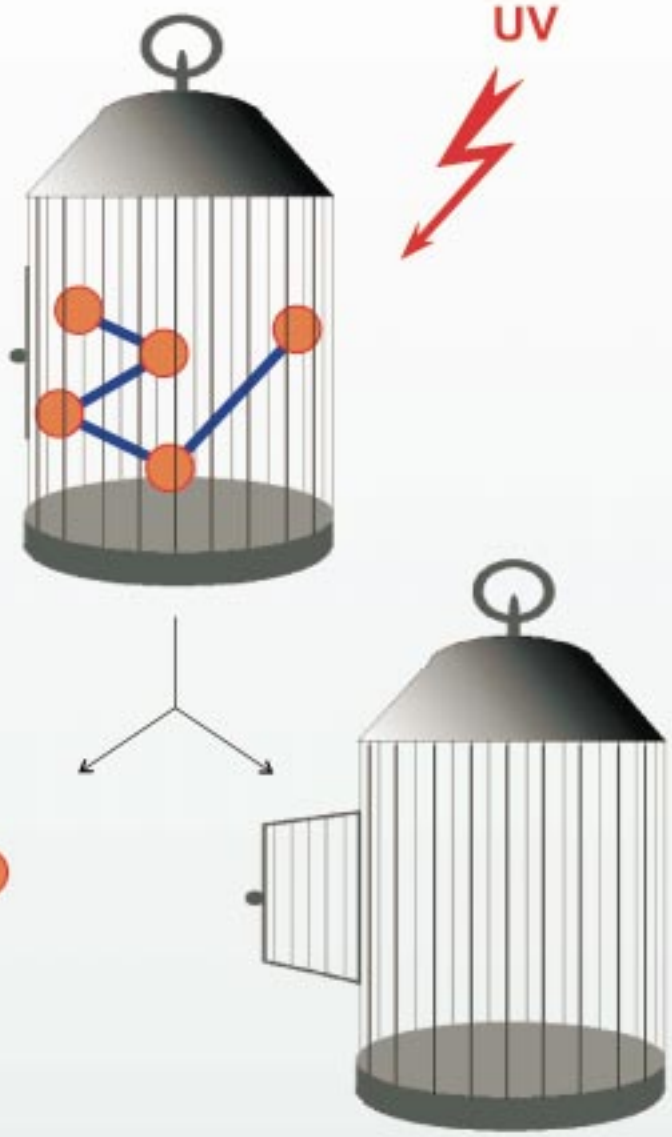
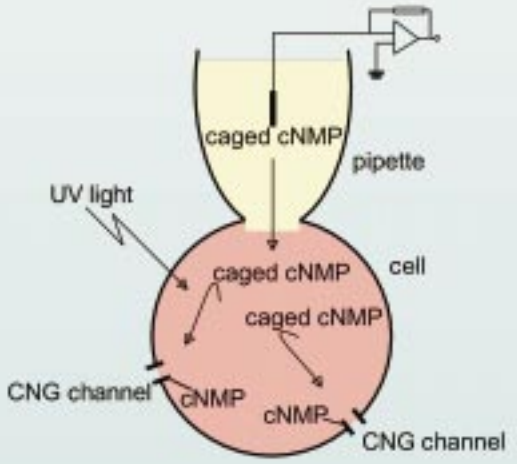
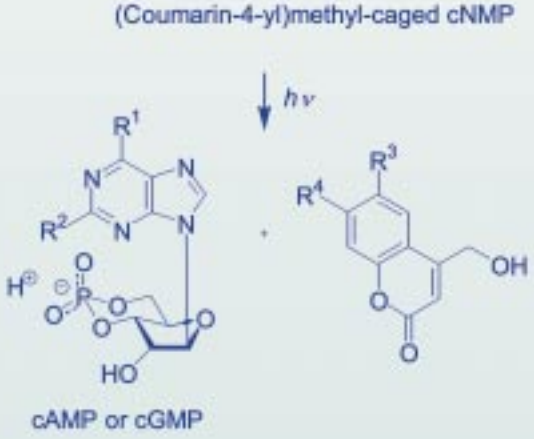
For the investigation of the space- and time-dependent aspects of signal transduction through cyclic nucleotide gated (CNG) ion channels, the ability to rapidly increase the intracellular concentration of cyclic nucleotide monophosphates (cNMPs) is required. This is achieved in an elegant manner by esterification of the cNMPs with photosensitive protecting groups to form biologically inactive caged cNMPs, which can then "smuggle" the cNMPs into the cell and set them free through UV light photolysis. New substituted coumarinylmethyl esters of the cNMPs are particularly suitable as the caging phototriggers.

