RESEARCH COMMUNICATION

Membrane permeability of coelenterazine analogues measured with fish eggs

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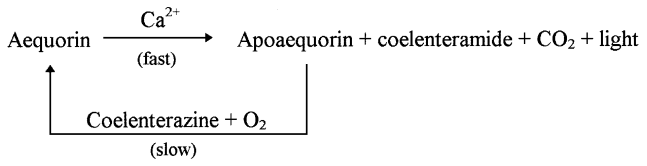
To determine the suitability of various coelenterazine analogues for the regeneration of aequorin in living cells, the membrane permeabilities of 11 analogues were measured using the eggs of the killifish *Fundulus grandis* by soaking the eggs in solutions containing the analogues. The results indicated that *e*-coelenterazine, which has an exceptionally high rate of *in vitro* regeneration of aequorin, not only permeated poorly into the eggs but also was highly unstable. All other analogues tested permeated sufficiently into the eggs. The highest permeability was found with *f*-coelenterazine; the concentration of *f*-coelenterazine in the eggs was about five times that in the surrounding medium, assuming that the distribution of the compound in the egg is uniform.

INTRODUCTION

The photoprotein aequorin emits light in the presence of a trace of Ca^{2+} by an intramolecular reaction, decomposing itself into apoaequorin, coelenteramide and carbon dioxide [1]. Apoaequorin can be regenerated into the original aequorin by incubation with coelenterazine or its various analogues in the presence of oxygen [2], as shown in Scheme 1.



Scheme 1

Because of its high sensitivity to Ca^{2+} and harmlessness to living cells, aequorin has been widely used as a probe of cellular Ca^{2+} for more than 25 years. The successful cloning of the apoaequorin gene about 10 years ago [3,4] has opened a new way of utilizing aequorin, by expressing recombinant apoaequorin (M_r 21 600) in various living cells; the recombinant apoaequorin produced in cells can be converted into aequorin by a coelenterazine added outside the cells [5,6]. This technique is highly valuable, because it does not require the use of the microinjection technique, which is difficult to perform with small cells. The success of this technique, however, depends on various factors: (1) the permeability of the cell membrane to coelenterazine; (2) the rate of the regeneration of aequorin from apoaequorin, a coelenterazine and oxygen; (3) the stability of the coelenterazine used; and (4) the intrinsic properties of the aequorin formed (such as quantum yield and Ca^{2+} sensitivity).

The relative rates of the regeneration *in vitro* of aequorin with various coelenterazine analogues were reported previously [7], and the results showed that *e*-coelenterazine has the highest rate in aequorin regeneration. The present study was aimed at gaining information mainly on the permeability of membranes to coelenterazines, to facilitate the selection of a coelenterazine suitable for experiments involving the intracellular regeneration of aequorin.

MATERIALS AND METHODS

The eggs of the killifish *Fundulus grandis* were purchased from Gulf Coast Minnows (Thibodaux, LA, U.S.A.), and they were used in experiments within 3 days after being laid. Coelenterazine and its analogues [8] were gifts from Dr. Y. Kishi (Harvard University, Cambridge, MA, U.S.A.) and Dr. K. Teranishi (Mie University, Tsu, Japan). *Oplophorus* luciferase had been previously prepared [9]. Light emission was measured with an integrating photometer (Model 8020; Pelagic Electronics, Falmouth, MA, U.S.A.). All experiments were carried out at 23–24 °C.

The eggs (0.1 g; 21–22 eggs) were suspended in 1 ml of the artificial seawater described by Lyman and Fleming [10], pH 7.8, and mixed with 10 μl of methanolic 0.1 mM coelenterazine analogue. The mixture was gently stirred every 2 min. After 15 min, the supernatant solution was separated from the eggs with a Pasteur pipette. The eggs were crushed in a test tube with a Teflon rod that closely fit inside the test tube, then were briefly ground with 1 ml of methanol; the layer of clear solution formed after the mixture was left standing for 2–3 minutes was used for the assay of coelenterazine. To determine the amounts of coelenterazine and analogues in the supernatant solution and the methanolic extract of eggs, 10 μl of each sample was added to 3 ml of 15 mM Tris/HCl, pH 8.3, containing 50 mM NaCl and 10 μg of *Oplophorus* luciferase, and the resulting light emission was measured.

