Photochemical Properties of New Photolabile cAMP Derivatives in a Physiological Saline Solution

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Three new photolabile esters of cAMP (2-anthraquinonyl)methyl (**1a**), (7-methoxycoumarinyl)methyl (**2a**), and 2-naphthylmethyl (**3a**), have been developed. The stability and photochemical properties of these derivatives were compared to the previously reported ones in a physiological saline solution (1% DMSO in Ringer's solution, pH 7.4). We found that **2a** had satisfactory stability ($t_{1/2}$ > 1000 h) in the dark and was photolyzed to release the parent cAMP on 340 nm irradiation (ϕ_{app} = 0.10, ϵ_{340} = 6730) more efficiently than previously reported caged cAMPs. A biological test using the melanophores of the medaka (*Oryzias latipes*) revealed that **2a** penetrated into the melanophores, inactive before irradiation and activated to release cAMP upon irradiation. We have developed a new caged cAMP which can be used in the investigation of biological responses regulated by intracellular cAMP concentrations using living cells.

Usage of protecting groups has progressed from protection of the reactive functional groups in synthetic organic chemistry to the protection of biological activities for the physiological investigation of cellular processes. In the latter case, photolabile protecting groups permit a greater range of applications because of the rapid deprotection rates afforded by photolysis and the ability to photoexcite these substrates in a wide variety of environment.[1] The protecting groups most frequently used for this purpose are 2-nitrobenzyl and its derivatives;[2] however, we observed a drawback for 2′-nitrophenethyl cAMP, known as caged cAMP, when we investigated the motile responses using melanophores. During incubation to melanophores, release of cAMP was observed before irradiation.[3] Additionally, the synthesis of optically pure (2′-nitrophenyl)ethanol is necessary in order to avoid the formation of unseparable diastereomers. We[4] and others[5] have reported several new and efficient photochemically labile protecting groups for phosphates to overcome such problems. However, almost all of these experiments have been performed in methanol or aqueous solution containing 50% organic solvents that could not be applied to the investigation of cellular processes using living cells. Therefore, we decided to explore the real stability and photochemical properties of these newly developed protecting groups for cAMP, a phosphate-containing cyclic nucleotide which plays important roles in many cellular processes as a second messenger, in a physiological saline solution. Our findings are the following: (1) stability of the protected cAMPs in 1% DMSO in Ringer's solution[6] was not comparable to methanol or 50% dioxane–50% water (v/v), (2) photochemical properties of protected cAMPs were different from protected diethyl phosphate used as a model compound, (3) the photorelease efficiency of cAMP depended on the solvent, and (4) two new protected cAMPs, (2-anthraquinonyl)methyl (**1a**) and (7-methoxycoumarinyl)methyl (**2a**), were stable in the dark and more efficiently released cAMP upon irradiation than previously reported ones. The preliminary result of application of **2a** for a mechanistic investigation of the dispersion and aggregation of pigment granules is also reported.

In the previous paper,[4a] we reported a 1-pyrenylmethyl group as a new photochemically labile protecting group for phosphates. The advantages of this group are high molar absorptivity (ϵ_{340} = 34 500), high quantum efficiency of disappearance (ϕ_{dis} = 0.22), and no additional stereogenic center that generates diastereoisomers when it is introduced to a homochiral molecule. However, even in the case of diethyl 1-pyrenylmethyl phosphate the low stability ($t_{1/2}$ = 3 h in 50% dioxane–Tris buffer solution) in the dark made it impossible to introduce this group to biologically important phosphates such as cAMP. Thus we investigated other protecting groups that have satisfactory stability in the dark and reactivity upon irradiation.

