ACS Pharmacology & Translational Science

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Article

Agonist-Dependent Coupling of the Promiscuous Adenosine A_{2B} Receptor to Gα Protein Subunits

Published as part of the ACS Pharmacology & Translational Science virtual special issue "GPCR Signaling".

Jan Hendrik Voss, Andhika B. Mahardhika, Asuka Inoue, and Christa E. Müller*

Cite This: *ACS Pharmacol. Transl. Sci.* 2022, 5, 373−386

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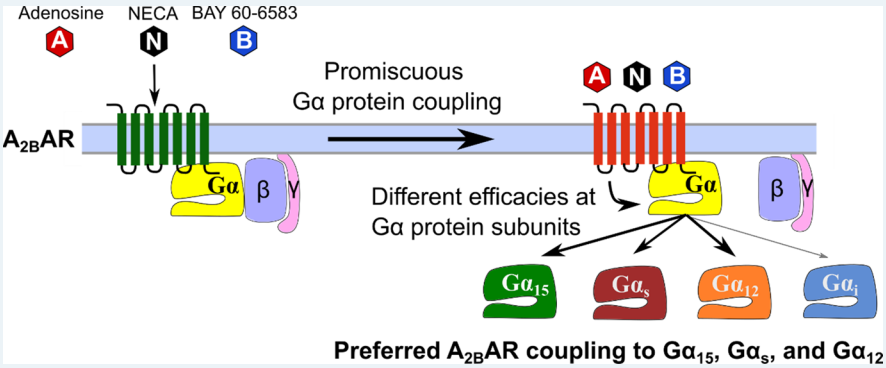

ABSTRACT: The adenosine A_{2B} receptor (A_{2B}AR) belongs to the rhodopsin-like G protein-coupled receptor (GPCR) family. It is upregulated under hypoxic conditions, in inflammation and cancer. Previous studies indicated the coupling of the A_{2B}AR to different G proteins, mainly G_s, but in some cases $G_{q/11}$ or G_i, depending on the cell type. We have now utilized novel technologies, (i) heterologous expression of individual members of the $G\alpha_{q/11}$ protein family ($G\alpha_q$, $G\alpha_{11}$, $G\alpha_{14}$, and $G\alpha_{15}$) in $G\alpha_{q/11}$ knockout cells, and (ii) the TRUPATH platform, allowing the direct observation of Gα protein activation for each of the Gα subunits by bioluminescence resonance energy transfer (BRET) measurements. Three structurally diverse A_{2B}AR agonists were studied: the cognate agonist adenosine, its metabolically stable analog NECA, and the non-nucleosidic partial agonist BAY 60-6583. Adenosine and NECA activated most members of all four Gα protein families ($G\alpha_s$, $G\alpha_{q/11}$, $G\alpha_i$, and $G\alpha_{12/13}$). Significant differences in potencies and efficacies were observed; the highest efficacies were determined at the $G\alpha_{15}$, $G\alpha_s$, and $G\alpha_{12}$ proteins, and for NECA additionally at the $G\alpha_{i2}$ protein. In contrast, the partial agonist BAY 60-6583 only activated $G\alpha_{15}$, $G\alpha_s$, and $G\alpha_{12}$ proteins. Adenosine deaminase, an allosteric modulator of ARs, selectively increased the potency and efficacy of NECA and BAY 60-6583 at the $G\alpha_{15}$ protein, while it had no effect or decreased efficacy at the other Gα proteins. We conclude that the A_{2B}AR is preferably coupled to the $G\alpha_{15}$, $G\alpha_s$, and $G\alpha_{12}$ proteins. Upon upregulation of receptor or Gα protein expression, coupling to further Gα proteins likely occurs. Importantly, different agonists can display different activation profiles.

KEYWORDS: *adenosine, BAY 60-6583, G protein coupling, $G_q/G_s/G_i/G_{12/13}$ proteins, HEK293 cells, NECA*

The adenosine A_{2B} receptor (A_{2B}AR) belongs to the α-branch of rhodopsin-like G protein-coupled receptors (GPCRs). Four different subtypes exist, designated A_1-, A_{2A}-, A_{2B}-, and A_3ARs. The alkaloid caffeine is a non-selective AR antagonist employed to improve lung function of pre-term babies and for pain management in combination with analgesics.[2,3] Moreover, the A_{2A}-selective antagonist istradefylline is used for the treatment of Parkinson's disease,[4,5] while the A_{2A}-selective agonist regadenoson and the cognate agonist adenosine are employed for cardiac imaging to induce vasodilation.[6] Moreover, adenosine is therapeutically applied to treat arrhythmia in paroxysmal supraventricular tachycardia.[1] All AR subtypes constitute promising drug targets, especially in the context of inflammation, immunity, and cancer.[2,3,7−12] The A_{2B}AR is mostly expressed in low density and only activated by relatively high, micromolar concentrations of adenosine, which are typically only present under pathological, e.g., inflammatory and hypoxic, conditions.[13,14] There are exceptions: e.g., some epithelial cells, particularly in the gut, display rather high expression levels of the A_{2B}AR.[15]

Received: February 5, 2022
Published: May 3, 2022

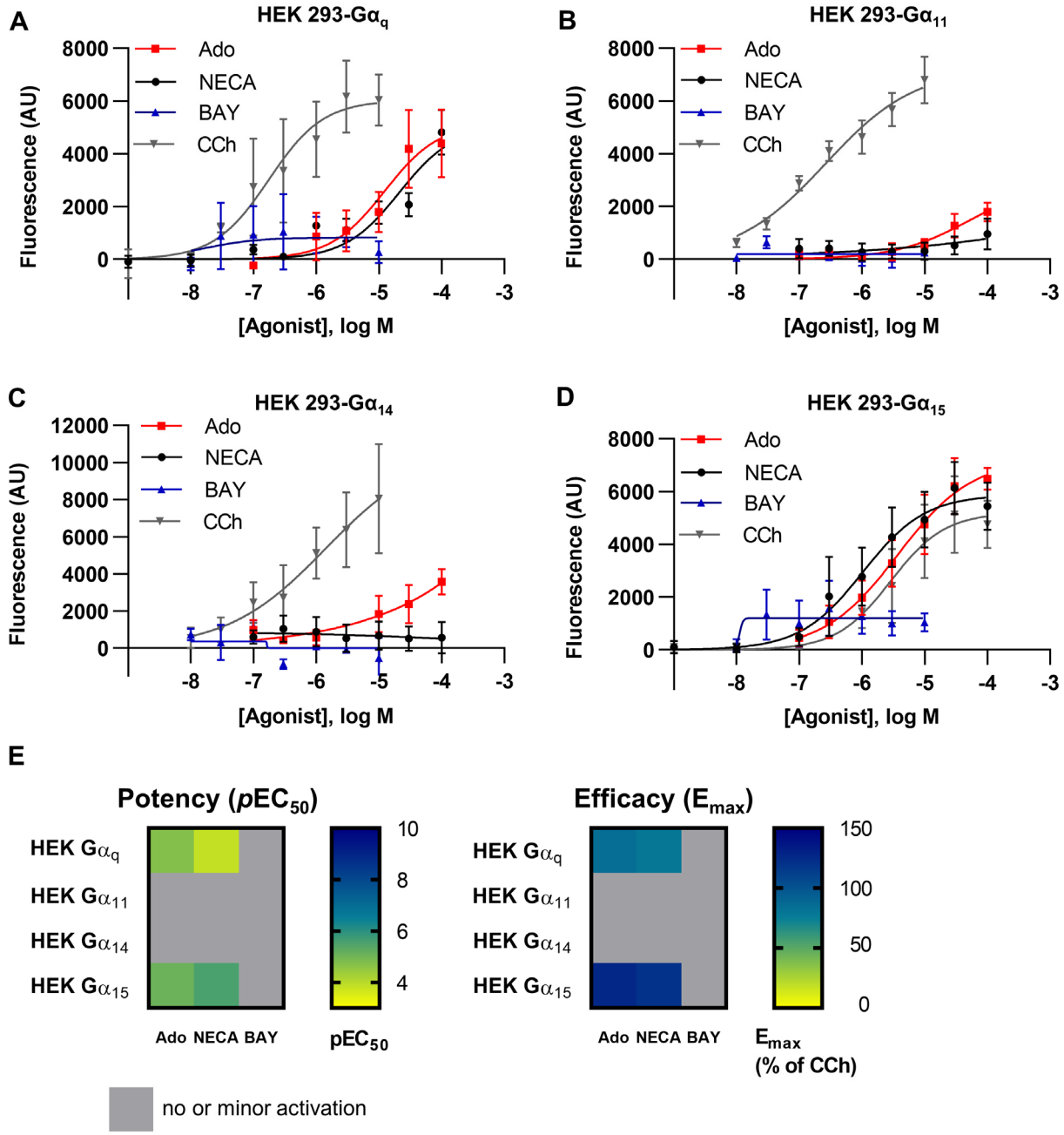

Figure 1. Concentration−response curves of NECA, adenosine (Ado), BAY 60-6583 (BAY), and carbachol (CCh) at HEK293 ΔG$\alpha_{q/11}$ cells recombinantly expressing (A) Gα_q, (B) Gα_{11}, (C) Gα_{14}, or (D) Gα_{15} proteins. Data points are from at least three separate experiments, each performed in triplicates. (E) Heatmaps of A_{2B}AR-mediated calcium signaling depicting potency (pEC$_{50}$, left) and efficacy (% of CCh signal at the highest tested concentration, right).