Find out more on the following pages.







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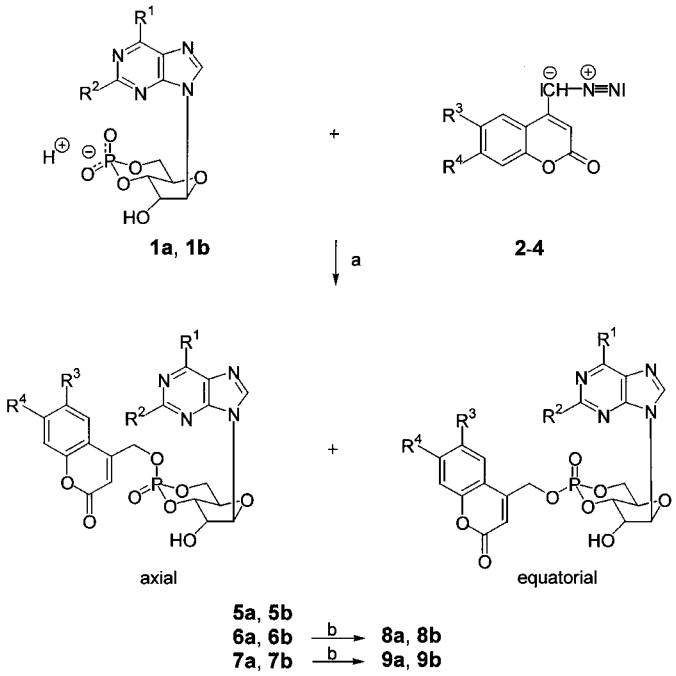
Highly Efficient and Ultrafast Phototriggers for cAMP and cGMP by Using Long-Wavelength UV/Vis-Activation**

Volker Hagen,* Jürgen Bendig, Stephan Frings, Torsten Eckardt, Siegrun Helm, Dirk Reuter, and U. Benjamin Kaupp

The release of the second messengers adenosine 3′,5′-cyclic monophosphate (cAMP, **1a**) and guanosine 3′,5′-cyclic monophosphate (cGMP, **1b**) from "caged" compounds has proven to be very useful for in situ studies of cyclic nucleotide-dependent cellular processes.[1] When caged, cAMP und cGMP are rendered biologically inactive by esterification of the phosphate moiety with a photolabile protecting or caging group. Caged biomolecules can be introduced into living cells without evoking any biological response. A flash of light is applied to cleave the caging group and to unmask the biologically active cyclic nucleotide. Thereby, a rapid increase in the intracellular concentrations of cAMP or cGMP can be achieved.

Caged compounds must meet specific requirements. They should be sufficiently soluble in aqueous solutions, stable toward solvolysis, and biologically inert. Furthermore, they should undergo fast and highly efficient photochemical reactions and display high molar absorptivities at wavelengths of >300 nm. The caged cAMPs and cGMPs commonly used up to now do not meet these requirements. The 2-nitrobenzyl,[2] 4,5-dimethoxy-2-nitrobenzyl (DMNB),[3] and 1-(2-nitrophenyl)ethyl (NPE) esters[4] photolyze relatively slowly and display rather low photoefficiencies.[5] Desoxybenzoinyl-caged (desyl-caged) cAMP[6] is very sensitive to solvolysis in aqueous buffer solutions[7] and the (7-methoxycoumarin-4-yl)methyl esters of cAMP and cGMP[7, 8] are poorly soluble.[9]

Here, we describe caged compounds of cAMP und cGMP exhibiting unprecedented favorable properties. In particular, we focus on the (7-diethylaminocoumarin-4-yl)methyl (DEACM), (7-carboxymethoxycoumarin-4-yl)methyl (CMCM), and [6,7-bis(carboxymethoxy)coumarin-4-yl]methyl (BCMCM) esters of cAMP and cGMP **5a**,**b**, **8a**,**b**, and **9a**,**b**. Their synthesis (Scheme 1) involves treatment of **1a** and **1b** with the diazoalkanes **2**–**4** followed by diastereomer separation using preparative reverse-phase HPLC and subsequent deprotection of the *tert*-butoxy groups with trifluoroacetic acid (only necessary for the *tert*-butyl esters). The yields were 11–34% and the diastereomers were formed in approximately 45:55 ratios (axial/equatorial). Isomeric species were assigned by ^{31}P NMR spectroscopy. (For preparative details as well as spectroscopic characterization, see Supporting Information.)



