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Dissociations in the Effects of β_2-Adrenergic Receptor Agonists on cAMP Formation and Superoxide Production in Human Neutrophils: Support for the Concept of Functional Selectivity

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Abstract

In neutrophils, activation of the β_2-adrenergic receptor (β_2AR), a G_s-coupled receptor, inhibits inflammatory responses, which could be therapeutically exploited. The aim of this study was to evaluate the effects of various β_2AR ligands on adenosine-3′,5′-cyclic monophosphate (cAMP) accumulation and N-formyl-L-methionyl-L-leucyl-L-phenylalanine (fMLP)-induced superoxide anion ($O_2^{\bullet-}$) production in human neutrophils and to probe the concept of ligand-specific receptor conformations (also referred to as functional selectivity or biased signaling) in a native cell system. This is an important question because so far, evidence for functional selectivity has been predominantly obtained with recombinant systems, due to the inherent difficulties to genetically manipulate human native cells. cAMP concentration was determined by HPLC/tandem mass spectrometry, and $O_2^{\bullet-}$ formation was assessed by superoxide dismutase-inhibitable reduction of ferricytochrome c. β_2AR agonists were generally more potent in inhibiting fMLP-induced $O_2^{\bullet-}$ production than in stimulating cAMP accumulation. (−)-Ephedrine and dichloroisoproterenol were devoid of any agonistic activity in the cAMP assay, but partially inhibited fMLP-induced $O_2^{\bullet-}$ production. Moreover, (−)-adrenaline was equi-efficacious in both assays whereas the efficacy of salbutamol was more than two-fold higher in the $O_2^{\bullet-}$ assay. Functional selectivity was visualized by deviations of ligand potencies and efficacies from linear correlations for various parameters. We obtained no evidence for involvement of protein kinase A in the inhibition of fMLP-induced $O_2^{\bullet-}$ production after β_2AR-stimulation although cAMP-increasing substances inhibited $O_2^{\bullet-}$ production. Taken together, our data corroborate the concept of ligand-specific receptor conformations with unique signaling capabilities in native human cells and suggest that the β_2AR inhibits $O_2^{\bullet-}$ production in a cAMP-independent manner.

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Introduction

Human neutrophils are crucial for the defense of the host organism against infectious agents such as bacteria, fungi, protozoa, viruses and tumor cells. After phagocytosis of invading agents neutrophils are able to destruct them, the respiratory burst NADPH oxidase being a major player [1]. This enzyme catalyzes the univalent reduction of molecular oxygen (O_2) to the superoxide anion ($O_2^{\bullet-}$) with NADPH as electron donor [2–5]. Activation of neutrophils is triggered by bacterial formyl peptides [6]. Upon binding of N-formyl-L-methionyl-L-leucyl-L-phenylalanine (fMLP) to the formyl peptide receptor, which is G_i-coupled [7–8], $O_2^{\bullet-}$ production in neutrophils increases [1]. fMLP-stimulated $O_2^{\bullet-}$ production in neutrophils is counteracted by compounds that increase the intracellular adenosine-3′,5′-cyclic monophosphate (cAMP) concentration [2]. These compounds include prostaglandins, the inhibitor of phosphodiesterases, 3-isobutyl-1-methylxanthine (IBMX), membrane-permeable analogs of cAMP as well as agonists of the β_2-adrenergic receptor (β_2AR) [9–14]. Furthermore, fMLP-stimulated $O_2^{\bullet-}$ formation is enhanced by the incubation of neutrophils with *N*-(2-{[(*E*)-3-(4-bromophenyl)prop-2-enyl]amino}ethyl)isoquinoline-5-sulfonamide (H-89), an inhibitor of cAMP-dependent protein kinase (PKA) [11]. Canonically, the β_2AR couples to G_s proteins in order to activate adenylyl cyclases (AC) resulting in increased intracellular cAMP concentration [2]. Nevertheless, the β_2AR can also couple to G_i proteins, G_q proteins and β-arrestin, triggering responses distinct from those activated through G_s proteins [15–19].

Table 1. Comparison of potencies and efficacies of standard β_2AR agonists, determined in three different test systems.

Cpd.	$O_2^{\bullet-}$ assay (β_2AR on neutrophil granulocytes)		cAMP assay (β_2AR on neutrophil granulocytes)		GTPase assay (recombinant protein hβ_2AR-$G_{s\alpha S}$)	
	$pIC_{50} \pm$ S.E.M. *(IC_{50} in μM)*	$E_{max} \pm$ S.E.M.	$pEC_{50} \pm$ S.E.M. *(EC_{50} in μM)*	$E_{max} \pm$ S.E.M.	$pEC_{50} \pm$ S.E.M. *(EC_{50} in μM)*	$E_{max} \pm$ S.E.M.
ISO	8.02±0.07 *(0.0096)*	1.00	7.42±0.10 *(0.038)*	1.00	7.50[b] *(0.032)*	1.00[b,c]
ADR	7.82±0.09 *(0.015)*	1.01±0.05	6.81±0.09 *(0.16)*	1.06±0.04	7.37[c] *(0.043)*	1.00[c]
SAL	7.16±0.12 *(0.069)*	0.77±0.04	6.74±0.15 *(0.18)*	0.35±0.03	6.70[b] *(0.20)*	0.74[b]
DOB	n.d.	0.15±0.03[a]	4.86±0.36 *(13.8)*	0.21±0.05	6.70[b] *(0.20)*	0.45[b]
EPH	5.95±0.23 *(1.12)*	0.34±0.04	<4 *(<100)*	0.01±0.01	4.69[b] *(20.5)*	0.31[b]
DCI	4.40±0.26 *(39.8)*	0.32±0.06	<4 *(<100)*	0.00±0.01	7.09[b] *(0.082)*	0.17[b]

On human neutrophil granulocytes, the $O_2^{\bullet-}$ assay (1×10^5 cells per well) and the cAMP assay (5×10^5 cells per cup) were performed as described under *Materials and Methods*. Data were analyzed by non-linear regression and were best fitted to sigmoidal concentration/response curves. Data shown are the means of four to nine independent experiments performed in triplicate. The efficacy (E_{max}) of ISO in each assay was set to 1.00 and the efficacies of other ligands were referred to this value. n.d. not determined. As DOB at concentrations higher than 500 nM caused reduction of ferricytochrome c *per se*, the reliable determination of pIC_{50} value was not possible. [a] Efficacy at concentration of 500 nM was taken as E_{max}. [b] The data were taken from [45]. The reported non-logarithmic EC_{50} values were converted into logarithmic pEC_{50} values. [c] The data were taken from [43]. The non-logarithmic EC_{50} values shown in μM in parentheses were converted into logarithmic pEC_{50} value. The literature data on GTPase assay are lacking S.E.M., because the original data are represented in non-logarithmic manner and/or S.D. or 95% confidence interval is indicated instead of S.E.M. Therefore, calculation of S.E.M. from available data was impossible.

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Classic models of G-protein-coupled receptor (GPCR) activation postulate the existence of a single active (R*) and an inactive (R) state [20–22]. In the active R* state, the receptor is assumed to activate its cognate G protein and regulate down-stream effectors. However, over the past 15–20 years, compelling evidence from various groups has accumulated that the R/R* dichotomy is too simplicistic. These studies comprise biochemical, pharmacological and biophysical approaches [19–39]. Accordingly, it is now generally assumed that any given ligand stabilizes a ligand-specific receptor conformation with unique signaling capabilities, resulting in ligand-specific activation of G-proteins and/or β-arrestin [27,28]. Stabilization of ligand-specific receptor conformations with unique signaling capabilities is also referred to as functional selectivity or biased signaling [20–22,29–33]. To this end, most of the evidence for ligand-specific receptor conformations has been obtained in studies with recombinant systems [34–36] or purified receptor proteins [31,37–39], but studies on the relevance of ligand-specific receptor conformations in native human cells are still largely missing. Reasons for this lack of knowledge are inherent difficulties to manipulate human cells genetically. In addition, after isolation, human blood cells such as neutrophils survive only for limited period of time [40]. However, since the concept of functional selectivity implies that certain ligands can be clinically more efficacious in a given setting while displaying less unwanted effects, it is of paramount importance to probe functional selectivity in native cells.

Recently, we have reported on the functional selectivity of another G_s-coupled receptor, the histamine H_2 receptor, in two native cell systems, human eosinophils and neutrophils [40]. The pharmacological profiles of H_2R agonists as well as H_2R antagonists do not match by comparing their effects on eosinophils and neutrophils as well as by comparing these parameters with data obtained in a recombinant test system. Moreover, even in the same cell type, differences were observed when ligands were characterized determining two different parameters. Each ligand triggers unique effects depending on the test system and parameters measured which is of importance for further drug development.

The aim of the present study was the characterization of the β_2AR on human neutrophils with a series of structurally diverse β_2AR ligands and thereby, to probe the concept of ligand-specific receptor conformations on one of the most important and best-characterized GPCRs in a physiologically relevant native human cell system. Two distinct parameters were chosen for the characterization of the β_2AR on neutrophils. The first parameter was measurement of the cAMP content in neutrophils. The second parameter was monitoring of the β_2AR-mediated effects on fMLP-stimulated $O_2^{\bullet-}$ production. We also examined the effects of various pharmacological tools including protein kinase inhibitors, AC inhibitors and activators and various cAMP analogues on fMLP-induced $O_2^{\bullet-}$ production in order to obtain further insights into the mechanisms underlying inhibition of NADPH oxidase.

