A Role for the Low-Affinity A_{2B} Adenosine Receptor in Regulating Superoxide Generation by Murine Neutrophils

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ABSTRACT

The formation of adenosine dampens inflammation by inhibiting most cells of the immune system. Among its actions on neutrophils, adenosine suppresses superoxide generation and regulates chemotactic activity. To date, most evidence implicates the G_s protein-coupled A_{2A} adenosine receptor (AR) as the primary AR subtype responsible for mediating the actions of adenosine on neutrophils by stimulating cAMP production. Given that the A_{2B}AR is now known to be expressed in neutrophils and that it is a G_s protein-coupled receptor, we examined in this study whether it signals to suppress neutrophil activities by using 2-[6-amino-3,5-dicyano-4-[4-(cyclopropylmethoxy) phenyl]pyridin-2-ylsulfanyl]acetamide (BAY 60-6583), a new agonist for the human A_{2B}AR that was confirmed in preliminary studies to be a potent and highly selective agonist for the murine A_{2B}AR. We found that treating mouse neutrophils with low concentrations (10^{-9} and 10^{-8} M) of BAY 60-6583 inhib- ited formylated-methionine-leucine-phenylalanine (fMLP)-stimulated superoxide production by either naive neutrophils, tumor necrosis factor-α-primed neutrophils, or neutrophils isolated from mice treated systemically with lipopolysaccharide. This inhibitory action of BAY 60-6583 was confirmed to involve the A_{2B}AR in experiments using neutrophils obtained from A_{2B}AR gene knockout mice. It is noteworthy that BAY 60-6583 increased fMLP-stimulated superoxide production at higher concentrations (>1 μM), which was attributed to an AR-independent effect. In a standard Boyden chamber migration assay, BAY 60-6583 alone did not stimulate neutrophil chemotaxis or influence chemotaxis in response to fMLP. These results indicate that the A_{2B}AR signals to suppress oxidase activity by murine neutrophils, supporting the idea that this low-affinity receptor for adenosine participates along with the A_{2A}AR in regulating the proinflammatory actions of neutrophils.

Introduction

Adenosine is a purine nucleoside generated in response to ischemia and inflammation, partly by the extracellular ca-

talysis by *ecto*-nucleotidases of ATP and ADP secreted by activated or injured cells (Haskó et al., 2008). During acute inflammation, adenosine functions in a autocrine/paracrine manner to suppress activity of essentially all cells of the immune system, including the neutrophil, thereby dampening and resolving the inflammatory reaction (Cronstein, 1994; Haskó et al., 2008). Among other actions, adenosine inhibits the superoxide burst produced by the NADPH complex in response to activating agents including host- and bacterial-derived formylated peptides, a process that is es-

ABBREVIATIONS: AR, adenosine receptor; ADA, adenosine deaminase; CCPA, 2-chloro-1,3-diethyl-N^6-cyclopentyldenosine; CGS 21680, 2-[p-(2-carboxyethyl)phenethylamino]-5′-N-ethylcarboxamidoadenosine; CP-532,903, (2S,3S,4R,5R)-3-amino-5-[6-(2,5-dichlorobenzylamino)purin-9-yl]-4-hydroxytetrahydrofuran-2-carboxylic acid methylamide; CVT-6883, 3-ethyl-1-propyl-8-(1-(3-trifluoromethylbenzyl)-1H-pyrazol-4-yl)-3,7-dihydropurine-2,6-dione; fMLP, formylated-methionine-leucine-phenylalanine; [^{125}I]-AB-MECA, N^6-(4-amino-3-[^{125}I]iodobenzyl)adenosine-5′-N-methylcarboxamide; APE, 2-[2-(4-aminophenyl)ethylamino]adenosine; [^{125}I]APE, 2-[2-amino-3-[^{125}I]iodophenyl)ethylamino]-adenosine; KO, knockout; MCLA, 2-methyl-6-(4-methoxyphenyl)-3,7-dihydroimidazol[1,2-a]pyrazin-3-one, hydrochloride; NECA, adenosine-5′-N-ethylcarboxamide; PSB-603, 8-[4-[4-(4-chlorophenyl)piperazide-1-sulfonyl)phenyl]]-1-propylxanthine; TNF-α, tumor necrosis factor-α; XAC, xanthine amine congener; BAY 60-6583, 2-[6-amino-3,5-dicyano-4-[4-(cyclopropylmethoxy)phenyl]pyridin-2-ylsulfanyl]acetamide; HEK, human embryonic kidney; HBSS, Hanks' balanced salt solution; G418, (2R,3S,4R,5R,6S)-5-amino-6-[(1R,2S,3S,4R,6S)-4,6-diamino-3-[(2R,3R,4R,5R)-3,5-dihydroxy-5-methyl-4-methylaminooxan-2-yl]oxy-2-hydroxycyclohexyl]oxy-2-(1-hydroxyethyl)oxane-3,4-diol; Ro 20-1724, 4-[(3-butoxy-4-methoxyphenyl)-methyl]-2-imidazolidinone; RLU, relative light units; LPS, lipopolysaccharide.

sential for killing phagocytosed bacteria but that also promotes oxidant-induced tissue injury in inflammatory diseases such as asthma, arthritis, inflammatory bowel disease, and ischemia/reperfusion injury (Cronstein, 1994; Haskó et al., 2008).

Among the four adenosine receptor (AR) subtypes (A$_1$, A$_{2A}$, A$_{2B}$, and A$_3$; Fredholm et al., 2000), most previous studies have implicated the G$_s$ protein-coupled A$_{2A}$AR in mediating the suppressive actions of adenosine on stimulated superoxide production (Sullivan et al., 2001; Haskó et al., 2008). However, it has become apparent in recent years that additional AR subtypes probably participate in this action. For example, it is now well established that the A$_3$AR is abundantly expressed in neutrophils, surprisingly at a level that exceeds that of the A$_{2A}$AR (Chen et al., 2006; van der Hoeven et al., 2008, 2010). Our data demonstrated that the A$_3$AR signals along with the A$_{2A}$AR to suppress the superoxide burst in murine neutrophils by a mechanism that seems to involve inhibition of the small GTPase Rac (van der Hoeven et al., 2008, 2010).

