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Pharmacological PKA Inhibition: All May Not Be What It Seems

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Signaling through the cyclic adenosine monophosphate–dependent protein kinase [protein kinase A (PKA)] is an important and widely studied area of signal transduction research. This signaling pathway is commonly investigated through the use of the pharmacological PKA inhibitors H89 and KT 5720. Both of these compounds are thought to block PKA actions through competitive inhibition of the adenosine triphosphate site on the PKA catalytic subunit. Recently, a number of studies have identified actions of H89 and KT 5720 that are independent of their effects on PKA. These nonspecific effects are widespread; they include actions on other protein kinases and signaling molecules and also on basic cellular functions, such as transcription. Here, I summarize the nonspecific effects of these two compounds and compare their actions with those of other PKA inhibitors.

Overview of cAMP Signaling

The transduction of extracellular signals to intracellular responses is one of the most important and complicated aspects of cellular life. The cyclic adenosine monophosphate (cAMP) signaling pathway is involved in numerous processes and is widely regarded as the "classical" second messenger signaling pathway. cAMP is synthesized from adenosine triphosphate (ATP) by adenylyl cyclase and is broken down to 5′ AMP by a class of proteins known as phosphodiesterases (PDEs) (*1*, *2*). Various stimuli activate adenylyl cyclase, but the best studied is ligand occupation of heterotrimeric guanine nucleotide-binding protein (G protein)–coupled receptors (GPCRs) coupled to G_s. Agonist occupation of G_s-coupled receptors catalyzes the exchange of guanosine diphosphate (GDP) to guanosine triphosphate (GTP) on the α subunit of the G protein, causing a conformational change and dissociation of this complex from the $\beta\gamma$ subunits. The α subunit can then interact with and activate adenylyl cyclases (Fig. 1). Receptors coupled to a different G protein, G_i, cause down-regulation of adenylyl cyclase activity and consequent lowering of cAMP concentrations (Fig. 1).

cAMP has three direct intracellular targets: protein kinase A (PKA), the exchange protein activated by cAMP (Epac), and cyclic nucleotide–gated ion channels (CNGCs). CNGCs, nonselective cation channels that open upon cyclic nucleotide binding, are particularly important in the olfactory and visual systems (*3*). Epac is a guanine nucleotide exchange factor for the small G protein Rap1 and has been implicated in a number of cellular processes such as insulin secretion, neurotransmitter release, and integrin-mediated cell adhesion (*4*–*6*). By far the best-studied aspect of cAMP signaling, though, involves cAMP-mediated activation of PKA.

Protein kinase A. PKA was discovered in the laboratory of Edwin G. Krebs in the 1960s (*7*). Since then it has been implicated in numerous cellular processes, including modulation of other protein kinases, regulation of intracellular calcium concentration, and regulation of transcription [reviewed in (*8*)]. Transcriptional responses to increased cAMP occur through activation of the cAMP response element–binding protein (CREB), cAMP response element modulator (CREM), and activating transcription factor 1 (ATF1) (*9*). Each of these transcription factors contains a kinase-inducible domain containing a conserved site for phosphorylation by PKA.

In its inactive state, PKA exists as a tetramer consisting of two regulatory and two catalytic subunits (Fig. 2). Four molecules of cAMP bind to the regulatory subunits to activate PKA, with two cAMP-binding sites, termed the A and B sites, that are present on each regulatory subunit. cAMP binding promotes a conformational change in PKA that initiates the dissociation of the catalytic subunits, leaving a dimer of the two regulatory subunits with four bound cAMP molecules. The two PKA catalytic monomers bind ATP; they then become catalytically active and can phosphorylate serine and threonine residues on proteins containing the appropriate substrate sequence (*1*) (Fig. 2). PKA signaling can occur in a very small defined domain because of the anchoring of PKA near its targets by A-kinase anchoring proteins (AKAPs), which tether PKA to particular cellular organelles and to the plasma membrane (*10*). PKA can also activate phosphodiesterases and promote cAMP breakdown in a negative feedback mechanism (*2*).