We synthesized three new cAMP derivatives, (2-anthraquinonyl)methyl (**1a**), (7-methoxycoumarinyl)methyl (**2a**), and 2-naphthylmethyl (**3a**), and two previously reported ones, 2′-nitrophenethyl (**4a**) and desyl (**5a**), by our silver oxide method.[4b] Givens et al. introduced (7-methoxycoumarinyl)methyl and 2-naphthylmethyl groups to diethyl phosphate and determined their photochemical properties.[7] Kemp et al. developed the (2-anthraquinonyl)-methyl group as a photocleavable carboxylic protecting

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(2) After the introduction by Engels et al. (Engels, J.; Schlaeger, E. J. *J. Med. Chem.* **1977**, *20*, 907–911.) many applications of 2-nitrobenzyl and its derivatives have been reported. For example (a) Nerbonne, J. M.; Richard, S.; Nargeot, J.; Lester, H. A. *Nature* **1984**, *310*, 74–76. See the following reviews: (b) Wootton, J. F.; Trentham, D. R. In *Photochemical Probes in Biochemistry*; Nielsen, P. E., Ed.; Kluwer Academic: Dordrecht, 1989; pp 277–296. (c) McCray, J. A.; Trentham, D. R. *Annu. Rev. Biophys. Biophys. Chem.* **1989**, *18*, 239–270. (d) Corrie, J. E. T.; Trentham, D. R. In *Bioorganic Photochemistry*; Morrison, H., Ed.; New York: John Wiley, 1993; Vol. 2, pp 243–305.

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(6) The composition of this solution is as follows: NaCl (125.3 mM), KCl (2.7), $CaCl_2$ (1.8), $MgCl_2$ (1.8), D-glucose (5.6), and Tris-HCl buffer (5.0) (pH 7.2).

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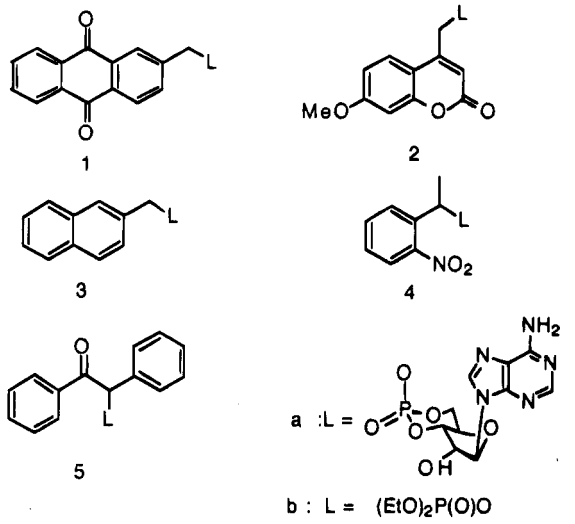

Figure 1. Caged cAMPs and caged diethyl phosphates. Only the axial isomer of **1a**–**5a** is presented.

Table 1. Half-Life for Caged cAMPs in the Dark

substrate	half-life (h) condition A[a]	condition B[a]
1a	>1000	ND[b]
2a (ax)	>1000	>1000
2a (eq)	>1000	60
3a (ax)	330	ND
3a (eq)	14	ND
4a	>1000	160
5a	220	0.5

[a] Condition A: 50% dioxane–50% water (v/v) (10^{-4} M). Condition B: 1% DMSO in Ringer's solution (10^{-4} M). [b] It could not be measured because of the low solubility.

group.[8] The stability of **1a**–**5a** in 50% dioxane–50% water (v/v) (condition A) and 1% DMSO in Ringer's solution (condition B) was determined by measurement of the half-lives in the dark. Under condition A, the order of decreasing stability is as follows: **4a** (mixture of four isomers) = **2a** (mixture of two isomers) = **1a** (mixture of two isomers) ($t_{1/2}$ > 1000 h) > **3a** (ax) ($t_{1/2}$ = 330 h) > **5a** (mixture of isomers) ($t_{1/2}$ = 220 h) ≫ **3a** (eq) ($t_{1/2}$ = 14 h). In contrast to these results, the stability of desyl cAMP (**5a**) markedly decreased in 1% DMSO in Ringer's solution ($t_{1/2}$ < 0.5 h) while others have comparable stability with that in dioxane in water (Table 1). Many metal salts such as NaCl, KCl, and $CaCl_2$ in Ringer's solution may accelerate hydrolysis of desyl cAMP (**5a**).