In our previous studies (van der Hoeven et al., 2008, 2010), we had also detected mRNA expression of the A$_{2B}$AR subtype at low levels in murine neutrophils that increased 15-fold during systemic inflammation (van der Hoeven et al., 2008). Considering that the A$_{2B}$AR is a G$_s$ protein-coupled receptor like the A$_{2A}$AR (Feoktistov and Biaggioni, 1997; Fredholm et al., 2000), in this investigation we tested the hypothesis that the A$_{2B}$AR couples to inhibition of stimulated superoxide production in neutrophils. We also examined whether the A$_{2B}$AR regulates neutrophil chemotaxis by using a Boyden chamber migration assay (van der Hoeven et al., 2008). Our studies involved the use of the A$_{2B}$AR agonist 2-[6-amino-3,5-dicyano-4-[4-(cyclopropylmethoxy)phenyl]pyridin-2-ylsulfanyl]acetamide (BAY 60-6385) (Beukers et al., 2004; Eckle et al., 2007) and A$_{2B}$AR gene knockout/β-galactosidase reporter gene knock-in mice (A$_{2B}$KO) (Yang et al., 2006). In preliminary studies the pharmacology of BAY 60-6583 for recombinant mouse ARs expressed in HEK 293 cells was extensively characterized in radioligand binding and functional cAMP accumulation assays.

Materials and Methods

Reagents. Dulbecco's modified Eagle's medium, fetal bovine serum, antibiotics, Hanks' balanced salt solution (HBSS), recombinant mouse tumor necrosis factor α (TNF-α), calcein-AM, and 2-methyl-6-(4-methoxyphenyl)-3,7-dihydroimidazol[1,2-a]pyrazin-3-one, hydrochloride (MCLA) were purchased from Invitrogen (Carlsbad, CA). Percoll was purchased from Amersham Biosciences (Piscataway, NJ), and adenosine deaminase (ADA) was from Roche Applied Science (Indianapolis, IN). 3-Ethyl-1-propyl-8-(1-(3-trifluoromethylbenzyl)-1H-pyrazol-4-yl)-3,7-dihydropurine-2,6-dione (CVT 6883) was purchased from ChemieTek (Indianapolis, IN), 8-[4-[4-(4-chlorophenzyl)piperazide-1-sulfonyl)phenyl]]-1-propylxanthine (PSB-603) was purchased from Tocris Bioscience (Ellisville, MO), xanthine amine congener (XAC) was from Research Biochemicals International (Natick, MA), and [^{3}H]cAMP and Na[^{125}I] was purchased from GE Healthcare (Chalfont St. Giles, Buckinghamshire, UK). BAY 60-6583 and 2-[2-(4-aminophenyl)ethylamino]adenosine (APE) were custom-synthesized as described previously (Luthin et al., 1995; Auchampach et al., 2009). All remaining reagents were purchased from Sigma-Aldrich (St. Louis, MO).

Mice. Male C57BL/6J wild-type mice (10–14 weeks old) were purchased from The Jackson Laboratory (Bar Harbor, ME). A$_{2B}$KO mice made congenic to the C57BL/6J mouse strain were as described

previously (Yang et al., 2006). All animal experiments were conducted with the approval of the Institutional Animal Care and Use Committee at the Medical College of Wisconsin.

Radioligand Binding Assays. Radioligand binding analysis was conducted with membranes prepared from HEK 293 cells lines expressing mouse ARs (Auchampach et al., 1997; Kreckler et al., 2006). The full-length cDNA sequences were obtained by reverse transcription-polymerase chain reaction using total RNA isolated from mouse brain (Kreckler et al., 2006). The cDNAs were subcloned into pCDNA3.1, transfected into HEK 293 cells using Lipofectamine, and selected with 2 mg/ml (2R,3S,4R,5R,6S)-5-amino-6-[(1R,2S,3S,4R,6S)-4,6-diamino-3-[(2R,3R,4R,5R)-3,5-dihydroxy-5-methyl-4-methylaminooxan-2-yl]oxy-2-hydroxycyclohexyl]oxy-2-(1-hydroxyethyl)oxane-3,4-diol (G418). In binding assays, crude membranes prepared from HEK 293 cells expressing mouse ARs (Kreckler et al., 2006) were incubated with various concentrations of radioligand and competitors in 100 μl of binding buffer (20 mM HEPES, 1 mM EDTA, 5 mM MgCl$_2$, 1 unit/ml adenosine deaminase). After incubation at 25°C for 3 h, bound and free radioligands were separated by rapid filtration over glass fiber filters. Nonspecific binding was measured in the presence of 100 μM adenosine-5′-N-ethylcarboxamide (NECA). N^6-(4-amino-3-[^{125}I]iodobenzyl)adenosine-5′-N-methylcarboxamide ([^{125}I]I-AB-MECA) (agonist) was used in assays with the mouse A$_1$ and A$_3$ ARs (Kreckler et al., 2006), and [^{125}I]APE (agonist) was used with mouse A$_{2A}$ARs (Luthin et al., 1995; Murphree et al., 2002). The radioligands were prepared by the chloramine-T method and purified by high-performance liquid chromatography (Luthin et al., 1995; Auchampach et al., 1997; Murphree et al., 2002).

In saturation binding assays, B_{max} and K_d values were obtained by fitting specific binding data to either single- or two-site binding models using nonlinear least-squares interpolation. An F test was used to identify the model that provided the best fit. In competition assays with compounds binding to a single site, specific binding data were fit to: binding = nonspecific binding + (total binding − specific binding)/$(1 + 10^{\times -\log IC_{50}})$. K_i values were calculated from IC$_{50}$ values using the Cheng-Prusoff equation (Cheng and Prusoff, 1973). In competition assays with compounds competing for two binding sites, two K_i values for competitors were calculated by solving the following four equations simultaneously (Auchampach et al., 1997): LB = $(B_{max1}[(L/K_{d1})/(1 + L/K_{d1} + C/K_{i1})]) + (B_{max2}[(L/K_{d2})/(1 + L/K_{d2} + C/K_{i2})]) + f \times L$; CB = $(B_{max1}[(C/K_{i1})/(1 + L/K_{d1} + C/K_{i1})]) + (B_{max2}[(C/K_{i2})/(1 + L/K_{d2} + C/K_{i2})]) + f \times C$; LT = L + LB; CT = C + CB, where LB is radioligand bound, CB is competitor bound, L is free radioligand, C is free competitor, and f is fraction of L or C nonspecifically bound (assumed to be equal). $K_{d1(high)}$ and $K_{d2(low)}$ were obtained from equilibrium saturation binding experiments in the absence of competitor. The other values were determined by simultaneously solving the four equations by interpolation within each iteration of nonlinear least-squares analysis (Auchampach et al., 1997).

cAMP Accumulation Assays. HEK 293 cells expressing recombinant mouse ARs were detached from cell culture plates using phosphate-buffered saline containing 5 mM EDTA, resuspended in serum-free Dulbecco's modified Eagle's medium containing 25 mM HEPES, pH 7.4, 1 unit/ml ADA, and 20 μM 4-[(3-butoxy-4-methoxyphenyl)-methyl]-2-imidazolidinone (Ro 20-1724), and then transferred to polypropylene tubes (200,000 cells/200 μl). The cells were incubated with ligands with or without forskolin (10 μM) for 15 min at 37°C with shaking, after which the assays were terminated by adding 500 μl of 0.15 N HCl. cAMP in the acid extract was determined by a competitive binding assay using [^{3}H]cAMP and protein extracts from bovine adrenal tissue (Nordstedt and Fredholm, 1990; Auchampach et al., 2010). The data were expressed as the amount of cAMP that accumulated during the 15-min incubation time (pmol/15 min/200,000 cells). EC$_{50}$ values were calculated by fitting the data to: $V = V_{min} + (V_{max} - V_{min})/(1 + 10^{\times -\log EC_{50}})$. The A$_{2B}$AR antagonists CVT-6883 (300 nM) and PSB-603 (300 nM) were included in assays with cells expressing the A$_1$, A$_{2A}$, or A$_3$ AR to inhibit A$_{2B}$ARs expressed endogenously in HEK 293 cells (Gao et al., 1999).