Pharmacological Blockade of PKA

The study of PKA function has been dominated by the use of pharmacological inhibitors of PKA. Their ease of use and ability to readily cross cell membranes has meant that two compounds in particular have been widely used to study PKA function: *N*-[2-(*p*-bromocinnamylamino)ethyl]-5-isoquinoline sulfonamide (H89) and (9*R*,10*S*,12*S*)-2,3,9,10,11,12-hexahydro-10-hydroxy-9-methyl-1-oxo-9,12-epoxy-1*H*-diindolo[1,2,3-fg:3′,2′,1′-kl]pyrrolo[3,4-i][1,6]benzodiazocine-10-carboxylic acid, hexyl ester (KT 5720). Separately or in combination, these two compounds have been used in more than 2000 separate studies.

H89 is an isoquinoline derivative that was developed from the nonspecific PKA and protein kinase G (PKG) inhibitor H8 (*11*); it has been reported to inhibit PKA with an inhibition constant (K_i) of 0.05 μM (*12*). KT 5720 is one of a family of compounds synthesized from the fungus *Nocardiopsis* sp.; it is thought to have a K_i of 60 nM for PKA inhibition (*13*). Both H89 and KT 5720 are thought to act through similar mechanisms (Fig. 3B), namely as competitive antagonists of ATP at its binding site on the PKA catalytic subunit (*13*, *14*). The catalytic subunit must bind ATP before it can phosphorylate appropriate serine or threonine residues on target proteins; therefore, blockade of this site prevents the cAMP-dependent phosphorylation of PKA substrates. Both H89 and KT 5720 are marketed as potent and specific inhibitors of PKA and are widely used as such.

Competition for ATP binding on protein kinases is a mechanism commonly exploited in developing pharmacological inhibitors. However, this approach presents two distinct and important problems. The

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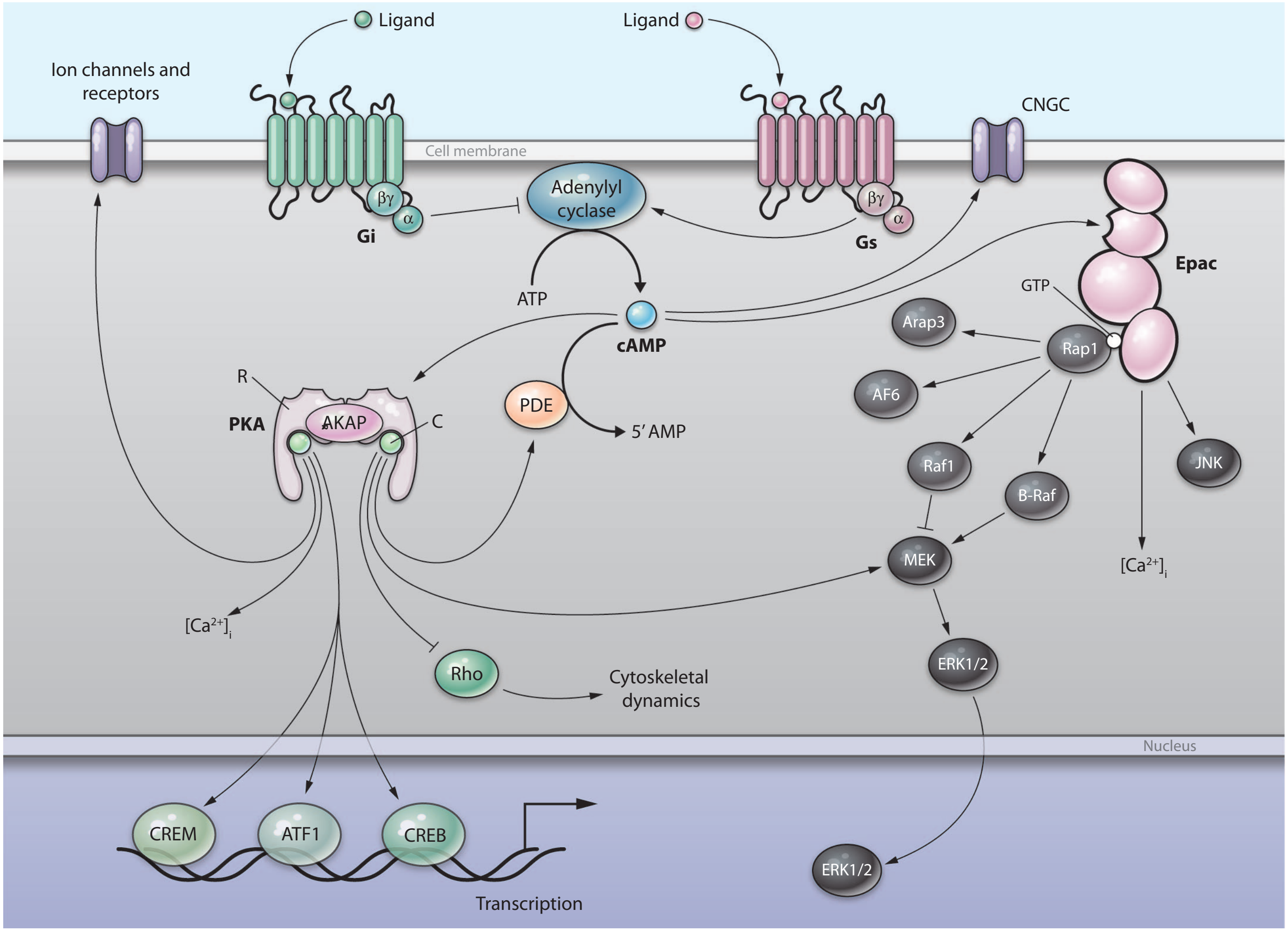


