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Electrostatically–driven fast association and perdeuteration allow detection of transferred cross–relaxation for G protein–coupled receptor ligands with equilibrium dissociation constants in the high–to–low nanomolar range

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Introduction

The mechanism of signal transduction mediated by G protein-coupled receptors is a subject of intense research in pharmacological and structural biology. Ligand association to the receptor constitutes a critical event in the activation process. Solution-state NMR can be amenable to high-resolution structure determination of agonist molecules in their receptor-bound state by detecting dipolar interactions in a transferred mode, even with equilibrium dissociation constants below the micromolar range. This is possible in the case of an inherent ultra–fast diffusive association of charged ligands onto a highly charged extracellular surface, and by slowing down the ^{1}H–^{1}H cross–relaxation by perdeuterating the receptor. Here, we demonstrate this for two fatty acid molecules (Fig. **1A**) in interaction with the leukotriene BLT2 receptor, for which both ligands display a submicromolar affinity.

Kinetic experiments performed by fluorescence

Kinetic experiments (Fig. **1B**) indicate that the dissociation rate constants k_{off} for LTB4 and 12–HHT are 50 and 18 s^{-1}, respectively. This translates into an approximately 3.6 times longer bound time for 12–HHT than LTB4:

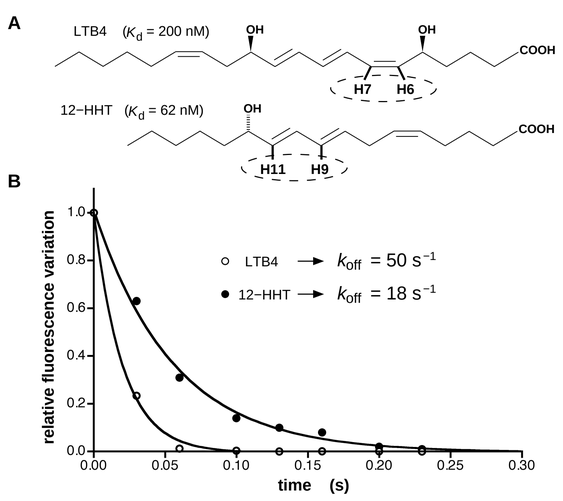

Fig. 1: (A) Chemical structures of LTB4 and 12–HHT with the corresponding *in vitro* affinities for BLT2. The two–spin systems used in Fig. 3 are circled with a dashed line. (B) Time–dependent decrease in fluorescence signal due to complex formation between LTB4–568 (*i.e.* LTB4 labeled with Alexa Fluor–568, *Sabirsh et al., 2005*) and BLT2 in the presence of either LTB4 (open circles) or 12–HHT (closed circles). The time–dependent changes in LTB4–568 fluorescence were monitored by exciting at 578 nm and detecting the emission intensity at 605 nM. The first–order rate constant describing the fluorescence change upon binding of LTB4–568 to BLT2 is the dissociation rate constant, k_{off}, of the unlabeled molecule from the receptor (*Bednar et al., 1997*; *Singh et al., 2011*).

Association rate constants k_{on} of 2.5×10^8 M^{-1}·s^{-1} for LTB4 and 2.9×10^8 M^{-1}·s^{-1} for 12–HHT can be derived from the experimentally measured K_d values of 200 and 62 nM. These values indicate that 12–HHT binds onto BLT2 slightly faster than LTB4. Both k_{on} exceed by ×3 the limit usually –but improperly– cited in the literature for biomolecular diffusional associations. These fast–associating k_{on} are not physically unrealistic, however, even for large biomolecules, cases of protein–protein association have been reported with k_{on} values close to or in excess of 10^9 M^{-1}·s^{-1} (*Schreiber & Fersht, 1996; Gabdoulline & Wade, 2002*). In this case, electrostatic interactions prevail because of their long–range nature, while they do not affect k_{off}, which is governed by short range interactions, including van der Waals and hydrophobic interactions, salt bridges and hydrogen bonds.

