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Sustained wash-resistant receptor activation responses of GPR119 agonists

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GSK-1292263 (PubChem CID: 24996872)
oleoylethanolamine (PubChem CID: 2583454)
AZ1 (PubChem CID: 51030901)
AZ2 (PubChem CID: 51029876)
AZ3 (PubChem CID: 56643412)
ARN-II (PubChem CID: 16036825)

ABSTRACT

G protein-coupled receptor 119 (GPR119) is involved in regulating metabolic homoeostasis, with GPR119 agonists targeted for the treatment of type-2 diabetes and obesity. Using the endogenous agonist oleoylethanolamide and a number of small molecule synthetic agonists we have investigated the temporal dynamics of receptor signalling. Using both a dynamic luminescence biosensor-based assay and an endpoint cAMP accumulation assay we show that agonist-driven desensitization is not a major regulatory mechanism for GPR119 despite robust activation responses, regardless of the agonist used. Temporal analysis of the cAMP responses demonstrated sustained signalling resistant to washout for some, but not all of the agonists tested. Further analysis indicated that the sustained effects of one synthetic agonist AR-231,453 were consistent with a role for slow dissociation kinetics. In contrast, the sustained responses to MBX-2982 and AZ1 appeared to involve membrane deposition. We also detect wash-resistant responses to AR-231,453 at the level of physiologically relevant responses in an endogenous expression system (GLP-1 secretion in GLUTag cells). In conclusion, our findings indicate that in a recombinant expression system GPR119 activation is sustained, with little evidence of pronounced receptor desensitization, and for some ligands persistent agonist responses continue despite removal of excess agonist. This provides novel understanding of the temporal responses profiles of potential drug candidates targetting GPR119, and highlights the importance of carefully examining the the mechanisms through which GPCRs generate sustained responses.

1. Introduction

GPR119 is a class A G protein-coupled receptor (GPCR) that signals predominantly through cAMP via the stimulatory G-protein ($G\alpha_s$) (Lauffer et al., 2009; Syed et al., 2012). GPR119 demonstrates a highly localized pattern of expression within the gastrointestinal tract and pancreas (Lan et al., 2009; Lauffer et al., 2009; Soga et al., 2005) consistent with a role in metabolic function. Endogenous agonists for GPR119 are lipids such as oleoylethanolamine and 2-oleoyl glycerol, further supporting this role (Cornall et al., 2013; Hansen et al., 2011; Syed et al., 2012). Indeed, stimulation of GPR119 is strongly linked with glucose homoeostasis with a direct effect on insulin release from pancreatic β-cells, as well as an indirect effect on insulin signalling via glucagon-like peptide 1 (GLP-1) release from enteroendocrine cells (Chu et al., 2008, 2007; Soga et al., 2005). For these reasons, the development of GPR119 agonists has attracted considerable interest as potential pharmacotherapies for type-2 diabetes and obesity (Cornall et al., 2013; Kang, 2013; Ohishi and Yoshida, 2012). A number of chemically diverse synthetic GPR119 agonists have been developed and characterized (Brocklehurst et al., 2011; Scott et al., 2012; Semple et al., 2008, 2011), with four having advanced to clinical trials (Ohishi and Yoshida, 2012).

Typically, upon chronic agonist exposure GPCRs undergo desensitization and internalization resulting in a loss of receptor responsiveness over time (Drake et al., 2006; Kelly et al., 2008). However, not all GPCR systems conform to this model of acute agonist-mediated regulation. Some receptors have been reported not to internalize and/or desensitize (Callander et al., 2009; Jensen

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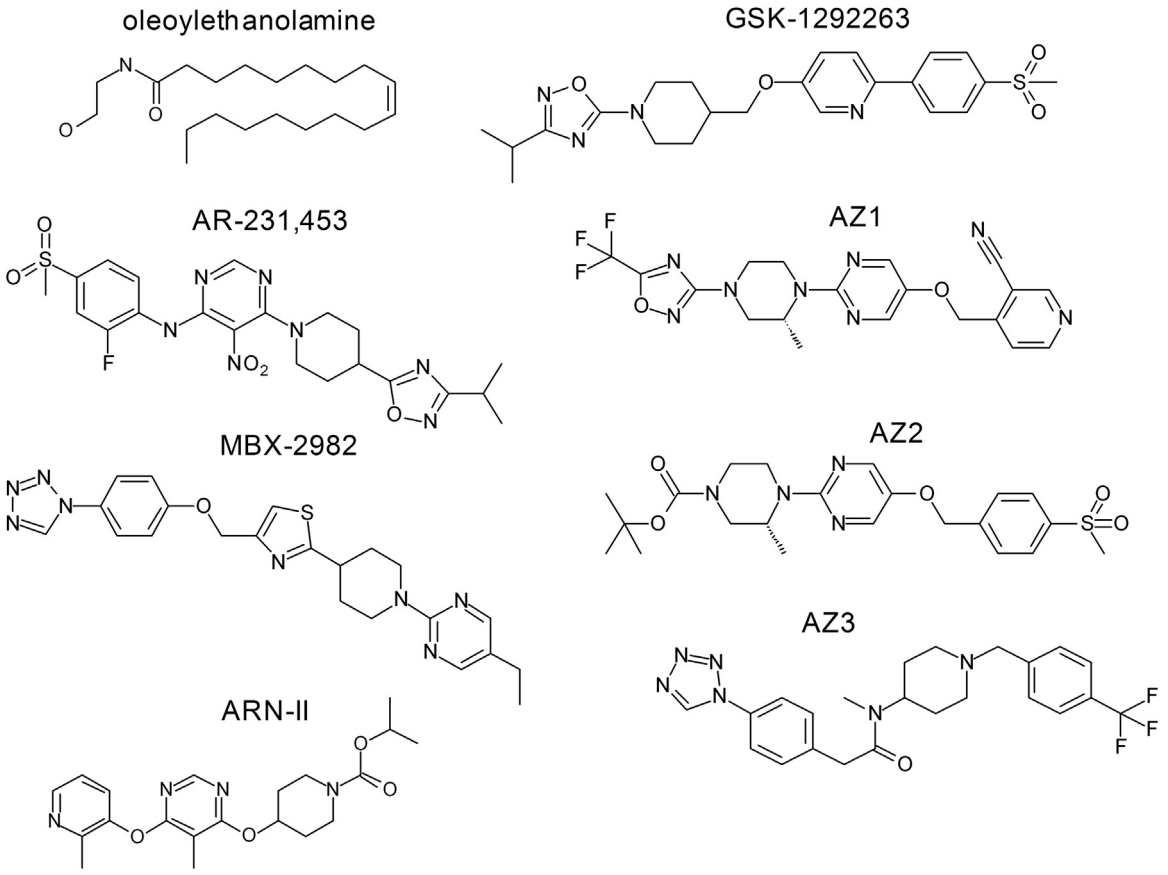

Fig. 1. Chemical structures of the agonists used in this study.

et al., 2013; Willars et al., 1999). Furthermore it has recently been demonstrated that when conventional desensitization mechanisms do not exert a major regulatory influence, sustained receptor responses can be maintained with persistent agonist–receptor interactions. Functionally, the consequences of this mechanism have been defined as sustained receptor activation even after removal of excess agonist (Calebiro et al., 2009; Ferrandon et al., 2009; Haskell-Luevano et al., 1996; Mullershausen et al., 2009). Whilst some evidence for GPR119 desensitization has been previously reported (Kumar et al., 2014; Lauffer et al., 2009; Zhang et al., 2014), a comprehensive characterization of desensitization of GPR119 cAMP signalling has not yet been conducted. This key gap in our understanding of GPR119 biology may be especially pertinent to observations of tachyphylaxis and lack of efficacy of GPR119 agonists in the clinic (Kang, 2013).

In the present study we have investigated the chronic effects of the endogenous GPR119 agonist oleoylethanolamide as well as the synthetic agonists AR-231,453, GSK-1292263, MBX-2982, AZ1, AZ2, and AZ3, as described previously (Brocklehurst et al., 2011; Scott et al., 2012; Semple et al., 2008) and shown in Fig. 1. Using a recombinant expression system we find that desensitization is not a major regulatory influence. Additionally, some agonists demonstrate sustained wash-resistant receptor activation, and we provide experimental evidence to support a role for slow dissociation kinetics or membrane deposition in mediating these responses.

2. Materials and methods

2.1. Materials

Cell culture materials were from Invitrogen. HTRF Dynamic cAMP assay kit was from CisBio. Assay plates were from Greiner Bio-One. GloSensor 22F plasmid DNA, GloSensor cAMP reagent, and Profection® mammalian transfection kit were from Promega. GLP-1 ELISA was from Millipore. All synthetic GPR119 agonists were synthesized at AstraZeneca. All other compounds and reagents were from Sigma-Aldrich (UK).

