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Residency time of agonists does not affect the stability of GPCR–arrestin complexes

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Background and Purpose: The interaction of arrestins with G-protein coupled receptors (GPCRs) desensitizes agonist-dependent receptor responses and often leads to receptor internalization. GPCRs that internalize without arrestin have been classified as "class A" GPCRs whereas "class B" GPCRs co-internalize with arrestin into endosomes. The interaction of arrestins with GPCRs requires both agonist activation and receptor phosphorylation. Here, we ask the question whether agonists with very slow off-rates can cause the formation of particularly stable receptor–arrestin complexes.

Experimental Approach: The stability of GPCR–arrestin-3 complexes at two class A GPCRs, the β_2-adrenoceptor and the μ opioid receptor, was assessed using two different techniques, fluorescence resonance energy transfer (FRET) and fluorescence recovery after photobleaching (FRAP) employing several ligands with very different off-rates. Arrestin trafficking was determined by confocal microscopy.

Key Results: Upon agonist washout, GPCR–arrestin-3 complexes showed markedly different dissociation rates in single-cell FRET experiments. In FRAP experiments, however, all full agonists led to the formation of receptor–arrestin complexes of identical stability whereas the complex between the μ receptor and arrestin-3 induced by the partial agonist morphine was less stable. Agonists with very slow off-rates could not mediate the co-internalization of arrestin-3 with class A GPCRs into endosomes.

Conclusions and Implications: Agonist off-rates do not affect the stability of GPCR–arrestin complexes but phosphorylation patterns do.

Our results imply that orthosteric agonists are not able to pharmacologically convert class A into class B GPCRs.

KEYWORDS
arrestin trafficking, FRAP, FRET

1 | INTRODUCTION

The arrestins are a small family of four cytosolic proteins that specifically bind to activated and phosphorylated G-protein coupled receptors (GPCRs) to prevent further G-protein coupling by sterically blocking the G-protein binding site. Furthermore, they also act as adaptors for the endocytic machinery and promote the internalization of activated receptors (Ferguson, 2001). Based on the apparent stability of arrestin–receptor complexes, GPCRs are classified into two groups (Oakley, Laporte, Holt, Caron, & Barak, 2000). Class A receptors such as the **β_2-adrenoceptor** or **μ opioid receptors** interact only transiently with arrestins resulting in the dissociation of arrestin during the endocytic process. In contrast, class B receptors like the vasopressin **V_2 receptor** form very stable complexes with arrestins that co-internalize into endosomes. Notably, agonists for class A receptors typically have fast off-rates whereas most agonists for class B receptors are peptides which dissociate slowly from the receptor under physiological conditions. However, it has been found that a sufficient number of phosphate residues in the correct spatial arrangement in the C-terminal tail of a receptor is required for high-affinity binding of arrestins (Mayer et al., 2019) and therefore determines which class a receptor belongs to. While the significance of phosphorylation sites for class A or B behaviour of a GPCR has been studied intensively (Latorraca et al., 2020; Mayer et al., 2019; Oakley, Laporte, Holt, Barak, & Caron, 1999; Zhou et al., 2017; Zindel et al., 2015, 2016), it is unknown whether the stability of arrestin–receptor complexes also depends on agonist affinity. While it was previously shown that arrestin binding increases agonist affinity to a receptor (Gurevich, Pals-Rylaarsdam, Benovic, Hosey, & Onorato, 1997), it is still unclear if agonists with prolonged receptor occupancies might stabilize the binding of arrestin at the receptor more than agonists with fast off-rates and whether arrestin actually dissociates from agonist-occupied receptors without previous agonist dissociation. Therefore, we compared the stability of arrestin–receptor complexes induced by several ligands with different off-rates by employing two different methods. Firstly, Foerster resonance energy transfer (FRET) was used to assess the interaction between arrestin-3 and receptor upon agonist application and withdrawal. In addition, the steady-state apparent affinity of arrestin-3 to receptors in the continuous presence of agonist was observed by dual-colour fluorescence recovery after photobleaching (FRAP). Furthermore, we imaged the co-internalization of arrestin-3 with activated receptors to find out whether agonist off-rates determine the class A or class B behaviour of GPCRs.

All experiments were conducted with two typical class A GPCRs, the β_2-adrenoceptor and the μ receptor. For the β_2-adrenoceptor, the high-affinity ligand BI-167107 which has a dissociation half-life of about 30 h (Rasmussen et al., 2011) was compared with **isoprenaline** and **adrenaline**, which both have dissociation half-lives of seconds (Krasel, Bünemann, Lorenz, & Lohse, 2005). For the μ receptor, we chose the synthetic opioid peptide **DAMGO**, the partial agonist **morphine**, the novel ligand SR17018 (Schmid et al., 2017) and **etorphine**, which was previously shown to induce long-lived receptor–arrestin complexes (McPherson et al., 2010).

What is already known

- Affinity of arrestins for agonist-activated GPCRs depends on the number and spacing of phosphorylation sites.
- Arrestin affinities have been compared using FRET and FRAP.

What does this study add

- Affinity and trafficking of arrestin is independent of agonist off-rates.
- Arrestin exchange on GPCRs occurs in the presence of bound agonists.

What is the clinical significance

- Receptor agonist occupancy times are unlikely to affect GPCR desensitization and trafficking.