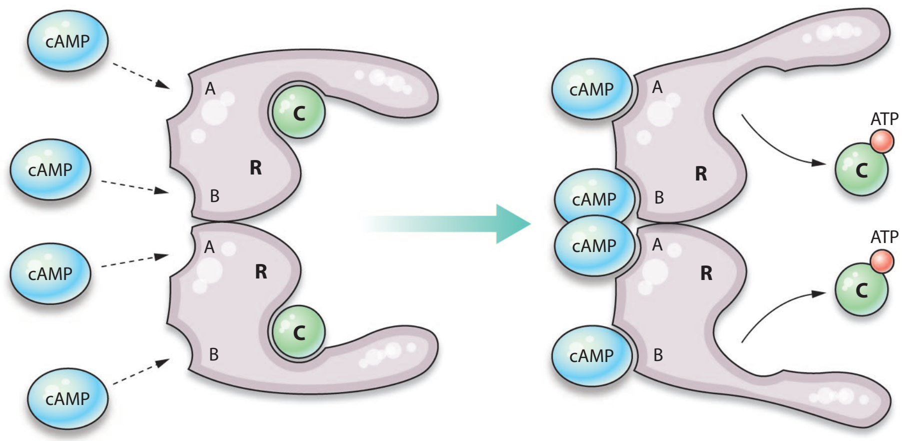
Fig. 1. Summary of the cAMP signaling cascade. cAMP is produced from ATP by adenylyl cyclase and is broken down by phosphodiesterases to 5′ AMP. Adenylyl cyclase activity can be modulated by agonist binding at GPCRs. cAMP acts directly on three targets: PKA, Epac, and CNGCs. These in turn regulate various cellular processes both directly and through intermediaries. For clarity, a number of pathways mediated by PKA, Epac, and Rap1 have been omitted. These pathways are discussed more fully in (*1*, *6*, *8*, *9*, *63*). AKAP, A-kinase anchoring protein; ATF1, activating transcription factor 1; C, protein kinase A catalytic subunit; cAMP, cyclic adenosine monophosphate; CNGC, cyclic nucleotide–gated ion channel; CREB, cAMP response element–binding protein; CREM, cAMP response element modulator; ERK1/2, extracellular signal–related kinase 1/2; JNK, Jun N-terminal kinase; PDE, phosphodiesterases; PKA, protein kinase A; R, protein kinase A regulatory subunit.