Electrostatic potential (Ep) of a BLT2 receptor model

Both agonists have a net charge of –1 and interact with the highly positively charged extracellular surface of the receptor (Fig. **2**). Indeed, The electrostatic potential was calculated for a model of the BLT2 receptor after a 0.5 μs molecular dynamics simulation in a fully hydrated lipid bilayer:

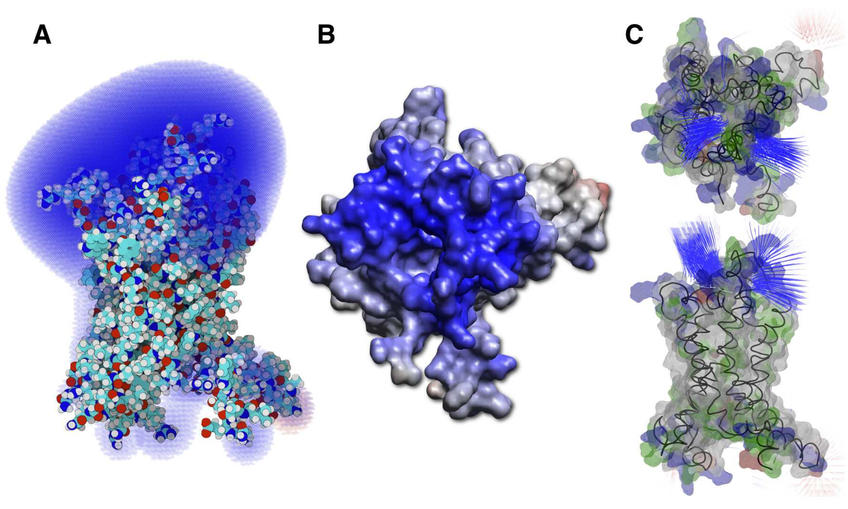

Fig. 2: Electrostatic potential (Ep) of a BLT2 receptor model calculated on a simulation snapshot. The Ep maps are colored from –400 kT/e in red to +400 kT/e in blue. In (A), the reach of the receptor's Ep is illustrated by a cloud. In this side view the extracellular ligand binding site is located at the top. (B) shows a top view of the binding site surface colored by Ep. (C) illustrates Ep field lines in a combined top/side view. (A) and (B) were prepared with Yasara (*Krieger et al., 2002*), and (C) with VMD (*Humphrey et al., 1996*).

Simultaneous graphical estimation of auto (ρ) and cross (σ) relaxation rate constants

Proton NMR relaxation rates of ^{1}H natural abundance in macromolecules are governed by indirect dipolar pathways. Deuteration of the receptor reduces spin diffusion, and, by doing so, substantially diminishes the rates of relaxation processes (*e.g. Markus et al., 1994*). As a consequence, dilution of the ^{1}H thermal bath allows the use of a longer NOESY mixing time (τ_m), the detection of longer interdipolar distances, and can shift the limit of trNOE observation towards higher affinities. To demonstrate that trNOE can be observed with tight–binding ligands, ρ and σ in the bound state can be estimated from 2D NOESY experiments in the presence of chemical exchange (*Ni, 1992*). One convenient way is to use a graphical approach where both ρ and σ can be estimated simultaneously from the experimental ratio of cross to diagonal peak volumes, Π_{exp}, knowing the k_{off}, the relative population of ligand *vs.* receptor, and ρ in the free state (*Catoire et al., 2011*). Fig. **3** illustrates two examples in the case of strong dipolar interactions, *i.e.* corresponding to short inter–proton distances in a rigid part of both molecules. In particular, Fig. **3** indicates values of σ close to 10 s^{-1}, *i.e.* below the respective dissociation rates of LTB4 and 12-HHT (Fig. **1**), fulfilling one of the most stringent criteria to observe trNOE when τ_c of large complexes become very long (*Clore & Gronenborn, 1982, 1983; Campbell & Sykes, 1993; Williamson, 2006*). Hence, BLT2/amphipol complexes in solution, which display a τ_c of ∼55 ns, are compatible with the observation of trNOE in the case of slow–intermediate (*Levitt, 2001*) chemical exchange.