Isolation of Mouse Bone Marrow Neutrophils. Morphologically mature neutrophils were purified from mouse bone marrow by isotonic Percoll gradient centrifugation as described previously (van der Hoeven et al., 2008, 2010). In brief, mice were euthanized by anoxia with carbon dioxide. Bone marrow cells were flushed from tibias and femurs of mice with neutrophil isolation buffer ($1\times$ Ca^{2+}- and Mg^{2+}-free HBSS containing 0.4% sodium citrate), washed once with neutrophil isolation buffer, and then layered onto a three-step Percoll gradient (72, 64, 52%). After centrifugation at $1060g$ for 30 min, cells at the 72/64% interface were removed and washed once with isolation buffer before use in experiments.

Neutrophil Superoxide Production Assay. Superoxide production was measured using the chemiluminescent probe MCLA (van der Hoeven et al., 2008). Bone marrow neutrophils (7×10^4 cells/ml) were preincubated in HBSS containing 1 unit/ml ADA at 37°C for 30 min with vehicle or BAY 60-6583 at the concentrations indicated. After the addition of MCLA (0.5 μM) and fMLP, chemiluminescence was measured using a luminometer (AutoLumat, LB 953; Berthold Technologies, Bad Wildbad, Germany), and the cumulative relative light units (RLU) over 3 min were obtained for each sample. RLUs of unstimulated cells were deducted from the sample RLUs, and superoxide produced was calculated as a percentage of that produced with vehicle-treated control samples. A cell-free xanthine/xanthine oxidase superoxide-generating system was used to determine the chemiluminescence enhancing/quenching properties of BAY 60-6583. Specificity of the probe for superoxide was verified in all of the assays by adding superoxide dismutase in control reactions (van der Hoeven et al., 2008).

Chemotaxis Assay. Neutrophil chemotaxis was assessed using a 48-well microchemotaxis chamber (van der Hoeven et al., 2008). Neutrophils were labeled with the fluorescent dye Calcein-AM (3 μM) in neutrophil isolation buffer for 30 min at 37°C. After washing twice, the cells were resuspended in HBSS containing 0.5% bovine serum albumin and 1 unit/ml ADA. Next, 5×10^4 neutrophils (in a volume of 100 μl) were transferred to the upper wells of the chemotaxis chamber, which were separated from the lower chamber by a porous (5 μm) polycarbonate membrane (Neuroprobe, Gaithersburg, MD). The lower wells were filled with media containing fMLP or BAY 60-6583 as indicated. The cells were allowed to migrate at 37°C for 35 min in a humidified cell culture incubator, after which fluorescence emitted by cells that adhered to the underside of the membrane was measured using a fluorimager (Molecular Dynamics Typhoon Imaging System; GE Healthcare). After densitometry analysis using Scion Image software (National Institutes of Health, Bethesda, MD), the chemotaxis index was calculated using the following formula: fluorescence density of cells migrated in the presence of chemoattractant/fluorescence density of cells migrated toward medium (van der Hoeven et al., 2008).

Data Analysis. Data are reported as means ± S.E.M. Superoxide data were analyzed by a one-way analysis of variance followed by post hoc contrasts using Dunnett's t test. Chemotaxis data were analyzed by a two-way analysis of variance to determine whether there was a main effect of the chemoattractant (i.e., fMLP), a main effect of pretreating with BAY 60-6583 or a chemoattractant-BAY 60-6583 treatment interaction. A p value <0.05 was considered statistically significant.

Results

Characterization of BAY 60-6583 for Mouse ARs. The potency and selectivity of BAY 60-6583 for murine ARs was initially assessed in cAMP accumulation assays and competitive radioligand binding assays using HEK 293 cells stably expressing recombinant mouse ARs. As shown in Fig. 1, BAY 60-6583 potently stimulated cAMP production in HEK 293 cells expressing mouse A_{2B}ARs with an EC_{50} value of 2.83 ± 1.03 nM. In comparison, BAY 60-6583 exhibited no agonist activity

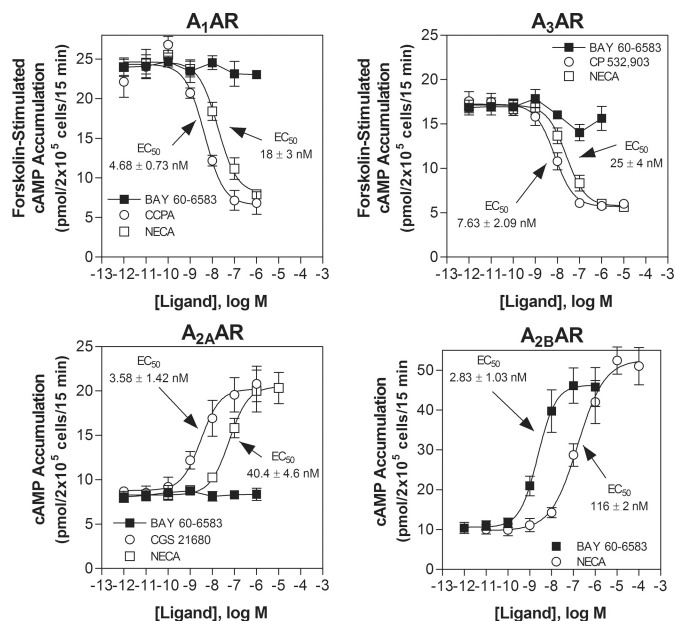**Fig. 1.** The selectivity of BAY 60-6583 for the mouse A_{2B}AR was confirmed in cAMP accumulation assays using HEK 293 cells stably expressing either the mouse A_1, A_{2A}, A_{2B}, or A_3 ARs. EC_{50} values calculated for BAY 60-6583 to stimulate cAMP accumulation (A_{2A} and A_{2B} ARs) or inhibit forskolin (10 μM)-stimulated cAMP accumulation are provided within the graphs. The potency of NECA, the selective A_1AR agonist CCPA, the selective A_{2A}AR agonist CGS 21680, and the A_3AR agonist CP 532,903 are included for the purpose of comparison. n = 3–5.

in assays using cells expressing A_1, A_{2A}, or A_3 ARs at concentrations as high as 1 μM. Inclusion of NECA and the subtype-selective agonists 2-chloro-1,3-diethyl-N^6-cyclopentyladenosine (CCPA; A_1), 2-[p-(2-carboxyethyl)phenethylamino]-5′-N-ethylcarboxamidoadenosine (CGS 21680) (A_{2A}), and (2S, 3S,4R,5R)-3-amino-5-[6-(2,5-dichlorobenzylamino)purin-9-yl]-4-hydroxytetrahydrofuran-2-carboxylic acid methylamide (CP-532,903) (A_3) were included as comparators in the assays (Fig. 1).