Materials and Methods

Materials

(*S**,*S**)-3-(Isopropylamino)-1-(7-methyl-2,3-dihydro-1*H*-inden-4-yloxy)butan-2-ol) ((±)-(*S**, *S**)-ICI 118551) (ICI) and (±)-bisoprolol (BIS) were obtained from Tocris Bioscience (Avonmouth, Bristol, UK). (−)-Isoproterenol (ISO), (−)-adrenaline (ADR), (±)-salbutamol (SAL), (±)-dobutamine (DOB), (±)-metoprolol (MET), (±)-alprenolol (ALP), (±)-atenolol (ATE) and forskolin (FSK) were from Sigma-Aldrich (St. Louis, MO, USA). (−)-Ephedrine (EPH) was from Mallinckrodt (St. Louis, MO, USA) and (±)-dichlorisoproterenol (DCI) from Aldrich (Milwaukee, WI, USA). Chemical structures of ligands are depicted in Fig. S1. Stock solutions of ISO, ADR, SAL, DOB, EPH and DCI (10 mM each) were prepared in 1 mM HCl and stock solutions of ICI, MET, ALP, BIS and ATE in Millipore water. Dilution series of all ligands were prepared in Millipore water. Dulbecco's PBS (DPBS, 10 x) without Ca^{2+} and Mg^{2+} (pH 6.5–7.0) was purchased from PAN Biotech (Aidenbach, Germany) and Biocoll separating solution from Biochrom (Berlin, Germany). Trypan blue solution, ferricytochrome c, cytochalasin B, fMLP and IBMX were from Sigma-Aldrich. Solvents for extraction and HPLC analysis were purchased as follows: HPLC-gradient grade water and methanol from J. T. Baker (Deventer, The Netherlands), ammonium acetate from Sigma-Aldrich and acetic acid from Riedel-de Haen (Hannover-Seelze, Germany). Tenofovir was obtained from the National

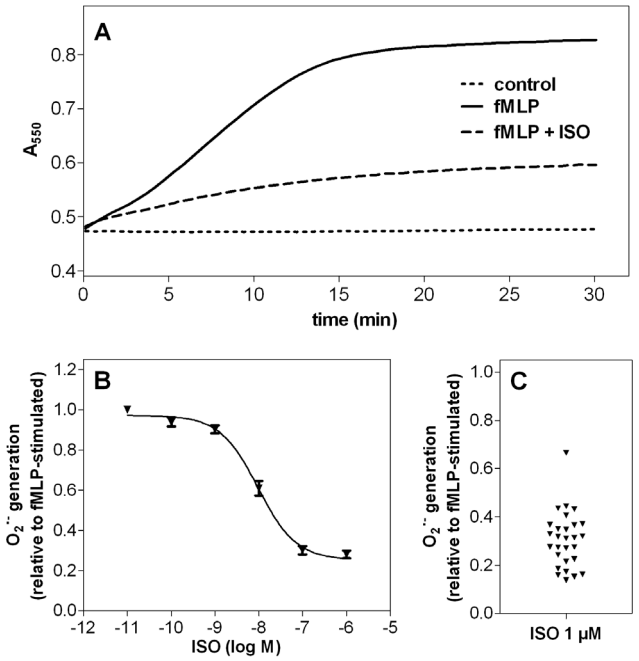

Figure 1. Superoxide anion generation assay ($O_2^{\bullet-}$ assay). The $O_2^{\bullet-}$ production in human neutrophil granulocytes (1×10^5 cells per well) was monitored by measuring the superoxide dismutase-inhibitable reduction of ferricytochrome c at 550 nm as described under *Materials and Methods*. (**A**) Continuous measurement of $O_2^{\bullet-}$ production for 30 min under control conditions (control), after stimulation with 1 µM fMLP (fMLP) and in the presence of 1 µM fMLP in combination with 1 µM ISO (fMLP+ISO). Data shown are from one representative experiment performed in triplicate. (**B**) Concentration-response curve for ISO in the $O_2^{\bullet-}$ assay. Data shown are from nine independent experiments, performed in triplicate (data points are means ± S.E.M.). Data were analyzed by non-linear regression and were best fitted to sigmoidal concentration/response curve. (**C**) Inter-experimental variability of inhibitory effect of 1 µM ISO on fMLP-stimulated $O_2^{\bullet-}$ production. Each data point represents one independent experiment. Increase in absorbance at 550 nm during 30 min after addition of fMLP was set to 1.00 and increase in absorbance in the presence of 1 µM ISO (+ fMLP) in each assay was compared to this value.
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Institutes of Health (Bethesda, MD, USA). N^6,2′-O-dibutyryladenosine-3′,5′-cyclic monophosphate (DB-cAMP) (>96%; <3% monobutyryl derivatives; <0.5% cAMP) was purchased from Sigma-Aldrich. cAMP (>99.9%), adenosine-3′,5′-cyclic monophosphorothioate, Rp-isomer (Rp-cAMPS) (>99.94%), N^6-monobutyryladenosine-3′,5′-cyclic monophosphate (6-MB-cAMP) (>99.56%), 2′-O-monobutyryladenosine-3′,5′-cyclic monophosphate (2′-O-MB-cAMP) (>99.69%), adenosine-3′,5′-cyclic monophosphorothioate, Sp-isomer (Sp-cAMPS) (>99.96%), and 8-(4-chlorophenylthio)-2′-O-methyladenosine-3′,5′-cyclic monophosphate (8-pCPT-2′-O-Me-cAMP) (>99.97%) were obtained from BIOLOG Life Science Institute (Bremen, Germany). Structures of cAMP analogs are shown in Fig. S2. Purities of cyclic nucleotides were determined by HPLC. Stock solutions of nucleotides (100 mM each) were prepared with Millipore water. H89 was obtained from Merck (Darmstadt, Germany) as a 10 mM solution in DMSO. KT5720 was from Enzo Life Sciences (Farmingdale, NY, USA) and a 10 mM stock solution was prepared in DMSO. SQ 22536 (Merck, Darmstadt, Germany) was prepared as a 10 mM solution in DMSO. FSK (10 mM) was dissolved in DMSO as well. Phorbol-12-myristate-13-acetate was prepared as a 10 mM solution in DMSO and was purchased from Sigma. Working solutions of all named substances were prepared by diluting stock solution with Millipore water.

Isolation of Human Neutrophils

This study and the consent procedure were approved by the Ethics Committee of the Medical School of Hannover. Written consent was obtained by all volunteers. The completed and signed consent forms are kept on file in the secretary of the Institute of Pharmacology of the Medical School of Hannover. Human neutrophils were isolated from venous blood of healthy volunteers of either sex (1.6 mg EDTA/ml blood as anticoagulant) or from buffy coat obtained from the Institute for Transfusion Medicine (Medical School of Hannover, Germany). Buffy coat preparations were also obtained from individual donors. All isolation steps were carried out at room temperature. Firstly, 7 ml of venous blood or 5 ml of buffy coat were diluted to 35 ml with 1×DPBS and carefully layered onto 15 ml of Biocoll separating solution (density 1.077 g/ml) in a 50 ml-Falcon tube. Following centrifugation (30 min, 400×g), the upper three layers were removed. The residual pellet (~2 ml), which contained erythrocytes and granulocytes, was resuspended in 18 ml of Millipore water and incubated for 1 min under gentle agitation in order to achieve selective lysis of erythrocytes. Afterwards, isotonicity was restored by adding 2.2 ml of 10×DPBS, and centrifugation at 300×g for 5 min followed. The lysis step was repeated once to remove residual erythrocytes. The cell pellet was re-suspended in 5 ml of 1×DPBS and sedimented by centrifugation at 300×g for 5 min. The resulting cell preparation consisted of viable neutrophils (>98%), as assessed by the trypan blue exclusion test. Finally, neutrophils were suspended in 1×PBS (1×10^6 cells/ml for the $O_2^{\bullet-}$ assay or 1×10^7 cells/ml for the determination of cAMP) and stored on ice until use. Experiments were performed within 4 h after completion of isolation because at later time points, viability of cells declined substantially as assessed by trypan blue dye uptake and declined responsiveness to receptor ligands (data not shown).

Superoxide Anion Generation ($O_2^{\bullet-}$ Assay)

Reactions were carried out in 96-well plates in triplicate. Standard reaction mixtures (total volume 200 µl) contained 1 mM $CaCl_2$, 100 µM ferricytochrome c, 0.3 µg/ml cytochalasin B (priming role by enhancing $O_2^{\bullet-}$ formation upon exposure to fMLP) [2], ligands at different concentrations (where indicated, additionally PKA inhibitors, AC inhibitors or cAMP analogs) and 1×10^5 neutrophils in 1×DPBS. After pre-incubation of the reaction mixtures for 3 min at 37°C, reactions were initiated by addition of fMLP (1 µM). Reference samples contained all components listed above except for fMLP. $O_2^{\bullet-}$ formation was continuously measured by monitoring the reduction of ferricytochrome c at 550 nm for 30 min at 37°C, using a Synergy 4 microplate reader (BioTek Instruments, Winooski, VT, USA). The difference in absorbance at 550 nm between 0 min (addition of fMLP) and 30 min was used for subsequent data analysis, in order to assess agonistic activity of examined ligands. With the exception of DOB, all examined test compounds did neither reduce ferricytochrome c nor stimulate $O_2^{\bullet-}$ production *per se* nor acted as radical scavenger as assessed by the lack of effect on phorbol ester-stimulated $O_2^{\bullet-}$ production (data not shown). As at DOB concentrations higher than 500 nM, ferricytochrome c reduction took place, the maximum concentration of DOB used in the $O_2^{\bullet-}$ assays was 500 nM.