We investigated the photorelease activity of cAMP first in 50% dioxane–50% water (v/v) (condition A) and next in 1% DMSO in Ringer's solution (condition B) upon 340 nm irradiation (10^{-4}M, using a 500 W Xe lamp through a JASCO CT-10 type monochrometer). Photolysis of **2a** and **3a** produced two products, cAMP and photoproduct (2-(hydroxymethyl)-7-methoxycoumarin for **2a** and 2-naphthylmethanol for **3a**). Photolysis of **1a** produced cAMP and unidentified mixtures of photoproducts. The progress of reaction was monitored by reversed phase HPLC (see Experimental Section) as a function of time (Figures 2 and 3). The order of the decreasing activity was found to be **1a** ≫ **2a** > **5a** > **4a** ≫ **3a** for condition A, **2a** ≫ **4a** = **1a** for condition B. The low solubility of **1a** in 1%

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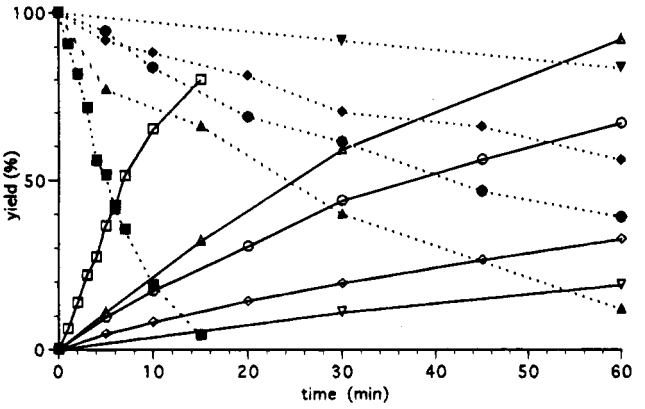

Figure 2. Photolysis of caged cAMPs in 50% dioxane–50% water (v/v). Photolytic consumption of **1a** (···■···), **2a** (···▲···), **3a** (···▼···), **4a** (···◆···), **5a** (···●···) and production of cAMP from **1a** (–□–), **2a** (–△–), **3a** (–▽–), **4a** (–◇–), **5a** (–○–).

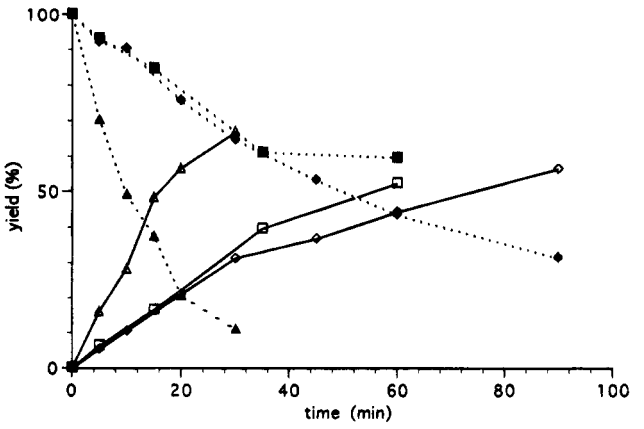

Figure 3. Photolysis of caged cAMPs in 1% DMSO in Ringer's solution. Photolytic consumption of **1a** (···■···), **2a** (···▲···), **4a** (···◆···) and production of cAMP from **1a** (–□–), **2a** (–△–), **4a** (–◇–).