IC_{50} value (the concentration at which a compound inhibits 50% of a given activity) of these compounds varies according to the ATP concentration. Because ATP concentrations vary widely in cells depending on the prevailing physiological conditions, the concentration of inhibitor required for effective protein kinase blockade is not always clear. For example, the IC_{50} of KT 5720 established at very low ATP concentrations is 56 nM; however, when tested at more physiological ATP concentrations, the IC_{50} is closer to 3 μM (*15*). Furthermore, the abundance of ATP and ATP receptors in cells means that PKA inhibitors that interact with ATP-binding sites could be prone to nonspecific actions (*16*). Indeed, various nonspecific effects of KT 5720 and H89 have been reported.

Non–PKA-Based Actions of KT 5720

KT 5720 actions on other protein kinases. One study of the specificity of a range of protein kinases (*15*) found that a number of protein kinases were inhibited by KT 5720; some of these were inhibited at substantially lower concentrations than was PKA. For example, KT 5720 inhibited phosphorylase kinase, a molecule activated by calcium that is important in regulation of glycogen homeostasis (*17*), more potently than it inhibited PKA by a factor of >100 (*15*). 3-Phosphoinositide-dependent kinase–1 (PDK1), a serine-threonine protein kinase that can activate other kinases, including protein kinase B (PKB), protein kinase C, and serum- and glucocorticoid-inducible kinase (*18*), was inhibited by KT 5720 with an IC_{50} of 300 nM, one-tenth the IC_{50} of KT 5720 for PKA (at identical ATP concentrations). KT 5720 also inhibited MEK [mitogen-activated protein kinase (MAPK) kinase], mitogen- and stress-activated protein kinase 1 (MSK1), PKBα, glycogen synthase kinase 3β, and AMP-activated

Fig. 2. Activation of PKA by cAMP. In its inactive state, PKA consists of a tetramer of two regulatory and two catalytic subunits. Each regulatory subunit has two cAMP-binding sites; cAMP binding releases the catalytic subunits, which become bound to ATP and go on to phosphorylate serine and threonine residues that possess the appropriate substrate sequence. The two cAMP-binding sites (A and B) on each regulatory subunit are shown. C, protein kinase A catalytic subunit; R, protein kinase A regulatory subunit.



protein kinase at least as effectively as it inhibited PKA (*15*). Thus, KT 5720, directly or indirectly, could alter a multitude of signaling pathways and could thereby potentially falsely implicate PKA in a number of cellular processes.

Other studies have also found non–PKA-based effects of KT 5720. A study in Chinese hamster ovary (CHO) cells (*19*) showed that KT 5720 inhibited MAPK with an IC_{50} of 1.0 μM, whereas it inhibited PKA with an IC_{50} of 1.4 μM. The effects of MAPK inhibition by KT 5720 were found to alter the cellular cytoskeleton and modify cell shape. PKA signaling has been implicated in cytoskeleton modification, mainly by altering actin and tubulin dynamics (*20*); this nonspecific effect could thus obscure interactions between PKA and the cytoskeleton. Indeed, the MAPK pathway is involved in many cellular processes that interact with PKA signaling pathways, providing additional opportunities for misinterpretation.

KT 5720 actions on transcription and cellular receptors. One of the major functions of cAMP and PKA is the regulation of transcription through phosphorylation of transcription factors such as CREB (*9*). One study (*21*) showed that application of 10 μM KT 5720 completely abolished transcription in CHO cells. Although this finding has been recently contested (*22*), the possibility remains that this compound could alter gene expression through mechanisms independent of PKA.