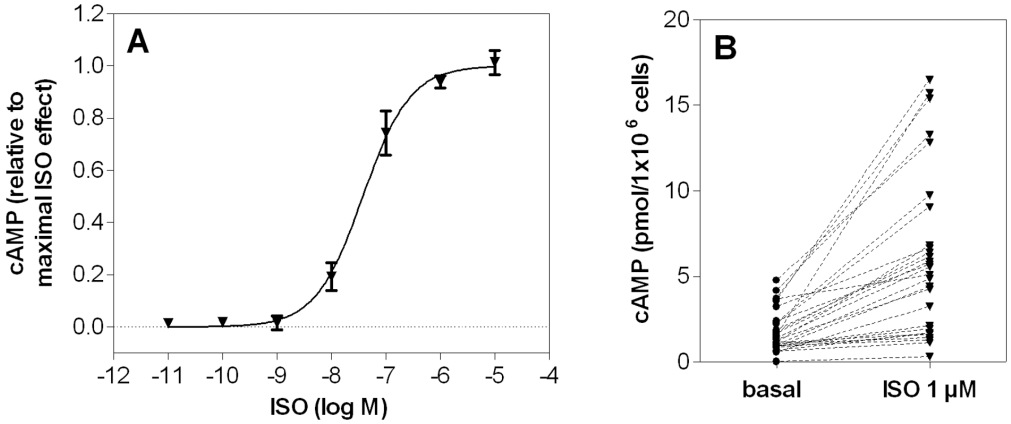

Figure 2. Measurement of cAMP content in neutrophil granulocytes (cAMP assay). cAMP accumulation in human neutrophil granulocytes (5×10^5 cells per sample) was monitored by HPLC-MS/MS system as described under *Materials and Methods*. (**A**) Concentration-response curve for ISO in the cAMP assay. Data shown are from four independent experiments, performed in triplicate (data points are mean ± S.E.M.). The maximal ISO-induced cAMP production was set to 1.00. Data were analyzed by non-linear regression and were best fitted to sigmoidal concentration/response curve. **(B)** Inter-experimental variability of basal cAMP concentration in neutrophil granulocytes (basal) and cAMP level after stimulation with 1 µM ISO (ISO 1 µM). Each data point represents one independent experiment. cAMP levels after stimulation with ISO increased by 30–1000% relative to basal cAMP levels.
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cAMP Accumulation and Extraction from Neutrophils (cAMP Assay)

Reactions were conducted in triplicate in 1.5 ml Eppendorf reaction vessels in a total volume of 100 µl. Fifty µl of the reaction mixture containing $CaCl_2$ (1 mM final concentration after addition of neutrophils), IBMX (non-selective phosphodiesterase inhibitor; 100 µM) and the respective ligand at different concentrations in 1 x DPBS were pre-incubated for 5 min at 37°C. Isolated neutrophils suspended in 1×DPBS were pre-incubated separately for 10 min at 37°C. Following the addition of 50 µl of neutrophils (5×10^5 cells/reaction vessel) to reaction mixture, samples were incubated for 10 min at 37°C. Afterwards, samples were incubated for 10 min at 95°C in order to denature cell proteins and then cooled to 4°C. One hundred µl of ice-cold internal standard (tenofovir; 100 ng/ml) in eluent A (3/97 MeOH/H_2O, 50 mM NH_4OAc, 0.1% HOAc) were added. The suspension was centrifuged at 20.800×g at 4°C for 5 min in order to remove denatured proteins. The cAMP concentration of the supernatant was determined by reversed phase HPLC coupled to mass spectrometry (HPLC-MS/MS).

Quantitation of cAMP by HPLC-MS/MS

In this study, cAMP levels were determined by HPLC-MS/MS which is characterized by extremely high sensitivity and specificity [41–42]. Since this method is not yet commonly known and used, we describe the experimental protocol in some detail. The chromatographic separation was performed on an Agilent 1100 Series HPLC System (Agilent Technologies, Santa Clara, CA, USA) equipped with a binary pump system and with a 100 µl sample loop. A combination of Supelco Column Saver (2.0 µm filter, Supelco Analytical, Bellafonte, CA, USA), Security Guard Cartridge (C18, 4×2 mm) in an Analytical Guard Holder KJO-4282 (Phenomenex, Aschaffenburg, Germany) and an analytical Zorbax Eclipse XDB-C16 column (50×4.6 mm, 1.8 µm particle size, Agilent Technologies), temperature controlled by a HPLC column oven at 25°C, were used. The binary pump system supplied eluent A (50 mM ammonium acetate and 0.1% (v/v) acetic acid in a methanol/water mixture (3/97 (v/v)) and eluent B (50 mM ammonium acetate and 0.1% (v/v) acetic acid in a methanol/water mixture (97/3 (v/v)). The injection volume was 50 µl and the flow rate of 0.4 ml/min remained constant throughout the chromatographic run. From 0 to 5 min, the gradient of eluent B was linearly increased from 0 to 50% of eluent B, and re-equilibrium of the column to 100% of eluent A was achieved from 5 to 8 min. Retention times of the analyte cAMP and the internal standard tenofovir were 6.2 and 5.4 min, respectively. The internal standard was used to mathematically correct the loss of cAMP during preparation as well as possible variabilities in HPLC-MS/MS measurement. Analyte detection was conducted on an AB Sciex QTRAP 5500 triple quadrupole mass spectrometer (AB Sciex, Foster City, CA, USA) using selected reaction monitoring (SRM) analysis in positive ionization mode. For this purpose nitrogen was used as collision gas. Using a 50 ms dwell time, SRM transitions were monitored as follows: cAMP +330/136 and +330/312, tenofovir +288/176 and +288/159. The transition +330/136 was the most intense transition of cAMP and therefore used for quantification. Additionally the +330/312 transition of cAMP was used as qualifier. The transition +288/176 of tenofovir was used as quantifier and the transition +288/159 as qualifier. The mass spectrometer parameters were as follows: ion source voltage: 4500 V, ion source temperature: 600°C, curtain gas: 30 psi and collision gas: 9 psi. cAMP in samples was quantified by applying the standard curve, obtained by analysis of known amounts of pure cAMP at: 0.0262, 0.066, 0.164, 0.41, 1.024, 2.56, 6.4, 16, 40, 100, 250 pmol/tube.

Miscellaneous (Data Analysis, Statistical Analysis and GTPase Assay)

Chromatograms, obtained by the HPLC-MS/MS analysis, were analyzed with the Analyst Software 1.5.1 (AB Sciex). Steady-state GTPase activity assay, using membrane preparations of Sf9 insect cells, expressing fusion protein β_2AR-$G_{s\alpha s}$, was performed as described previously [43]. Data from the $O_2^{\bullet-}$, cAMP and GTPase assays were analyzed with the Prism 5.01 software (GraphPad, San Diego, CA, USA). The means ± S.E.M. were always determined by the analysis of at least three

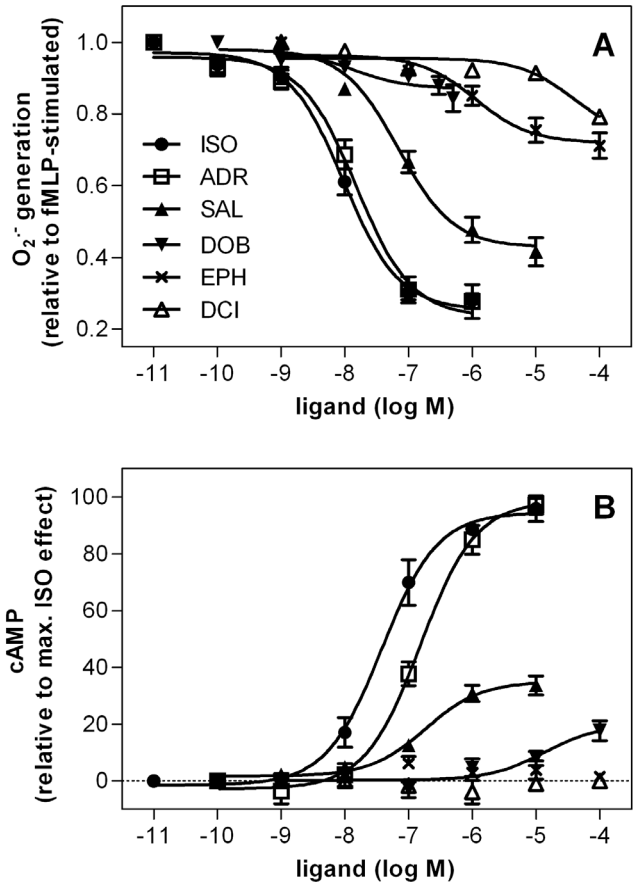

Figure 3. Concentration-response curves for β_2AR agonists determined in the $O_2^{\bullet-}$ assay (A) and cAMP assay (B). The $O_2^{\bullet-}$ assay (1×10^5 cells per well) and the cAMP assay (5×10^5 cells per cup) were performed as described in *Materials and Methods*. Data were analyzed by non-linear regression and were best fitted to sigmoidal concentration/response curves. Data shown are the means ± S.E.M. of four to nine independent experiments performed in triplicate. As DOB at concentrations higher than 500 nM caused reduction of ferricytochrome c *per se*, the maximal DOB concentration examined in the $O_2^{\bullet-}$ assay was 500 nM.
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independent experiments, performed in triplicate, if not indicated otherwise.

