THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 275, No. 6, Issue of February 11, pp. 4429–4434, 2000

Printed in U.S.A.

Disruption of the A_3 Adenosine Receptor Gene in Mice and Its Effect on Stimulated Inflammatory Cells*

(Received for publication, June 2, 1999, and in revised form, November 17, 1999)

Christopher A. Salvatore‡§, Stephen L. Tilley§¶‖, Anne M. Latour¶, Daniel S. Fletcher, Beverly H. Koller¶‡‡, and Marlene A. Jacobson‡§§**

*From the ‡Department of Pharmacology, Merck Research Laboratories, West Point, Pennsylvania 19486, the **Department of Animal Pharmacology, Merck Research Laboratories, Rahway, New Jersey 07065, and the ¶Department of Medicine, University of North Carolina, Chapel Hill, North Carolina 27599*

The A_3 adenosine receptor (A3AR) is one of four receptor subtypes for adenosine and is expressed in a broad spectrum of tissues. In order to study the function of A3AR, a mouse line carrying a mutant A_3 allele was generated. Mice homozygous for targeted disruption of the *A3AR* gene, *A3AR*$^{-/-}$, are fertile and visually and histologically indistinguishable from wild type mice. The lack of a functional receptor in the *A3AR*$^{-/-}$ mice was confirmed by molecular and pharmacological analyses. The absence of A3AR protein expression in the *A3AR*$^{-/-}$ mice was demonstrated by lack of N^6-(4-amino-3-[^{125}I]iodobenzyl)adenosine binding to bone marrow-derived mast cell membranes that were found to express high levels of A3AR in wild type mice. In *A3AR*$^{-/-}$ mice, the density of A_1 and A_{2A} adenosine receptor subtypes was the same as in *A3AR*$^{+/+}$ mice as determined by radioligand binding to brain membranes. Additionally, A_{2B} receptor transcript expression was not affected by ablation of the *A3AR* gene. *A3AR*$^{-/-}$ mice have basal heart rates and arterial blood pressures indistinguishable from *A3AR*$^{+/+}$ mice. Functionally, in contrast to wild type mice, adenosine and the A3AR-specific agonist, 2-chloro-N^6-(3-iodobenzyl)-adenosine-5′-*N*-methylcarboxamide (2-Cl-IB-MECA), elicit no potentiation of antigen-dependent degranulation of bone marrow-derived mast cells from *A3AR*$^{-/-}$ mice as measured by hexosaminidase release. Also, the ability of 2Cl-IB-MECA to inhibit lipopolysaccharide-induced tumor necrosis factor-α production *in vivo* was decreased in *A3AR*$^{-/-}$ mice in comparison to *A3AR*$^{+/+}$ mice. The A_{2A} adenosine receptor agonist, 2-*p*-(2-carboxyethyl)phenylamino)-5′-*N*-ethylcarboxamidoadenosine, produced inhibition of lipopolysaccharide-stimulated tumor necrosis factor-α production in both *A3AR*$^{-/-}$ and *A3AR*$^{+/+}$ mice. These results show that the inhibition *in vivo* can be mediated by multiple subtypes, specifically the A_3 and A_{2A} adenosine receptors, and A3AR activation plays an important role in both pro- and anti-inflammatory responses.

Adenosine is a naturally occurring nucleoside that exhibits diverse and potent physiological responses in the central nervous system and the cardiovascular, renal, pulmonary, and immune systems. The actions of adenosine are mediated through G-protein-coupled receptors classified into four subtypes, A_1, A_{2A}, A_{2B}, and A_3, on the basis of their affinity order profiles for agonists and antagonists (1–3). The A_3 adenosine receptor (A3AR)[1] is the most recent subtype identified, since it had remained pharmacologically obscure until its gene was cloned in the early 1990s. The A3AR has been cloned from multiple species including rat (4, 5), human (6, 7), sheep (8), canine (9), and rabbit (10).

The physiological importance of the A_3 adenosine receptor has not been established. A3AR is expressed in a broad spectrum of tissues. The most abundant expression in human is found in lung and aorta (7). The evaluation of the functional role of the A3AR has been hampered by the presence of multiple adenosine receptor subtypes in target tissues and lack of selective A3AR agonists and antagonists. In addition, the unique species difference between rat and human receptors in their ability to bind xanthine antagonists and differences in tissue distribution of expression has contributed to the difficulty in selecting an appropriate model to study the function of the A3AR (3). Despite these obstacles, a number of studies have been reported in which the A3AR has been linked to a variety of physiological processes including degranulation of antigen-stimulated rat basophilic leukemic cell line, RBL-2H3 (11), mast cell-dependent constriction of hamster cheek pouch arterioles (12), mast cell mediation of hypotension in rats (13, 14), induction of apoptotic cell death (15), inhibition of TNFα production from LPS-stimulated murine (16) and human (17) macrophage-like cell lines, inhibition of platelet-activating factor-induced chemotaxis of human eosinophils (18), neural (19) and cardiac (20, 21) protection, and depression of locomoter activity in mice (22).

The generation of a mouse line that does not express the A3AR subtype provides a useful tool to dissect out the role of this receptor from other adenosine receptor subtypes. By complementing pharmacological analyses with targeted gene disruption, evaluation of the functional role of the A3AR in various physiological processes can be more readily addressed. In this report, we describe the initial characterization of the *A3AR*$^{-/-}$ mouse, and we evaluate the consequence of *A3AR*

* The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

§ Both authors contributed equally to this work

‖ Supported by an American Lung Association Research Training Fellowship award.

‡‡ Supported in part by National Institutes of Health Grant HL-58554.

§§ To whom correspondence should be addressed: Merck Research Laboratories, WP26-265, West Point, PA 19486. Tel.: 215-652-7122; Fax: 215-652-0800; E-mail: marlene_jacobson@merck.com.

[1] The abbreviations used are: A3AR, A_3 adenosine receptor; *A3AR*$^{-/-}$, A_3 adenosine receptor-deficient; *A3AR*$^{+/+}$, wild type; BMMC, bone marrow-derived mast cells; CGS21680, 2-(*p*-(2-carboxyethyl)phenylamino)-5′-*N*-ethylcarboxamidoadenosine; 2-Cl-IB-MECA, 2-chloro-N^6-(3-iodobenzyl)-adenosine-5′-*N*-methylcarboxamide); DPCPX, 8-cyclopentyl-1,3-dipropylxanthine; ES, embryonic stem; I-ABA, N^6-(4-amino-3-iodobenzyl)adenosine; IB-MECA, N^6-(3-iodobenzyl)-9-[5-(methylcarbamoyl)-β-ribofuranosyl]adenosine; LPS, lipopolysaccharide; TNFα, tumor necrosis factor-α; kb, kilobase pair; Pipes, 1,4-piperazinediethanesulfonic acid APNEA, N^6-2-(4-aminophenyl)ethyladenosine.

gene ablation on adenosine receptor-mediated bone marrow-derived mast cell degranulation and *in vivo* inhibition of LPS-stimulated TNFα production. Our findings illustrate an important role for the A3AR on stimulated inflammatory cells.

