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A_3 Adenosine Receptor Activation Triggers Phosphorylation of Protein Kinase B and Protects Rat Basophilic Leukemia 2H3 Mast Cells from Apoptosis

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ABSTRACT

Adenosine accumulates to high levels in inflamed or ischemic tissues and activates A_3 adenosine receptors (ARs) on mast cells to trigger degranulation. Here we show that stimulation of rat basophilic leukemia (RBL)-2H3 mast-like cells with the A_3 AR agonists N^6-(3-iodo)benzyl-5′-*N*-methylcarboxamidodoadenosine (IB-MECA; 10 nM) or inosine (10 μM) stimulates phosphorylation of protein kinase B (Akt). IB-MECA (1 μM) also causes a >50% reduction in apoptosis caused by exposure of RBL-2H3 cells to UV light. Akt phosphorylation is not stimulated by 100 nM N^6-cyclopentyladenosine (A_1-selective) or CGS21680 (A_{2A}-selective) and is absent in cells pretreated with wortmannin or pertussis toxin. The K_i values of the AR antagonists BW-1433 and 8-sulfophenyltheophylline (8-SPT) were determined in radioligand binding assays for all four subtypes of rat ARs: BW-1433 (A_1, 5.8 ± 1.0 nM; A_{2A}, 240 ± 37; A_{2B}, 30 ± 10; A_3, 12,300 ± 3,700); 8-SPT (A_1, 3.2 ± 1.2 μM; A_{2A}, 57 ± 4; A_{2B}, 2.2 ± 0.8; A_3, >100). BW-1433 and the A_3-slective antagonist MRS1523 (5 μM), but not 8-SPT (100 μM), block IB-MECA-induced protection from apoptosis, confirming the A_3 AR as the mediator of the antiapoptotic response. The data suggest that adenosine and inosine activate Gi-coupled A_3 ARs to protect mast cells from apoptosis by a pathway involving the $\beta\gamma$ subunits of Gi, phosphatidylinositol 3-kinase β, and Akt. We speculate that activation of A_3 ARs on mast cells or other cells that express A_3 ARs (e.g., eosinophils) may facilitate their survival and accumulation in inflamed tissues.

Activation of the serine/threonine kinase, Akt, also called protein kinase B (PKB) inhibits programmed cell death (Chan et al., 1999; Kandel et al., 1999; Stambolic et al., 1999). Phosphoinositides generated by activated phosphatidylinositol 3-kinases (PI3Ks) bind to the plextrin homology domain on Akt and stimulate its translocation to the plasma membrane, where it is phosphorylated on both Ser473 and Thr308 and activated by phosphoinositide-dependent kinase-1 (Chan et al., 1999). PI3Ks can be activated by tyrosine kinase growth factor receptors, or in some cells by activation of G protein-coupled receptors. Activation of PI3K via heterotrimeric G proteins occurs selectively in cells that express PI3Kβ (Murga et al., 2000). Hence, it is possible that Akt is involved in the regulation of apoptosis that has been noted in astrocytes and cardiomyocytes by G protein-coupled A_3 ARs (Jacobson, 1998). The A_3 AR is known to regulate the degranulation of rodent perivascular mast cells (Jin et al., 1997) and RBL-2H3 mast-like cultured cells (Ramkumar et al., 1993). Activation of mast cell A_3 ARs increases mast cell degranulation to release histamine and other allergic mediators. This prompted us to determine in this study whether A_3 AR activation protects RBL-2H3 mast cells from apoptosis. We show that IB-MECA, Cl-IB-MECA, and inosine signal via A_3 ARs to stimulate phosphorylation of Akt and to reduce RBL-2H3 cell apoptosis induced by UV light.

Experimental Procedures

Materials. CPA, 5′-*N*-ethylcarboxamidoadenosine, Cl-IB-MECA, CGS21680, 8-SPT, theophylline, and enprofylline were purchased

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ABBREVIATIONS: Akt or PKB, protein kinase B; PI3K, phosphatidylinositol 3-kinases; AR, adenosine receptor; RBL, rat basophilic leukemia; IB-MECA, N^6-(3-iodobenzyl)-5′-*N*-methylcarboxamidoadenosine; CPA, N^6-cyclopentyladenosine; CGS21680, 2-*p*-(2-carboxyethyl)phenethylamino-5′-ethylcarboxaminoadenosine; 8-SPT, 8-sulfophenyltheophylline; enprofylline, 3-propylxanthine; MRS1523, 5-propyl 2-ethyl-4-propyl-3-(ethylsulfanylcarbonyl)-6-phenylpyridine-5-carboxylate; WRC-0571, C^8-(*N*-methylisopropyl)-amino-N^6-(5-endohydroxy)-endonorbornan-2-yl-9-methyladenine; BW-1433, 8-(4-carboxyethenylphenyl)-1,3-dipropylxanthine; ABA, N^6-aminobenzyladenosine; HEK, human embryonic kidney; HE, HEPES/EDTA; ZM241385, 4-(2-[7-amino-2-[2-furyl][1,2,4]triazolo[2,3-*a*][1,3,5]triazin-5-yl-amino]ethyl)phenol; TUNEL, terminal deoxynucleotidyl-transferase-mediated dUTP-fluorescein isothiocyanate nick end labeling; DMSO, dimethylsulfoxide.