The efficacy (E_{max}) of ISO in each assay was set to 1.00 and the efficacies of other ligands were referred to this value. The pK_B values for β_2AR antagonists were calculated according to Cheng and Prusoff [44] using the following equation: $pK_B = -\log(IC_{50antagonist}/(1+(c_{ISO}/x)))$; $IC_{50antagonist}$ – IC_{50} value of an antagonist, determined in antagonist mode, c_{ISO} – used concentration of ISO, ×– IC_{50} ($O_2^{\bullet-}$ assay) or EC_{50} value (cAMP assay and GTPase assay) for ISO, determined in agonist mode (data from Table 1).

Data for receptor ligands were analyzed using one-way ANOVA, followed by Bonferroni's multiple comparison test, in order to compare pK_B values of the examined β_2AR antagonists between the $O_2^{\bullet-}$, cAMP and GTPase assay. Data for AC- and PKA inhibitors were analyzed using one-way ANOVA, followed by Dunnett's multiple comparison test. Statistical significance was defined as $p < 0.05$ (95% confidence interval).

Results

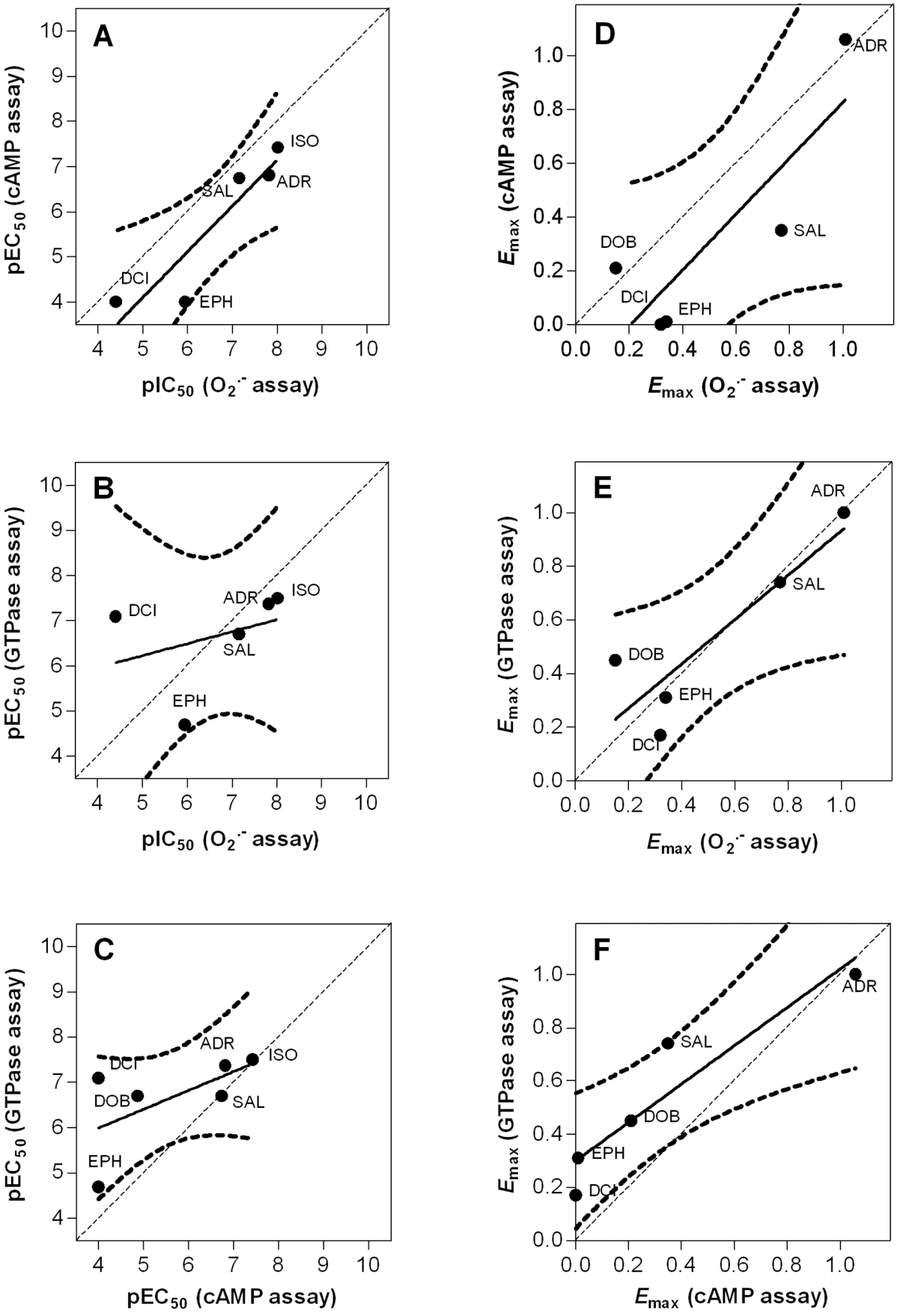
Characterization of the β_2AR on Human Neutrophils with β_2AR Agonists

β_2AR agonists with efficacies varying from very weak partial to full agonism [45] were examined. The effects of β_2AR agonists were measured as inhibition of fMLP-stimulated $O_2^{\bullet-}$ production (Fig. 1) and as cAMP accumulation (Fig. 2). In both cases, the inter-experimental variability was high (Figs. 1 and 2). Note that in Figs. 1C and 2B, data from different individuals are depicted. High inter-individual variability of human neutrophil function was observed previously [46]. However, when neutrophils from a given donor were analyzed on different days, data in the two test systems generally varied by less than 20% (data not shown). Thus, interindividual data variability is a much greater issue with neutrophils than intraindividual day-to-day variability. Accordingly, in order to allow comparison of results from experiments with different donors in this study, data were normalized with 1.00 representing the maximal effect of the β_2AR agonist ISO and 0.00 representing the basal activity.

Potencies and efficacies of the examined β_2AR agonists in the $O_2^{\bullet-}$ and the cAMP assay are listed in Table 1 and concentration-response curves are depicted in Fig. 3. Additionally, the EC_{50} and E_{max} values of ligands determined in steady-state GTPase activity assays using membrane preparations of Sf9 insect cells expressing the β_2AR-$G_{s\alpha S}$ fusion protein [43,45] are listed in Table 1. The β_2AR-$G_{s\alpha S}$ fusion protein is artificial but has become a standard system for the pharmacological analysis of the β_2AR [43,45]. For a detailed discussion on the advantages and disadvantages of the fusion protein technique as well as additional references relevant for this approach, the reader is referred to ref. 43. Potencies of ISO, ADR and SAL were higher in the $O_2^{\bullet-}$ assay than in the cAMP assay. EPH and DCI were lacking agonistic activity in the cAMP assay at concentrations up to 100 μM, whereas inhibitory effects of both ligands on fMLP-stimulated $O_2^{\bullet-}$ production were readily observed. The efficacy of ADR was comparable in both test systems, but the efficacy of SAL more than two times higher in the $O_2^{\bullet-}$ assay relative to the cAMP assay. When the data from the recombinant test system were included in the comparison, the rank order of potency of ligands was cAMP assay<GTPase assay<$O_2^{\bullet-}$ assay, and the rank order of efficacy was cAMP assay<$O_2^{\bullet-}$ assay ≈ GTPase assay (Fig. 4).

In case of the two-state model postulating a single active state, we would have expected linear correlations for agonists with respect to efficacies and potencies, regardless of which parameters are compared. However, Fig. 4 shows that the correlations are, in general, rather poor, regardless of which comparisons are being made. The worst correlations in terms of deviation from the theoretically expected slope of 1.00 in case of identity of parameters were observed for the comparison of pEC_{50} values in the GTPase and $O_2^{\bullet-}$ assay (Fig. 4B) and pEC_{50} values in the GTPase and cAMP assay (Fig. 4C). A limitation of our study is that we studied only a limited number of agonists, but an advantage is that the ligands cover a broad range of efficacies and potencies so that clustering of the data in one spot is avoided. In fact, this type of two-dimensional comparison of ligand potencies and efficacies has been repeatedly used to support the concept of ligand-specific receptor conformations in various test systems [24,27,32,40].

