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Negative cooperativity across β₁-adrenoceptor homodimers provides insights into the nature of the secondary low-affinity CGP 12177 β₁-adrenoceptor binding conformation

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ABSTRACT At the β₁-adrenoceptor, CGP 12177 potently antagonizes agonist responses at the primary high-affinity catecholamine conformation while also exerting agonist effects of its own through a secondary low-affinity conformation. A recent mutagenesis study identified transmembrane region (TM)4 of the β₁-adrenoceptor as key for this low-affinity conformation. Others suggested that TM4 has a role in β₁-adrenoceptor oligomerization. Here, assessment of the dissociation rate of a fluorescent analog of CGP 12177 [bordifluoropyrromethane-tetramethylrhodamine-(±)CGP 12177 (BODIPY-TMR-CGP)] at the human β₁-adrenoceptor expressed in Chinese hamster ovary cells revealed negative cooperative interactions between 2 distinct β₁-adrenoceptor conformations. The dissociation rate of 3 nM BODIPY-TMR-CGP was 0.09 ± 0.01 min⁻¹ in the absence of competitor ligands, and this was enhanced 2.2- and 2.1-fold in the presence of 1 μM CGP 12177 and 1 μM propranolol, respectively. These effects on the BODIPY-TMR-CGP dissociation rate were markedly enhanced in β₁-adrenoceptor homodimers constrained by bimolecular fluorescence complementation (9.8- and 9.9-fold for 1 μM CGP 12177 and 1 μM propranolol, respectively) and abolished in β₁-adrenoceptors containing TM4 mutations vital for the second conformation pharmacology. This study suggests that negative cooperativity across a β₁-adrenoceptor homodimer may be responsible for generating the low-affinity pharmacology of the secondary β₁-adrenoceptor conformation.—Gherbi, K., May, L. T., Baker, J. G., Briddon, S. J., Hill, S. J. Negative cooperativity across β₁-adrenoceptor homodimers provides insights into the nature of the secondary low-affinity CGP 12177 β₁-adrenoceptor binding conformation. FASEBJ. 29, 2859–2871 (2015). www.fasebj.org

Key Words: dissociation · receptor dimerization · GPCR · allostery

The β₁-adrenoceptor has been reported to exist in 2 active conformations (1–6), although the nature of the secondary β₁-adrenoceptor conformation has been the subject of speculation for many years (7–11). The endogenous ligands adrenaline and noradrenaline exhibit their agonist effects through the orthosteric catecholamine site of the β₁-adrenoceptor. These agonist responses are antagonized by β-blockers such as CGP 20712A, CGP 12177, and propranolol with high affinity (6, 12–14). However, CGP 12177 (15) was also found to exert partial agonist effects at 100-fold higher concentrations than were needed to antagonize catecholamine-mediated agonist responses (4, 12).

According to classic receptor theory, the EC₅₀ of a partial agonist is expected to be similar to its binding affinity. The discrepancy between these 2 values for CGP 12177 and also other β-adrenoceptor ligands, such as pindolol, led to the classification of nonconventional β-adrenoceptor agonists (16). Furthermore, the CGP 12177 agonist effect appeared resistant to β-blocker antagonism at concentrations normally sufficient to block catecholamine-mediated agonist effects (4, 6, 12, 14, 17). As such, the affinities of a range of β-adrenoceptor antagonists have been reported to be >1 order of magnitude lower when inhibiting responses mediated by CGP 12177 at the β₁-adrenoceptor compared with those mediated by catecholamines in both animal and human tissue preparations (10, 14, 18). It is noteworthy that the use of recombinant cell systems and cardiac tissue isolated from β₁/β₂-adrenoceptor knockout mice clearly showed that the β₁-adrenoceptor alone was responsible for the observed CGP 12177 pharmacology (1, 3, 4). This led to the proposal that there are 2 active conformations of the β₁-adrenoceptor: a primary high-affinity endogenous catecholamine site and a secondary low-affinity CGP 12177 site (1, 4).

Abbreviations: BiFC, bimolecular fluorescence complementation; BODIPY-TMR-CGP (or BOD-CGP), bordifluoropyrromethane-tetramethylrhodamine-(±)CGP 12177; CHO, Chinese hamster ovary; ID, infinite dilution; ROI, region of interest; TM, transmembrane; YFP, yellow fluorescent protein

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Site-directed mutagenesis has been used in previous studies to investigate the nature of the secondary low-affinity \( \beta_1 \)-adrenoceptor conformation (7, 11). Initial work suggested some overlap of the 2 proposed \( \beta_1 \)-adrenoceptor binding conformations (7). However, more recently, Baker et al. (11) demonstrated that residues L195 and W199 in transmembrane domain (TM) 4 are essential for the secondary \( \beta_1 \)-adrenoceptor conformation (11). Furthermore, TM4 may have a role in oligomerization (19), because the formation of \( \beta_1 \)-adrenoceptor homodimers has been reported previously (20–22), and an important interface for this appears to be between TM4 and TM5 (19).

A \( \beta_1 \)-adrenoceptor homodimer complex would possess 2 structurally identical orthosteric \( \beta_1 \)-adrenoceptor sites, to which ligands would be expected to bind with similar affinities. However, negative cooperative interactions between the 2 orthosteric \( \beta_1 \)-adrenoceptor binding sites may provide an explanation of the lower affinity observed for the secondary \( \beta_1 \)-adrenoceptor protomer, if indeed this occurs as a dimer (23). Negative cooperativity across a homodimer interface has previously been described for the human A3 adenosine receptor (23). In this example, negative cooperativity was demonstrated in single living cells by following the impact of orthosteric unlabeled ligands binding to one protomer of an A3-homodimer on the dissociation of a fluorescently labeled agonist (which was enhanced) from the orthosteric site of the other A3-receptor protomer (23).

We previously showed that the fluorescent CGP 12177 analog bordifluoropyromethane-tetramethylrhodamine-(±) CGP 12177 (BODIPY-TMR-CGP) can be used to label both conformations of the \( \beta_1 \)-adrenoceptor (24). In this study, we used this fluorescent CGP 12177 analog to investigate the potential for allosteric interactions across a homodimer interface of the \( \beta_1 \)-adrenoceptor using kinetic measurements of BODIPY-TMR-CGP binding in single living cells.

