. We proposed that the attachment of a hydrophobic moiety, such as an alkyl group, to the peptide would improve its uptake through the lipophilic microenvironment of the plasma membranes. Toward this goal, we used the steartated form of the Ht-31-derived peptide (St-Ht31) and its corresponding control St-Ht31P to study their effect on mammalian sperm motility.

Mammalian sperm serve as an excellent model to study the role of AKAP on cell motility since this activity is stimulated within 10 minutes after an increase in cAMP level, and sperm possess a predominant localization of type II PKA on the outer membrane of the mitochondria. Overlay analysis of bovine, human and monkey sperm using ³²P-labeled RII α or RII β as probes detected a single dominant AKAP in each species (Figure 1, Panel A). The bovine and the human AKAP migrated with a MW of 110kDa, and the monkey AKAP was slightly larger (115kDa). Sequence analysis of this AKAP revealed that it contains eight functionally conserved positions within an amphipathic helix structure that interact with RII. It is exclusively present in testis and primarily in round spermatids suggesting its exclusivity to male germ cells (20). When the Ht-31-derived peptide, which contains the amphipathic helical motif that is required for binding to RII, was added to the overlay assay, it blocked RII binding to AKAP 110 (Figure 1, Panel B) suggesting that this sperm AKAP also contains an amphipathic helix-binding domain. A cell-permeable steartated Ht-31 counterpart (St-Ht31) also inhibited in vitro binding of RII to AKAP 110. As observed in earlier studies, the control peptide Ht-31P and its steartated form (St-Ht31P) had no effect on RII binding.

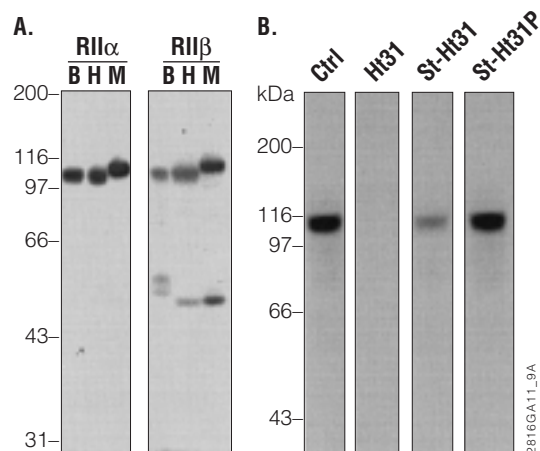


Figure 1. Identification of PKA subunits and AKAPs in mammalian sperm. Sperm from bovine (B), human (H) and monkey (M) were lysed, and the proteins were separated by SDS-PAGE and analyzed by Western blotting and overlay analysis. **Panel A:** A single predominant AKAP was detected in bovine, human and monkey sperm using either RII α or RII β probes. **Panel B:** Disruption of RII binding to sperm AKAPs by InCELLect™ AKAP St-Ht31 Inhibitor Peptide (Cat.# V8211). Ht31, nonsteartated form of AKAP Ht31; St-Ht31, InCELLect™ AKAP St-Ht31 Inhibitor Peptide (Cat.# V8211); and St-Ht31P, InCELLect™ St-Ht31P Control Peptide. (Reproduced from reference 14 with permission.)

INCELLECT™ CELL-PERMEABLE PEPTIDES IN A SPERM MOTILITY ASSAY

To test the effect of the St-Ht31 and its control (St-Ht31P) on sperm motility, we incubated the peptides with vigorously motile sperm under a variety of conditions. Sperm motility was measured as the forward motility index (FMI), which is a product of percent motility (percent of sperm with velocity >20mm/sec) and average velocity (14). As shown in Figure 2, Panel A, the basal motility was inhibited by the St-Ht31 peptide in a concentration- and time-dependent manner, and complete arrest of motility was achieved at concentrations of 5–50 μ M within 3–5 minutes after addition of the peptide St-Ht31. When motility was measured at five minutes post-treatment, the concentration of the peptide required for 50% inhibition was approximately 1 μ M (Figure 2, Panel B). The control peptide, St-Ht31P, which is ineffective in disrupting PKA anchoring to AKAPs, had no effect on sperm motility at concentrations of up to 100 μ M, suggesting that motility inhibition by the active peptide was due to a disruption of PKA anchoring.