A trivial explanation for the differing effects of β_2AR agonists in the $O_2^{\bullet-}$ assay and cAMP assay could be that the agonists exhibit $O_2^{\bullet-}$ scavenging properties on fMLP-stimulated $O_2^{\bullet-}$ production. However, when $O_2^{\bullet-}$ production in neutrophils was triggered with phorbol-12-myristate-13-acetate (activator of protein kinase C,


A
pEC50 (cAMP assay)
pIC50 (O2·- assay)
ISO
ADR
SAL
DCI
EPH
B
pEC50 (GTPase assay)
pIC50 (O2·- assay)
DCI
ADR
ISO
SAL
EPH
C
pEC50 (GTPase assay)
pEC50 (cAMP assay)
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Emax (cAMP assay)
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DOB
EPH
DCI

Figure 4. Pair-wise comparison of the potencies (A–C) and efficacies (D–F) of the β_2AR agonists in the $O_2^{\bullet-}$ assay, the cAMP assay and the GTPase assay. The data for comparison were taken from Table 1 and were analyzed by linear regression. The dashed lines represent the 95% confidence intervals in the regression line. The diagonal dotted line indicates a theoretical line for identical values (slope = 1). Slopes (95% confidence interval) and r^2 of the calculated correlations are as follows; **A.** 1.02 (0.22 to 1.81), 0.85; **B.** 0.27 (−1.05 to 1.58), 0.12; **C.** 0.42 (−0.32 to 1.15), 0.38; **D.** 1.04 (−0.14 to 2.21), 0.73; **E.** 0.83 (0.02 to 1.63), 0.78; **F.** 0.72 (0.22 to 1.22), 0.89.
doi:10.1371/journal.pone.0064556.g004

100 nM) instead of fMLP, the examined β_2AR agonists had no effect on $O_2^{\bullet-}$ production at all (data not shown).

Characterization of the β_2AR on Human Neutrophils with β_2AR Antagonists

According to conventional models of GPCR activation, potency of an antagonist for a given receptor is constant irrespective of the tissue or recombinant system selected for the characterization, the agonist used for the stimulation of GPCR and downstream signaling event monitored [20–22,47]. However, by monitoring the cAMP accumulation and cAMP response element-mediated reporter gene transcription in Chinese hamster ovary (CHO) cells, different K_B values were determined for β_2AR antagonists [48]. These data indicate that antagonists, like agonists, may stabilize functionally distinct receptor conformations. Likewise, we obtained evidence for functional selectivity of antagonists at various recombinant histamine receptor subtypes [49]. Hence, the question arose whether parameter-dependent β_2AR antagonist potency is also apparent in a native test system, namely in neutrophils.

The pK_B values for ICI, MET, ALP, BIS and ATE were determined in the cAMP and $O_2^{\bullet-}$ assay by applying a submaximally effective concentration of ISO and increasing concentrations of β_2AR antagonists. Concentration-response curves for antagonists are shown in Fig. 5. In Table 2, the results are summarized and compared with antagonistic activity of the same ligands in the recombinant test system (GTPase assay using membranes of Sf9 insect cells expressing β_2AR-$G_{s\alpha S}$). The statistical analysis of the obtained data revealed no difference of pK_B values between the two different parameters in neutrophils, BIS being an exception. However, in comparison with data on neutrophils, the potencies of all antagonists were significantly reduced on the recombinantly expressed β_2AR-$G_{s\alpha S}$. Fig. 6 shows correlations of the pK_B values of antagonists for the various parameters analyzed. It is evident that the correlations for antagonists are much better than the corresponding correlations for agonists shown in Fig. 5.

Do AC- and PKA-activation Interfere with fMLP-stimulated $O_2^{\bullet-}$ Production?

As already mentioned in the introduction, β_2AR-signaling is very complex, depending on ligand and test system [15,16,18,26,27]. β_2AR-mediated inhibition of fMLP-stimulated $O_2^{\bullet-}$ production is thought to be due to cAMP production and PKA activation [50–51]. In order to address this question we studied the effects of three structurally distinct and well-established PKA inhibitors. Among the inhibitors are an isoquinoline sulfonamide [52], a cAMP analog [53] and a microbial metabolite [54]. However, in our experiments, we failed to obtain evidence for the hypothesis that activation of PKA after β_2AR stimulation is a crucial event for inhibition of fMLP-stimulated $O_2^{\bullet-}$ production (Fig. 7). Specifically, compounds H89 and KT5720, widely-used and effective cell-permeable competitive inhibitors of the ATP-binding to the ATP-binding pocket of the PKA in other test systems [55–58], did not reverse ISO-induced inhibition of fMLP-stimulated $O_2^{\bullet-}$ production (also when neutrophils were pretreated 15 min with H89 or KT5720). Even the cAMP antagonist Rp-cAMPS which competes with cAMP for the binding to the regulatory subunits of PKA [57,59], did not interfere with the effect of ISO of $O_2^{\bullet-}$ production in human neutrophils (Fig. 7).

Moreover, we tried to assess the involvement of the cAMP signaling pathway in the fMLP-stimulated $O_2^{\bullet-}$ production by applying the AC inhibitor SQ 22536 [60]. Surprisingly, SQ 22536 enhanced rather than diminished the inhibitory effect of ISO on fMLP-induced $O_2^{\bullet-}$ production (Fig. 8A). Additionally, SQ 22536 exhibited unexpected inhibitory effects on fMLP-induced $O_2^{\bullet-}$ production on its own. SQ 22536 by itself did not increase cAMP levels in neutrophils, and the compound also did not inhibit the ISO-induced cAMP increase (Fig. 8B). Pleiotropic and AC-

Table 2. Comparison of pK_B values of the β_2AR antagonists, determined in three different test systems.

Cpd.	$O_2^{\bullet-}$ assay (β_2AR on neutrophil granulocytes)	cAMP assay (β_2AR on neutrophil granulocytes)	GTPase assay (recombinant protein β_2AR-$G_{s\alpha S}$)[a]
	pK_B ± S.E.M. (K_B *in* μM)	pK_B ± S.E.M. (K_B *in* μM)	pK_B ± S.E.M. (K_B *in* μM)
ICI	9.51 ±0.09 *(0.00031)*	9.97 ±0.07 *(0.00011)*	8.55 ±0.14$^{***,+++}$ *(0.0028)*
MET	7.18 ±0.15 *(0.066)*	7.19 ±0.12 *(0.065)*	6.08 ±0.17$^{**,++}$ *(0.83)*
ALP	9.40 ±0.10 *(0.00040)*	9.71 ±0.06 *(0.00020)*	8.56 ±0.14$^{***,+++}$ *(0.0028)*
BIS	6.58 ±0.19 *(0.26)*	7.17 ±0.11* *(0.068)*	5.77 ±0.10$^{**,+++}$ *(1.70)*
ATE	5.89 ±0.12 *(1.29)*	5.99 ±0.20 *(1.02)*	5.12 ±0.17$^{*,+}$ *(7.59)*

On human neutrophil granulocytes, the $O_2^{\bullet-}$ assay (1×10^5 cells per well) and the cAMP assay (5×10^5 cells per sample) were performed as described under *Materials and Methods*. Steady-state GTPase activity assay, using membrane preparations of Sf9 insect cells, expressing fusion protein β_2AR-$G_{s\alpha S}$, was performed as described in [43]. $O_2^{\bullet-}$ and cAMP production as well as GTP hydrolysis were determined at submaximally effective concentration of ISO (100 nM in the $O_2^{\bullet-}$ and cAMP assay, 10 nM in the GTPase assay) in the presence of increasing concentrations of β_2AR antagonists. Data were analyzed by non-linear regression and were best fitted to sigmoidal concentration/response curves. Data shown are from four to five independent experiments performed in triplicate. The pK_B values were calculated from the IC_{50} values according to Cheng and Prusoff [44]. pK_B values were compared with each other using one-way ANOVA, followed by Bonferroni's multiple comparison test (pK_B significantly different to: *$O_2^{\bullet-}$ assay, $^+$cAMP assay; one symbol: $p<0.05$, two symbols: $p<0.01$, three symbols: $p<0.001$). Non-logarithmic K_B values in μM are shown in parentheses.
doi:10.1371/journal.pone.0064556.t002

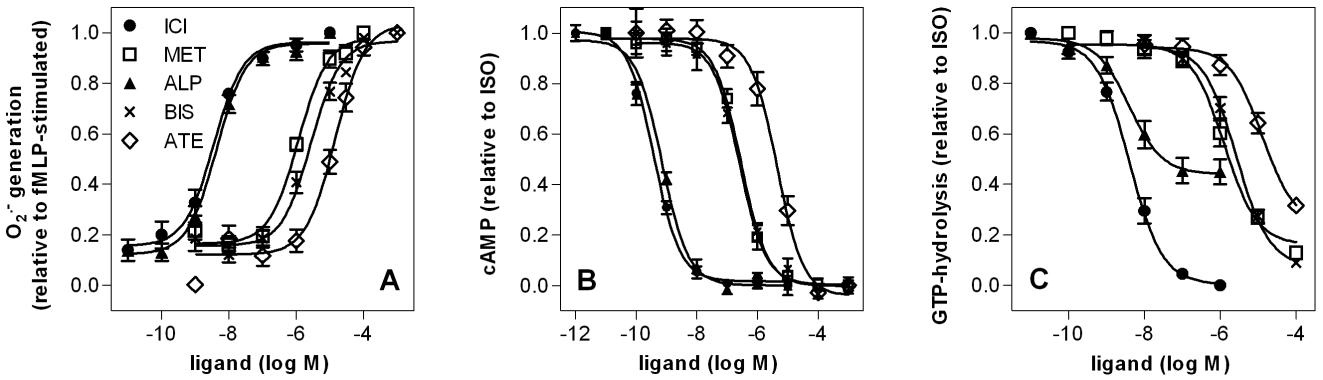

Figure 5. Concentration-response curves for β_2AR antagonists determined in the $O_2^{\bullet-}$ (A), cAMP (B) and GTPase assay (C). The $O_2^{\bullet-}$ assay (1×10^5 cells per well) and the cAMP assay (5×10^5 cells per cup) were performed as described in sections 2.3 and 2.4, respectively. Steady-state GTPase activity assay, using membrane preparations of Sf9 insect cells, expressing fusion protein β_2AR-$G_{s\alpha S}$, was performed as described in [43]. Data were analyzed by non-linear regression and were best fitted to sigmoidal concentration/response curves. Data shown are the means ± S.E.M. of four to five independent experiments performed in triplicate. $O_2^{\bullet-}$ and cAMP production as well as GTP hydrolysis were determined at submaximally effective concentration of ISO (100 nM in the $O_2^{\bullet-}$ and cAMP assay, 10 nM in the GTPase assay) in the presence of increasing concentrations of β_2AR antagonists.
doi:10.1371/journal.pone.0064556.g005

independent effects of SQ 22536 have been observed repeatedly [61].

