

β_1 -adrenergic receptor antagonists signal via PDE4 translocation

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It is generally assumed that antagonists of G_s-coupled receptors do not activate cAMP signalling, because they do not stimulate cAMP production via G_s-protein/adenylyl cyclase activation. Here, we report a new signalling pathway whereby antagonists of β_1 -adrenergic receptors (β_1 ARs) increase cAMP levels locally without stimulating cAMP production directly. Binding of antagonists causes dissociation of a preformed complex between β_1 ARs and Type-4 cyclic nucleotide phosphodiesterases (PDE4s). This reduces the local concentration of cAMP-hydrolytic activity, thereby increasing submembrane cAMP and PKA activity. Our study identifies receptor/PDE4 complex dissociation as a novel mechanism of antagonist action that contributes to the pharmacological properties of β_1 AR antagonists and might be shared by other receptor subtypes.

Keywords: cAMP; cyclic nucleotide phosphodiesterase; PDE; β -adrenergic receptor; antagonist

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INTRODUCTION

In the classical model of G_s protein-coupled receptor (G_sPCR) activation, the unoccupied receptor resides in an ‘inactive’ state that does not induce G protein activation. Binding of an agonist causes conformational changes that stabilize the receptor in the ‘active’ state that promotes interaction with, and activate, G α_s . In this two-state model, an antagonist is defined as a ligand that can bind to the receptor without promoting the conformation required for G_s activation. Therefore, an antagonist might prevent binding and signalling of an agonist, but does not trigger a biological response *per se*. However, it is now well established that the two-state model is an oversimplification that does not accurately describe G_sPCR function [1,2]. GPCRs might interact with more

than one type of G protein and might induce G protein-independent signalling events. The β_2 AR, for example, induces biological effects via activation of G_s protein, G_i protein as well as via β -arrestin [1–3]. Importantly, it is now established that specific receptor conformations show distinct efficacies in the activation of the different signalling mechanisms. As an extension of this concept, receptor ligands can be biased in that they stabilize conformations of the receptors that show distinct activities towards downstream signalling pathways [2,3]. A β AR ligand might function antagonistically on receptor signalling via G_s but as an agonist of β -arrestin signalling. Despite this complexity, antagonists to the β AR receptors are generally thought to reduce cAMP signalling, because they do not induce G_s coupling of the receptor *per se*, but might lower basal and agonist-stimulated activity of the receptor.

We have shown previously that the β_1 -adrenergic receptor (β_1 AR) forms a signalling complex with a Type-4 cyclic nucleotide phosphodiesterase (PDE4) and that occupancy of the receptor by an agonist induces dissociation of the β_1 AR/PDE4 complex [4]. This regulation of the β_1 AR/PDE4 complex differs from that of the related β_2 AR, which is characterized by a β -arrestin-dependent recruitment of PDE4 to the occupied receptor [5], and differential interaction with PDEs might contribute to the distinct physiological roles of β_1 AR and β_2 AR, for instance in the regulation of cardiac excitation–contraction coupling.

Here, using several independent approaches, we provide evidence that antagonist occupancy of the β_1 AR receptor triggers a previously undetected signal at the cell membrane by promoting the release of a PDE4. This release results in a local increase in cAMP levels and activation of PKA in the proximity of the receptor without a concurrent stimulation of cAMP synthesis via the classical G_s/adenylyl cyclase (AC) pathway by the antagonist-occupied β_1 AR itself. These local effects are in opposition to the dampening effect of β_1 AR antagonists on bulk cellular cAMP and global PKA activation. Our findings might explain some observations on the pharmacological effects of β_1 AR antagonists.

RESULTS AND DISCUSSION

Antagonists dissociate β_1 AR/PDE4 complexes

To study the dynamics of β_1 AR/PDE4 association/dissociation, we tested a series of β -adrenergic ligands for their effect on the interaction between recombinant PDE4D8 and β_1 AR.