Table 2. Photochemical Properties of Caged cAMPs

substrate	ϵ_{340}[a]	condn[b]	Φ_{dis}[c]	Φ_{app}[c]	$\Phi_{dis}\epsilon_{340}$[d]
1a	3920	A	0.30	0.20	1176
	ND	B	0.04	0.03	ND
2a	9600	A	0.07	0.06	672
	6730	B	0.12	0.10	808
3a (ax)	34	A	0.66	ND	22
3a (eq)	70	A	0.61	0.38	43
4a	400	A	0.27	0.16	108
	720	B	0.16	0.16	115
5a	140	A	0.74	0.66	104

[a] Molar absorptivity (L mol^{-1} cm^{-1}). [b] Condition A: 50% dioxane–50% water (v/v) (10^{-4} M), condition B: 1% DMSO in Ringer's solution (10^{-4} M). [c] Both quantum efficiencies are determined using ferrioxalate actinometry.[11] [d] Index of reactivity that indicates the order of the reactivity.[12]

DMSO in Ringer's solution resulted in the decrease in the reactivity, because the resulting white suspension prevented light transmittance. Quantum yields for the disappearance of the starting materials and the appearance of cAMP are presented in Table 2. High molar absorptivities (9600 for condition A and 6730 for condition B) and modest quantum yields (ϕ_{dis} = 0.07 for condition A and ϕ_{dis} = 0.12 for condition B) are the characteristic features of **2a**. Although the desyl ester of diethyl phosphate (**5b**) was photolyzed much more efficiently than the (7-methoxycoumarinyl)methyl ester (**2b**),[9] the order was reversed in the case of cAMP. These results

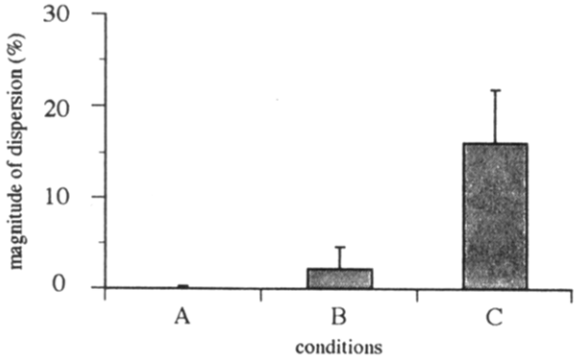

Figure 4. Dispersing responses of pigment granules after UV irradiation. A: Ringer's solution (control); B: 2-(hydroxymethyl)-7-methoxycoumarin, photoproduct of **2a**; C: **2a**.

indicated that photorelease efficiency depended on both phosphate structures and solvents so that it was necessary to use the target molecule itself under physiological condition for evaluation of the protecting groups. On the basis of these analyses, we concluded that the observed stability in the Ringer's solution and efficiency for photoreaction of **2a** are advantageous for the investigation of biological processes that are regulated by cAMP concentration.

Studies of the biological application were performed on the motile response of fish melanophores. The dispersion response of melanin pigments in melanophores is known to be mediated by an increase in the concentration of intracellular cAMP.[10] Melanophores in scales isolated from the dorsal trunk of the wild-type medaka fish (*Oryzias latipes*) were immersed in the experimental solutions (A: Ringer's solution, B: 10^{-4} M of 2-(hydroxymethyl)-7-methoxycoumarin, C: 10^{-4} M of **2a**) for 1 h at room temperature (100% dispersion). These scales were then transferred into 10 μL of 1 μM norepinephrine hydrochloride in a well of an HT coating slide and incubated for 2 min. After the aggregation of melanin pigments (0% dispersion), UV irradiation for 10 s was applied through an objective lens of a fluorescent microscope. The motile responses of the melanophores were observed and photographed with the same microscope. The results of quantitative analysis are presented in Figure 4. Effect of UV irradiation (10 s) for motility was negligible because no dispersion was observed for sample solution A. A photoproduct of **2a**, 2-(hydroxymethyl)-7-methoxycoumarin, slightly induced dispersing response (2% dispersion) after UV irradiation (sample solution B). The dispersing response observed for sample solution C (16% dispersion) indicated that **2a** penetrated into the melanophores and released a sufficient amount of cAMP to induce a motile response of pigment granules upon UV irradiation. Hydrolysis of **2a** before irradiation was also negligible by making comparison of the 0% dispersion pictures. This is a preliminary result. However, it can be said that we have developed a new caged cAMP which can be used in the investigation of biological responses regulated by intracellular cAMP concentrations using living cells.