EXPERIMENTAL PROCEDURES

Generation of A3R$^{-/-}$ Mice—The mouse genomic DNA utilized to construct the targeting vector was obtained by polymerase chain reaction screening (5′-GCTGCCATCGGGCTCTGTG and 5′-GCAGGCATAGAAGTGCATCT) of a P1 129 Mouse ES Library (Genome Systems, Inc.). The targeting vector was introduced into the embryonic stem (ES) line E14Tg2a (23) by electroporation, and G418-resistant colonies were identified by established protocols (24). Two *A3AR*-targeted ES cell clones were injected into 3.5-day-old blastocysts from C57BL/6 mice and reimplanted into pseudopregnant foster mothers. Resultant progeny were screened initially by coat color chimerism as the indicator of ES cell contribution. Chimeras were bred to B6D2 and C57BL/6 mice and heterozygote mice determined by genomic Southern blot of tail biopsy DNA. Heterozygote mice were intercrossed to produce homozygote *A3AR*$^{-/-}$ animals and wild type (*A3AR*$^{+/+}$) littermate controls.

Southern Blot Analysis—Genomic DNA was isolated from ES cell clones and tail biopsies by modifications of standard procedures (25) and analyzed by genomic Southern blot for targeted recombination. Ten micrograms of genomic DNA was digested with the restriction enzyme *Bam*HI, electrophoresed on a 0.7% agarose gel, transferred to Hybond-N, and UV cross-linked according to the manufacturer's suggestion (Amersham Pharmacia Biotech). Filters containing the immobilized DNA were incubated with 2×10^6 cpm/ml of radiolabeled probe in RapidHyb (Amersham Pharmacia Biotech) solution for 3 h at 65 °C. The A3 targeting probe corresponds to a 0.75-kb *Xho*I-*Hin*dIII fragment located immediately upstream of the short arm of the targeting construct. Filters were washed at 65 °C in a solution containing 0.1× SSC and 0.1% SDS and analyzed by autoradiography.

Purification of Bone Marrow-derived Mast Cell (BMMCs)—BMMCs were isolated from the femurs of 8–12-week-old A3AR$^{-/-}$ and *A3AR*$^{+/+}$ mice according to established procedures (26). BMMCs were cultured in the presence of interleukin-3 and passed weekly to eliminate adherent cells as described (26). Experiments were carried out within 4–8 weeks of culture initiation.

Northern Blot Analysis—Total brain RNA was isolated from A3AR$^{-/-}$ and *A3AR*$^{+/+}$ mice using the Trizol Reagent (Life Technologies, Inc.). Poly(A)$^+$ mRNA was isolated by subjecting total brain RNA to two cycles of oligo(dT)-cellulose chromatography. Total RNA was isolated from *A3AR*$^{-/-}$, *A3AR*$^{+/-}$, and *A3AR*$^{+/+}$ cultured BMMCs using RNAzol (Tel-Test, Inc.) according to the manufacturer's recommendations. Fifteen micrograms of total BMMC RNA or 5 μg of poly(A)$^+$ brain RNA was fractionated on a 1% agarose/formaldehyde gel (27), transferred to Hybond-N, and hybridized in RapidHyb solution containing 2×10^6 cpm/ml radiolabeled probe. Mouse A_1, A_{2A}, and A_{2B} cDNA probes were a kind gift from Dr. Diana Marquardt (University of California, San Diego). The mouse A_3 probe corresponds to a 431-base pair *Sac*I fragment containing part of the 2nd extracellular loop and stopping 18 amino acids upstream of the stop codon. Filters were washed at 65 °C in a solution containing 0.1× SSC and 0.1% SDS and analyzed by autoradiography.

Radioligand Binding—Membranes were prepared from cultured bone marrow-derived mast cells from A3AR$^{-/-}$, *A3AR*$^{+/-}$, and *A3AR*$^{+/+}$ mice, and [^{125}I]ABA binding was carried out according to Salvatore *et al.* (7). Membranes were prepared from mouse whole brain as described (28) and utilized to measure the binding of [^{3}H]DPCPX (8-cyclopentyl-1,3-dipropylxanthine) to A_1 adenosine receptors and [^{3}H]CGS21680 (2-(*p*-(2-carboxyethyl)phenylamino)-5′-*N*-ethylcarboxamidoadenosine) to A_{2A} adenosine receptors. Binding to A_1 and A_{2A} receptors was performed in 50 mM Tris, pH 7.4, 10 mM $MgCl_2$ at 25 °C for 2 h. Assays were terminated by rapid filtration on to Skatron glass fiber filters (Skatron Inc., Sterling, VA) with three washes of ice-cold binding buffer. Filter bound radioactivity was determined using a Packard Tri Carb liquid scintillation counter. Equilibrium dissociation constants (K_D) and maximal binding densities were obtained using the program EBDA (BioSoft, Ferguson, MO).

Hemodynamic Measurements—Mice were anesthetized with ketamine, xylazine mixture (3.8 mg/ml ketamine, 0.46 mg/ml xylazine at 0.1 ml per 15 g mouse body weight) and placed on a circulating water heating pad. A micro-ranethane catheter, filled with heparinized saline, was inserted into the right carotid artery and held in place with suture and veterinary adhesive. The catheter was sealed and then tunneled subcutaneously and exteriorized between the scapulae. The catheter was protected by a small plastic tube glued to the mouse back. After the mouse recovered from the anesthesia, it recovered overnight with food and water, *ad libitum*. All surgeries were done in accordance with AICUC standards. On day of the experiment, the arterial catheter was connected by a pressure transducer to a data acquisition system (MI_2, Malvern, PA). After a quieting down period, control heart rate and blood pressure measurements were made every 15 min for a period of an hour. The final reading is reported as the basal heart rate and mean arterial pressure value. Means (± S.E.) were done for each group ($n = 16$).