Moreover, A_{2B}AR expression is upregulated in inflamed tissues and on many cancer cells.[16−18] Therefore, A_{2B}ARs have significant potential as future drug targets for a range of diseases. Agonists have, e.g., been proposed for the treatment of stroke, obesity-induced diabetes, atherosclerosis, and wound healing, while antagonists have shown potential for treating inflammatory diseases (e.g., asthma, pulmonary and liver fibrosis, inflammatory bowel disease, multiple sclerosis), pain, cancer, and infections.[3,12] On the other hand, anti-inflammatory effects induced by A_{2B}AR activation have also been described.[14]

Agonists on GPCRs trigger intracellular signaling cascades by activation of heterotrimeric guanine nucleotide-binding proteins (G proteins), consisting of α-, β-, and γ-subunits. The Gα-subunits are crucial for the activation of various second messenger systems. They are subdivided into four families: Gα_s, G$\alpha_{i/o}$, G$\alpha_{q/11}$, and G$\alpha_{12/13}$.[19,20] During G protein activation, the helical and the Ras-like domains of the Gα-subunit separate from each other, thereby allowing the dissociation of the bound guanosine diphosphate (GDP), which is replaced by guanosine triphosphate (GTP).[21] The GTP-bound Gα-subunit then dissociates from the associated G$\beta\gamma$-dimer and subsequently interacts with its effector proteins. Depending on the Gα protein subunit, specific effects are triggered in the cell, e.g., stimulation of adenylate cyclase (AC) by Gα_s proteins, inhibition of AC by G$\alpha_{i/o}$

https://doi.org/10.1021/acsptsci.2c00020
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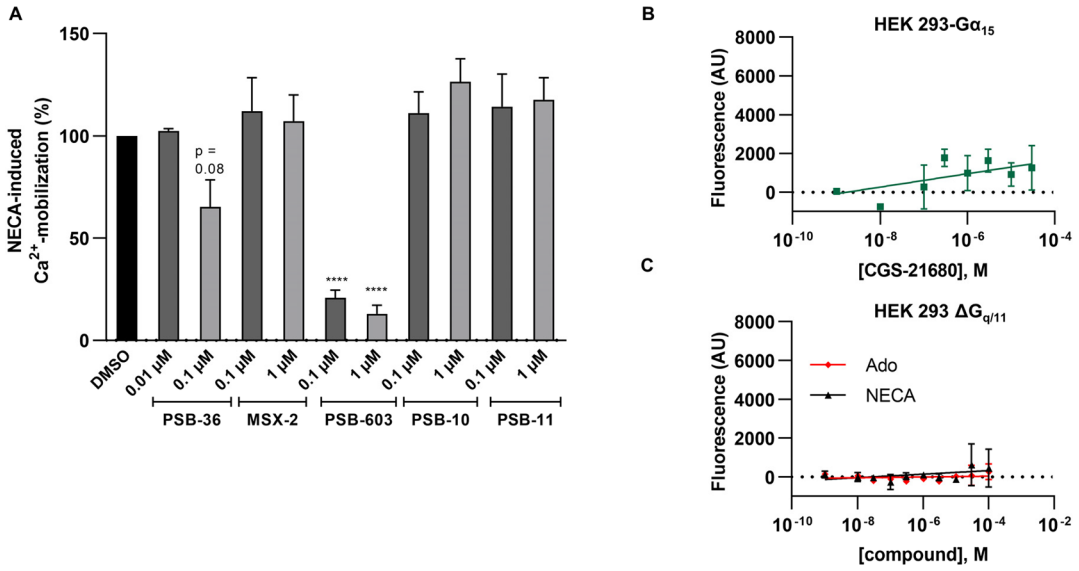

Figure 2. NECA-induced calcium mobilization in HEK293-Gα_{15} cells is inhibited by A_{2B}-selective AR antagonists. (A) NECA-induced calcium mobilization (8.8 μM NECA corresponding to its EC_{80} value) in the presence of the AR antagonists PSB-36 (A_1AR), MSX-2 (A_{2A}AR), PSB-603 (A_{2B}AR), PSB-10, and PSB-11 (both A_3AR) in HEK293-Gα_{15} cells. Cells were preincubated with the indicated concentrations of AR antagonist for 30 min before measurement. Values were normalized to controls in the absence of antagonist (100%) and in the absence of agonist (0%). Three (PSB-36) or four (all other) independent experiments were performed in triplicates; bars represent means ± SEM. (B) The A_{2A}AR-selective agonist CGS-21680 did not induce calcium mobilization in HEK293-Gα_{15} cells at concentrations of up to 30 μM. (C) Adenosine (Ado) and NECA did not induce calcium mobilization in HEK293-ΔG$\alpha_{q/11}$ cells.

proteins, calcium mobilization by G$\alpha_{q/11}$ proteins, or activation of Rho guanine exchange factors (Rho-GEFs) by G$\alpha_{12/13}$ proteins.[20,22]

In the past, it was challenging to investigate the activation of specific Gα protein subunits by GPCR subtypes and their agonists. Recent technological advances, namely the clustered regularly interspaced short palindromic repeats (CRISPR)-Cas9 technology,[23] and especially the development of novel biosensors for measuring G protein activation, have provided tools to unambiguously investigate the activation of individual Gα protein subunits. Human embryonic kidney (HEK) 293 cells depleted of Gα proteins by CRISPR-Cas9 knockout followed by heterologous expression of individual Gα protein subunits have enabled specific studies.[24−28] BRET sensors, e.g., the TRUPATH assay and similar previously described biosensors,[29,30] allow the measurement of BRET ratios between renilla luciferase-8 (RLuc8)-fused Gα protein subunits and a green fluorescent protein 2 (GFP2)-tagged Gγ subunit. This ratio decreases upon G protein activation due to the dissociation of the α- and β,γ-subunits, resulting in an increased distance between BRET donor and acceptor.[31] Further recently established methods for direct measurement of G protein activation include (i) split-luciferase assays (where Gα and, e.g., Gγ proteins are labeled by luciferase fragments that dissociate upon receptor activation, resulting in a decrease in luminescence),[32−35] (ii) effector-membrane translocator assays (EMTAs, in which luciferase-tagged effectors interact exclusively with GTP-bound Gα subunits and are thereby brought into close proximity to a membrane-anchored BRET acceptor),[36,37] and transforming growth factor-α (TGF-α) shedding assays (in which G$\alpha_{q/11}$ protein activation results in cleavage and release of a soluble alkaline phosphatase (AP) fragment from a membrane-bound TGF-α-AP fusion protein by the metalloprotease ADAM17).[34,38]

The A_{2B}AR was reported to couple to Gα_s and Gα_q proteins; however Gα_q protein coupling has only been observed in some of the investigated cell types,[39−41] and the reason for this has remained unclear. Few individual publications indicated additional coupling to G$\alpha_{i/o}$ proteins.[40,42,43] Thus, the A_{2B}AR appears to be endowed with an amazing, and at the same time confusing, promiscuity.[40,42] In the present study, we utilized novel techniques, that only recently became accessible, to monitor the activation of individual Gα protein subunits by the A_{2B}AR upon stimulation with structurally diverse agonists—namely the endogenous agonist adenosine, its metabolically stable analog NECA, and the A_{2B}-selective partial agonist BAY 60-6583[39] (for structures see Figure S1A). Our first approach was to stably express each G$\alpha_{q/11}$ protein family member (Gα_q, Gα_{11}, Gα_{14}, and Gα_{15}) in G$\alpha_{q/11}$-deficient HEK293 cells (HEK293-ΔG$\alpha_{q/11}$) that natively express a low level of the human A_{2B}AR[40,44] and then directly measure intracellular calcium mobilization (see Figure S1B). Next, we employed the BRET-based biosensor platform TRUPATH[31] to probe A_{2B}AR-induced activation of virtually each of the Gα protein subunits, directly at the G protein level (see Figure S1C). We discovered that the A_{2B}AR is preferably coupled to Gα_{15}, Gα_s, and Gα_{12} proteins. While the full A_{2B}AR agonists activated almost all Gα protein subunits, their potencies and efficacies were significantly different. Efficacy in particular appears to play a decisive role for the resulting physiological and pharmacological effects.