2 | METHODS

2.1 | Plasmids

cDNAs for human β_2-adrenoceptor-YFP, human GRK2 (Krasel, Bünemann, Lorenz, & Lohse, 2005), human YFP-β_2-adrenoceptor (Dorsch et al., 2009), bovine Arr3-mTurquoise and mouse μ receptor-SYFP2 (Miess et al., 2018) have been described elsewhere. β_2-adrenoceptor-mCherry and μ receptor-mCherry were cloned by replacing the sequence encoding the fluorescent protein in β_2-adrenoceptor-YFP and μ receptor-SYFP2, respectively, with mCherry using PCR. Arr3-GFP was cloned from Arr3-CFP (Krasel, Bünemann, Lorenz, & Lohse, 2005) using the same strategy. The μ receptor tagged N-terminally with YFP (YFP-μ receptor) was cloned from the YFP-β_2-adrenoceptor by replacing the β_2-adrenoceptor receptor sequence with the mouse μ receptor sequence.

2.2 | Reagents

DMEM, FBS, penicillin/streptomycin, L-glutamine and trypsin–EDTA were purchased from Capricorn Scientific (Ebsdorfergrund, Germany). Effectene Transfection Reagent was from Qiagen (Hilden, Germany). SR17018 was a kind gift from Stefan Schulz and Andrea Kliewer (University of Jena, Germany) (Miess et al., 2018) (Gillis et al., 2020). BI-167107 was a gift from Boehringer Ingelheim through their opnme program (https://opnme.com/). Etorphine hydrochloride (Captivon 98®) was obtained from Wildlife Pharmaceuticals

(White River, South Africa) through Chilla CTS GmbH (Georgsmarienhütte, Germany). All other reagents were purchased from Merck (Darmstadt, Germany).

2.3 | Cell culture

HEK293T cells were cultured in Dulbecco's modified Eagle's medium (4.5 g·L^{-1} glucose) supplemented with 10% fetal calf serum, 2-mM L-glutamine, penicillin (50 mg·ml^{-1}), and streptomycin (50 mg·ml^{-1}) at 37°C in a humidified atmosphere with 5% CO_2. Cells were transfected using Effectene® (Qiagen, Hilden, Germany) according to the manufacturer's protocol. Twenty-four hours after transfection, cells were plated on coverslips coated with poly-L-lysine and experiments were performed the next day.

2.4 | Foerster resonance energy transfer (FRET)

FRET between fluorescently tagged arrestin and receptors was measured as described previously (Krasel, Bünemann, Lorenz, & Lohse, 2005). HEK293T cells were transiently transfected with YFP-labelled receptors (0.7 μg), mTurquoise-labelled arrestin-3 (0.9 μg) and non-fluorescent GRK2 (0.4 μg). Measurements were either performed on an inverted eclipse Ti Nikon (Düsseldorf, Germany) microscope as described by Zindel et al. (2015) or on an inverted Zeiss (Oberkochen, Germany) Axiovert 100 microscope as described by Wolters, Krasel, Brockmann, and Bünemann (2015). In both cases, the illumination frequency was set to 2 Hz and the illumination time to 30–60 ms, depending on the brightness of the cells. During all measurements, cells were continuously superfused with FRET buffer (137-mM NaCl, 5.4-mM KCl, 2-mM $CaCl_2$, 1-mM $MgCl_2$ and 10-mM HEPES, pH 7.3) or buffer containing agonist in different concentrations using a pressure-driven fast-switching eight-channel valve-controlled pressurized perfusion system (VC3-8xP Series; ALA Scientific Instruments, Farmingdale, NY, USA). Data were corrected for background fluorescence, spillover of CFP into the YFP channel and direct YFP excitation. The resulting emission ratios F_{YFP}/F_{CFP} were normalized to baseline and plotted as mean ± SEM. For quantification, kinetics were quantified by a monophasic exponential fit of the full (μ receptor) washout phase or the first 200 s upon agonist withdrawal (β$_2$-adrenoceptor).

2.5 | Fluorescence recovery after photobleaching (FRAP)

FRAP was used to assess the stability of the receptor-arrestin interaction similar to the protocol described previously (Zindel et al., 2015, 2016). For this type of experiment, it is important that the receptor is present in excess over the arrestin because otherwise arrestin mobility will only be partially altered. HEK293T cells were transfected with mTurquoise-tagged arrestin3, GRK2 and receptors labelled at the N-terminus with SYFP2. Receptors were immobilized by crosslinking them with a polyclonal anti-GFP antibody (Rockland Cat# 600-101-215, RRID:AB_218182; Rockland, Gilbertsville, PA) at a dilution of 1:100 for 30 min at 37°C. Subsequently, cells were stimulated with agonist and imaged using a Leica TCS SP5 scanning confocal microscope equipped with a Lambda Blue 63×/1.4NA oil immersion lens (Leica, Wetzlar, Germany). Image size was 256 × 256 pixels, and scan speed was 1000 Hz. mTurquoise was bleached with a 405-nm diode laser and SYFP2 with the 514-nm line of an argon laser. A 3 × 1 μm area was bleached, and the fluorescence recovery was observed for almost 3 min. Resulting fluorescence intensity values were corrected for photobleaching and normalized by setting the initial fluorescence intensities (pre-bleach) to 100%. Cells were excluded if they moved throughout the experiment (making the determination of fluorescence recovery impossible) or if fluorescence recovery in the YFP channel was >40% (suggesting that immobilization of the receptor had not worked). Normalized FRAP curves were averaged and plotted as mean ± SEM. For quantification, post-bleach values were fitted using GraphPad 8.3 (GraphPad Software, San Diego, CA, USA) with a mono-exponential curve to obtain recovery time constants, with $t = 0$ s set for the beginning of the bleach. Results from fits were not included in the statistical analysis if curve fitting could not determine a 95% confidence interval for the time constant and/or the plateau.

