CAFLUX, a simplified version of the CALUX assay for Ah receptor (ant)agonists, based on enhanced green fluorescent protein (EGFP) reporter gene expression

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1. Introduction

Polyhalogenated dibenzo-p-dioxins (PCDDs), dibenzofurans (PCDFs), biphenyls (PCBs) and various other environmental and food chain contaminants are potent toxic substances producing adverse health effects in humans and wild life species, which are mostly mediated by Ah receptor activation [1]. There is a great demand for convenient and cheap biological effect assays to quantify the potency of complex mixtures of Ah receptor (ant)agonists.

In recent years, we have developed a method to measure the biological potency of complex mixtures of Ah receptor (ant)agonists which is based on chemical-activated luciferase reporter gene expression (CALUX) in recombinant H4IIE rat and Hepa-1c1c7 mouse hepatoma cells [2, 3, 4]. This CALUX method, complemented with a simple acid silica clean-up procedure, has since then been applied to various matrices, such as human milk and blood plasma [5], cows milk [6], pore water and sediment [7].

Here, we present the CAFLUX-assay, an adaptation of the CALUX approach based on chemical-activated fluorescent protein expression in recombinant H4IIE rat hepatoma cells. The CAFLUX utilizes the enhanced green fluorescent protein (EGFP) gene as a reporter gene for Ah receptor activation instead of the firefly luciferase gene used in the CALUX. EGFP is a protein derived from the jellyfish *Aequoria victoria* carrying a cyclic tripeptide acting as a fluorophore [8]. The use of EGFP eliminates the need to lyse the cells and to add the expensive luciferin as a substrate to determine luciferase activity.

2. Materials and methods

2.1. Construction of a H4IIE CAFLUX cell line

The pMDREegfp1.1 reporter plasmid, conferring Ah receptor-controlled EGFP expression, was constructed from pEGFP-1 (Clontech) using standard methods [9]. H4IIE cells were stably transfected with pMDREegfp1.1 by co-transfection with pSV2-neo [10] using DOTAP transfection reagent as prescribed by the manufacturer (Boehringer Mannheim).

2.2. CAFLUX-assay

H4IIE CAFLUX cells were plated in 96-well plates (Greiner; 2×10^4 cells/well) using α-MEM (GIBCO) supplemented with 10% heat-inactivated foetal calf serum (GIBCO). After reaching confluency the cells were exposed to TCDD and PCB congeners using DMSO as a vehicle (final concentration 0.4%) for 48 hours, unless indicated otherwise. Subsequently, EGFP expression was measured using a Cytofluor 2350 fluorometer (Millipore) and 485/530 nm excitation/emission filters. To preserve sterility and allow repeated measurements, the lid was always left on the plates during measurement.

2.3. CALUX-assay

CALUX-assays were performed in white 96-well view plates (Packard) as described elsewhere [7]. Luciferase was measured using flash kinetics and a Luminoskan RS luminometer (Labsystems) equipped with an automatic substrate injector.

2.4. Data processing

Dose-response curves were fitted using the equation $y = a_0x/(a_1 + x)$; y = EGFP induction; a_0 = maximal induction level; $a_1 = EC_{50}$; x = concentration of Ah receptor agonist. Statistical significance of EGFP induction was determined using Student's t-test ($p < 0.05$).

3. Results and Discussion

3.1. Construction of a H4IIE CAFLUX reporter cell line.

A 1829 bp *Hind* III fragment from pGudLuc1.1 [4] carrying mouse mammary tumour virus (MMTV) long terminal repeat (LTR) promoter sequences, and four dioxin responsive elements (DREs) derived from the upstream regulatory region of the mouse cytochrome P4501A1 gene, was inserted in the *Hind* III site of pEGFP-1 upstream from the EGFP-encoding sequence. The resulting plasmid, called pMDREegfp1.1, was stably transfected into H4IIE rat hepatoma cells. In three consecutive rounds of selecting the best inducible clones, an H4IIE cell line was obtained showing Ah receptor-controlled EGFP expression.

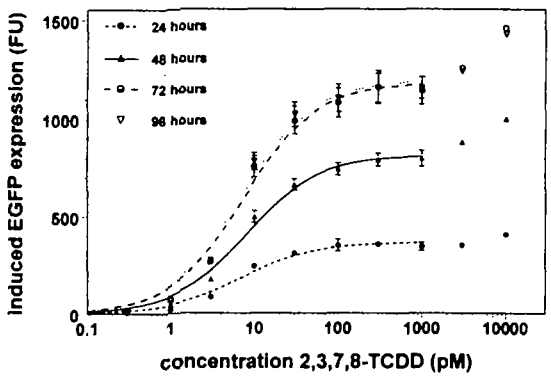

Fig. 1. Dose-response relation for EGFP induction by 2,3,7,8-TCDD in H4IIE CAFLUX cells after 24, 48, 72 and 96 hours of exposure. The EGFP expression, corrected for the solvent (DMSO) control, was plotted as arbitrary fluorescence units (FU).

