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Inhibitory effects of benzodiazepines on the adenosine A_{2B} receptor mediated secretion of interleukin-8 in human mast cells

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ABSTRACT

The activation of adenosine A_{2B} receptors in human mast cells causes pro-inflammatory responses such as the secretion of interleukin-8. There is evidence for an inhibitory effect of benzodiazepines on mast cell mediated symptoms in patients with systemic mast cell activation disease. Therefore, we investigated the effects of benzodiazepines on adenosine A_{2B} receptor mediated interleukin-8 production in human mast cell leukaemia (HMC1) cells by an enzyme linked immunosorbent assay. The adenosine analogue N-ethylcarboxamidoadenosine (NECA, 0.3–3 μM) increased interleukin-8 production about 5-fold above baseline. This effect was attenuated by the adenosine A_{2B} receptor antagonist MRS1754 (N-(4-cyanophenyl)-2-{4-(2,3,6,7-tetrahydro-2,6-dioxo-1,3-dipropyl-1H-purin-8-yl) phenoxy}-acetamide) 1 μM. In addition, diazepam, 4′-chlorodiazepam and flunitrazepam (1–30 μM) markedly reduced NECA-induced interleukin-8 production in that order of potency, whereas clonazepam showed only a modest inhibition. The inhibitory effect of diazepam was not altered by flumazenil 10 μM or PK11195 (1-(2-chlorophenyl)-N-methyl-N-(1-methylpropyl)-3-isoquinolinecarboxamide) 10 μM. Diazepam attenuated the NECA-induced expression of mRNA encoding for interleukin-8. Moreover, diazepam and flunitrazepam reduced the increasing effects of NECA on cAMP-response element- and nuclear factor of activated t-cells-driven luciferase reporter gene activities in HMC1 cells. Neither diazepam nor flunitrazepam affected NECA-induced increases in cellular cAMP levels in CHO Flp-In cells stably expressing recombinant human adenosine A_{2B} receptors, excluding a direct action of benzodiazepines on human adenosine A_{2B} receptors. In conclusion, this is the first study showing an inhibitory action of benzodiazepines on adenosine A_{2B} receptor mediated interleukin-8 production in human mast (HMC1) cells. The rank order of potency indicates the involvement of an atypical benzodiazepine binding site.

1. Introduction

Mast cells play important roles in inflammatory and allergic diseases (for review see Hofmann and Abraham, 2009). Activation of mast cells leads to their proliferation and secretion of various biological mediators like vasoactive compounds, nitric oxide and different cytokines. Among a large number of activators like IgE and cytokines, mast cells respond to adenosine (Kuehn and Gilfillan, 2007; Feoktistov and Biaggioni, 2011). Adenosine causes its effects via activation of four different G-protein coupled receptors, namely adenosine A_1-, A_{2A}-, A_{2B}- and A_3-receptors (Fredholm et al., 2011). Adenosine A_1- and A_3-receptors are coupled to Gi-proteins, whereas adenosine A_{2A}-receptors couple to Gs-proteins and adenosine A_{2B} receptors to both Gs- and Gq-proteins (Fredholm et al., 2011). In human mast cells adenosine A_{2B} receptors mediate pro-inflammatory responses to adenosine including the production and secretion of interleukin-8 (Feoktistov and Biaggioni, 1995, 2011; Feoktistov et al., 1999, 2003; Meade et al., 2002; Buceta et al., 2008; Ryzhov et al., 2008). The adenosine analogue NECA (N-ethylcarboxamido-adenosine) activates adenosine A_{2B} receptors and the xanthine derivative MRS1754 blocks adenosine A_{2B} receptor-mediated responses (Ji et al., 2001; Jacobson and Gao, 2006).

In canine, murine and rat mast cells, benzodiazepines including diazepam and midazolam inhibit pro-inflammatory responses (Suzuki-Nishimura et al., 1989; Bidri et al., 1999; Fujimoto et al., 2005). Several members of the group of 1,4-benzodiazepines such as flunitrazepam, lorazepam and alprazolam have been reported to be effective in the treatment of mast cell mediator-induced symptoms in patients suffering from mast cell activation disease

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(MCAD; Homann et al., 2010; Alvarez-Twose et al., 2012; Torrelo et al., 2012). MCAD is a heterogenous group of disorders characterised by abnormal mast cell accumulation and pathologically elevated secretion of mast cell mediators (see Molderings et al., 2011). High affinity binding sites for benzodiazepines on mast cells have been demonstrated (Miller et al., 1988), but the molecular site of action of benzodiazepines in mast cells has not been identified yet.

In the present study, we therefore studied effects of benzodiazepines in a human mast cell line (HMC1 cells). Benzodiazepine compounds acting at the binding sites of $GABA_A$-receptors as well as compounds acting at the peripheral benzodiazepine binding sites (Gavish et al., 1999; Beurdeley-Thomas et al., 2000) were included in the experiments. Effects on the adenosine A_{2B} receptor-induced expression of mRNA encoding for interleukin-8 in HMC1 cells, on the adenosine A_{2B} receptor-mediated secretion of interleukin-8 from HMC1 cells and on adenosine A_{2B} receptor-mediated changes in reporter gene activities in these cells were analysed. Interleukin-8 (CXCL8) is a chemoattractant and mitogen mediator promoting inflammation by activation of the G-protein coupled chemokine receptors CXCR1 and CXCR2 (Murphy et al., 2000). Interleukin-8 has also been shown to play roles in control of cancer cells (Waugh and Wilson, 2008; Singh et al., 2010) and in cardiovascular diseases (Apostolakis et al., 2009). Some of the present results have been presented in abstract form (Meis et al., 2009; Altarcheh Xifró et al., 2010).

