Photoactivation of a Signal Transduction Pathway in Living Cells

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Received May 22, 1998

Light-based chemical bond cleavage of masked functional groups is a simple, noninvasive, and effective technique that has been employed to generate concentration jumps of such species as Ca^{2+}, cAMP, NO, ATP, and others in vivo.[1] In addition to small caged ions and molecules, several caged proteins have been described.[2] However, until recently, there have not been any studies describing caged regulators of protein kinase signal transduction cascades. Such species should prove to be of decided utility in assessing the role of individual protein members of signal transduction pathways for several reasons. First, since caged species can be photochemically liberated with temporal precision, the presumed strict temporal control of signaling cascades can be easily assessed. Second, caged species can be readily loaded into cells in an innocuous form and then unleashed only when needed. Third, it should be possible to control subcellular spatial photorelease by employing appropriately designed caged species.[1] We recently reported the design and synthesis, as well as the in vitro and in vivo behavior of a caged protein kinase inhibitor, a species that blocks the signal transduction cascade controlled by the cAMP-dependent protein kinase (PKA).[3] We now describe the corresponding functional antipode, a caged PKA.

PKA is one of the simplest and certainly the best understood of all protein kinases.[4] The inactive holoenzyme is a tetramer consisting of two regulatory and two catalytic subunits. Upon exposure to cAMP, the active catalytic subunit is released and is now free to catalyze the phosphorylation of serine and threonine residues in appropriate protein substrates. Several studies have demonstrated that covalent modification of a near active site cysteine (Cys-199) residue results in nearly complete abolition of protein kinase activity.[5] PKA also contains a second cysteine moiety (Cys-343); however, this residue is remote from the active site. With these features in mind, we prepared the active-site-directed peptide **3** in two steps via the protocol outlined in Scheme 1 (see Supporting Information for experimental details). The design of **3** was based on two criteria. First, we have previously described peptide-based affinity labels for PKA that contain an electrophilic appendage two residues removed from the analogous Ala residue in **3**.[6] Upon the basis of the known three-dimensional structure of PKA,[7] this should position the electrophile adjacent to the active site Cys-199. Second, the amino acid sequence in this peptide was chosen to promote active site binding, but to do so only weakly. This design feature ensures that, once the peptide is photochemically released from the active site under in vivo conditions, it will be unable to serve as an effective reversible inhibitor of PKA. Indeed, an analogue of the photochemically released peptide (AcGRRNA-$NHCH_2CH_2NHCOCOC_6H_5$) was prepared and exhibits an IC_{50} of 1.2 mM, 3 orders of magnitude greater than the in vivo concentration of PKA. The affinity label **3** inactivates PKA in a time-dependent fashion ($K_I = 1.5 \pm 0.3$ mM). The caged PKA was subsequently purified and displays the expected molecular weight as assessed via electrospray ionization mass spectrometry (Supporting Information).

Caged PKA exhibits less than 2% of the activity displayed by its native counterpart. Photochemical liberation of caged PKA restores 50% of the activity exhibited by the unmodified enzyme. The mechanism of uncaging presumably proceeds via a photochemically driven oxygen transfer from the nitro group to the benzylic carbon, followed by breakdown of the hemithioacetal intermediate to release the free sulfhydryl. Once the thioether bridge has been cleaved, the poorly bound active-site-embedded peptide (AcGRRNA-$NHCH_2CH_2NHCOCOC_6H_4$-*o*-NO) should dissociate from the enzyme, thereby restoring catalytic activity. With caged PKA in hand, we next turned our attention to in vivo studies.

Hofmann and his colleagues have previously demonstrated that rat embryo fibroblasts (REFs) undergo dramatic morphological changes upon microinjection of the active catalytic subunit of PKA.[8] These changes include membrane ruffling, disruption of actin-containing stress fibers, and an overall transmogrification of cell shape from flat and extended to rounded. We have been able to recapitulate these transformations in a photochemically dependent fashion with caged PKA. Microinjection solutions contained 3−7 μM caged PKA and an inert protein (mouse IgG; 2 mg/mL). The latter allows microinjected cells to be distinguished from nonmicroinjected ones via subsequent treatment with a fluorescein-conjugated anti-mouse IgG (FITC-IgG). After microinjection, REFs were either directly returned to the incubator for 1 h or first photolyzed and then incubated. After incubation, the cells were fixed, permeablized, blocked, and then stained with a combination of rhodamine-conjugated phalloidin (which binds to F-actin) and FITC-IgG. Both microinjected (green with a red halo) and nonmicroinjected (solid red) REFs are displayed in Figure 1. Microinjected cells which were not exposed to UV irradiation (Figure 1A) retain their stress fibers, indicating that the PKA-induced pathway had not been activated. In contrast, microinjection followed by photolysis (Figure 1B) not only results in the loss of stress fibers but also generates a rounded morphological appearance with membrane ruffling. Finally, direct microinjection of native PKA (Figure 1C) generates a cellular appearance that reproduces the results obtained with the photochemically liberated caged PKA.