2.2. Cell culture and transfection

HEK-293 cells stably expressing human GPR119 (HEK-GPR119) were maintained in Dulbecco's Modified Eagle Medium supplemented with 25 mM D-glucose, 4 mM L-alanyl-L-glutamine, 1 mM sodium pyruvate, 10% foetal calf serum and 800 μg/ml G-418 in an atmosphere of 5% CO_2 at 37 °C. GLUTag cells were maintained in Dulbecco's Modified Eagle Medium supplemented with 5.6 mM D-glucose, 4 mM L-glutamine, 1 mM sodium pyruvate, 10% foetal calf serum, 100 μg/ml streptomycin, and 100 I.U/ml penicillin. Cells were transfected by the calcium phosphate method using the Profection® kit (Promega), as per the manufacturer's instructions. In short, cells were plated onto 25 cm^2 flasks the day prior to transfection to achieve approximately 75% confluency. Culture media was replaced 3 h before addition of DNA/$CaPO_4$ complexes (30 μg DNA per flask) and then cells were incubated for a further 20 h at 37 °C. Culture media was replaced again before experimentation 5–10 h later.

2.3. Dynamic GloSensor cAMP assay

2.3.1. Agonist responses

HEK-GPR119 cells were transfected with GloSensor 22F plasmid (Promega) and used for dynamic cAMP measurements 24–30 h later. Cell suspensions were made by dislodging the cells using PBS

wash and Accutase treatment followed by resuspension in culture media. Cells were then washed twice by pelleting through centrifugation (300*g*, 5 min) and resuspension in assay buffer (Hank's Balanced Salt Solution supplemented with 20 mM HEPES and 0.01% fatty acid free BSA, pH 7.4). Cells were then counted and diluted to 600,000 cells/ml in buffer, before GloSensor cAMP reagent was added (2% v/v) and equilibrated with the cells for 2 h at 20 °C with periodic mixing. 50 μl/well of cells were added to white-bottomed 384 well plates (30,000 cells/well) in triplicate and baseline luminescence was measuring using an Envision plate-reader. 5 μl of test compound (serially diluted in DMSO and then diluted 1:100 in assay buffer to obtain × 10 concentrated solution) was manually added to the assay wells to achieve the stated final concentration. Plates were incubated at 20 °C with luminescence read at regular intervals to detect dynamic cAMP changes over time within the same wells. cAMP responses at each time-point were expressed as fold over control (vehicle-treated cells).

2.3.2. GloSensor desensitization assay

Cells prepared as above were incubated with agonist (at indicated concentrations) for 10 min at 20 °C. cAMP levels were then measured by reading luminescence to determine maximal control stimulation. In the continued presence of agonist, cells were then incubated at 37 °C for 60 min, subsequently re-equilibrated to 20 °C (15 min), and cAMP levels then measured once more to determine responsiveness after chronic agonist exposure in the same population of cells.

2.4. Endpoint HTRF cAMP assay

2.4.1. Preparation of cells

HEK-GPR119 cells were grown to confluency in flasks, and cell suspensions were made by dislodging cells using PBS wash and accutase treatment followed by resuspension in culture media. Cells were then washed twice by pelleting through centrifugation (227*g*, 7 min, 20 °C) and resuspension in warm assay buffer (Hank's Balanced Salt Solution supplemented with 20 mM HEPES and 0.01% fatty acid free BSA, pH 7.4), with a 5 min incubation at 37 °C after the second wash. Cells were then counted and diluted to 200,000 cells/ml in warm assay buffer.

2.4.2. cAMP capture and measurement

In cells prepared as above, cAMP accumulation was captured by addition of the phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine (IBMX) at 1 mM with or without co-incubation of test agonist for 45 min (unless otherwise stated) at 20 °C. Cellular cAMP was then measured using the HTRF Dynamic competitive immunoassay kit (CisBio), following the manufacturer's instructions. In short, 10 μl/well of cells were added to black-bottomed 384 well plates (2000 cells/well) in quadruplicate before addition of 5 μl/well of dye-labelled cAMP diluted in lysis buffer, then addition of 5 μl/well of anti-cAMP antibody labelled with cryptate diluted in lysis buffer, followed by incubation for 1–16 h (20 °C). To construct a standard curve, samples of known cAMP concentration were also incubated in the same way. Fluorescence was then read at 665 and 620 nm using an Envision plate-reader, and a 665/620 ratio derived. Cellular cAMP concentration was calculated by comparing these values (normalized to background fluorescence) to the standard curve for each plate.

2.4.3. "Chronic incubation/washout" protocol

To investigate the effects of chronic treatment with GPR119 agonists followed by removal of excess ligand by washing, the "chronic incubation/washout" protocol was applied: 12 ml of cells prepared as above (Section 2.4.1) were aliquoted into 15 ml tubes and test agonist was added (1:1000 dilution of compound serially diluted in DMSO) before incubation at 37 °C for 60 min (in the absence of IBMX), with periodic mixing. To remove excess ligand, cells were washed three times by pelleting through centrifugation (227*g*, 7 min, 4 °C) and resuspension in ice-cold buffer. Cells were then resuspended in ice-cold buffer at 200,000 cells/ml and cAMP capture and measurement was performed as described above. Where indicated, a smaller volume of agonist pre-treatment buffer was used (0.05 ml).

2.4.4. Infinite dilution protocol

As an alternative approach to remove excess ligand after chronic pre-treatment, infinite dilution was used. This involved chronically treating the cells with agonists (as above; Section 2.4.3) followed by centrifugation (227*g*, 7 min, 20 °C) and removing the bulk of the drug-containing buffer, followed by another centrifugation step (227*g*, 4 min, 20 °C) and carefully removing the remaining liquid. Cells were then resuspended in 12 ml fresh buffer (20 °C) at approximately 200,000 cells/ml.

2.5. GLP-1 secretion in GLUTag cells

GLUTag cells were seeded onto poly-D lysine coated 96 well plates at 8000 cells/well and grown for approximately 48 h. Cells were incubated in assay buffer (Hank's Balanced Salt Solution supplemented with a final concentration of 10 mM (D)-glucose, 20 mM HEPES and 0.1% BSA, pH 7.4) containing test ligand (1:1000 dilution of compound serially diluted in DMSO) for 1 h at 37 °C. Cells were then washed by four exchanges of fresh buffer, before exposure to buffer containing the diepetidyl peptidase IV inhibitor K-579 (100 nM) ± test compound for 2 h at 37 °C. Supernatant was then removed and GLP-1 concentration measured by the Millipore ELISA kit, as per the manufacturers instructions. Fluorescence was measured at abs/em of 355/460 nm using a Tecan Safire, and samples compared to a standard curve. Responses were normalized to signal in control-treated cells.

2.6. Data analysis and statistics

Graphs were constructed and statistical analysis performed using GraphPad Prism 4. Data are expressed as mean of a least three separate experiments performed in triplicate (GloSensor assay) or quadruplicate (Endpoint assay) plus and minus standard error of mean. *, **, and *** indicate $P < 0.05$, 0,01 and 0.001, respectively with the indicated statistical test. ANOVA used one-way ANOVA with Bonferroni post test. Concentration–response analysis used the sigmoidal dose–response (variable slope) function.

3. Results

3.1. GPR119 responses to the endogenous agonist oleoylethanolamide

To investigate GPR119 responses we used HEK293 cells stably expressing the human GPR119 receptor (HEK-GPR119) as a model system. Two complementary cAMP assays were used to monitor receptor activation responses in these cells to more directly probe specific aspects of chronic agonist effects. Firstly, the GloSensor assay allowed the temporal analysis of cellular cAMP changes to be measured in the same population of cells, supporting the characterization of dynamic changes in receptor function with continued agonist treatment (without the inclusion of a phosphodiesterase inhibitor). The endpoint assay was used to measure total cellular cAMP accumulation over a single, defined time-

Table 1
pEC50 values derived from concentration–response analysis in different GPR119 cAMP assays. All values represent pEC_{50} values ± standard deviation ($n=3$–6) derived from sigmoidal concentration response using nonlinear regression with variable slope. GloSensor responses were measured for 40 min at 20 °C (as shown in Figs. 2A and 3A). Endpoint method 1 measured responses with 45 min agonist plus IBMX incubation at 20 °C (Fig. 2A). Endpoint method 2 measured responses with 60 min agonist incubation at 37 °C plus an additional 45 min plus IBMX at 20 °C (Fig. 5 A–D). Sustained responses were measured after 60 min agonist incubation at 37 °C followed by multiple washing stages and then incubation with IBMX for 45 min at 20 °C (Fig. 5 A–D). "nt" indicates curves that were not tested.

Agonist	GloSensor	Endpoint method 1	Endpoint method 2	Sustained response
Oleoylethanolamide	5.94 ± 0.05	6.72 ± 0.17	nt	nt
AR-231,453	8.03 ± 0.13	7.78 ± 0.09	8.93 ± 0.17	8.67 ± 0.11
MBX-2982	8.33 ± 0.11	8.12 ± 0.12	8.79 ± 0.12	7.03 ± 0.13
GSK-1292263	7.84 ± 0.10	nt	nt	nt
AZ1	8.64 ± 0.10	8.50 ± 0.09	8.61 ± 0.09	6.63 ± 0.17
AZ2	7.75 ± 0.10	nt	nt	nt
AZ3	7.16 ± 0.05	nt	nt	nt

course within a population of cells by inclusion of a phosphodiesterase inhibitor (1 mM IBMX), and supported the characterization of receptor function after ligand pre-exposure followed by washout.

Initially, we investigated cAMP responses upon GPR119 stimulation with the endogenous agonist oleoylethanolamide. HEK-GPR119 cells transiently expressing GloSensor were incubated with a range of concentrations of oleoylethanolamide for 40 min at 20 °C. Concentration–response analysis derived a pEC_{50} of 5.94 ± 0.05 (Table 1) and a maximal response of 4.05 ± 0.45 fold over basal ($n=3$) (Fig. 2A).

