Embryogenesis

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A Caged Retinoic Acid for One- and Two-Photon Excitation in Zebrafish Embryos**

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The embryogenesis of a multicellular organism relies on the spatiotemporal control of the concentrations of signaling molecules and the way cells respond to them.[1] One of these, retinoic acid (RA), plays a key role in patterning the body axis and in the formation of many organs.[2] RA concentration is finely regulated both spatially and temporally and its alteration results in developmental defects. An ability to artificially tune the RA concentration at the single-cell level would bring new insight into these phenomena.

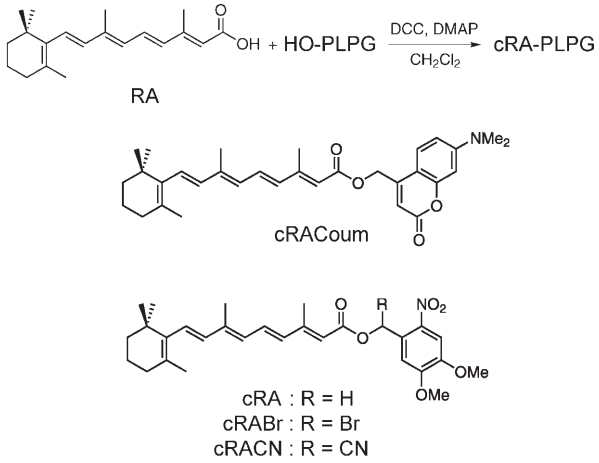
Caged compounds,[3,4] which are molecules with a photolabile moiety, combined with two-photon excitation[5] provide an attractive possibility to fulfill this goal, while the zebrafish presents a particularly promising model organism:[6] its embryo is transparent, its genome is known, and mutants are available. Herein, we introduce a caged retinoic acid (cRA) that can be photoactivated in zebrafish with both one- and two-photon excitation. We characterize its two-photon uncaging kinetics in vivo, which allows tuning of the RA concentration at the single-cell level in a live organism.

In principle, a carboxylic acid like RA can be photoreleased from a carboxylic ester obtained after reaction with an appropriate alcohol acting as a photolabile group. We considered two putative series of photoactive RA esters relying on benzylic coumaryl[7] or *ortho*-nitrobenzyl moieties.[8] Thus, we first prepared the 7-dimethylaminocoumarin-4-ylmethyl all-*trans* retinoic acid ester (cRACoum) by condensation of RA with 7-dimethylaminocoumarin-4-yl methanol[9] in the presence of DCC with 84% yield. We also similarly synthesized from the corresponding 2-nitrobenzyl alcohols[10] three other RA esters: cRA, cRABr, and cRACN (see Scheme 1 and the Supporting Information).

The RA esters were preliminarily screened for their potency by injection into zebrafish embryos. While none of them was active in the dark, cRA was retained as the most appropriate for further studies: 1) it was the most soluble compound; 2) it produced the most pronounced teratogenic effects upon UV (365 nm) illumination; and 3) it can be readily synthesized from commercially available materials.

We first characterized the uncaging of cRA in vitro by UV/Vis absorption and capillary electrophoresis (CE) to estimate the typical time associated not only with uncaging (τ_2) but also with photoisomerization (τ_1) and photodegradation (τ_3), processes known to occur in the parent compound RA under UV illumination.[11] A drop in absorbance at approximately 350 nm of a cRA solution after UV illumination for typically 20 s (Figure 1a) is also observed in RA solution. This phenomenon is associated with relaxation toward a photostationary state involving a dynamic exchange between different RA stereoisomers, among which the *trans*-RA isomer represents 20% (see Supporting Information).

In contrast, the blue shift of the absorbance at intermediate times (100–1000 s) significantly departs from the behavior of RA (Figure 1b). The shift is associated with uncaging, as



Scheme 1. Synthesis and structures of the investigated caged esters incorporating a photolabile protecting group (PLPG), obtained from reaction of all-*trans* RA with the corresponding photolabile alcohols HO-PLPG. DCC = *N*,*N*′-dicyclohexylcarbodiimide, DMAP = *N*,*N*′-dimethylamino-4-pyridine.