Before conducting competition binding assays, we performed preliminary diagnostic experiments with GTPγS to determine the characteristics of agonist radioligand binding to mouse ARs expressed in HEK 293 cells. As shown in Fig. 2, binding of [^{125}I]I-AB-MECA to the A_1AR was reduced significantly in the presence of GTPγS (100 μM). In the case of the A_1AR, specific binding was essentially abolished, whereas with the A_3AR specific binding was reduced ~50%. In saturation assays (Fig. 2), specific [^{125}I]I-AB-MECA binding to the mouse A_1AR fit best to a one-site binding model with a K_d value of 7.27 ± 1.8 nM and a B_{max} value of 956 ± 51 fmol/mg protein. On the other hand, specific binding of [^{125}I]I-AB-MECA to the mouse A_3AR fit best to a two-site binding model ($K_{d(high)}$ = 0.51 ± 0.08 nM; B_{max} = 374 ± 24 fmol/mg protein; $K_{d(low)}$ = 23.8 ± 5.6 nM; B_{max} = 504 ± 85 fmol/mg protein) that was converted to a one-site relationship in the presence of GTPγS (Fig. 2). These data indicate that [^{125}I]I-AB-MECA is able to detect the high-affinity, G protein-coupled states of the mouse A_1 and A_3 ARs when expressed in HEK 293 cells. In the case of the A_1AR, [^{125}I]I-AB-MECA exclusively detects the G protein-coupled state of the receptor, whereas it detects both the coupled and uncoupled states of the A_3AR. In contrast, specific binding of [^{125}I]APE to the mouse A_{2A}AR was not appreciably reduced by GTPγS (Fig. 2). Moreover, sat-
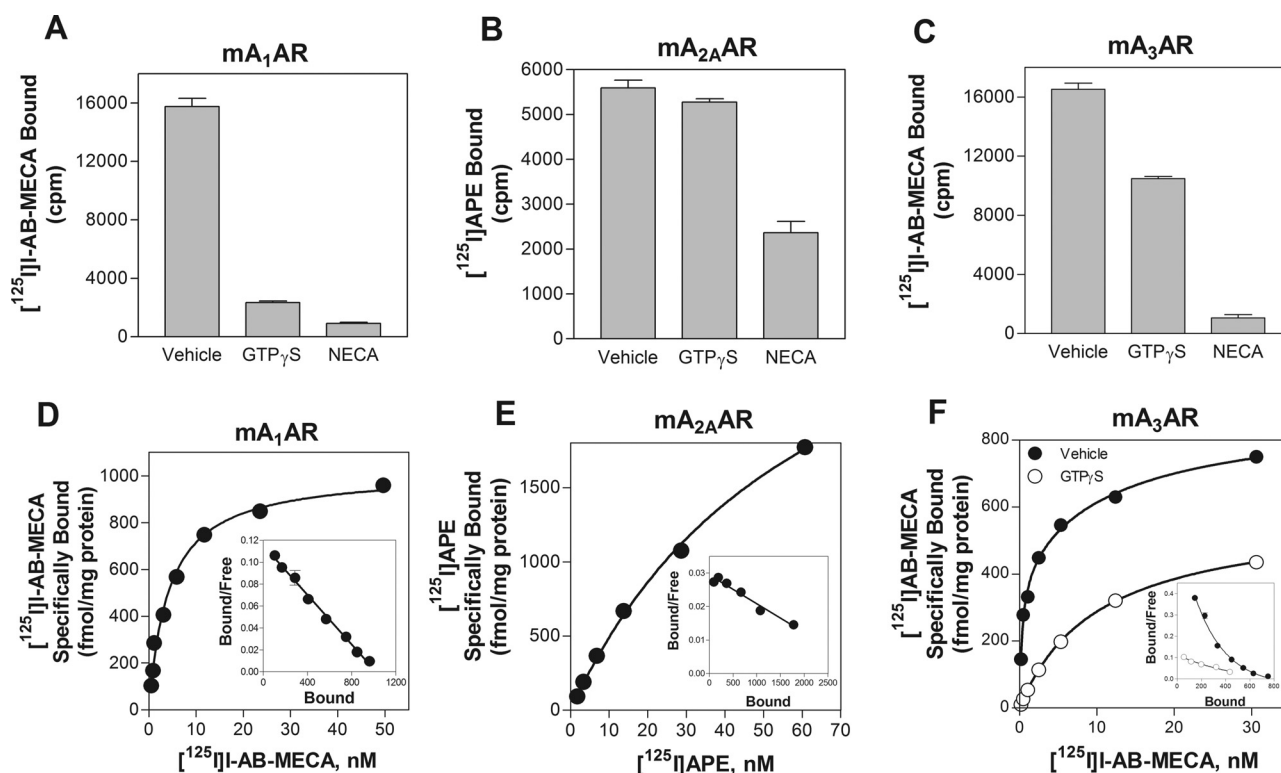
Fig. 2. Agonist radioligand binding to HEK 293 cells expressing recombinant mouse ARs. A to C, inhibition of agonist radioligand binding to HEK 293 cell membranes expressing recombinant mouse ARs by GTPγS (100 μM). Agonist radioligands were included at a concentration of ~0.2 nM. D to F, specific binding of the agonist radioligands to HEK 293 cell membranes expressing recombinant mouse ARs in equilibrium saturation binding assays. In studies of the A$_3$AR, the assays were conducted in the presence and absence of 100 μM GTPγS. Scatchard transformation of the specific binding data is presented in the insets. Each assay contained 50 μg of membrane protein, and nonspecific binding was revealed by the presence of NECA (100 μM). $n = 3$ for the binding assays with GTPγS. For saturation bindings assays, specific binding data from a representative experiment performed in triplicate of a total of three independent experiments is presented.

uration binding analysis revealed that [^{125}I]APE bound to a single receptor population with relatively low affinity (46 ± 6 nM and a B_{max} value of 4235 ± 782 fmol/mg protein). Similar results were obtained with the tritiated radioligand [^{3}H]CGS 2180 (data not shown). Collectively, these data indicate that mouse A$_{2A}$ARs expressed in HEK 293 cells fail to form high-affinity receptor-G protein complexes to an appreciable extend that can be accurately detected with agonist radioligands.