from RBI/Sigma (Natick, MA). MRS1523 was a gift from Dr. K.A. Jacobson (National Institutes of Health, Bethesda, MD). IB-MECA was obtained from Dr. Saul Kadin (Pfizer, Groton, CT), and WRC-0571 was obtained from Dr. Pauline Martin (Discovery Therapeutics, Richmond, VA). BW-1433, 3-(4-amino-3-iodobenzyl)-8-oxyacetate-1-propyl-xanthine and N^6-aminobenzyladenosine (ABA) were from Dr. Susan Daluge (Glaxo-Wellcome, Research Triangle Park, NC). Wortmannin was from Calbiochem (San Diego, CA); pertussis toxin was from Sigma Chemical Co. (St. Louis, MO); adenosine deaminase was from Boehringer-Mannheim Biochemicals (Indianapolis, IN); cell culture medium was from Life Technologies (Gaithersburg, MD); phospho-AKT (Ser473 or Thr308) antibodies were from New England BioLabs (Beverly, MA); and polyclonal anti-AKT antibody was a gift from Dr. John C. Lawrence (University of Virginia, Charlottesville, VA).

Cell Culture. Rat basophilic leukemia 2H3 clonal cells (RBL-2H3) were from Dr. R.P. Siraganian (National Institutes of Health, Bethesda, MD) and were grown as monolayers in Eagle's minimum essential medium with Earle's balanced salts without glutamine, supplemented with 10% fetal calf serum, 200 mM glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, 50 μg/ml gentamicin, and 25 μg/ml fungizone. Cells were subcultured every 3 days.

Radioligand Binding Studies. Rat A_{2B} receptor cDNA was prepared by reverse transcription polymerase chain reaction from rat bladder and sequenced on both strands in the University of Virginia Biomolecular core laboratory. The A_{2B} cDNA was subcloned into pDoubleTrouble (Robeva et al., 1996). The plasmids were amplified in competent JM109 cells and plasmid DNA isolated using Wizard Megaprep columns (Promega Corporation, Madison, WI). Rat A_{2B} and A_3 cDNAs were introduced into HEK-293 cells by means of Lipofectin. Stable clones were selected using 500 μg/ml G418 (Life Technologies) and maintained in 250 μg/ml G418. HEK-293T cells were transiently transfected with rat A_{2A} ARs. HEK-293 cells expressing recombinant receptors or rat cortex (a rich source of A_1 receptors) were homogenized in HE buffer (10 mM HEPES, 1 mM EDTA, pH 7.4) with protease inhibitors (10 μg/ml benzamidine, 100 μM phenylmethylsulfonyl fluoride, and 2 μg/ml each of aprotinin, pepstatin, and leupeptin). The membranes were homogenized in a Polytron Homogenizer (Brinkmann Instruments, Westbury, NY) for 20 s, centrifuged at 30,000*g*, and the pellets washed twice in HE buffer with protease inhibitors. The final pellet was resuspended in HE supplemented with 10% sucrose and frozen in aliquots at −80°C. For binding assays, membranes were thawed and diluted 5- to 10-fold with HE to a final membrane protein concentration of approximately 1 mg/ml.

Saturation binding assays for rat adenosine receptors were performed, respectively, with ^{125}I-ABA for A_1 and A_3 ARs (Linden et al., 1985; Salvatore et al., 1993), ^{125}I-ZM241385 for A_{2A} AR (Sullivan et al., 1999), and [^{125}I]3-(4-amino-3-iodobenzyl)-8-oxyacetate-1-propyl-xanthine for A_{2B} AR (Linden et al., 1999). Nonspecific binding was defined using 100 μM 5′-*N*-ethylcarboxamidoadenosine. Radioligand binding experiments were performed in triplicate with 10 to 25 μg of membrane protein in a total volume of 0.1 ml HE buffer supplemented with 1 U/ml adenosine deaminase and 5 mM $MgCl_2$. The incubation time was 3 h at 21°C. Competition experiments were carried out using 0.5 to 1 nM radioligands and seven concentrations of competing ligands. Membranes were filtered on Whatman GF/C filters using a Brandel cell harvester (Gaithersburg, MD) and washed three times over 15 to 20 s with ice-cold buffer (10 mM Tris, 1 mM $MgCl_2$, pH 7.4). K_I values were derived from IC_{50} values of competition binding assays as described previously (Linden, 1982).