Hexosaminidase Release Assay—BMMCs were incubated overnight at 37 °C with a monoclonal IgE directed against human dinitrophenyl albumin (Sigma) at a concentration of 100 ng/ml/10^6 cells to give 100% occupancy of IgE receptors. After removal of unbound IgE by washing twice in a glucose/saline, Pipes-buffered solution (119 mM NaCl, 5 mM KCL, 25 mM Pipes, 5.6 mM glucose, 1 mM $CaCl_2$, 0.4 mM $MgCl_2$, 0.1% bovine serum albumin), 5×10^5 cells were transferred to 96-well microtiter plates. Cells were incubated in the presence and absence of adenosine (10 nM to 100 μM) or the adenosine analogue 2-Cl-IB-MECA (2-chloro-*N*^{6}-(3-iodobenzyl)-adenosine-5′-*N*-methyl-carboxamide) (0.1 nM to 1 μM) for 1 min prior to stimulation with antigen (dinitrophenyl albumin, 10–100 ng/ml) at 37 °C. Reactions were terminated after 20 min by centrifugation at $2000 \times g$ for 5 min at 4 °C. The extent of mast cell degranulation was determined by comparing levels of hexosaminidase activity in the supernatant and cell pellets. Hexosaminidase activity was determined by incubating supernatant and cell lysate with 1 mM *p*-nitrophenyl-*N*-acetyl-B-D-glucosaminidine (M_r 342.3, Sigma) for 1 h at 37 °C. The reaction was terminated by adding 0.1 M Na_2CO_3/$NaHCO_3$, and the absorbance was measured at 410 nm. Hexosaminidase release was expressed as a percentage of the total amount of hexosaminidase present in the cells as described previously (29).

Measurement of TNFα Production—Mice had access to food and water *ad libitum*. At zero time, mice were injected intravenously with 0.2 ml of saline vehicle with or without test compound, followed immediately by intraperitoneal injection of 0.5 ml of saline containing 10 μg of LPS (lipopolysaccharide B from *Escherichia coli* 0111:B4, Difco) and 800 mg/kg D-galactosamine. Mice were sacrificed by CO_2 asphyxiation 90 min later, and heparinized blood was obtained by cardiac puncture. Plasma was prepared by centrifugation at $1000 \times g$ for 15 min, and plasma TNFα levels were determined by enzyme-linked immunosorbent assay as described in Molineaux *et al.* (30). TNFα levels are expressed as the mean ± S.E. Results were compared statistically using the Student's *t* test, and differences were considered to be significant at or below $p = 0.05$.

Materials—Adenosine was purchased from Roche Molecular Biochemicals. CGS21680 was purchased from RBI (Natick, MA). 2-Cl-IB-MECA from RBI NIMH synthesis program. [^{125}I]ABA was synthesized according to Ref. 31. [^{3}H]CGS21680 and [^{3}H]DPCPX was from NEN Life Science Products.

RESULTS

Cloning of the Mouse A3AR Gene and Generation of Targeting Plasmid—The mouse genomic DNA encoding the A_3 adenosine receptor was cloned by polymerase chain reaction screening from a P1 129 mouse ES library. The mouse *A3AR* coding sequence lies on two exons separated by an approximately 2.1-kb intron interrupting the second intracellular loop. The deduced amino acid sequence encodes a 319- amino acid protein and exhibits 89% overall identity to the rat (4, 5), 76% to the sheep (8), and 73% to the human (7) A3ARs. A targeting construct designed to disrupt the A_3 adenosine receptor gene was generated containing 1.5 kb of mouse genomic DNA immediately upstream of the *A3AR* coding sequence, a neomycin resistance gene replacing the initiation codon through the third transmembrane domain and part of the intron, and 7.5 kb of genomic DNA downstream containing the remaining intron and the remainder of the *A3AR* coding sequence (Fig. 1*A*). This DNA construct was electroporated into embryonic stem cells (line E14Tg2a), and transfectants were selected with G418.

Targeted Disruption of the Mouse A3AR Gene—Homologous recombination with the endogenous *A3AR* gene results in disruption of the *A3AR* gene by replacement of the 5′-half of the receptor with a neomycin resistance gene. Southern blot analysis was used to screen *Bam*HI digested genomic DNA prepared from 60 G418-resistant ES cell clones for targeted re-

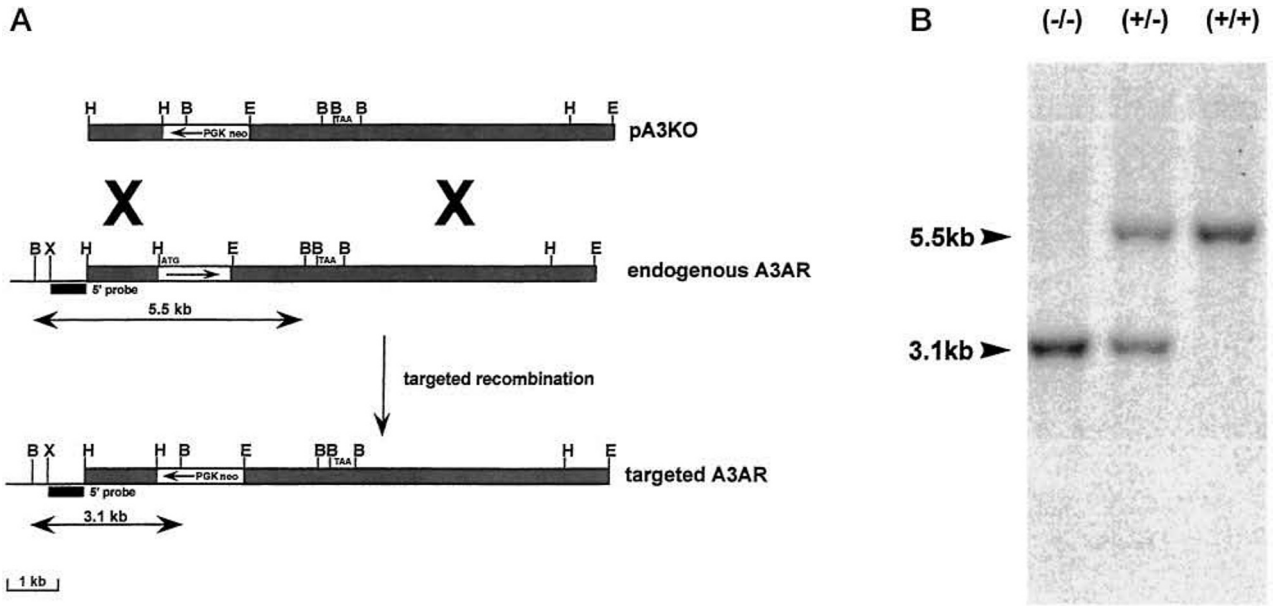

FIG. 1. *A,* schematic representation of targeted recombination of the mouse A_3 adenosine receptor. A_3 replacement vector pA3KO (*top panel*), endogenous A_3 adenosine receptor (*middle panel*), disrupted A_3 adenosine receptor (*bottom panel*) are shown. Restriction sites *Bam*HI (*B*), *Eco*RI (*E*), *Hin*dIII (*H*), and *Xho*I (*X*) are as indicated. The position of 0.75-kb *Xho*I-*Hin*dIII 5′ probe used as diagnostic for targeted recombination and expected fragment sizes is indicated. The putative start site of the A_3 adenosine receptor is indicated by *ATG* and the stop site by *TAA*. *B,* genomic Southern blot representing the three A3AR genotypes ($A3AR^{-/-}$ (−/−), $A3AR^{+/-}$ (+/−), $A3AR^{+/+}$ (+/+)).

combination. Four ES cell clones were positive for targeted recombination as determined by a decrease in the size of the *Bam*HI fragment from 5.5 kb in wild type to 3.1 kb in the mutant allele (Fig. 1*B*). Two of the targeted clones were injected into 3.5-day-old blastocytes and produced highly chimeric mice. Male chimeras were bred to B6D2 and C57BL/6 mice, and transmission of the mutant allele was determined by coat color and genomic Southern blot of DNA obtained from tail biopsies.