2.6 | Confocal microscopy

HEK293T cells were transiently transfected with mCherry-tagged receptors (0.6 μg), GFP-tagged arrestin-3 (1.0 μg) and non-fluorescent GRK2 (0.4 μg). Cells were treated with agonists for 30 min at 37°C and immediately examined for receptor–arrestin colocalization by confocal microscopy (Leica TCS SP5, Lambda Blue 63×/1.4NA oil immersion lens). GFP was excited at 488 nm with an argon laser and emission was collected at 500–540 nm. mCherry was excited at 543 nm with a helium/neon laser, and mCherry emission was collected at 600–680 nm. Pearson's colocalization coefficient was calculated from individual images using ImageJ 1.47 and the Colocalization Test plugin as described previously (Zindel et al., 2015).

2.7 | Statistics

The data and statistical analysis in this study complies with the recommendations on experimental design and analysis in pharmacology (Curtis et al., 2018). Results are represented as means ± SEM from at least three independent transfections of *n* (at least five) individual randomly selected cells. No statistical methods were used to predetermine group size. Experiments were designed with equal group sizes, but sometimes, data from individual cells had to be excluded; for example, if the data were too noisy to allow a reliable curve fit as explained above. Otherwise, all outliers were included in the data analysis and presentation. Statistical analysis was performed using

GraphPad Prism 8.3. Unpaired Student's t tests were used for comparison of two groups. To compare more than two groups and to test each condition against the mean of every other, one-way ANOVAs followed by Tukey's post hoc tests were used when the standard deviations of each group were not significantly different, whereas Brown–Forsythe tests followed by Dunnett's T3 multiple comparisons test were used for significantly different standard deviations. Post hoc tests were run only performed if F achieved $P < 0.05$ and there was no significant variance inhomogeneity. Differences were considered statistically significant for $P < 0.05$.

2.8 | Nomenclature of targets and ligands

Key protein targets and ligands in this article are hyperlinked to corresponding entries in http://www.guidetopharmacology.org, and are permanently archived in the Concise Guide to PHARMACOLOGY 2021/22 (Alexander, Christopoulos et al., 2021; Alexander, Fabbro, et al., 2021).

3 | RESULTS

In this study, we compared two different methods for the assessment of GPCR-arrestin interactions in single living cells, FRET and FRAP, using two receptors, the β_2-adrenoceptor and the μ receptor.

Interactions between β_2-adrenoceptor-YFP and arrestin-3-mTurquoise in the presence of co-transfected GRK2 were measured by FRET in single HEK293T cells. During the measurement, cells excited at 430 nm were continuously superfused with buffer or buffer containing the indicated agonist while the YFP/CFP emission ratio was obtained simultaneously. Stimulation of the β_2-adrenoceptor with saturating concentrations of isoprenaline (10 μM), adrenaline (15 μM) or BI-167107 (1 μM) resulted in a similar recruitment of arrestin-3 to the receptor which was demonstrated by comparable amplitudes for these full agonists (Figure 1a). However, the dissociation kinetics upon agonist withdrawal demonstrated remarkable differences (Figure 1b) which were quantified by mono-exponential curve fits of the first 200 s of the washout phase. Arrestin-3 dissociated significantly faster from the β_2-adrenoceptor after washout of adrenaline compared to isoprenaline as previously reported (Krasel, Bünemann, Lorenz, & Lohse, 2005). In contrast, the washout of BI-167107 did not lead to any dissociation of arrestin from the β_2-adrenoceptor which can be explained by the long dissociation half-life of the ligand of approximately 30 h (Rasmussen et al., 2011). To enable statistical comparison between the exponential decays of adrenaline, isoprenaline and the apparent non-dissociation of BI-167107, the area under the curve (AUC) of traces normalized to the maximum was calculated (Figure 1c). As the AUC values for BI-167107 were markedly higher than for adrenaline or isoprenaline, the FRET measurements demonstrated that arrestin-3 dissociation from the β_2-adrenoceptor depends on the specific agonist. These data suggest that the dissociation kinetics of arrestin in FRET assays are more suited as a proxy for agonist

off-rates from the receptor as discussed by Krasel, Bünemann, Lorenz, & Lohse (2005). We have used agonist off-rates before as a measure of the apparent affinity of arrestin to a receptor; however, in these experiments, we did not compare different agonists but always used the same agonist (isoprenaline) and examined different receptor phosphorylation patterns (Zindel et al., 2015).

We also measured agonist-induced interactions between β_2-adrenoceptor-YFP and arrestin-2-mTurquoise in the presence of GRK2 (Figure S1a). The FRET amplitude was notably smaller than with arrestin-3-mTurquoise, consistent with a lower affinity of arrestin-2 to class A receptors. While isoprenaline washout led to a dissociation of the β_2-adrenoceptor–arrestin-2 complex, BI-167107 could not be washed out, analogous to the results with arrestin-3. Furthermore, we investigated the agonist-induced interaction between β_2-adrenoceptor-YFP and arrestin-3-mTurquoise in the absence of GRK2 (Figure S1b). As expected, the binding of arrestin-3 was slower in the absence of GRK2 than in the presence of GRK2, but the final amplitude and the dissociation rate of the complex upon isoprenaline washout were identical to those in the presence of GRK2. This reiterates that the rate-limiting step for the formation of the β_2-adrenoceptor–arrestin complex is β_2-adrenoceptor phosphorylation, as previously shown (Krasel, Bünemann, Lorenz, & Lohse, 2005). There was no significant difference in complex dissociation rates upon agonist washout (Figure S1c).