RESULTS

Calcium Mobilization Studies. Signaling via the $G_{q/11}$ protein family was initially studied by calcium mobilization assays in HEK293 cells, which natively express the A_{2B}AR.[40,44] In previously published experiments, Hinz et al.[39] and Gao et al.[40] demonstrated calcium mobilization upon A_{2B}AR stimulation with the adenosine analog NECA, while the A_{2B}AR-

https://doi.org/10.1021/acsptsci.2c00020
ACS Pharmacol. Transl. Sci. 2022, 5, 373−386

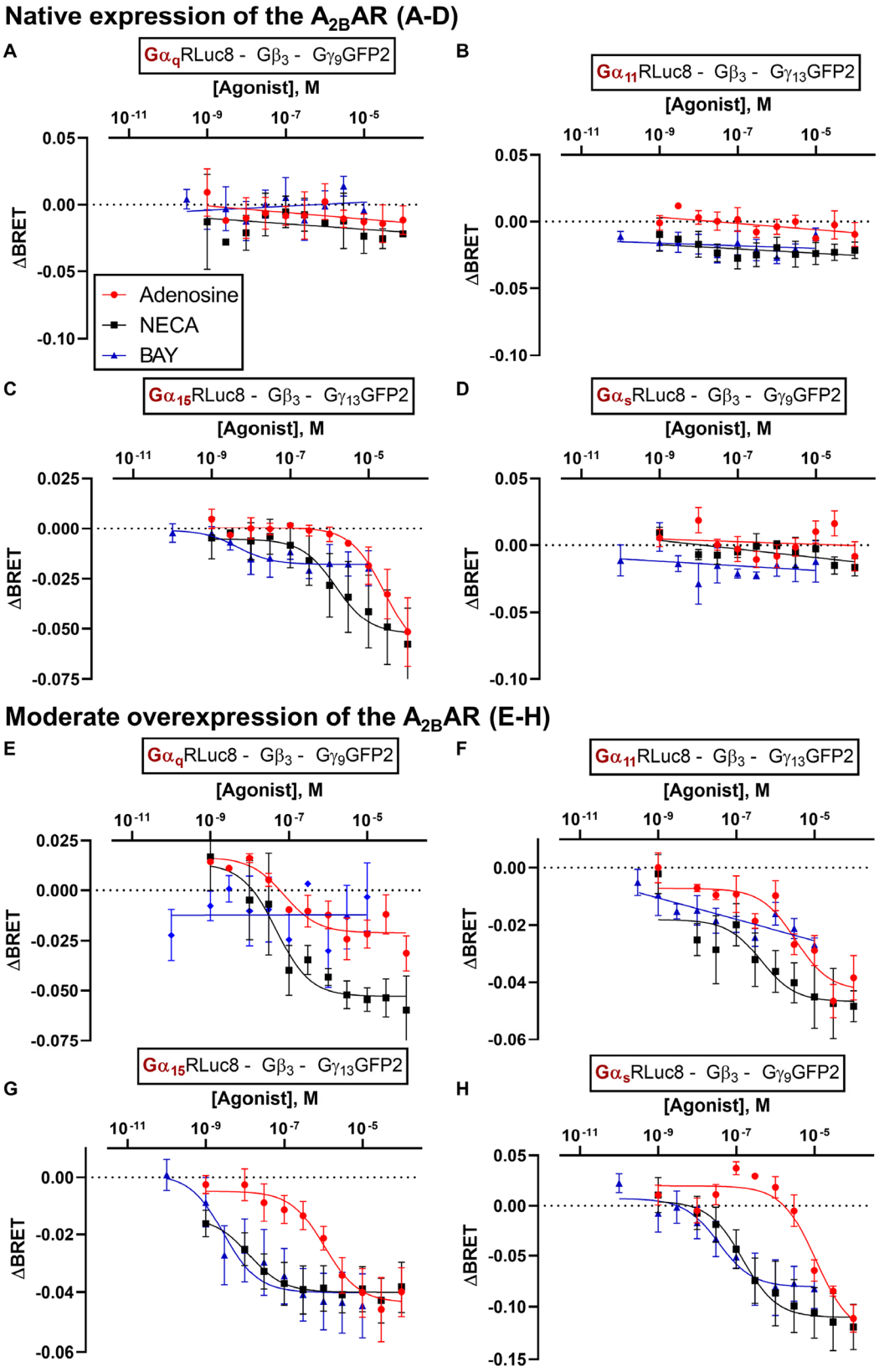

Figure 3. Concentration−response curves of the agonists adenosine, NECA and BAY 60-6583 at HEK293 cells transiently transfected with TRUPATH biosensors for the indicated G protein subunits. **A-D**. ΔBRET values measured in HEK293 cells with native A_{2B}AR expression levels and overexpression of the indicated biosensors **A**. Gα_q, **B**. Gα_{11}, **C**. Gα_{15}, or **D**. G$\alpha_{s\text{-}s}$. **E-H**. ΔBRET values measured in HEK293 cells with overexpression of the A_{2B}AR and the indicated biosensors Gα_q (e), Gα_{11} (f), Gα_{15} (g), or G$\alpha_{s\text{-}s}$ (h) biosensors. Data points are presented as means ± SEM of three or more independent experiments; pEC_{50} values and efficacy values are listed in Tables S2 and S3 of Supporting Information.

https://doi.org/10.1021/acsptsci.2c00020
ACS Pharmacol. Transl. Sci. 2022, 5, 373−386

selective partial agonist BAY 60-6583 had not induced calcium mobilization in those studies. In order to investigate this discrepancy in more detail, we generated stable (polyclonal) cell lines that exclusively expressed a single Gα protein subunit of the Gα_q protein family, either Gα_q, Gα_{11}, Gα_{14}, or Gα_{15}, by retroviral transfection of HEK293-ΔG$\alpha_{q/11}$ cells, resulting in similar expression levels for Gα_q, Gα_{11}, and Gα_{14} proteins (the expression level of Gα_{15} could not be quantified on a protein level).[25] These cells were subsequently used to identify the Gα protein(s) involved in A_{2B}AR-induced intracellular calcium release. Our aim was to examine (i) whether the A_{2B}AR favors signaling via a specific Gα_q subfamily member and/or (ii) if structurally diverse A_{2B}AR agonists would result in specific or selective activation of certain Gα protein subunits and thus show biased signaling. Carbachol (CCh)-mediated activation of the muscarinic M_3 receptor (M_3R), which is endogenously expressed in HEK293 cells, was used as a control (for results see Figure 1A−E and Table S1).

In HEK293-ΔG$\alpha_{q/11}$ cells not expressing any G$\alpha_{q/11}$ family protein, calcium mobilization was neither induced by adenosine nor by NECA (see below). CCh exhibited a preference for signaling via Gα_q and Gα_{11} proteins, both of which were activated by submicromolar concentrations of the muscarinic receptor agonist. Higher CCh concentrations (20- to 30-fold) were required to activate calcium mobilization in HEK-Gα_{14} and HEK-Gα_{15} cells.

The physiological agonist adenosine and its closely related derivative NECA displayed similar activity showing a preference for Gα_{15}-induced intracellular calcium release (pEC_{50} adenosine: 5.52; NECA, 5.79) (see Figure 1A,D and Table S1) followed by Gα_q-mediated calcium mobilization (pEC_{50} adenosine: 4.84, NECA: 4.37). The efficacy also appeared to be higher for Gα_{15}- as compared to Gα_q-induced calcium release. Only minor or negligible activation of calcium mobilization via Gα_{11} or Gα_{14} proteins was observed (see Figure 1B,C). These results indicate that adenosine preferably activates Gα_{15} within the G$\alpha_{q/11}$ protein family. Interestingly, the A_{2B}-selective non-nucleosidic partial agonist BAY 60-6583 did not activate any of the G$\alpha_{q/11}$ family proteins in these calcium mobilization experiments (fluorescence monitored at the highest tested concentration of BAY 60-6583 did not significantly differ from basal values).

In order to confirm that the observed effects were actually due to the activation of A_{2B}ARs endogenously expressed in the HEK293 cells, we preincubated the cells with AR subtype-selective antagonists. Calcium mobilization induced by NECA was almost completely blocked by the A_{2B}AR-antagonist PSB-603 (Figure 2A), but not by selective A_1-, A_{2A}-, and A_3AR antagonists. The A_1AR-selective antagonist PSB-36 only inhibited calcium mobilization moderately at a concentration of 100 nM, but not at 10 nM, consistent with its affinity for the A_{2B}AR (K_i A_{2B}, 187 nM; K_i A_1 0.7 nM).[45] The A_{2A}AR-selective agonist CGS-21680 did not induce calcium mobilization (Figure 2B), nor did adenosine or NECA in G$\alpha_{q/11}$ knockout cells (Figure 2C).

Since the observed effects downstream of A_{2B}AR-mediated Gα protein activation might be modulated by unknown factors, we next measured direct activation of Gα protein subunits.