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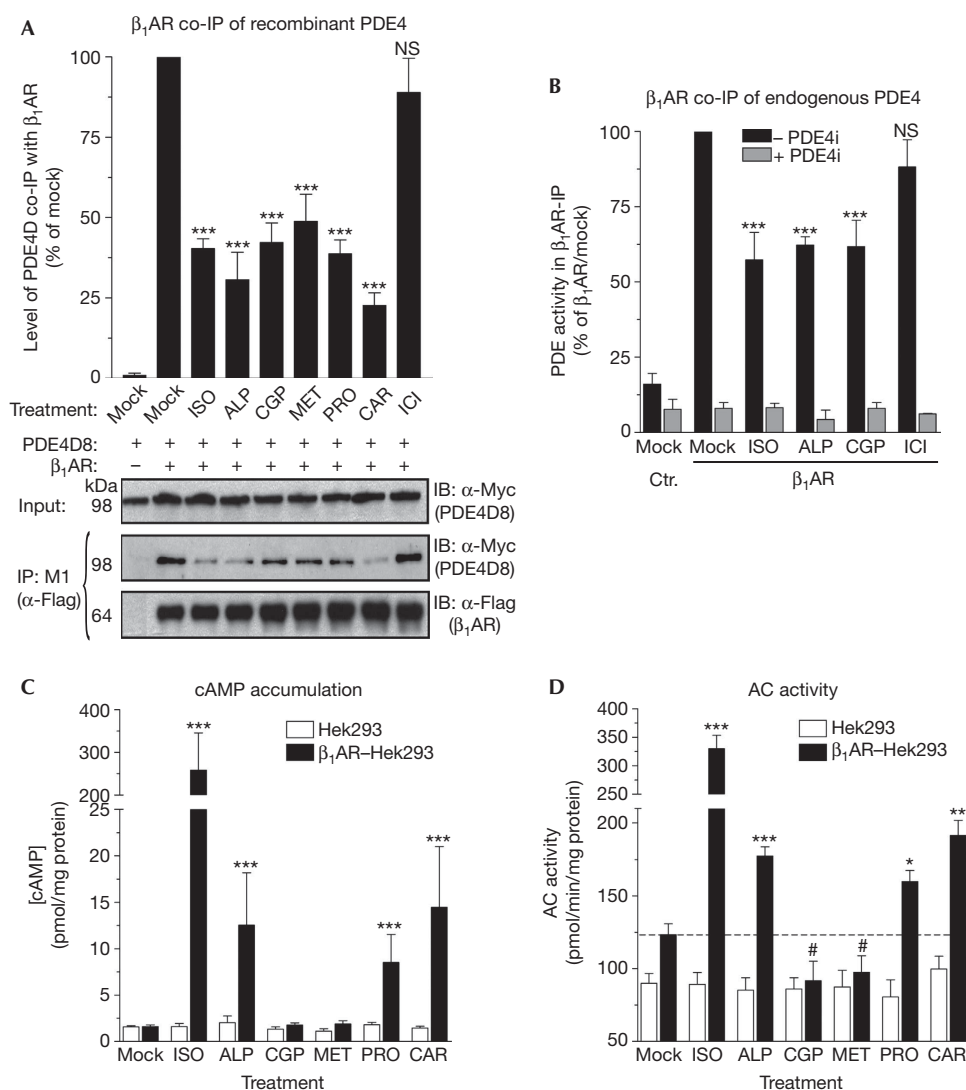


Fig 1 | β_1 AR antagonists dissociate the β_1 AR/PDE4 complex. (A) Hek293 cells expressing Flag-tagged β_1 AR and Myc-tagged PDE4D8 were pretreated for 5 min with the β AR agonist ISO (10 μ M), the β_1 AR antagonists ALP, CGP, MET, PRO and CAR, or the β_2 AR-antagonist ICI (1 μ M each) and then lysed and subjected to IP of β_1 AR. The amount of exogenous PDE4D8 recovered in the IP pellets is quantified. (B) Parental or Flag- β_1 AR-expressing Hek293 cells were treated for 5 min with ISO (10 μ M), ALP and CGP or ICI (each at 1 μ M) before cell lysis and β_1 AR-IP. Shown is the amount of endogenous cAMP-PDE activity recovered in β_1 AR-IP pellets measured in the absence (– PDE4i) or presence (+ PDE4i) of the PDE4-selective inhibitor rolipram (10 μ M). (C) cAMP accumulation by parental or β_1 AR-expressing Hek293 cells treated for 5 min with ISO (100 nM), or with various β_1 AR antagonists (each at 1 μ M) measured by RIA. (D) Adenylyl cyclase (AC) activity in membrane preparations from parental or β_1 AR-expressing Hek293 cells measured in the presence or absence of β_1 AR antagonists (each at 1 μ M) or ISO (100 nM). Experiments were performed in the presence of the β_2 AR-selective antagonist ICI (1 μ M) to block signalling of endogenous β_2 ARs. Data are the mean \pm s.e.m. of at least three experiments. NS (not significant; $P > 0.05$); * ($P < 0.05$); ** ($P < 0.01$); *** ($P < 0.001$); # (significantly decreased; $P < 0.05$). ALP, alprenolol; CAR, carvedilol; CGP, CGP-20712A; ICI, ICI-118551; IP, immunoprecipitation; ISO, isoproterenol; MET, metoprolol; NS, not significant; PDE4, type-4 cyclic nucleotide phosphodiesterase; PRO, propranolol; RIA, radioimmunoassay; β_1 AR, β_1 -adrenergic receptor.

Unexpectedly, we found that not only agonists, but also treatment with various β_1 AR antagonists, including alprenolol (ALP), CGP-20712A (CGP), metoprolol (MET), propranolol (PRO) and carvedilol (CAR) (Fig 1A), induced β_1 AR/PDE4 complex dissociation. Conversely, the β_2 AR-selective antagonist ICI-118551 (ICI) had no effect.

