A Caged Protein Kinase Inhibitor

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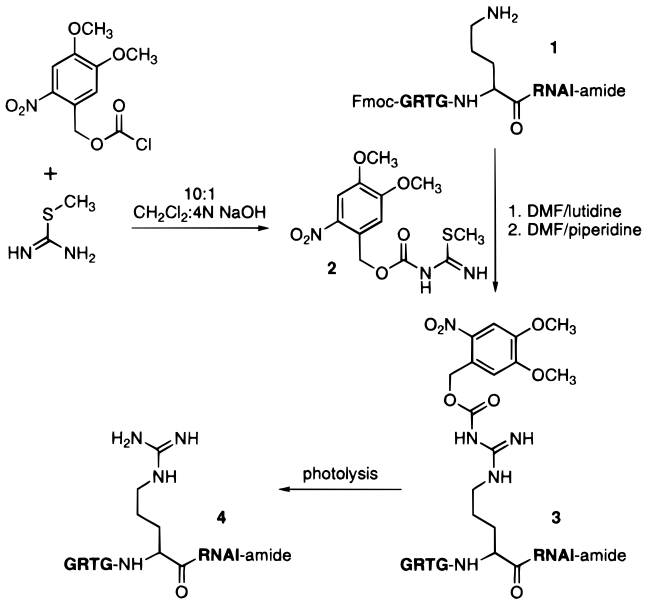
Kinase-mediated protein phosphorylation is a crucial component of the signal transduction pathways by which extracellular signaling molecules influence their target cells. For example, mitosis is ultimately dependent upon the interaction of extracellular growth factors with their appropriate membrane-embedded receptors, an interaction that is transmitted to the cell nucleus via kinase-dependent signal transduction cascades.[1] There has been and continues to be a significant effort devoted to the identification and characterization of individual protein members of these signaling cascades. However, recently it has become evident that these transduction pathways are significantly more complex than simple linear arrays of enzyme-catalyzed reactions that run from the cell surface to the nucleus. Instead, there exists a network of interlacing and interacting pathways that are under both spatial and temporal control.[2] For example, activation of the signal transduction pathway(s) responsible for synaptic plasticity in cerebellar Purkinje cells is exquisitely dependent upon the coincident timing of signals from two distinct presynaptic neurons.[3] What, if any, temporal cross-talk is there between individual members of bisecting signaling pathways? How long must a specific protein kinase remain active in a particular transduction cascade to ensure that the message results in the appropriate cellular response? Questions such as these can be addressed with caged compounds, species that can be rapidly unleashed in living cells at specific time points during the cellular response to environmental stimuli.[3,4] We report herein a caged inhibitor directed against the cAMP-dependent protein kinase (PKA).[5]

The cellular effects of the second messenger adenosine cyclic 3′,5′-phosphate (cAMP) are primarily mediated through PKA.[6] In 1982, Whitehouse and Walsh purified to homogeneity a heat-stable protein, PKI, that serves as a powerful inhibitor of PKA.[7] Peptides, whose sequences encompass the region of PKI that binds to the PKA active site, have been synthesized and found to exhibit impressive inhibitory activity against PKA as well.[8] The Arg residue in the active site-directed sequence (-Gly-Arg-Thr-Gly-Arg-Arg-Asn-Ala-Ile-) is known to be an important contributor to the inhibitory activity of these PKI-based peptides.[8b] Therefore, covalent modification of this residue should afford a relatively poor PKA inhibitor. With these features in mind, we synthesized the ornithine-containing peptide **1** (Scheme 1) via solid-phase peptide synthesis using the Fmoc protocol. The novel guanylating agent **2** was prepared from *S*-methylisothiourea and 6-nitroveratryl chloroformate in 85% yield. Subsequent guanylation of **2** in DMF/lutidine, followed by removal of the Fmoc moiety with piperidine, afforded the desired peptide **3** in 50% yield. Peptide **3** (K_i = 20 μM) is a nearly 50-fold poorer inhibitor than its uncaged counterpart, Gly-Arg-Thr-Gly-Arg-Arg-Asn-Ala-Ile-amide (**4**) (K_i = 420 nM).[9,10] Both peptides are competitive inhibitors versus the peptide substrate Leu-Arg-Arg-Ala-Ser-Leu-Gly (see the Supporting Information). Photolysis of **3** generates **4**[11] with a quantum efficiency of 0.023,[12] a process which presumably proceeds through a mechanistic pathway that includes (i) photoinduced oxygen transfer from the nitro group to the benzylic carbon position, (ii) hemiacetal decomposition, and (iii) decarboxylation to furnish the free Arg side chain. Each of these individual steps is well precedented.[13]

Scheme 1



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(10) The K_i for peptide **4** has been previously reported to be 36 nM,[8d] an order of magnitude smaller than the value we obtained. We believe that this discrepancy is due to the ionic strength of our assay buffer, which is significantly higher than that utilized in ref 8d. The higher ionic strength more closely approximates the intracellular environment. For a detailed discussion of the effect of assay conditions on inhibitory potency, see: Lawrence, D. S.; Niu, J. *Pharmacol. Ther.* **1998**, *77*, 81−114.