As an additional tool we examined the diterpene, FSK, a direct activator of membranous ACs [61]. However, FSK did neither significantly reduce fMLP-stimulated $O_2^{\bullet-}$ production (Fig. 8A) nor robustly increase cAMP levels (Fig. 8B).

In order to provide proof of principle that an increase in intracellular cAMP concentration is capable of inhibiting fMLP-stimulated $O_2^{\bullet-}$ production, we examined the effects of cAMP and various cAMP analogs on NADPH oxidase activation. DB-cAMP is lipophilic and penetrates the plasma membrane. Inside the cell, DB-cAMP is converted to the biologically active 6-MB-cAMP [62]. In accordance with previous data [9], DB-cAMP reduced fMLP-stimulated $O_2^{\bullet-}$ production, whereas the control compound sodium butyrate was ineffective (Fig. 9). In addition, the mono-butyrylated control compound 6-MB-cAMP did not robustly inhibit NADPH oxidase, most likely due to inefficient membrane penetration. Sp-cAMPS is less lipophilic than DB-cAMP but does not require bioactivation [59]. Sp-cAMPS was similarly effective at inhibiting $O_2^{\bullet-}$ production as DB-cAMP. These data show that cAMP does have the potential to inhibit NADPH oxidase. However, we also noted that very high concentrations of DB-cAMP are required to elicit inhibition, probably exceeding the intracellular cAMP concentrations achieved following β_2AR stimulation.

cAMP itself also slightly inhibited $O_2^{\bullet-}$ production. This could be due to extracellular degradation of cAMP to adenosine by phosphodiesterases and ectonucleotidases and subsequent activation of adenosine A2-receptors by adenosine [63]. Import of cAMP via multidrug resistance protein transporters (MRPs) into cells and subsequent PKA activation could be involved as well [64]. Likewise, the small inhibitory effects of the PKA inhibitor Rp-cAMPS could be due to adenosine liberation from the parent compound. We do not have a satisfactory explanation for the small but significant stimulatory effect of the mono-butyrylated control compound 2′-O-MB-cAMP on $O_2^{\bullet-}$ production. However, we confirmed that the compound per se did not activate $O_2^{\bullet-}$ production or reduced ferricytochrome c independently of NADPH oxidase (data not shown). The activator of the cAMP effector protein Epac, 8-pCPT-2′-O-Me-cAMP [65], did not inhibit fMLP-induced $O_2^{\bullet-}$ production, arguing against an involvement of Epac in NADPH oxidase regulation. We also observed that the PKA inhibitors H89, KT5720 and Rp-cAMPS showed no reversing effect on the inhibition of $O_2^{\bullet-}$ production caused by DB-cAMP (Fig. 7). These data raise questions whether a hitherto unidentified cAMP-binding protein is involved in the inhibition of $O_2^{\bullet-}$ production by DB-cAMP and Sp-cAMPS.

Discussion

The two-state model of receptor activation implying an active (R*) and an inactive (R) state has now been superseded by a more complex model involving multiple active receptor conformations that lead to ligand-specific receptor activation, also referred to as functional selectivity or biased agonism [19–33]. Functional selectivity has been reported for numerous GPCRs such as dopamine D_1 and D_2 receptors, the histamine H_2 and H_4 receptor, adenosine A_1 and A_3 receptors, the α_{2A}-adrenoceptor and the β_2AR [15–33,40,49]. So-called biased ligands can differently activate G protein-dependent and -independent signaling such as the β-arrestin pathway [27,32,34,35], can discriminate between G_s, G_i, G_q and other G protein-mediated pathways [15,66] or even selectively modulate e.g. G_{i1}, G_{i2} and G_{i3} protein subtype activities [67]. Therefore, it is not surprising that any given ligand possesses multiple potencies and efficacies depending on the down-stream pathway analyzed [33]. This concept was also confirmed in our study with β_2AR agonists in human neutrophils using the cAMP assay and the $O_2^{\bullet-}$ assay as parameters and by comparison of the results with literature data obtained in recombinant test system (Fig. 4). If only a single active β_2AR conformation existed, we would have expected linear correlations following the dotted lines in Fig. 4 between potencies and efficacies (relative to the reference compound ISO) of agonists, regardless of which parameters are considered. However, this was not the case. In accordance with our data, fluorescence studies with purified β_2AR nuclear magnetic resonance studies provided evidence for ligand-specific conformations [31,37,39].

With respect to β_2AR antagonists, effects were similar in the cAMP and $O_2^{\bullet-}$ assay on neutrophils (Table 2 and Fig. 5), indicating that in neutrophils, functional selectivity is predominantly observed for β_2AR agonists. In contrast to neutrophil parameters, potencies of antagonists were generally lower at the

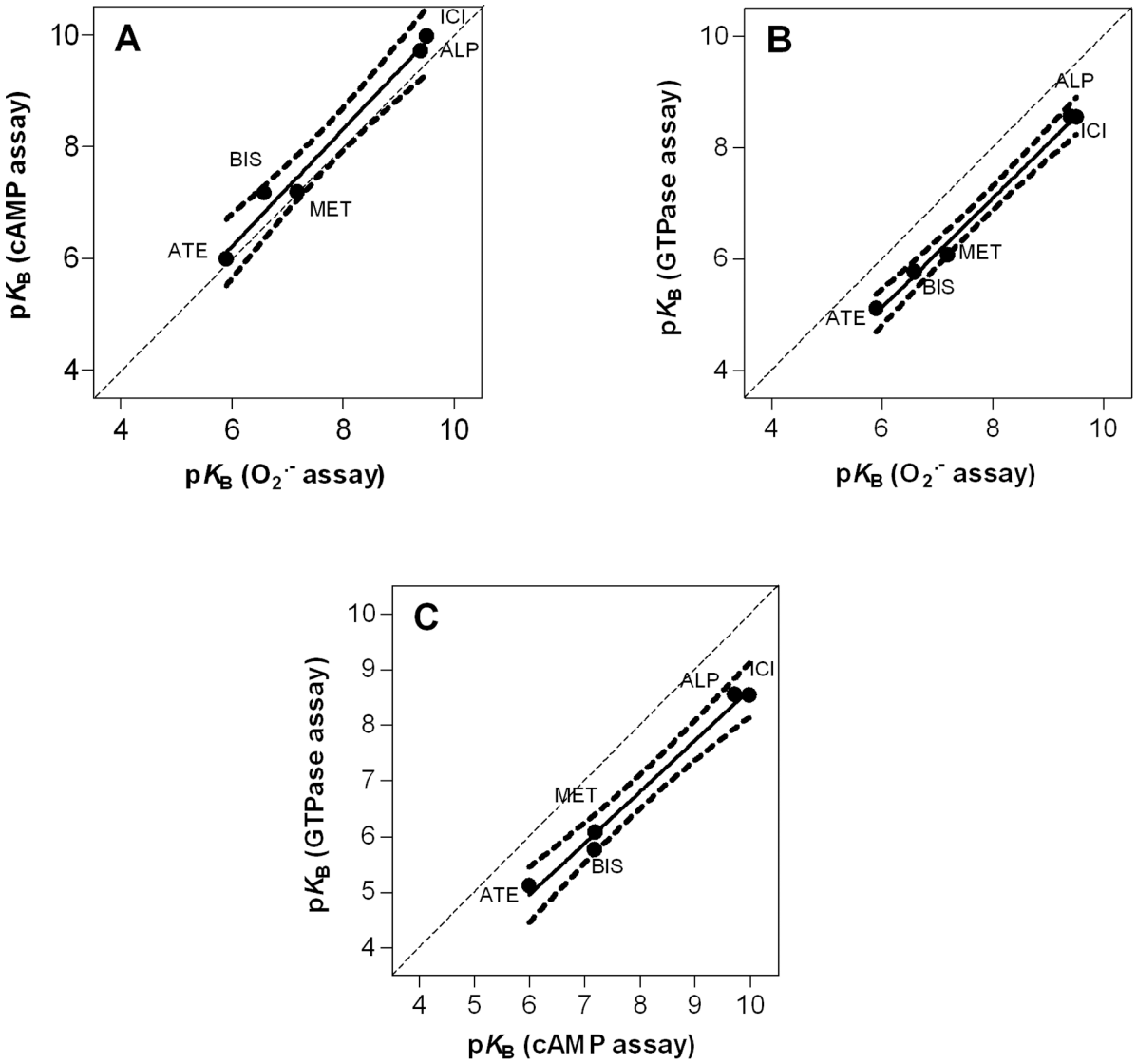

Figure 6. Pair-wise comparison of the pK_B values of the β_2AR antagonists in the $O_2^{\bullet-}$ assay, the cAMP assay and the GTPase assay. The data for comparison were taken from Table 2 and were analyzed by linear regression. The dashed lines represent the 95% confidence intervals in the regression line. The diagonal dotted line indicates a theoretical line for identical values (slope = 1). Slopes (95% confidence interval) and r^2 of the calculated correlations are as follows; **A.** 1.05 (0.79 to 1.30), 0.98; **B.** 0.98 (0.84 to 1.12), 0.99; **C.** 0.93 (0.73 to 1.12), 0.99.
doi:10.1371/journal.pone.0064556.g006

recombinant β_2AR than at the native β_2AR (Table 2), and there were also ligand-specific differences. The trend towards lower antagonist (inverse agonist) potencies at the recombinant β_2AR could be due to higher constitutive activity of the recombinant than of the native system [20].

