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Acyloxycoumarinylmethyl-Caged cAMP, the Photolabile and Membrane-Permeable Derivative of cAMP That Effectively Stimulates Pigment-Dispersion Response of Melanophores

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Two acyloxycoumarinylmethyl-caged cAMPs (ACM- and PCM-cAMP) have been synthesized using a silver (I) oxide promoted method. Introduction of the acyloxy group to the 7-position on the coumarin ring not only enhanced the membrane permeability but diminished the photolability of the coumarin-cage. Because intracellular enzymatic hydrolysis of the 7-acyloxy group would produce the 7-hydroxy moiety which is more hydrophilic and photolabile, application of acyloxycoumarinylmethyl-caged cAMPs in biological studies would be expected to be efficient. Thus, the effect of extracellularly applied ACM- and PCM-cAMP had been investigated using the motile response of fish melanophores. After irradiation, a significant enhancement in the motility responses was observed. The observed magnitudes of the dispersions are comparable to that of Bt_2cAMP/AM which is known as a membrane permeable cAMP derivative.

Photolabile precursors of biologically interesting molecules, such as second messengers, neurotransmitters and enzyme substrates, are potential tools for the investigation of the dynamics of biological processes (1, 2). To date, one of the most interesting classes of compounds is the phosphate-containing second messengers. Among them, we have focused our attention on adenosine 3′,5′-cyclic monophosphate (cAMP) which is commercially available, has only one phosphate to be protected and is known to regulate many biological processes.

We have already synthesized (3) three new photolabile cAMP derivatives, (2-anthraquinonyl)-methyl (AQ), 4-(7-methoxycoumarinyl)methyl (MCM), and 2-naphthylmethyl (NM), and found that MCM-cAMP was superior in stability and photoreactivity to the others, including 1-(2-nitrophenyl)ethyl cAMP (NPE-cAMP) (4) and desyl cAMP (5). Despite these advantages, MCM-cAMP showed poor biological activity when it was applied to the study of the motile responses of fish melanophores. Since its solubility in Ringer's solution is poor, we expected the intracellular concentration of MCM-cAMP to be low. This would be the reason for its poor biological availability.

In order to enhance the intracellular concentration of coumarin-caged cAMP, we plan to modify the 7-position of the coumarin ring with an acyloxy (RCOO-) group. The following advantages are expected from acyloxycoumarin-caging groups. Like other caging groups, acyloxycoumarins will make cAMP more lipophilic which can be controlled by the chain length of the RCOO- moiety. Besides, once it enter the cells, these can be hydrolyzed by cytosolic esterases to produce more of the hydrophilic 7-hydroxycoumarin derivative which hardly leaks out from the cells. Here we report the results of the synthesis, photochemistry and

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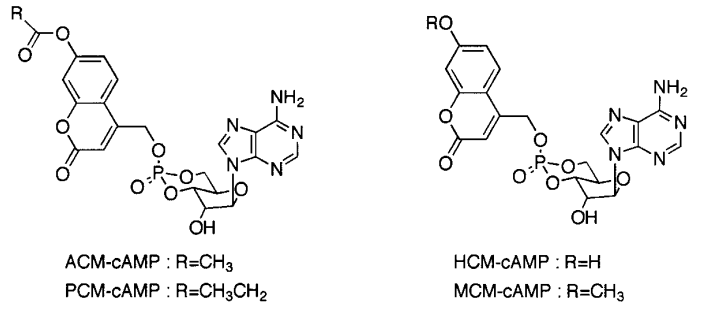


FIG. 1. Chemical structures of caged cAMPs. ACM- and PCM- are acyloxycoumarin-caged cAMP, HCM- and MCM- are hydroxy and methoxycoumarin derivatives, respectively. Only the axial isomers are presented.

biological application of two acyloxycoumarin-caged cAMPs, 4-(7-acetoxycoumarinyl)methyl cAMP (ACM-cAMP) and 4-(7-propanoyloxycoumarinyl)-methyl cAMP (PCM-cAMP) (Fig. 1), which show significantly higher biological activity compared to MCM-cAMP.

MATERIALS AND METHODS

Synthesis. Coumarin-caged cAMPs were synthesized by derivatizing cAMP with corresponding 4-(7-substituted-coumarinyl)methyl halides using the previously described silver (I) oxide promoted method (6). The detailed synthetic method and spectral properties of the new compounds will be described elsewhere.