(11) Photochemically generated **4** exhibits HPLC, mass spectral, and enzymological properties identical to those obtained with **4** prepared via standard solid-phase peptide synthesis. See the Supporting Information.

(12) Quantum yield determination performed via the method described in the following: Adams, S. R., Kao; J. P. Y.; Tsien, R. Y. *J. Am. Chem. Soc.* **1989**, *111*, 7957−7968.

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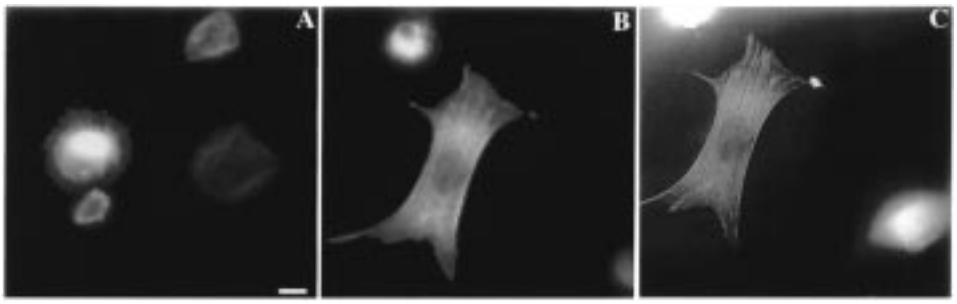

Figure 1. Overlay images of rhodamine−phalloidin staining for F-actin (red) and FITC-IgG staining for microinjected cells (green) in (a) CPT-cAMP-exposed REFs microinjected with **3** but not photolyzed and (b) CPT-cAMP-exposed REFs microinjected with **3** and subsequently photolyzed. (c) z scan image of part b taken in the rhodamine channel illustrating the presence of stress fibers in the PKA-blocked cell. 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. Scale bar = 10 μm.

cAMP, as well as several membrane permeant analogues, have been shown to exert a pronounced effect on cellular morphology.[14] This includes the rapid disruption of actin-containing stress fibers leading ultimately to a distinctive rounded morphology. Microinjection of activated PKA likewise produces this effect.[15] In contrast, microinjection of a truncated analogue of PKI blocks these 8-bromo-cAMP-induced morphological changes.[16] We assessed the in vivo inhibitory activity of **3** in rat embryo fibroblasts (REFs), a cell line known to exhibit PKA-dependent changes in shape upon exposure to cAMP analogues.[15]

REFs exposed to the membrane permeable PKA activator, 8-(4-chlorophenylthio)-cAMP (CPT-cAMP), undergo the expected morphological transformation. Furthermore, neither photolysis nor microinjection of an inert protein (mouse IgG) interferes with this process. We included mouse IgG in all subsequent microinjection experiments so that microinjected cells could be differentiated from other, noninjected, cells by staining with a fluorescein-conjugated anti-mouse IgG (FITC-IgG). Two additional control experiments were performed. First, REFs are unable to achieve a rounded appearance in the absence of CPT-cAMP. Specifically, microinjection of a 50 μM solution of **3** alone fails to produce any morphological changes (note: all subsequent injections employed 50 μM **3**, resulting in an estimated intracellular concentration of 5 μM[17]). Second, microinjection of REFs with **3**, but in the absence of photolysis, does not block CPT-cAMP-induced changes in morphology (Figure 1A). However, REFs that have been microinjected with **3**, *and subsequently photolyzed*, are unable to respond to the CPT-cAMP stimulus. This effect is illustrated in Figure 1B, in which two photolyzed cells, one lacking inhibitor (red) and the other microinjected with inhibitor (green), lie within the optical field. CPT-cAMP activation of the PKA pathway is clearly blocked in the microinjected REF. Furthermore, the presence of actin-containing stress fibers are visible in Figure 1C, a z scan image taken in the rhodamine channel. These results demonstrate that photolysis is required for uncaging the in vivo inhibitory activity of **3**, results entirely consistent with our in vitro data.

To the best of our knowledge, this communication describes the first example of a caged arginine moiety. Arginine not only serves as an important substrate recognition element for a number of protein kinases, but commonly serves as a key participant in a variety of protein−protein inetractions. Furthermore, given the important role of multiple arginine residues in substrate recognition by PKA,[8] a multiply caged analogue of **3** should exhibit a significantly more pronounced change in inhibitory potency (i.e., ≫50-fold) than its singly caged counterpart (**3**) upon photolytic conversion to **4**. We also note that multiply caged inhibitors could be useful in confining inhibitor photorelease to specific intracellular sites (for a discussion of photorelease spatial control, see ref 4). These experiments, as well as related studies, are in progress.

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Supporting Information Available: Experimental details and Lineweaver−Burk plots (6 pages, print/PDF). See any current masthead page for ordering information and Web access instructions.

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