Direct Measurement of G Protein Activation. G protein activation by A_{2B}AR agonists was monitored using the TRUPATH assay platform, a BRET2-based method that allows to observe the dissociation of Gα from G$\beta\gamma$ protein subunits.[31] Biosensors are available for all Gα protein subunits with the exception of transducins, Gα_{14}, and Gα_{olf}. For each biosensor, the RLuc8-Gα subunit was paired with an optimized G$\beta\gamma$-GFP2 combination to yield maximum BRET2 ratio shifts upon GPCR activation.[31] The biosensors were transiently expressed in native HEK293 cells together with the receptor of interest, and the BRET ratio shifts in response to agonist stimulation were measured. To validate the assay, we expressed the Gα_{i1}-, and the Gα_q biosensor, respectively, together with a GPCR that is known to couple to the respective Gα protein subunits (Figure S2), namely the thromboxane receptor (TP) for the Gα_q biosensor, and the A_3AR for the Gα_{i1} biosensor. Transfected cells were activated by a full agonist of the respective receptor. The U46619-activated TP receptor elicited a maximum ΔBRET shift of −0.2. Using the Gα_{i1} biosensor, we measured a maximum ΔBRET shift of −0.23 for the NECA-activated A_3AR. The E_{max} values for the TP receptor and the A_3AR were nearly identical to the E_{max} values reported for model receptors (isoprotenerol-activated β_2 adrenoceptor for the Gα_s family, DAMGO-activated μ-opioid receptor for the G$\alpha_{i/o}$ family, neurotensin-activated neurotensin 1 receptor for the G$\alpha_{q/11}$ and G$\alpha_{12/13}$ families) in the original publication.[31] To assess the efficacy of receptor−G protein coupling we therefore decided to compare E_{max} values determined in the present study for the A_{2B}AR upon coupling to various Gα protein subunits to E_{max} values for the same subunits observed for full agonists with standard GPCRs reported in the original publication using the same procedures and conditions.[31]

We first investigated direct activation of the G$\alpha_{q/11}$ family members induced by A_{2B}AR agonists to enable comparison with our previous results from calcium mobilization assays. Next, we studied the A_{2B}AR's canonical effector, Gα_s (short isoform Gα_{s-s}). Endogenous A_{2B}AR expression in HEK293 cells was found to be not sufficient to trigger measurable activation of Gα_q, Gα_{11}, and Gα_{s-s} biosensors in this system (Figure 3A-D; similarly, carbachol, an agonist at the endogenously expressed M_3R, failed to activate Gα_q, Gα_{11}, and Gα_{15} biosensors in the TRUPATH BRET2 assays, data not shown). Only the Gα_{15} biosensor was activated in these cells by adenosine and NECA in a concentration-dependent manner. BAY 60-6583 showed a very small effect typical of a weak partial agonist (Figure 3C).

Next, the A_{2B}AR was (moderately) overexpressed in HEK293 cells as recommended by the TRUPATH assay protocol[31] (100 ng pCDNA3.1-human ADORA2B per 10^6 cells, transient expression) (see Figure 3E-H; and Tables S2 and S3 in Supporting Information). Now, adenosine and NECA activated all four investigated Gα proteins, Gα_q, Gα_{11}, Gα_{15} and Gα_{s-s}, in a concentration-dependent manner, while BAY 60-6583 activated Gα_{15} and Gα_{s-s} only. NECA appeared to be significantly more potent than adenosine in all cases, while BAY 60-6583 was equipotent to adenosine at Gα_{15} and Gα_{s-s} proteins, where it showed activation. At the Gα_q protein, NECA was more efficacious than adenosine while at all other investigated Gα subunits both nucleosidic agonists displayed similar efficacy. BAY 60-6583 was as efficacious as adenosine at the Gα_{15} and Gα_{s-s} subunit.

Since we had not observed any calcium mobilization induced by BAY 60-6583 in Gα_{15}-expressing HEK293 cells, we wondered whether this was due to the low A_{2B}AR expression level in this cell line combined with the partial agonistic properties of BAY 60-6583. Thus, we studied calcium mobilization in HEK293 cells overexpressing A_{2B}ARs by BAY

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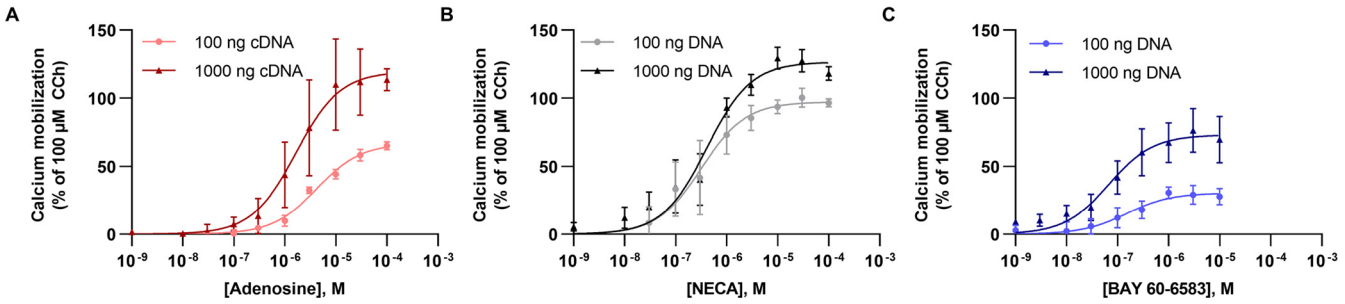

Figure 4. Calcium mobilization induced by A_{2B}AR agonists in HEK293-Gα_{15} cells transiently overexpressing A_{2B}ARs. The human A_{2B}AR was transiently overexpressed with either a low amount of cDNA (100 ng/well) or a high amount of cDNA (1000 ng/well). A_{2B}AR activation by **A**. adenosine (E_{max} adenosine: 100 ng DNA/well, 76 ± 10%; 1000 ng DNA/well, 119 ± 5%; pEC_{50} adenosine: 100 ng DNA/well, 5.38 ± 0.19; 1000 ng/well, 5.72 ± 0.21), **B**. NECA (E_{max} NECA: 100 ng DNA/well, 111 ± 3%; 1000 ng DNA/well, 122 ± 7%; pEC_{50} NECA: 100 ng DNA/well, 6.72 ± 0.36; 1000 ng/well, 6.37 ± 0.11), and **C**. BAY 60-6583 (E_{max} BAY 60-6583:100 ng DNA/well, 27 ± 1%; 1000 ng/DNA well, 71 ± 17%; pEC_{50} BAY 60-6583:100 ng DNA/well, 6.73 ± 0.11; 100 ng/well, 6.95 ± 0.15), normalized to the effect of 100 μM CCh. Data are means ± SEM of three individual experiments performed in duplicates.

60-6583 (Figure 4). To this end, we transiently overexpressed the A_{2B}AR using two different cDNA concentrations, and recorded concentration-dependent intracellular calcium release induced by adenosine, NECA, and BAY 60-6583. Adenosine showed an increase in efficacy with increasing amounts of cDNA (compared to the maximal effect of CCh) and a moderate increase in potency (E_{max} adenosine: 100 ng DNA/well, 76%; 1000 ng DNA/well, 119%; pEC_{50} adenosine: 100 ng DNA/well, 5.38; 1000 ng/well, 5.72; Figure 4A). NECA displayed a minor increase in efficacy and a similar potency with increasing cDNA concentrations (E_{max} NECA: 100 ng DNA/well, 111%; 1000 ng DNA/well, 122%; pEC_{50} NECA: 100 ng DNA/well, 6.72; 1000 ng/well, 6.37; Figure 4B). In HEK-Gα_{15} cells with overexpression of the A_{2B}AR, BAY 60-6583 was able to induce calcium mobilization; both potency and efficacy of BAY 60-6583 increased with increasing cDNA amounts used for transfection (E_{max}: BAY 60-6583 100 ng DNA/well = 27%, 1000 ng/DNA well = 71%; pEC_{50} BAY 60-6583 100 ng DNA/well = 6.73, 100 ng/well = 6.95, Figure 4C). Thus, the partial agonist BAY 60-6583 can induce calcium mobilization via Gα_{15}-activation in HEK cells, but requires high A_{2B}AR expression levels.

Effect of adenosine deaminase. The signaling molecule adenosine is ubiquitous and may be released by the cells or formed from ATP by ectonucleotidases.[46,47] To avoid an interfering effect of adenosine potentially present in the HEK293 cell culture, we additionally performed TRUPATH assays with the agonists NECA and BAY 60-6583 in the presence of adenosine deaminase (ADA) which converts adenosine to inosine, but has no effect on NECA or BAY 60-6583. NECA-induced Gα_q and Gα_{11} biosensor activation was not significantly different in terms of potency and efficacy in the presence and absence of ADA (Figure 5; Supp. Tables 2,3). At the Gα_{15} protein, the potency of NECA and BAY 60-6583 was enhanced by ADA (~10-fold for NECA, ~ 120-fold for BAY), while the efficacy was concomitantly increased by about 2-fold for BAY as well as for NECA. Potencies at the G$\alpha_{s\text{-}s}$ biosensor observed in the presence of ADA were again similar to those without ADA, but ADA reduced the efficacy of NECA- and BAY 60-6583-induced G$\alpha_{s\text{-}s}$ activation by approximately 2-fold (see Table S3). In summary, ADA selectively increased potency and/or efficacy of NECA and BAY 60-6583 in Gα_{15} protein activation, while it had no effect or decreased efficacy at the other Gα proteins.