2. Material and methods

2.1. Culturing of human mast (HMC1) cells

Human mast cell leukaemia cells (HMC1.2; see Molderings et al., 2007) were grown in OptiMEM I-medium (Life Technologies, Karlsruhe, Germany) supplemented with 2% fetal bovine serum at 37 °C and 5% CO_2. Cells were split twice a week by mechanical dissociation at a rate of approximately 1 to 5.

2.2. Measurement of interleukin-8 levels in the supernatant

Interleukin-8 levels in the supernatant of HMC1 cells in response to adenosine-receptor agonists were quantified by the use of a sandwich enzyme linked immunosorbent assay (ELISA, PeliKine compact, Sanquin, Amsterdam, Netherlands). HMC1 cells were seeded to a density of about 1.7×10^6 cells per well of a 24-well cell culture plate using serum-free OptiMEM I-medium. After 30 min the experiment was started by addition of the compounds with incubation times indicated below. Benzodiazepines and adenosine-receptor antagonists (or solvents) were added 30 min or 4 h before the adenosine analogue N-ethylcarboxamido-adenosine (NECA). At the end of the experiment, the content of every well was collected and centrifuged (5 min, 300 g). The supernatant was then diluted and transferred to a microtiter plate; the assay using antibodies directed against human interleukin-8 was carried out as described by the manufacturer. The microtiter plate was read in an ELISA reader at 450 nm (BMG Labtech, Offenburg, Germany).

2.3. Reporter gene assays in HMC1 cells

For analysis of effects of benzodiazepines on signalling transduction pathways, changes in cAMP response element (CRE)-, activator protein-1 (AP1)- and nuclear factor of activated t-cells (NFAT)-dependent luciferase expression in HMC1 cells were assessed. HMC1 cells were transiently transfected with the pCRE-luc, pNFAT-luc or pAP1-luc vectors (Stratagene, Amsterdam, Netherlands) by the use of lipofectamine 2000 (Life Technologies). 24 h after transfection, cells were centrifuged (5 min, 100 g), resuspended in serum-free OptiMEM I-medium and seeded on a 24-well culture plate. 30 min later, the experiment was started by addition of the compounds, followed by incubation at 37 °C and 5% CO_2. After 3 h stimulation by NECA the reaction was stopped by adding bright-Glo luciferase reagent (Promega, Mannheim, Germany). 3 min later, luciferase activity was analysed by the use of a single photon luminometer (Berthold, Wildbad, Germany).

2.4. Expression of recombinant human adenosine A_{2B} receptors in CHO Flp-In cells

The sequence encoding for the human adenosine A_{2B} receptor was subcloned into the pcDNA5/FRT/V5-His TOPO TA expression vector (Life Technologies) using standard methods. Chinese hamster ovary (CHO) Flp-In cells (Life Technologies) were stably transfected by the use of the pcDNA5 vector encoding the human adenosine A_{2B} receptor, the pOG44 vector (Life Technologies) and lipofectamine 2000. Then the cells were grown at 37 °C and 5% CO_2 in Ham's F12-GlutaMAX medium (Life Technologies) supplemented with 10% fetal bovine serum and 500 μg/ml hygromycin B (Life Technologies). Cells were split every 3 days by treating with 0.05% Trypsin-EDTA (EDTA 0.54 mM). Cells from passages 3 to 40 were used for experiments.

2.5. Analysis of cellular cAMP accumulation and reporter gene assays in CHO cells

Adenosine A_{2B} receptor function was assessed by quantification of changes in cellular cAMP. CHO Flp-In cells stably expressing recombinant human adenosine A_{2B} receptors were cultured for 24 h on 24-well-plates. After removal of culture medium, cells were washed with HBSS buffer and then incubated for 2 h at 37 °C and 5% CO_2. Cellular cAMP production was stimulated by addition of NECA [0.3–10 μM] for 10 min. Flunitrazepam or diazepam (both 30 μM) were added 10 min prior to NECA. The reaction was stopped by removal of reaction buffer and addition of a hot lysis solution (500 μl, 90 °C, Na_2EDTA 4 mM, Triton X 100 0,1‰, Sigma, Deisenhoven, Germany, pH 7.5). After 5 min at room temperature, the plates were shaken on ice for 1 h. cAMP levels were quantified by incubation of an aliquot with cAMP binding protein and [^{3}H]-cAMP (Amersham, Freiburg, Germany), followed by liquid scintillation counting after removal of unbound cAMP by charcoal as previously described (Hoffmann et al., 2008). cAMP levels were calculated by linear regression from a standard curve determined for each experiment. cAMP-accumulation was expressed as pmol cAMP/well. Reporter gene assays in CHO Flp-In cells stably expressing recombinant human adenosine A_{2B} receptors were performed as described above (2.3) after transfection of the cells using the pCRE-luc vector.

2.6. Real-time PCR

Cells were cultured in 6-well plates. After 24 h the cells were pre-incubated with diazepam or its solvent for 30 min; then NECA or its solvent was added for additional 60 min. After washing and centrifugation cells were lysed in 200 μl of lysing buffer (Qiagen, Hilden, Germany). Reverse transcription of total RNA was carried out by random priming using a commercial kit (Quantitec Reverse Transcription, Qiagen), PCR reactions were set up using Quantifast SYBR Green Master Mix (Qiagen). Amplification was performed over 40 cycles with denaturation at 95 °C for 10 s and annealing/elongation at 60 °C for 30 s in a 96-well plate on an OneStepPlus system (Life Technologies). The primer pairs used are