**MATERIALS AND METHODS**

**Materials**

Cell culture plastics were purchased from Thermo Fisher Scientific (Loughborough, United Kingdom), and cell culture reagents were from Sigma-Aldrich (Gillingham, United Kingdom) except for fetal calf serum, which was obtained from PAA Laboratories (Pasching, Austria). Lipofectamine 2000 transfection reagent and Opti-MEM medium were from Invitrogen (Paisley, United Kingdom), and SNAP-Surface 488 was from New England Biolabs (Ipswich, MA, USA). BODIPY-TMR-CGP was from Molecular Probes (Leiden, The Netherlands), and unlabeled CGP 12177 and propranolol were from Tocris Cookson (Paisley, United Kingdom), and SNAP-Surface 488 was from Molecular Probes (Leiden, The Netherlands). All reagents were from Sigma Chemicals (Poole, United Kingdom). Cell culture Chinese hamster ovary (CHO)-K1 cells were used for all transient transfections. CHO-K1 cells stably expressing the secreted placent al alkaline phosphatase reporter gene under the transcriptional control of a 6-CAMP response element promoter (CHO-CS cells) were used as a control, as appropriate. CHO-CS cell lines either expressing human wild-type \( \beta_1 \)-adrenoceptors (CHO-\( \beta_1 \) cells; 1147 fmol/mg protein) (6) or human \( \beta_1 \)-adrenoceptors containing 11 amino acid mutations (G177V, L178I, V179I, C180L, T181M, A184I, A185V, A187G, V189T, L195Q, and W199Y that convert TM4 to the equivalent residues in the \( \beta_2 \)-adrenoceptor; CHO-\( \beta_2 \)TM4 cells) (11) were used. CHO-K1, CHO-CS, CHO-\( \beta_1 \), and CHO-\( \beta_2 \)TM4 cells were grown at 37°C in CHO growth medium [DMEM/Ham’s nutrient mixture F12 containing 10% (v/v) fetal calf serum and 2 mM l-glutamine] in a humidified 5% \( \text{CO}_2/95\% \) air atmosphere.

**Generation of \( \beta_1 \)-adrenoceptor constructs**

The \( \beta_1 \)-yellow fluorescent protein (YFP\( _N \)) and \( \beta_1 \)-YFP\( _C \) receptor constructs were generated by fusing either the N-terminal fragment of YFP (YFP\( _N \); amino acids 1–155) or the C-terminal fragment of YFP (YFP\( _C \); amino acids 156–239) to the C-terminal end of the full-length wild-type human \( \beta_1 \)-adrenoceptor. The SNAP-\( \beta_1 \) construct was generated by fusing the SNAP-tag (New England Biolabs, Ipswich, MA, USA) to the N-terminal end of the wild-type human \( \beta_1 \)-adrenoceptor. The D138A mutation (7) was introduced into the \( \beta_1 \)-YFP\( _C \) and the SNAP-\( \beta_1 \) sequence using the QuickChange site-directed mutagenesis kit (Agilent Technologies, Cheshure, United Kingdom). All sequences were confirmed by DNA sequencing. All receptor constructs were subcloned into pcDNA3.1 vectors.

**SNAP-tag labeling and confocal imaging**

Confocal microscopy was performed using a Zeiss LSM710 laser scanning microscope with a ×40 1.3 NA oil immersion lens. CHO-K1 cells were grown to 70% confluence in 8-well Labtek borosilicate chambered-cover glasses (Nalgene Nunc International, Fisher Scientific) and transiently transfected with SNAP-\( \beta_1 \) or SNAP-\( \beta_1 \)D138A recombinant DNA using Lipofectamine 2000 and Opti-MEM medium according to the manufacturer’s instructions. The next day, a 1 μM concentration of the benzyl-guanine labeled SNAP-tag substrate SNAP-Surface 488 (BG-488) was prepared in fresh cell culture medium, added to these cells, and incubated in the dark for 30 min (room temperature). The cells then were washed twice in imaging buffer (147 mM NaCl, 24 mM KCl, 1.3 mM CaCl\( _2 \), 1 mM MgSO\( _4 \), 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, 2 mM sodium pyruvate, 1.43 mM NaHCO\( _3 \), 4.5 mM d-glucose, pH 7.4). After this, 3 mM BODIPY-TMR-CGP was added to the cells in the dark for 10 min (room temperature), after which the cells were imaged immediately (1024 × 1024 pixels, averaging at 4 frames, pinhole diameter 1 airy unit); 561 nm diode and 488 nm argon lasers were used to excite BODIPY-TMR-CGP and BG-488, respectively. A variable spectral fluorescence imaging was performed on the Zeiss LSM510 laser scanning confocal microscope using a Zeiss Plan-Apochromat 63×/1.3 oil immersion lens. CHO-K1 cells were grown at 37°C in 4% newborn calf serum and 2 mM l-glutamine; CHO-K1 cells were grown at 37°C in the dark for 30 min (room temperature), after which the cells were imaged immediately (1024 × 1024 pixels, averaging at 4 frames, pinhole diameter 1 airy unit); 561 nm diode and 488 nm argon lasers were used to excite BODIPY-TMR-CGP and BG-488, respectively. A variable spectral detection system was used to capture emission at 565–565 and 495–535 nm, respectively. Confocal settings for laser power, offset, and gain were kept constant throughout each experiment set.

**BODIPY-TMR-CGP kinetic studies using the confocal perfusion system**

**BODIPY-TMR-CGP association and dissociation kinetics**

Live cell fluorescence imaging was performed on the Zeiss LSM510 laser scanning confocal microscope using a Zeiss Plan-Neofluar ×40 1.3 NA oil-immersion objective in conjunction with a temperature-controlled (37°C) perfusion system to allow the visualization and quantification of BODIPY-TMR-CGP association kinetics under infinite dilution (ID) conditions in single living cells (25). Kinetic experiments were performed as described by May et al. (25). In brief, cells were grown to near confluence on 32 mm glass coverslips in 6-well plates 1 d prior to experimentation. On the day of experimentation, the coverslip was placed into a tightly closed imaging chamber on a heated (37°C) microscope stage, where it was connected to tubes to facilitate the flow of imaging buffer through the imaging chamber in the absence and presence of BODIPY-TMR-CGP and/or unlabeled ligands at a constant flow rate of ≈4 ml/min.
For association and dissociation kinetic experiments of 10–100 nM BODIPY-TMR-CGP in CHO-β₁ and CHO-CS cells, the cells were exposed to imaging buffer only (30 s baseline fluorescence recording), then BODIPY-TMR-CGP (4.5 min association), and followed again by imaging buffer only (4.5 min dissociation). BODIPY-TMR-CGP was excited using a 543 nm helium-neon laser with emission collected through a 565 nm long-pass filter every 3 s throughout each experiment (512 × 512 pixels, averaging at 2 frames). The pinhole diameter (1 airy unit), laser power (2%), offset, and gain remained constant between the 3 BODIPY-TMR-CGP concentrations and the 2 cell lines used. Membrane-associated BODIPY-TMR-CGP fluorescence was measured by drawing regions of interest (ROIs) around the membranes of 10 single cells of each imaged coverslip, and changes in the average pixel intensity values for each ROI over time were analyzed to obtain association and dissociation rates for each experiment.