Animals heterozygous for the mutant allele were intercrossed, and the progeny of these matings yielded wild type mice, heterozygotes, and mice homozygous for the targeted allele with the expected Mendelian frequency (Fig. 1*B*). No difference was observed between the A3AR-deficient and wild type littermates by visual inspection or routine histological analysis of all organs. In addition, despite high levels of the *A3AR* expression in the testes (4), both male and female $A3AR^{-/-}$ mice demonstrated normal fertility. Body mass was identical between $A3AR^{+/+}$ and $A3AR^{-/-}$ mice. The cardiovascular effects produced by adenosine, coronary vasodilation, hypotension, and bradycardia, are well documented (32). *A3AR* transcripts are expressed in heart; however, the involvement of the A3AR in maintaining basal blood pressure or heart rate has not been explored. The mean arterial blood pressure and resting heart rate was evaluated in $A3AR^{+/+}$ and $A3AR^{-/-}$ mice and found to be virtually identical. Values measured for basal mean arterial pressure were 116.71 ± 4.61 mm Hg for $A3AR^{+/+}$ and 115.87 ± 5.03 mm Hg for $A3AR^{-/-}$ mice and for basal heart rate, 537.59 ± 26.06 beats/min for $A3AR^{+/+}$ and 578.20 ± 21.71 beats/min for $A3AR^{-/-}$ mice.

mRNA Expression of Adenosine Receptor Subtypes in $A3AR^{+/+}$ and $A3AR^{-/-}$ Mice—It has been previously shown that A_{2A} and A_{2B} adenosine receptor subtype mRNAs, but not A_1 adenosine receptor subtype mRNAs, are expressed in murine BMMCs (33). A3 transcript expression was not determined in this initial analysis of BMMCs. In cultured BMMCs isolated from $A3AR^{+/+}$ mice, high levels of *A3AR* transcripts were detected (Fig. 2*A*). In comparison, no *A3AR* mRNA was detected in cultured BMMCs isolated from $A3AR^{-/-}$ mice. To determine whether the expression of A_1, A_{2A}, and A_{2B} adenosine receptor subtypes were affected by the elimination of A3AR expression, mRNA levels were determined by Northern blot analysis. No significant change in levels of either A_{2A} or A_{2B} adenosine receptor transcript was seen in cultured BMMCs from wild type and $A3AR^{-/-}$ mice (Fig. 3). Consistent with previous reports (33), no expression of A_1 receptor transcript was detectable in cultured BMMC mRNA from wild type and $A3AR^{-/-}$ mice (data not shown). In order to evaluate A_1 receptor expression in $A3AR^{+/+}$ and $A3AR^{-/-}$ mice, RNA was isolated from whole brain and analyzed by Northern blots. Similar to A_{2A} and A_{2B} expression in BMMCs, no differences were observed between $A3AR^{+/+}$ and $A3AR^{-/-}$ mice (Fig. 3).

Binding Analysis of Tissues from $A3AR^{+/+}$ and $A3AR^{-/-}$ Mice—The lack of functional A3AR protein expression was confirmed by binding assays on BMMC membranes utilizing the A3AR agonist [^{125}I]ABA (Fig. 2*B*). BMMC membranes were utilized for the binding analysis as they express high levels of A3AR. Membranes prepared from $A3AR^{+/+}$ mice exhibited specific [^{125}I]ABA binding. Heterozygote mice exhibited a 50% decrease in specific [^{125}I]ABA binding. In comparison, no specific [^{125}I]ABA binding was measured on membranes from $A3AR^{-/-}$ mice (Fig. 2*B*). The density of A_1 and A_{2A} adenosine receptors expressed in whole brain from $A3AR^{+/+}$ and $A3AR^{-/-}$ mice was measured by radioligand binding of [^{3}H] DPCPX, 8-cyclopentyl-1,3-dipropylxanthine for A_1 adenosine receptors, and [^{3}H]CGS21680 for A_{2A} receptors. No differences were observed in the density or affinities of A_1 and A_{2A} receptors between $A3AR^{-/-}$ and $A3AR^{+/+}$ mice. K_D and B_{max} values for [^{3}H]DPCPX binding to A_1 receptors on $A3AR^{-/-}$ brain membranes was measured as 0.70 ± 0.31 nM and 1040 ± 106 fmol/mg of protein and 0.90 ± 0.45 nM and 910 ± 82 fmol/mg of protein on $A3AR^{+/+}$ brain membranes. K_D and B_{max} values for [^{3}H]CGS21680 binding to A_{2A} receptors was measured as 16.02 ± 1.8 nM and 200.3 ± 23.7 fmol/mg of protein for binding to $A3AR^{-/-}$ brain membranes, and 15.33 ± 2.5 nM and 214.5 fmol/mg of protein on $A3AR^{+/+}$ brain membranes were measured.

Degranulation of BMMCs from $A3AR^{+/+}$ and $A3AR^{-/-}$

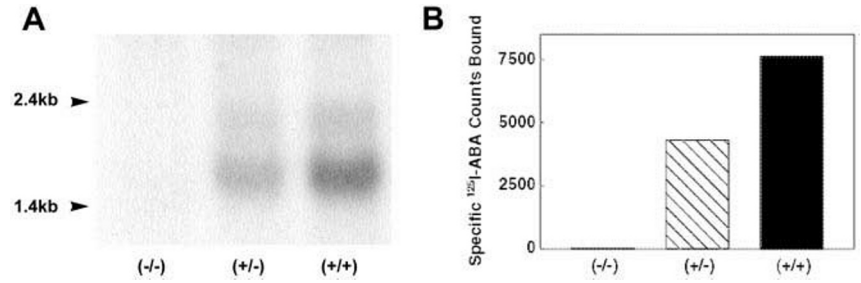

FIG. 2. *A,* Northern blot analysis of cultured bone marrow-derived mast cell total RNA (7 μg) from *A3AR*$^{-/-}$(−/−), *A3AR*$^{+/-}$ (+/−), and *A3AR*$^{+/+}$ (+/+) mice for the A3 adenosine receptor transcript. The mouse A_3 probe corresponds to a 431-base pair *Sac*I fragment encoding part of the second extracellular loop and stopping 18 amino acids upstream of the stop codon. *B,* specific binding of [^{125}I]ABA to BMMC membranes from *A3AR*$^{-/-}$ (−/−), *A3AR*$^{+/-}$ (+/−), and *A3AR*$^{+/+}$ (+/+) mice. Data are representative of three experiments.