Use of the GloSensor assay permitted measurement of dynamic changes in cAMP over the time-course of agonist stimulation (Fig. 2B). At the maximal concentration of 30 μM there was a 4.35 ± 0.73 fold increase in cAMP after 5 min exposure, and this remained stable throughout the experiment. Responses to all concentrations of oleoylethanolamide tested demonstrated a similar activation profile; there was no significant loss of cAMP signal over the time-course of the experiment ($P>0.05$; ANOVA; $n=3$; comparing responses at 5 min to those thereafter).

These stable cAMP responses are consistent with a lack of GPR119 desensitization upon oleoylethanolamide stimulation. However, the measurements were made at a non-physiological temperature which may influence normal cellular processes of desensitization and internalization. Therefore, as a positive control for putative GPCR desensitization responses in these cells, we investigated the temporal profile of endogenous prostaglandin receptors signalling (Fig. 2C). Upon stimulation with 10 μM prostaglandin E_2 this receptor demonstrated a significant loss of signal over time ($P<0.05$ or 0.01; ANOVA comparing responses after 5 min with those subsequent; $n=3$) consistent with a fully intact desensitization mechanism. Importantly, this PGE desensitization occurred despite a lower level of cAMP generation (Fig. 2C) compared to oleoylethanolamide responses (Fig. 2B) and therefore does not appear to be a function of the magnitude of cAMP generated.

Further analysis of dynamic oleoylethanolamide responses at 37 °C demonstrated no loss of signal with time (Sup. Fig. S1). However, the detection of robust and reproducible luminescence signals were challenging at this temperature. To confirm the lack of desensitization with GPR119 at physiological temperature, we designed an experiment that allowed measurement of GloSensor cAMP responses at 20 °C before and after chronic incubation at 37 °C in the continued presence of agonist (GloSensor desensitization assay). In this experiment, responses to oleoylethanolamide (5 μM) were not lost after chronic exposure, (Fig. 2D), indicating that no desensitization occurred even at physiological temperature. In fact, there was a small but significant increase ($P<0.05$; ANOVA; $n=3$) in cAMP levels comparing the before and after responses to oleoylethanolamide. In marked contrast, prostaglandin E_2 (10 μM) responses demonstrated robust desensitization with cAMP signals significantly reduced ($P<0.001$; ANOVA; $n=3$; Fig. 2D) after chronic exposure.

To further confirm these effects, an endpoint cAMP assay was also used to investigate GPR119 responses to oleoylethanolamide. Concentration–response analysis in this assay after incubation with oleoylethanolamide in the presence of IBMX for 45 min at 20 °C generated a pEC_{50} of 6.72 ± 0.17 M (Fig. 2A; Table 1). This concentration–response curve is somewhat different to the GloSensor assay in both its potency (6.03 fold shift in EC_{50}) and profile (slight "bell-shaped").

To corroborate a lack of oleoylethanolamide-mediated GPR119 desensitization with the endpoint assay, we assessed receptor responsiveness after chronic pre-treatment with a maximally stimulating concentration of oleoylethanolamide (1 μM) at 37 °C using the "chronic incubation/washout" protocol (described in Section 2). Compared to control cells, no loss of acute responsiveness was observed after chronic pre-treatment with a maximal concentration of oleoylethanolamide ($P>0.05$; ANOVA; $n=3$), consistent with no desensitization (Fig. 2E). In oleoylethanolamide pre-treated cells without subsequent re-stimulation, cAMP levels were not significantly different ($P>0.05$; ANOVA; $n=3$) to basal cAMP levels in control cells, indicating that oleoylethanolamide was effectively removed by washing. For comparison with the GloSensor assay, we also tested the effect of chronic pre-treatment with 5 μM oleoylethanolamide (which is supramaximal in the endpoint assay but submaximal in the GloSensor assay). Acute cAMP responses were significantly lower than in oleoylethanolamide pre-treated cells than in control cells ($P<0.001$; ANOVA; $n=4$), indicating desensitization upon chronic exposure to this concentration of oleoylethanolamide (Fig. 2F). Therefore, it appears that at high concentrations there is an assay-specific observation of GPR119 desensitization by oleoylethanolamide, although importantly in both assays no desensitization is observed at maximally stimulating concentrations (relevant to that assay). PGE responses also demonstrated robust desensitization in the endpoint assay (Sup. Fig. S2), indicating its applicability to measure GPCR desensitization responses.

3.2. Acute GPR119 responses and lack of GPR119 desensitization upon chronic exposure to synthetic agonists

Since GPR119 did not desensitize upon stimulation with an endogenous agonists, we also wished to investigate whether this was the case with a panel of synthetic agonists (AR-231,453, MBX-2982, GSK-1292263, AZ1, AZ2, and AZ3; Fig. 1). We therefore first characterized the acute effects of these ligands by performing concentration–response analysis in the GloSensor assay (40 min incubation at 20 °C) (Fig. 3A). Derived pEC_{50} values are summarized in Table 1. Time-courses of dynamic cAMP responses to these agonists at 20 °C (Sup. Fig. S3) or 37 °C (Sup. Fig. S4) showed stable responses, consistent with little desensitization at maximally stimulating concentrations. Concentration–response analysis was also determined for some agonists using the endpoint assay after 45 min incubation with IBMX (Table 1), and was found to be largely consistent with GloSensor.

We next directly investigated whether the synthetic agonists could cause desensitization of GPR119 responses. Chronic exposure at 37 °C of all six agonists at a supramaximal concentration of 1 μM using the GloSensor desensitization assay did not cause any significant loss of response (Fig. 3B; $P>0.05$; ANOVA; $n=3$), indicating an apparent lack of desensitization.

Additionally, we investigated desensitization with these ligands (at 1 μM) in "chronic incubation/washout" experiments using the endpoint assay. Comparing acute re-stimulation responses in