**Measuring the influence of unlabeled ligands on the BODIPY-TMR-CGP dissociation rate**

Live cell fluorescence imaging using 3 nM BODIPY-TMR-CGP was performed on the Zeiss LSM710 laser scanning confocal microscope using a Zeiss Plan-Neofluar 40×1.3 NA oil-immersion objective in conjunction with a perfusion system as described above for the Zeiss LSM510 laser scanning confocal microscope. For association and dissociation kinetic experiments using 3 nM BODIPY-TMR-CGP in CHO-β₁ and CHO-CS cells, the cells were exposed to imaging buffer only (30 s baseline fluorescence recording), then BODIPY-TMR-CGP (4 min association), and followed again by imaging buffer only (4 min dissociation). Influences of unlabeled ligands on the BODIPY-TMR-CGP dissociation rate in CHO-β₁ cells were determined by perfusion of imaging buffer (30 s baseline read), 3 nM BODIPY-TMR-CGP (4 min association), and imaging buffer (4 min dissociation) in the absence or presence of CGP 12177 (0.01–10 μM) or propranolol (0.1–10 μM).

In experiments using CHO-β₁/TM4 cells, the cells were first exposed to 3 nM BODIPY-TMR-CGP for 3.5 min in a 6-well plate prior to placing the coverslip into the imaging chamber to achieve a significant but low level of labeling of the receptor. Once placed onto the microscope stage, the cells were perfused with BODIPY-TMR-CGP (30 s baseline), before perfusing imaging buffer in the absence or presence of 1 μM CGP 12177 or 1 μM propranolol (dissociation).

In bimolecular fluorescence complementation (BiFC) experiments, CHO-K1 cells were seeded onto coverslips on day 1 and transiently transfected with YFP<sub>N</sub> and YFP<sub>C</sub>-tagged β₁-adrenoceptor recombinant DNA (750 ng total) using Lipofectamine 2000 and Opti-MEM medium according to the manufacturer’s instructions on day 2. The following day, the transfection medium was removed and replaced with fresh CHO growth medium, before the cells were placed back into the cell culture incubator (37°C, 5% CO₂/95% air atmosphere). After ~6 h, the cells were moved into a 30°C incubator overnight (5% CO₂/95% air atmosphere) to allow the maturation of the fluorophore following correct protein folding (26). On day 4, the cells were used for experimentation, and the dissociation of 3 nM BODIPY-TMR-CGP in the absence and presence of unlabeled ligands was determined as described above.

**BODIPY-TMR-CGP and YFP (when used)** were excited using a 561 nm diode and 488 argon laser, respectively, every 2 s throughout each experiment (512 × 512 pixels, averaging at 2 frames). A variable spectral detection system was used to capture BODIPY-TMR-CGP and YFP emission at 565–605 and 495–535 nm, respectively. The pinhole diameter (1 airy unit) and laser power (5%) remained constant between all experiments, but the gain and offset were adjusted for each experiment for optimal detection of 3 nM BODIPY-TMR-CGP, and kinetic data were expressed in percentage fluorescent intensity to allow data to be grouped and compared.

ROIs were drawn around the membranes of 3–10 single cells of each imaged coverslip, and changes in average pixel intensity values for each ROI over time were analyzed to obtain BODIPY-TMR-CGP dissociation rates for each experiment. In BiFC experiments, membrane-associated BODIPY-TMR-CGP fluorescence was measured by drawing ROIs around membranes of cells that were identified to express BiFC-constrained homodimers by examination of the YFP fluorescence.

**Data analysis**

GraphPad Prism 5.0 (GraphPad Software, San Diego, CA, USA) was used to fit all data presented in this study. Association kinetic data were fitted using the following monoeXponential association equation:

\[ Y = Y_0 + (\text{Plateau} - Y_0)(1 - e^{-k_{\text{on}} t}) \quad (1) \]

where \( Y_0 \) is the level of BODIPY-TMR-CGP binding at time \( t = 0 \) (i.e., baseline fluorescence), \( \text{Plateau} \) is the level of BODIPY-TMR-CGP binding at infinite time, and \( k_{\text{on}} \) is the rate of observed association.

**BODIPY-TMR-CGP** fluorescence intensities measured at time \( t = 0 \) in CHO-β₁ cells and CHO-CS cells were plotted against the BODIPY-TMR-CGP concentrations used and fitted to a 1-site total binding saturation equation (Eq. 2) or a nonspecific binding linear regression equation (Eq. 3), respectively.

\[
\text{Total binding} = \frac{B_{\text{MAX}} \times [B]}{K_D + [B]} + M \times [B] + C \quad (2)
\]

\[
\text{NS binding} = M \times [B] + C \quad (3)
\]

where \( B_{\text{MAX}} \) is the maximum specific BODIPY-TMR-CGP binding, \([B]\) is the BODIPY-TMR-CGP concentration, \( K_D \) is the BODIPY-TMR-CGP concentration that achieves 50% specific binding, \( M \) denotes the slope of the linear regression component, and \( C \) is the background fluorescence intensity.

The dissociation kinetic data were analyzed using the following monoeXponential decay equation, if nonspecific binding of <10% of total binding was observed for a given BODIPY-TMR-CGP concentration:

\[
Y = (Y_0 - \text{Plateau}) \times e^{-k_{\text{off}} t} + \text{Plateau} \quad (4)
\]

where \( Y_0 \), Plateau, and \( t \) are the same as defined above with \( k_0 \) representing the start of dissociation in \( Y_0 \) (i.e., the binding of BODIPY-TMR-CGP at time zero). The \( k_{\text{off}} \) is the dissociation rate of BODIPY-TMR-CGP. Where a level of nonspecific binding >10% of total binding was observed, the dissociation kinetic data were fitted to a 2-phase exponential decay function

\[
Y = \text{Plateau} + \text{Span}_{\text{fast}} \times e^{-k_{\text{off(fast)}} t} + \text{Span}_{\text{slow}} \times e^{-k_{\text{off(slow)}} t} \quad (5)
\]

where Plateau and \( t \) are as defined above, and \( k_{\text{off(fast)}} \) and \( k_{\text{off(slow)}} \) represent the proportion of \( Y_0 \)-Plateau accounted for by the fast \( k_{\text{off(fast)}} \) and slow \( k_{\text{off(slow)}} \) dissociation rate, respectively. Within this analysis, \( k_{\text{off(fast)}} \) and Plateau were constrained to the average rate of dissociation and the average Plateau (in %) reached by BODIPY-TMR-CGP in control CHO-CS cells (i.e., cell not expressing the receptor of interest to determine the nonspecific BODIPY-TMR-CGP binding component). Equation 6 was then used to calculate the association rate constants \( k_{\text{on}} \) using the \( k_{\text{on}} \) and \( k_{\text{off(slow)}} \) determined above for each BODIPY-TMR-CGP concentration \([B]\)

\[
k_{\text{on}} = \frac{k_{\text{on}} - k_{\text{off(slow)}}}{[B]} \quad (6)
\]

The negative logarithms of the equilibrium dissociation constant \( (pK_D) \) were obtained using the above determined kinetic parameters in the following equation:
Experiments investigating the kinetic parameters of 3 nM BODIPY-TMR-CGP binding to CHO-β1 cells were analyzed using a global fit of its association (Eq. 8a) and dissociation traces (Eq. 8b)

\[
Y = \frac{B_{\text{MAX}} \times (3 \times 10^{-9})}{(3 \times 10^{-9}) + K_D} \times \left(1 - e^{-\left[(3 \times 10^{-9}) \times k_{\text{on}} + k_{\text{off}}\right] \times t}\right) + \text{NS}
\]  