a: cAMP and coumarinylmethyl-caged cAMPs: $R^1 = NH_2$, $R^2 = H$
b: cGMP and coumarinylmethyl-caged cGMPs: $R^1 = OH$, $R^2 = NH_2$

2, **5**: $R^3 = H$, $R^4 = NEt_2$
3, **6**: $R^3 = H$, $R^4 = OCH_2COOtBu$
4, **7**: $R^3 = R^4 = OCH_2COOtBu$
8: $R^3 = H$, $R^4 = OCH_2COOH$
9: $R^3 = R^4 = OCH_2COOH$

Scheme 1. Synthesis of **5**–**9**: a) DMSO/CH_3CN (1/4), 60 °C, 24 h; b) TFA/CH_2Cl_2/H_2O (75/24/1), RT, 20 min. TFA = trifluoroacetic acid.

The CMCM and BCMCM moieties greatly increase the overall hydrophilicity, and so render the corresponding caged cyclic nucleotides highly soluble in water. This is advantageous for their incorporation into cells, for instance, by standard patch-clamp techniques. The DEACM-protecting group, on the other hand, exhibits remarkable photochemical properties. All novel caged compounds are released very quickly[10] (within a few nanoseconds) and show very high efficiencies of photocleavage to yield the cyclic nucleotide and the respective strongly fluorescent 4-(hydroxymethyl)coumarin (Scheme 2), due to a combination of high quantum yields and relatively intense absorptivities at >300 nm (Table 1).

Relative to the values for **8a**,**b**, the long-wavelength absorption maxima of **9a**,**b** are red-shifted by approximately 20 nm and those of **5a**,**b** by as much as 70 nm. Therefore, the optimal wavelengths for the photolysis of **8a**,**b**, **9a**,**b**, and **5a**,**b** are 300–340 nm, 330–370 nm, and 360–440 nm, respectively. As a result, the adaption to conventional continuous wave and

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[**] This work was supported by the Deutsche Forschungsgemeinschaft, the European Union, and the Fonds der Chemischen Industrie. We thank B. Dekowski and J. Loßmann for technical assistance and S. Hecht for proof reading.

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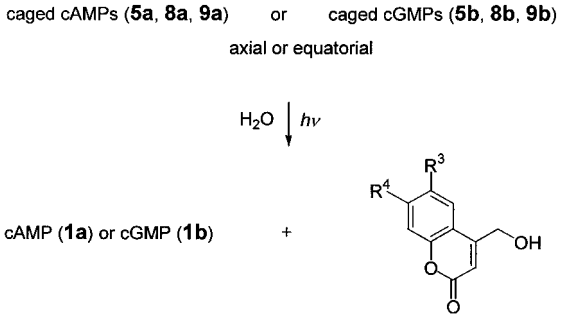

Scheme 2. Photolysis of **5a**,**b**, **8a**,**b**, and **9a**,**b**.

Table 1. Long-wavelength absorption λ_{max}, quantum yield Φ of photolysis, and solubility s of the coumarinylmethyl-caged derivatives **5a**,**b**, **8a**,**b**, and **9a**,**b**.

Compound	λ_{max} [nm] (ε [$M^{-1}cm^{-1}$])	Φ[c]	s [μM]
5a (axial)[a]	402 (18600)	0.21[d]	135
5a (equatorial)[a]	396 (20200)	0.23[d]	15
5b (axial)[a]	403 (19300)	0.25[d]	120
5b (equatorial)[a]	396 (19100)	0.26[d]	15
8a (axial)[b]	326 (12500)	0.12	900
8a (equatorial)[b]	324 (12400)	0.10	200
8b (axial)[b]	326 (11700)	0.16	350
8b (equatorial)[b]	325 (11200)	0.10	> 1000
9a (axial)[b]	346 (10700)	0.10	500
9a (equatorial)[b]	347 (12400)	0.08	1000
9b (axial)[b]	348 (11000)	0.14	550
9b (equatorial)[b]	347 (11200)	0.09	> 1000