Akt/PKB Phosphorylation Assay. RBL-2H3 cells were serum-starved for 18 h and AKT activation assays were carried out on monolayers of cells in serum-free Eagle's minimal essential medium in a 37°C, 5% CO_2 incubator. The reactions were terminated by placing the cells on ice and washing them with ice-cold PBS. Cells were then lysed in Triton lysis buffer [50 mM Tris·HCl, pH 7.5, 100 mM NaCl, 50 mM NaF, 5 mM EDTA, 1% (v/v) Triton X-100, 40 mM β-glycerophosphate, 40 mM paranitrophenylphosphate, 200 μM sodium orthovanadate, 100 μM phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin, 1 μg/ml pepstatin A, and 1 μg/ml aprotinin]. The lysate was mixed and clarified by centrifugation (15 min, 14,000 rpm, 4°C) in an Eppendorf microcentrifuge. The supernatant was subjected to SDS-polyacrylamide gel electrophoresis followed by transfer to nitrocellulose and immunoblotting. Phosphorylation and activation of PKB was detected by immunoblotting using rabbit polyclonal anti-phospho-Akt (Ser473 or Thr308) antibody and visualized by enhanced chemiluminescence with horseradish peroxidase conjugated goat anti-rabbit IgG as the secondary antibody (1:10,000 dilution). The membranes were then stripped by incubating in stripping buffer (62.5 mM Tris·HCl, 2% SDS, and 100 mM β-mercaptoethanol, pH 6.7 at 65°C) in a shaking water bath, and reprobed with polyclonal anti-PKB antibodies to quantify the total PKB loaded onto each lane.

Apoptosis Assay. Protection from UV-induced apoptosis was carried out essentially as described previously (Murga et al., 1998). Cells were starved overnight in Eagle's minimal essential medium containing 10 mM HEPES. Subsequently, starved cells were subjected to UV irradiation (150 mJ; UV-Stratalinker 2400; Stratagene, La Jolla, CA). After UV treatment, fresh serum-free medium with and without test compounds was added to cells and they were maintained in the incubator for an additional 10 h. The cells were fixed in 4% paraformaldehyde and apoptotic cells were detected by terminal deoxynucleotidyltransferase-mediated dUTP-fluorescein isothiocyanate nick end labeling (TUNEL), following the manufacturer's instructions (Boehringer Mannheim). The frequency of apoptosis was scored by counting several hundred positive TUNEL-stained cells from 25 different fields per coverslip. Results from four independent experiments are reported as the mean ± S.E.M.

Results

IB-MECA activates A_3 ARs to degranulate RBL-2H3 cells half-maximally at a concentration of 4.2 nM (Jin et al., 1997). To study the influence of A_3 AR activation on Akt phosphorylation, we initially used a concentration of IB-MECA (100 nM) sufficient to trigger maximal mast cell degranulation. Activation of Akt requires phosphorylation of both Ser473 in the C-terminal regulatory domain and Thr308 in the activation loop of the kinase domain. We initially used the specific anti-phospho(Ser473)-Akt antibody to study the regulation of Akt phosphorylation by A_3 AR. Subsequent studies using the specific anti-phospho(Thr308)-Akt antibody yielded essentially the same results. For simplicity, we report hereafter only the results from the experiments using anti-phospho(Ser473) antibody. IB-MECA significantly stimulated Akt phosphorylation within 2 min, and the response peaked in 5 min (Fig. 1A). The dose-dependence of IB-MECA to stimulate Akt phosphorylation at 5 min is shown in Fig. 1B. Maximal Akt phosphorylation was produced by 100 nM IB-MECA. Similar results were obtained with another A_3 AR-selective agonist, Cl-IB-MECA (data not shown). The addition of 100 nM CPA, an A_1 AR-selective agonist, or 100 nM CGS21680, an A_{2A} AR-selective agonist, failed to significantly stimulate Akt phosphorylation (Fig. 2A); inosine, however, a weak but selective agonist of rat A_3 ARs, did stimulate Akt phosphorylation in the range of 10 to 100 μM (Fig. 2B). Xanthines are weak antagonists of rodent A_3 ARs. However, the nonselective xanthine antagonist BW-1433 blocks the rat A_3 AR with a K_I value of approximately 20 μM (Jin et al., 1997). As shown in Fig. 2, B and C, BW-1433 reduced IB-MECA-stimulated Akt phosphorylation in a dose-dependent manner, but concentrations of WRC-0571, ZM241385, theophylline, enprofylline, and 8-SPT sufficient to block A_1, A_{2A},

or A_{2B} receptors singly or in combination failed to block Akt phosphorylation (Fig. 2B). Furthermore, a selective antagonist of the rat A_3 receptor, MRS1523 (5 μM) also blocked IB-MECA-induced Akt phosphorylation. Therefore, we conclude that the A_3 AR subtype is responsible for IB-MECA-mediated stimulation of Akt phosphorylation in RBL-2H3 cells.