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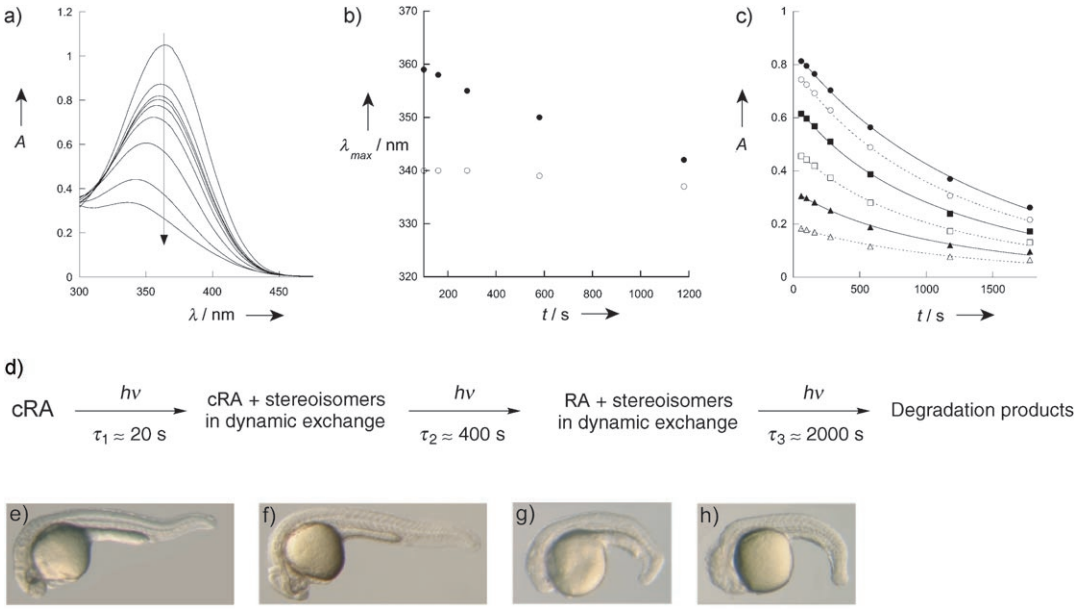

Figure 1. UV illumination (365 nm) of a cRA solution. a–c) In vitro irradiation of a 25 μM cRA solution in acetonitrile/20 mM Tris pH 9 buffer (1:1, v/v) as a function of time ($t = 0$, 20, 60, 100, 160, 280, 580, 1180, 1780 s). a) Evolution of the UV/Vis absorption spectra; b) the maximal wavelength of absorption (cRA, ●; RA, ○; see Supporting Information, Figure 2S a); and c) the absorbance at several wavelengths (364, ●; 374, ○; 384, ■; 394, □; 404, ▲; 414 nm, △; lines, global biexponential fit $A_2e^{-t/\tau_2} + A_3e^{-t/\tau_3}$). Using $\tau_3 = 1700$ s from the kinetics of RA photodegradation (see Supporting Information Figure 2S b), we extracted $\tau_2 = 400$ s which yielded 35 $M^{-1}cm^{-1}$ for the uncaging action cross section for cRA in the 350-nm range. d) Photochemical processes occurring upon cRA photoactivation. e–h) In vivo experiments: 128-cell dechorionated embryos were incubated at 25 °C for 90 min in 10 μM cRA. They were then washed with embryo medium and illuminated (or not). The embryos were subsequently observed at 30 h post-fertilization. The nonilluminated embryos (e) develop normally as do the untreated ones (f). The embryos illuminated for 80 s (g) exhibit strong teratogenic effects similar to those observed in embryos (h) incubated for 90 min in 1 μM RA.

expected from the longer wavelength of maximal absorption in cRA (364 nm) than in RA (343 nm) and confirmed by direct CE observations of several photoreleased intermediates: RA and also 13-*cis*- (C13RA) and 9-*cis*-RA (C9RA), which are involved in RA photoisomerization. Eventually, the drop in absorbance at long irradiation times (beyond 1000 s) as a result of photodegradation is in line with similar observations on RA (see Supporting Information).

The temporal evolution of the absorbance (between 364 and 414 nm) was analyzed with a simple kinetic model that accounts for the observed photoinduced processes. We deduced a typical cRA uncaging rate of $(2.5 \pm 1) \times 10^{-3}\,s^{-1}$ (Figure 1 c), which compares well with the value of $(3.5 \pm 0.5) \times 10^{-3}\,s^{-1}$ obtained with a model compound and with CE data (see Supporting Information). Hence, UV illumination of cRA causes three types of photochemical reactions with well-separated timescales (Figure 1 d): first, photoisomerization along the polyenic backbone ($\tau_1 \approx 20$ s); then uncaging ($\tau_2 \approx 400$ s) to yield a mixture of continuously interconverting RAs; and finally, photodegradation of the various compounds ($\tau_3 \approx 2000$ s). To manifest themselves, cRA-photoinduced effects may thus require enough but not too many photons.

In view of biological application in a whole organism, cRA has to fulfill several constraints. It should 1) enter the cells passively, 2) be chemically stable under physiological conditions, 3) be biologically inactive and nontoxic, and 4) be uncaged at light levels that do not damage the cell. Having already established during the screening step that cRA was nonteratogenic during zebrafish development, we proceeded to determine its effects upon UV (365 nm) illumination. Dechorionated 128-cell embryos were incubated for 90 min in a 10 μM cRA solution. Consistent with the injection experiment, nonilluminated embryos developed normally (Figure 1 e and f). In contrast, embryos illuminated for 80 s exhibited the typical phenotype (Figure 1 g and h) induced by RA, C13RA or C9RA, at a concentration of about 1 μM (the UV dose alone does not induce any defects, see the Supporting Information). This result shows that cRA passively diffuses into dechorionated embryos.

