Selective regulation of nuclear orphan receptors 4A by adenosine receptor subtypes in human mast cells

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Abstract Nuclear orphan receptors 4A (NR4A) are early responsive genes that belong to the superfamily of hormone receptors and comprise NR4A1, NR4A2 and NR4A3. They have been associated to transcriptional activation of multiple genes involved in inflammation, apoptosis and cell cycle control. Here, we establish a link between NR4As and adenosine, a paradoxical inflammatory molecule that can contribute to persistence of inflammation or mediate inflammatory shutdown. Transcriptomics screening of the human mast cell-line HMC-1 revealed a sharp induction of transcriptionally active NR4A2 and NR4A3 by the adenosine analogue NECA. The concomitant treatment of NECA and the adenosine receptor A_{2A} (A_{2A}AR) selective antagonist SCH-58261 exaggerated this effect, suggesting that upregulation of these factors in mast cells is mediated by other AR subtypes (A_{2B} and A₃) and that A_{2A}AR activation counteracts NR4A2 and NR4A3 induction. In agreement with this, A_{2A}AR-silencing amplified NR4A induction by NECA. Interestingly, a similar A_{2A}AR modulatory effect was observed on ERK1/2 phosphorylation because A_{2A}AR blockage exacerbated NECA-mediated phosphorylation of ERK1/2. In addition,

PKC or MEK1/2 inhibition prevented ERK1/2 phosphorylation and antagonized AR-mediated induction of NR4A2 and NR4A3, suggesting the involvement of these kinases in AR to NR4A signaling. Finally, we observed that selective A_{2A}AR activation with CGS-21680 blocked PMA-induced ERK1/2 phosphorylation and modulated the overexpression of functional nuclear orphan receptors 4A. Taken together, these results establish a novel PKC/ERK/nuclear orphan receptors 4A axis for adenosinergic signaling in mast cells, which can be modulated by A_{2A}AR activation, not only in the context of adenosine but of other mast cell activating stimuli as well.

Keywords Nuclear orphan receptor 4A · Mast cells · Adenosine · Adenosine receptor

Abbreviations

AR	Adenosine receptor
HMC-1	Human mast cell line 1
NBRE-LUC	Nerve-growth factor I-B responsive element-luciferase reporter gene
NR4A1	Nuclear orphan receptor 4 A1
NR4A2	Nuclear orphan receptor 4 A2
NR4A3	Nuclear orphan receptor 4 A3
PKC	Protein kinase C
RT-PCR	Reverse transcriptase polymerase chain reaction

Concise summary Non-selective AR engagement triggers robust induction NR4A orphan receptors in mast cells. Induction of these early responsive genes can be blocked by selective activation of the anti-inflammatory A_{2A}AR not only in the context of adenosine but also of PMA signaling, suggesting a general mechanism of mast cell regulation.

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Introduction

NR4A orphan receptors are transcription factors that belong to the superfamily of steroid nuclear hormone receptors that have been associated with different cellular processes, including inflammation (Murphy et al. 2001; Pei et al. 2005), steroidogenesis (Manna et al. 2002), apoptosis (Winoto and Littman 2002), development of dopaminergic

neurons (Zetterstrom et al. 1997) and glucose metabolism (Pei et al. 2006b). This subfamily of nuclear orphan receptors is constituted by 3 members: NR4A1 (also known as Nur77 or nerve growth factor inducible B, NGFI-B), NR4A2 (also known as Nurrl) and NR4A3 (also known as NOR1). In the context of inflammation, these factors represent NF- κ B and LPS-inducible genes in macrophages (Pei et al. 2005) and NR4A1 expression leads to induction of inflammatory genes, potentiating NF- κ B pro-inflammatory signaling by IKKi kinase upregulation (Pei et al. 2006a). In addition, NR4A2 has been recognized as an important inducible factor in inflamed synovium and as a target of anti-inflammatory effects of methotrexate (Ralph et al. 2005).

Adenosine is a purine nucleoside normally present in the nanomolar range but its concentration in the extracellular space rises with increased oxygen consumption during hypoxia, tissue injury and inflammation. The rapid accumulation of adenosine is followed by biological responses through activation of 4 types of G-coupled adenosine receptors (ARs): A₁, A_{2A}, A_{2B} and A₃. Each of the receptor subtypes has a different pharmacological profile, tissue distribution and effector coupling profile. During chronic inflammatory processes the sustained formation of adenosine has been associated with deleterious effects. Elevated adenosine concentrations can be found, for example, in bronchoalveolar lavage and exhaled breath condensate of human patients with asthma (Driver et al. 1993; Huszar et al. 2002) where it perpetuates inflammation and contributes to airway hyperresponsiveness. However, activation of the A_{2A}AR has been associated to the suppression of inflammation and tissue remodelling (Fozard 2003; Ohta and Sitkovsky 2001) and inhibition of histamine and tryptase release from human mast cells (Suzuki et al. 1998). However, the transcriptional effectors downstream of AR activation responsible for adenosine's pro or anti-inflammatory effects remain to be characterized.