We next sought to examine the signaling pathway by which A_3 AR activation triggers Akt phosphorylation. Pretreatment of RBL-2H3 cells with 100 ng/ml pertussis toxin for 6 h nearly abolished IB-MECA-induced Akt phosphorylation (Fig. 3A). Pretreatment of cells for 30 min with 10 nM wortmannin, an inhibitor of PI3Ks, abolished IB-MECA-induced Akt phosphorylation (Fig. 3B). These data suggest that the A_3 AR signals through a pathway including Gi/o and PI3K. To determine whether A_3 AR activation inhibits programmed cell death in RBL-2H3 cells, apoptosis was stimulated by exposing cells to UV light. As shown in Fig. 4, 100 nM IB-MECA failed to significantly reduce UV light-induced apoptosis, but 1 or 10 μM IB-MECA reduced apoptosis by more than 50%. Comparable results were also obtained with Cl-IB-MECA (data not shown). At concentrations $\leq$ 10 μM, IB-MECA alone (in the absence of UV treatment) did not cause any change in apoptosis in RBL-2H3 cells. However, 100 μM IB-MECA did induce apoptosis (20~25% above control) in RBL-2H3 cells by unknown mechanisms (data not shown). This is consistent with the data published previously showing that high concentrations of IB-MECA (>10 μM) induce apoptosis in various cell types (Jacobson, 1998; Shneyvays et al., 1998). The greater potency of IB-MECA in stimulating Akt phosphorylation (Fig. 1B) than in inhibiting apoptosis (Fig. 4) may be related to the time courses of the two assays, (minutes for the phosphorylation assay versus hours for the apoptosis assay) because A_3 receptors have been shown to undergo rapid desensitization (Palmer and Stiles, 2000). Alternatively, activation of Akt alone may not be sufficient to inhibit apoptosis, and higher concentrations of IB-MECA may activate additional anti-apoptotic pathways.

To ensure that the protection from apoptosis caused by IB-MECA is mediated by A_3 ARs, we compared the effects of BW-1433, a xanthine that has been reported to block all rat ARs including the A_3 AR (Jin et al., 1997), with 8-SPT, a xanthine that is thought to block all subtypes of rat ARs except A_3 (Fozard et al., 1996). As shown in Fig. 5A and B, BW-1433 (100 μM) but not 8-SPT (100 μM) reversed the ability of 10 μM IB-MECA to reduce apoptosis, suggesting

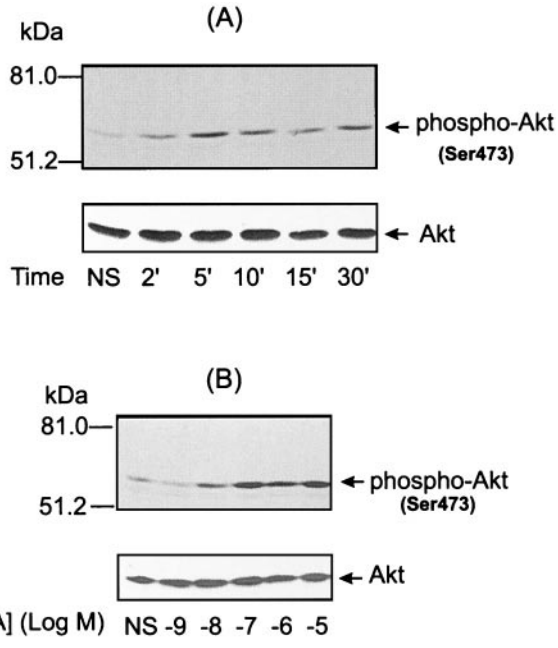

Fig. 1. Time course and dose-dependence of IB-MECA-induced AKT phosphorylation in RBL-2H3 cells. A, serum-starved RBL-2H3 cells were incubated at 37°C with DMSO vehicle (NS, not stimulated) or 100 nM IB-MECA for the times indicated. AKT phosphorylation (top) and total AKT (bottom) was determined by immunoblotting. B, serum-starved RBL-2H3 cells were stimulated for 5 min with either vehicle or various concentrations of IB-MECA before determination of AKT phosphorylation. The data are representative of triplicate experiments.