The M1 muscarinic acetylcholine receptor is a GPCR that is expressed widely in the nervous system. Agonist occupation of M1 receptors stimulates phospholipase C activity, releasing calcium from intracellular stores, and can also modulate activity of the epidermal growth factor receptor (*23*). KT 5720 is a potent allosteric modulator of this receptor; at concentrations as low as 10 nM, KT 5720 increases the affinity of acetylcholine for M1 receptors by 40% (*24*). This suggests that KT 5720 may be inappropriate for studies in the nervous system or in neuronal cells expressing this receptor.

These studies indicate that KT 5720 has numerous actions unrelated to its ability to inhibit PKA. This suggests that other PKA inhibitors should be used in its place. However, the actions of H89 may also not be entirely restricted to PKA.

Non–PKA-Based Actions of H89

H89 actions on other protein kinases and calcium. cAMP and PKA stimulate neurite outgrowth in cultured neurons and neuronal cell lines and also promote the regeneration of damaged axons in vivo (*25*, *26*). One of the main mechanisms whereby cAMP-PKA is thought to promote neurite and axon growth is through inhibition of the small GTPase RhoA, which destabilizes the cytoskeleton through activation of Rho-associated kinase (ROCK) (*27*). H89 inhibited ROCK in two independent studies. H89 blocked ROCK activity completely when used at 10 μM in a kinase assay, a more potent effect than its inhibition of PKA (*15*). H89 (again at 10 μM) was also found to block ROCK activation in a neuroblastoma-glioma cell line (*28*). Application of H89 promoted the formation of neurite-like extensions and also prevented process retraction initiated by RhoA activation. Both of these actions were identical to the actions of a specific ROCK inhibitor and were not mimicked by another PKA inhibitor (*28*). As PKA itself has been shown to inhibit the actions of ROCK (*1*), these studies indicate a possible situation whereby both PKA and the PKA inhibitor H89 could have identical effects on this molecule, leading to obvious opportunities for misinterpretation of results.

Other important intracellular signaling molecules are also affected by H89. A study on isolated ventricular myocytes showed that H89 reduced Ca^{2+} uptake by the sarcoplasmic reticulum through a direct action on the Ca^{2+}-ATPase (*29*), perhaps by lowering this transporter's affinity for calcium (*30*). Another study showed that 20 μM H89 prevented increases in cytosolic calcium in response to glucose in pancreatic islets and reduced calcium release from intracellular stores in a differentiated β cell line (INS-1), an effect that was not mimicked by other PKA inhibitors and was thought to be independent of PKA inhibition (*31*). Recently, H89 has been shown to inhibit insulin-like growth factor–I (IGF-I) activation of the MAPK pathway (*32*). In a study on myelin basic protein expression in oligodendrocytes, 20 μM H89 prevented extracellular signal–regulated kinase 1 and 2 (ERK 1/2; part of the MAPK pathway) phosphorylation in response to IGF-I, independently of any actions on PKA (*32*). The numerous functions of calcium and the MAPK pathway in intracellular signaling processes make these observations particularly important.

In kinase assays (*15*), 10 μM H89 has been shown to inhibit the activity of several protein kinases by 80 to 100%, including MSK1, protein kinase B, serum- and glucocorticoid-induced kinase, AMP-activated kinase, checkpoint kinase, ribosomal protein s6 kinase (S6K1), and MAPK-activated protein kinase 1. Additionally, three kinases (ROCK-II, MSK1, and S6K1) were inhibited far more potently than PKA itself (*15*). S6K1 is a downstream target of mammalian target of rapamycin (mTOR) and has been implicated in processes as diverse as insulin signaling (*33*), regulation of cell size (*34*), and oncogenesis (*35*). Furthermore, S6K1 can interact with both the regulatory and catalytic subunits of PKA (*36*) and can directly phosphorylate and activate both CREB (*37*) and CREM (*38*); that is, just by inhibiting this one other kinase (S6K1), H89 could substantially obscure studies of PKA signaling. Thus, similarly to KT 5720, H89 can potentially affect a wide variety of signaling molecules.

