Glow-type Cellular Aequorin Assay

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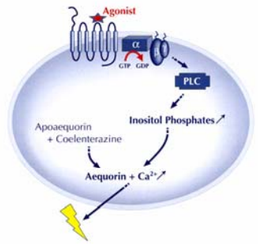
1 Introduction



Aequorin-based Ca^{2+} assays represent a new paradigm in drug discovery research for Ca^{2+}-coupled GPCRs and ion channels cell-based assays. In the aequorin assay, cells co-expressing apo-aequorin and the target receptor are first incubated with the co-factor coelenterazine in order to reconstitute the active aequorin enzyme.

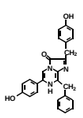
Reconstitution of an active aequorin, using native coelenterazine or its derivative coelenterazine h, yields an enzyme having a fast luminescent response to increasing calcium concentrations, and a high level of signal intensity. Amongst the other types of coelenterazine derivatives characterized in *in vitro* assays, coelenterazine i has retained our attention, due to the slow reaction kinetics it confers to the aequorin enzyme.

***In vitro* properties of semi-synthetic aequorins prepared from natural aequorin**

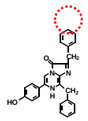
(reviewed by Shimomura, 2006. Bioluminescence: chemical principles and methods. World Scientific Publishing Co.)

Coelenterazine	Luminescence Max (nm)	Relative Total Light Amount	Half-total Light Time* (s)
native	465	100 %	0.4 - 0.8
h	464	82 %	0.4 - 0.8
i	476	70 %	8

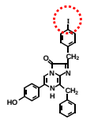
* The time required to emit 50% of the total light in 10 mM calcium acetate at 25 °C



Native coelenterazine



Coelenterazine h

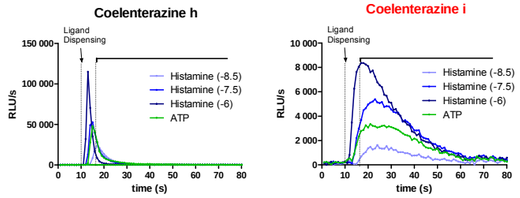


Coelenterazine i

Here we show that, when using the coelenterazine i derivative in a cell-based assay, despite the temporary nature of the intracellular calcium wave, the kinetics of the system are considerably delayed compared to the ones obtained with other coelenterazines. While the first seconds after dispensing must be measured when working with coelenterazine h, they can be omitted while keeping the correct rank order of potency of agonists or antagonists when working with coelenterazine i. This opens the possibility of using separated dispensing and reading devices in an automated assay environment

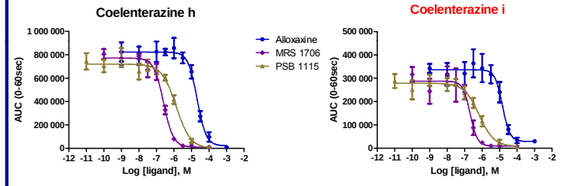
2 Use of "coelenterazine i" derivative delays the Aequorin signal in cellular assays

CHO-H_1



Kinetics of the cellular response to Histamine H_1 receptor stimulation by an agonist, when aequorin was reconstituted with coelenterazine h or with coelenterazine i. The response to 10 µM ATP, acting on an endogenous P2Y receptor present in CHO cells is also presented. This kinetic measurement was acquired by the LumiLux®, which is a decidated "dispense-and-read" luminometer. The horizontal bar indicates the part of the signal that could be recorded by using a robotic station dispensing followed by plate reading with a "non-dispensing" luminometer such as the ViewLux®. In the case of coelenterazine h, most of the signal would be lost, while most of the signal can still be recorded when using coelenterazine i.

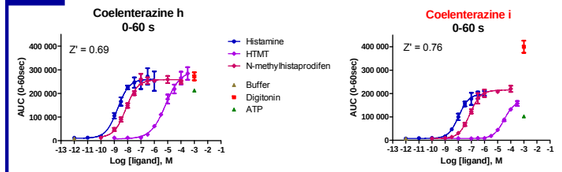
3A HEK-A_{2B} – Antagonist Assay



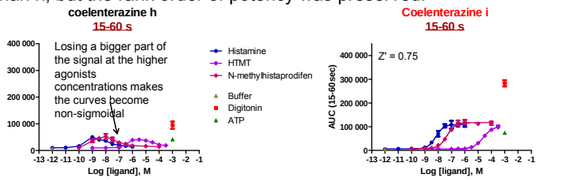
pIC_{50}	coelenterazine h	coelenterazine i
Alloxazine	4.68	4.84
MRS 1706	6.59	6.69
PSB 1115	5.87	6.17

Inhibition by 3 antagonists of the stimulation of the Adenosine A_{2B} receptor by 10 µM NECA. IC_{50} values were similar when aequorin was reconstituted with coelenterazine h or i.