Photolysis. To a 10-mL volumetric flask were added 100 μL of coumarin-caged cAMP stock solution (10 mM in DMSO) and normal Ringer's solution (NaCl, 125.3 mM; KCl, 2.7 mM; $CaCl_2$, 1.8 mM; $MgCl_2$, 1.8 mM; D-glucose, 5.6 mM; Tris-HCl buffer, 5.0 mM (pH 7.2)) to fill. Aliquots (20 μL) were placed in a well of an HT coating slide. UV radiation for 10 s was applied through an objective lens of a fluorescent microscope (Olympus BHT-RFC type fluorescent microscope equipped with a BH2-RFC). The light of 334-365nm was selected with a U-filter. Aliquots of 10 μL were removed and analyzed directly using HPLC as previously described.

Biological study. Members of both sexes of the wild-type medaka (*Oryzias latipes*), each 2.5-3.5 cm in total length, were used as the experimental material. Scales isolated from the dorsal trunk were rinsed in CMF (Ca^{2+} and Mg^{2+} free) Ringer's solution to remove the overlaying epidermal layer, and then were immersed in the experimental solution for 60 min at room temperature. After rinsing in normal Ringer's solution, these scales were transfered into 10 μL of 1 μM norepinephrine and 100 μM IBMX solution in a well of a HT coating slide and incubated for 90 s. After aggregation of the melanin granules, UV irradiation for 10 s was applied through an objective lens of a fluorescent microscope (Olympus BHT-RFC type fluorescent microscope equipped with a BH2-RFC). The light of 334-365 nm was selected with a U-filter. Observed motile responses of melanophores were photographed at 60 s after irradiation with the same microscope. For the quantitative analysis of the response of the melanophores, the area occupied by the melanin pigments within a definite area (0.09 mm^2) in individual scales was measured as previously described. The magnitude of the dispersion response of the 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. The means of two groups were compared using an unpaired Student's *t*-test. Differences at $p<0.05$ were considered to be significant.

RESULTS

The introduction of the 4-(7-acyloxycoumarinyl)methyl moiety to cAMP has been accomplished by the silver (I) oxide promoted condensation reaction with the corresponding halides (6). After chromatographic purification, ACM-cAMP and PCM-cAMP were obtained as a mixture of axial and equatorial isomers in 14 and 22% isolated yields, respectively. Because, before photolysis, the ACM and PCM moiety would be converted to the 7-hydroxy derivative inside the cells, 4-(7-hydroxycoumarinyl)methyl cAMP (HCM-cAMP) was also synthesized in 16% isolated yield using the same method.

Photolysis of coumarin-caged cAMP. The photolysis of four coumarin-caged cAMPs were performed through the objective lens of a fluorescent microscope in Ringer's solution con-

TABLE 1
Photolytic Consumption of Caged cAMPs

Caged cAMP	ACM-cAMP	PCM-cAMP	HCM-cAMP	MCM-cAMP	NPE-cAMP
Conversion[a] (%)	23	9	64	60	32

[a] Caged cAMPs in 1% DMSO in Ringer's solution (100 μM) were irradiated through an objective lense of a fluorescent microscope for 10 s. Consumption of the starting material was determined by reversed phase HPLC analysis.

taining 1% DMSO. Table 1 summarizes the consumption of the starting materials measured by reversed phase HPLC after 10 s irradiation. While both ACM- and PCM-cAMP showed less efficient photoreactivity (23% and 9% conversion, respectively), HCM-cAMP could be photolyzed in comparable efficiency to MCM-cAMP (64% and 60% conversion, respectively). The reactivity of these two coumarin-caging groups are superior to the traditional NPE-cAMP (32% conversion under the same conditions).