We subsequently proceeded to assess the affinity of BAY 60-6583 and other ligands used in the investigation for the two mouse AR subtypes in competition binding assays wherein it was possible to detect high-affinity agonist binding (i.e., A$_1$ and A$_3$ ARs). The results of these experiments are shown in Table 1. The K_i values reported for the A$_3$AR represents binding of the competitors for the high-affinity,

G protein-coupled state of the mouse A$_3$AR calculated using nontraditional analysis taking into account competition of the radioligand and competitors for two affinity states (Auchampach et al., 1997). The binding data indicate that BAY 60-6583 displays relatively low binding affinity for the functional, G protein-coupled state of the mouse A$_1$ and A$_3$ ARs.

Activation of the A$_{2B}$AR Inhibits fMLP-Induced Superoxide Production by Murine Neutrophils. Treating neutrophils with BAY 60-6583 produced a biphasic effect on fMLP-stimulated superoxide production (Fig. 3A). At low concentrations (1–10 nM), BAY 60-6583 reduced fMLP-stimulated superoxide production with a maximal inhibitory effect of ~50% at 10 nM. At higher concentrations (1–10 μM), however, BAY 60-6583 stimulated fMLP-induced superoxide production, producing a >50% increase over vehicle-treated cells at a concentration of 10 μM. In the absence of fMLP, treating neutrophils with BAY 60-6583 did not directly stimulate superoxide production (i.e., in the absence of fMLP) at any of the concentrations tested (Fig. 3B). BAY 60-6583 (10^{-11} to 10^{-5}) also did not directly scavenge superoxide anions or interfere with light production by MCLA as determined in a cell-free superoxide-generating system composed of xanthine/xanthine oxidase (data not shown).

In assays using neutrophils isolated from A$_{2B}$KO mice, the inhibitory effect of BAY 60-6583 on fMLP-induced superoxide generation observed at low nanomolar concentrations was no longer evident, although it continued to potentiate superoxide
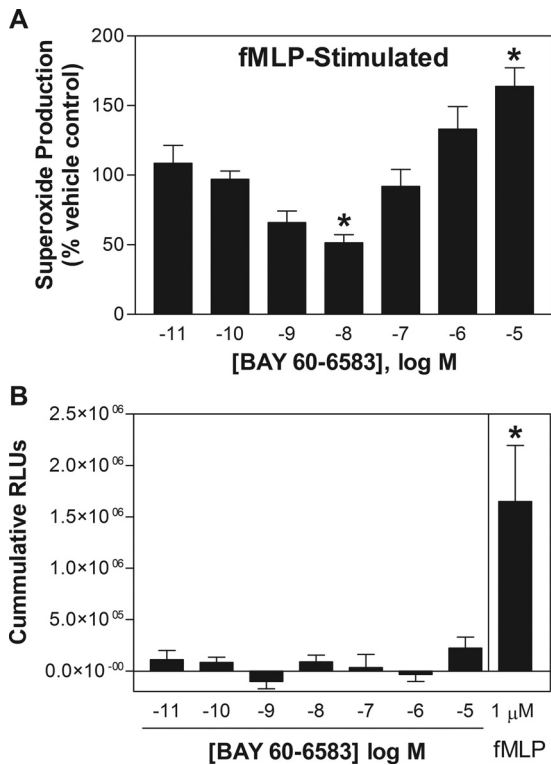
TABLE 1
Binding affinities (nM) of BAY 60-6583 and other drugs used in the investigation to the high-affinity, G protein-coupled state of recombinant mouse ARs determined by competition radioligand binding analysis using membranes prepared from HEK 293 cells expressing mouse ARs and [^{125}I]I-AB-MECA

	mA$_1$	mA$_3$
BAY 60-6583	232 ± 19	>10 μM
NECA	0.45 ± 0.13	14 ± 7
CCPA	0.21 ± 0.10	17 ± 5
CGS 21680	193 ± 38	N.D.
CP 532,903	889 ± 43	8.5 ± 2.2

N.D., not determined.

Fig. 3. Effect of increasing concentrations of BAY 60-6583 on superoxide production by murine neutrophils. A, effect of BAY 60-6583 expressed as a percentage of vehicle-treated cells. Neutrophils were pretreated with BAY 60-6583 for 30 min before stimulation with fMLP (1 μM). B, direct effect of treating with BAY 60-6583 (no stimulation with fMLP) on neutrophil superoxide production, expressed as cumulative RLUs. Superoxide production was measured as described under *Materials and Methods* using the chemiluminescent probe MCLA. ∗, $P < 0.05$ versus vehicle-treated cells; $n = 4$–6.
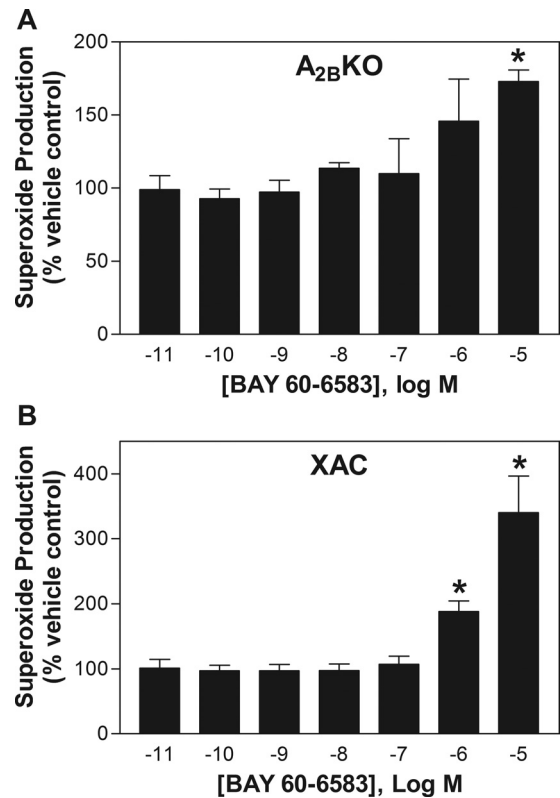

Fig. 4. Effect of increasing concentrations of BAY 60-6583 on fMLP-stimulated superoxide production of murine neutrophils obtained from $A_{2B}KO$ mice (A) or neutrophils obtained from wild-type mice in the presence of the nonselective AR antagonist XAC (1 μM) (B). Neutrophils were pretreated with BAY 60-6583 for 30 min before stimulation with fMLP (1 μM). Superoxide production is expressed as the percentage of vehicle-treated cells. ∗, $P < 0.05$ versus vehicle-treated cells; $n = 3$.

production at higher concentrations (Fig. 4A). A similar profile with BAY 60-6583 was observed with wild-type neutrophils in the presence of the nonselective AR antagonist XAC (1 μM; Fig. 4B). These results indicate that the inhibitory effect of BAY 60-6583 on fMLP-stimulated superoxide production at lower concentrations was mediated via the $A_{2B}AR$, whereas the stimulatory effect at higher concentrations was caused by a nonspecific, AR-independent mechanism.