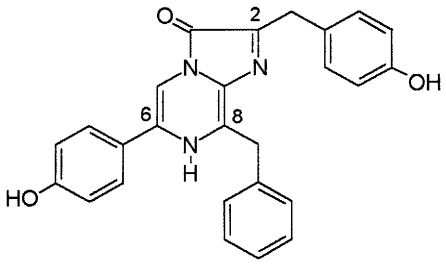
RESULTS AND DISCUSSION

The eggs of the killifish *F. grandis* are nearly transparent and quite uniform in size (diameter approx. 1.5 mm). To measure the permeability of the outer membrane to various coelenterazine analogues, the eggs were soaked for 15 min in a solution containing 1 nmol of an analogue, then the amounts of the analogue in the solution and in the eggs were separately assayed. The results are summarized in Table 1. For the convenience of evaluating the coelenterazine analogues for use in the intracellular regeneration of aequorin, previously reported data on the Ca^{2+} sensitivity and the regeneration rate *in vitro* of aequorins are included in Table 1.

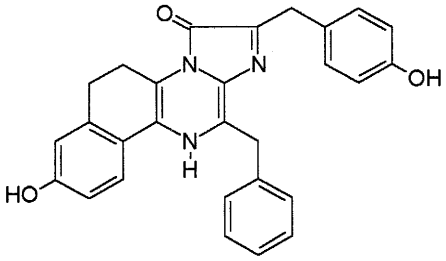
The permeation of a coelenterazine analogue into eggs appeared to reach a plateau within 10–15 min (results not shown). *e*-Coelenterazine, which has the highest rate in the regeneration *in vitro* of aequorin, was found to be poorly permeable, and

Table 1 Measurement of the membrane permeability of coelenterazine analogues with the eggs of *F. grandis*

Eggs (0.1 g) were soaked in 1 ml of artificial seawater containing 1 nmol of a coelenterazine analogue; coelenterazines were assayed with *Oplophorus* luciferase; the data on Ca^{2+} sensitivity and regeneration rate are included for comparison. The substitution positions 2 and 8 are indicated on the coelenterazine structure shown. The asterisk indicates compounds that are commercially available from Molecular Probes (Eugene, OR, U.S.A.).



Coelenterazine



e -Coelenterazine

Coelenterazine analogues		Coelenterazine found after 15 min		Regenerated aequorin	
Prefix	Substitution	Total (%)	In eggs (%)	Relative Ca^{2+} sensitivity [11]	Relative regeneration rate [7]
(Coelenterazine)*	None	79	26	1	1
h-*	2: -$CH_2C_6H_5$	65	25	16	0.105
f-*	2: -$CH_2C_6H_4F$ (*p*)	95	45	20	0.157
f_2-	2: -$CH_2C_6H_3F_2$ (*m,p*)	82	32	30	0.20
Cl-	2: -$CH_2C_6H_4Cl$ (*p*)	77	36	0.6	0.92
Br-	2: -$CH_2C_6H_4Br$ (*p*)	68	32	0.5	0.88
n-*	2: -CH_2-naphthyl (β)	76	39	0.15	0.07
ch-	8: -CH_2-cyclohexyl	94	29	15	0.49
cp-	8: -CH_2-cyclopentyl	70	25	28	0.24
f(8)-	8: -$CH_2C_6H_4F$ (*p*)	92	20	5	0.31
e-	8: -CH_2CH_2-bridge	15	4	6	2.75

it also rapidly decomposed in solution. The results clearly indicated that *e*-coelenterazine is unsuitable for use in the intracellular regeneration of aequorin, contrary to a previous report [7]. All other analogues tested appear to be sufficiently membrane permeable for the purpose of regenerating aequorin, although there were considerable variations among them in Ca^{2+} sensitivity and the regeneration rate of aequorin, which must be taken into account in the selection of a coelenterazine analogue. The most permeable was found to be *f*-coelenterazine. Considering that the volume of the soaking solution was ten times that of the eggs, the concentration of *f*-coelenterazine in the eggs must be nearly five times that in the soaking solution, assuming that the distribution of *f*-coelenterazine in the egg is uniform. Although such an assumption is certainly unrealistic, because the cells are not homogeneous chemically and structurally, the results may imply the existence of areas or compartments in cells where the concentration of *f*-coelenterazine is exceedingly high.

In summary, the data presented in Table 1 may suggest the use of the following analogues for intracellular regeneration of aequorin: (1) native coelenterazine when a fast regeneration of aequorin is important; (2) *f*-coelenterazine or f_2-coelenterazine when a high Ca^{2+} sensitivity of regenerated aequorin is needed; (3) *ch*-coelenterazine if both high Ca^{2+} sensitivity and fast regeneration are preferable; (4) *Cl*-coelenterazine or *Br*-coelenterazine when lower Ca^{2+} sensitivity of aequorin is sufficient for the purpose.

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