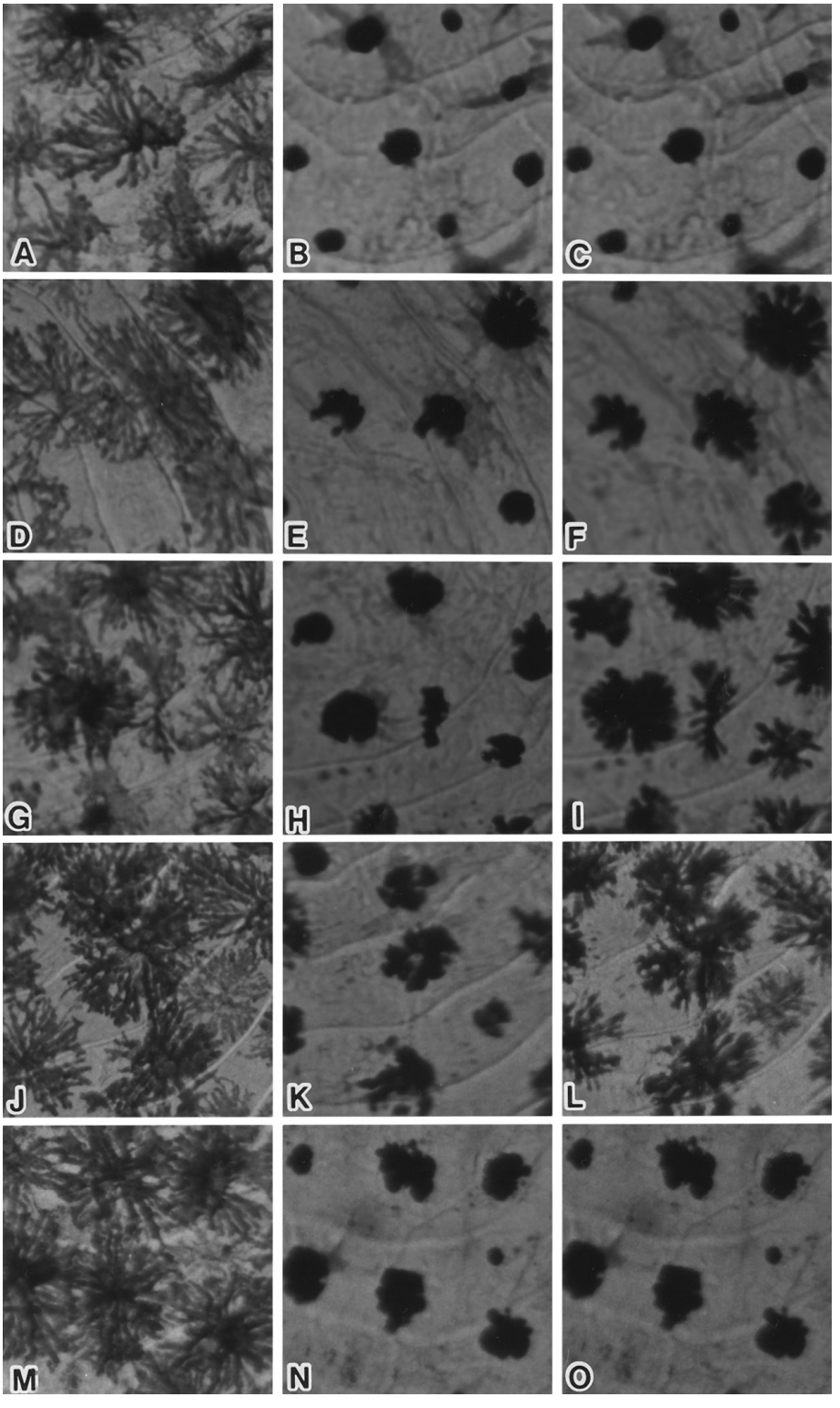
Motile responses in fish melanophores. Melanophores in scales isolated from the dorsal trunk of the wild-type medaka fish (*Oryzias latipes*) were used to demonstrate the utility of acyloxycoumarin-caged cAMP. In order to clarify the effect of extracellularly applied caged cAMP, the melanophores were pretreated with an α_2-adrenergic agonist (norepinephrine) together with a phosphodiesterase inhibitor, 3-isobutyl-1-methylxanthine (IBMX), which subsides the reaggregation of the dispersing melanin pigments by the intracellular phosphodiesterase (see experimental procedures). The results of the dispersing responses for each coumarin-caged cAMP (100 μM in 1% DMSO in Ringer's solution) are shown in Fig. 2. Both acyloxycoumarins, ACM- and PCM-cAMP, are effectively dispersed melanin pigments upon illumination, while MCM- and HCM-cAMP are less effective. 1 mM cAMP could not stimulate pigment dispersion of melanophores after the application of norepinephrine (deta not shown). Fig. 3 represents the quantitative analysis of the motile responses and clearly shows the reactivity difference between the acyloxy series and alkoxy series. While no significant difference was observed between ACM- (38.5$\pm$5.8%) and PCM-cAMP (53.6$\pm$4.4%), the difference between PCM- and MCM-cAMP (23.3$\pm$6.9%) was regarded as significant ($p<0.05$). The dependence of the PCM-cAMP dose on the motile responses is shown in Fig. 4. A concentration of 100 μM was necessary to cause significant dispersion.