Another peptide, St-AKAP79, containing the amphipathic helix RII binding motif, also inhibited sperm motility (Figure 2, Panel C). In contrast, St-CaNBP and St-PKI peptides, which do not have the amphipathic helical motif but inhibited the activity of calcineurin and PKA, respectively, had no effect on motility. The reduced potency of St-AKAP79 compared to St-Ht31 might be due to difficulty in solubilizing this peptide in aqueous solution, and thus, we are uncertain of its final concentration. These results are consistent with the model where RII/AKAP interaction is required for motility.

Sperm viability and membrane integrity in the presence of cell-permeable peptides were similar to those of control treatments as shown by dye uptake tests with SYBR[®]-green and rhodamine 123, and Fura 2 release, respectively. The addition of St-PKI (up to 100 μ M) to sperm had no effect on motility as shown earlier (Figure 2, Panel C). Similarly, the addition of high levels of another PKA inhibitor H-89 (50 μ M) only inhibited the basal sperm motility by about 50% or less. However, H-89 had no effect on sperm motility stimulated by the adenosine analog CDA or agents that increase intracellular cAMP, such as IBMX or 8-bromo-cAMP instead of CDA. Thus, stimulation of sperm motility that is induced by a cAMP-generating system does not require an increase in the catalytic activity of PKA and occurs even when PKA kinase activity is substantially inhibited.