When expressed in Hek293 cells, recombinant β_1 ARs also form complexes with endogenous PDE4 (Fig 1B). Treatment of

β_1 AR-Hek293 cells with β AR-antagonists ALP, CGP or ICI did not affect total PDE or PDE4 activity in cell extracts (supplementary Fig S1 online). Conversely, treatment with ALP, CGP or the β_1 AR-agonist isoproterenol (ISO), but not the β_2 AR antagonist ICI, significantly reduced the amount of PDE4 activity associated with the β_1 AR. This finding confirms a ligand-dependent dissociation of β_1 AR complexes with endogenous PDE4 similar to that observed with the β_1 AR/PDE4D8 overexpression

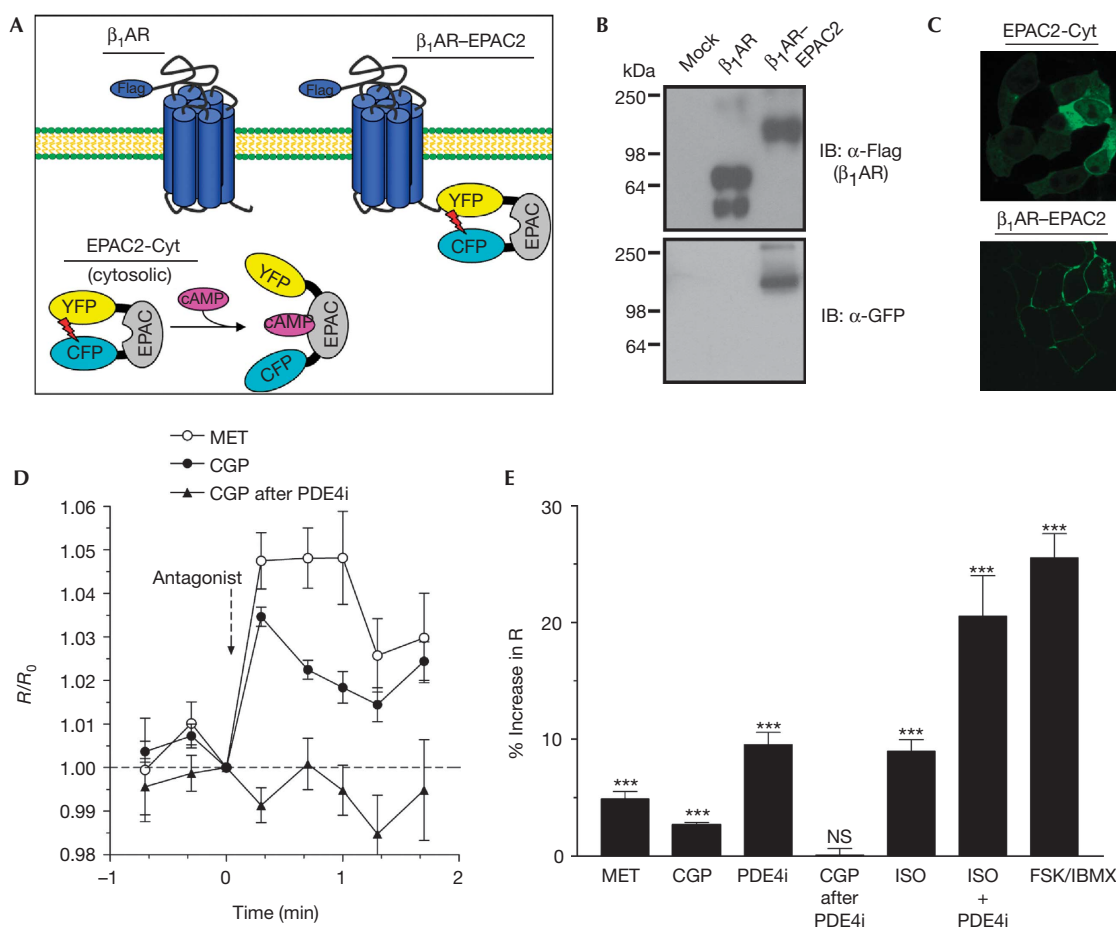


Fig 2 | Antagonist treatment increases cAMP levels in the vicinity of the β_1 AR. (A) Scheme illustrating the design of the cytosolic EPAC2-Cyt sensor and the β_1 AR-EPAC2 chimera. (B) Representative western blots showing the expression of Flag-tagged β_1 AR and β_1 AR-EPAC2 in Hek293 cells. (C) The images show YFP emissions in Hek293A cells expressing the cytosolic EPAC2-Cyt or the β_1 AR-EPAC2 sensor. (D,E) Live-cell cAMP measurements in Hek293 cells expressing the β_1 AR-EPAC2 sensor. Shown are average traces of R/R_0 , defined as the CFP/FRET emission ratio (R) at each time point divided by the CFP/FRET ratio at time 0 min (R_0), for cells treated with MET (1 μ M; $n = 15$), CGP (1 μ M; $n = 54$) or for cells treated with CGP following a 5-min pretreatment with a PDE4 inhibitor (PDE4i; rolipram; 10 μ M; $n = 30$). An increase in R/R_0 indicates an increase in intracellular cAMP levels. (E) Percentage increase in R (CFP/FRET) after treatment with β_1 AR antagonists MET ($n = 15$) or CGP ($n = 54$), rolipram (PDE4i, 10 μ M; $n = 30$), CGP after pretreatment with PDE4i ($n = 30$), ISO (100 nM; $n = 19$), combined treatment with ISO and PDE4i ($n = 16$) or combined treatment with the non-selective PDE inhibitor IBMX (100 μ M) and the adenylyl cyclase activator FSK (50 μ M) ($n = 37$). NS (not significant; $P > 0.05$); ***($P < 0.001$). CGP, CGP-20712A; FSK, forskolin; IB, immunoblotting; ISO, isoproterenol; MET, metoprolol; PDE4, type-4 cyclic nucleotide phosphodiesterase; β_1 AR, β_1 -adrenergic receptor.

system (Fig 1A). The efficacy of antagonists in this signalling mechanism suggests that β_1 AR/PDE4 complex dissociation is induced regardless of whether or not β_1 AR ligands promote receptor interaction with a G_s protein. In addition, antagonist-dependent β_1 AR/PDE4 complex dissociation does not require β -arrestin recruitment as shown in supplementary Fig S2 online.