The vast majority of reports about functional selectivity originate from studies with recombinant test systems or purified receptors (see, e.g., [31,34–36,38–39]). On the contrary, functional selectivity in native test systems has been rarely studied so far, e.g. for the histamine H_4 receptor on isolated human eosinophils [68] and for the histamine H_2 receptor on isolated human eosinophils and neutrophils [40]. Here, we report on functional selectivity of the β_2AR in human neutrophils. In accordance with our data, there is evidence for functional selectivity of β_2AR ligands in cardiomyocytes [69]. In this system, stereoisomers of fenoterol differentially activate G_i- and G_s-proteins.

Unfortunately, in-depth analysis of functional selectivity in neutrophils is hampered by limited possibilities to block coupling of the β_2AR to coupling partners. GPCR-G_i protein coupling can be interrupted with pertussis toxin [70], whereas there is no pharmacological tool available for the selective inhibition of G_s and G_q coupling or the β-arrestin pathway in native test systems. Furthermore, since the formyl peptide receptor is coupled to G_i-proteins and an essential stimulatory component in the $O_2^{\bullet-}$ assay, we could not use pertussis toxin to differentiate between β_2AR ligands in this assay and in the cAMP assay. Moreover, in neutrophils, difficulties for effective genetic manipulation, inter-individual variability and relatively short life time impede with more detailed analysis of functional selectivity in this native test system.

Our data obtained by measuring GTP hydrolysis in the recombinant test system reflect coupling of the β_2AR solely to the short splice variant of the G_s protein [43,45]. There is no

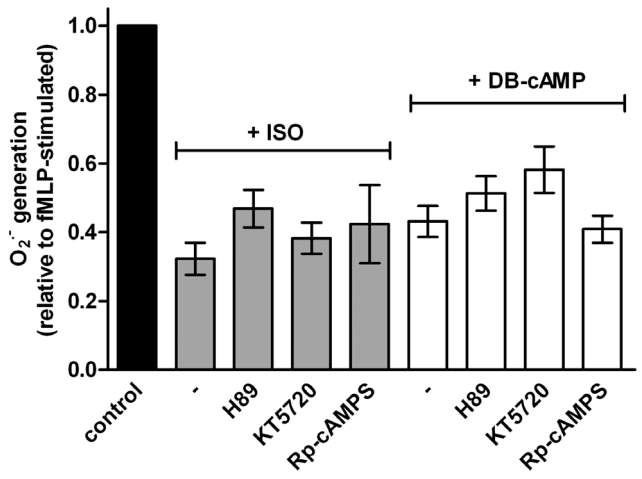

Figure 7. Effects of different PKA inhibitors on the ISO- and DB-cAMP-induced inhibition of fMLP-stimulated $O_2^{\cdot-}$ production. $O_2^{\cdot-}$ production in human neutrophil granulocytes (1×10^5 cells per well) was monitored by measuring the superoxide dismutase-inhibitable reduction of ferricytochrome c at 550 nm. Data shown (mean ± S.E.M.) are from three independent experiments, performed in triplicate. Concentrations used are as follows; ISO 100 nM, DB-cAMP 500 μM, H89 1 μM, KT5720 10 μM and Rp-cAMPS 100 μM. H89, KT5720 and Rp-cAMPS had no significant reversal effect on the inhibitions caused by ISO and DB-cAMP.
doi:10.1371/journal.pone.0064556.g007

doubt that the β_2AR couples to G_s in neutrophils, but no information is available regarding the involvement of specific G_s splice variants. The question remains what the reason for the generally increased potency of the examined β_2AR agonists in the $O_2^{\cdot-}$ assay compared to the cAMP assay is. Lack of correlation between the cAMP accumulation and inhibition of fMLP-induced $O_2^{\cdot-}$ production in neutrophils has been described also by other research groups using other stimuli [71–74]. Among other reasons, better coupling efficiency of the β_2AR to the $O_2^{\cdot-}$ pathway than to the cAMP pathway is of relevance for the divergence in data. Differences in strength of coupling to different signaling pathways have been reported for other receptors [75]. Additionally, we have no evidence for the involvement of Epac in the signal transduction pathway of the β_2AR leading to inhibition of NADPH oxidase since an effective Epac activator failed to inhibit fMLP-stimulated $O_2^{\cdot-}$ production (Fig. 9).

We failed to support an involvement of AC, cAMP and PKA in β_2AR-mediated inhibition of fMLP-stimulated $O_2^{\cdot-}$ production. Thus, it appears that the two measured events (cAMP accumulation and $O_2^{\cdot-}$ production) in neutrophils are independent of each other. The lack of effect of DCI and EPH on cAMP accumulation despite inhibitory effects of these ligands on $O_2^{\cdot-}$ production supports the concept of cAMP-independent inhibition of NADPH oxidase. Moreover, SAL is more effective at inhibiting NADPH oxidase than at increasing cAMP. Our failure to detect stimulatory effects of DCI and EPH on cAMP accumulation and ineffective stimulation of cAMP accumulation by SAL are not due to cAMP degradation since we included a phosphodiesterase inhibitor into the cAMP assay. Moreover, we applied a highly sensitive and specific MS method to detect cAMP, avoiding notorious cross-reactivity problems of antibodies widely applied in cyclic nucleotide detection [41,42,76]. Thus, our study addressing ligand-specific receptor conformations also casted doubt about the dogma of cAMP-dependent inhibition of NADPH oxidase by the β_2AR although, in principle, cAMP can inhibit $O_2^{\cdot-}$ production (Fig. 9). Even in case of inhibition of $O_2^{\cdot-}$ production by cAMP analogs, we failed to obtain positive evidence for an involvement of PKA (Fig. 7).

Analysis of the signaling pathways responsible for inhibition of $O_2^{\cdot-}$ production in neutrophils is hampered by unsuitability of

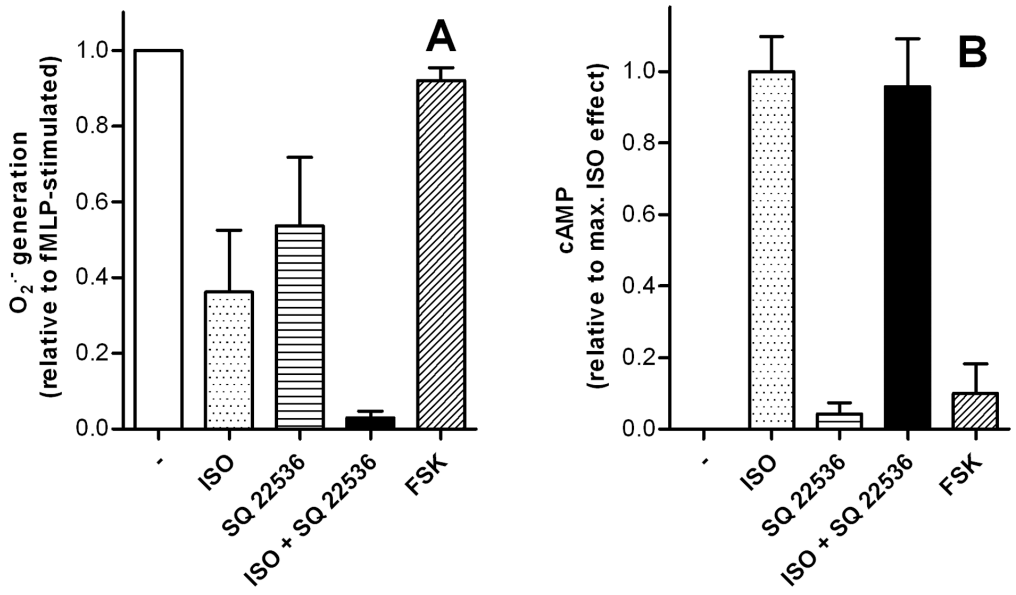

Figure 8. Effects of SQ 22536 and FSK on the fMLP-stimulated $O_2^{\cdot-}$ production (A) and cAMP accumulation (B). The $O_2^{\cdot-}$ assay (1×10^5 cells per well) and the cAMP assay (5×10^5 cells per cup) were performed as described in Materials and Methods. Data shown (mean ± S.E.M.) are from three to six independent experiments, performed in triplicate. Concentrations used are as follows; ISO 100 nM (**A**) and 1 μM (**B**), SQ 22536 100 μM. For comparison, we also studied the effect of the direct AC activator FSK (10 μM). In all tubes, a final concentration of 1% (v/v) DMSO (unavoidable for dissolving SQ 22536 and FSK) was present to achieve comparable results. SQ 22536 had no significant reversal effect on the inhibition caused by ISO in the $O_2^{\cdot-}$ assay, and FSK had no significant inhibitory effect on fMLP stimulation in this assay.
doi:10.1371/journal.pone.0064556.g008