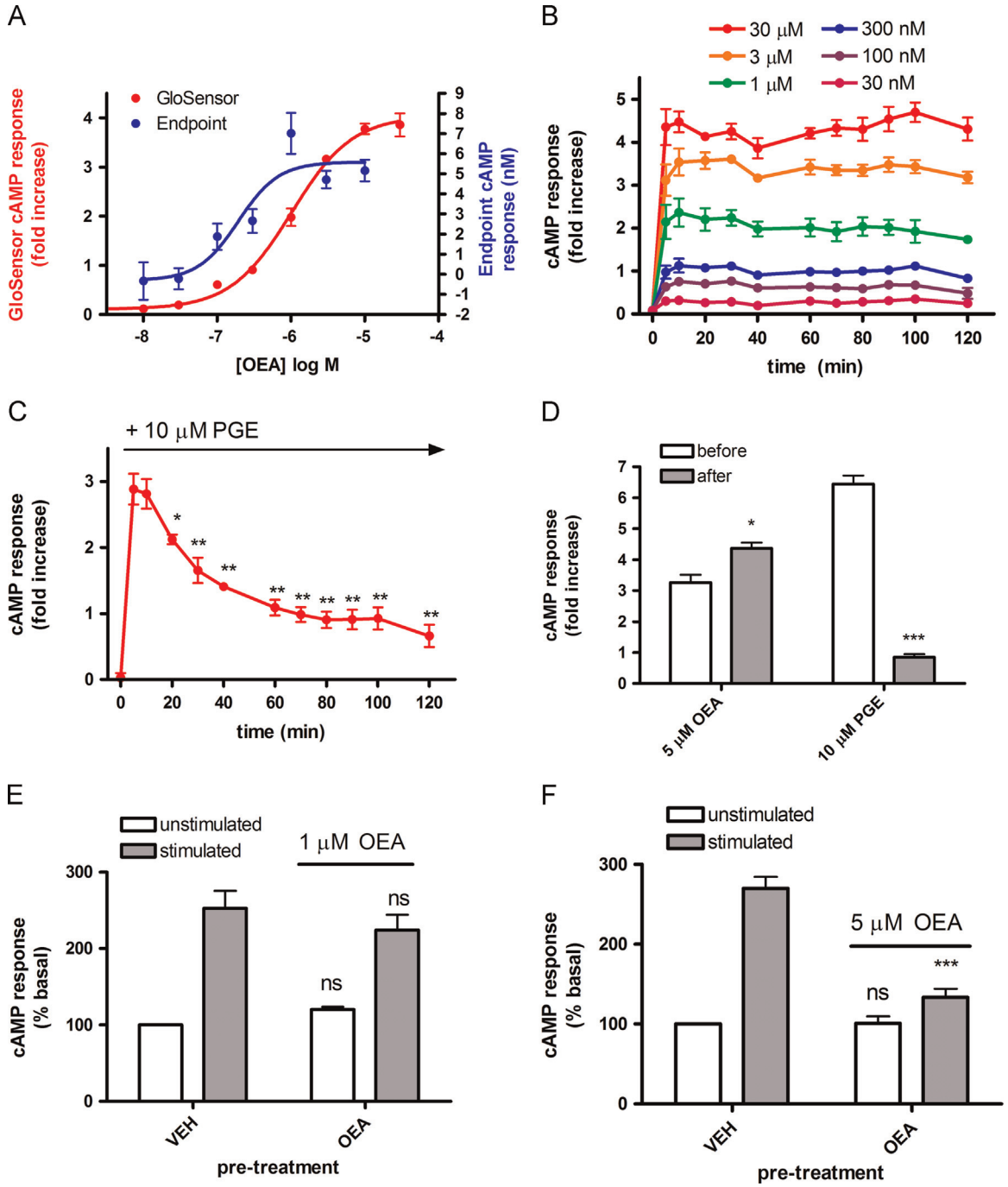

Fig. 2. GPR119 responses to stimulation by oleoylethanolamide. (A) Concentration–response analysis of oleoylethanolamide in the GloSensor (red curve) and endpoint (blue curve) cAMP assays. In the GloSensor assay HEK-GPR119 cells transiently transfected with GloSensor plasmid were incubated with the indicated concentrations of oleoylethanolamide for 40 min at 20 °C. In the endpoint assay, HEK-GPR119 cells were incubated with oleoylethanolamide and IBMX for 45 min at 20 °C. (B) Time-courses of oleoylethanolamide responses using the GloSensor assay. Cells were incubated with the indicated concentrations of oleoylethanolamide at 20 °C and cAMP responses were measured at the indicated time-points. Shown are representative concentrations of the full response curve presented in A. (C) Time-course of prostaglandin E_2 responses using the GloSensor assay. HEK-GPR119 cells transiently expressing GloSensor plasmid were incubated with 10 μM prostaglandin E_{22} at 20 °C and cAMP responses measured over time. (D) GloSensor desensitization assay using r. Cells were incubated with 5 μM oleoylethanolamide or 10 μM prostaglandin E_2 for 10 min at 20 °C and cAMP levels measured (before; white bars). In the continued presence of agonist, cells were then incubated at 37 °C for 60 min before re-equilibration to 20 °C and cAMP levels measured again (after; grey bars). (E, F) Desensitization assay using the endpoint cAMP assay. HEK-GPR119 cells were incubated with 1 μM (E) or 5 μM (F) oleoylethanolamide or vehicle control (VEH) for 60 min at 37 °C before excess ligand was removed by washing. Cells were then re-stimulated with the same concentration of oleoylethanolamide (stimulated; grey bars) or vehicle control (unstimulated; white bars) in the presence of IBMX for 45 min at 20 °C. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

control and agonist pre-treated cells, no significant difference ($P > 0.05$; ANOVA; $n = 3–6$) was observed for all agonists tested, indicating no loss of responsiveness after chronic agonist treatment.) (Fig. 3C). In summary, no evidence for GPR119 desensitization was found for all the agonists tested.

3.3. Sustained wash-resistant cAMP responses to some GPR119 agonists

As GPR119 agonist responses are stable over time, factors that prolong agonist/receptor interactions may lead to continual

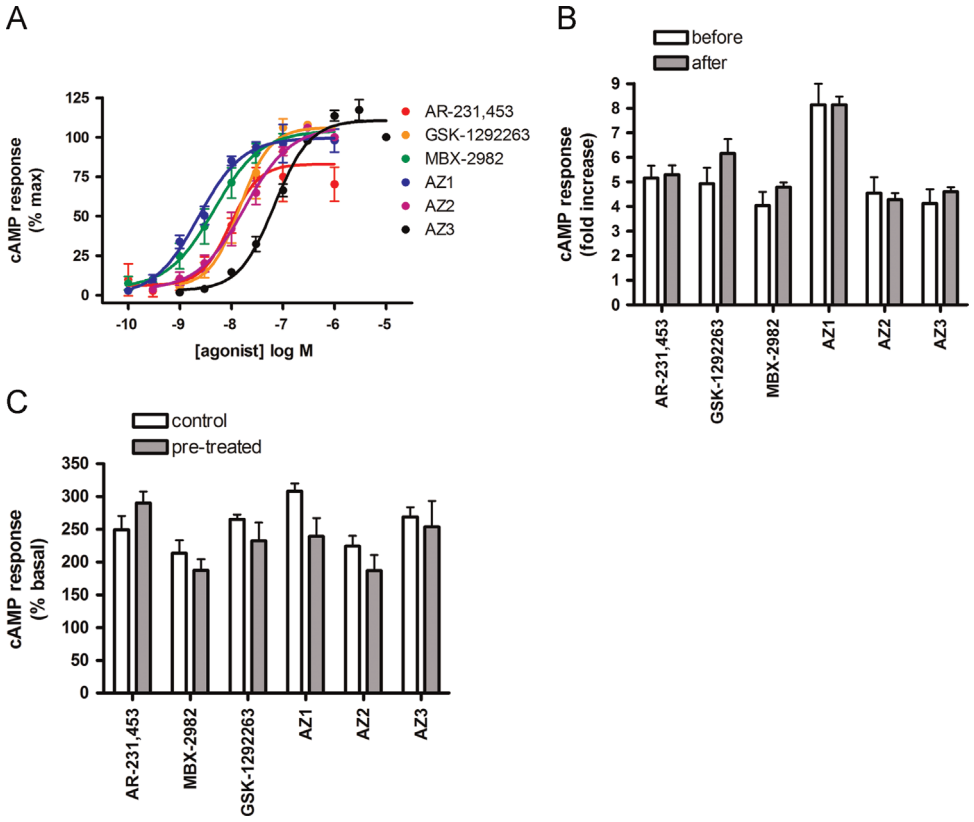

Fig. 3. GPR119 responses to stimulation by synthetic agonists. (A) Concentration–response analysis of AR-231,453 (red), GSK-1292263 (orange), MBX-2982 (green), AZ1 (blue), AZ2 (indigo), and AZ3 (black) using the GloSensor cAMP assay with a 40 min incubation at 20 °C. (B) GloSensor desensitization assay. Cells were incubated with 1 μM of the indicated agonist for 10 min at 20 °C and cAMP levels measured (before; white bars). In the continued presence of agonist, cells were then incubated at 37 °C for 60 min before re-equilibration to 20 °C and cAMP levels were measured again (after; grey bars). (C) Desensitization assay using the endpoint cAMP assay. Cells were incubated with vehicle control (white bars) or 1 μM of the indicated agonist (grey bars) for 60 min at 37 °C before excess ligand was removed by washing. Cells were then re-stimulated with 1 μM of the same agonist and responses in agonist pre-treated cells expressed as a percentage of basal cAMP levels. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

activation even after removal of free ligand by washing. Therefore, we investigated if cAMP responses evoked by GPR119 agonists were resistant to washing and thus sustained in nature. In the same "chronic incubation/washout" experiments as described above (Section 2.4.3), sustained responses were identified by an increase in cAMP levels in cells that were pre-treated with agonist but did not experience subsequent re-stimulation with agonist; i.e. a residual effect of the agonist after washing (Fig. 4A). In cells pre-treated with AR-231,453, MBX-2982, or AZ1 (1 μM) in "chronic incubation/washout" experiments, cAMP accumulation captured by IBMX inclusion was significantly increased compared to control cells ($P < 0.01$; ANOVA; $n = 3–6$) despite extensive washing to remove excess agonist. In contrast, unstimulated cAMP levels in cells pre-treated with GSK-1292263, AZ2, or AZ3 (1 μM) were not different to control, indicating that washing had successfully removed the effect of these ligands. Compared to the acute cAMP responses evoked by AR-231,453, MBX-2982, and AZ1 in control cells, the sustained responses after "chronic incubation/washout" were high at 101.38 ± 25.04%, 84.62 ± 20.22%, and 51.75 ± 18.61% of acute, respectively ($n = 3–6$). Therefore, it appears that maximal or near-maximal cAMP generation is achieved by prior exposure to these agonists, despite removal of excess ligand by washing.

To directly assess whether these sustained wash-resistant cAMP responses were caused by continual activation of the receptor after agonist washout we investigated the potential contribution of newly synthesized cAMP to the total signal by monitoring the temporal profile of cAMP accumulation in the presence of IBMX after agonist washout. Cells pre-treated with 1 μM AR-231,453 or vehicle control (0.1% DMSO) in "chronic incubation/washout" experiments were incubated post-washout for 60 min at 20 °C in the presence of IBMX to enable the temporal profile of cAMP accumulation to be measured (Fig. 4B). Low cAMP levels were observed in the absence of IBMX (6.94 ± 1.91% of maximal response generated in the presence of IBMX at 60 min). This indicates efficient degradation of cAMP via phosphodiesterase mechanisms in these cells that is prevented by IBMX. Upon addition of IBMX there was a robust time-dependent increase in cAMP accumulation in AR-231,453 pre-treated cells, whilst in control cells this increase was modest. These observations are consistent with de novo cAMP synthesis occurring over the time-course of IBMX addition and thus after removal of excess agonist. Therefore, it appears that the receptor is continually activated by AR-231,453 after it has been washed out.