In this study, we employed the human mast cell HMC-1 to characterize subtype specific effectors of AR activation and we established a link between adenosine receptor activation and nuclear orphan receptors. Furthermore, we determined that activation of the A_{2A}AR counterbalances the induction of these transcription factors and that this effect is not limited to adenosinergic signaling but that it is also conserved for other mast cell activating stimuli. These observations describe a novel regulatory mechanism by A_{2A}AR, with implications in the progression of inflammation and related pathologies.

Materials and methods

Reagents and cell culture

All chemicals were obtained from Sigma-Aldrich (Switzerland) unless otherwise indicated. The human mast cell line-1

(HMC-1) was a kind gift from Dr. J. H. Butterfield, Mayo Clinic, Rochester, MN, USA and was grown in Iscove's Modified Dulbecco's Medium (IMDM, Invitrogen) supplemented with 10% iron supplemented fetal bovine serum (Invitrogen), 1.2 mM α -hydroxyglycerol (Sigma) and 100'000 U/l Penicillin and 100 mg/l Streptomycin (complete IMDM) at 37°C in 5% CO₂.

Cell treatments

HMC-1 cells were seeded at 8×10^5 cells/ml and allowed to settle overnight. They were then treated at the indicated concentrations with the following chemicals: the adenosine analogue NECA (5'-N-Ethylcarboxamido-adenosine), the A_{2A}AR agonist CGS-21680 (9-Chloro-2-(2-furanyl)-[1,2,4]triazolo[1,5-c]quinazolin-5-amine), the A_{2A}AR antagonist SCH-58261 (7-(2-phenylethyl)-5-amino-2-(2-furyl)-pyrazolo-[4,3-e]-1,2,4-triazolo[1,5-c]pyrimidine) and PMA (phorbol 12-myristate 13-acetate). The length of treatment varied between 30 min and 72 h. Cells were pretreated with 1 U/ml adenosine deaminase (Roche) to remove any pre-existing endogenous adenosine for 20 min.

RNA extraction and cDNA synthesis

Cells were collected by centrifugation at the corresponding time points and total RNA was recovered using the Qiashredder and Rneasy mini kit (Qiagen). Concentration and quality of total RNA were measured with the Ultraspec 2100 pro spectrophotometer (Amersham Biosciences). Samples with a UV absorbance 260/280 ratio of 1.8–2.1 were considered to be suitable for cDNA synthesis. RNA samples were stored at –20°C until use. Complementary DNA (cDNA) was synthesized using the one-cycle cDNA synthesis kit followed by a sample cleanup to optimize volumes and concentration of the cDNA (GeneChip sample cleanup module, Affymetrix).

Genome wide gene expression analysis

Global gene expression analysis was done in 4 independent experiments, consisting of the treatment with 10 μ M NECA and untreated cells as a baseline control. After 3 h treatment of HMC-1 cells total RNA was extracted as described in the previous section. The quality of RNA was determined on the Agilent Lab-on-a-chip Bioanalyzer 2000 (Palo Alto, USA). Samples with a total area under 28S and 18S bands of less than 65% of total RNA, as well as a 28S/18S ratio of less than 1.5, were considered to be degraded and therefore excluded from microarray analysis. cRNA was synthesized from cDNA with the IVT labeling kit (Affymetrix). cRNA quality was assessed with the Agilent Lab-on-a-chip

Bioanalyzer 2000. The biotin-labeled cRNA was fragmented and hybridized on Human Genome U122 plus 2.0 microarrays (Affymetrix), which cover sequences of 47'000 human transcripts, following the manufacturer's instructions. After hybridization periods of 16 h the microarrays were automatically washed and stained on the Affymetrix Fluidics Station 450. Staining of the hybridized probes was performed with fluorescent streptavidin-phycoerythrin conjugates (1 mg/ml; Molecular Probes). The subsequent scanning of DNA microarrays was carried out on an Affymetrix scanner 3000 7 G. The generated data was then normalized and subsequently filtered using a significance value of $P < 0.05$ using the GeneSpring 7.3.1 software (Agilent, Palo Alto, CA, USA). We employed pair-wise analysis based on B - Fabric infrastructure tool (Functional Genomics Center Zurich, University of Zurich-Irchel, Switzerland) and the GeneGo Metacore (www.genego.com) integrated software for data mining and functional analysis of experimental data. Hierarchical clustering was performed with gplots library from CRAN.