In the FRET experiments, arrestin dissociation was examined upon agonist washout which leads to a conformational change of the receptor from the active back to the inactive conformation. To examine the interaction of arrestin-3 with the β_2-adrenoceptor in the active conformation, we also investigated the stability of arrestin–receptor complexes in the continuous presence of agonist by measuring dual-colour FRAP (Zindel et al., 2016). In this assay, HEK293T cells transiently transfected with N-terminally YFP-labelled β_2-adrenoceptor, arrestin-3-mTurquoise and GRK2 were preincubated with a polyclonal anti-GFP antibody for 30 min at 37°C to crosslink the receptors before applying the indicated agonist. After photobleaching a small spot of the plasma membrane, the fluorescence recovery of mTurquoise-labelled arrestin-3 and YFP-labelled β_2-adrenoceptor was monitored simultaneously as a measure of the mobility of the two fluorescent proteins. Since the receptor is crosslinked with antibody, fluorescence recovery in the receptor channel is low and fluorescence recovery in the arrestin channel depends mostly on the affinity of arrestin binding to the agonist-activated receptor. Weak interactions between arrestin and the receptor lead to a fast dissociation of photobleached arrestin-3 and the subsequent exchange with a fluorescent arrestin molecule from the unbleached environment, resulting in a fluorescence increase in the formerly bleached area of the cell membrane. Strong interactions between arrestin and the receptor prevent the dissociation of photobleached arrestin and thus also the rebinding of fluorescent arrestin. Therefore, the extent and speed of the arrestin-3 fluorescence recovery reflects a combination of the dissociation rate of bleached arrestin from the agonist-bound receptor and the association rate of unbleached arrestin to this receptor; this allows an estimation of the stability of the arrestin–receptor complex.
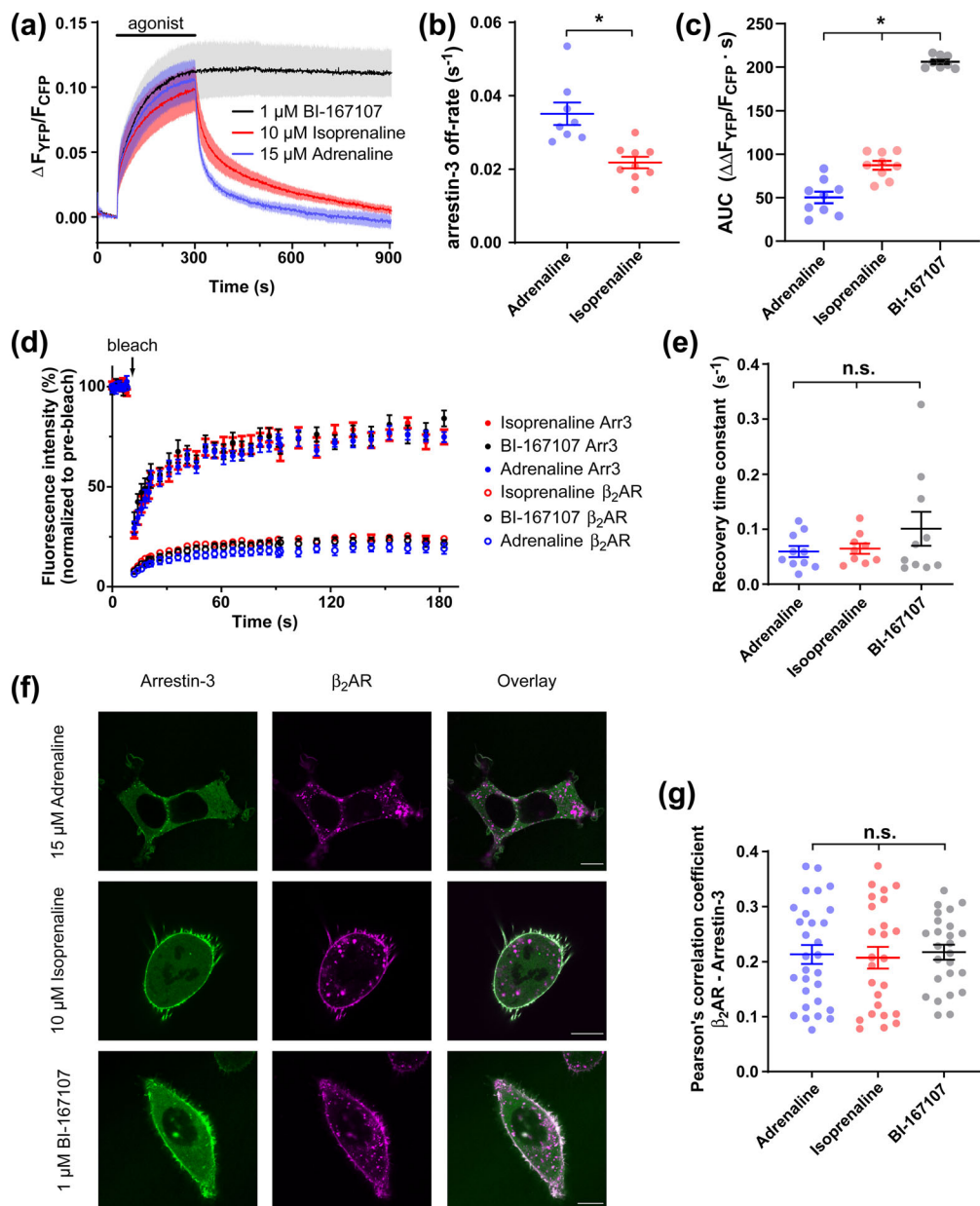
FIGURE 1 Interaction of arrestin-3 with the β_2-adrenoceptor. (a) Arrestin-3 recruitment to the β_2-adrenoceptor induced by isoprenaline, adrenaline or BI-17018 was measured by FRET as described in Section 2. (b) Dissociation kinetics upon withdrawal of isoprenaline (Iso) or adrenaline (Adr) agonist were quantified by fitting the first 200 s of the washout phase with a monophasic exponential decay whereas the very slow off-rate for BI-167107 did not permit an exponential curve fit. Statistical analyses were performed using an unpaired Student's t test (*$P < 0.05$). (c) Additionally, dissociation kinetics were quantified using the area under the curve (AUC) of traces for the first 200 s of the washout phase normalized to the maximum to compare linear and exponential off-rates. Statistical analyses were performed using a one-way ANOVA followed by Tukey's multiple comparisons test (*$P < 0.05$). Data are shown as means ± SEM with $n = 9$ for isoprenaline or adrenaline and $n = 8$ for BI-167107. (d) The stability of β_2-adrenoceptor–arrestin-3 complexes was analysed by dual-colour FRAP as described in Section 2. Traces were normalized to pre-bleach fluorescence intensities (100%) and represent means ± SEM with $n = 13$ for isoprenaline and BI-167107 and $n = 17$ for adrenaline. Filled symbols show the fluorescence intensities of arrestin-3 (arr3) whereas open symbols show the fluorescence intensities of the β_2-adrenoceptor (β_2AR). (e) Time constants for the recovery of β_2-adrenoceptor and arrestin-3 were obtained by mono-exponential curve fits of the post-bleach values. Statistical analyses were performed using a one-way ANOVA followed by using a Brown–Forsythe test followed by Dunnett's T3 multiple comparisons test (n.s. if $P \geq 0.05$). (f) Cellular trafficking of arrestin-3 with the β_2-adrenoceptor was analysed by confocal microscopy. HEK293T cells transiently expressing β_2-adrenoceptor-mCherry, Arr3-GFP and GRK2 were stimulated with the indicated agonists for 30 min at 37°C before imaging. Colocalized β_2-adrenoceptor (β_2AR, magenta) and arrestin-3 (green) are observed as white in the overlay image. Scale bars represent 10 μm. (g) Colocalization of arrestin-3 and β_2-adrenoceptor was quantified by calculating Pearson's correlation coefficients. Statistical analyses were performed using a Brown–Forsythe test followed by Dunnett's T3 multiple comparisons test (n.s. if $P \geq 0.05$). Data are shown as means ± SEM ($n = 25$)