(8a)

\[
Y = \frac{B_{\text{MAX}} \times (3 \times 10^{-9})}{(3 \times 10^{-9}) + K_D} \times \left(1 - e^{-\left[(3 \times 10^{-9}) \times k_{\text{on}} + k_{\text{off}}\right] \times T(\text{ID})}\right) \times e^{-k_{\text{off}} \times (t - T(\text{ID}))} + \text{NS}
\]  

(8b)

where \(B_{\text{MAX}}\) is the maximum specific BODIPY-TMR-CGP binding, \(K_D\) is the BODIPY-TMR-CGP concentration that achieves 50\% specific binding, NS is nonspecific binding, \(T(\text{ID})\) is the time at which infinite dilution was commenced, and \(k_{\text{on}}, k_{\text{off}}, \text{ and } t\) are as described above.

Concentration-dependent cooperative effects of unlabeled ligands CGP 12177 and propranolol on the dissociation rate of 3 nM BODIPY-TMR-CGP were fit to the following equation:

BODIPY – TMR – CGP dissociation rate = \(\frac{(E_{\text{MAX}} \times [B])}{([B] + K_{D(\text{site 2})})}\)

(9)

where \(E_{\text{MAX}}\) is the maximal increase in BODIPY-TMR-CGP dissociation rate, \([B]\) is the concentration of unlabeled ligand, and \(K_{D(\text{site 2})}\) is the concentration of the unlabeled ligand that achieves 50\% binding to a secondary binding site that exerts cooperative effects on the BODIPY-TMR-CGP dissociation from the primary binding site. A measure of cooperativity between 2 binding sites is provided by the cooperativity factor \(\alpha\), which was calculated using the following equation:

\[
\alpha = \frac{K_{D(\text{site 1})}}{K_{D(\text{site 2})}}
\]

(10)

where \(K_{D(\text{site 1})}\) is the equilibrium dissociation constant of a given ligand for the primary (site 1) binding site of an unbound receptor, and \(K_{D(\text{site 2})}\) is the equilibrium dissociation constant of the same ligand for the secondary (site 2) binding site of a ligand-bound (at site 1) receptor.

All data are represented as means ± SEM from \(n\) separate experiments. Statistical analysis was performed where appropriate and as detailed in the text, with \(P < 0.05\) reflecting statistical significance.

**RESULTS**

**Kinetic parameters of BODIPY-TMR-CGP at the human β1-adrenoceptor**

The association and dissociation of 10, 30, and 100 nM BODIPY-TMR-CGP at the human β1-adrenoceptor were examined in CHO-β1 and CHO-CS cells to determine total and nonspecific binding levels, respectively. Using the same microscope settings for all BODIPY-TMR-CGP concentrations in both cell lines allowed direct comparison of fluorescence intensities and thus BODIPY-TMR-CGP binding levels (Fig. 1). The fluorescence intensity levels increased with increasing BODIPY-TMR-CGP concentrations in both CHO-β1 and CHO-CS cells; however, the nonspecific membrane-associated fluorescence intensity in CHO-CS cells was markedly lower than the total binding cell membrane-associated fluorescence intensity measured in CHO-β1 cells (Fig. 2A, B). A plot of the binding levels against BODIPY-TMR-CGP concentration was best described by a saturable and linear component following 4.5 min association in CHO-β1 cells (Fig. 2C). In contrast, the increase in BODIPY-TMR-CGP binding levels with increasing concentration of fluorescent label in CHO-CS cells was best described by a linear relationship (Fig. 2D), which is characteristic of

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**Figure 1.** Concentration-dependent increase of BODIPY-TMR-CGP binding levels in CHO-β1 and CHO-CS cells. Confocal images of 10, 50, and 100 nM BODIPY-TMR-CGP (BOD-CGP) binding levels following 4.5 min association measured in CHO-β1 and CHO-CS cells. The microscope settings were kept constant for the recordings of fluorescence intensities of all 3 BODIPY-TMR-CGP concentrations in both CHO-β1 and CHO-CS cells to allow for direct comparison of the BODIPY-TMR-CGP binding levels. Images are representatives of a total of 5, 6, and 4 separate experiments (for 10, 30, and 100 nM BODIPY-TMR-CGP, respectively) using CHO-β1 cells, and 3, 6, and 3 separate experiments (for 10, 30, and 100 nM BODIPY-TMR-CGP, respectively) using CHO-CS cells. Scale bars, 50 μm.
nonspecific binding components. Interestingly the nonspecific binding component appeared smaller in the cells expressing the human β₁-adrenoceptor (Fig. 2C).

The association and dissociation traces obtained in CHO-CS could only be accurately analyzed for 30 and 100 nM BODIPY-TMR-CGP, and revealed rapid observed association (rate determined for 100 nM BODIPY-TMR-CGP, and revealed rapid observed association rates (fast component)) and dissociation rates during BODIPY-TMR-CGP perfusion (first 4.5 min), and dissociation rates during perfusion of imaging buffer only (subsequent 4.5 min). The fluorescence intensity values measured in (C) CHO-β₁ and (D) CHO-CS cells following 4.5 min association of 10, 30, and 100 nM BODIPY-TMR-CGP were plotted against the corresponding BODIPY-TMR-CGP concentration and highlight the saturable binding of BODIPY-TMR-CGP to CHO-β₁ but not CHO-CS cells at the concentrations used. Data shown are means ± SEM of 3–6 separate experiments per BODIPY-TMR-CGP concentration in each cell line. Each experimental replicate reflects the average fluorescent intensity of the plasma membrane of 10 cells. Summary data and statistical analysis are provided in Table 1.

![Image of fluorescence intensity over time for CHO-β₁ and CHO-CS cells](image-url)

**Figure 2.** Characterization of BODIPY-TMR-CGP binding properties in CHO-β₁ and CHO-CS cells. The fluorescence intensity of 10, 30, and 100 nM BODIPY-TMR-CGP (BOD-CGP) was monitored every 3 s (shown for every 6 s for better visualization) in (A) CHO-β₁ and (B) CHO-CS cells to determine association rates during BODIPY-TMR-CGP perfusion (first 4.5 min), and dissociation rates during perfusion of imaging buffer only (subsequent 4.5 min). The fluorescence intensity values measured in (C) CHO-β₁ and (D) CHO-CS cells following 4.5 min association of 10, 30, and 100 nM BODIPY-TMR-CGP were plotted against the corresponding BODIPY-TMR-CGP concentration and highlight the saturable binding of BODIPY-TMR-CGP to CHO-β₁ but not CHO-CS cells at the concentrations used. Data shown are means ± SEM of 3–6 separate experiments per BODIPY-TMR-CGP concentration in each cell line. Each experimental replicate reflects the average fluorescent intensity of the plasma membrane of 10 cells. Summary data and statistical analysis are provided in Table 1.