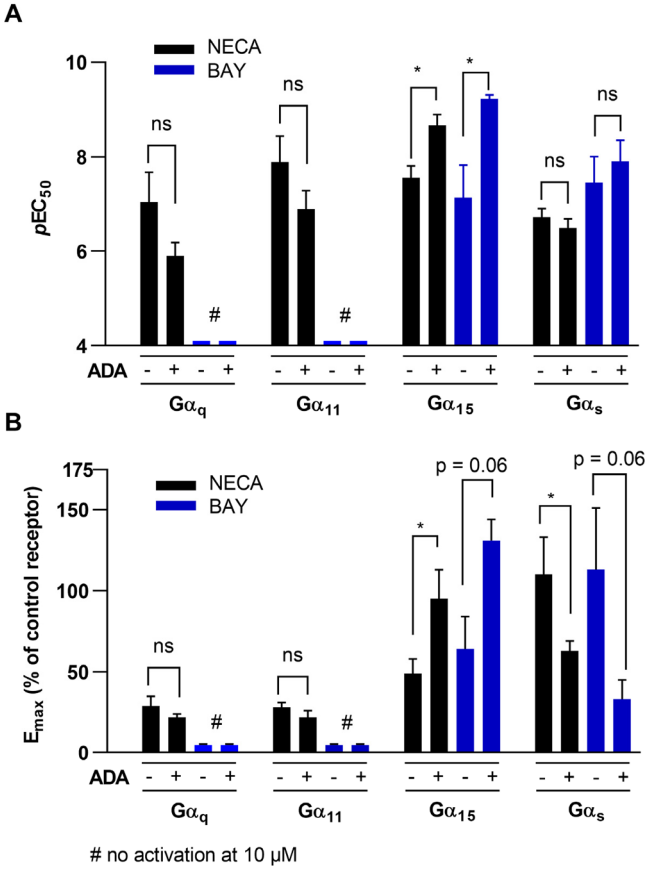

Figure 5. **A**. Potency and **B**. efficacy of NECA (black) or BAY 60-6583 (blue) for A_{2B}AR stimulation determined in TRUPATH BRET^2 assays using the Gα_q, Gα_{11}, Gα_{15}, or the G$\alpha_{s\text{-}s}$ biosensor in the absence (−) or presence (+) of ADA. Data are presented as means ± SEM of three or more independent experiments; pEC_{50} and E_{max} values are summarized in Table S2 and S3. Statistical analysis was performed with multiple unpaired *t*-tests corrected for multiple comparisons by the Holm-Sidak-method (as implemented in GraphPad PRISM v. 8.0; ns − $p > 0.05$, * − $p < 0.05$, *** − $p < 0.001$).

The promiscuous A_{2B}AR couples to all Gα protein families. Next, we investigated the potential coupling of the A_{2B}AR to further, noncanonical Gα protein subunits, specifically to members of the G$\alpha_{12/13}$ and the G$\alpha_{i/o}$ families

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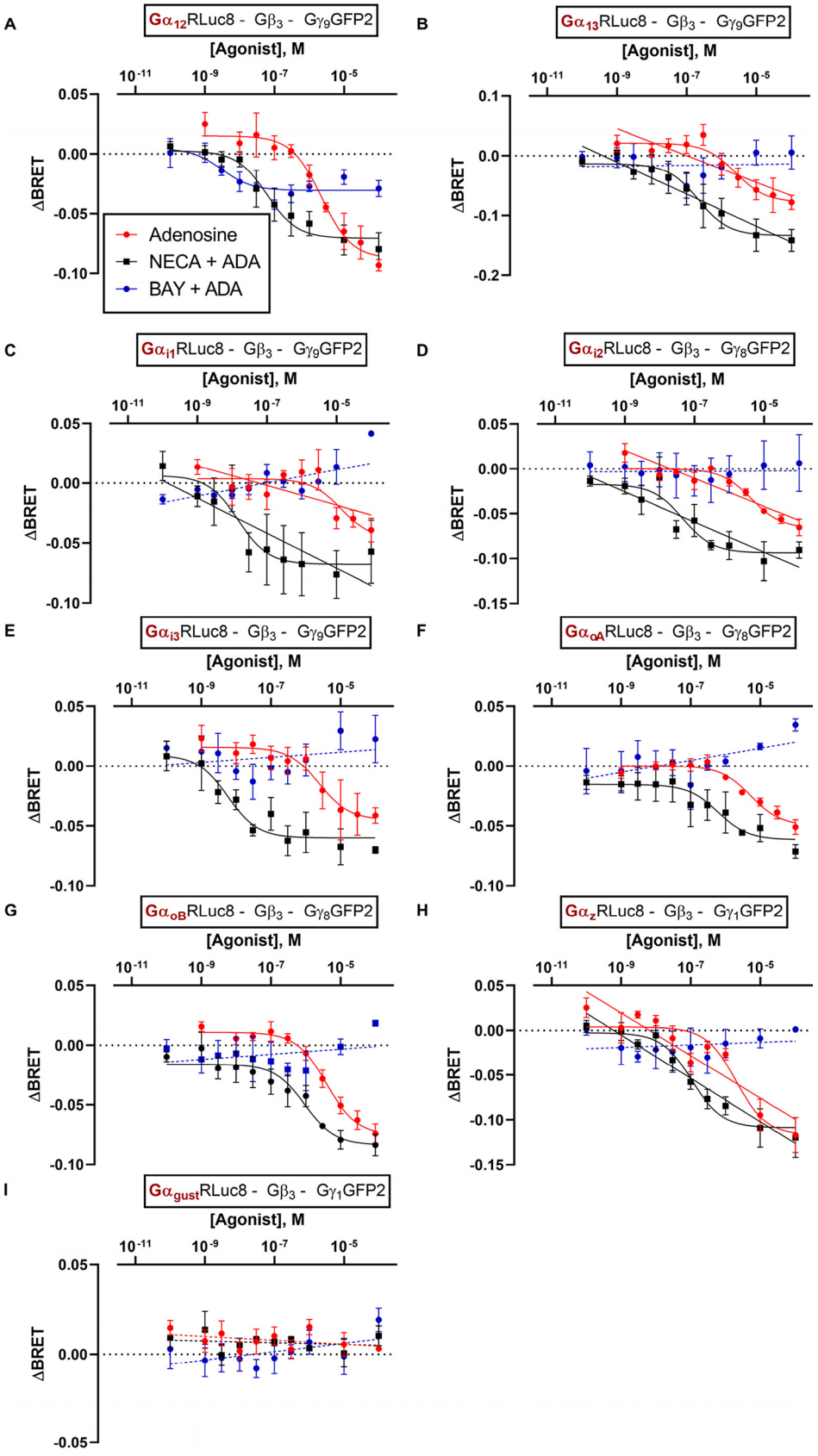

Figure 6. ΔBRET curves of the A_{2B}AR-agonists adenosine, NECA, and BAY 60-6583 at HEK293 cells transiently transfected with the A_{2B}AR and the indicated TRUPATH biosensors: A. Gα_{12}, **B.** Gα_{13}, **C.** Gα_{i1}, **D.** Gα_{i2}, **E.** Gα_{i3}, **F.** Gα_{oA}, **G.** Gα_{oB}, **H.** Gα_{z}, **I.** Gα_{gust}. Curves for NECA and BAY 60-6583 were determined in the presence of 2 U/ml ADA. Data points were obtained in 3−7 independent experiments, each performed with two technical replicates, and are represented as mean ± SEM. All pEC_{50} and E_{max} values are collected in Tables S2 and S3.

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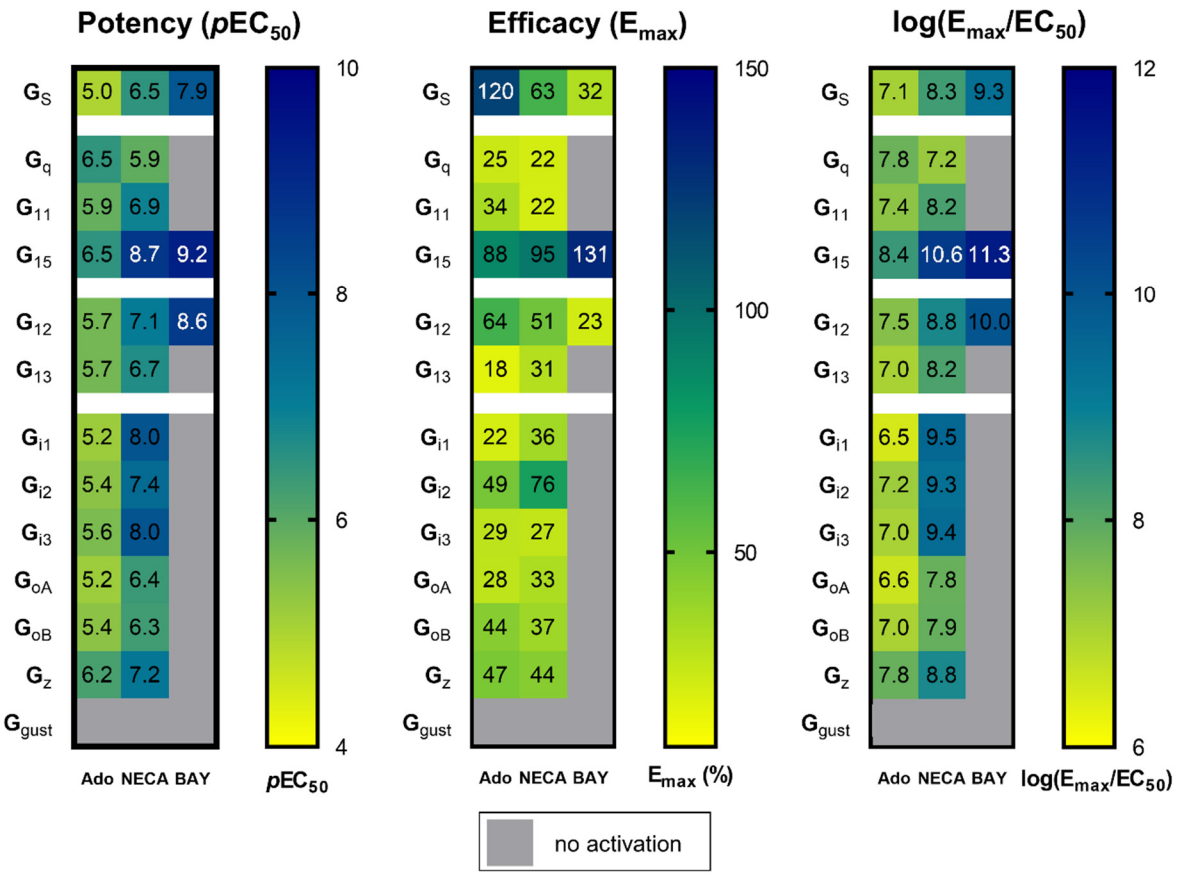