In summary, we have designed and synthesized an active-site-directed PKA affinity label that directly generates a caged protein kinase. The activity of the latter is restored upon exposure to UV irradiation. Finally, we have shown that caged PKA can be photochemically unleashed in vivo, leading to morphological

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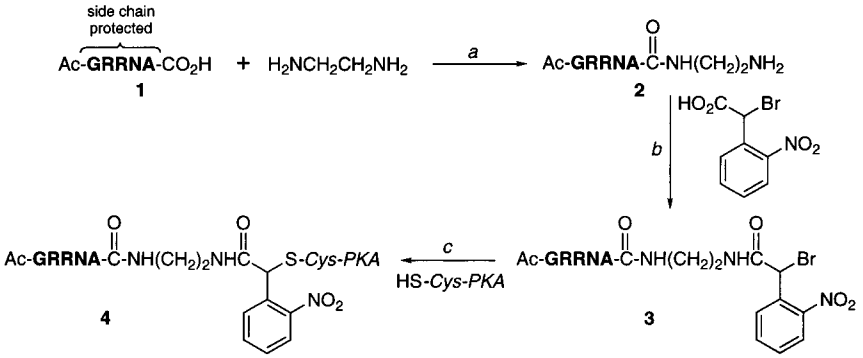
S0002-7863(98)01786-7 CCC: $15.00
Published on Web 08/07/1998

Scheme 1. Chemical Synthesis of Caged PKA[a]



[a] (a) (i) Benzotriazol-1-yloxytris(dimethylamino)phosphonium hexafluorophosphate, 1-hydroxybenztriazole, DMF, *N*-methylmorpholine, 20-fold excess of ethylenediamine, 4 h, room temperature (RT); (ii) 95% trifluoroacetic acid, 2.5% thioanisole, 2.5% H_2O; 25% yield (includes peptide synthesis); (b) dicyclohexylcarbodiimide, DMF, 18 h, RT, 60% yield; (c) 20 mM phosphate (pH 7.2), 5 mM KCl, 300−500-fold excess of **3**, 6 h, RT.

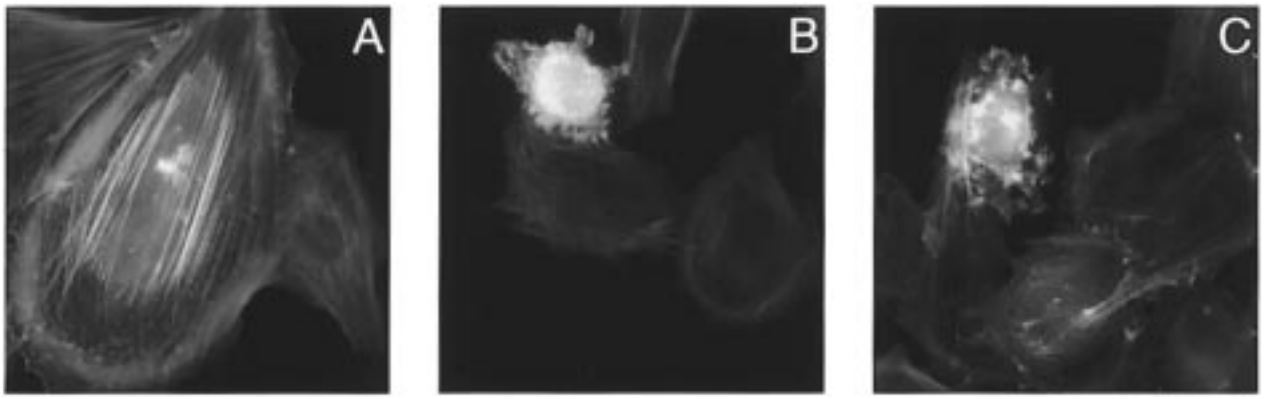


Figure 1. Overlay images of rhodamine−phalloidin staining for F-actin (red) and FITC-IgG staining for microinjected cells (green) in (A) REFs microinjected with caged PKA but not photochemically activated, (B) REFs microinjected with caged PKA and photochemically activated, and (C) REFs microinjected with native PKA. Images were collected on a Photometrics KAF1400 cooled CCD camera (1 × 1 binning) attached to an Olympus IX70 microscope with an infinity corrected 60× N.A. 1.4 phase 3 objective. Image overlay was performed with Photoshop 4.0.1.

changes that have been previously attributed to PKA activation. Caged protein kinases and caged inhibitors of protein kinases constitute a new family of synthetic modulators of signal transduction pathways and, as such, should prove useful in helping to decipher the role of individual members of signaling cascades.

Acknowledgment. We are pleased to acknowledge Professor Dennis Rousseau for the use of his Hg arc lamp apparatus. We also thank Dr. Jason Wood for his assistance with the microinjection experiments and image analyses, which were performed in the Albert Einstein College of Medicine's Analytical Imaging Facility. This work was funded by the National Institutes of Health (GM45989). K.C. acknowledges a Ladies Auxiliary of the Veterans of Foreign Wars Postdoctoral Fellowship.

Supporting Information Available: Experimental details and electrospray ionization mass spectra (9 pages, print/PDF). See any current masthead page for ordering information and Web access instructions.

JA981786S