**TABLE 1. Kinetic parameters of 10, 30, and 100 nM BODIPY-TMR-CGP at CHO-β₁ and CHO-CS cells**

<table>
<thead>
<tr>
<th>BODIPY-TMR-CGP</th>
<th>10 nM</th>
<th>30 nM</th>
<th>100 nM</th>
</tr>
</thead>
<tbody>
<tr>
<td>CHO-CS (nM)</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>10</td>
<td>NA</td>
<td>1.40 ± 0.26</td>
<td>1.62 ± 0.13</td>
</tr>
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<td>30</td>
<td>1.40 ± 0.26</td>
<td>2.46 ± 0.48</td>
<td>2.47 ± 0.13</td>
</tr>
<tr>
<td>100</td>
<td>1.62 ± 0.13</td>
<td>2.47 ± 0.13</td>
<td>2.47 ± 0.13</td>
</tr>
<tr>
<td>CHO-β₁ (nM)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>0.63 ± 0.08</td>
<td>0.63 ± 0.08</td>
<td>0.63 ± 0.08</td>
</tr>
<tr>
<td>30</td>
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<td>1.40 ± 0.08*</td>
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</tr>
<tr>
<td>100</td>
<td>3.08 ± 0.11*,#</td>
<td>2.47</td>
<td>2.47</td>
</tr>
</tbody>
</table>

Data are means ± SEM with n representing the number of separate experiments carried out. In each experiment, ROIs were drawn around the membrane of 10 cells. NA, not applicable. *Statistical significance (P < 0.05) from the value determined for 10 nM and $^*P < 0.05$ from 30 nM BODIPY-TMR-CGP in CHO-β₁ cells (1-way ANOVA followed by Tukey’s multiple comparisons test).
Figure 3. Association and dissociation kinetics of 10, 30, and 100 nM BODIPY-TMR-CGP in CHO-β2 cells. The fluorescence intensity of 10, 30, and 100 nM BODIPY-TMR-CGP (BOD-CGP) during perfusion of BODIPY-TMR-CGP (association) and imaging buffer only (dissociation) was monitored every 3 s (shown for every 6 s for better visualization). A and B) Normalized association (A) and dissociation data (B) of BODIPY-TMR-CGP (BOD-CGP) in CHO-β2 cells are means ± SEM of 5, 6, and 4 separate experiments for 10, 30, and 100 nM BODIPY-TMR-CGP, respectively. Association data were normalized to the predicted maximum binding level of each BODIPY-TMR-CGP concentration used (determined using a monoexponential association equation; Eq. 1). Specific dissociation data were normalized to the fluorescence intensity level measured before initiation of imaging buffer only perfusion. C and D) Single cell analysis of observed association rates (C) and dissociation rate (D) constants for 10, 30, and 100 nM BODIPY-TMR-CGP, with each replicate representing the kinetic parameter of 1 single cell. Data shown are means ± SEM of 50, 60, and 40 separate cells for 10, 30, and 100 nM BODIPY-TMR-CGP, respectively, and represent the parameter estimates of single cells from 5, 6, and 4 separate experiments for 10, 30, and 100 nM BODIPY-TMR-CGP, respectively. Summary data and statistical analysis are provided in Table 1.

TMR-CGP binds to the secondary low affinity conformation of the β1-adrenoceptor, and as such may cause allosteric effects that result in an enhanced dissociation rate of 100 nM BODIPY-TMR-CGP (i.e., negative cooperativity) from the primary high-affinity β1-adrenoceptor conformation. This was not evident for 10 and 30 nM BODIPY-TMR-CGP, which is likely caused by lower occupancy levels of these BODIPY-TMR-CGP concentrations at the secondary β1-adrenoceptor conformation compared with 100 nM BODIPY-TMR-CGP (24). The concentration independence of the dissociation rates of 10 and 30 nM BODIPY-TMR-CGP and the concentration dependence of the observed association rates of 10, 30, and 100 nM BODIPY-TMR-CGP at the β1-adrenoceptor were clearly seen for normalized grouped data (Fig. 3A, B), as well as single cell data (Fig. 3C, D). The observed association (konobs) and β1-adrenoceptor–specific dissociation rate (kdisslow) of each BODIPY-TMR-CGP concentration were used to determine the association rate constants (kon). The kon and kdissslow were then used to calculate the equilibrium dissociation constant (Kd) for each BODIPY-TMR-CGP concentration and are summarized in Table 1.

Cooperative interactions between the high- and low-affinity conformation of the human β1-adrenoceptor

The dissociation rate of a ligand should not be altered in the presence of a second ligand if the two compete for the same binding site. However, if the second ligand binds to a separate second binding site, a resulting conformational change could lead to cooperative (allosteric) effects and thus affect the dissociation rate of the first ligand (23, 28–30). To assess whether the faster dissociation rate observed for 100 nM BODIPY-TMR-CGP was in fact caused by cooperative interactions between the high- and low-affinity conformation of the β1-adrenoceptor, we investigated the dissociation rates of 3 nM BODIPY-TMR-CGP in the absence and presence of increasing concentrations of CGP 12177 and propranolol. We previously showed that 3 nM BODIPY-TMR-CGP predominantly labels the high-affinity conformation over the secondary low-affinity conformation of the β1-adrenoceptor [~86% and 3% occupancy, respectively, based on the affinity of BODIPY-TMR-CGP for the high- and low-affinity β1-adrenoceptor conformations determined in functional assays (24)]. This limits competition of labeled and unlabeled ligands at the secondary β1-adrenoceptor conformation and therefore ensures that any observed effects on the BODIPY-TMR-CGP dissociation rate are caused by the unlabeled ligand used.

First, we assessed the association and dissociation kinetics of 3 nM BODIPY-TMR-CGP at CHO-β1 and CHO-CS cells (Fig. 4). The fluorescence intensities measured in CHO-CS cells were too low to accurately determine observed association and dissociation rates. In line with this, the nonspecific binding component in the 3 nM BODIPY-TMR-CGP dissociation trace obtained in CHO-β1 cells was also too low to be detected and therefore was analyzed as β1-adrenoceptor–specific dissociation using a 1-phase dissociation equation. This gave a dissociation rate of 0.09 ± 0.01 min⁻¹ (n = 9) in the absence of unlabeled ligands, which was similar to the dissociation rate obtained for 10 and 30 nM BODIPY-TMR-CGP (P > 0.05, 1-way ANOVA followed by Tukey’s multiple comparisons test). To selectively label the high-affinity conformation of the β1-adrenoceptor, we chose a concentration of 3 nM BODIPY-TMR-CGP (24) for subsequent dissociation experiments and limited the association of fluorescent ligand to 4 min. As a consequence, the association of 3 nM BODIPY-TMR-CGP did not reach a plateau within this time period, and the association rate could not be accurately determined.
from these data alone. However, when globally analyzed in combination with the dissociation trace, an association rate constant \( k_{on} \) of \( 5.27 \pm 0.53 \times 10^7 \text{M}^{-1}\text{min}^{-1} \) \((n = 9)\), a dissociation rate \( k_{off} \) of \( 0.08 \pm 0.01 \text{min}^{-1} \) \((n = 9)\), and a \( pK_d \) of \( 8.83 \pm 0.06 \) \((n = 9)\) were obtained (Fig. 4C).