Figure 7. Heatmaps depicting potency (pEC_{50} values) and efficacy (E_{max} values given as % of maximal efficacy (E_{max}) of control receptors: isoprotenerol-activated β_2 adrenoceptor for the $G\alpha_s$ family, DAMGO-activated μ-opioid receptor for the $G\alpha_{i/o}$ family, neurotensin-activated neurotensin 1 receptor for the $G\alpha_{q/11}$ and the $G\alpha_{12/13}$ family), and a transduction coefficient expressed as log (E_{max}/EC_{50}).[31] Data for NECA and BAY 60-6583 (BAY) were determined in the presence of ADA. Units for log(E_{max}/EC_{50}): E_{max} is in %, EC_{50} in mol/L.

(see Figure 6; pEC_{50} and E_{max} values are collected in Tables S2 and S3).

All three agonists were able to activate the $G\alpha_{12}$ protein (Figure 6A). BAY 60-6583 acted as a partial agonist displaying ca. 36% efficacy relative to adenosine. Similar to the situation at $G\alpha_{15}$ and $G\alpha_{s\text{-}s}$ proteins, BAY 60-6583 and NECA were much more potent than adenosine. At the $G\alpha_{13}$ protein, adenosine and NECA displayed similar potency as at the $G\alpha_{12}$ protein, but adenosine was less efficacious than NECA, and BAY 60-6583 was inactive under the employed conditions (Figure 6B).

In further experiments, the $G\alpha_{i/o}$ protein family was investigated. Again, both adenosine and NECA displayed concentration-dependent $G\alpha_i$ protein activation. NECA was about 2 orders of magnitude more potent than adenosine in activating the $G\alpha_{i1-3}$, both agonists being similarly efficacious. In contrast, BAY 60-6583 did not activate any of the $G\alpha_i$ proteins (Figure 6C-E). Likewise, $G\alpha_{oA}$ and $G\alpha_{oB}$ biosensors were activated exclusively by adenosine and NECA, but not by BAY 60-6583; however, the potency of NECA was markedly lower at these $G\alpha$ protein subunits than at $G\alpha_{i1-3}$, while the potency of adenosine was similar in both cases (Figure 6F-G).

At the $G\alpha_z$ protein, a ubiquitously expressed, PTX-insensitive member of the $G\alpha_{i/o}$-family, adenosine displayed a slightly higher potency (Figure 6H) than at other $G\alpha_{i/o}$ proteins while the gustatory G protein $G\alpha_{gust}$ was not activated by any of the investigated A_{2B}AR agonists (Figure 6I).

In summary, all $G\alpha$ protein subunits (with the exception of the $G\alpha_{gust}$ protein) could be activated by adenosine-stimulated A_{2B}ARs. The agonist NECA generally displayed higher potencies than adenosine, especially regarding the activation of $G\alpha_s$, $G\alpha_{i1-3}$, and $G\alpha_{15}$ proteins. BAY 60-6583 exclusively activated $G\alpha_s$, $G\alpha_{15}$, and $G\alpha_{12}$ protein subunits.

The extremely high promiscuity of the A_{2B}AR, activating virtually all $G\alpha$ protein subunits with pEC_{50} values for adenosine ranging from 5.0 to 6.5 as determined in the TRUPATH assay, raises the question: How does this actually translate into signal transduction? For example, $G\alpha_s$ proteins activate adenylate cyclase, while $G\alpha_{i/o}$ proteins inhibit the enzyme. Many of these G proteins, including $G\alpha_s$ and $G\alpha_i$, are coexpressed by a large number of cells. Why does G_s signaling in most cells win over G_i signaling induced by A_{2B}ARs? Besides differences in expression levels, different efficacies in activation of distinct $G\alpha$ proteins by the agonist-stimulated A_{2B}AR might play a decisive role.

Efficacy of A_{2B}AR-Induced G Protein Activation. As demonstrated using the TRUPATH assay, the A_{2B}AR induces activation, measured by dissociation from the $G\beta\gamma$-subunits, of nearly all $G\alpha$ protein subunits. However, the A_{2B}AR is generally considered to be primarily a $G\alpha_s$-coupled and secondarily a $G\alpha_{q/11}$-coupled receptor. Besides potency (pEC_{50}), we additionally calculated efficacy (E_{max}) of agonist-induced A_{2B}AR-$G\alpha$ protein interactions. Thus, we compared the E_{max} values determined in the A_{2B}AR-dependent assays with the E_{max} values measured for model receptors, the

https://doi.org/10.1021/acsptsci.2c00020
ACS Pharmacol. Transl. Sci. 2022, 5, 373−386

isoprotenerol-activated β_2 adrenoceptor for the Gα_s family, the DAMGO-activated μ-opioid receptor for the G$\alpha_{i/o}$ family, and the neurotensin-activated neurotensin 1 receptor for the G$\alpha_{q/11}$ and the G$\alpha_{12/13}$ families.[31] The adenosine-activated A_{2B}AR was observed to couple to Gα_s proteins (efficacy: 120%) and also to the Gα_{15} protein (88%) with an efficacy close to that of the control receptors (set at 100%); the efficacy in activating the Gα_{12} protein was also still relatively high (64%) (see Figure 7 and Table S3; see Figure S3 for statistical analysis). A similar efficacy fingerprint was obtained for the A_{2B}AR activated by NECA, but here we observed higher efficacy at the Gα_{15} protein than at Gα_s and Gα_{12} proteins. NECA additionally activated the Gα_{i2} subunit with relatively high efficacy (76%). At all other Gα proteins, the adenosine- and NECA-activated A_{2B}AR displayed an efficacy below 50% of controls. The partial agonist BAY 60-6583 exclusively activated subunits displaying a high coupling efficacy for adenosine and NECA. It partially activated Gα_s (32% efficacy) and the Gα_{12} proteins (23%), and fully activated the Gα_{15} protein (in comparison to the endogenous agonist adenosine as well as the control receptors).

The log(E_{max}/EC_{50}) calculation is used to describe ligand bias toward a specific signaling pathway to provide a coefficient that includes both potency and efficacy.[48] These data are presented in Figure 7 for comparison. When this term is applied, the EC_{50} values appear to predominate while the efficacy values are underappreciated. Thus, we conclude that it is important to consider potencies and efficacies separately, in agreement with other authors.[31] Nevertheless, these data confirm the superior importance of the Gα_{15} signaling pathway for all three investigated A_{2B}AR agonists.

DISCUSSION

Previous studies on A_{2B}AR signaling indicated that it could signal via multiple Gα proteins depending on the cell line studied.[14,39−42] However, only recently, novel methods have become available to unambiguously study the direct activation of specific Gα protein subunits upon stimulation with agonists.[31,35,37,38] In the present study, we determined agonist-dependent coupling of the human A_{2B}AR to specific Gα protein subunits using two different approaches: (i) calcium mobilization assays utilizing CRISPR-Cas9-G$\alpha_{q/11}$-KO cells with re-expression of specific Gα_q protein subunits (Gα_q, Gα_{11}, Gα_{14}, or Gα_{15}),[25] and (ii) TRUPATH BRET2 assays measuring dissociation of the G protein heterotrimer.[31] We investigated the coupling behavior induced by three different A_{2B}AR agonists, the cognate agonist adenosine, its metabolically stable analog NECA, and the non-nucleosidic A_{2B}-selective partial agonist BAY 60-6583.[39] Based on our data, we conclude and confirm that the A_{2B}AR is a promiscuous receptor. Upon activation with the full agonists adenosine or NECA it potentially interacted with all Gα protein subunits, with the exception of the Gα_{gust} and the Gα_{14} protein (the latter could only be tested in the calcium assays since a TRUPATH biosensor for Gα_{14} has not been available). Activation of the A_{2B}AR with the non-nucleosidic (partial) agonist BAY 60-6583, however, resulted exclusively in Gα_{15}, Gα_s, and Gα_{12} coupling, with high efficacy at Gα_{15}, but low efficacy at the other Gα proteins.

Interestingly, the treatment of HEK293 cells with the adenosine-metabolizing enzyme ADA increased the potency of the agonists NECA (10-fold) and BAY 60-6583 (120-fold) exclusively at the Gα_{15} subunit in TRUPATH BRET2 assays, while the efficacy of both agonists was approximately doubled (Figure 5). This may be explained by an allosteric modulation of the A_{2B}AR by ADA as previously postulated.[49−51] ADA is proposed to act as an allosteric modulator facilitating a receptor conformation that exhibits high affinity for the Gα_{15} protein. Another explanation could be that in cases where ADA appears to increase the potency of NECA, this could be due to the enzymatic activity of the enzyme.