Real time polymerase chain reaction (RT-PCR)

Specific primers for the selected transcripts as well as TaqMan probes and TaqMan master mix were obtained from Applied Biosystems. 40 ng of cDNA were mixed with 1 μ l of forward and reverse primers and 10 μ l of master mix supplemented with 25 nM of the corresponding TaqMan probe in a final volume of 20 μ l. The reactions were performed in a 7500 Fast Real-time PCR-System ABI 7500 (Applied Biosystems) in 40 cycles (95°C for 3 s, 60°C for 30 s) after an initial 20 s incubation at 95°C, and was analyzed with the 7500 Fast System SDS Software System (Applied Biosystems). The fold change in expression of each gene was calculated with the 2-Delta Delta C(T) method. Each of these values had been normalized to the endogenous housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

Western blot

Thirty microgram of total protein in 1× Laemmli buffer were separated on a 10% polyacrylamide gel by standard SDS-PAGE technique from each sample, followed by transfer onto Immune-Blot polyvinylidene difluoride (PVDF) membranes (0.2 μ m pore size, Bio-Rad) and blocking 16 h at 4°C with blocking solution (5% non-fat dry milk, 3% BSA in TBST (10 mM Tris, 150 mM NaCl, 0.05% Tween 20, pH 7.4). NR4A2 and total ERK protein levels were assessed employing a monoclonal antibody (Alpha Diagnostic and R&D, respectively) and

phosphorylated ERK1/2 by a specific T185/Y187 phospho-antibody (R&D, USA). GAPDH and β -actin antibodies (Ambion, USA) were employed as internal loading controls. Enhanced chemiluminescence was performed with SuperSignal West Femto Maximum (Thermo Fisher Scientific, Switzerland) and images were acquired on a LAS-3000 image reader (Fujifilm Life Science, Japan). NR4A2 induction was quantified by calculating the ratio of intensity signals to GAPDH (Quantity 1 software, BioRad) and compared to untreated controls (value=1).

siRNA silencing of A_{2A}AR

0.4×10⁶ HMC-1 cells in 1 ml were seeded in 24 well-plates. Cells were transfected with 20nM A_{2A}AR siRNA (Santa Cruz, Cat. sc-39850) using 4 μ l lipofectamine RNAiMax (Invitrogen) according to the manufacturer's instructions in Opti-MEM serum and antibiotics-free medium. After 5 h of incubation, the medium was replaced with complete medium. As a scramble negative control, control (FITC Conjugate)-A siRNA (Santa Cruz, sc-36869) was employed. Silencing of ARs was tested by RT-PCR with specific TaqMan probes (Applied Biosystems) as indicated above, and was maximal at 36 h. Therefore, this time point was selected for further analysis of AR signaling.

Transient transfection and luciferase reporter gene assay

1.5×10⁶ HMC-1 cells in 1 ml were co-transfected with the tk-NBREx3-luc plasmid, which was a kind gift Dr. R Evans (Howard Hughes Medical Institute, San Diego, CA) and the pRL-TK vector (Promega, USA) at a ratio 30:1 in serum- and antibiotics-free Iscove's medium containing 8 μ l of Lipofectamine 2000 reagent (Invitrogen). After 5 h of incubation, the medium was replaced with complete medium and were allowed to recover at 37°C for 24 h and were subsequently stimulated as indicated. For luciferase activity assay, cell lysates were prepared and assayed using the Luciferase Assay System (Promega, USA), according to the manufacturer's instructions. All luminescent measurements were performed automatically in a 96 well-plate in a Luminescence Spectrometer (MLX, Dynex).

Data analysis

Statistical analysis was performed using the PASW Statistics software (SPSS Inc., USA). Data were analyzed by nonparametric tests (Mann-Whitney U-test) and differences between groups were considered significant when p-values were less than 0.05.

Results

Transcriptional profiling of human mast cells reveals upregulation of NR4A family members by adenosine receptor activation

The human mast cell line HMC-1 exhibits a phenotype, which in several aspects is similar to tissue resident mast cells (Nilsson et al. 1994). However, these cells do not degranulate and therefore represent a suitable system for studying degranulation-independent de novo synthesis of inflammatory mediators.