CGP and MET do not stimulate cAMP production

Next, we probed the effect of antagonist-dependent β_1 AR/PDE4 complex dissociation on cAMP signalling. Treatment of β_1 AR-Hek293 cells with PRO, ALP and CAR triggered a significant increase in global cellular cAMP as measured by radioimmunoassay (RIA) (Fig 1C). This increase is due to partial

agonist activity of these compounds at this concentration as confirmed by their ability to stimulate AC activity (Fig 1D). Conversely, CGP and MET did not stimulate global cAMP levels (Fig 1C), suggesting that they do not promote receptor coupling to G_s . Indeed, and in line with previous reports [6], CGP and MET act as inverse agonists and lower basal activity of the receptor as reflected by reduced basal AC activity (Fig 1D). Thus, CGP and MET are suitable β_1 AR-selective ligands to probe the effect of PDE4 displacement on cAMP levels because they do not stimulate cAMP production via G_s protein/AC activation. Fig 1C also demonstrates that dissociation of β_1 AR/PDE4 signalling complexes does not affect global cAMP levels because CGP and MET do not increase total cAMP.

Complex dissociation augments local cAMP levels

To determine whether antagonist-dependent complex dissociation affects local cAMP levels in the vicinity of the receptors, we generated a live-cell sensor that detects cAMP levels in this specific compartment (Fig 2A). To this end, we fused a FRET-based cAMP sensor (EPAC2-Cyt), which has been characterized previously [7], to the C-terminus of the β_1 AR. The β_1 AR–EPAC2 chimera was expressed at similar levels as the wild-type β_1 AR (Fig 2B). Fusion with the receptor causes membrane localization of the EPAC2 sensor, which by itself distributes throughout the cytosol (Fig 2C). Importantly, fusion of β_1 AR and the EPAC2 probe does not impair the function of the receptor (supplementary Figs S3,S4 online) nor, as shown below, the function of the EPAC2 sensor.

Next, we used the β_1 AR–EPAC2 sensor to determine whether antagonist-dependent β_1 AR/PDE4 complex dissociation affects local cAMP levels. As shown in Fig 2D, treatment with β_1 AR antagonists CGP or MET induced a significant increase in R (the ratio of CFP emission over FRET), which indicates an increase in cAMP levels in the vicinity of the sensor. Treatment with a PDE4-selective inhibitor also induced a significant increase in local cAMP levels (Fig 2E). On pretreatment with PDE4 inhibitor, β_1 AR antagonists have no further effect on cAMP levels in the vicinity of the β_1 AR–EPAC2 sensor suggesting that the antagonist-dependent increase in local cAMP is due to reduced PDE4 activity in this compartment. The antagonist-dependent increase in cAMP levels is spatially restricted to the vicinity of the receptor and is not detected with cAMP sensors that are widely distributed throughout the cell membrane or in the bulk cytosol (supplementary Fig S5 online). Whereas treatment with MET and/or CGP does not stimulate cAMP production (Fig 1D), basal constitutive cAMP production is required to detect the effect of antagonist-dependent β_1 AR/PDE4 complex dissociation as demonstrated by AC inhibition (supplementary Fig S6 online). This finding also confirms that the antagonist-induced changes in CFP/FRET emissions reflect changes in local cAMP levels and not an artifact resulting from conformational changes of the β_1 AR–EPAC2 chimera upon ligand binding.

Complex dissociation increases PKA activity locally

To determine whether the local increase in cAMP levels measured with the β_1 AR–EPAC2 probe is mirrored by local increases in PKA activity, we determined the effect of antagonist-dependent β_1 AR/PDE4 complex dissociation on PKA phosphorylation of the receptor itself. Treatment with antagonists induced a significant increase in β_1 AR phosphorylation by PKA (Fig 3A; supplementary Fig S7 online) in spite of their inverse agonist activity that lowers basal AC activity (Fig 1D). Several observations indicate that the increased PKA phosphorylation of the receptor is due to antagonist-dependent β_1 AR/PDE4 complex dissociation. First, there is a positive correlation between the time course of antagonist-dependent complex dissociation and that of increased PKA phosphorylation of the receptor. Both are induced within the first minute of treatment (supplementary Fig S8 online). Similarly, the dose–response curve for the antagonist-dependent β_1 AR/PDE4 complex dissociation compares well with that of increased PKA phosphorylation of the receptor (Fig 3B,C). Finally, treatment with a PDE4 inhibitor also increases PKA phosphorylation of the receptor in line with the idea that PDE4 controls local PKA activity (Fig 3D). Importantly, upon pretreatment with a PDE4 inhibitor,

β_1 AR antagonists have no further effect on PKA phosphorylation levels of β_1 AR (Fig 3D) confirming that the effect of antagonists is mediated via altered PDE4 activity in the vicinity of the β_1 AR.