In order to address whether the sustained effects of AR-231,453 were modulated specifically through the GPR119 receptor agonist binding, we investigated whether it was feasible to competitively antagonize the sustained responses driven by other GPR119 ligands. For this study we used AZ2 which represents a potent agonist (Table 1; Fig. 3A) that does not demonstrate a wash-resistant activation profile (Fig. 4A). The presence of saturating concentrations of AZ2 would be predicted to occupy the GPR119 binding site thereby competitively inhibiting the binding of other ligands during chronic pre-treatment. Pre-incubation with AZ2 (1 μM, 60 min, 37 °C) followed by treatment with 5 nM AR-231,453 in the continued presence of AZ2 (60 min, 37 °C), demonstrated that AZ2 could inhibit the ability of AR-231,453 to exhibit a sustained wash-resistant cAMP response (Fig. 4C). In cells pre-incubated with AZ2 compared to control cells, sustained cAMP levels

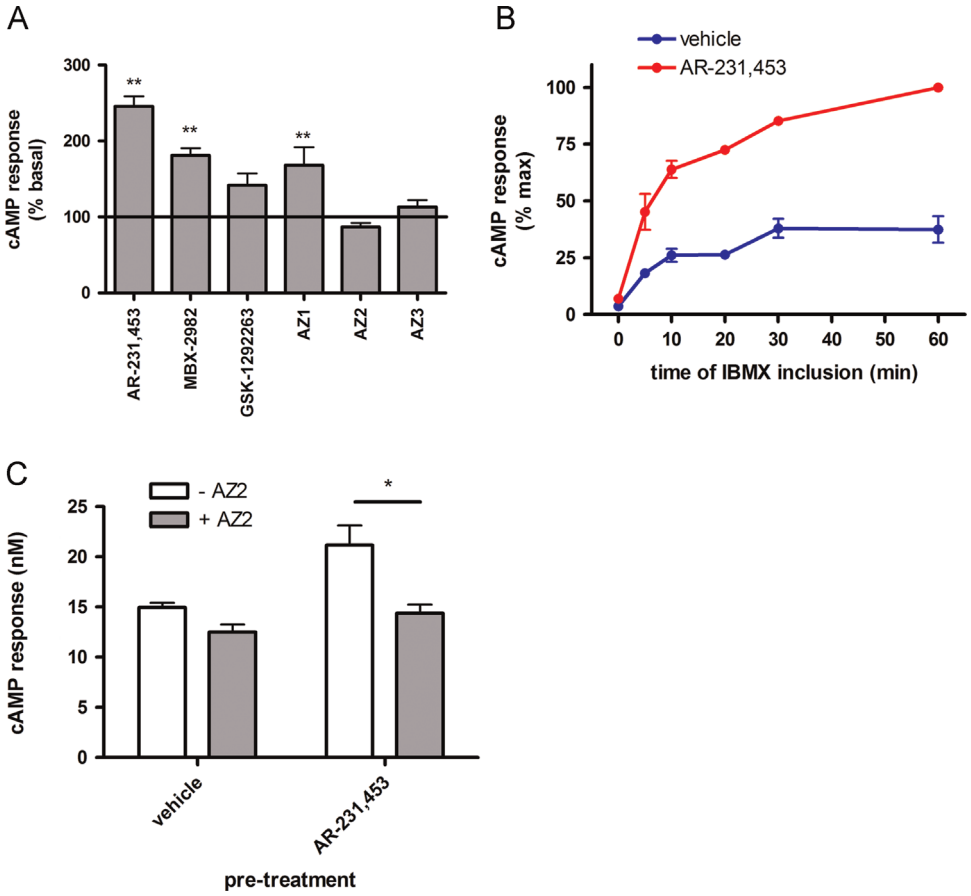

Fig. 4. Sustained cAMP responses evoked by GPR119 agonists. (A) "Chronic incubation/washout" experiments using the endpoint cAMP assay. Cells were chronically pre-treated with 1 μM of the indicated agonist for 60 min at 37 °C or with vehicle control. Excess ligand was then removed by washing and cells were subsequently incubated with IBMX for 45 min at 20 °C to measure unstimulated cAMP levels. Responses are expressed as percentage of basal (unstimulated cAMP levels in control cells) (B) Time-course of IBMX effects after removal of excess AR-231,453. Cells were pre-treated with or without AR-231,453 (1 μM, 60 min, 37 °C) and washed as above. Cells were then incubated for 60 min with IBMX included for the indicated times during this period. cAMP responses are expressed as a percentage of maximum (the AR-231,453effect with 60 min IBMX inclusion). (C) Competitive antagonism of sustained agonist effects. Cells were pre-incubated with (grey bars) or without (white bars) AZ2 (1 μM, 60 min, 37 °C) before subsequent chronic pre-treatment with AR-231,453 (5 nM, 60 min, 37 °C) or vehicle control. Excess ligand was removed by washing and cAMP was then captured in the presence of IBMX (45 min) without subsequent re-stimulation to detect sustained responses.

evoked by AR-231,453 (following the "chronic incubation/washout" protocol) were significantly reduced ($P<0.05$; ANOVA; $n=3$) and were comparable to basal levels of cAMP accumulation. These data suggest that the sustained response driven by AR-231,453 can be competitively inhibited by other GPR119 ligands, which is likely consistent with the response being mediated specifically by the same GPR119 binding site.

3.4. The stability and longevity of sustained GPR119 agonist responses

To gain further insight into the stability of the agonist/receptor interactions after washout we measured the concentration-dependency of the sustained responses to AR-231,453, MBX-2982 and AZ1 in "chronic incubation/washout" experiments using the endpoint assay and compared these to their acute stimulatory effects (as shown previously in Fig. 5A–C and Table 1 method 2). Under the same cAMP capture conditions, concentration–response curves were generated after either previous exposure to agonist followed by washout (sustained) or in the continued presence of agonist without washout (acute). Based on the assumption that concentration–response curves for acute and sustained responses are reflective of agonist-mediated receptor activation before and after washout, respectively, then the relationship between these curves will be dependent on how much agonist/receptor interaction is lost by washing (Table 1). AR-231,453 produced sustained responses in a similar concentration range to those observed with acute stimulation (a small 1.82 fold shift), with pEC_{50} values of 8.67 ± 0.11 and 8.93 ± 0.17, respectively. This suggests that very little binding is lost upon washout of this ligand. In marked contrast, AZ1 required higher concentrations to evoke a sustained response relative to acute activation (a 95.50 fold shift in the curves) with pEC_{50} values of 6.63 ± 0.17 and 8.61 ± 0.09, respectively. Likewise, a large but less severe shift in concentration responses (57.54 fold) was observed for MBX-2982 with respective sustained and acute pEC_{50} values of 7.03 ± 0.13 and 8.79 ± 0.12. This suggests that a large proportion of binding interactions with AZ1 and MBX-2982 are lost by washing as for each concentration applied at the beginning of the experiment there are less agonist activated receptors at end.

As an alternative approach, time-course experiments were conducted to examine the sustained effect duration of a single concentration of agonist after its rapid removal by infinite dilution. We calculated that a single wash step (as described by the infinite dilution protocol in methods) could effectively dilute the agonist when applied at maximally stimulating concentrations (30 nM; based on acute responses; Fig. 5A–C) to a concentration without a functional effect on receptor activation. This provided a means to more directly measure the time-course of sustained effects without complications of the time delay of multiple wash stages.