Figure S2 shows representative raw data from the FRAP experiments. As the lateral mobility of the crosslinked receptors was restricted, the recovery of the receptor fluorescence was reduced to about 20% for all agonists (Figures 1 and S2, right, yellow circles). Mono-exponential curve fits of the post-bleach values showed no significant differences in the kinetics of the arrestin-3 recovery regardless of the applied agonist (Figure 1e). This shows that BI-167107 did not cause the formation of more stable arrestin-3–β_2-adrenoceptor complexes than the other two full agonists. Thus, the slow dissociation in the FRET experiments (Figure 1b) can rather be attributed to stable agonist binding than to stable arrestin binding. This was further supported by confocal imaging which revealed that stimulation of the β_2-adrenoceptor with BI-167107 for 30 min at 37°C did not induce co-internalization of arrestin-3 (green) with β_2-adrenoceptors (magenta). Figure 1f illustrates that stimulation with any of the three agonists resulted in membrane translocation of arrestin-3 (green), whereas the receptors (magenta) were internalized into endosomes. Therefore, an overlay of the single fluorescence images showed barely any colocalization of arrestin-3 and β_2-adrenoceptor in intracellular compartments. Quantification of the colocalization with the Pearson's correlation coefficient resulted in values fairly close to 0, indicating little colocalization of the two fluorescent proteins. Furthermore, there was no significant difference in the Pearson's correlation coefficients between the three agonists (Figure 1g). In conclusion, these data indicate that the ligand off-rate does not have an impact on the stability of arrestin-3–receptor complexes and does not influence arrestin-3 trafficking.

FRAP experiments with arrestin2 also did not reveal significant differences between isoprenaline and BI-167107 (Figure S3a,b). The recovery rates were apparently faster for arrestin-2 than for arrestin-3 (compare Figure 1e with Figure S3b), confirming the lower affinity of arrestin-2 to the β_2-adrenoceptor.

To test this assumption on another receptor, similar experiments were also performed with the μ opioid receptor. In the FRET assay, the observed peak amplitudes upon agonist stimulation were remarkably smaller for the partial agonists morphine and SR17018 compared to DAMGO and etorphine (Figure 2a). The arrestin recruitment also seemed to be slower for SR17018 than for DAMGO or etorphine, consistent with its slower phosphorylation kinetics (Gillis et al., 2020). Normalization of the traces to the maximum revealed that the dissociation kinetics for DAMGO and morphine were quite similar, whereas SR17018 and etorphine showed considerably slower off-kinetics (Figure 2b) which was also confirmed by lower time constants (Figure 2c). However, the FRET experiments again did not correlate with the stability of arrestin complexes in continuous presence of agonist in dual-colour FRAP measurements (Figure 2d). In these experiments, we initially omitted treatment with morphine as this very rarely leads to visible arrestin translocation to the plasma membrane (Zhang et al., 1998). The recovery kinetics of fluorescent arrestins into the bleached area did not show statistical differences between the applied agonists (Figure 2e), similar to the β_2-adrenoceptor. Furthermore, none of the μ receptor agonists could induce co-internalization of arrestin-3 with

the receptors (Figure 2f). Again, morphine was omitted in these experiments as it does not cause μ receptor internalization in HEK293 cells (Keith et al., 1996). Quantification of the colocalization of arrestin-3 with the μ receptor using the Pearson's correlation coefficient did not reveal any agonist-specific difference (Figure 2g) as seen before for the β_2-adrenoceptor. Taken together, ligands with longer binding times do not stabilize receptor–arrestin complexes and therefore do not induce co-internalization of arrestin-3 with the receptor.