3B CHO-H_1 – Agonist Assay



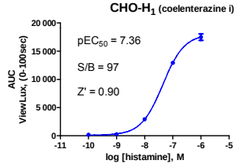
Response to 3 agonists of the Histamine H_1 receptor. There was some right-shift of EC_{50} values when using coelenterazine i rather than h, but the rank order of potency was preserved.



When using coelenterazine i – not h – the first 15 seconds after dispensing may be omitted from the signal recording, as shown by this simulation using different integration time windows

pEC_{50}	coelenterazine	integration 0-60 s	integration 15-60 s
Histamine	h	8.75	-
	i	7.97	8.17
HTMT	h	5.15	-
	i	4.50	4.62
N-methylhistaprodifen	h	8.12	-
	i	7.12	7.33

4 AequoScreen® assay using the ViewLux®



While dedicated, highly sensitive readers such as the LumiLux® (PerkinElmer), the MicroBetaJet® (PerkinElmer), the FDSS (Hamamatsu Photonics) and the Cybi®Lumax (Cybio) have already been in use for several years to perform drug discovery using aequorin assays, not all the labs are equipped with readers having simultaneous dispense and read capabilities. The transformation of the "Flash"-aequorin assay into a "Glow-" assay opens the possibility of using separated dispensing and reading devices in an automated environment. The result of an aequorin assay performed using the ViewLux® reader (PerkinElmer) is shown here. The huge sensitivity and good accuracy of the ViewLux® allowed to get excellent signal window and Z' values.

5 AequoScreen® assay

Suspension cells assay: All LumiLux® and ViewLux® measurements presented here were performed with suspension cells, in 384-well format, and at a cell density of 5 000 cells/well. After thawing or detaching from culture plates, cells were resuspended in 10-ml of assay buffer containing 5µM coelenterazine h or i. This cell suspension was put in a 10 ml Falcon tube, fixed onto a rotating wheel and incubated for 4h or overnight at RT° in the dark (8 rpm; 45° angle). Cells were diluted with Assay Buffer to 5 000 cells/20 µL (LumiLux®) or 5 000 cells/40 µL (ViewLux®). For LumiLux® experiments, ligands (20 µL/well), diluted in Assay Buffer, were prepared in black, clear bottom assay plates, and the cell suspension was dispensed on the ligands using the LumiLux®. For ViewLux® experiments, a 12-channel pipette was used to dispense manually 40 µL of cell suspension (5 000 cells) onto wells of the assay plate containing 40 µL of various agonist concentrations, and the assay plate was then immediately inserted in the ViewLux® for reading the emitted light. The time between dispensing and start of ViewLux® measurement was around 8 seconds. Digitonin at a final concentration of 100 µM diluted in Assay Buffer was used to measure the receptor-independent cellular calcium response (cell membrane permeabilization).

ViewLux® settings: A single reading frame of 100 s was acquired as soon as possible after insertion of the assay plate into the ViewLux®, using a clear emission filter, 8x image binning, slow CCD readout speed and high gain, yielding a single value per well. For automation, plates can be placed by a robotic arm onto the robotic handler, where cells can be loaded to the compound plate in case of a suspension assay. The plate can then be automatically loaded inside, maintaining a constant time between loading of the cells and start of the measurement.

Double transfectant aequorin cell lines, stably expressing both mitochondria-targeted aequorin and a GPCR were used in this study: CHO Histamine H_1 AequoScreen® cell line (PerkinElmer Product # ES-390-A) and HEK Adenosine A_{2B} AequoScreen® cell line (PerkinElmer Product # ES-013-A).

Reagents: Assay Buffer was DMEM/Ham's F12 culture medium (with 15 mM HEPES, L-glutamine, without phenol red; Invitrogen Cat n°11039) + 0.1% protease-free BSA

Coelenterazine h was from Promega (Cat n° S2011) and coelenterazine i was from Biotium (Cat n° 10121): both were prepared as 500 µM stock solution in methanol.

Digitonin was from Sigma (Cat n°37006): 50 mM stock solution in DMSO.

6 Conclusion

We have shown here that, using the same AequoScreen® cell lines, it is possible to slow down the kinetics of the luminescent response by using coelenterazine derivative i. Therefore recording the few seconds after dispensing is no more needed, which allows performing AequoScreen® assays with readers that are devoid of built-in dispensing system, as demonstrated with the ViewLux®.

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