DISCUSSION

In a previous paper, we reported that the (7-methoxycoumarinyl)methyl group was a potential candidate as a caging group for cyclic nucleotides (3). The only observed disadvantage was its poor membrane permeability when it was applied to living cells. Because the one practical feature of caged compounds would be their applicability to live cells, we should enhance the uptake of coumarin-caged cAMP into fish melanophores.

Hydroxycoumarines have been known as fluorescent probes for intracellular pH measurements (7,8). To enhance their uptake into cells, the 7-hydroxy group has been protected as its acetyl ester which had a lipophilicity and could be hydrolyzed by intracellular esterases to regenerate hydrophilic 7-hydroxy coumarins inside the cells. Such a concept, the intracellular esterases mediated deprotection, has been proved to be useful for enhancing the uptake of small molecules into cells (9).

In our case, the photoreactivity of the coumarin-cage was diminished by introducing the acyloxy moiety into the 7-position of the coumarin ring. Nevertheless, a significant difference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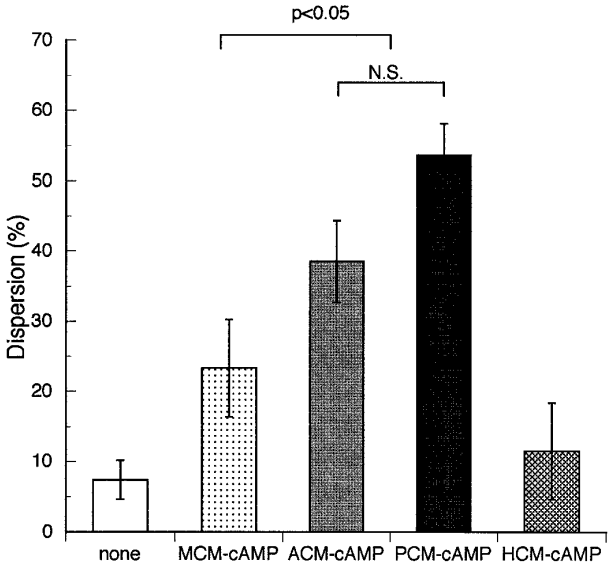

FIG. 3. Dispersing responses of pigment granules after UV irradiation. The magnitude of dispersion was quantitatively analyzed according to the method described under Materials and Methods after incubation with the following solutions, none : Ringer's solution, MCM-cAMP : 100 μM MCM-cAMP in Ringer's solution, ACM-cAMP : 100 μM ACM-cAMP in Ringer's solution, PCM-cAMP : 100 μM PCM-cAMP in Ringer's solution, HCM-cAMP : 100 μM HCM-cAMP in Ringer's solution. The values are means$\pm$S.D. of three separate experiments.

in motility response between MCM- and acyloxycoumarin-caged cAMP (ACM- and PCM) was observed. The hydrolysis of the acyloxycoumarin-cage produced HCM-cAMP which has a comparable photoreactivity to MCM-cAMP. Moreover, application of HCM-cAMP itself to melanophores showed poor dispersing ability after irradiation. These results strongly suggest that both acyloxycoumarin-cages, after incubation, would be enzymatically hydrolyzed to produce hydrophilic and highly photolabile HCM-cAMP which would be efficiently trapped inside the cells. PCM-cAMP showed a comparable dose dependency to acetoxymethyl ester of Bt_2cAMP (Bt_2cAMP/AM), which was reported to be 1000 times more efficient than Bt_2cAMP (9). Compared with these non-photolyzable derivatives, one of the principal advantages of the acyloxycoumarin-caged cAMPs is that cAMP can be released in the time-controlled manner at anywhere in the cells upon UV irradiation. Because the motile responses of fish melanophores can not be cotrolled by extracellularly applied cAMP, acyloxycoumarin-caged cAMPs with increased membrane permeability are the promising tools to investigate the dynamics of the biological processes known to be regulated by cAMP (10, 11) using living cells. Furthermore, in the present study, we disclosed that the modification of the photolabile protecting groups by esters which can be enzymatically hydrolyzed affords caged compounds of new type that act efficiently inside the cells.