Next, the influence of unlabeled ligands on the BODIPY-TMR-CGP dissociation rate was examined, and the dissociation rate of 3 nM BODIPY-TMR-CGP was significantly enhanced in the presence of 100 nM \((0.21 \pm 0.02 \text{min}^{-1}, n = 5)\), 1 \( \mu \text{M}\) \((0.20 \pm 0.02 \text{min}^{-1}, n = 7)\), and 10 \( \mu \text{M}\) \((0.22 \pm 0.03 \text{min}^{-1}, n = 5)\) CGP 12177 \((P < 0.05, 1\text{-way ANOVA followed by Dunnett’s multiple comparisons test; Fig. 5A and Table 2)}\). A similar increase in BODIPY-TMR-CGP dissociation rate was observed in the presence of 1 \( \mu \text{M}\) \((0.19 \pm 0.01 \text{min}^{-1}, n = 6)\) and 10 \( \mu \text{M}\) \((0.22 \pm 0.03 \text{min}^{-1}, n = 5)\) propranolol (Fig. 5B). The effect of the enhanced dissociation rate was concentration dependent and saturable, which is characteristic of allosteric interactions (28). A concentration-response curve was fitted through the grouped BODIPY-TMR-CGP dissociation rates (listed in Table 2) plotted against the concentrations of unlabeled CGP 12177 (Fig. 5C) and propranolol (Fig. 5D) used, with the midpoint of the curve providing affinity estimates \( (pK_d) \) of the unlabeled ligands for a secondary conformation on the B1-adrenoceptor with the fluorescent ligand already bound to the primary orthosteric conformation, which were determined to be 7.79 and 6.65 for CGP 12177 and propranolol, respectively. These values are consistent with those determined from inhibition of functional CGP 12177 responses via the secondary conformation of the B1-adrenoceptor (11, 24).

**Influence of TM4 on cooperative interactions involving the B1-adrenoceptor**

To further investigate the cooperative effects observed above on the BODIPY-TMR-CGP dissociation rate from the catecholamine conformation by the action of propranolol and CGP 12177 acting at the secondary conformation, we examined the effect of CGP 12177 on the BODIPY-TMR-CGP dissociation kinetics in CHO-B1 TM4 cells. These cells express B1-adrenoceptors that have been mutated such that the residues in TM4 are those of the B2-adrenoceptor (11). Importantly, this mutant B1-adrenoceptor does not exhibit the secondary CGP 12177 conformation (11). In the absence of unlabeled ligands, the dissociation rate of 3 nM BODIPY-TMR-CGP in CHO-B1 TM4 cells was \( 0.066 \pm 0.005 \text{min}^{-1} \) \((n = 8)\) in these cells. Interestingly, the dissociation rates of 3 nM BODIPY-TMR-CGP in the presence of 1 \( \mu \text{M}\) CGP 12177 and 1 \( \mu \text{M}\) propranolol were \( 0.089 \pm 0.010 \) \((n = 6)\) and \( 0.080 \pm 0.007 \text{min}^{-1} \) \((n = 10)\), respectively, which are comparable to the dissociation rate in the absence of ligands \((P > 0.05, 1\text{-way ANOVA followed by Dunnett’s multiple comparisons test; Fig. 6)}\).

**Effects of enhancing and disrupting B1AR homodimer interactions on cooperative interactions**

The cooperative effects observed above at the wild-type B1-adrenoceptor clearly highlight the presence of 2 distinct binding conformations to which \( \beta \)-adrenoceptor ligands can bind. Baker et al. (7) conducted mutagenesis studies in which selected mutations \( (e.g., \text{D138A}) \) in the orthosteric ligand binding domain disrupted both the high- and low-affinity binding conformation (7). Two key mutations in the B1-adrenoceptor TM4 region completely
abolished the secondary conformation (L195Q and W199Y) (11). Interestingly, these are thought to lie within the TM4-TM5 heterodimer interface of the β1-adrenoceptor (19), suggesting a potential role of β1-adrenoceptor homodimerization in the secondary conformation CGP 12177 pharmacology.

Furthermore, this study showed that the cooperative interactions at the wild-type β1-adrenoceptor are completely prevented in CHO-β1TM4 cells (Fig. 6). Homodimerization of β1-adrenoceptors has been reported to be transient (20, 21). Thus, to detect β1-adrenoceptor homodimers and allow their pharmacological investigation, we

<table>
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<th>TABLE 2. Dissociation rate constants of 3 nM BODIPY-TMR-CGP in the absence and presence of CGP 12177 and propranolol</th>
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Data are means ± SEM of n separate experiments. ND, not determined. *Statistical significance (P < 0.05) in CHO-β1 cells from control conditions (infinite dilution) for each unlabeled ligand used (1-way ANOVA followed by Dunnnett’s multiple comparisons test). **Statistical significance (P < 0.05) from the equivalent value in CHO-β1 cells and *P < 0.05 from control conditions (infinite dilution) within each cell line (2-way ANOVA followed by Tukey’s multiple comparisons test).
used BiFC (31, 32) to irreversibly trap and stabilize β-adrenoceptor homodimers that formed at any given time. We hypothesized that constraining dimers using BiFC would increase the percentage of β1-adrenoceptors dimers and as such enhance any dimer-mediated allosteric effects. It has been shown previously that a D138A mutation in TMS disrupted lipid binding to both the catecholamine and the secondary conformation of the β-adrenoceptor (7). Consequently, any enhanced allosteric effects should be prevented by constraining dimers containing 1 nonligand-binding protomer (β1D138A) (7).

BiFC uses 2 nonfluorescent fragments of a fluorescent protein, which reconstitute the functional (i.e., fluorescent) full-length fluorescent protein when in close proximity to one another (32). The N-terminal fragment and the C-terminal fragment of the YFP (YFPN and YFPC, respectively) were fused to the C-terminal end of the wild-type or D138A β1-adrenoceptor to generate the β1YFPN, β1YFPC, and β1D138AYFPC receptor constructs. The β1YFPN/β1YFPC and β1YFPN/β1D138AYFPC constructs were transiently cotransfected into CHO-K1 cells, and clear membrane fluorescence of reconstituted YFP and BODIPY-TMR-CGP binding could be seen (Fig. 7), confirming cell surface expression of wild-type/wild-type and wild-type/D138A β1-adrenoceptor homodimers that each contain at least 1 BODIPY-TMR-CGP binding conformation. To confirm that the D138A mutation abolished lipid binding to the β1-adrenoceptor, we examined the binding of 3 nM BODIPY-TMR-CGP to a SNAP-tagged D138A β1-adrenoceptor. Indeed, no binding of 3 nM BODIPY-TMR-CGP could be seen in CHO-K1 cells transiently transfectected with the SNAP-β1D138A construct, but clear membrane fluorescence was observed following labeling of the SNAP-tag with 1 μM BG-488, confirming the expression of the non–ligand-binding receptor at the cell surface (Fig. 8, lower left panel). A SNAP-tagged wild-type β1-adrenoceptor was transiently transfected as a positive control, and clear fluorescence of the BG-488 labeled SNAP-tag and 3 nM BODIPY-TMR-CGP binding to the wild-type receptor can be seen (Fig. 8, upper panel). This indicates that the lack of BODIPY-TMR-CGP fluorescence seen for the SNAP-β1D138A–transfected cells is caused by the D138A mutation introduced into the β1-adrenoceptor.