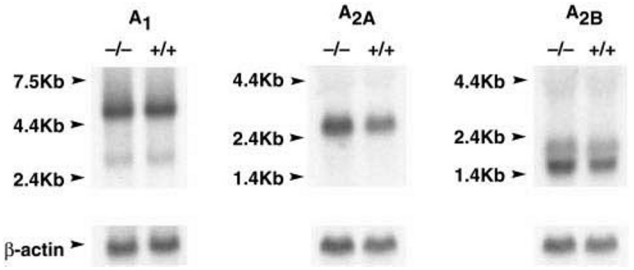

FIG. 3. **Northern blot analysis of tissue from *A3AR*$^{-/-}$ and *A3AR*$^{+/+}$ mice for A_1, A_{2A}, and A_{2B} adenosine receptor subtypes.** *A_1 panel* contains 5 μg of brain poly(A)$^+$ RNA. *A_{2A}* and *A_{2B} panels* contain 15 μg of bone marrow-derived mast cell total RNA. Blots were stripped and reprobed for β-actin.

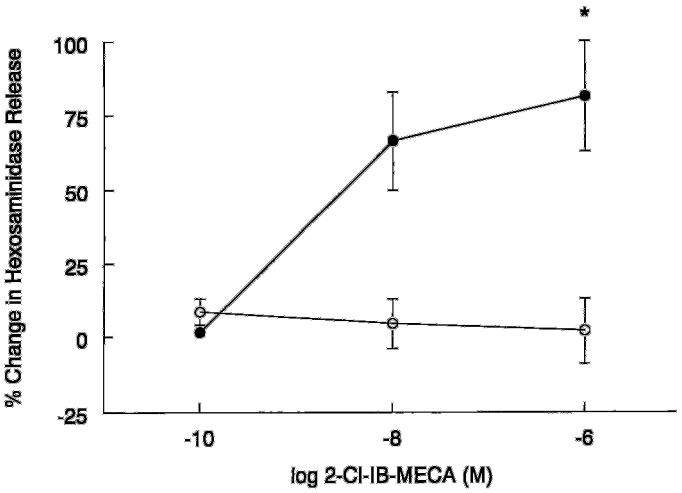

FIG. 4. **2-Cl-IB-MECA-stimulated secretion of hexosaminidase in BMMCs from *A3AR*$^{-/-}$ mice (○) and *A3AR*$^{+/+}$ (●) littermate controls.** Antigen-induced secretion without agonist averaged 24% of total cell hexosaminidase. Mean % increase with 2-Cl-IB-MECA ± S.E. from two experiments performed in duplicate is depicted.

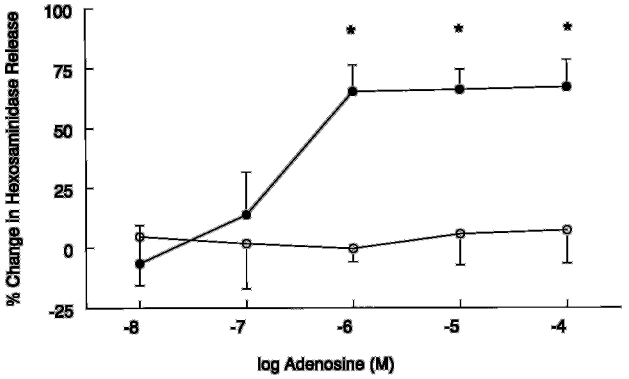

FIG. 5. **Adenosine-stimulated secretion of hexosaminidase in BMMCs from *A3AR*$^{-/-}$ mice (○) and *A3AR*$^{+/+}$ (●) littermate controls.** Mean percent increase in hexosaminidase release in response to adenosine ± S.E. from 3 experiments performed in duplicate is depicted. *, significantly greater hexosaminidase release than from cells challenged with antigen alone ($p < 0.05$).

Mice—Adenosine has been shown to potentiate mediator release murine from BMMCs after stimulation with antigen or calcium ionophore (33). The A_{2A} adenosine receptor agonist, CGS21680, failed to enhance mediator release from BMMCs, and it was postulated that the A_{2B} receptor and/or another receptor subtype modulated the secretory process. To determine which adenosine receptor subtype mediates the response in BMMCs, degranulation experiments were carried out in BMMCs from *A3AR*$^{-/-}$ and *A3AR*$^{+/+}$ mice. The ability of a selective A3AR agonist, 2-chloro-*N*^{6}-(3-iodobenzyl)-adenosine-5′-*N*-methyl-carboxamide (2-Cl-IB-MECA), to potentiate mast cell degranulation was evaluated on antigen-stimulated BMMCs isolated from *A3AR*$^{-/-}$ and *A3AR*$^{+/+}$ mice. BMMCs were preincubated with varying concentrations of 2-Cl-IB-MECA (0.1 nM to 1 μM) prior to antigen challenge. BMMCs from wild type mice demonstrated a dose-dependent increase in hexosaminidase release (81.5 ± 11.5% at 1 μM, $p = 0.05$), whereas BMMCs from *A3AR*$^{-/-}$ mice showed no change over antigen alone (Fig. 4). In comparison BMMCs from *A3AR*$^{-/-}$ mice exhibited no difference to wild type mice in the potentiation of antigen-induced degranulation by prostanoid prostaglandin E_2 (115 ± 35 and 113 ± 28%, respectively). To determine whether activation of the A_{2A} or A_{2B} adenosine receptor expressed on BMMCs could also potentiate mast cell degranulation, BMMCs from *A3AR*$^{+/+}$ and *A3AR*$^{-/-}$ mice were preincubated with varying concentrations of adenosine prior to antigen challenge. BMMCs from wild type mice displayed a dose-dependent potentiation of hexosaminidase release with adenosine preincubation, whereas *A3AR*$^{-/-}$ mice showed no potentiation over antigen alone (Fig. 5). Mean percent hexosaminidase release with antigen alone in *A3AR*$^{+/+}$ and *A3AR*$^{-/-}$ was 27 ± 3 and 27 ± 5%, respectively. Preincubation with 1×10^{-4} M adenosine prior to antigen challenge resulted in an increase in hexosaminidase release by 67.3 ± 11% ($p = 0.028$) in wild type mice, whereas *A3AR*$^{-/-}$ mice showed no change (28 ± 5%) over antigen alone. These results support the key role of A3AR activation in enhancing mast cell mediator release.