(9) In ref 7, Givens et al. reported the quantum efficiencies of **2b** (in methanol) and **5b** (in benzene) as 0.038 and 0.28, respectively. We also reported in ref 4a the decreasing reactivity of photolysis in methanol as **5b** > **2b** > **4b**.

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Experimental Section

General Methods. (−)−Adenosine cyclic 3′,5′-monophosphate (Janssen), 4-(bromomethyl)-7-methoxycoumarin (nacalai), and silver(I) oxide (WAKO) were used without further purification. (Hydroxymethyl)anthraquinone (TCI) was purified by silica gel chromatography. Acetonitrile was distilled from P_2O_5 and stored over 4-Å molecular sieves. Chromatography was performed using EM Kieselgel 60 0.063−0.200-mm grade silica gel for column chromatography and 0.042−0.063-mm grade for the flash column. HPLC chromatography was monitored at 254 nm. The mobile phase was 50% methanol−50% water (v/v). The flow rate was 0.8 mL/min except for **2a** and cAMP (0.6 mL/min). The retention time (min) for each compounds were determined as follows: **1a**: 9.91 (ax), 17.6 (eq); **2a**: 9.85 (ax), 12.3 (eq); **3a**: 6.27 (ax), 11.96 (eq); **4a**: 10.41, 11.70, 15.83; **5a**: 16.23 (ax), 20.20 (eq); cAMP: 2.97.

Synthesis of [4-(7-Methoxycoumarinyl)]methyl Adenosine Cyclic 3′,5′-Monophosphate (2a). General Procedure for Caged cAMP. In an oven-dried, 30 mL, two-necked, round bottom flask equipped with a magnetic stirring bar and a rubber septum were placed cAMP (33 mg, 0.10 mmol) and 4-(bromomethyl)-7-methoxycoumarin (80.7 mg, 0.300 mmol). The flask was evacuated under vacuum and then was flushed with argon. Dry acetonitrile (6 mL) and DMSO (1 mL) were added via syringe. After the addition of solid silver(I) oxide (44 mg, 0.20 mmol), the resulting black suspension was stirred at 60 °C for 45 h under argon. The reaction mixture was filtered through filter paper, and the residue was washed with chloroform. The combined filtrate was evaporated under reduced pressure. The oily residue was purified by column chromatography (30 g of SiO_2, 4.7% MeOH−CH_2Cl_2 and 6.2% MeOH−CH_2Cl_2) to give 74 mg of yellow oil. Further purification by flush chromatography (30 g of SiO_2, 4.7% MeOH−CH_2Cl_2) gave **2a** (23 mg, 44%) as a mixture of two stereoisomers (ax/eq = 2.3/1). From the residual black solid was obtained silver salt of cAMP (12.5 mg, 29%). **2a** (mixture of axial and equatorial isomers) mp 260−263 °C (dec.); 1H NMR (5% $CD_3OD/CDCl_3$) axial isomer δ/ppm 3.85 (3H, s), 3.90 (3H, s), 4.48 (1H, d, ,J 6 Hz), 4.68 (1H, m), 4.85 (1H, d, J 5 Hz), 5.41 (2H, d, J 6.5 Hz), 5.51 (1H, m), 5.99 (1H, s), 6.03 (1H, s), 6.49 (1H, t, J 1 Hz), 6.54 (1H, t, J 1 Hz), 6.89−6.95 (2H, m), 7.47 (1H, dd, J 2 and 6 Hz), 7.74 (1H, dd, J 2 and 6 Hz), 7.96 (1H, s), 7.99 (1H, s), 8.25 (1H, s), 8.27 (1H, s); IR (KBr) ν_{max}/cm^{-1} 1719, 1618, 1296, 1212, 1069, 1017; UV (50% dioxane/H_2O) λ_{max}/nm (ϵ) 325 (13300), 259 (14600).