The experiments with the μ receptor described so far were all conducted in the presence of co-transfected GRK2. To investigate whether the FRAP assay can detect differences in receptor phosphorylation, we tried to conduct FRAP assays with DAMGO and morphine in the absence of co-transfected GRK2. Whereas DAMGO causes a full phosphorylation of the μ receptor leading to fairly robust μ receptor–arrestin interaction, morphine causes only a phosphorylation of the μ receptor at Ser375 (Just et al., 2013) leading to a very weak μ receptor–arrestin interaction (McPherson et al., 2010; Zhang et al., 1998). As a negative control, we also included untreated cells in the assay; no receptor–arrestin interaction should occur under these conditions, and therefore, a very fast fluorescence recovery was expected. Even though we could not see any visible arrestin-3 translocation to the membrane in our confocal images, the FRAP kinetics indeed showed a slight delay of fluorescence recovery in morphine-treated cells compared to untreated cells (Figure 3a). It appears from the raw data that the arrestin in untreated or morphine-treated cells is less efficiently bleached than in DAMGO-treated cells (Figure 3a). However, we believe that this is caused by a very fast recovery that occurs during the bleach. Our confocal microscope takes about 1.7 s from the start of the bleach to the start of the acquisition of the first post-bleach image. Since the recovery is so fast, it was impossible to fit mono-exponential kinetics to the individual morphine and untreated data, even if constraints were taken into account. Therefore, we quantitated the area under the curve within the first 10 s after bleaching and could demonstrate significant differences between DAMGO, morphine and untreated cells (Figure 3b). These results are to our knowledge the first that demonstrate ligand-dependent changes in arrestin affinity to a class A GPCR in cells.