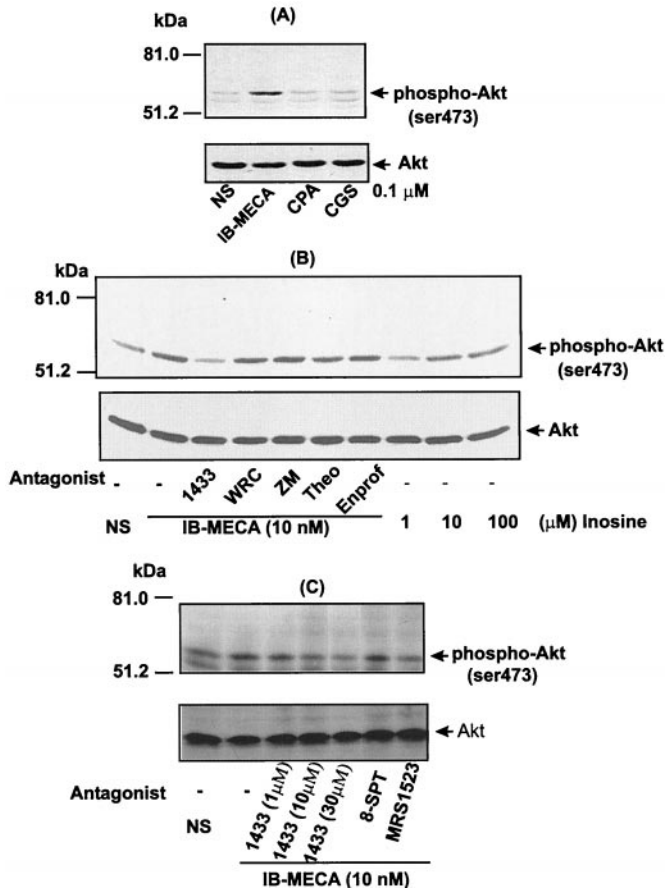

Fig. 2. IB-MECA-stimulated AKT phosphorylation in RBL-2H3 cells is mediated by A_3 ARs. A, AKT phosphorylation in response to various AR agonists. Serum-starved RBL-2H3 cells were stimulated for 5 min with either vehicle (DMSO), or 100 nM AR subtype-selective agonists: CPA (A_1AR), CGS21680 (A_{2A}AR), or IB-MECA (A_3AR) before the determination of AKT phosphorylation. B, AKT phosphorylation is stimulated by inosine and blocked by BW-1433 (30 μM) but is not blocked by theophylline (100 μM), enprofylline (100 μM), WRC-0571 (1 μM, A_1AR selective), or ZM241385 (100 nM, A_{2A}AR selective). Serum-starved RBL-2H3 cells were treated with 10 nM IB-MECA in the absence or presence of various AR antagonists or inosine of increasing concentrations as indicated in the figure before the determination of AKT phosphorylation by immunoblotting. C, effects of increasing concentrations (1–30 μM) of BW-1433, 20 μM 8-SPT, and 5 μM MRS1523 on IB-MECA (10 nM) induced-Akt phosphorylation. Serum-starved RBL-2H3 cells were treated with 10 nM IB-MECA in the absence or presence of various AR antagonists as indicated in the figure before the determination of AKT phosphorylation by immunoblotting. The data are representative of triplicate experiments with similar results.

the involvement of A_3 ARs in the IB-MECA-mediated antiapoptotic effect. To further confirm this pharmacology, we determined the K_I values of these two xanthines for the four subtypes of rat adenosine receptors (Table 1). The data confirm that at 100 μM BW-1433 effectively blocks all four subtypes, whereas 100 μM 8-SPT blocks rat A_1, A_{2A}, and A_{2B}, but only weakly blocks rat A_3 ARs. Furthermore, the effect of 10 μM IB-MECA in reducing apoptosis was also blocked by the A_3-selective antagonist MRS1523 in a dose-dependent manner (Fig. 5C). In radioligand binding studies (Fig. 6), we determined that the affinity of MRS1523 for rat A_3 AR is 519 $\pm$ 86 nM (mean $\pm$ S.E.M., N = 6). This K_I value is somewhat higher (~5-fold) than the K_I value (113 nM) reported by Li et al. (1998), but it is consistent with our observation that 5 μM MRS1523 almost completely blocked the effect of IB-MECA on apoptosis in RBL-2H3 cells, whereas 1 μM MRS1523 had only a small effect. Collectively, these data suggest that the antiapoptotic effect of IB-MECA is mediated by A_3 ARs.

We evaluated the effect of wortmannin on the antiapoptotic effect of IB-MECA. As is the case for IB-MECA-induced Akt phosphorylation, the IB-MECA-induced inhibition of apoptosis is reversed by pretreatment of RBL-2H3 cells with 10 or 50 nM wortmannin (Fig. 5 C). This suggests that PI3K is involved in A_3AR-mediated inhibition of apoptosis in RBL-2H3 cells.

Discussion

Adenosine and various adenosine analogs have been reported to both stimulate and inhibit apoptosis in various cells (Jacobson, 1998). In human mononuclear cells, apoptosis is stimulated by adenosine analogs through activation of A_{2A} or A_3 receptors or by non-receptor-mediated effects of intracellular nucleosides (Barbieri et al., 1998). In cultured newborn rat cardiomyocytes, high concentrations of IB-MECA trigger apoptosis (Jacobson, 1998; Shneyvays et al., 1998). In cells of astroglial lineage, apoptosis is stimulated by high concentrations of IB-MECA and inhibited by low concentrations (Franceschi et al., 1996). In this study, we set out to investigate possible A_3 AR-mediated effects on programmed cell death in RBL 2H3 cells, a line in which the existence of functional A_3 ARs has been firmly established (Ramkumar et al., 1993; Jin et al., 1997). We also investigated the signaling pathway through which apoptosis is regulated in these cells.

Pharmacology of Adenosine Receptors to Inhibit Apoptosis in RBL-2H3 Cells. Because various adenosine analogs either stimulate or inhibit apoptosis through mechanisms involving different AR subtypes and receptor-independent mechanisms, there is some uncertainty about the mechanism of the effects of IB-MECA on apoptosis in various cells. We reasoned that the effects of A_3 AR receptor activation on apoptosis should be examined using cells in which a physiological effect of A_3 AR activation has been clearly established. In RBL-2H3 cells, we were able to detect Akt activation not only by 10 nM concentrations of the selective A_3 AR agonists IB-MECA and Cl-IB-MECA, but also by inosine in the range of 10 to 100 μM, the same range over which inosine activates A_3 receptors. In ischemic tissues, inosine accumulates to concentrations in excess of 10 μM,