3.2. EGFP expression in H4IIE CAFLUX cells is Ah receptor-mediated

Upon exposure to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), the H4IIE CAFLUX cells showed concentration-dependent and essentially saturable induction of EGFP expression, which is accurately described by the 1-site ligand binding equation (§2.4) up to 1000 pM of TCDD (Fig. 1), as expected for an Ah receptor-mediated process. The significance of the slight biphasic increase in fluorescence at concentrations above 1000 pM is not yet clear, but it was consistently observed (Fig. 1). Less than 1 pM of TCDD produced a

significant ($p < 0,05$) induction of EGFP already after 24 hours, although the EGFP expression level increased up to 72 hours of exposure, allowing even less than 0.3 pM of TCDD to be detected ($p < 0.05$).

Furthermore, the potency as an Ah receptor agonist of the polychlorinated biphenyls (PCBs) 77, 156, and 52 (respectively non-*ortho*-, mono-*ortho*- and di-*ortho*-substituted), as indicated by their toxic equivalency factor (TEF), decreases in this order, which was correctly reflected by their relative potency to induce EGFP expression in the CAFLUX (Table 1).

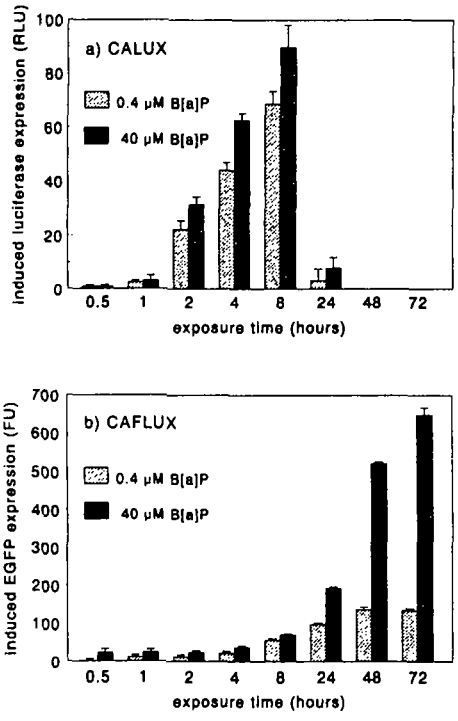

Fig. 2. Time course of EGFP induction and luciferase induction by 0.4 µM and 40 µM of benzo[*a*]pyrene in H4IIE CALUX (a) and CAFLUX (b) cells, respectively. The plotted luciferase and EGFP expression were corrected for background measured in the solvent control.

Table 1. Relative potency of PCB77, PCB156 and PCB52 as compared to 2,3,7,8-TCDD to induce EGFP expression in H4IIE CAFLUX cells.

AhR agonist	CAFLUX		TEF-value [1]
	EC50 (pM)	relative potency	
2,3,7,8-TCDD	10.7	1	1
PCB77	20600	0.00052	0.01
PCB156	414000	0.000026	0.0004
PCB52	-	$<< 10^{-7}$	-

3.3. Detection of Ah receptor-antagonistic activity

The di-*ortho*-substituted PCB, 2,2',5,5'-tetrachlorobiphenyl (PCB52), was found to display species-specific Ah receptor antagonism by CALUX analysis [3]. Similarly, the CAFLUX assay revealed strong and concentration-dependent antagonistic interaction of PCB52 with the EGFP induction by TCDD (data not shown).

3.4. Detection of readily metabolized Ah receptor agonists

In vivo, luciferase is directed to the peroxisomes and rapidly inactivated. As a result, metabolically unstable Ah receptor agonists, such as benzo-[*a*]-pyrene, cause a transient response in the CALUX assay. EGFP, on the other hand, is far more stable than luciferase. Accordingly, we observed a continuous accumulation of EGFP protein in the CAFLUX up to 72 hours, whereas the CALUX response (luciferase) had almost completely disappeared after 24 hours (Fig. 2).

3.5. Evaluation of the CAFLUX assay

The enhanced green fluorescent protein (EGFP) reporter gene, as applied in the CAFLUX assay, was found to present a sensitive and very easy-to-measure quantitative reporter, which was suitable to determine Ah receptor activation.

The CAFLUX appeared at least as sensitive as the CALUX assay (limit of detection less than 1 pM of TCDD [7]), even after 24 hours of exposure, although higher levels of EGFP expression were attained, and thus increased sensitivity and accuracy, at 48 hours of exposure. In addition, the CAFLUX is less complicated and cheaper to perform, since there is no expensive substrate or luminometer needed and a standard fluorometer is sufficient to quantify the expression of EGFP. Furthermore, the CAFLUX is a non-destructive method, which allows to follow the expression of the reporter gene almost on a real-time basis.

Unlike in the CALUX assay, a cumulative signal is generated in the CAFLUX assay by persistent as well as by non-persistent Ah receptor agonists. When the focus is on low levels of labile Ah receptor agonists, this would be an advantage, since they might become detectable by recurrent exposure of CAFLUX cells, whereas the steady-state level of luciferase activity reached in CALUX cells, might still be insufficient to allow detection. On the other hand, this might be a disadvantage if only the effect of persistent compounds is of interest, or when the contribution of persistent and non-persistent compounds to the level of Ah receptor activation has to be distinguished. However, parallel CAFLUX/CALUX analysis would allow separate quantification of the (metabolically) labile and the persistent class of Ah receptor-active compounds.

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5. References

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