From the in vitro experiments, which gave an uncaging rate constant of $2.5 \times 10^{-3}\,s^{-1}$ for cRA, we evaluated the extent of uncaging after 80 s of illumination to be about 20 %. As both *cis*-RAs produce phenotypes similar to those observed with RA at the same concentration, we conclude that the initial cRA concentration within the embryo before illumination was close to the external incubating concentration of cRA (10 μM). Moreover, the teratogenic effects disappeared after too long a UV illumination of cRA (see Supporting Information), as expected from the in vitro studies.

We then attempted to control the RA concentration at the single-cell level using two-photon uncaging. To determine the appropriate laser parameters and characterize the targeted cells, a caged fluorophore sharing the same caging group as cRA was used. As revealed by the rise in fluorescence intensity, illumination at 750 nm (1.8 mW; below 5 mW, two-photon illumination alone does not induce any defects, see Supporting Information) results in uncaging within a typical time $\tau_2 \approx 25$ s. From the distribution of τ_2, we also computed

the average size of a retina cell as (13.5 ± 6) μm, consistent with values in the literature[12,13]. Equipped with a quantitative characterization of two-photon uncaging of a model compound, we investigated the kinetics of RA photorelease in a retina cell and the photoinduction of a RA-induced phenotype in the embryo retina (Figure 2a).

Although RA is almost nonfluorescent in solution, it becomes fluorescent when bound to endogenous cytoplasmic retinol or RA binding protein (CRBP or CRABP).[14,15] The photorelease and complexation of RA can thus be monitored through the change in fluorescence induced by its binding to CRABP and CRBP as it competes with the more fluorescent endogenous retinol. We did indeed observe a biphasic change in fluorescence (Figure 2b). The first regime of decreasing fluorescence was attributed to retinol unbinding from CRBP; its rate constant $k_{off} = 0.06\ s^{-1}$ is consistent with reported values (0.01–0.1 s^{-1} [16,17]) and the 23% decrease in fluorescence is also in line with the 26% drop reported when RA displaces retinol from its complex with CRBP.[15] The second regime of fluorescence increase is caused by RA binding to CRABP. It is limited by uncaging and gives an uncaging rate constant of 0.015 s^{-1} at 1 mW power and 730 nm excitation, akin to that measured on a similar caged fluorophore (0.013 s^{-1}). Thus, this experiment suggests that RA is released upon two-photon cRA uncaging and remains confined to the illuminated cell volume within the time span of the measurement (about 3 min).

Having estimated the cRA two-photon uncaging rate, we examined whether two-photon cRA uncaging could cause a RA-induced phenotype. We relied on retina malformations induced by RA application on the dorsal part of the retina.[18] Nondetrimental two-photon excitation (750 nm, 4.5 mW) was applied in four dorsal cells of the retina of zebrafish incubated in cRA (see Supporting Information). Figure 2d shows the typical phenotype observed 15 h later. In comparison with the nonilluminated control eye (Figure 2c), the illuminated retina is larger, more elongated, and its dorsal part is slightly invaginated (see reference [18]). Thus, two-photon uncaging of cRA reproduces a known specific RA-induced phenotype.

In conclusion, we have shown that cRA exhibits attractive features for use in a biological context: it permeates cells and is stable in a zebrafish embryo where it is not biologically active. Upon one- or two-photon excitation, RA can be released in a controlled way using nondetrimental light intensities. In particular, by confinement of two-photon uncaging to a single cell, cRA could be a useful tool to alter and investigate key developmental events, such as somitogenesis, where comparison of models with experiments has been hampered by the lack of means to precisely set RA gradients.[19]

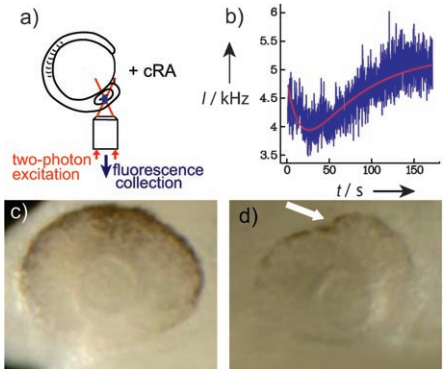

Figure 2. Two-photon excitation in vivo. a) Experimental principle: a single cell (b) or four cells (d) of the right retina of a manually dechorionated 4–14 somite embryo previously incubated for 90 min in 10 μM cRA is (are) illuminated, and either the temporal variation of the fluorescence signal (b) or the eye malformation phenotype 15 h after treatment are observed. b) Variation in fluorescence intensity $I(t)$ upon cRA uncaging at a laser power of 1 mW. The changes are caused by retinol unbinding from CRBP, which results in a decrease in fluorescence, and by RA binding to CRABP, which increases the fluorescence. Continuous line: double exponential fit $I(t) = I_0 - \Delta I^{off}(1 - e^{-k_{off}t}) + \Delta I^{on}(1 - e^{-k_{on}t})$, where $k_{off} = 0.06\ s^{-1}$ is the rate of retinol unbinding and $k_{on} = 0.015\ s^{-1}$ is the uncaging rate at 1 mW power, from which we deduce a two-photon action cross section of 25 mGM at 750 nm, compatible with reported values.[10] c) Control and d) illuminated retina of the same embryo. The illuminated eye exhibits an invagination (arrow) which is characteristic of a RA-induced phenotype.

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