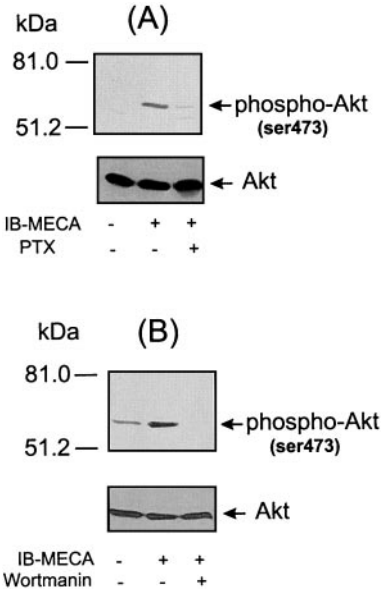

Fig. 3. A_3AR-induced AKT phosphorylation is mediated by pertussis-toxin (PTX)-sensitive G proteins and wortmannin-sensitive PI-3 kinases. A, IB-MECA-stimulated AKT phosphorylation is abolished by PTX treatment. For the final 6 h of serum starvation, RBL-2H3 cells were treated with 100 ng/ml PTX before stimulation with 100 nM IB-MECA. B, blockade of IB-MECA-stimulated AKT phosphorylation by the PI-3 kinase inhibitor wortmannin. Serum-starved RBL-2H3 cells were preincubated at 37°C for 30 min with 10 nM wortmannin before stimulation with 100 nM IB-MECA for 5 min. AKT phosphorylation (top) and total AKT (bottom) were determined by immunoblotting.

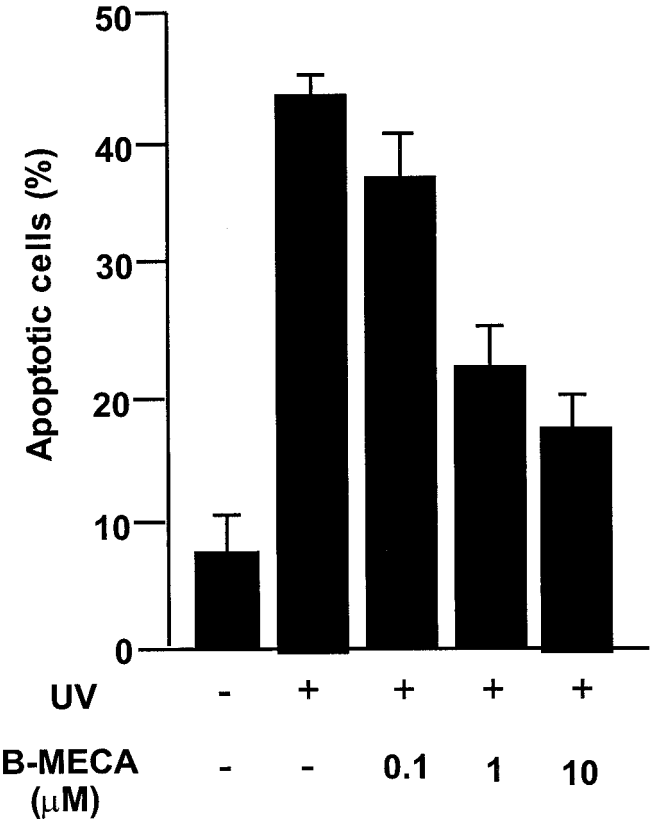

Fig. 4. IB-MECA protects RBL-2H3 cells from UV-induced apoptosis. RBL-2H3 cells were grown on coverslips in six-well plates and were left untreated or irradiated with UV light and stimulated with different concentrations of IB-MECA as indicated. The percentage of apoptosis was determined by TUNEL staining. Results from four independent experiments are shown as the mean $\pm$ S.E.M.

sufficient to selectively activate rat A_3 ARs (Jin et al., 1997). Note, however, that inosine is only a weak, partial agonist of human A_3 ARs (X. Jin and J. Linden, unpublished observations). The rat A_3 AR is only weakly blocked by xanthine antagonists or A_3- selective nonxanthine antagonists of human A_3 ARs (Kim et al., 1996; Baraldi et al., 1999). MRS1191 (>1 μ M) has been reported to compete with radioligands for rat A_3 ARs in binding assays in which solubility is increased by the inclusion of DMSO (Jiang et al., 1997), but we found that MRS1191 (10 or 30 μM) fails to block IB-MECA effects in intact RBL-2H3 cells at its limits of aqueous solubility (data not shown). MRE308F20 has recently been reported as a potent and selective antagonist of human A_3 receptors (Varani et al., 2000) but this compound does not bind well to A_3 receptors of other species. Hence, in this study, we compared the efficacy of BW-1433 and 8-SPT as evidence that IB-MECA-induced effects on Akt phosphorylation and apoptosis are mediated by A_3 ARs. A similar strategy was used to support the conclusion that A_3 ARs trigger mast cell degranulation in rats (Fozard et al., 1996). To bolster our conclusions, we determined the K_I values of these xanthines in inhibiting radioligand binding to the four subtypes of rat ARs. The binding data indicate that 100 μM 8-SPT selectively fails to block rat A_3 ARs, whereas 100 μM BW-1433 blocks all rat AR subtypes. Differential blockade by BW-1433 and not by 8-SPT of IB-MECA-induced inhibition of apoptosis is consistent with the conclusion that this effect is mediated by A_3 ARs on RBL-2H3 cells. This conclusion is further supported by the finding that 5 μM MRS1523, a recently identified selective antagonist of rat A_3 receptors, blocks the effects of IB-MECA on both Akt phosphorylation and apoptosis in RBL-2H3 cells.