BAY 60-6583 also produced a biphasic response on fMLP-induced superoxide production in assays using TNF-α-primed neutrophils (100 ng/ml for 30 min) or neutrophils isolated from mice pretreated 4 h earlier with 10 mg/kg of lipopolysaccharide (LPS) that results in neutrophil priming and a 10- to 15-fold increase in mRNA expression of A_{2A} and A_{2B} ARs (van der Hoeven et al., 2008; Fig. 5). The maximal inhibitory effect of BAY 60-6583 in these studies (∼20%) was less compared with assays with naive cells (see Fig. 3).

Activation of the $A_{2B}AR$ Does Not Influence Chemotaxis. In transwell chemotaxis assays, adding BAY 60-6583 (10^{-12} to 10^{-5}) into the lower wells did not directly stimulate migration or influence migration toward 1 μM fMLP of either naive or TNF-α-primed neutrophils (Fig. 6). Treating neutrophils with BAY 60-6583 (10 nM for 30 min) before the addition of the cells to upper wells also had no influence on migration toward increasing concentrations of fMLP in lower wells (Fig. 7).

Discussion

We previously identified expression of the low-affinity $A_{2B}AR$ subtype in murine neutrophils that is transcriptionally induced during systemic inflammation (van der Hoeven et al., 2008). In this study, we demonstrated that the $A_{2B}AR$ signals in murine neutrophils to inhibit superoxide production, but does not seem to modulate chemotaxis. Our results indicate that the $A_{2B}AR$ participates in mediating the inhibitory actions of adenosine on neutrophils. Considering that the $A_{2B}AR$ is a low-affinity receptor for adenosine and its expression is induced in response to hypoxia or inflammatory stimuli (Eckle et al., 2007; van der Hoeven et al., 2008; Haskó et al., 2009), we propose that $A_{2B}AR$ signaling may become increasingly important during inflammation resolution and when adenosine levels in tissues are high.

Based on the results of this investigation and previous work from our group and others, it is now apparent that three different AR subtypes expressed in murine neutrophils (A_{2A}, A_{2B}, and A_3) are capable of regulating stimulated superoxide production (Cronstein et al., 1985, 1992; Cronstein, 1994; Fredholm et al., 1996; Sullivan et al., 2001; van der Hoeven et al., 2008, 2010). The participation of multiple different AR subtypes may serve to increase the potency and efficacy of adenosine to dampen neutrophil activity at various stages of the inflammatory reaction. The expression of multiple different AR subtypes in neutrophils also raises the possibility for complex regulation of neutrophil activities through receptor
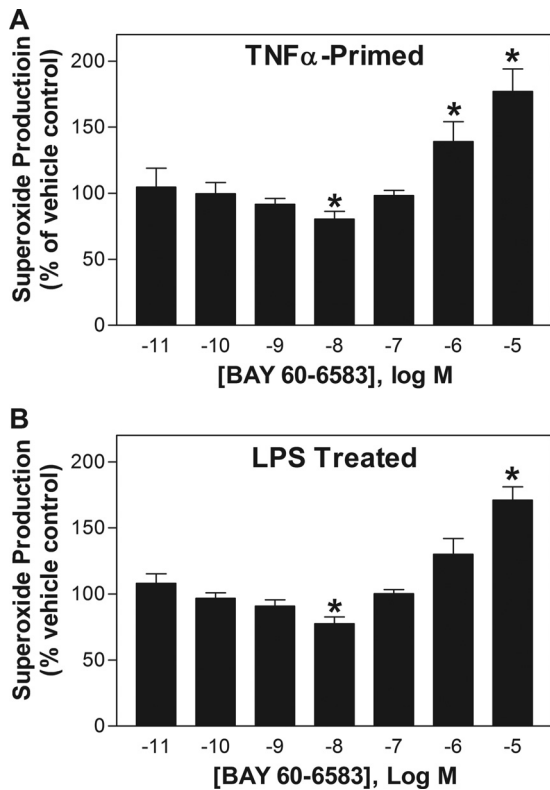
Fig. 5. Effect of increasing concentrations of BAY 60-6583 on fMLP (1 μM)-stimulated superoxide production of murine neutrophils primed by pretreatment with TNF-α (100 ng/ml for 30 min) (A) or neutrophils isolated from mice treated 4 h earlier with LPS (10 mg/kg i.p.) (B). Neutrophils were pretreated with BAY 60-6583 for 30 min before stimulation with fMLP (1 μM). Superoxide production is expressed as the percentage of vehicle-treated cells. ∗, $P < 0.05$ versus vehicle-treated cells; $n = 4$–6.

Fig. 6. Effect of BAY 60-6583 on chemotaxis of mouse neutrophils. Fluorescently labeled naive (A) or TNF-α-primed (100 ng/ml for 30 min) (B) neutrophils were placed into the upper wells of a 48-well chemotaxis chamber separated by a polycarbonate membrane (5-μm pores). The lower wells contained increasing concentrations of BAY 60-6583 in combination with vehicle or fMLP (1 μM). The cells were allowed to migrate at 37°C for 35 min, and the chemotaxis index was calculated as described under *Materials and Methods.* $n = 3$–5.

interactions that may involve receptor heterodimeration and cross-talk between signal transduction pathways. It has previously been demonstrated that human neutrophils express the A$_{2B}$AR, along with the A$_{2A}$, A$_3$, and potentially the A$_1$ AR (Chen et al., 2006; Fortin et al., 2006; Zhang et al., 2006). It will be important to determine whether the findings from this work with murine cells hold true with human neutrophils.