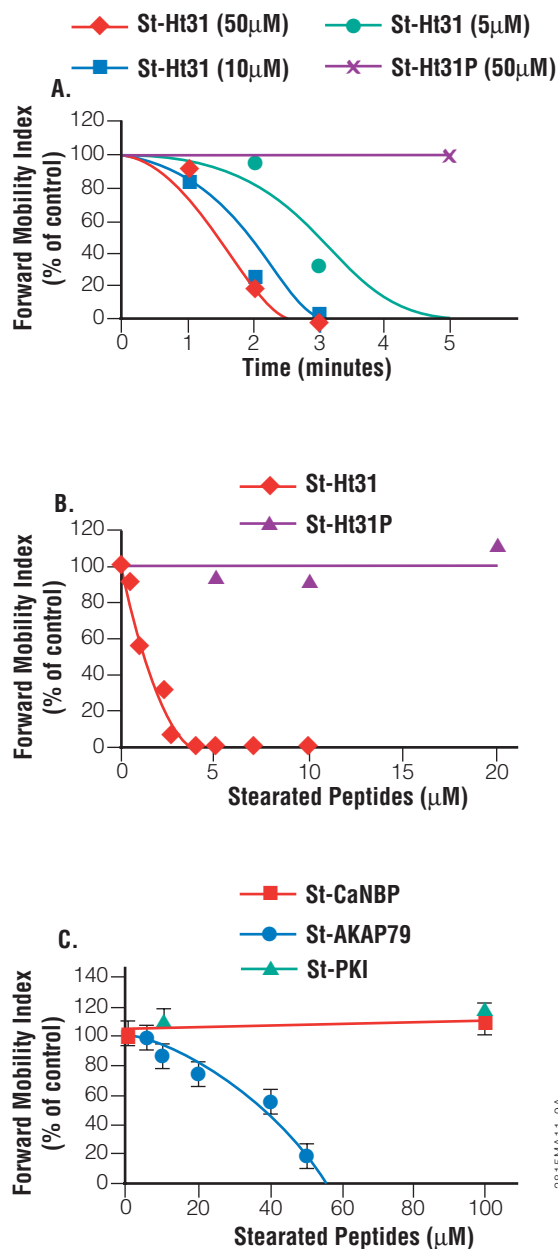


Figure 2. Anchoring inhibitor peptides arrest bovine sperm motility in a time- and dose-dependent manner. **Panel A:** Sperm were incubated in buffer containing 5 μM (●), 10 μM (■) or 50 μM (◆) of InCELLect™ AKAP St-Ht31 Inhibitor Peptide (Cat.# V8211) or 50 μM (×) InCELLect™ St-Ht31P Control Peptide (Cat.# V8221). **Panel B:** Sperm were incubated for 5 minutes in buffer containing increasing concentrations of InCELLect™ AKAP St-Ht31 Inhibitor Peptide (◆) or InCELLect™ St-Ht31P Control Peptide (▲) and motility was assessed. **Panel C:** Sperm were incubated for 5 minutes in buffer containing increasing concentrations of anchoring inhibitor peptide St-AKAP79 (●) or control peptides St-CaNBp (■) or St-PKI (▲), and motility was assessed. (Reproduced from reference 14 with permission.)

Table 1. Analysis of the Effects of St-Ht31 and St-Ht31P Peptides and H89 on Forskolin-Induced AQP-2 Translocation in IMCD Cells.

Treatment of Cells	Absolute Fluorescence Intensity	
	Intracellular	Cell Membrane
Nonstimulated	72.7 ± 5.5	44.9 ± 3.9
Forskolin	35.9 ± 2.5	113.2 ± 3.1
St-Ht31 + forskolin	79.5 ± 5.2	55.9 ± 5.2
St-Ht31P + forskolin	79.7 ± 5.3	137.2 ± 4.4
H89 + forskolin	73.7 ± 5.2	44.6 ± 3.5

IMCD cells were incubated with or without synthetic peptide or H89 for 30 minutes. If indicated, forskolin (100 μM) was added for a further 15 minutes. Thereafter, cells were fixed, permeabilized and incubated with anti-AQP-2 antibodies and secondary Cy³-conjugated anti-rabbit antibodies. Immunofluorescence was visualized and analyzed quantitatively by laser scanning microscopy. (Data reproduced from reference 13 by permission of the authors and the American Society for Biochemistry and Molecular Biology.)

RECENT APPLICATIONS

More recent studies with the AKAP St-Ht31 Inhibitor peptide showed that it strongly inhibited the forskolin-induced translocation of the water channel, aquaporin-2 (AQP-2), from intracellular vesicles into apical cell membranes of renal principal cells (Table 1) while the St-Ht31P control peptide was ineffective (13). The St-Ht31 was also shown to significantly stimulate the progesterone-initiated mammalian sperm acrosome reaction (AR). The addition of St-PKI5-24, a cell-permeable PKA inhibitor, prior to St-Ht31 addition significantly inhibited AR (16). These results together with our own studies support the use of the St-Ht31 peptide and its control peptide, St-Ht31P, to study PKA-mediated responses that are anchoring dependent.

Correction: This article originally appeared as *Application Note* (AN074). In AN074, the key to Figure 3, Panel C, contained an error. The symbols representing St-AKAP79 and St-CaNBp were switched. We apologize for the error, which has been corrected in the article as it appears here.

CONCLUSIONS

We have shown that the InCELLect™ Cell-Permeable Peptides, steared forms of AKAP-derived peptides, serve as excellent tools to explore the role of PKA and PKA-mediated responses in cell signaling. The cell-permeable peptides are active at relatively low concentrations, are easy to use and offer high selectivity for studies of PKA-mediated functions in the cell.

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Ordering Information

Product	Size	Cat.#
InCELLect™ AKAP St-Ht31 Inhibitor Peptide ^(a)	150µl	V8211
InCELLect™ St-Ht31P Control Peptide ^(a)	150µl	V8221

Related Products

Product	Size	Cat.#
cAMP-Dependent Protein Kinase, Catalytic Subunit	2,500 units	V5161
cAMP-Dependent Protein Kinase, Regulatory Subunit (Type II)	2,500 units	V5221
cAMP-Dependent Protein Kinase, Peptide Inhibitor	1mg	V5681
SignaTECT® cAMP-Dependent Protein Kinase (PKA) Assay System ^(a)	96 reactions	V7480
PepTag® Non-Radioactive cAMP-Dependent Protein Kinase Assay ^(b)	120 reactions	V5340
Kemptide PKA Peptide Substrate	1mg	V5601
cAMP	500µl	V6421

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^(a)Patent Pending.

^(b)U.S. Pat. No. 5,580,747.