FIG. 2. Photomicrographs showing responses of melanophores to extracellularly applied caged cAMP before and after UV irradiation. A–C, D–F, G–I, J–L and M–O show the effect of Ringer's solution, MCM-cAMP, ACM-cAMP, PCM-cAMP and HCM-cAMP, respectively. In each series, photographs were taken with transmission optics of the same part of a given scale. A, D, G, J and M : 60 min after incubation in the experimental solutions. B, E, H, K and N : 60 s after the transfer into 1 μM norepinephrine and 100 μM IBMX. C, F, I, L and O : 60 s after UV irradiation. $\times$ 235. 1 mM cAMP could not stimulate pigment dispersion of melanophores after the application of norepinephrine (deta not shown).

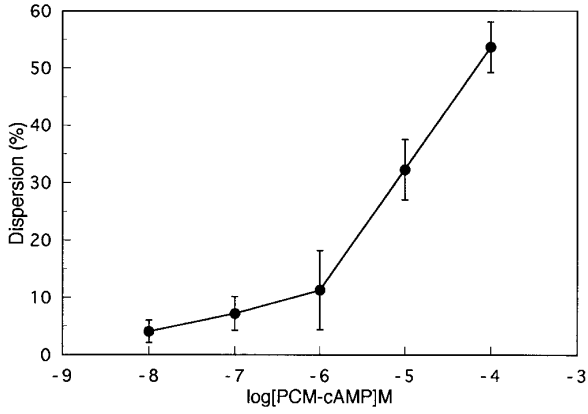

FIG. 4. Dispersing responses of pigment granules to PCM-cAMP dose. Melanophores were incubated for 60 min with various concentrations of PCM-cAMP in 1% DMSO in Ringer's solution. The magnitude of dispersion was quantitatively analyzed according to the method described under Materials and Methods. The values are means±S.D. of three separate experiments.

REFERENCES

1. Adams, S. R., and Tsien, R. Y. (1993) *Annu. Rev. Physiol.* **55,** 755–784.
2. Corrie, J. E. T., and Trentham, D. R. (1993) *in* Bioorganic Photochemistry, Vol. 2, Biological Applications of Photochemical Switches (Morrison, H., Ed.), pp. 243–305, Wiley, New York.
3. Furuta, T., Torigai, H., Sugimoto, M., and Iwamura, M. (1995) *J. Org. Chem.* **60,** 3953–3956.
4. Wootton, J. F., and Trentham, D. R. (1989) *in* Photochemical Probes in Biochemistry (Nielsen, P. E., Ed.), NATO ASI Ser. C, Vol. 272, pp. 277–296, Kluwer Academic, Dordrecht.
5. Givens, R. S., Athey, P. S., Matuszewski, B., Kueper, L. W., III, Xue, J.-Y., and Fister, T. (1993) *J. Am. Chem. Soc.* **115,** 6001–6012.
6. Furuta, T., Torigai, H., Osawa, T., and Iwamura, M. (1993) *J. Chem. Soc., Perkin Trans.* **1,** 3139–3142.
7. Chance, B., and Mela, L. (1966) *J. Biol. Chem.* **241,** 4588–4599.
8. Gerson, D. F., and Burton, A. C. (1977) *J. Cell Physiol.* **91,** 297–303.
9. Schultz, C., Vajanaphanich, M., Harootunian, A. T., Sammak, P. J., Barrett, K. E., and Tsien, R. Y. (1993) *J. Biol. Chem.* **268,** 6316–6322.
10. Hassoni, A. A., and Gray, P. T. (1994) *Pflugers Arch.* **428,** 269–274.
11. Nakashima, Y., and Ono, K. (1994) *Am. J. Physiol.* **267** (*Heart Circ. Physiol.* **36**), H1514–H1522.