It should be kept in mind that the employed test systems are highly artificial, the TRUPATH assays requiring overexpression of the A_{2B}AR and control receptors. In calcium mobilization assays using HEK cells with low native A_{2B}AR expression and recombinant expression of a single Gα_q protein subunit, the full agonists adenosine and NECA sufficiently activated Gα_{15} and Gα_q proteins to induce calcium mobilization, but not Gα_{11} and Gα_{14} (see Figure 1). The partial agonist BAY 60-6583 only induced a calcium signal in cells recombinantly expressing a higher A_{2B}AR level via the Gα_{15} protein subunit (see Figure 4). These results point to a prominent role of the efficacy of a particular A_{2B}AR agonist to activate a specific Gα protein subunit (see Figure 8). It appears

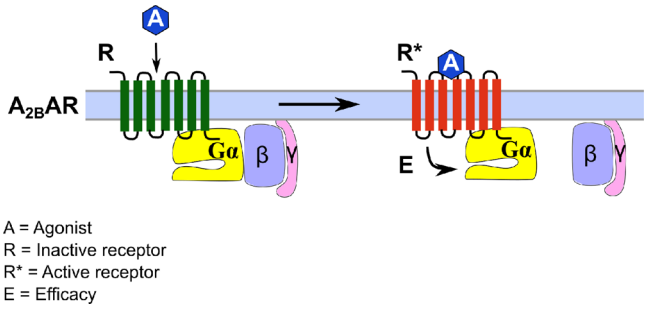

Figure 8. G protein dissociation induced by A_{2B}AR activation. The agonist (A) binds to the receptor (R) with an agonist-specific affinity and induces an active conformation (R*). Different active conformations are conceivable depending on the employed agonist. The activated receptor binds to the heterotrimeric G protein (G$\alpha\beta\gamma$) with a receptor (R*)- and Gα-subunit-dependent affinity, and induces dissociation of the Gα- from the $\beta\gamma$-subunit with a specific efficacy (E) that depends on the nature and concentration of the agonist A, the receptor R*, and the Gα protein. The maximal efficacy (E_{max}) corresponds to the maximal effect observed for a specific agonist in a defined system.

that the efficacy fingerprint is an excellent indicator for the actual G protein coupling observed in native cells. Efficacies, however, can hardly be predicted at present, which may be the reason why computer programs have failed to correctly predict the G protein-coupling for the A_{2B}AR (see precog.russel-lab.org).[52] Focus on potency rather than efficacy, or log(E_{max}/EC_{50}) as previously proposed,[53] may not provide optimal results, at least for the A_{2B}AR.

The A_{2B}AR couples most efficaciously to Gα_s (E_{max} 120%), Gα_{15} (88%) and Gα_{12} proteins (64%) when activated by adenosine, and is therefore biased toward these signaling pathways. The (partial) agonist BAY 60-6583 only activated these three G proteins at all, with low efficacy in case of Gα_s and Gα_{12} (see Figure 7). Notably, the adenosine analog NECA additionally activated the Gα_{i2} protein with relatively high efficacy (E_{max} 76%, compared to 49% for adenosine). Thus, the activation of specific G proteins is dependent on the employed agonist, and synthetic agonists do not necessarily imitate the activity of the physiological ligand.

https://doi.org/10.1021/acsptsci.2c00020
ACS Pharmacol. Transl. Sci. 2022, 5, 373−386

The preferential potent and efficacious coupling of the A_{2B}AR to $G\alpha_{15}$ proteins is of great importance in the context of immunology and immunotherapy since $G\alpha_{15}$ proteins are exclusively expressed on hematopoietic cells including immune cells.[54]

Studies on the $G\alpha$ protein coupling of a large number of GPCRs have been recently published utilizing biochemical probes, such as the TRUPATH BRET2 assay,[31] the enhanced bystander BRET or EMTA assays,[37] and the TGFα shedding assay[34,38]). Inoue et al. employed the TGF-α shedding assay and found concentration-dependent coupling of the adenosine-activated A_{2B}AR to all investigated $G\alpha$ protein subunits ($G\alpha_{i1}$, $G\alpha_{i3}$, $G\alpha_{o}$, $G\alpha_{z}$, $G\alpha_{12}$, $G\alpha_{13}$, $G\alpha_{s}$, $G\alpha_{olf}$, $G\alpha_{q}$, $G\alpha_{14}$) with the exception of the $G\alpha_{15}$ protein.[34] In contrast, the $G\alpha_{15}$ protein was potently and efficaciously activated by A_{2B}AR stimulation, both in TRUPATH BRET2 and calcium mobilization assays in the present study. The previously published study relied on chimeric $G\alpha_{q}$ proteins, which only harbored the six C-terminal residues of the $G\alpha_{15}$ protein to investigate its coupling. It appears likely that the interaction between the A_{2B}AR and the $G\alpha_{15}$ protein is based on other $G\alpha_{15}$-specific protein−protein interactions apart from the C-terminal residues. Thus, the $G\alpha_{15}$ protein is presumed to have a unique mode of engagement with and activation by the GPCR. In agreement with the present results, a study by Avet et al. using ebBRET/EMTA assays identified exclusively the $G\alpha_{s}$ and $G\alpha_{15}$ proteins as coupling partners for the adenosine-activated A_{2B}AR.[37] These proteins were likewise identified as the most efficacious coupling partners for the A_{2B}AR in the present study using TRUPATH BRET^2 assays (Figure 7).

At increasing A_{2B}AR or G protein density, e. g. in pathological scenarios such as inflammation and cancer,[12,14,55] A_{2B}AR activation by adenosine could result in efficacious activation of additional G proteins, such as $G\alpha_{q}$, $G\alpha_{11}$, $G\alpha_{i/o}$ or $G\alpha_{13}$ proteins.

In summary, each investigated agonist displays a characteristic signaling fingerprint regarding potency and especially efficacy at individual $G\alpha$ protein subunits. The efficacy of the agonist-activated A_{2B}AR at certain $G\alpha$ proteins appears to be decisive for biological significance of the respective signaling pathway. Since the cellular response to A_{2B}AR activation is dependent on the investigated cell type and the employed agonist, results from biological studies have to be interpreted with great care. BAY 60-6583 being a partial agonist does not imitate adenosine action, but nevertheless activates $G\alpha_{15}$, $G\alpha_{s}$, and $G\alpha_{12}$ proteins—presumably the most important downstream effectors of the A_{2B}AR. A highly potent A_{2B}-selective agonist that imitates adenosine action is currently not available, but would be most useful.

MATERIALS AND METHODS

Materials and Reagents. HEK293 $G\alpha_{q/11}$-KO cells were prepared by A. Inoue as previously described.[56] Dulbecco's modified Eagle medium (DMEM) and Hank's balanced salt solution (HBSS) buffer, supplemented with $CaCl_2$ and $MgCl_2$, were purchased from Sigma-Aldrich (Merck, Darmstadt, Germany). Fetal calf serum (FCS), G418, and penicillin-streptomycin were ordered from PAN Biotech (Aidenbach, Germany). Recombinant HEK293 cell lines stably expressing exclusively $G\alpha_{q/11}$ family subunits were created from CRISPR-Cas9-modified HEK293-$\Delta G\alpha_{q/11}$ cells as previously described.[25] Fluo-4-AM was purchased from Invitrogen (ThermoFisher, Waltham, MA, USA). Adenosine and ATP disodium salt were from Sigma (St. Louis, MO, USA); NECA was purchased from SantaCruz (Dallas, TX, USA), carbachol was purchased from AlfaAesar (Haverhill, MA, USA), BAY 60-6583 was purchased from Tocris (Bristol, UK). PSB-10,[57] PSB-11,[58] PSB-36,[59] and PSB-603[60] were synthesized as previously described. Lipofectamine 2000 was ordered from ThermoFisher Scientific (Waltham, MA, USA). U46619 was ordered from SantaCruz.[61] Flat white-bottom 96-well plates were purchased from Greiner BioOne disposable materials such as pipet tips and cell culture flasks were purchased from Sarstedt (Nümbrecht, Germany).

Cultivation of Cells. Cells were cultured at 37 °C and 5% CO_2 in DMEM supplemented with 10% FCS, 0.1 mg/mL streptomycin, and 100 U/ml penicillin (growth medium). If cells stably expressed recombinant genes, G418 (200 μg/mL) was added to the medium. At 70−80% confluency, cells were detached by trypsination, diluted with growth medium, and transferred into a fresh cell culture flask. Retroviral transfection of HEK293-$\Delta G\alpha_{q/11}$ cells was performed as previously described.[25]

Calcium Mobilization Assay. Calcium mobilization was measured as previously described for the $P2Y_2$ receptor.[62] In short, cells were trypsinized from cell culture flasks and seeded into a black clear-bottom 96-well plate (Corning 3340, Corning, Amsterdam, NL) in a final volume of 200 μL per well (40,000 cells per well) 1 day before the assay. Cells were incubated overnight in 96-well plates. On the next day, the supernatant medium was discarded and the following solution (40 μL) was added: HBSS buffer containing 3 μM fluo-4-AM + 0.075% w/v Pluronic F-127 ("dye solution"). All steps of the assay were performed at room temperature. The cells were incubated with the dye solution for 60 min while gently shaking. Then, the dye solution was removed and a mixture of 178 μL HBSS + 2 μL DMSO (without or with inhibitor) was added. If no inhibitor was present, the measurement was performed after a 2 min equilibration period. All assays in the presence of inhibitory compounds were preincubated for 30 min with the inhibitor before the measurement started. Cells were transferred to a plate reader (NovoStar, BMG Labtech, Offenburg, Germany). The A_{2B}AR was activated by the addition of 20 μL of agonist solution in HBSS (negative control: pure HBSS buffer). Fluorescence at 525 nm was measured for 40 s per well. Raw data were obtained in arbitrary fluorescence units (AU) and were corrected for the background signal (fluorescence intensity in the absence of agonist). If data were normalized, the normalization procedure was performed as indicated (see Figures). Each data point was recorded in duplicate or triplicate in three or more independent experiments. Dose−response curves were generated with Graph Pad Prism 8.0 (GraphPad, San Diego, CA, USA) using the "sigmoidal dose-response, variable slope" function. The assay principle is visualized in Figure S1B.