Antagonists alter subcellular cAMP distribution

Although antagonist-dependent β_1 AR/PDE4 complex dissociation is rapid, reassociation of the complex after antagonist washout is relatively slow. Only a fraction of β_1 AR/PDE4 complexes reformed at 10 min after MET washout (supplementary Figs S9, S10 online). This allowed us to compare agonist signalling of receptors associated with different amounts of PDE4, using the experimental protocol outlined in Fig 4A. As shown in Fig 4B, pretreatment and subsequent washout of β_1 AR–EPAC2-expressing Hek293 cells with MET or CGP results in a significant reduction in ISO (10 nM)-stimulated global cAMP production compared with mock-treated cells. Incomplete washout of CGP (see supplementary Fig S9 online) is likely responsible for the more severe suppression of ISO-induced cAMP accumulation after washout of CGP compared with MET. In contrast to the suppressed global cAMP signals, cAMP levels in the vicinity of the receptor, as measured by changes in FRET, are significantly elevated upon antagonist pretreatment (Fig 4C), confirming receptor-associated PDE4 activity as the main regulator of cAMP levels in this compartment. The distinct effect of antagonist pretreatment on local and global cAMP levels is mirrored by experiments with the wild-type β_1 AR. Total cellular cAMP accumulation as measured by enzyme immunoassay (EIA) was reduced by antagonist pretreatment (supplementary Fig S11A online). The reduced global cAMP accumulation was associated with significantly reduced global PKA phosphorylation levels upon antagonist pretreatment (Fig 4D,E; supplementary Fig S11B online). Conversely, basal and ISO-induced PKA phosphorylation of the receptor itself was increased following antagonist pretreatment and washout. These data are consistent with the hypothesis that antagonist pretreatment causes an increase in cAMP in the vicinity of the receptors via PDE4 dissociation.

Complex dissociation affects antagonist action

To determine whether antagonist pretreatment augments responses of endogenous β_1 ARs, we measured its effect in neonatal mouse cardiac myocytes expressing the plasma membrane-targeted EPAC2 cAMP sensor (EPAC2-PM; Fig 4F; supplementary Fig S12 online; [8,9]). Pretreatment followed by washout of MET potentiated ISO-induced cAMP levels in this compartment, whereas cAMP levels in the bulk cytosol measured with the cytosolic EPAC2-Cyt sensor (Fig 4G) were not affected. Consistent with the hypothesis that the antagonist-dependent increase in submembrane cAMP levels is due to PDE4 translocation, treatment with a PDE4-selective inhibitor also potentiated submembrane cAMP signals (supplementary Fig S12 online) and MET pretreatment had no further effect in the presence of this inhibitor. Taken together, these data suggest that antagonist-dependent β_1 AR/PDE4 complex dissociation also affects signalling of endogenous β_1 ARs in cardiac myocytes. An increase in cAMP after antagonist treatment is detected only with the β_1 AR–EPAC2 sensor, suggesting a restricted effect in proximity of the receptor (supplementary Fig S5 online), whereas the response to an agonist after antagonist pretreatment/washout can be detected with both membrane probes (β_1 AR–EPAC2 as well as EPAC2-PM) but not