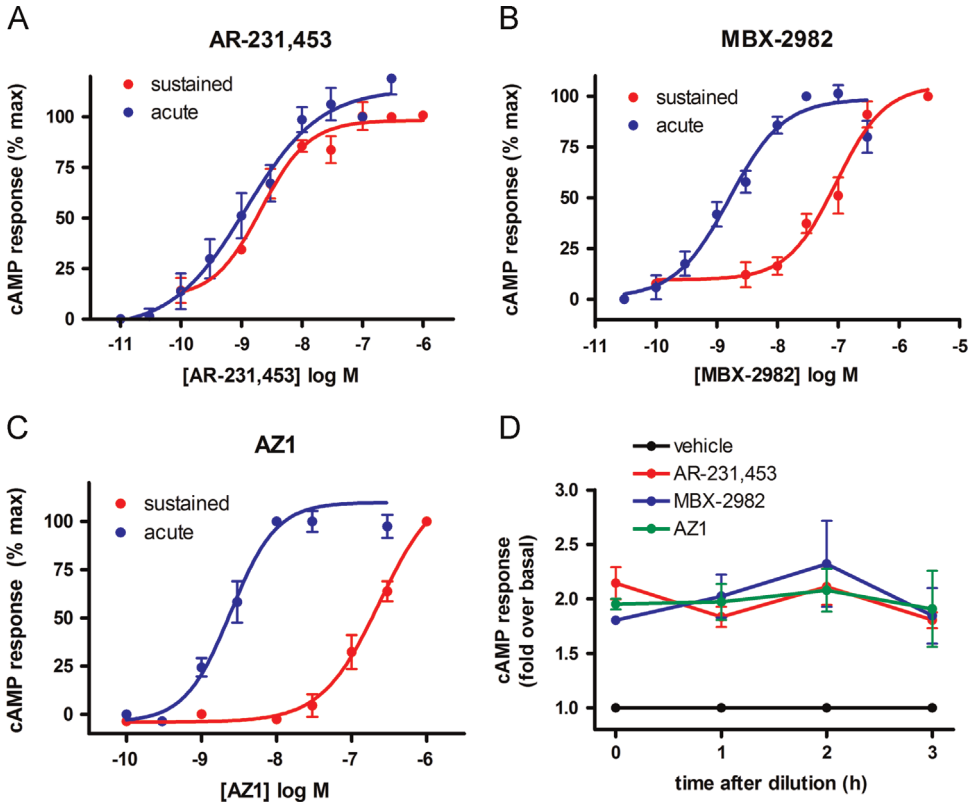

Fig. 5. Examining the longevity of wash-resistant cAMP responses to GPR119 agonists. (A–C) Concentration-dependent cAMP responses to AR-231,453 (A), MBX-2982 (B) and AZ1 (C) were measured in HEK-GPR119 cells. Acute responses (blue curves) were measured by incubation of the indicated agonist concentrations for 60 min at 37 °C before addition of IBMX for a further 45 min at 20 °C. Sustained responses (red curves) were measured by pre-treatment with a range of agonist concentrations for 60 min (37 °C), followed by washout of excess ligand and subsequent inclusion of IBMX for 45 min (20 °C). Responses are expressed as percentage of maximal (defined as the concentration giving the largest cAMP increase) (D) Infinite dilution experiments with GPR119 agonists to investigate the time-course of sustained effects. HEK-GPR119 cells were pre-treated with vehicle control (black), 30 nM AR-231,453 (red), MBX-2982 (blue) or AZ1 (green) for 60 min at 37 °C. Excess ligand was then removed by infinite dilution using a single wash step, and cells were incubated for the indicated times before addition of IBMX for a further 45 min to capture cAMP. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Suspension cells were pre-treated with AR-231,453, MBX-2982, or AZ1 (30 nM, 60 min, 37 °C) before agonist was removed by infinite dilution. Subsequently cells were incubated in drug-free buffer for 0, 1, 2, or 3 h at 20 °C before cellular cAMP levels were captured by the addition of IBMX and measured by the endpoint assay (Fig. 5D). AR-231,453, MBX-2982 and AZ1 pre-treated cells all demonstrated significantly elevated cAMP levels compared to control cells for up to 3 h after infinite dilution ($P < 0.05$ or 0.01; ANOVA; $n = 3$), indicating that sustained responses were maintained for at least this length of time.

3.5. *Evidence for a role of membrane deposition in sustained responses to MBX-2982 and AZ1*

The sustained wash-resistant GPR119 cAMP responses could be explained by either slow dissociation kinetics (a direct receptor-specific mechanism), rebinding (upon dissociation the ligand may be more likely to re-associate with the receptor rather than diffuse away and discontinue its influence (Vauquelin and Charlton, 2010)), and/or a membrane deposition (continual access of a ligand to the binding site due to plasma membrane accumulation in the immediate vicinity of the receptor). Given the relatively high log *D* for some of the compounds tested (Sup. Table S1) it was predicted that the potential would exist for them to become incorporated into the membrane.

Differences in the sustained responses of MBX-2982 and AZ1 (but not AR-231,453) between chronic washout and infinite dilution experiments provided the first clues that rebinding/membrane deposition may be important with these ligands. When the ligand is removed by infinite dilution, sustained receptor activation is maintained at a constant level for a number of hours (Fig. 5D), whereas responses were clearly lost with the "chronic incubation/washout" protocol (Fig. 5B and C). Indeed, we find that MBX-2982 and AZ1 concentrations incapable of causing a large sustained effect in the "chronic incubation/washout" experiments (30 nM; concentration–response analysis in Fig. 5B and C) can cause a robust and sustained response in infinite dilution experiments (Fig. 5D). Therefore, it appears that multiple washes over time ("chronic incubation/washout") are more effective at removing the effects of these agonists than single rapid wash step (infinite dilution), despite both approaches infinitely diluting the ligand. Consequently, the sustained responses to MBX-2982 and AZ1 are not entirely dependent on free concentration of the agonist in the surrounding buffer.

To more directly probe a role for such membrane interactions, we reduced the potential for deposition of agonist molecules into the membrane environment. By significantly reducing the number of molecules of agonist whilst maintaining the same agonist concentration as well as number of cells, this approach will limit the propensity for significant membrane binding effects (Teschemacher and Lemoine, 1999). Through a minor adaptation to the infinite solution protocol described previously we pre-treated cells with a greatly reduced volume of agonist-containing buffer at 0.05 ml instead of 12 ml (30 nM, 60 min, 37 °C) (Fig. 6A). Under these conditions MBX-2982 and AZ1 failed to exhibit a sustained wash-resistant response as cAMP accumulation captured after infinite dilution were not significantly different to control cells ($P > 0.05$; ANOVA; $n = 3$), indicating that the effect of these agonist

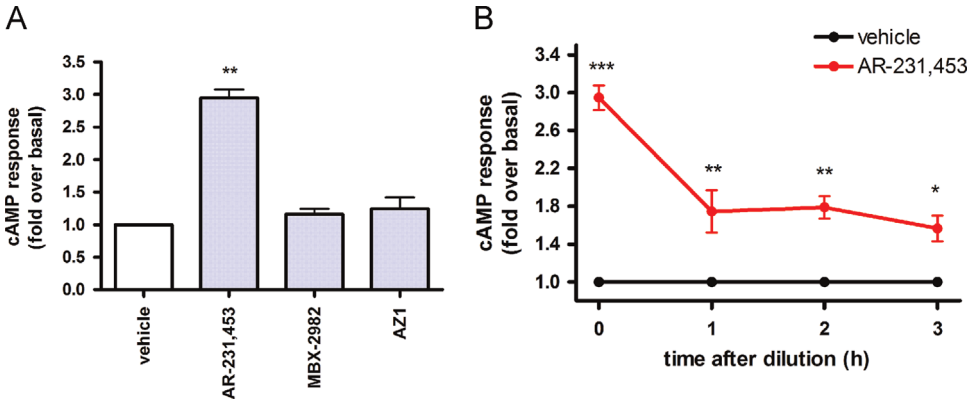

Fig. 6. Infinite dilution experiments with pre-treatment of GPR119 agonists in a small volume. (A) Cells were pre-treated with vehicle control, 30 nM AR-231,453, MBX-2982 or AZ1 in a small volume of buffer (0.05 ml) for 60 min at 37 °C. Excess ligand was then removed using infinite dilution by addition of a large volume of buffer, and then cells were immediately exposed to IBMX to capture cAMP (45 min). (B) Cells were pre-treated with vehicle control or 30 nM AR-231,453 in a small volume of buffer (0.05 ml, 60 min, 37 °C), and cells washed via infinite dilution by addition of fresh buffer. Cells were then incubated for the indicated times before addition of IBMX for a further 45 min to capture cAMP.

was immediately lost. In contrast, AR-231,453 was still able to evoke a sustained response with cAMP levels significantly higher than control ($P<0.01$; ANOVA; $n=3$). Moreover, when the longevity of these sustained effects of AR-231,453 were investigated (by incubating in drug-free buffer for a range of times after infinite dilution) it was observed that cAMP levels remained significantly raised for at least 3 h ($P<0.05$; ANOVA compared to control cells at each time-points; $n=3$) (Fig. 6B), comparable to responses in a large volume of buffer (Fig. 5D). These data are consistent with a requirement for membrane deposition in the sustained wash-resistant effects for MBX-2982 and AZ1, but not AR-231,453.

3.6. Evidence for a role of slow binding kinetics in sustained responses to AR-231,453

As discussed above, membrane deposition does not appear to drive the sustained signalling of AR-231,453. It is feasible that slow dissociation kinetics and/or rebinding play a role. In the absence of a suitable kinetic radioligand binding assay we indirectly assessed agonist dissociation at the level of functional cAMP responses. By measuring the rate of reversal of agonist responses by an antagonist we could estimate agonist off-rate because competitive displacement by antagonist requires agonist dissociation. As agonist responses are relatively stable over time (Figs. 2 and 3), this