Inhibition of TNFα Production in A3AR$^{+/+}$ *and A3AR*$^{-/-}$ *Mice*—Adenosine has been shown to inhibit production of TNFα from stimulated J774.1 murine macrophage-like cells (16) and from the human cell line U937 (17). These reports have suggested that the A3AR mediates the response to adenosine as the inhibition of TNFα in response to adenosine receptor agonists exhibited an affinity order profile similar to the cloned rat A_3 receptor. The involvement of the A3AR in the inhibition of TNFα production from LPS-stimulated inflammatory cells was evaluated *in vivo* in *A3AR*$^{-/-}$ mice (Fig. 6). Mice injected with vehicle before LPS stimulation had TNFα concentrations of 10.58 ± 1.7 ng/ml ($n = 15$) and 10.50 ± 2.02 ng/ml ($n = 14$) for *A3AR*$^{+/+}$ and *A3AR*$^{-/-}$ mice, respectively. Intravenous administration of 50 μg/kg 2-Cl-IB-MECA immediately before LPS injection prevented elevations in plasma TNFα concentrations in *A3AR*$^{+/+}$ mice (2.21 ± 0.51 ng/ml, $n = 15$) but

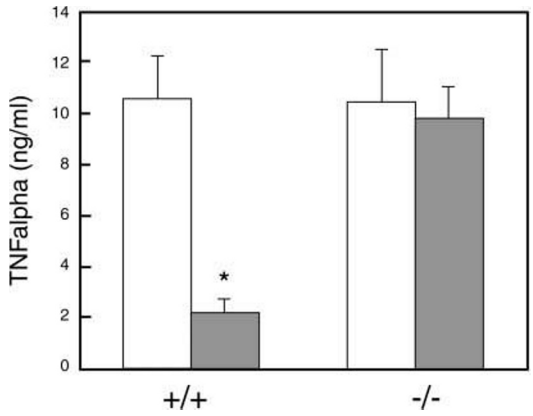

FIG. 6. **Effect of administration of 2-Cl-IB-MECA on LPS-induced plasma elevations in *A3AR*$^{+/+}$ (+/+) and *A3AR*$^{-/-}$ (−/−) mice.** Mice were injected intravenously with vehicle (*open bars*) or 50 μg 2-Cl-IB-MECA (*shaded bars*) immediately following intraperitoneal injection of LPS/D-Gal. TNFα levels were determined in plasma samples from blood obtained 90 min later. *, $p < 0.001$ compared with vehicle control group.

not in *A3AR*$^{-/-}$ mice (9.8 ± 1.23 ng/ml, $n = 15$). These results are in agreement with results reported from *in vitro* measurements in cultured mouse J774.1 and human U937 cells where A3AR activation was correlated with inhibition of LPS-stimulated TNFα production.

The effect of the A_{2A} adenosine receptor agonist, CGS21680, on inhibition of LPS-stimulated TNFα production was compared in A3AR$^{+/+}$ and A3AR$^{-/-}$ mice. In contrast to the observation with the A3AR agonist, 2Cl-IB-MECA, CGS21680 produced inhibition of LPS-stimulated TNFα production in both A3AR-deficient and wild type mice. Mice injected with vehicle before LPS stimulation had TNFα concentrations of 7.29 ± 1.02 ng/ml ($n = 8$) and 9.65 ± 1.15 ng/ml ($n = 12$) for *A3AR*$^{+/+}$ and *A3AR*$^{-/-}$ mice, respectively. Administration of 100 μg/kg CGS21680 immediately before LPS prevented elevations in plasma TNFα concentrations in both *A3AR*$^{+/+}$ (3.17 ± 0.54 ng/ml; $n = 8$) and *A3AR*$^{-/-}$ (4.35 ± 0.50 ng/ml; $n = 10$) mice. These results are comparable to those measured in BALB/c mice where CGS2180 produced inhibition of LPS-stimulated TNFα production *in vivo* (34). The results obtained in the A3AR$^{+/+}$ and A3AR$^{-/-}$ mice with 2Cl-IB-MECA and CGS21680 suggest that inhibition of LPS-stimulated TNFα production is mediated via multiple adenosine receptor subtypes, specifically the A_3 and A_{2A} adenosine receptor subtypes.

DISCUSSION

In this study, mice deficient in the expression of A3AR were generated. The *A3AR*$^{-/-}$ mice were determined to be healthy and fertile and exhibited no differences in the development of any organ systems. In addition, the expression of the other adenosine receptor subtypes, A_1, A_{2A}, and A_{2B}, was not affected by the ablation of the *A3AR* gene. The physiological consequence of the loss of A3AR expression was evaluated by measuring the response of antigen-stimulated BMMC degranulation to adenosine and inhibition of LPS-stimulated TNFα production *in vivo* in *A3AR*$^{-/-}$ and *A3AR*$^{+/+}$ mice. Our results support the essential role of the A3AR as a mediator in these processes as both functions were found to be eliminated in mice lacking A3AR. The ablation of adenosine or 2-Cl-IB-MECA potentiation of antigen-stimulated BMMC degranulation and decrease in 2-Cl-IB-MECA inhibition of LPS-stimulated TNFα production are the first phenotypic differences to be reported for the *A3AR*$^{-/-}$ mice.

Adenosine has been reported to potentiate mediator release from multiple types of mast cells including human lung mast cells (35), rat mast cells (36), murine BMMCs (33), and the rat basophilic leukemic cell line, RBL-2H3 (11, 37), after stimulation with antigen or calcium ionophore. Adenosine has been implicated to contribute to the pathophysiology of asthma as inhaled adenosine provokes bronchoconstriction in asthmatic but not normal patients (38). The response to adenosine is believed to be predominantly mediated through mast cell activation (39). Attempts to elucidate definitively the adenosine receptor subtype that mediates mast cell activation has been pharmacologically difficult due to lack of agonists and antagonists that display sufficient selectivity to discriminate between multiple adenosine receptor subtypes present on these cells. Recent studies employing the rat mast cell-derived basophilic leukemia cell line, RBL-2H3, a model for mast cells, have suggested that the A3AR is the adenosine receptor subtype that mediates mast cell degranulation.

The relationship between A3AR activation and mast cell degranulation was first postulated by the detection of A3AR on RBL-2H3 cells, using the A3AR agonist ^{125}I-labeled aminophenylethyladenosine N^6-2-(4-aminophenyl) (APNEA) (11). It was further shown that enhancement of antigen-induced secretion from RBL-2H3 cells correlated with an affinity order profile for adenosine receptor agonists similar to that established for the cloned rat A3AR (11). Additional support for the A3AR role in enhancement of antigen-induced degranulation of RBL-2H3 cells came from the observation that dexamethasone increased the amount of ^{125}I-APNEA binding, possibly due to an increase in A3AR expression, and the A3AR-dependent release of hexosaminidase (37).