[a] In acetonitrile/HEPES-KCl buffer (5/95), pH 7.2; $\lambda_{exc} = 333$ nm. [b] In HEPES-KCl buffer, pH 7.2; $\lambda_{exc} = 333$ nm. [c] Error limit ±0.01. [d] In MeOH/HEPES-KCl buffer (20/80).

laser light sources is straightforward. Solvolytic stability of the novel caged compounds is extremely high with half-lives of > 1000 h in HEPES buffer at pH 7.2 (HEPES = 2-[4-(2-hydroxyethyl)-1-piperazinyl]ethanesulfonic acid). The respective diastereomers of the novel coumarinylmethyl-caged compounds differ only in their solubility in aqueous buffer solutions (Table 1), and at least in the case of **5a**,**b**, the use of the more soluble isomer is recommended.

In Table 2 the efficiencies of photorelease of the axial isomers of **5b**, **8b**,and **9b** in comparison to DMNB- and NPE-caged cGMP in aqueous buffer solution at different wavelengths are illustrated. The results show that the coumarinylmethyl-caged compounds are far superior to the nitrobenzyl-caged derivatives. The highest release of cGMP is achieved with the following caged compounds: at 337 nm (HBO, nitrogen laser) with **8b**, at 365 nm (HBO, argon-ion laser) with **9b** and **5b**, and at 405 nm (HBO) with **5b** (HBO = high pressure lamp, Osram).

The excellent properties of the novel caged compounds were confirmed in studies on cyclic nucleotide-gated (CNG) channels using the patch-clamp method. In HEK293 cells expressing cAMP-gated channels (CNCα3), submaximal activation of the channels was obtained with **9a** and illumination at 360 nm by flashes of 20 ms duration; maximal activation was achieved by a 100 ms flash (Figure 1). In Figure 2 the photorelease of cyclic nucleotides from **9a** is

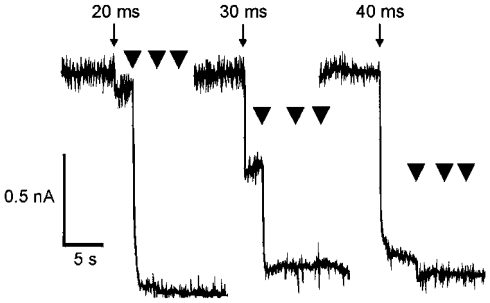

Figure 1. Determination of the concentration of photolytically released cAMP from the activation of cAMP-gated ion channels in HEK293 cells. (Whole-cell current recordings at −70 mV obtained from three cells that were loaded with 500 μM of the equatorial isomer of **9a**.) Brief flashes of UV light (20–40 ms, ↓) do not liberate sufficient cAMP to maximally activate the channels. Longer flashes (100 ms, ▼) induce maximal channel activity. The relative current amplitude at submaximal activation is used to calculate the concentration of photoreleased cAMP using the known dose–response relationship for channel activation by cAMP.[11]

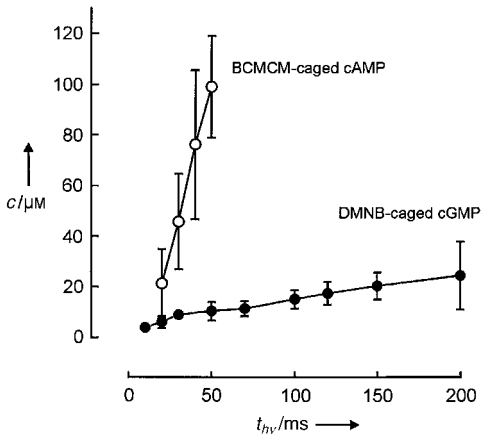

Figure 2. Comparison of photolytic liberation of cyclic nucleotides from **9a** and DMNB-caged cGMP in HEK293 cells at various flash durations. Due to better solubility and quantum yield, BCMCM derivatives can produce tenfold higher concentration steps. c = concentration of free nucleotide, $t_{h\nu}$ = duration of flash.

Table 2. Comparison of the efficiencies (in %) of the release of cGMP from caged compounds (axial isomers) with various irradiation times at different wavelengths.