H89 actions on cellular receptors and channels. The β_1- and β_2-adrenergic receptors (β_1AR and β_2AR) are seven-transmembrane receptors that are activated by binding of epinephrine or norepinephrine. These receptors are G protein–coupled and can alter adenylyl cyclase activity and thereby modulate cAMP production (*39*). They have multiple functions in the body, but are particularly important in the heart (*40*) and brain (*41*). A study on human airway cells (*42*) showed that H89 was a potent antagonist of both β_1AR and β_2AR, binding with K_i values of just 500 nM for β_1AR and 240 nM for β_2AR. In accordance with these receptors' role in modulating cAMP production, preapplication of H89 blocked cAMP accumulation induced by isoproterenol (a β-adrenergic agonist) in these cells. H89 could therefore alter cellular responses to adrenergic receptor binding and influence intracellular concentrations of cAMP. This would interfere with signaling through not only the PKA pathway but also other cAMP effectors such as Epac and CNGCs.

H89 alters conductance in a number of different classes of ion channel. In a study in alveolar type II epithelial cells, H89 was found to potentiate sodium transport by activating amiloride-sensitive sodium channels, an effect similar to that seen with the application of cAMP analogs but opposite to that of other PKA inhibitors (*43*). In experiments examining potassium currents in rat cardiac myocytes, H89 was found to lower potassium currents through voltage-dependent potassium channels, independently of PKA inhibition (*44*). In a separate study, H89 decreased currents through Kv1.3 K^+ channels expressed in CHO cells with an IC_{50} value of 1.7 μM (*45*).

The potency of both H89 and KT 5720 in a variety of cellular signaling molecules, processes, and receptors means that their use can no longer be accepted as evidence for PKA's involvement in cellular functions. There are, however, a number of other methods that can be used to study PKA signaling in intact cells.

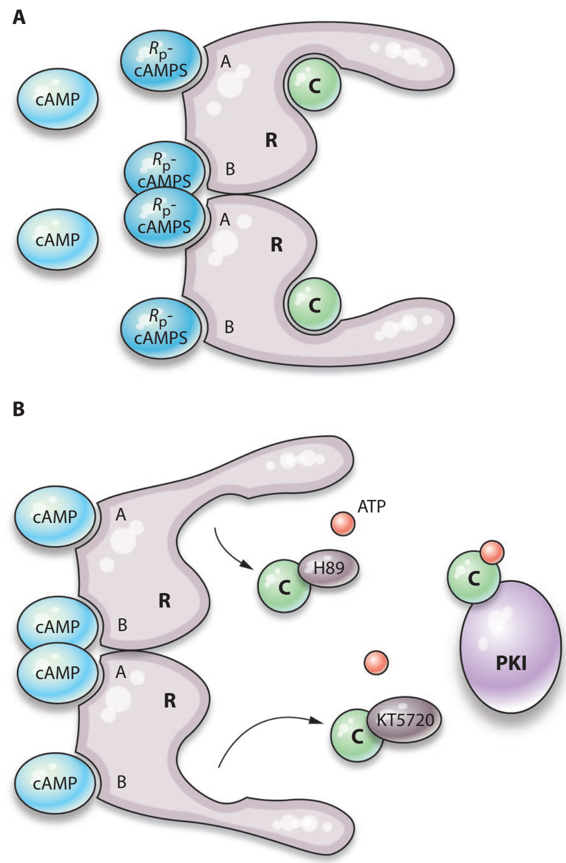


Fig. 3. Mechanisms of actions of PKA inhibitors. (**A**) R_p-cAMPS is a competitive antagonist of the cAMP-binding sites on PKA; binding of R_p-cAMPS to PKA prevents the catalytic subunits from being released. PKI peptide, H89, and KT 5720 all act one stage later. After cAMP binding and catalytic subunit release, PKI peptide binds the catalytic subunits and prevents them from phosphorylating targets. (**B**) H89 and KT 5720 are both competitive antagonists of the ATP sites on the catalytic subunits; without ATP binding, the catalytic subunits are unable to phosphorylate target proteins. C, protein kinase A catalytic subunit; PKI, protein kinase inhibitor peptide; R, protein kinase A regulatory subunit.