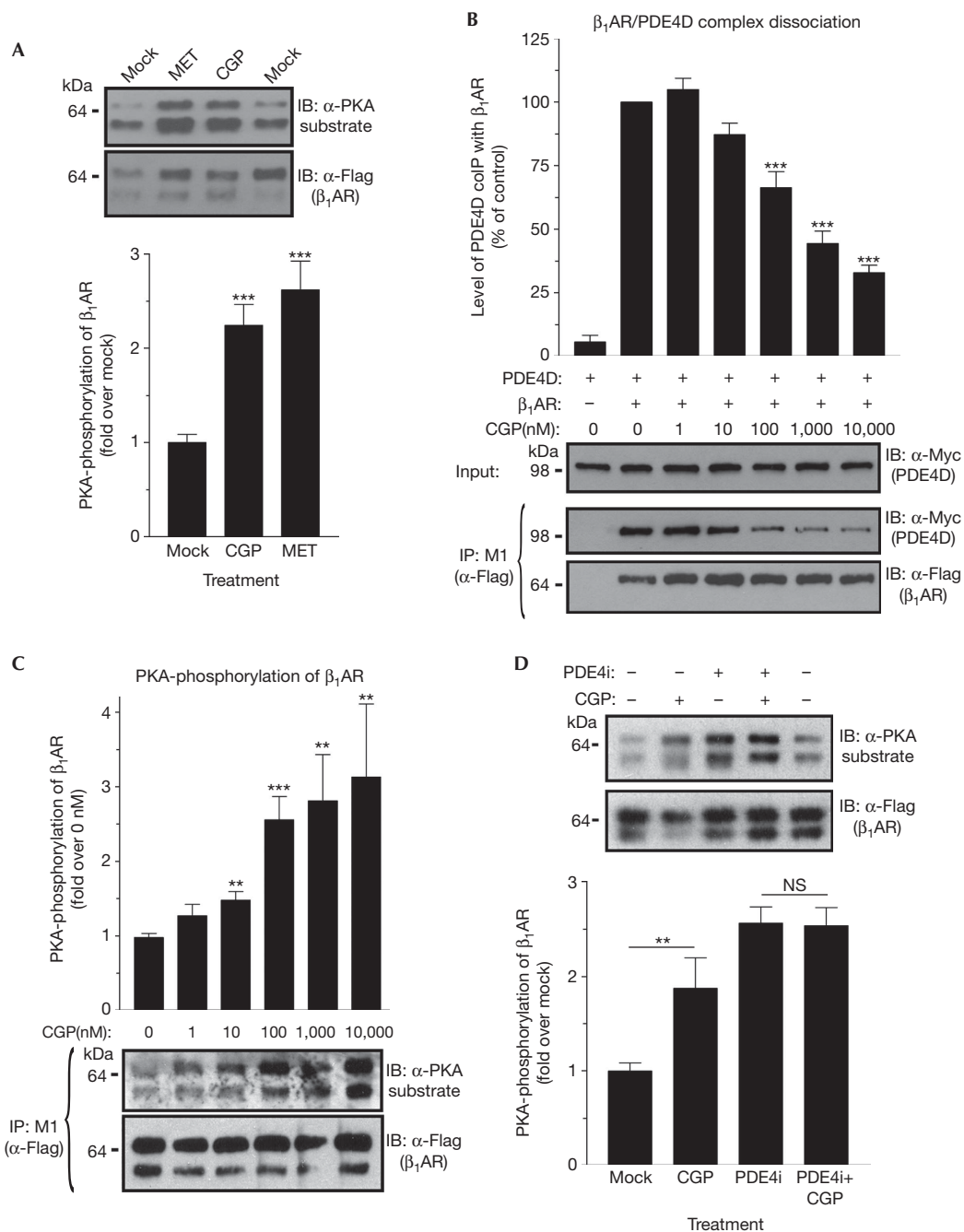


Fig 3 | Antagonist-dependent β_1 AR/PDE4 complex dissociation increases local PKA activity. (A) Hek293 cells expressing Flag-tagged β_1 AR were treated for 5 min with β_1 AR antagonists CGP or MET (each 1 μ M). Detergent extracts prepared from these cells were subjected to α -Flag(M1)-IP and the phosphorylation levels of the β_1 AR recovered in IP pellets was determined by western blot using a PKA-substrate antibody. (B) Hek293 cells expressing Flag-tagged β_1 AR and Myc-tagged PDE4D8 were treated for 5 min with different concentrations of CGP, before the cells were lysed and subjected to β_1 AR IP. The amount of exogenous PDE4D8 recovered in the IP pellets is quantified. (C) Dose-dependent increase in PKA phosphorylation of β_1 AR expressed in Hek293 cells in response to a 5-min treatment with different concentrations of CGP. (D) Hek293 cells expressing Flag-tagged β_1 AR were treated for 5 min with CGP (1 μ M) and/or rolipram (PDE4i; 10 μ M). Detergent extracts were subjected to α -Flag(M1)-IP and the phosphorylation levels of the β_1 AR recovered in IP pellets was determined by western blot with a PKA-substrate antibody. Data are the mean \pm s.e.m. of at least three experiments. **($P < 0.01$); ***($P < 0.001$); NS (not significant; $P > 0.05$). CGP, CGP-20712A; IB, immunoblotting; IP, immunoprecipitation; MET, metoprolol; PDE4, type-4 cyclic nucleotide phosphodiesterase; β_1 AR, β_1 -adrenergic receptor.