4 | DISCUSSION

In this study, we assessed the interaction between arrestin-3 and both the β_2-adrenoceptor and the μ receptor upon application of various agonists by two different methods. Ligands with prolonged off-rates significantly slowed the dissociation kinetics of arrestin-3 from the receptor upon agonist withdrawal in FRET measurements as expected from previous experiments (Krasel, Bünemann, Lorenz, & Lohse, 2005). However, the stability of arrestin–receptor complexes in the continuous presence of these agonists (as assessed by FRAP measurements) did not differ at all. Furthermore, the ligand off-rates at the β_2-adrenoceptor or μ receptor did not affect the co-trafficking of
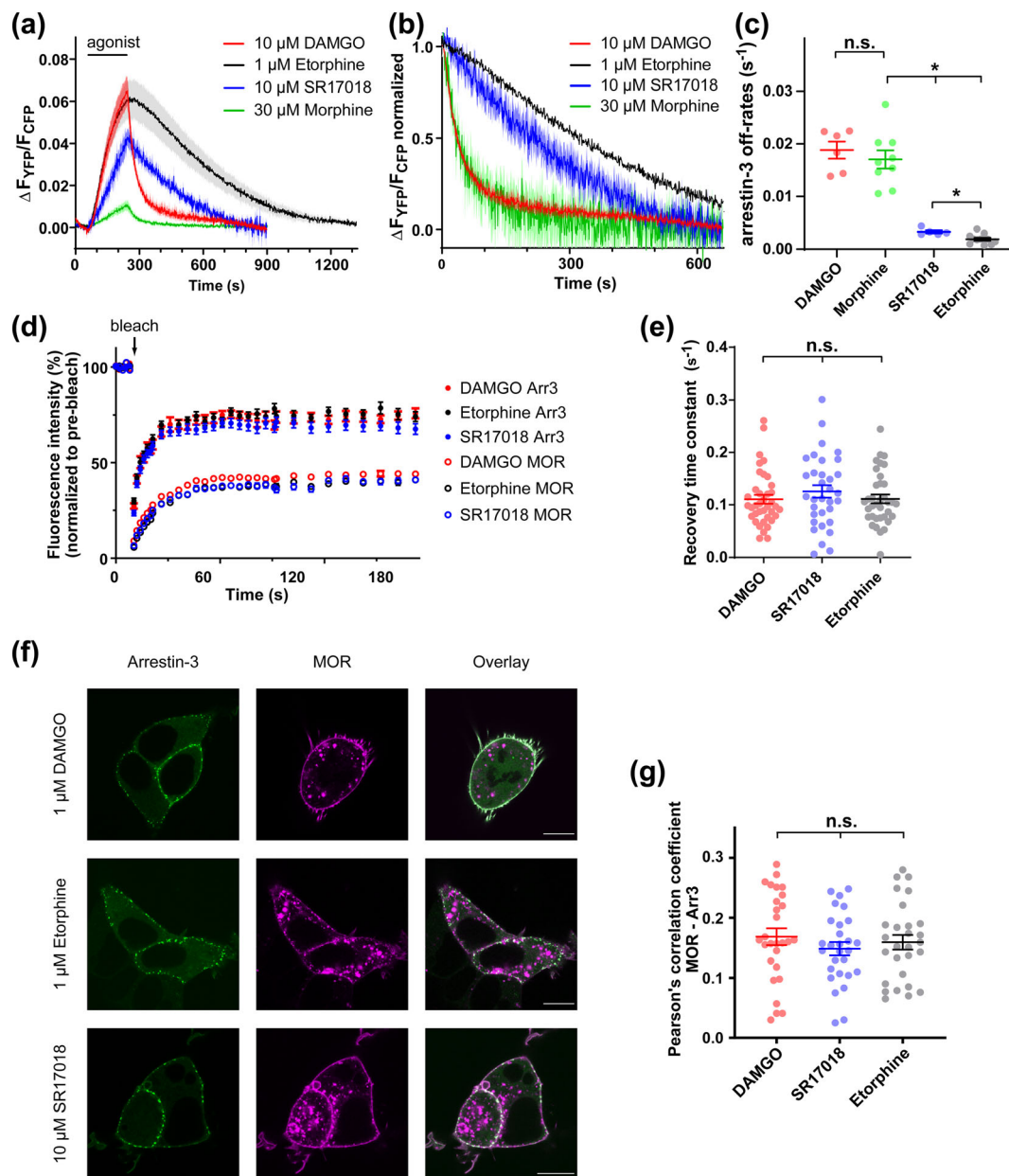
FIGURE 2   Interaction of arrestin-3 with the μ opioid receptor. (a) Arrestin-3 recruitment to the μ receptor induced by DAMGO, etorphine, SR17018 or morphine was measured by FRET as described in Section 2. (b) Traces were normalized to the maximum peak to facilitate direct comparison of the dissociation kinetics upon agonist withdrawal. (c) Kinetics were quantified by a monophasic exponential fit. Statistical analyses were performed using a Brown–Forsythe test followed by Dunnett's T3 multiple comparisons test (n.s. if $P \geq 0.05$, *$P < 0.05$). Data represent means ± SEM with $n = 6$ for DAMGO or SR17018, $n = 9$ for morphine and $n = 11$ for etorphine. (d) The stability of μ receptor–arrestin-3 complexes was analysed by dual-colour FRAP as described in Section 2. Traces were normalized to pre-bleach fluorescence intensities (100%) and are shown as means ± SEM with $n = 36$ for DAMGO or etorphine and $n = 33$ for SR17018. Filled symbols show the fluorescence intensities of arrestin-3 whereas open symbols show the fluorescence intensities of the μ receptor (MOR). (e) Time constants for the recovery of μ receptor and arrestin-3 were obtained by mono-exponential curve fits of the post-bleach values. Statistical analyses were performed using a one-way ANOVA followed by Tukey's multiple comparisons test (n.s. if $P \geq 0.05$). (f) Cellular trafficking of arrestin-3 with the μ receptor was analysed by confocal microscopy. HEK293T cells transiently expressing μ receptor-mCherry, Arr3-GFP and GRK2 were stimulated with the indicated agonists for 30 min at 37°C before imaging. Colocalized μ receptor (MOR, magenta) and arrestin-3 (green) are observed as white spots in the overlay image. Scale bars represent 10 μm. (g) Colocalization of arrestin-3 and μ receptor was quantified by calculating Pearson's correlation coefficients. Statistical analyses were performed using a one-way ANOVA followed by Tukey's multiple comparisons test (n.s. if $P \geq 0.05$). Data are shown as means ± SEM ($n = 28$)
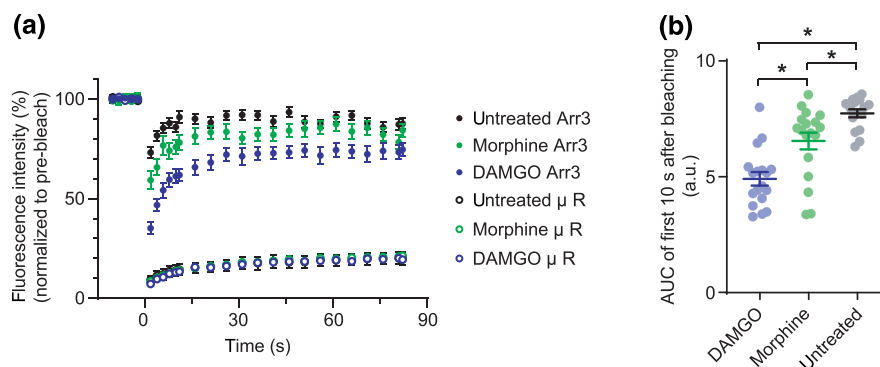
(a)

(b)

FIGURE 3 Stability of μ receptor (μ R)–arrestin complexes induced by morphine. (a) FRAP measurements were conducted as described in Section 2. Traces normalized to pre-bleach fluorescence intensities (100%) are shown as means ± SEM with $n = 18$ for DAMGO, $n = 19$ for morphine and $n = 17$ for untreated. Filled symbols show the fluorescence intensities of arrestin-3 whereas open symbols show the fluorescence intensities of the μ receptor. (b) Recovery kinetics of arrestin-3 were quantified using the AUC of the first 10 s after photobleaching. Statistical analyses were performed using Brown–Forsythe tests followed by Dunnett's T3 post hoc tests (*$P < 0.05$)

arrestin-3 as none of the agonists induced co-internalization of arrestin with these class A receptors.