Alternative PKA Inhibitors

R_p-cAMPS. R_p-adenosine-3′,5′-cyclic monophosphorothioate (R_p-cAMPS) is the R_p isomer of the cAMP analog S_p-adenosine-3′,5′-cyclic monophosphorothioate (S_p-cAMPS) and acts as a competitive antagonist of the cyclic nucleotide–binding domains on PKA (46) (Fig. 3A). Several modifications of R_p-cAMPS have been synthesized that are more resistant to hydrolyzation by phosphodiesterases than is cAMP, and they are also membrane-permeable. The mechanisms of action and structure (closely related to cAMP) of the R_p-cAMPS family of compounds suggest that they are unlikely to have substantial effects outside of the cAMP signaling cascade. However, as R_p-cAMPS is a competitive inhibitor of the cAMP-binding site, its effects are diminished when endogenous levels of cAMP are extremely high, and in this situation cAMP may still be able to bind to and activate PKA. Additionally, although often considered selective inhibitors of PKA, these compounds should be used cautiously in studies of PKA function, as a number of other molecules contain cyclic nucleotide–binding sites similar in structure to those found on PKA. For example, whether R_p-cAMPS acts on the cAMP-binding site of Epac is controversial; although it has been shown that R_p-cAMPS has a high affinity for PKA, it is also known to bind to Epac (*47*, *48*). Cyclic nucleotide–gated ion channels contain similar domains, and it is conceivable that R_p-cAMPS could also interfere with their function, although this has not yet been reported.

Protein kinase inhibitor peptide. Protein kinase inhibitor peptide (PKI) is an endogenous molecule that participates in the regulation of PKA activity. PKI binds to the free catalytic subunit of PKA and prevents phosphorylation of PKA targets, in a manner similar to how the catalytic subunits are "housed" by the regulatory subunits of PKA in the absence of cAMP (Fig. 3B). Three distinct PKI isoforms have been identified (α, β, and γ), and each is expressed in various tissues and cell types (*49*). Synthetic forms of PKI have been developed, such as PKI-(6-22)-amide and PKI-(Myr-14-22)-amide, and these have been used to examine the role of PKA in various cell processes (*49*). As a result of its mechanisms of action, PKI is likely to be a more specific inhibitor of PKA than is either H89 or KT 5720; indeed, endogenous PKI is thought to be completely specific for PKA. However, higher concentrations of the synthetic PKIs affect PKG signaling (*50*), and so these compounds still need to be used with caution.

Molecular techniques. RNA interference (RNAi) has revolutionized the study of signal transduction molecules and led to the 2006 Nobel Prize in Physiology or Medicine for its discoverers (*51*). Briefly, the process involves the production of small interfering RNAs (siRNAs), sequences 20 to 25 nucleotides in length that bind to specific mRNA molecules and prevent protein synthesis. This has the effect of "knocking down" levels of particular proteins. Commercially available siRNAs are readily accessible and can be introduced into intact cells through various transfection techniques. Inhibition of PKA activity can be achieved with knockdown of the catalytic subunits of PKA. Three different isoforms of the catalytic subunit have been identified (α, β, and γ); α and β are expressed in various cell types, whereas γ is thought to be expressed only in the testis (*52*). Therefore, for effective inhibition, at least two isoforms (α and β) of this subunit need to be targeted with siRNA (*53–55*). Furthermore, the recent coupling of RNAi technology with adeno-associated viral vectors allows for the study of PKA signaling in vivo in both a spatially restricted and cell type–restricted manner (*56*). Although some off-target effects of RNAi have been reported (*57*), these molecules still represent a far more specific approach than H89 and KT 5720.