When the A_{2B}AR was transiently overexpressed for calcium mobilization assays, cells were transfected for 12 h with 100 or 1000 ng of hADORA2B-pcDNA3.1 per well in a 6-well plate (denoted as 100 ng DNA/well and 1000 ng DNA/well, respectively), 4 h after seeding the cells, using Lipofectamine 2000 according to the manufacturer's protocol. At the end of the 12 h period, the medium was exchanged. Transfected cells were harvested and transferred to 96-well plates 24 h after the end of the transfection, and from thereon the assay procedure was continued as described above.

https://doi.org/10.1021/acsptsci.2c00020
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BRET2 TRUPATH Assay. G protein heterotrimer dissociation was measured in HEK293 cells using the TRUPATH assay kit.[31] TRUPATH plasmids were a gift from Bryan Roth (University of North Carolina on Chapel Hill, NC, USA) and shipped via Addgene (Addgene kit no. 1000000163). The measurements were performed as indicated in the original publication with minor modifications to the original protocol. On the first day, HEK293 cells cultivated in growth medium were detached from cell culture flasks by trypsination. Cells were seeded into 6-well plates at a density of approximately 500 000 cells per well in a volume of 2 mL, and incubated at 37 °C for 2 h before transfection. Transient transfection of HEK293 cells with the TRUPATH biosensors (100 ng of each pcDNA5/FRT/TO-Gα-RLuc8, pcDNA3.1-Gβ, and pcDNA3.1-Gγ-GFP2 per well) and the receptor of interest (100 ng receptor cDNA in pcDNA3.1 per well) was performed with Lipofectamine 2000 (2.5 μL per μg cDNA, ThermoFisher, Waltham, MA, USA). Lipofectamine was diluted in OptiMEM, incubated at room temperature for 10 min, and subsequently added to the DNA mixture in OptiMEM to a final volume of 500 μL per well. The mixture was added to the cells and the transfection was performed overnight. On the second day, media were removed, and cells were detached by pipetting and transferred to 96-well white bottom plates (Greiner BioOne, Frickenhausen, Germany) at a density of 30 000 cells per well in 60 μL of growth medium. On the third day, the medium was carefully aspirated, and cells were gently washed with assay buffer (HBSS + 20 mM HEPES pH 7.4). Assay buffer (60 μL per well; in experiments with adenosine deaminase (ADA), the buffer was supplemented with 5 μg ADA (Roche, Basel, Switzerland) per ml of buffer) and luciferase solution (50 μM coelenterazine 400a (Biomol, Hamburg, Germany) in assay buffer) were added to the cells. After a 5 min equilibration period, agonist solution (30 μL; 1% final DMSO concentration diluted in assay buffer) was added to the cells. After another 5 min equilibration period, luminescence and fluorescence were measured on a Mithras LB940 plate reader, using 395 and 510 nm emission filters for the RLuc8 and GFP2 signals, respectively. During assay optimization, the measurments were performed for several time points between 5 and 15 min after substrate addition.While BRET ratios remained stable, the absolute RLuc and GFP2 counts decreased over time probably due to degradation of the substrate. The ratio between the GFP2 and the RLuc8 signal intensity was computed and corrected for the baseline signal to obtain ΔBRET values, which were then fit to the "log (agonist) vs. response (three parameters)" equation in GraphPad PRISM 8 (GraphPad, San Diego, CA, USA) as suggested by Olsen et al.[31] to determine pEC_{50} values and maximum efficacy (top-bottom of the sigmoidal concentration−response curve). The assay principle is visualized in Figure S1C.

DATA ANALYSIS

All data are expressed as mean ± standard error of the mean (SEM) of at least three independent experiments performed with at least two replicates per experiment. To test for significant differences between two groups, an unpaired *t*-test was employed. To assess significant differences among three or more groups, a one-way analysis of variance (ANOVA) was used. Significant thresholds are defined as follows: not significant (ns) $p > 0.05$, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. Data analysis was performed with GraphPad PRISM v8.0 (GraphPad, San Diego, CA, USA).

To assess differences in potency and efficacy for each individual agonist across all Gα protein subunits for all results obtained in the TRUPATH BRET2 assays, mean efficacies and potencies of each agonist (depicted in Figure 7) were compared to each other by a one-way ANOVA. The mean of each column was compared with the mean of each other column and corrected for multiple comparisons by Turkey's test. All p-values, depicted in Figure S3, are multiplicity-adjusted.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsptsci.2c00020.

Tables S1−S3, listing all determined potencies and efficacies in calcium and BRET^2 assays; Figures S1 and S2, displaying structures of the employed agonists, the assay principles, and reference curves obtained during establishment of TRUPATH BRET^2 assays (PDF)

AUTHOR INFORMATION

Corresponding Author

Christa E. Müller − *PharmaCenter Bonn, Pharmaceutical Institute, Pharmaceutical & Medicinal Chemistry, University of Bonn, D-53121 Bonn, Germany; Research Training Group GRK1873, University of Bonn, D-53121 Bonn, Germany;* orcid.org/0000-0002-0013-6624; Phone: +49-(0)-228-73-2301; Email: christa.mueller@uni-bonn.de; Fax: +49-(0)-228-73-2567

Authors

Jan Hendrik Voss − *PharmaCenter Bonn, Pharmaceutical Institute, Pharmaceutical & Medicinal Chemistry, University of Bonn, D-53121 Bonn, Germany;* orcid.org/0000-0003-0595-4607

Andhika B. Mahardhika − *PharmaCenter Bonn, Pharmaceutical Institute, Pharmaceutical & Medicinal Chemistry, University of Bonn, D-53121 Bonn, Germany; Research Training Group GRK1873, University of Bonn, D-53121 Bonn, Germany;* orcid.org/0000-0002-0456-1353

Asuka Inoue − *Graduate School of Pharmaceutical Sciences, Tohoku University, Sendai, Miyagi 980-8578, Japan*

Complete contact information is available at: https://pubs.acs.org/10.1021/acsptsci.2c00020

Funding

J.H.V., A.B.M., and C.E.M. were supported by the Deutsche Forschungsgemeinschaft (DFG, FOR2372, GRK1328). J.H.V., A.B.M., and C.E.M. are grateful for funding by the BMBF-funded Bonn International Graduate School Drug Sciences (BIGS DrugS). C.E.M. was supported by the EU COST Action ERNEST CA18133. A.B.M. is grateful for funding by the Ministry of Finance of Indonesia in the scheme of Indonesia Endowment Fund for Education (Lembaga Pengelola Dana Pendidikan (LPDP). A.I. was funded by the Japan Society for the Promotion of Science (JSPS) KAKENHI grants 21H04791 and 21H05113, FOREST JPMJFR215T from the Japan Science and Technology Agency (JST), LEAP JP20gm0010004 and BINDS JP20am0101095 from the Japan Agency for Medical Research and Development (AMED), the

Takeda Science Foundation, the Daiichi Sankyo Foundation of Life Science, and The Uehara Memorial Foundation.

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

We thank Dr. Bryan Roth, University of North Carolina School of Medicine, Chapel Hill, NC, USA, for sharing the TRUPATH Biosensor Platform.

■ ABBREVIATIONS

A_1AR, adenosine A_1 receptor; A_{2A}R, adenosine A_{2A} receptor; A_{2B}AR, adenosine A_{2B} receptor; A_3R, adenosine A_3 receptor; AC, adenylate cyclase; ADA, adenosine deaminase; Ado, adenosine; AR, adenosine receptor; ATP, adenosine triphosphate; $BRET^2$, bioluminescence resonance energy transfer type 2; CCh, carbachol; CRISPR-Cas9, clustered regularly interspaced short palindromic repeats - CRISPR-associated protein 9; EMTA, effector-membrane translocator assays; GDP, guanosine diphosphate; GFP2, green fluorescent protein-2; GPCR, G protein-coupled receptor; GTP, guanosine triphosphate; HEK293, human embryonic kidney type 293; M_3R, muscarinic M3 receptor; NECA, 5′-*N*-ethylcarboxamidoadenosine; Rluc8, Renilla luciferase; TGF-α, transforming growth factor-α; TP, thromboxane receptor

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https://doi.org/10.1021/acsptsci.2c00020
ACS Pharmacol. Transl. Sci. 2022, 5, 373−386