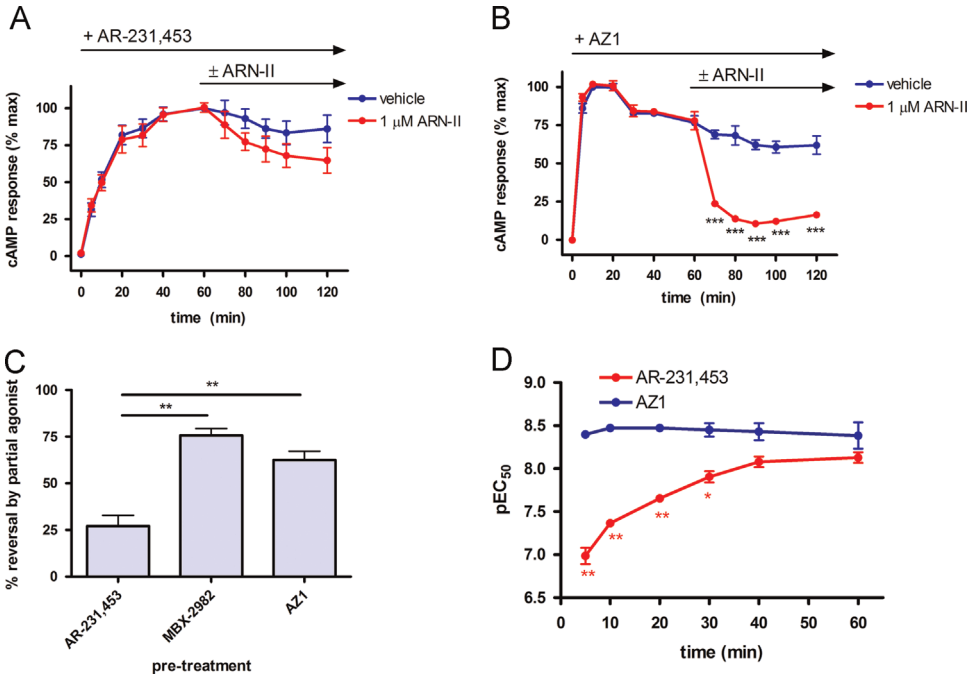

Fig. 7. Dynamic cAMP measurements to examine the binding kinetics of GPR119 agonists. (A, B) Antagonist-mediated reversal of GPR119 agonist mediated acute cAMP responses. HEK-GPR119 cells transiently expressing GloSensor were incubated with 30 nM AR-231,453 (A) or AZ1 (B) for 60 min before addition of 1 μM ARN-II (red lines) or vehicle control (blue lines) for a further 60 min, with cellular cAMP levels measured at the indicated times. cAMP responses are expressed as percentage of maximal responsiveness (at 10 and 60 min for AR-231,453 and AZ1, respectively). (C) Antagonist-mediated reversal of sustained cAMP responses using the endpoint assay. Cells were pre-treated with 30 nM AR-231,453, MBX-2982, or AZ1 (60 min, 37 °C) before washing by infinite dilution. Cells were then incubated with or without 1 μM ARN-II for 60 min before IBMX added for a further 45 min to capture cAMP. Normalized data are expressed as percentage reduction of cAMP responses by inclusion of ARN-II compared to control cells for each pre-treatment. (D) The effect of incubation time of AR-231,453 (red line) or AZ1 (blue line) on potency measurements was investigated. HEK-GPR119 cells transiently expressing GloSensor were incubated with a range of concentrations of AR-231,453 or AZ1 and cAMP levels were measured at the indicated times. pEC_{50} values were derived from the concentration–response curves analyzed at each time-point. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

provides a good response window with which to observe reversal responses. In the absence of a suitable neutral antagonist we used the weak partial agonist ARN-II (ligand 9a in Semple et al. (2012)).

Cells were incubated with AR-231,453 (30 nM, 60 min, 20 °C) before addition of 1 μM ARN-II or vehicle control (0.1% DMSO) for a further 60 min, and cAMP levels measured at regular intervals (Fig. 7A). Whilst ARN-II caused a small drop in cAMP levels evoked by AR-231,453 stimulation, this was not significantly different to control at all time-points ($P>0.05$; ANOVA; $n=3$). In marked contrast, when the receptor was activated with AZ1 (30 nM; approximately equipotent with applied AR-231,453 concentration), cAMP responses were rapidly and robustly reversed by ARN-II (Fig. 7B), with a significant reduction in cAMP levels compared to control after 10 min onwards exposure to ARN-II ($P<0.001$; ANOVA; $n=3$). Reversal was nearly complete with AZ1 with responses at $10.58\pm3.16\%$ of maximal after 30 min antagonist exposure. These findings are consistent with a much slower and less complete dissociation of AR-231,453 compared to AZ1. Moreover, they demonstrate that GPR119 cAMP responses measured using this assay can be rapidly reversed under appropriate circumstances and the sustained GPR119 responses observed (Figs. 2B, D, 3B and 7A) are not an experimental artefact.

Using the endpoint assay, we next investigated the ability of ARN-II to reverse the sustained responses of these ligands in "chronic incubation/washout" experiments. In cells pre-treated with AR-231,453, MBX-2982 or AZ1 (30 nM, 60 min, 37 °C), excess agonist was removed by infinite dilution and then incubated with or without ARN-II (1 μM, 60 min, 20 °C). Sustained responses to each agonist with and without subsequent ARN-II addition were compared to calculate a percentage reversal (Fig. 7C). The sustained response to AR-231,453 was reversed by $27.03\pm10.04\%$, whilst MBX-2982 and AZ1 were reversed to a significantly greater extent ($P<0.01$; ANOVA; $n=3$) to $75.70\pm6.34\%$ and $62.56\pm8.00\%$, respectively. These findings are consistent with slower dissociation kinetics in AR-231,453 promoting its sustained response, as little AR-231,453 is competitively displaced by ARN-II in this setting. Moreover, the more robust reversal of MBX-2982 and AZ1 responses suggest faster dissociation kinetics, which is consistent with membrane deposition being a prominent factor in their sustained response profile (the prolonged effects cannot be explained by a single long-lived binding event, but instead by multiple short-lived binding events that can be promoted by accumulation near the receptor).

To further assess the kinetic profiles of these agonists the influence of incubation time on potency was measured by performing concentration–response analysis at different time-points using the GloSensor assay. It would be expected that a system whereby the slow kinetics of receptor activation/deactivation played a significant role in the measurement potency would be more sensitive to the effects of incubation time. Fig. 7D demonstrates the relationship between pEC_{50} and time for AR-231,453 or AZ1 in HEK-GPR119 cells using the GloSensor assay format highlighting a significant shift of the pEC_{50} of AR-231,453from 6.99 ± 0.16 (102 nM) at 5 min to 8.13 ± 0.10 (7 nM) at 60 min. Potency measured for AR-231,453 was significantly higher at 60 min compared to 5, 10, 20 ($P<0.01$), and 30 ($P<0.05$) min (ANOVA; $n=3$). In direct contrast, no significant effect of time was observed for potency measurements of AZ1 (60 min vs. all other time-points; $P>0.05$; ANOVA; $n=3$). Moreover, little time effect on pEC_{50} was observed for MBX-2982 (not shown). These potency shifts with AR-231,453 are indicative of this ligand taking longer to reach equilibrium, and therefore having slower binding kinetics. Indeed dynamic traces of receptor activation observed in GloSensor experiments demonstrate slower responses for AR-231,453 compared to AZ1 (Fig. 7A and B).

3.7. Sustained responses in a physiologically relevant test system

We next investigated whether the observation of sustained wash-resistant signalling also occurs at the level of physiologically relevant functional responses in an endogenous cellular expression system. We therefore measured GLP-1 secretion in murine GLUTag cells, which has previously been characterized as a GPR119-mediated response (Lan et al., 2012). To confirm GPR119 responses, plated cells were exposed to agonists (1 μM) in the presence of the diepetidyl peptidase IV inhibitor K-579 (100 nM) for 2 h at 37 °C, and ELISA was used to measure GLP-1 concentration in the supernatant. AR-231,453, MBX-2982, and AZ2 evoked a statistically significant increase in GLP-1 secretion compared to control cells ($P<0.01$ or 0.05; ANOVA; $n=4$; Fig. 8A). However, AZ1 failed to evoke a response. A likely explanation for this is the species-specific pharmacology of some GPR119 agonists (Scott et al., 2013); we are comparing mouse (GLUTag) and human (HEK-GPR119) receptor responses.

We next investigated the propensity for sustained wash-resistant receptor activation by pre-treating cells with 1 μM of these agonists (60 min, 37 °C) and then washing out excess ligand. Post-wash GLP-1 secretion was then measured by subsequent incubation in agonist-free buffer in the presence of K-579 for 2 h at 37 °C. In these experiments, only AR-231,453 evoked a residual receptor response that was higher than control cells ($P<0.05$; ANOVA; $n=3$; Fig. 8B). Although there was a trend towards increased

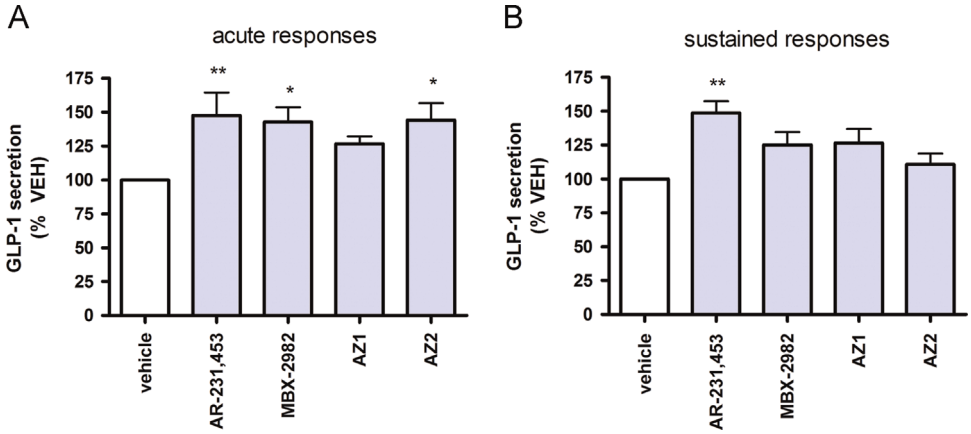

Fig. 8. Acute and sustained GPR119-mediated GLP-1 secretion responses in GLUTag cells. (A) Acute responses were monitored by measuring GLP-1 secretion stimulated by inclusion of 1 μM agonist for 120 min at 37 °C without washout. (B) Sustained responses were monitored by measuring GLP-1 secretion over 120 min at 37 °C after agonist pre-treatment (1 μM, 60 min, 37 °C) followed by washout. Responses are normalized to vehicle control (white bars) in each case.

response for MBX-2982, this was not statistically significant. Therefore, sustained wash-resistant receptor activation can translate to a functional read-out in an endogenous expression system, although not all agonists identified as sustained in our recombinant system displayed this behaviour. Perhaps this is linked to AR-231,453 responses being more robust, durable and less susceptible to exposure conditions (e.g. incubation volume) compared to MBX-2982. The responses window for GPR119 signalling in this GLUTag cell assay was small, which may hinder the observation of low-level sustained responses.