Introduction of a nonfunctioning PKA mutant, such as a dominant negative version of PKA, into cells allows researchers to specifically perturb signaling through PKA with a far-decreased likelihood of nonspecific effects. Dominant negative forms of PKA have been used to examine PKA's role in cell anchorage (*58*), protein expression in epithelial cells (*59*), and differentiation of tumor cells (*60*). PKA signaling can also be perturbed by transfection of cDNA that prevents binding of the regulatory subunits to AKAPs and therefore prevents the localization of PKA to specific cellular organelles. This strategy has been used, for example, in the study of PKA signaling in neuronal receptor expression (*61*). The major drawback of these strategies is the difficulty of transfecting constructs into cells in vitro or in vivo with high efficiency. This is especially problematic with postmitotic cells such as neurons. However, transfection technology is improving constantly, and a number of different methods now exist, such as viral vectors and numerous commercially available lipofection reagents. The extremely high specificity of these techniques relative to pharmacological agents makes it likely that molecular techniques will continue to set the standard in signal transduction research.

Future Strategies for PKA Inhibition

The above outlined studies indicate that neither KT 5720 nor H89 should be used alone to study the function of PKA. As these compounds are so commonly used, it will therefore be necessary to devise strategies that can overcome their shortcomings. One possibility is to use H89 or KT 5720 along with R_p-cAMPS, PKI, or both. If these distinct sets of inhibitors give similar results, the investigator can be more confident of PKA involvement. (Additionally, the use of H89 and KT 5720 could be accompanied by tests of inhibitors of other kinases to assess whether any of the observed effects could have been through inhibition of other signaling molecules.) PKA inhibitors could also be used along with specific activators of PKA. Recently, a number of cAMP analogs that can specifically activate PKA (rather than other cAMP targets) have become available (*62*). Demonstration that these activators have effects opposite to those of inhibitors such as R_p-cAMPS would enable the experimenter to have more confidence that PKA mediated the functions under investigation. Finally, and perhaps most preferentially, pharmacological inhibitors of PKA could be used in combination with one or more genetic methods for blocking PKA signaling. Mimicking the effect of H89 or KT 5720 with siRNA or dominant negative PKA would provide the most reliable indication of PKA function.

This combinatorial approach to PKA inhibition should provide a new benchmark for analysis of PKA function and hopefully will lead to a more discriminate use of ATP site–directed inhibitors.

Summary

The PKA inhibitors H89 and KT 5720 have been widely used in the study of signal transduction and have provided invaluable insights into the function of PKA in various cell types. However, a substantial body of evidence has now accumulated that indicates that both H89 and KT 5720 can have effects independent of PKA inhibition. These actions are extremely varied; some of the most worrisome actions are the substantial effects on the MAPK and calcium signaling pathways, which interact with the PKA pathway and mediate multiple cellular functions. Furthermore, many of these non–PKA-based actions of H89 and KT 5720 occur at concentrations that have been widely used to investigate PKA function. Despite these nonspecific actions, both of these compounds are still widely used; PubMed searches reveal that in the past 2 years alone, they have been used in more than 200 studies. The identification of a distinct target for cAMP (Epac) has complicated the cAMP signaling cascade. Many of the pathways involved in Epac and PKA signaling are related, and some cellular processes that have been previously attributed to PKA may actually involve Epac. Therefore, the use of H89 and KT 5720 could give false indications of PKA function by acting both within and without the cAMP signaling cascade. A number of other methods exist, both pharmacological and molecular, for studying the function of PKA, and many of these are more specific. Furthermore, the molecular bases of some cellular processes attributed to PKA solely through the use of these compounds may have to be reevaluated. Thus, although H89 and KT 5720 have been extremely useful in examining the roles of PKA in cell signaling, it may now be time for them to be superseded by other methods.

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