A_3 AR Signaling in RBL 2H3 cells. Recombinant A_3 ARs expressed in HEK-293 cells are coupled to Ca^{2+} mobilization and inhibition of adenylyl cyclase by a pertussis toxin-sensitive pathway (Linden et al., 1999). In this study, we observed that IB-MECA-stimulated Akt phosphorylation is abolished in RBL-2H3 cells treated with pertussis toxin. This is consistent with the conclusion that IB-MECA-mediated Akt phosphorylation involves pertussis-toxin sensitive Gi/o proteins.

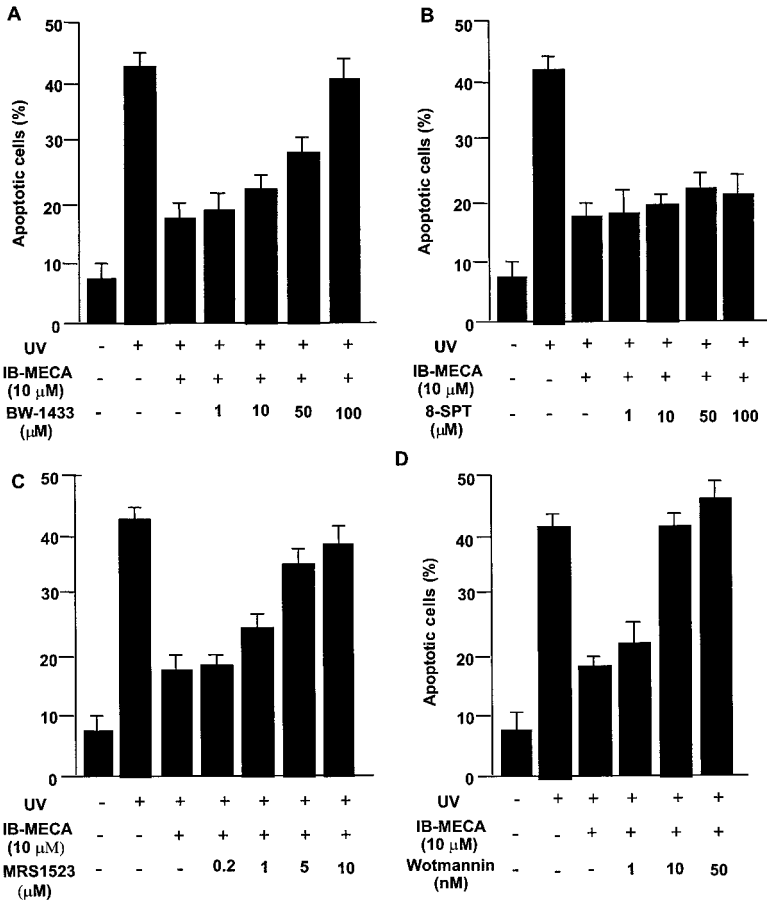

Fig. 5. IB-MECA-induced inhibition of apoptosis in RBL-2H3 cells is blocked by BW-1433, MRS1523, and wortmannin, but not by 8-SPT. RBL-2H3 cells were grown, irradiated, and analyzed for apoptosis as described in the legend to Fig. 4. Apoptosis was measured in cells pretreated with 10 μM IB-MECA and pretreated with the indicated concentrations of BW-1433 (A), 8-SPT (B), MRS1523 (C), and wortmannin (D). Results from four independent experiments are shown as means ± S.E.M.

TABLE 1

Binding affinities of BW-1433 and 8-SPT for rat AR subtypes.

Results are the means ± S.E.M. of triplicate radioligand binding experiments each consisting of seven concentrations of inhibitor assayed in triplicate. See *Experimental Procedures* for details.

Xanthine AR Antagonist	A_1	A_{2A}	A_{2B}	A_3
BW-1433 (K_I, nM)	5.8 ± 1.0	239 ± 37	30.3 ± 10.1	12,300 ± 3,660
% Occupancy by 100 μM	>99	>99	>99	89
8-SPT (K_I, μM)	3.2 ± 1.2	57 ± 4	2.2 ± 0.8	>100
% Occupancy by 100 μM	96	64	97	<10

The pathway may involve the activation of PI3K by G protein $\beta\gamma$ subunits (Hawes et al., 1996). Our observation that wortmannin blocks IB-MECA-induced Akt phosphorylation is consistent with this hypothesis. The PI3Ks consist of a 110-kDa catalytic domain and a regulatory subunit encoded by the p85α, p85β, or p55 γ genes. In mast cells, phosphorylation of Akt stimulated by activation of FcεRI is blocked by wortmannin (an inhibitor of all classes of PI3Ks), but not by disruption of the p85α gene (Lu-Kuo et al., 2000). This suggests that PI3Kβ could be involved in activation of Akt by FcεRI in mast cells. Murga et al. (2000) have recently suggested that the $\beta\gamma$ subunits of heterotrimeric G proteins use PI3Kβ to activate Akt. These considerations suggest that A_3 ARs of RBL 2H3 cells may activate Akt by a pathway including $\beta\gamma$ subunits of Gi and PI3Kβ.