Synthesis of 2-(bromomethyl)anthraquinone. To a solution of 2-(hydroxymethyl)anthraquinone (119 mg, 0.50 mmol) in 8 mL of CH_2Cl_2 were added carbon tetrachloride (249 mg, 0.75 mmol) and triphenylphosphine (157 mg, 0.60 mmol). The reaction mixture was stirred for 11 h at room temperature, 8 mL of sat. $NaHCO_3$ was added, and the mixture was stirred. The layers were separated, and the organic layer was washed with sat. $NaHCO_3$ and brine. Drying over $MgSO_4$, concentration under vacuum, and purification by column chromatography (30 g of SiO_2, CH_2Cl_2) gave 2-(bromomethyl)anthraquinone (93 mg, 62%) as a yellow solid: mp 196 °C; ^{1}H NMR ($CDCl_3$) δ/ppm 4.60 (2H, s), 7.80−7.86 (3H, m), 8.29−8.35 (4H, m); IR (KBr) ν_{max}/cm^{-1} 3376, 1678, 1593, 1325, 1296, 934, 712.

Synthesis of (2-Anthraquinonyl)methyl Adenosine Cyclic 3′,5′-Monophosphate (1a). This reaction was performed as described above except that 2-(bromomethyl)-anthraquinone (61 mg, 0.23 mmol) was used as a halide. Purification by flash chromatography (18 g of SiO_2, 4.7% MeOH−CH_2Cl_2 and then 6.2% MeOH−CH_2Cl_2) gave **1a** (7.3 mg, 14%) as a mixture of two stereoisomers (ax/eq = 1.8/1). Silver salt of cAMP (22 mg, 50%) was obtained from the residual black solid. **1a** (mixture of axial and equatorial isomers) mp 243−248 °C (dec.); 1H NMR (5% $CD_3OD/CDCl_3$) δ/ppm 4.46 (2H, dd, J 3.5 and 7 Hz), 4.55−4.75 (2H, m), 4.85 (1H, d, J 4 Hz), 5.83 (2H, m), 5.96 (1H, s), 6.03 (1H, s), 7.80−7.86 (4H, m), 7.87 (1H, s), 7.91 (1H, s), 8.17 (1H, s), 8.18 (1H,

s), 8.25–8.36 (4H, m); IR (KBr) ν_{max}/cm^{-1} 3336, 1678, 1638, 1597, 1330, 1294, 1131, 1006, 913, 837, 712; UV (50% dioxane/H_2O) λ_{max}/nm (ϵ) 329 (4714), 256 (51283).

2-Naphthylmethyl Adenosine Cyclic 3′,5′-Monophosphate (3a). This reaction was performed as described above except that 2-naphthylmethyl bromide (110.5 mg, 0.500 mmol) was used as halide. Purification by column chromatography (30 g of SiO_2, 4.7% MeOH–CH_2Cl_2 and then 6.2% MeOH–CH_2Cl_2) gave the axial isomer of **3a** (13 mg, 27%) and the equatorial isomer (14 mg, 31%). Silver salt of cAMP (2 mg, 1%) was obtained from the residual black solid.