4. Discussion

In this study we have investigated the temporal dynamics of GPR119 signalling and have found that chronic incubation of agonists permits stable activation of the receptor. Moreover, agonist-specific factors that we propose can delay the termination of agonist/receptor interactions (slow off-rate or membrane deposition) cause sustained wash-resistant receptor activation, persisting for at least a number of hours.

Classic understanding of GPCR signalling is described by a gradual loss of receptor responsiveness after prolonged agonist exposure caused by desensitization and internalization via β-arrestin-dependent mechanisms (Drake et al., 2006; Kelly et al., 2008). However, we find that in our test system, desensitization is not a major regulatory influence on GPR119 function as little/no loss of cAMP response is observed with chronic agonist exposure. Direct reports of GPR119 desensitization in the literature are limited, although a recent study demonstrated desensitization of calcium responses by novel and existing GPR119 agonists using re-stimulation experiments (Zhang et al., 2014). This need not be inconsistent with our findings as pathway-specific or agonist-specific desensitization mechanisms potentially play. Additionally, the kinetics of the calcium response and levels of receptor reserve may also contribute to the observed differences in desensitization profile between these systems. A second recent report suggested that both AR-231,453 and oleoylethanolamide evoke complete desensitization of GPR119 cAMP responses (Kumar et al., 2014). However, in these experiments the investigators did not appear to wash out excess ligand before challenging with a second stimulus, which may preclude the observation of re-stimulation responses and therefore confound the observation of true desensitization. To our knowledge, this is the first direct and comprehensive investigation of agonist-mediated desensitization of GPR119 cAMP responses. A number of other GPCR systems have also been reported to show no loss of responsiveness upon chronic stimulation with agonist (Calebiro et al., 2009; Callander et al., 2009; Ferrandon et al., 2009; Jensen et al., 2013; Mullershausen et al., 2009; Willars et al., 1999).

We cannot rule out desensitization at very high agonist concentrations, as this was observed in some cases with supramaximal stimulation in an assay-specific manner. Lauffer et al. (2009) speculated that bell-shaped concentration–responses curves to GPR119 agonists indicate receptor desensitization at high concentrations. Indeed, we also observe bell-shaped curves for some agonists. It is plausible that supramaximal concentrations of agonists evoke desensitization by either non-conventional mechanisms or due to low affinity interactions with β-arrestin-coupled conformations of the receptor. Nevertheless, robust receptor activation is still observed after chronic exposure to pharmacologically relevant concentrations of our test agonists.

For some agonists, these stable GPR119 responses were resistant to washing. Thus, sustained activation could continue, for at least a number of hours, even after removal of free agonist. These data are consistent with reports for other GPCR systems that do not desensitize (Calebiro et al., 2009; Ferrandon et al., 2009; Mullershausen et al., 2009).

The simplest explanation for the observation of sustained wash-resistant responses is slow agonist dissociation kinetics, where a single long-lived binding interaction continues long after removal of unbound ligand by washing. Our findings are consistent with an involvement of binding kinetics in the sustained effects of AR-231,453. The strongest evidence came from an inability of antagonist to reverse AR-231,453-mediated GPR119 activation. In the presence of a saturating concentration of antagonist it would be expected that if agonist molecules dissociate, the binding site would become occupied by antagonist thereby preventing future receptor activation. Since the antagonist fails to interrupt receptor activation, this suggests that AR-231,453 remains bound to the receptor, without dissociating, throughout the generation of the sustained responses. These experiments indirectly monitor agonist binding kinetics as a downstream functional response. Therefore, it would be advantageous to perform follow up studies that directly probe occupation of the binding site (e.g. radioligand binding), when these become available. Comparison of the concentration–response relationship before and after washing demonstrates little effect of removal of unbound ligand. This suggests the presence of stable agonist interactions within the receptor binding site, rather than a secondary requirement for nonspecific interactions with membrane sites which if involved would likely impact the temporal characteristics of receptor activation. In support of this assumption we found little evidence for a requirement for membrane deposition of AR-231,453. Whilst a direct analysis of the binding kinetics of AR-231,453 would be highly desirable to validate a role for long residence time in its sustained effects, we currently lack the tools to establish such an assay.

Much of the emphasis in the literature is focused on the role of receptor binding kinetics in antagonist function. However, whilst an understanding and role for GPCR agonist binding kinetics is largely overlooked, there is some precedent for this effect in the literature. Sustained receptor responses linked to agonists with slow dissociation rates have been reported for the calcitonin (Andreassen et al., 2014), parathyroid hormone (Dean et al., 2008; Ferrandon et al., 2009; Okazaki et al., 2008), mealocortin (Haskell-Luevano et al., 1996) and muscarinic M1 (Jakubik et al., 2002) receptors.

An inability of washing/dilution to successfully remove the agonist from the vicinity of the receptor, rather than persistent occupation of the receptor binding site per se is an alternative mechanism to explain sustained wash-resistant signalling. Rebinding, where upon dissociation from the receptor an agonist is more likely to bind again than diffuse away and discontinue its influence (Vauquelin and Charlton, 2010); or membrane deposition, where cellular membranes act as a reservoir of the agonist that can later access the receptor binding site (Anderson et al., 1994) have both been postulated to play a role in sustained wash-resistant agonist responses. A key distinction between the roles of binding kinetics and rebinding/membrane deposition is that the former involves pre-established binding interactions formed during the initial agonist incubation that remain after removal of unbound ligand, whilst the latter relies on new binding interactions being made after removal of excess ligand as unbound ligand is not effectively removed by dilution of drug in the surrounding fluid. These post-washing binding events can be identified by responses that are readily reversed by antagonist. As the responses to MBX-2982 and AZ1 were reversed by antagonist, this suggests that agonist dissociation events occur over the time-course of the experiments as receptor binding sites are left unoccupied for antagonist binding. Our observations that rapid washing (infinite dilution protocol) was less effective than many washes over time

("chronic incubation/washout" experiments) were also consistent with rebinding/membrane deposition being involved in the sustained effects of the agonists MBX-2982 and AZ1 as they highlighted that free concentration of the ligand was not the only factor driving the effect. Moreover, these findings indicate that receptor-independent mechanisms are involved, and cannot be explained by binding kinetics alone.

Evidence that membrane deposition of MBX-2982 and AZ1 may play a prominent role over rebinding come from findings that the sustained effects are sensitive to a reduction in buffer volume during chronic pre-treatment, suggesting that a large number of agonist molecules are needed to evoke the response via their accumulation in cell membranes (Teschemacher and Lemoine, 1999). High lipophilicity is often cited as a key determinant in ligand membrane deposition as it favours interactions with lipid membranes. Indeed we find that the lipophilicity (log *D* values) and protein binding of MBX-2982 and AZ1 are high (Sup. Table S1) and could accommodate this role. However, other agonists that do not exert a sustained wash-resistant effect also have high lipophilicity (e.g. AZ2), so this is unlikely to be the sole mechanism. Membrane deposition has been proposed as a mechanism of the prolonged cellular and clinical effects of long-acting β_2-adrenergic receptor agonists such as salmeterol (Anderson et al., 1994; Szczuka et al., 2009; Teschemacher and Lemoine, 1999).

Whilst a number of GPR119 agonists, including GSK-1292263 and MBX-2982, have advanced to phase II clinical trials for type-2 diabetes, it appears that a common problem with these ligands may be a lack of clinical efficacy (Kang, 2013). Indeed, clinical development of GSK-1292263 has been discontinued. It is tempting to speculate that efficacy could be improved by targeting ligands with enhanced sustained effects, as this could provide more prolonged receptor signalling. There is a growing appreciation that targeting ligands with a long residence time could be useful strategy in drug discovery for increased target coverage and decreased toxicity (Copeland et al., 2006; Guo et al., 2014). We find that the sustained effects of GSK-1292263 are weak and not statistically significant, and may therefore limit its ability to activate GPR119 in a physiological setting. Although MBX-2982 did cause robust sustained wash-resistant receptor activation, the effects with AR-231,453 were higher in magnitude (Fig. 4A), as well as being more stable, more potent and less susceptible to exposure conditions (Figs. 5 and 6). Encouragingly, for AR-231,453 we also observe sustained responses downstream of cAMP in an endogenous expression system, which supports the potential (patho) physiological relevance of this phenomena.

This study highlight the importance of carefully examining the mechanisms through which G protein-coupled receptor agonists generate their temporal response profiles and could identify exciting opportunities to exploit these responses with pharmacological agents. Clearly, further work is needed to assess the chronic effects of GPR119 agonists on their clinical efficacy and to define the desired temporal profile of target activation as well mechanism of action (binding kinetics vs. rebinding/membrane deposition), with the potential to develop improved pharmacotherapies for this target.

5. Conclusions

We have demonstrated that in a recombinant expression system GPR119 responses at the level of cAMP signalling are under minimal influence from desensitization at pharmacologically relevant agonists concentrations. Furthermore we identified a compound specific (AR-231,453, MBX-2982, and AZ1) sustained GPR119 activation profile that was insensitive to extensive washing to remove unbound ligand. Our data support that multiple mechanisms, including slow dissociation kinetics (AR-231,453), or membrane deposition and rebinding (MBX-2982 and AZ1) may contribute to wash-resistant signalling. Importantly, sustained effects of AR-231,453 were also observed at the level of physiological responses in an endogenous cellular expression system.

Acknowledgements

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.ejphar.2015.06.031.

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