We then examined the dissociation rate of 3 nM BODIPY-TMR-CGP at irreversibly constrained stable wild-type/wild-type β1-adrenoceptor homodimers under ID conditions, which was determined to be 0.02 ± 0.01 min−1 (n = 5; Fig. 9A). This was significantly slower than the dissociation rate measured in CHO-β1 cells (P < 0.05, 2-way ANOVA analysis followed by Tukey’s multiple comparisons test). The dissociation of 3 nM BODIPY-TMR-CGP binding was enhanced in the presence of 1 μM CGP 12177 and 1 μM propranolol with dissociation rates of 0.186 ± 0.008 (n = 6) and 0.189 ± 0.007 min−1 (n = 6), respectively (Fig. 9A). This was significantly faster than the dissociation rate determined in the absence of unlabeled ligands (P < 0.05, 2-way ANOVA followed by Tukey’s multiple comparisons test; Fig. 9A). The change in the 3 nM BODIPY-TMR-CGP dissociation rate in the absence and presence of unlabeled ligands was ~24-fold in CHO-β1 cells but was ~10-fold in CHO-K1 cells expressing β1YFPN/β1YFPC homodimers.

The transient expression of BiFC constructs yielded a mixed population of cells with different expression levels
To investigate whether higher expression levels of \( \beta_1 \)-adrenoceptor homodimers affected the BODIPY-TMR-CGP dissociation rate measurements in this study, we compared the measurements taken in cells with both high and low expression levels (Supplemental Fig. S1). These data confirmed that the dissociation kinetics were very similar at both expression levels (Supplemental Fig. S1).

The dissociation rate of 3 nM BODIPY-TMR-CGP in CHO-K1 cells transiently transfected with 1 wild-type and 1 nonligand-binding \( \beta_1 \)-adrenoceptor construct (\( \beta_1YFPN/\beta_1D138AYFPC \)) under ID conditions was determined to be \( 0.054 \pm 0.011 \text{ min}^{-1} \) \( (n = 5) \). This dissociation rate was increased in the presence of 1 mM CGP 12177 \( (k_{\text{off}, \text{CGP}} = 0.169 \pm 0.010 \text{ min}^{-1}; n = 6) \) and 1 mM propranolol \( (k_{\text{off}, \text{propranolol}} = 0.144 \pm 0.009 \text{ min}^{-1}; n = 5; P < 0.05, \) 2-way ANOVA followed by Tukey’s multiple comparisons test; Fig. 9B). Interestingly, the difference in the 3 nM BODIPY-TMR-CGP dissociation rate in the presence of unlabeled ligands compared with in the absence of unlabeled ligands was ~3-fold in cells expressing constrained wild-type/nonligand-binding \( \beta_1 \)-adrenoceptor homodimers.

**DISCUSSION**

In this study, we used a confocal microscopy approach in conjunction with a perfusion system to investigate the dissociation kinetics of BODIPY-TMR-CGP from the human \( \beta_1 \)-adrenoceptor under ID conditions in single living cells. Using this technique, we revealed negative cooperative...
interactions between the high- and low-affinity $\beta_1$-adrenoceptor conformations, which are facilitated by $\beta_1$-adrenoceptor homodimerization.

In CHO-$\beta_1$ cells, the observed association rates for 10, 30, and 100 nM BODIPY-TMR-CGP increased in a concentration-dependent manner. The dissociation rate was monophasic for 10 nM fluorescent ligand, but biphasic for higher concentrations (30 and 100 nM) of BODIPY-TMR-CGP. The fast dissociation component was clearly identified as a nonspecific component as it was comparable to the dissociation rate observed in CHO-CS cells lacking the $\beta_1$-adrenoceptor. The $\beta_1$-adrenoceptor–specific dissociation rates were comparable for 10 and 30 nM BODIPY-TMR-CGP, but not for 100 nM BODIPY-TMR-CGP, where a significantly faster dissociation rate was observed. According to classic receptor theory, dissociation rates of a ligand should be independent of the ligand concentration used. However, this analysis assumes that the ligand only binds to 1 receptor binding conformation (25). We recently described BODIPY-TMR-CGP binding to the ligand only binds to 1 receptor binding conformation through which CGP 12177 and propranolol binding to a secondary conformation and may be a consequence of negative cooperativity occurring between these 2 ligand-bound conformations.

To further explore the potential for negative cooperativity between different conformations within the $\beta_1$-adrenoceptor, we examined the dissociation rate of 3 nM BODIPY-TMR-CGP in the absence and presence of increasing concentrations of unlabeled ligands. The affinity value derived from the association and dissociation parameters obtained for 3 nM BODIPY-TMR-CGP (~2.6 nM) compared well to the affinity value of BODIPY-TMR-CGP for the orthosteric binding conformation determined in functional studies (0.6 and 87 nM, respectively) (24). Thus, the observation of a faster dissociation rate for 100 nM BODIPY-TMR-CGP may be caused by BODIPY-TMR-CGP binding to both the high- and low-affinity $\beta_1$-adrenoceptor conformations and may be a consequence of negative cooperativity occurring between these 2 ligand-bound conformations.