Compound	t ($\lambda_{exc} = 333$ nm)			t ($\lambda_{exc} = 365$ nm)			t ($\lambda_{exc} = 405$ nm)		
	6 s	60 s	600 s	6 s	60 s	600 s	6 s	60 s	600 s
NPE ester[a]	2	7	38	< 1	2	10	< 1	< 1	1
DMNB ester[a]	< 1	3	10	< 1	3	11	< 1	< 1	< 1
5b (DEACM ester)[a]	3	15	75	16	80	> 98	38	98	> 98
8b (CMCM ester)[b]	16	75	> 98	4	19	93	< 1	1	3
9b (BCMCM ester)[b]	10	51	98	12	65	> 98	1	3	20

[a] $c = 25$ μM in MeOH/HEPES (1/4). [b] $c = 25$ μM in HEPES.

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compared with that from DMNB-caged cGMP in HEK293 cells expressing cAMP-gated (CNCα3) and cGMP-gated (CNCα2) channels. The cells were loaded with 500 μM of the equatorial isomer of **9a** or 200 μM (maximal solubility) of the axial isomer of DMNB-caged cGMP using a patch pipette and illuminated with UV rays. The nucleotide concentrations released by the flash were estimated using the known dose–response relation for activation of the channels by cAMP and cGMP.[11] The experiment demonstrates that **9a** liberates the cyclic nucleotide about one order of magnitude more efficiently than the DMNB-caged derivative and that only **9a** is able rapidly to produce high concentrations of cyclic nucleotide inside cells. In Figure 3 the photorelease at $\lambda =$ 405 nm of 8-Br-cGMP from the axial isomer of DEACM-caged8-Br-cGMP[12] is compared with that from the axial isomer of DMNB-caged 8-Br-cGMP[11] in HEK293 cells expressing CNCα2 channels. While every light flash released approximately 0.5 μM of cGMP from the DEACM-caged compound, no measurable release could be detected with the DMNB-caged compound.

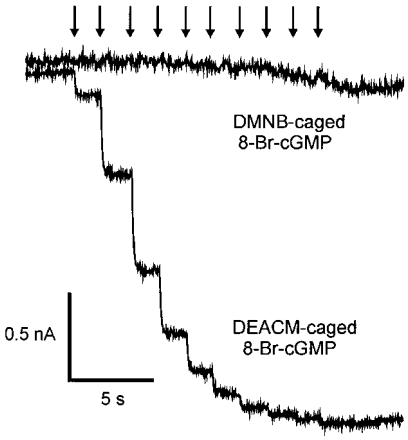

Figure 3. Activation of cGMP-gated cation channels by 8-Br-cGMP photolytically liberated from caged derivatives through a series of 5-ms UV light flashes (405 ± 20 nm, ↓). (Whole-cell current recordings from two HEK293 cells at −50 mV with 20 μM of the respective caged compound.)

The properties of the caged compounds inside cells correspond well with their performance in solution and confirm that the described coumarinylmethyl-caged compounds are indeed highly efficient phototriggers for cAMP and cGMP. The advantage of the carboxymethoxy-substituted coumarinylmethyl esters **8a**,**b** and **9a**,**b** arises from their superior solubility allowing the instantaneous liberation of high concentrations of the cyclic nucleotides. With respect to the DEACM-caged compounds **5a** and **5b**, the cyclic nucleotides are efficiently released under nondamaging light conditions. In summary, these novel caged compounds show great potential for the study of spatial- and time-dependent aspects of cellular signaling at a quantitative level.

Received: November 22, 2000 [Z16158]

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Analysis of the Topology of the Chromophore Binding Pocket of Phytochromes by Variation of the Chromophore Substitution Pattern

Uwe Robben, Ingo Lindner, Wolfgang Gärtner,* and Kurt Schaffner

Dedicated to Professor Henning Hopf on the occasion of his 60th birthday

We have recently reported[1] on the use of synthetic linear tetrapyrroles with modified substitution patterns on ring D in recombinant phytochromes (65 kDa oat phyA),[2] in order to spectroscopically explore the interactions between the binding pocket of the protein and the chromophores phytochromobilin (**1**; Scheme 1), isophytochromobilin (**4**), and phycocyanobilin (**3a**). The first unnatural chromophore investigated (**4**)[1] has been found, upon assembly with the recombinant apoprotein (apophyA65),[3] to undergo a $P_r \rightarrow P_{fr}$ photoisomerization which is characteristic of phytochromes such as phyA65-**1**. The absorption maximum for the P_{fr} form of phyA65-**4** is hypsochromically shifted by about 14 nm. This shift occurs selectively in the P_{fr} form and is even larger (by

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