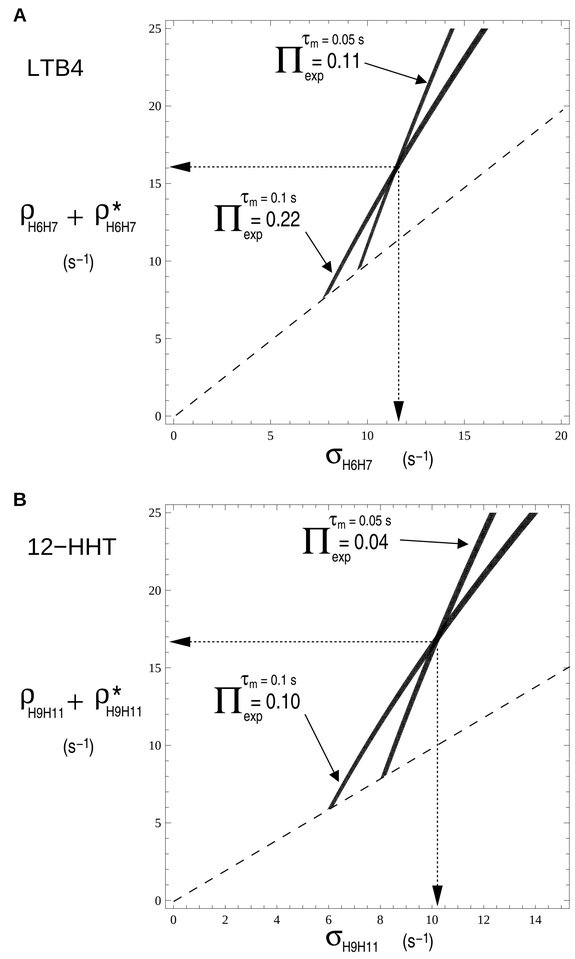

Fig. 3: Simultaneous graphical estimation of auto (ρ) and cross (σ) relaxation rate constants of LTB4 and 12–HHT in their BLT2–bound states in the presence of chemical exchange (see respective k_{off} values in Fig. 1B). (A) and (B) correspond to superimposed projections along the cross to diagonal NOESY peak volume ratio, Π, axis of contour plots of theoretical Π taken at experimental Π values. NOESY volumes are measured at two mixing times (τ_m) (see corresponding LTB4/BLT2 and 12–HHT/BLT2 NOESY spectra in *Catoire et al., 2010* and *2011*, respectively). Illustration with the dipolar interactions between nuclei H6 and H7 of LTB4 (A) and H9 and H11 of 12–HHT (B) in the presence of ∼9–fold excess of ligand over u–^{2}H–BLT2. ρ^* represents other non-dipolar relaxation contributions and/or a contribution from some other spins of the lattice. Contour lines are drawn for ρ+ρ^* greater than or equal to σ (above the dashed line) (see *Catoire et al., 2011* for any details on this graphical method).

Conclusion

Without a significant coulombic contribution to the interaction, *i.e.* with k_{on} of ∼10^8 M^{-1}·s^{-1}, and/or unhindered access of the ligand to the binding site, perdeuteration would not be sufficient. Fortunately, this accelerated diffusive association does not seem to be specific of BLT2. For instance, the β_2 adrenergic receptor, which is also characterized by a highly positively charged extracellular surface, has diffusive agonists that associate with k_{on} close to or higher than those measured here (*Hegener et al., 2004*). On the nuclear longitudinal relaxation timescale, this provides the opportunity to study structures of tight–binding ligands, *i.e.* with K_d of a few tens of nM, bridging the gap between pharmacology and NMR.

References

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For more information:
Catoire *et al.* 2011, *Journal of Biomolecular NMR*, DOI: 10.1007/s10858–011–9523–3 & **http://www.youtube.com/marcbaaden**