A correlation between A3AR activation and nonimmunologically dependent mast cell mediator release has recently been demonstrated *in vivo*. The hypotensive response to A3AR activation by APNEA in the anesthetized rat is qualitatively similar to the mast cell degranulation agent compound 48/80. Additionally, the hypotensive response to APNEA was suppressed by mast cell degranulation inhibitors, and depletion of mast cell mediators with compound 48/80 decreased the effectiveness of APNEA-induced hypotension (14). Van Schaick *et al.* (40) have shown that intravenous administration of the A_3 agonist, 2-Cl-IB-MECA, in conscious rats resulted in a hypotensive response coincident with an increase in plasma histamine concentrations indicating the involvement of the A3AR in mediator release from mast cells *in vivo*.

In this report, we demonstrate high levels of A3AR expression on murine BMMCs. This is the first report of A3AR expression on native mast cells in contrast to established cell lines such as RBL-2H3 cells. In BMMCs from *A3AR*$^{-/-}$ mice, the potentiation of antigen-dependent degranulation by either adenosine or the A3AR agonist, 2-Cl-IB-MECA, was found to be eliminated. The observation of the absence of adenosine or 2-Cl-IB-MECA potentiation of BMMC degranulation in *A3AR*$^{-/-}$ mice supports the conclusions drawn from previous studies with RBL-2H3 cells and in rats *in vivo* in which the A3AR was implicated in mediating mast cell degranulation.

Previous reports have implicated the A_{2B} adenosine receptor to mediate BMMC degranulation through demonstration of an inability of pertussis toxin to affect adenosine-induced calcium mobilization (41). In other species, specifically, canine and human, the A_{2B} receptor has also been reported to mediate mast cell degranulation (42–44). This discrepancy in identifying which adenosine receptor subtype mediates mast cell degranulation may be due to a number of variables including differences in the levels of adenosine receptor subtype expression between cells selected for evaluation, the tissue source for mast cells, species differences or whether mast cells are immunologically primed prior to degranulation measurements. For exam-

ple, RBL-2H3 cells and mouse BMMCs express high levels of A3ARs as measured by both transcript expression and radiolabeled binding. BMMCs are most similar phenotypically to the rat RBL-2H3 tumor cell line in which the A3AR has been reported to predominate the potentiation of mast cell mediator release. In contrast, dog BR mastocytoma cells express low levels of A3ARs. Degranulation of non-immunologically stimulated BR mastocytoma cells in response to adenosine receptor agonists was found to exhibit an affinity order profile most consistent with the A_{2B} adenosine receptor (42). In the human mast cell line, HMC-1, A_{2B} receptor activation has been shown to couple to mobilization of intracellular calcium and interleukin-8 secretion (43, 44). The adenosine receptor subtype that mediates these responses has not been defined on HMC-1 cells and warrants further study with selective agonists, such as 2Cl-IB-MECA, to determine if the A3AR is involved as we have shown in the mouse. Recently, it has been reported that calcium mobilization in HMC-1 cells is stimulated with a potency order of 5′-*N*-ethylcarboxamidoadenosine > N^6-(2-iodo)benzyl-5′-*N*-methylcarboxamidoadenosine (IB-MECA) consistent with activation of A_{2B} receptors (45). It is important to note that native human mast cells have not been evaluated for A3AR-mediated responses.

In this report, we also evaluated the potential role of A3AR activation in the inhibition of LPS-stimulated TNFα production through a comparison of the responses of $A3AR^{+/+}$ and $A3AR^{-/-}$ mice. A number of published reports have linked A3AR activation with inhibition of LPS-stimulated TNFα production *in vitro* from cultured human monocytes and mouse and human macrophage-like cell lines. The possible involvement of A3AR was first postulated when adenosine receptor agonists were shown to inhibit TNFα production in endotoxin-stimulated human monocytes with a pharmacological profile uncharacteristic of either A_1 or A_{2A} adenosine receptor subtypes (46). In J774.1 cells, a murine macrophage-like cell line, LPS-induced TNFα secretion and TNFα gene expression were inhibited by adenosine receptor agonists with an affinity order profile similar to the cloned rat A_3 receptor (16). In human U937 cells, micromolar concentrations of the A_3 agonist, IB-MECA, decreased LPS-stimulated TNFα production, and it was concluded that A3AR activation mediated this response (17). Recently, inhibition of LPS-stimulated TNFα production by high concentrations (1 μM) IB-MECA was confirmed on human monocytes; however, the inhibition could be completely blocked with an A_{2A} adenosine receptor-selective antagonist indicating that IB-MECA was acting via A_{2A} and not A_3 receptor activation (47). The availability of the $A3AR^{-/-}$ mouse provided the opportunity to evaluate specific receptor activation *in vivo*. Our results suggest that activation of either A_3 or A_{2A} adenosine receptors can mediate inhibition of LPS-stimulated TNFα production *in vivo* in mice. These results are consistent with published reports obtained with murine cell lines where A3AR activation has been implicated to mediate the inhibition of LPS-stimulated TNFα production (16) and with *in vivo* studies in BALB/c mice where A_{2A} receptor activation was demonstrated to mediate inhibition of TNFα production (34).

In summary, our characterization of the $A3AR^{-/-}$ mouse has shown that A3AR activation can produce both pro-inflammatory and anti-inflammatory effects. Specifically, we have demonstrated that the A3AR is the adenosine receptor subtype mediating potentiation of antigen-induced degranulation in murine BMMCs. We have also shown that A_3 and A_{2A} receptor activation inhibits LPS-stimulated increases in TNFα *in vivo*. Further characterization of the $A3AR^{-/-}$ mouse may provide important information about the functional role of the A3AR in more complex inflammatory responses.

Acknowledgment—We thank Ann Scott for performing the hemodynamic measurements.