Neutrophil oxidant production is catalyzed by the multicomponent NADPH oxidase complex composed of cytosolic subunits (p47phox, p67phox, p40phox, and Rac2) that migrate to the membrane and associate with membrane subunits (p22phox and gp91phox) upon activation (Groemping and Rittinger, 2005). The complex catalyzes NADPH-dependent reduction of oxygen to superoxide anion (Groemping and Rittinger, 2005). The extent of neutrophil superoxide production is enhanced anywhere from ~4- to 15-fold after exposure to TNF-α and a variety of other proinflammatory cytokines, a process that is termed neutrophil "priming" (Dewas et al., 2003; Boussetta et al., 2010). The mechanism of priming by TNF-α is not completely understood, but seems to involve hyperphosphorylation of the cytosolic p47phox subunit of the NADPH complex that may be facilitated after isomerization of a phosphorylated serine residue within the carboxyl-terminal region of the protein (Dewas et al., 2003; Boussetta et al., 2010). Priming serves to amplify the destructive actions of neutrophils selectively within regions that are inflamed.
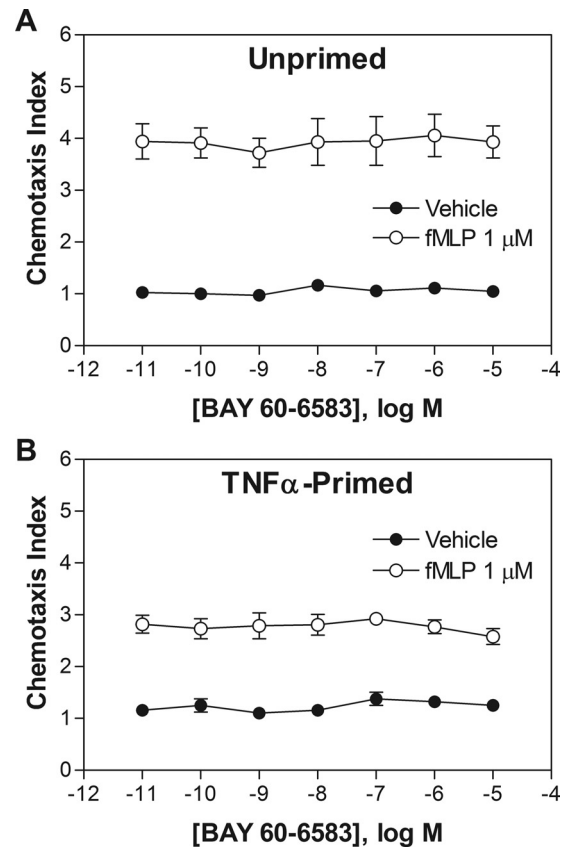
Our data indicate that activation of the A$_{2B}$AR is able to continue to suppress fMLP-stimulated superoxide by neutrophils that had been primed by TNF-α, albeit to a lesser extent compared with naive cells. With priming, BAY 60-6583 produced a maximal inhibitory effect of ~25% compared with a maximal inhibitory effect of 50% with nonprimed cells. A similar reduction in efficacy was observed in experiments using neutrophils isolated from LPS-treated mice whereby priming occurs in vivo before isolation (van der Hoeven et al., 2008). In this experiment, the efficacy of BAY 60-6583 was reduced even though expression of the A$_{2B}$AR was induced. At a molecular level, the reduced efficacy with TNF-α priming may be related to previous reports that AR activation does not inhibit neutrophil superoxide production in response to phorbol esters that directly activate protein kinase C, and that the molecular mechanism of TNF-α priming involving p47phox hyperphosphorylation is protein kinase C-dependent (Cronstein et al., 1985; van der Hoeven et al., 2008; Boussetta et al., 2010). It is notable that in our previous studies of murine neutrophils we also observed that the extent of inhibition of stimulated superoxide production provided by A$_{2A}$ and A$_3$ AR activation was similarly reduced after priming by TNF-α (van der Hoeven et al., 2008).

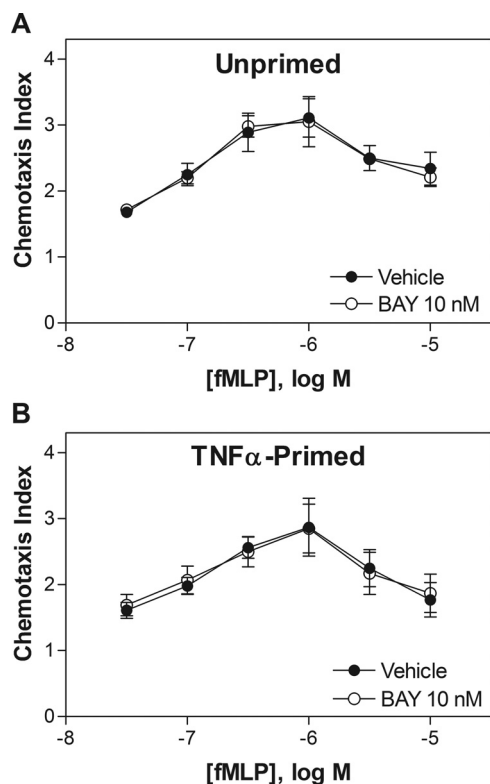BAY 60-6583 is member of a new series of 6-amino-3,5-dicyano-4-phenylpyridine derivatives that has been identi-

Fig. 7. Effect of pretreating mouse neutrophils with BAY 60-6583 on chemotaxis induced by fMLP. Fluorescently labeled naive (A) or TNF-α-primed (100 ng/ml for 30 min) (B) neutrophils were incubated with 10 nM BAY 60-6583 for 30 min and then placed into the upper wells of a 48-well chemotaxis chamber separated by a polycarbonate membrane (5-μm pores). The lower wells contained increasing concentrations of fMLP. The cells were allowed to migrate at 37°C for 35 min, and the chemotaxis index was calculated as described under *Materials and Methods*. $n = 4$–5.

fied as selective agonists for the human $A_{2B}AR$ (Beukers et al., 2004; Eckle et al., 2007). In preliminary studies, we carefully examined the binding affinity and functional potency of BAY 60-6583 for murine ARs. Comparisons were made with the adenosine analog NECA, which has been used extensively as a pharmacological agonist for the $A_{2B}AR$ in past studies. We found that BAY 60-6583 potently stimulated cAMP accumulation in cells transfected with the murine $A_{2B}AR$ with an EC_{50} value of 2.83 $\pm$ 1.03 nM while exhibiting no agonist activity for mouse A_1, A_{2A}, or A_3 ARs at concentrations as high as 1 μM. Correlating with the cAMP data, our radioligand binding data demonstrated that BAY 60-6583 binds to the G protein-coupled, high-affinity states of the A_1 and A_3 AR with low affinity. In comparison, NECA was essentially equipotent at activating all four subtypes of murine ARs being 40 times less potent than BAY 60-6583 as an agonist of the murine $A_{2B}AR$. Thus, our data agree with previous work with human ARs (Eckle et al., 2007) and confirm that BAY 60-6583 is a potent and remarkably selective agonist for the murine $A_{2B}AR$. Our data further reveal that NECA functions as a nonselective agonist for mouse ARs. In fact, among the four mouse AR subtypes, NECA displays the lowest potency for the $A_{2B}AR$, indicating that this agent should not be used as a selective probe for the $A_{2B}AR$ in mouse studies. One notable observation comparing the binding data in Table 1 and the cAMP data in Fig. 1 is that BAY 60-6583 binds to the mouse A_1AR with a K_i value of 232 nM (Table 1), yet it does not inhibit forskolin-stimu-

lated cAMP accumulation in assays with A_1AR-overexpressing HEK 293 cells even at a concentration of 1 μM, suggesting that it may act as an antagonist of the murine A_1AR. This turns out not to be the case, however, because we observed in additional experiments that including BAY 60-6583 up to a concentration of 1 μM did not shift the concentration-response curve of CCPA to inhibit forskolin-stimulated cAMP accumulation (Fig. 8). A clear explanation for this discrepancy between binding potency and functional potency is not readily apparent. One possibility is that BAY 60-6583 may exert a noncompetitive effect that influences radioligand binding to the A_1AR in assays involving isolated membrane preparations that does not occur with intact cells. For instance, BAY 60-6583 at high concentrations may in some way disrupt the formation of A_1AR-G protein complexes in isolated membranes.