The TM4 has been highlighted to play a role in dimerization of various class A GPCRs (35), including the $\beta_1$-adrenoceptor (19, 36), indicating a potential role of $\beta_1$-adrenoceptor homodimerization in the secondary conformation. Homodimers of $\beta_1$-adrenoceptors have been reported to be transient in nature (20). In an attempt to generate more stable $\beta_1$-adrenoceptor dimers, we used a BiFC approach to lock $\beta_1$-adrenoceptor homodimers into constrained stable dimers of defined composition. Although BiFC does not affect the rate of homodimerization (26), the prevention of dimer dissociation as a consequence of the irreversible nature of BiFC will increase the percentage of $\beta_1$-adrenoceptors that exist as homodimers. The successful trapping of BiFC-constrained $\beta_1$-adrenoceptor dimers was demonstrated in CHO cells cotransfected with YFP$^+$ and YFP$^-$tagged $\beta_1$-adrenoceptor constructs by the clear membrane labeling observed with the reconstituted YFP. In cells expressing these wild-type ($\beta_2$YFP$^+$/$\beta_2$YFP$^-$) BiFC-constrained $\beta_1$-adrenoceptor homodimers, the BODIPY-TMR-CGP dissociation rate was enhanced ~10-fold by 1 $\mu$M CGP 12177 and 1 $\mu$M propranolol. In contrast, only a ~3-fold difference in dissociation rate was observed in CHO-$\beta_1$ cells (i.e., transient unconstrained dimers), suggesting that the cooperative effects of unlabeled ligands on the dissociation rate of BODIPY-TMR-CGP may be mediated across a $\beta_1$-adrenoceptor homodimer interface (Fig. 10A). It was also notable that the dissociation of 3 nM BODIPY-TMR-CGP from native transient $\beta_1$-adrenoceptor dimers ($k_{off}$, 0.09 min$^{-1}$) was faster than that from wild-type BiFC-constrained $\beta_1$-adrenoceptor homodimers ($k_{off}$, 0.02 min$^{-1}$). This suggests that the
negative cooperativity (α<1)
equil. rate increased K_{D,2}
no cooperativity
no change K_{D,2}
no cooperativity
no change K_{D,2}
no cooperativity
no change K_{D,2}

Figure 10. Schematic diagram describing negative cooperative interactions at β1-adrenoceptor homodimers. A) A β1-adrenoceptor homodimer possesses 2 endogenous (i.e., structurally identical) ligand-binding sites for which β1-adrenoceptor ligands have the same affinity. However, following binding of a β1-adrenoceptor ligand to 1 β1-adrenoceptor site (primary, orthosteric binding site; red filled circles) with high affinity, negative cooperativity between the 2 β1-adrenoceptor binding sites in a β1-adrenoceptor homodimer results in a markedly reduced affinity (increased K_{D,2}) of a secondary β1-adrenoceptor ligand for the secondary β1-adrenoceptor site (secondary, allosteric binding site; black filled circles), which is often described as the low-affinity CGP 12177 binding site. This negative cooperativity is reciprocal between the 2 β1-adrenoceptor binding sites, and ligand binding to the secondary site causes an enhanced dissociation rate of the ligand already bound to the primary site. The cooperativity factor α provides a quantitative estimate of the degree and direction of cooperativity between 2 binding sites and is defined as the ratio of a ligand’s affinity for the free receptor over the affinity of the same ligand for the already ligand-occupied receptor (23, 28). Negative cooperativity is indicated by a cooperativity factor smaller than unity. The low-affinity β1-adrenoceptor site therefore represents the binding affinity of ligand A for a receptor dimer where 1 protomer is already occupied, and the dissociation constant is given by K_{D}α/α (see equation). Effective removal of the orthosteric site from one of the protomers through (B) a point mutation that abolishes ligand binding or (C) disruption of the TM4/TM5 β1-adrenoceptor dimerization interface removes any cooperative effects between 2 β1-adrenoceptor sites across the homodimer interface. In these 2 situations, the binding equation reverts to a simple mass action equilibrium between ligand A and receptor R.

formation of stable homodimers itself leads to significant basal allosteric influences on ligand binding kinetics.

To further test whether the secondary (allosteric) β1-adrenoceptor conformation is facilitated by a second β1-adrenoceptor protomer in a homodimer complex, BODIPY-TMR-CGP dissociation kinetics were determined in cells expressing BiFC-constrained β1-adrenoceptor homodimers of I wild-type β1-adrenoceptor (β1YFP_S) and 1 protomer containing a point mutation that abolishes binding of β-adrenoceptor ligands at both the primary catecholamine and secondary conformations (β1D188A/YFP_C) (7). The removal of one of the orthosteric binding conformations in a homodimeric β-adrenoceptor pair should remove the potential for negatively cooperative affects across the dimer interface (Fig. 10B). Indeed, in cells expressing β1YFP_N/β1D188A/YFP_C, BiFC-constrained homodimers, the effects of CGP 12177 and propranolol on the BODIPY-TMR-CGP dissociation kinetics were reduced and reflected more closely the pharmacology observed in CHO-β1 cells (i.e., transient wild-type unconstrained dimers). This residual cooperativity is most likely caused by the cooperative effects that can still occur across transient wild-type β1YFP_S/β1YFP_N dimers that will be present in the cell population. Two dimerization interfaces have been reported for the β1-adrenoceptor involving transmembrane regions 1 and 2 and helix 8 (TM1/TM2/H8) in the first interface and transmembrane regions 4 and 5 and intracellular loop 2 (TM4/TM5/ICL2) in the second interface, the latter of which has been described to make structural rearrangements during receptor activation (19). Key residues (L195, W199) identified by Baker et al. (11) that are responsible for the secondary conformation lie within the dimer interface region of TM4. Using a stable cell line containing the reported β1-adrenoceptor TM4 mutations that result in no secondary conformation (11), we observed no effects on the dissociation rate of 3 nM BODIPY-TMR-CGP in the presence of 1 μM CGP 12177 and 1 μM propranolol. The loss of the enhanced dissociation rate in cells expressing β1-adrenoceptor TM4 mutations is consistent with a role of a dimerization interface involving TM4 in the negative cooperativity observed at the β1-adrenoceptor (Fig. 10C).

In summary, these data suggest that the secondary low-affinity conformation of the β1-adrenoceptor may be a consequence of negative cooperative interactions between 2 orthosteric binding conformations within a β1-adrenoceptor homodimer. This can then lead to a reduced apparent affinity of ligands for the second protomer of an already ligand-occupied (on the first protomer) dimer [see Supplemental Fig. S1 in May et al. (23)]. The contribution of these cooperative interactions is provided by the cooperativity factor α, and the ligand affinity for the already ligand-bound receptor is described as a ratio of the ligand affinity for the unbound receptor and the cooperativity factor α (K_{D}/α). As such, the cooperativity factor at β1-adrenoceptor dimers can be determined by taking the ratio of the apparent K_{D} value determined for binding to the first protomer (orthosteric β1-adrenoceptor conformation 1 K_{D}, i.e., unbound receptor) and that determined for modulating the dissociation rate of BODIPY-TMR-CGP from conformation 1 by an unlabeled ligand binding to the second protomer (allosteric β1-adrenoceptor conformation 2 K_{D}, i.e., ligand-bound receptor). Negative cooperativity that leads to an increase in the apparent dissociation observed is reflected in a cooperativity factor smaller than unity. Indeed, the cooperativity factors (α) for CGP 12177 and propranolol were estimated to be 0.015 and 0.010, respectively, using the binding affinities for the orthosteric β1-adrenoceptor conformation determined in Gherbi et al. (24). A mechanistic framework for the secondary β1-adrenoceptor conformation based on homodimer formation opens up new insights into the role of dimerization in altering the molecular pharmacology of GPCRs.

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NEGATIVE COOPERATIVITY ACROSS β1-ADRENOCEPTOR HOMODIMERS