In FRET measurements, the dissociation of arrestin from the receptor is induced by the dissociation of the ligand upon agonist washout which forces the receptor back to its inactive state. Arrestin senses this conformational change so that its dissociation kinetics from the receptor depends on the ligand off-rate (Krasel, Bünemann, Lorenz, & Lohse, 2005). During FRAP experiments, the agonist is continuously present and therefore the receptor remains in an active conformation. This enabled the assessment of the apparent affinity of arrestin to activated GPCRs without the interference of ligand dissociation kinetics. Therefore, we conclude that our dual-colour FRAP assay is more suitable for this analysis than the FRET assay. On the other hand, FRET but not FRAP assays should be able to sense a transition from the "core" to the "tail" conformation of a GPCR–arrestin complex (Shukla et al., 2014). Surprisingly, we could use the FRAP assay to assess the relative apparent affinity of morphine for the μ receptor even though morphine causes very little visible membrane recruitment of arrestins (Zhang et al., 1998). These results again demonstrate that agonist off-rate is irrelevant for arrestin affinity as DAMGO and morphine display roughly the same off-rate in the arrestin FRET assay (Figure 2b) but clearly the morphine–arrestin complex is less stable (Figure 3); this is probably also reflected by the lower amplitude that can be achieved in the FRET assay (Figure 2c). An open question that is not clarified by our experiments is the role of agonist efficacy for the stability of the receptor–arrestin complex. In other words, is the lower stability of the morphine–arrestin complex only caused by altered phosphorylation (Just et al., 2013), or is it also caused by lower agonist efficacy?

For some agonist–receptor pairs, the kinetics of receptor dephosphorylation upon agonist washout has been investigated (Doll et al., 2012; Fritzwanker, Schulz, & Kliewer, 2021; Krasel, Bünemann, Lorenz, & Lohse, 2005). For all these pairs, arrestin dissociation is faster than receptor dephosphorylation. This is particularly apparent for the SR17018-stimulated μ receptor which shows little

dephosphorylation within 1 h of agonist washout (Fritzwanker, Schulz, & Kliewer, 2021). We are not aware of other publications correlating the lifetime of GPCR–arrestin complexes with the kinetics of GPCR dephosphorylation.

Finally, our results demonstrate that a class A GPCR like the β2-adrenoceptor or the μ receptor cannot be converted to a class B receptor by using orthosteric agonists with prolonged off-rates. In contrast, it has been shown for the CCR7 which is a class B GPCR that upon stimulation with an agonist that leads to reduced receptor phosphorylation, arrestin-3 can no longer co-internalize with the CCR7 (Zidar, Violin, Whalen, & Lefkowitz, 2009). Numerous studies have shown that a sufficient number of phosphate residues in an appropriate pattern in the C-terminal tail is required for a receptor to bind arrestin with higher affinity (Dwivedi-Agnihotri et al., 2020; Latorraca et al., 2020; Mayer et al., 2019; Zhou et al., 2017; Zindel et al., 2015, 2016). Assuming that a full agonist causes maximal phosphorylation of a GPCR, we cannot envisage how orthosteric agonists could increase the number of phosphorylated residues. Allosteric compounds, in contrast, may generate entirely novel receptor conformations that bind arrestins with higher affinity. We did not observe differences in the stability of receptor–arrestin complexes when GRK2 was overexpressed, probably because this "blurs" the phosphorylation pattern; this has been demonstrated at least for the μ receptor. When we did not overexpress GRK2, we could observe differences at the μ receptor between morphine and DAMGO which cause different phosphorylation patterns in the absence of overexpressed GRKs (Just et al., 2013).

In our study, we used the novel μ receptor ligand SR17018 (Schmid et al., 2017) that has been reported to be G-protein-biased. In our hands, this compound is able to recruit arrestin to the μ receptor under conditions of GRK overexpression (see also Gillis et al., 2020) and shows apparent arrestin affinity comparable to the full agonists DAMGO and etorphine. Compared to other agonists at the μ receptor, recruitment is delayed which suggests a slowed phosphorylation

kinetics of the μ receptor as described recently (Gillis et al., 2020). We also used etorphine which we have previously described as causing quasi-irreversible arrestin binding (McPherson et al., 2010). However, in the experiments described here, we could observe arrestin dissociation from the μ receptor upon etorphine washout. The main difference to our previous experiments is that we used a tenfold lower concentration of etorphine in the present study. It is possible that the lack of dissociation in our previous experiments was caused by a residual low concentration of etorphine that was not washed away by the perfusion system. The dissociation kinetics of the μ receptor–arrestin complex triggered by washout of DAMGO and morphine are in the same range as those determined with a μ receptor biosensor in vitro (Livingston, Mahoney, Manglik, Sunahara, & Traynor, 2018) (they report 23 s for DAMGO and 21 s for morphine whereas we find dissociation half-lives of about 37 s) whereas our dissociation kinetics for the μ receptor–arrestin complex formed by etorphine are approximately 10-fold slower (27 s reported by Livingston, Mahoney, Manglik, Sunahara, & Traynor, 2018, compared to approximately 300 s here).

The physiological, let alone clinical, significance of class A versus class B GPCR behaviour has not been investigated. If there is a relevant difference, our study shows that class A behaviour of a GPCR cannot simply be converted into class B behaviour by increasing the residency time of an orthosteric agonist.

Taken together, our study reveals that the ligand off-rate does not influence arrestin–receptor interactions or the co-trafficking of arrestin-3 with GPCRs and points to the importance of receptor phosphorylation for both of these processes. In addition, we demonstrate that dual-colour FRAP measurements are more suitable than FRET experiments to assess arrestin–receptor interactions induced by different ligands.

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AUTHOR CONTRIBUTIONS

NM and CK designed the experiments. NM, LMGP, AF and CK performed the experiments. NM and CK wrote the manuscript. MB and CK supervised the project. All authors read and edited the final manuscript.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

DECLARATION OF TRANSPARENCY AND SCIENTIFIC RIGOUR

This Declaration acknowledges that this paper adheres to the principles for transparent reporting and scientific rigour of preclinical research as stated in the BJP guidelines for Design and Analysis, and as recommended by funding agencies, publishers and other organizations engaged with supporting research.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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