We believe it is important to emphasize the results of our binding data with mouse A_2ARs. In preliminary assays, we found that agonist binding to mouse $A_{2A}ARs$ expressed in HEK 293 cells is almost completely insensitive to GTPγS, indicating that the observed binding is primarily to the low-affinity, G protein-uncoupled form of the receptor. As previously suggested by Murphree et al. (2002), this is probably explained by the limited ability of $A_{2A}ARs$ to form receptor-G protein complexes when expressed in HEK 293 cells. As a result, heterologous overexpression of the $A_{2A}AR$ in HEK 293 cells results in a large pool of uncoupled receptors whose presence interferes with detection of the much smaller pool of high-affinity binding sites using readily available radioligands. Poor receptor coupling is probably caused by limited expression of required subtypes of heterotrimeric G proteins (Murphree et al., 2002). Thus, it was not possible to accurately assess the binding affinity of BAY 60-6583 for the murine $A_{2A}AR$ in the present investigation. For this same reason, we believe it is also not possible to accurately assess high-affinity agonist binding to the $A_{2B}AR$ when expressed in HEK 293 cells as described previously (Auchampach et al., 2009).

We did not explore the signaling mechanisms by which the $A_{2B}AR$ inhibits superoxide production. Considering that the $A_{2B}AR$ is coupled to G_s proteins, it is likely that it functions similarly to the $A_{2A}AR$, namely through either the elevation of cAMP (Sullivan et al., 2001) or G_s protein-mediated un-
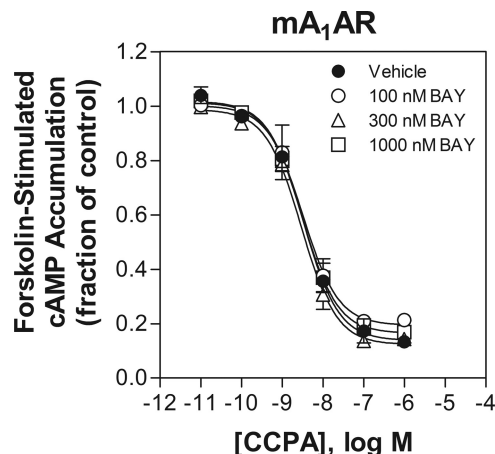

Fig. 8. Lack of effect of BAY 60-6583 up to a concentration of 1 μM to inhibit CCPA-mediated inhibition of forskolin (10 μM)-stimulated cAMP accumulation in HEK 293 cells overexpressing the mouse A_1AR. $n = 3$.

coupling of chemoattractant receptors (Cronstein et al., 1992). The A$_{2B}$AR differs from the A$_{2A}$AR, however, in being capable of dually coupling to both G$_s$ and G$_q$ proteins in some cell types (Feoktistov and Biaggioni, 1997; Fredholm et al., 2000). Thus, the participation of additional or alternative signaling mechanisms remains possible.

Our evidence for involvement of the A$_{2B}$AR is based on the observation that BAY 60-6583 inhibited fMLP-stimulated superoxide generation at low concentrations that correlated with its potency at activating the murine A$_{2B}$AR. At concentrations between 1 and 10 nM, BAY 60-6583 inhibited fMLP-stimulated superoxide production of naive cells as well as neutrophils primed with TNF-α or systemic exposure to LPS. This potent effect of BAY 60-6583 was not apparent in studies using neutrophils isolated from A$_{2B}$KO mice. It is noteworthy, however, that the effect of BAY 60-6583 was biphasic, producing a significant increase in fMLP-stimulated superoxide production at micromolar concentrations; this stimulatory action of BAY 60-6583 persisted in the presence of the nonselective A$_{2B}$AR antagonist XAC and in studies using neutrophils obtained from A$_{2B}$KO mice. Thus, BAY 60-6583 may have additional pharmacological actions other than A$_{2B}$AR agonism.

In addition to suppression of the NADPH oxidase burst, adenosine regulates chemotactic activity of neutrophils. However, this action of adenosine remains equivocal. It has been reported that adenosine functions to both promote and inhibit neutrophil chemotaxis, with the participation of multiple different AR subtypes including A$_1$, A$_{2A}$, and A$_3$ ARs (Cronstein et al., 1990; Chen et al., 2006; Zhang et al., 2006; van der Hoeven et al., 2008). To address the possible involvement of the A$_{2B}$AR, we examined the effect of BAY 60-6583 on chemotaxis of murine neutrophils in a standard Boyden chamber assay and found that it had no effect to directly stimulate chemotaxis or modulate chemotactic activity produced by fMLP. Although these data provide evidence that the A$_{2B}$AR probably does not participate in chemotaxis of murine neutrophils, it will be important to investigate this issue further using dynamic assays that can accurately detect effects on direction sensing and migration velocity (Chen et al., 2006).

The results of this investigation demonstrate that selective activation of the A$_{2B}$AR in murine neutrophils inhibits fMLP-stimulated superoxide production. This observation supports the concept that the A$_{2B}$AR probably participates along with other AR subtypes in transducing some of the inhibitory actions of adenosine on neutrophils. Studies using A$_{2B}$KO mice and BAY 60-6583 have provided evidence that the A$_{2B}$AR plays a protective role in various models of acute tissue injury in the heart, lungs, kidneys, gastrointestinal tract, and blood vessels (Eckle et al., 2007, 2008a,b; Grenz et al., 2008; Yang et al., 2008; Chen et al., 2009; Eltzschig et al., 2009; Hart et al., 2009; Maas et al., 2010; Schingnitz et al., 2010; Zhou et al., 2011). It has been proposed that the protective action of the A$_{2B}$AR in these models may involve anti-inflammation related to reduced proinflammatory cytokine expression, inhibition of platelet aggregation, and promotion of barrier function within the vasculature and alveoli (Eckle et al., 2007, 2008a,b; Grenz et al., 2008; Yang et al., 2008; Chen et al., 2009; Eltzschig et al., 2009; Hart et al., 2009; Maas et al., 2010; Schingnitz et al.; Zhou et al., 2011). The data reported herein provide evidence that an additional contributing mechanism may involve suppression of oxidant injury by neutrophils.

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