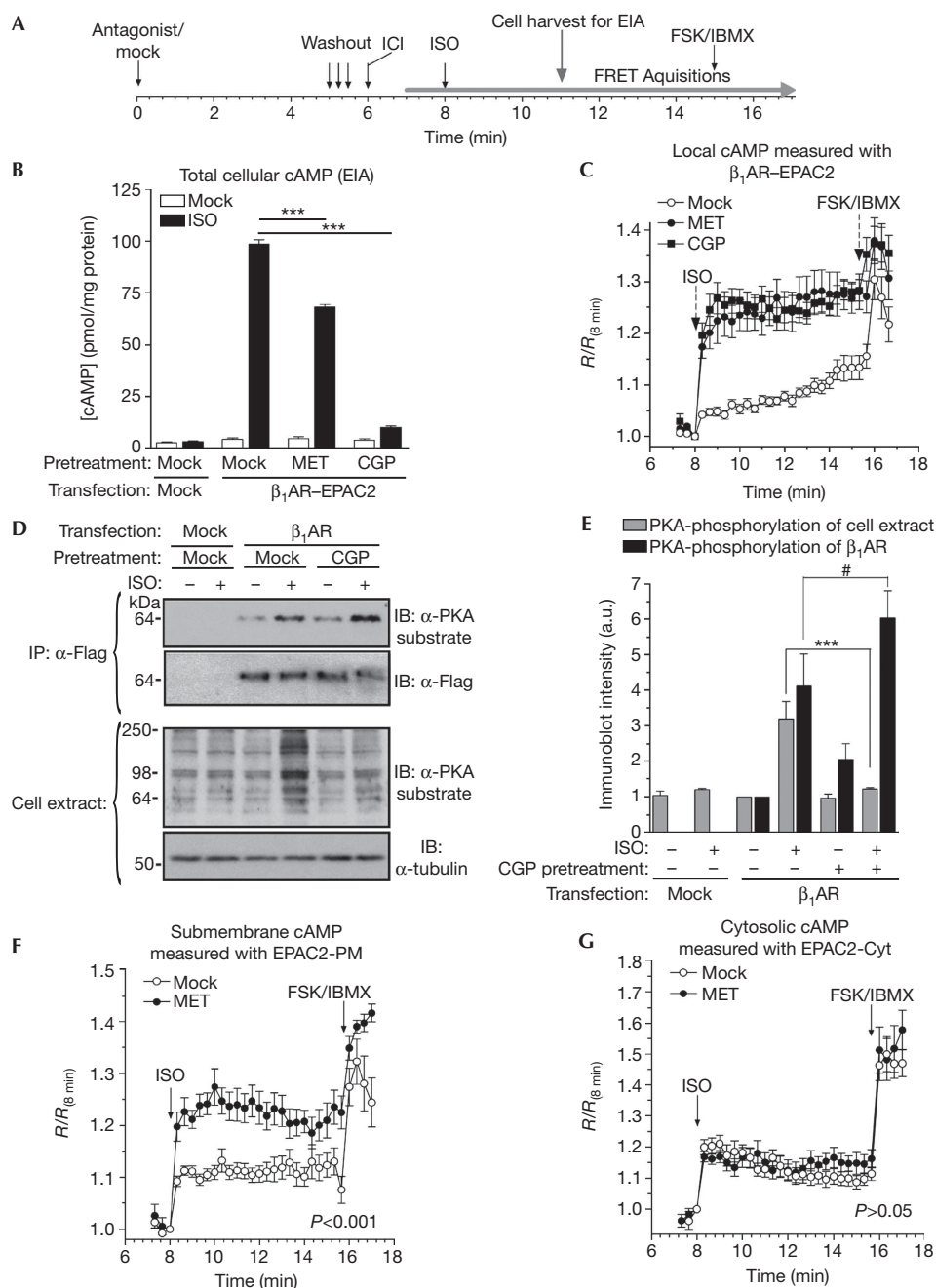


Fig 4 | Antagonist-dependent β_1 AR/PDE4 complex dissociation differentially affects submembrane and global cytosolic cAMP levels. (A) Scheme of the treatment protocol for experiments shown in (B–G). Cells were pretreated for 5 min with MET or CGP (1 μ M each) or solvent. Cell monolayers were then rapidly rinsed three times to wash out the antagonists, treated with ICI (1 μ M) to block signalling of endogenous β_2 ARs and then stimulated with 10 nM ISO. For measurement of global cAMP levels (B) and PKA-phosphorylation patterns (D,E), cells were harvested 3 min after addition of ISO. For FRET measurements, images were acquired every 20 s for \sim 7 min, after which cells were treated with FSK (50 μ M) and IBMX (100 μ M). (B) Total cellular cAMP accumulation in Hek293 cells expressing β_1 AR-EPAC2 in response to ISO as measured by EIA. (C) Average traces of FRET from β_1 AR-EPAC2-expressing cells stimulated with ISO after pretreatment with MET ($n=28$), CGP ($n=19$) or solvent (mock; $n=21$). Data are expressed as R (ratio of CFP/FRET emissions at each time point) divided by the R value acquired before ISO addition ($R_{(8 \text{ min})}$). (D,E) Detergent extracts prepared from control or β_1 AR-expressing Hek293 cells were subjected to α -Flag IP. Total cell extracts and β_1 ARs recovered in Flag-IP pellets were subjected to immunoblot analysis using a PKA substrate antibody. (F) Average traces of FRET from EPAC2-PM-expressing neonatal cardiac myocytes stimulated with ISO (10 nM) after pretreatment and washout of MET ($n=19$) or solvent (mock; $n=20$). (G) Pretreatment with MET does not affect ISO-induced cAMP levels in the bulk cytosol measured with the EPAC2-Cyt probe ($n_{\text{mock}}=15$; $n_{\text{MET}}=20$). Data represent the means \pm s.e.m. of at least three experiments. *** (significantly reduced; $P < 0.001$); # (significantly increased; $P < 0.05$). CGP, CGP-20712A; EIA, enzyme immunoassay; FSK, forskolin; ICI, ICI-118551; IP, immunoprecipitation; ISO, isoproterenol; MET, metoprolol; PDE4, type-4 cyclic nucleotide phosphodiesterase; β_1 AR, β_1 -adrenergic receptor.

with the cytoplasmic sensor (Fig 4). We explain this broader effect of the agonist as a consequence of the large increase in cAMP owing to the activation of cAMP synthesis and decreased local degradation owing to dissociation of the PDE4/ β_1 AR complex. These two events synergize to cause spillover of cAMP from a compartment close to the receptor to other membrane domains.

To determine whether antagonist-dependent β_1 AR/PDE4 complex dissociation might occur at therapeutic concentrations, dose–response curves for two compounds used in the treatment of heart failure, MET and CAR, were determined in Hek293 cells. β_1 AR/PDE4 complex dissociation is induced by MET concentrations >100 nM, whereas CAR induces β_1 AR/PDE4 dissociation at concentrations of as little as 10 nM (supplementary Fig S13 online). Reported peak plasma concentrations for MET and CAR range from 100–1,000 nM and from 20–400 nM, respectively, depending on dosage and preparation (see supplementary Fig S13 online). Thus, MET is more likely to signal via β_1 AR/PDE4 complex dissociation in individuals receiving high doses of the drug (that is, ≥ 200 mg/dose) compared with individuals receiving low doses. Conversely, we predict a significant and long-lasting effect of CAR on β_1 AR/PDE4 complexes in all patients, because it induces complex dissociation at concentrations lower than therapeutic doses (supplementary Fig S13 online). In addition, the drug has a very slow dissociation constant resulting in quasi-irreversible binding to the β_1 AR [10] (supplementary Fig S9 online). Thus, individual antagonists likely show distinct efficacies in signalling via β_1 AR/PDE4 complex dissociation in human heart.