REFERENCES

1. Bruns, R. F., Lu, G. H., and Pugsley, T. A. (1986) *Mol. Pharmacol.* **29,** 331–346
2. Jacobson, K. A., van Galen, P. J. M., and Williams, M. (1992) *J. Med. Chem.* **35,** 407–422
3. Linden, J. (1994) *Trends Pharmacol. Sci.* **15,** 298–306
4. Meyerhof, W., Muller-Brechlin, R., and Richter, D. (1991) *FEBS Lett.* **284,** 155–160
5. Zhou, Q.-Y., Li, C., Olah, M. E., Johnson, R. A., Stiles, G. L., and Civelli, O. (1992) *Proc. Natl. Acad. Sci. U. S. A.* **89,** 7432–7436
6. Sajjadi, F. G., and Firestein, G. S. (1993) *Biochim. Biophys. Acta* **1179,** 105–107
7. Salvatore, C. A., Jacobson, M. A., Taylor, H. E., Linden, J., and Johnson, R. G. (1993) *Proc. Natl. Acad. Sci. U. S. A.* **90,** 10365–10369
8. Linden, J., Taylor, H. E., Robeva, A. S., Tucker, A. L., Stehle, J. H., Rivkees, S. A., Fink, J. S., and Reppert, S. M. (1993) *Mol. Pharmacol.* **44,** 524–532
9. Auchampach, J. A., Jin, X., Wan, T. C., Caughey, G. H., and Linden, J. (1997) *Mol. Pharmacol.* **52,** 846–860
10. Hill, R. J., Oleynek, J. J., Hoth, C. F., Ravi Kiron, M. A., Weng, W., Webster, R. T., Tracey, W. R., Knight, D. R., Buchholz, R. A., and Kennedy, S. P. (1997) *J. Pharmacol. Exp. Ther.* **280,** 122–128
11. Ramkumar, V., Stiles, G. L., Beaven, M. A., and Ali, H. (1993) *J. Biol. Chem.* **268,** 16887–16890
12. Doyle, M. P., Linden, J., and Duling, B. R. (1994) *Am. J. Physiol.* **266,** H2042—H2050
13. Fozard, J. R., and Carruthers, A. M. (1993) *Br. J. Pharmacol.* **109,** 3–5
14. Hannon, J. P., Pfannkuche, H. J., and Fozard, J. R. (1995) *Br. J. Pharmacol.* **115,** 945–952
15. Yao, Y., Sei, Y., Abbracchio, M. P., Jiang, J.-L., Kim, Y.-C., and Jacobson, K. A. (1997) *Biochem. Biophys. Res. Commun.* **232,** 317–322
16. Bowlin, T. L., Borcherding, D. R., Edwards, C. K., III, and McWhinney, C. D. (1997) *Cell. Mol. Biol.* **43,** 345–349
17. Sajjadi, F. G., Takabayashi, K., Foster, A. C., Domingo, R. C., and Firestein, G. S. (1996) *J. Immunol.* **156,** 3435–3442
18. Walker, B. A. M., Jacobson, M. A., Knight, D. A., Salvatore, C. A., Weir, T., Zhou, D., and Bai, T. R. (1997) *Am. J. Respir. Cell Mol. Biol.* **16,** 531–537
19. Von Lubitz, D. K., Lin, R. C. S., Popik, P., Carter, M. F., and Jacobson, K. A. (1994) *Eur. J. Pharmacol.* **263,** 59–67
20. Armstrong, S., and Ganote, C. E. (1995) *Cardiovasc. Res.* **29,** 647–652
21. Liang, B. T., and Jacobson, K. A. (1998) *Proc. Natl. Acad. Sci. U. S. A.* **95,** 6995–6999
22. Jacobson, K. A., Nikodijevic, O., Shi, D., Gallo-Rodriguez, C., Olah, M. E., Stiles, G. L., and Daly, J. W. (1993) *FEBS Lett.* **336,** 57–60
23. Hooper, M., Hardy, K., Handyside, A., Hunter, S., and Monk, M. (1987) *Nature* **326,** 292–295
24. Dombrowicz, D., Flamand, V., Brignan, K., Koller, B., and Kinet, J. (1993) *Cell* **75,** 969–976
25. Miller, S. A., Dykes, D. D., and Polesky, H. F. (1988) *Nucleic Acids Res.* **16,** 1215
26. Rottem, M., Barbieri, S., Kinet, J. P., and Metcalfe, D. D. (1992) *Blood* **79,** 972–980
27. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual,* 2nd Ed., pp. 7.43–7.45, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
28. Terai, T., Kita, Y., Kusunoki, T., Shimazaki, T., Ando, T., Horiai, H., Akahane, A., and Yoshida, K. (1995) *Eur. J. Pharmacol.* **279,** 217–225
29. Schwartz, L. B., Austen, F. K., and Wasserman, S. I. (1979) *J. Immunol.* **123,** 1445–1450
30. Molineaux, S. M., Casano, F. J., Rolando, A. M., Peterson, E. P., Limjuco, G., Chin, J., Griffin, P. R., Calaycay, J. R., Ding, G. J., and Yamin, T. T. (1993) *Proc. Natl. Acad. Sci. U. S. A.* **90,** 1809–1813
31. Linden, J., Patel, A., and Sadek, S. (1985) *Circ. Res.* **56,** 279–284
32. Bellardinelli, L., Linden, J., and Berne, R. (1989) *Prog. Cardiovasc. Dis.* **32,** 73–97
33. Marquardt, D. L., Walker, L. L., and Heinemann, S. (1994) *J. Immunol.* **152,** 4508–4515
34. Hasko, G., Szabo, C., Nemeth, Z. H., Kvetan, V., Pastores, S. M., and Vizi, E. S. (1996) *J. Immunol.* **157,** 4634–4640
35. Peachell, P. T., Columbo, M., Kagey-Sobotka, A., Lichtenstein, L. M., and Marone, G. (1988) *Am. Rev. Respir. Dis.* **138,** 1143–1151
36. Marquardt, D. L., Parker, C. W., and Sullivan, T. J. (1978) *J. Immunol.* **120,** 871–878
37. Ramkumar, V., Wilson, M., Dhanraj, D. N., Gettys, T. W., and Ali, H. (1995) *J. Immunol.* **154,** 5436–5443
38. Cushley, M. J., Tattersfield, A. E., and Holgate, S. T. (1984) *Am. Rev. Respir. Dis.* **129,** 380–384
39. Rafferty, P., Beasley, C. R., and Holgate, S. T. (1987) *Am. Rev. Respir. Dis.* **136,** 369–373
40. Van Schaick, Jacobson, K. A., Kim, H. O., Ijzerman, A. P., and Danhof, M. (1996) *Eur. J. Pharmacol.* **308,** 311–314
41. Marquardt, D. L., and Walker, L. L. (1988) *Biochem. Pharmacol.* **37,** 4019–4025
42. Auchampach, J. A., Jin, X., Wan, T. C., Caughey, G. H., and Linden, J. (1997) *Mol. Pharmacol.* **52,** 846–860
43. Feoktiskov, I., and Biaggioni, I. (1995) *J. Clin. Invest.* **96,** 1979–1986
44. Feoktiskov, I., and Biaggioni, I. (1998) *Biochem. Pharmacol.* **55,** 627–633
45. Linden, J., Thai, T., Figler, H., Jin, X., and Robeva, A. S. (1999) *Mol. Pharmacol.* **56,** 705–713
46. LeVraux, V., Chen, Y. L., Masson, I., DeSousa, M., Giroud, J. P., Florentin, I., and Chauvelot-Moachon, L. (1993) *Life Sci.* **52,** 1917–1924
47. Sullivan, G. W., and Linden, J. (1998) *Drug Dev. Res.* **45,** 103–112