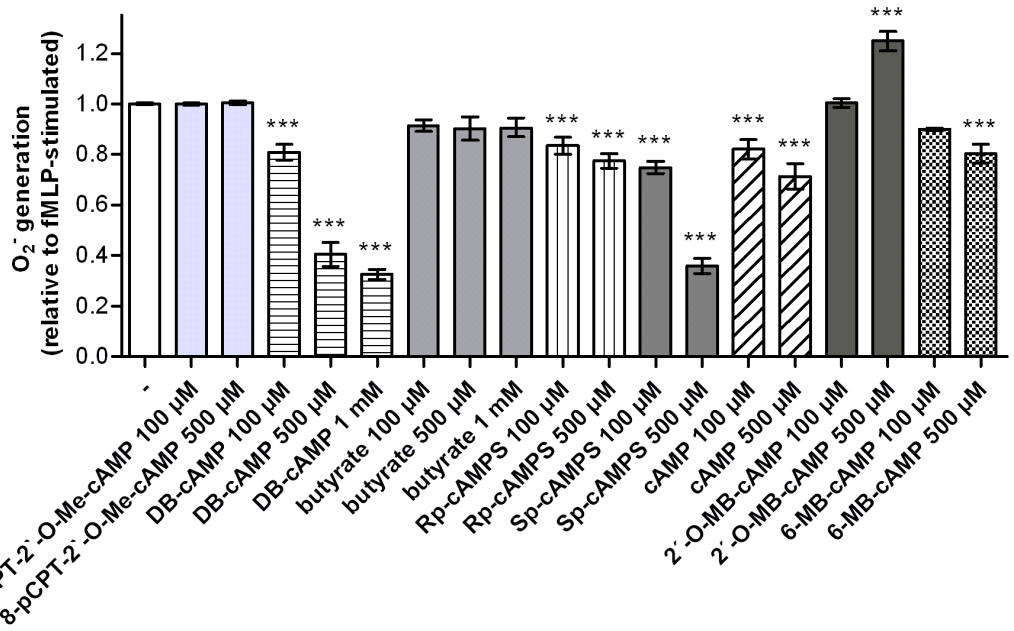

Figure 9. Effects of cAMP, butyrate and cAMP analogs on fMLP-stimulated $O_2^{\bullet-}$ production. $O_2^{\bullet-}$ production in human neutrophil granulocytes (1×10^5 cells per well) was monitored by measuring superoxide dismutase-inhibitable reduction of ferricytochrome c at 550 nm as described in *Materials and Methods*. Data shown (mean ± S.E.M.) are from three to seven independent experiments, performed in triplicate. Data were analyzed for statistical significance relative to the control (-, set to 1.0) using one-way ANOVA, followed by Dunnett's multiple comparison test (***$p<0.001$).
doi:10.1371/journal.pone.0064556.g009

experimental tools available. Most strikingly, the widely used AC inhibitor SQ 22536 failed to reduce the stimulatory effect of ISO on cAMP levels but further augmented the inhibitory effect of ISO on $O_2^{\bullet-}$ production (Fig. 8). Non-specific and pleiotropic effects of SQ 22536 have been subject of a recent review [61]. Quite striking too was the lack of inhibitory effect of FSK on $O_2^{\bullet-}$ production and lack of stimulatory effect of FSK on cAMP production (Fig. 8). These data could be explained by a model according to which the FSK-insensitive AC isoform 9 [61] is the functionally predominant AC in neutrophils. This hypothesis needs to be tested in future studies. Again, this is not a trivial task since the quality of AC antibodies is generally poor [61], and we are not aware of the availability of specific AC9 antibodies.

Stimulation of cAMP accumulation and reduction of $O_2^{\bullet-}$ production mediated by ISO, was inhibited according to monophasic competition isotherms by ICI, a highly potent and selective β_2AR antagonist with very low potency on the β_1-adrenergic receptor [77]. In case of an exclusive involvement of the β_1-adrenergic receptor we would have expected low potency of ICI, and in case of an involvement of both β-adrenergic receptors, we would have expected biphasic isotherms. This was clearly not the case (Fig. 5), and moreover, the potency of ICI at the native β_2AR was even higher than at the recombinant β_2AR (Fig. 5 and Table 2). Thus, the data obtained with ICI provide strong evidence for the notion that only the β_2AR but not the β_1-adrenergic receptor is functionally expressed in human neutrophils. Moreover, we excluded the possibility that ISO as representative β_2AR agonist cross-reacts with the histamine H_2 receptor, which is also expressed on human neutrophils [40]. Specifically, the effect of ISO on fMLP-induced $O_2^{\bullet-}$ production was not reduced by the histamine H_2 receptor antagonists famotidine, tiotidine and zolantidine (data not shown).

Stallaert and coworkers [78] demonstrated on HEK293S cells that β_2AR-dependent impedance response to ISO is the result of activation of multiple signaling pathways, including G_s and G_i coupling, Gβγ-dependent signaling, cAMP production, extracellular signal-regulated kinase (ERK) 1/2 activation as well as Ca^{2+} mobilization. Therefore, when stimulation of the β_2AR does not activate the PKA-dependent pathway in human neutrophils or this pathway does not interfere with the NADPH oxidase signaling, modulation of the e.g. ERK1/2-pathway could be the explanation for the inhibition of the NADPH oxidase. Interestingly, in other studies on human neutrophils, a correlation between activation of cAMP/PKA signaling pathway and inhibition of ERK phosphorylation was observed, resulting in reduced fMLP-induced $O_2^{\bullet-}$ production [79,80]. Intriguingly, the β_2AR was reported to activate ERK signaling pathway *via* interaction with G_i, Src and/or arrestin proteins in addition to G_s proteins in other systems [81]. As it is evident that β_2AR-signalling strongly depends on the cell system used [16], there is a need to address the correlation between β_2AR activation, ERK activation and fMLP-stimulated $O_2^{\bullet-}$ production in future studies as well. This could provide an explanation for the observed biased effects of some examined β_2AR ligands on neutrophils.

In preliminary studies we examined a number of pharmacological inhibitors to explore alternative signaling pathways of the β_2AR; e.g. we tested the p38 inhibitor SB203580, the JNK inhibitors SP600125 and SP600123, the ERK inhibitor PD980598, the PI3 kinase inhibitor LY294002 and the protein kinase C inhibitor Goe 6978. Unfortunately, these compounds per se inhibited fMLP-stimulated $O_2^{\bullet-}$ production (data not shown) so that separate effects of these compounds on ISO actions could not be properly dissected. An alternative approach will be the examination of the effects of β_2AR ligands on protein phosphorylation in neutrophils, using screening approaches encompassing numerous protein kinases as starting point. Lastly, it will also be very important to explore the possibility that the new signaling

molecules cyclic CMP (cCMP) and cyclic UMP (cUMP) [82] are involved in β_2AR-mediated signal transduction.

Data from the literature suggest a feasible use of β_2AR agonists as anti-inflammatory agents [10,83]. In order to extend the data on functional selectivity in native test systems, we compared the effect of β_2AR ligands on cAMP accumulation and $O_2^{\bullet-}$ production in human neutrophils. However, there are many native test systems described, which would afford an expansion of our knowledge about functional selectivity of β_2AR ligands. One example could be the parallel measurement of cAMP concentration and contractility of cardiomyocytes or endothelial cells as functional parameter [16,69]. Moreover, a comparison of cAMP accumulation and ERK phosphorylation in e.g. mouse embryonic fibroblasts [84] could be used for screening of β_2AR ligands. All in all, there is a need to assess biased signaling through the β_2AR in a wide spectrum of native test systems in order to improve the desired therapeutic effect of developed compounds on the one and to minimize side effects on the other. Furthermore, β_2AR agonists that have been used for many years in the therapy of humans, e.g. as bronchodilators in patients with asthma or chronic obstructive pulmonary disease, should be reassessed using various native test systems, as there is a potential to improve already existing therapies, particularly by minimizing unwanted effects.

As a general approach to study functional selectivity in native cells, it is necessary to construct a systematic data matrix in which multiple ligands are examined at multiple concentrations (enabling determination of precise potencies and efficacies) and for multiple parameters. Previous studies with native human cells may have largely overlooked functional selectivity at GPCRs because there was no appreciation of the necessity to generate such a systematic data collection in order to understand cell biology. It is evident that availability of human cells is an issue for comprehensive pharmacological studies. The human neutrophil, despite its inherent problems, i.e. short survival time after isolation, variable responsiveness and poor accessibility to genetic manipulation, is a suitable model system to test functional selectivity for several reasons. Specifically, neutrophils can be obtained in large quantities, express multiple receptors, display numerous cell functions that can be assessed quantitatively and are pathophysiologically relevant for inflammation.

Supporting Information

Figure S1 Structures of the β_2AR agonists and antagonists examined in this study.
(PPTX)

Figure S2 Structures of cAMP and cAMP analogs examined in this study.
(PPTX)

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Author Contributions

Conceived and designed the experiments: IBH RS. Performed the experiments: IBH MTR SK HB. Analyzed the data: IBH SK HB MTR FS AB RS. Contributed reagents/materials/analysis tools: HB FS. Wrote the paper: IBH MTR AB RS.

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