Taken together, our studies reveal the presence of a novel intracellular signal generated when a neutral antagonist or an inverse agonist interacts with the β_1 AR (supplementary Fig S14 online). By inducing the dissociation of a β_1 AR/PDE4 complex, antagonist binding results in a local increase in cAMP sufficient to activate PKA locally. These local effects are opposite to the conventional effects of antagonists on the bulk cytoplasmic cAMP where they lower cAMP levels and do not require increased cAMP production via the classical G_s /AC pathway. Thus, these findings provide a new mechanism of antagonist action that might explain some pharmacological properties attributed to this class of β -adrenergic ligands. The related β_2 AR has also been shown to sequester a PDE4, although via distinct modes of interaction. Nevertheless, this example raises the possibility that other G_s PCRs or non-GPCR receptors dynamically interact with PDEs, and that formation and dissociation of these complexes and thereby submembrane cAMP/PKA levels might be regulated by receptor occupancy.

METHODS

Immunoprecipitation of Flag-tagged β_1 ARs from cell lysates.

Flag-tagged β_1 ARs were immunoprecipitated as described previously [4]. In brief, cells were lysed in buffer containing 20 mM HEPES, 150 mM NaCl, 2 mM EDTA, 10% glycerol, 1% *n*-dodecyl- β -D-maltopyranoside (Anatrace), 1 μ M microcystin-LR (Calbiochem) and Complete protease inhibitor cocktail (Roche Applied Sciences). Lysates were cleared by centrifugation at 20,000g and 4 °C, and preincubation with ProteinG Sepharose. Flag-tagged receptors were then immunoprecipitated using M1-affinity resin (α -Flag antibody resin; Sigma-Aldrich).

PDE assay. PDE activity was measured as described previously [11]. In brief, samples were assayed in a reaction mixture containing 40 mM Tris–HCl (pH 8.0), 1 mM MgCl₂,

1.4 mM β -mercaptoethanol, 1 μ M cAMP, 0.75 mg/ml bovine serum albumin and 0.1 μ Ci of [³H]cAMP for 10 min at 33 °C. The reaction was terminated by boiling for 1 min. The PDE reaction product 5'-AMP was then hydrolysed by incubation of the assay mixture with 50 μ g of *Crotalus atrox* snake venom (Sigma-Aldrich) for 20 min at 33 °C, and the resulting adenosine was separated by anion exchange chromatography using 1 ml of AG1-X8 resin (BioRad, Hercules, CA) and quantitated by scintillation counting.

Measurement of global cellular cAMP levels. Global intracellular cAMP levels were measured by EIA using a kit from Cayman Chemicals following the manufacturer's protocol or by RIA as described previously [12].

Measurement of cAMP levels with EPAC2 sensors. Cells grown on collagen-coated glass coverslips were transfected with plasmids or infected with adenoviruses encoding EPAC2 sensors. After overnight culture, cells were serum-starved for 2 h. FRET microscopy was then performed as described previously [12]. In brief, coverslips were placed in a modified Sykes–Moore Chamber and kept in 500 μ l of Locke's medium (5 mM HEPES (pH 7.4), 154 mM NaCl, 5.6 mM KCl, 1 mM CaCl₂, 1 mM MgCl₂, 3.6 mM NaHCO₃, 5 mM glucose and 0.05% bovine serum albumin) at 37 °C. Images were acquired with a Nikon TE2000 inverted fluorescence microscope using a $\times 60$ fluorescence objective. CFP (donor) fluorescence was viewed by exciting at 430–455 nm and measuring emission at 470–490 nm. YFP (acceptor) fluorescence was viewed by exciting at 500–520 nm and measuring emission at 535–565 nm. FRET was viewed by exciting at 430–455 nm (donor excitation) and measuring fluorescence at 535–565 nm (acceptor emission).

AC activity assay. AC activity was measured as described [13]. In brief, samples were assayed in a reaction mixture containing 40 mM Tris–HCl (pH 7.4), 5 mM MgCl₂, 0.2 mM cAMP, 10 mM phosphoenol pyruvate, three units of pyruvate kinase, 10 μ M GTP, 1 mM ATP and 2 μ Ci of [α -³²P]-ATP for 15 min at 37 °C. The reaction was terminated by boiling for 2 min. Cyclic AMP was then separated from the substrate ATP by column chromatography using 2.5 cm³ Alumina WN-6. The column was eluted into scintillation vials with 5 ml of 0.1 M ammonium acetate (pH 6.5), the eluate was mixed with 12 ml of Aquasol-2 scintillation fluid (PerkinElmer, Waltham, MA) and the eluted cAMP quantified by scintillation counting.

Supplementary information is available at EMBO reports online (<http://www.emboreports.org>).

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Author contributions: W.R., D.M., P.D. and E.B. performed the experiments and analysed the data. W.R. and M.C. designed the study and wrote the manuscript.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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