Adenosine Regulation of Apoptosis in Inflammatory Cells. Variability in the expression of PI3Kβ among cells may account for inconsistent effects of A_3 AR activation to influence cell survival. In CHO cells transfected with A_3 ARs, Cl-IB-MECA inhibits cell proliferation, but this effect is not caused by stimulation of apoptosis (Brambilla et al., 2000). In mice A_3, ARs are highly expressed in bone marrow-derived mast cells and these receptors play a role in potentiating antigen-dependent degranulation (Salvatore et al., 2000). In contrast, A_{2B} rather than A_3 ARs regulate canine BR mast cell and human HMC-1 mast cell function (Auchampach et al., 1997; Linden et al., 1999). The role of the $\beta\gamma$ subunits of heterotrimeric G proteins suggests a possible lesser effect on mast cell survival of A_{2B} ARs coupled to Gs and Gq than of A_3 ARs coupled to Gi, which is more abundant than Gs or Gq. Additional experiments will be required to determine whether adenosine has a more profound effect on survival of inflammatory cells expressing A_3 ARs than in cells expressing A_{2B} ARs. There is evidence that A_3 ARs are highly expressed on eosinophils in human lung (Walker et al., 1997). Eosinophils accumulate in the lungs of patients with asthma and at sites of parasitic invasion. Inhibited apoptosis might contribute to expansion of cells that have A_3 receptors in inflamed tissues. The amount of adenosine required to activate A_3 receptors remains an important question. It is always difficult to estimate the adenosine concentration required to produce any response because of its rapid metabolism. However, Doyle et al. (1994) have shown that 1 μM adenosine acting at A_3 receptors produces a mast cell-dependent constriction of isolated microvessels. Hence, we speculate that 1 μM adenosine is sufficient to activate A_3 receptors of rat mast cells. Inosine is a partial agonist of the human receptor but a full agonist of the rat receptor (Jin et al., 1997). We speculate that this species difference may cause different apoptotic responses to inosine in cells that express rat versus human A_3 receptors. In fact, inosine would be expected to possibly attenuate inhibition of apoptosis in human cells exposed to a combination of adenosine and inosine. Additional experimentation will be necessary to determine whether the accumulation of adenosine and inosine at inflamed sites contributes to inflammatory cell expansion by binding to A_3 ARs to inhibit apoptosis and whether there are species differences in these responses.

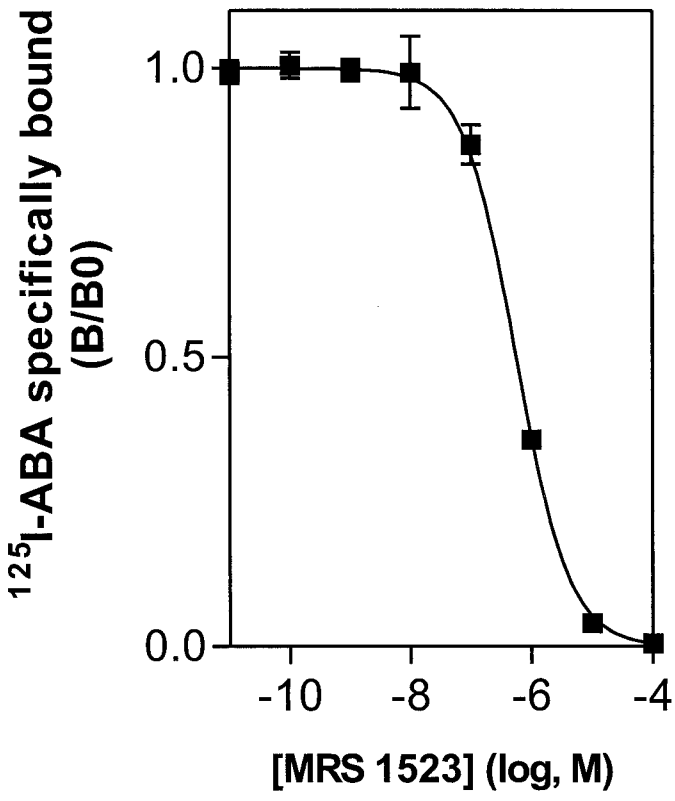

Fig. 6. Competition by MRS1523 for [^{125}I]ABA binding to membranes prepared from HEK-293 cells stably transfected with the rat A_3 adenosine receptor. Each tube contained 0.3 nM [^{125}I]ABA and 10 to 25 μg of membrane protein. Nonspecific binding was <5% of total binding. The average K_I value of MRS1523 for rat A_3 AR is 519 ± 86 nM (mean ± S.E.M., $N = 6$). The K_D value for [^{125}I]ABA binding to rat A_3 AR that was used in calculation of the K_I value was 5.2 nM.

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