Application of NanoBRET to monitor ligand binding kinetics at the histamine H1 receptor

A full understanding of the kinetics of ligand-protein interactions is important in the design of new drugs and in delineating their mode of action in vivo. For G protein-coupled receptors the techniques that are often used to determine ligand binding kinetics use cell membranes or remove the receptor from its native membrane environment entirely. It is not known if kinetic parameters measured in membranes are the same as those measured in a whole cell environment. To address this we have utilised a recently described bioluminescence resonance energy transfer (BRET) technique named NanoBRET that uses a modified luminescent protein Nanoluc as the energy donor and fluorescently tagged ligands as the acceptors (Stoddart et al., 2015). Using the histamine H_1receptor (H_1R) as a model system we have determined kinetic parameters for labelled and unlabelled ligands in both whole cells and cell membranes.

Full length cDNA encoding the human histamine H_1 receptor was amplified and fused in frame into a vector containing Nluc to generate Nluc-H_1 and a HEK293 cell line stably expressing this construct generated. For kinetic studies, cells grown to confluence or cell membranes were incubated with the Nluc substrate (10 µM furimazine) for 10 min prior to the addition of the fluorescent ligand in the presence or absence of unlabelled ligands. Non-specific binding was determined in the presence of 10 µM doxepin. Luminescence and the resulting BRET was measured every minute for 60 minutes using the PHERAstar FS plate reader (BMG labtech). Filtered light emissions were measured at 460 nm (80 nm bandpass) and >610 nm (longpass). All data are mean ± SEM of four separate experiments performed in triplicate.

Using the NanoBRET assay, the kinetic parameters of the fluorescent antagonist mepyramine-alanine-alanine-BODIPY630/650 (mep-AA-BY630) at the H_1R was determined in whole cells with low levels of non-specific binding. When using membranes, both a physiological buffer (HEPES-buffered saline solution) and a traditional membrane binding buffer was used and there was no difference in the kinetic and affinity values obtained (Table 1).

	Buffer	**K_{on} ($M^{-1}min^{-1}$)**	**K_{off} (min^{-1})**	**K_d**	***n***
Intact cells	HBSS	$4.58 \pm 0.59 \times 10^7$	0.225 ± 0.03	8.31 ± 0.01	4
Membranes	$NaKPO_4$	$3.95 \pm 2.10 \times 10^7$	0.255 ± 0.10	8.11 ± 0.20	5
Membranes	HBSS	$8.79 \pm 3.10 \times 10^7$	0.116 ± 0.02	8.80 ± 0.09	4

Table 1 Kinetic parameters of mep-AA-BY630 under different conditions at Nluc-H_1

The NanoBRET assay was subsequently used to determine kinetic parameters of a panel of eight unlabelled H_1-R antagonists. The kinetic parameters obtained were similar in whole cells as in cell membranes. We also determined the kinetics of six H_1-R antagonists in whole cells with a structurally different fluorescent ligand (VUF131816-ala-ala-ala-BY630/650). In comparison to the kinetic values determined using mep-AA-BY630 there was no change in the association rates but for three antagonists differences in the dissociation rates were observed.

The NanoBRET assay can be used to determine kinetic parameters in whole cells and cells membranes using the H_1R. The association and dissociation rates appear to not be influenced by the whole cell environment but may be dependent on the fluorescent ligand used.

Stoddart LA et al. (2015). *Nat Methods* **12**:661–663.

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