3a (axial): mp 222–224 °C dec; 1H NMR (5% CD_3OD/$CDCl_3$) 4.33 (1H, d J 8 Hz), 4.40 (1H, dd, J 8 and 4 Hz), 4.52 (1H, dd, J 8 and 4 Hz), 4.62 (1H, d, J 4.5 Hz), 5.19 (1H, ddd, J 9, 5 and 1 Hz), 5.34 (2H, d, J 9 Hz), 5.90 (1H, s), 6.10 (2H, s), 7.43–7.50 (2H, m), 7.58 (1H, dd, J 8 and 1.5 Hz), 7.75–7.86 (4H, m), 7.89 (1H, s), 8.11 (1H, s); IR (KBr) ν_{max}/cm^{-1} 2368, 1649, 1334, 1294, 1133, 1009, 917, 837, 727; UV (50% dioxane/H_2O) λ_{max}/nm (ϵ) 262 (15300), 226 (86400). (equatorial): 1H NMR (5% $CD_3OD/CDCl_3$) 4.49–4.62 (3H, m), 4.76 (1H, d, J 5 Hz), 5.30 (1H, m), 5.34 (2H, d, J 9 Hz), 5.97 (1H, s), 6.16 (2H, s), 7.47–7.57 (3H, m), 7.75–7.88 (4H, m), 7.85 (1H, s), 8.26 (1H, s); IR (KBr) ν_{max}/cm^{-1} 2366, 1651, 1334, 1263, 1129, 1013, 913, 822, 729; UV (50% dioxane/H_2O) λ_{max}/nm (ϵ) 262 (15900), 222 (64600).

Half-Life Measurement for 2a. General Procedure for Caged cAMP. To each of two sample tubes was added 20 μL of the internal standard stock solution (10 μM), 3.3 mg (14 μmol) of 2-(hydroxymethyl)anthraquinone in 1.385 mL of DMSO, 20 mL of **2a** stock solution, 3.22 mg (6.22 μmol) of **2a**, and 0.62 mL of DMSO. Each tube was brought to 2 mL by addition of either 50% dioxane–50% water (v/v) or Ringer's solution. The solutions were placed in the dark at room temperature and monitored by HPLC at various time intervals.

Photolysis of 2a in 50% Dioxane–50% Water (v/v). General Procedure for Photolysis of Caged cAMPs. To a 10-mL volumetric flask were added 0.82 mg (1.59 μmol) of **2a**, 5 mL of 1,4-dioxane, and distilled water to fill. To a Pyrex cell (4 mL) was added 4 mL of the above solution. The Pyrex cell was sealed with a rubber septum, deaerated with argon for 5 min, and irradiated at 340 nm using 500 W Xe lamp through JASCO CT-10 type monochrometer. Aliquots of 10 μL were removed periodically and analyzed by HPLC.

Photolysis of 2a in 1% DMSO in Ringer's Solution. To a 10-mL volumetric flask were added 0.1 mL of the **2a** stock solution, 3.22 mg (6.22 μmol) of **2a** in 0.62 mL of DMSO, and Ringer's solution to fill. This solution was irradiated at 340 nm as described above and analyzed by HPLC.

Biological Study. Melanophores in scales isolated from the dorsal trunk of the wild-type medaka (*Oryzias latipes*) were immersed in the experimental solutions for 1 h at room temperature. These scales were then transferred into 10 μL of 1 μM norepinephrine hydrochloride in a well of an HT coating slide and incubated for 2 min. After the agregation of melanin pigments, UV radiation for 10 s was applied through an objective lens of a fluorescent microscope (Olympus BHT-RFC type fluorescent midroscope equipped with a BH2-RFC). The motile response of melanophores were observed and photographed with the same microscope. For the quantitative analysis of the response of melanophores, the area occupied by melanin pigments within a definite area (0.09 mm^2) in individual scales was measured by a video-image processing system, which consisted of an industrial color video camera module (Sony, XC-O09), a processing unit (Nireco, Luzex-F), and a TV monitor (Mitsubishi, XC-98V3). The magnitude of the dispersion response of melanophores was expressed as a percentage of the change in the area, taking the reading for maximal aggregation as zero and that for full dispersion as 100. Three measurements on different animals were done and the mean value with standard error was obtained.

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