Previous studies have successfully used this cell line to analyze adenosine signaling on human mast cells (Ryzhov et al. 2006; Ryzhov et al. 2004), which prompted us to employ a systematic genome-wide approach for the identification of novel effector molecules involved in AR signaling. For this, we stimulated HMC-1 cells to the adenosine analogue NECA and 3 h after exposure the total fraction of RNA transcripts was extracted, processed and hybridized to Affymetrix microarrays (4 replicates). To identify differentially expressed genes, the data sets of each replicate were analysed in a pair-wise fashion with the untreated controls as described in the methods section. This analysis yielded 19 NECA-induced transcripts with fold changes of three or higher (Table 1). The resulting heat map reflects the degree of similarity between the individual replicates (vertical dendrogram, Fig. 1a). Gene ontology analysis of these transcripts revealed that most of these

transcripts encode protein products involved in transcription regulation (NR4A2, NR4A3, CREM, EGR1, 2 and 3, FOSB, ELL2, ZEB1), signal transduction (HOMER1, ITK, IL2RB), hormone activity (STC1, CGA), DNA metabolism and repair (JMY, TP53INP2, ITK), cell cycle regulation (RGC32), apoptosis (BCL2L11) and tissue remodeling (PLAUR). Remarkably, two nuclear orphan receptors (NR4A2 and NR4A3), which had not been associated to adenosine signaling before, were among the highest upregulated genes. Interestingly, the third member of orphan receptor family, NR4A1, was induced only marginally (2.05 fold). These results were confirmed by real time RT-PCR (Fig. 1b) and suggest that NR4A2 and NR4A3 could mediate proinflammatory signaling by adenosine in mast cells.

A_{2A}AR inactivation amplifies NECA-mediated induction of NR4A2 and NR4A3

Human mast cells express *A_{2A}*, *A_{2B}*, and *A₃*ARs (Feoktistov and Biaggioni 1998; Feoktistov et al. 2003). Therefore, the observed response to NECA reflects the composite effect of activation of all 3 receptor subtypes. Because AR subtypes can promote (*A_{2B}* and *A₃*ARs) or downregulate (*A_{2A}*AR) inflammation, we wanted to determine the influence of *A_{2A}*AR in NR4A induction. To address this issue cells were stimulated either with NECA, with the selective *A_{2A}*AR agonist CGS-21680, or with a combination of NECA plus the *A_{2A}*AR antagonist SCH-58261, and NR4A induction

Table 1 Top regulated transcripts by non-selective AR stimulation. Cells were treated with 10 μ M NECA and fold changes (FC) calculated against untreated control as described (fold change cut-off=3, $P<0.005$)

Gene Name	Description	NECA FC
NR4A2	Nuclear receptor subfamily 4, group A, member 2	33.52
STC1	Stanniocalcin 1	25.37
NR4A3	Nuclear receptor subfamily 4, group A, member 3	24.13
CREM	CAMP responsive element modulator	13.57
EGR3	Early growth response 3	10.16
RGC32	Response gene to complement 32	9.54
HOMER1	Homer homolog 1 (Drosophila)	7.95
FOSB	FBJ murine osteosarcoma viral oncogene homolog B	6.91
CGA	Glycoprotein hormones, alpha polypeptide	6.14
ELL2	Elongation factor, RNA polymerase II, 2	6.14
JMY	Junction-mediating and regulatory protein	5.96
ZEB1	Transcription factor 8	5.42
EGR1	Early growth response 1	5.07
BCL2L11	BCL2-like 11 (apoptosis facilitator)	4.99
ITK	IL2-inducible T-cell kinase	4.76
PLAUR	Plasminogen activator, urokinase receptor	4.58
TP53INP2	p53 induced nuclear protein 2	3.88
IL2RB	Interleukin 2 receptor, beta	3.78
EGR2	Early growth response 2 (Krox-20 homolog, Drosophila)	3.72

controls respectively. These observations point out that the induction of these two transcription factors rely on the activation of *non-A_{2A} ARs*. Interestingly, NECA plus SCH-58261 did not upregulate NR4A1 further when compared to NECA (4.2-fold, Fig. 2c), indicating that this interplay between non-selective and selective A_{2A}AR activation is not conserved to the third member of this family of receptors. Also, induction of the cAMP-response element modulator (CREM, a top-regulated gene from the transcriptomics screening, see Table 1) reaches 55-fold when treated with NECA, while CGS-21680 or the combination of NECA plus SCH-58261 induce CREM only marginally, indicating that simultaneous activation of all ARs is required to achieve maximal CREM induction (Fig. 2d).

Adenosine receptor activation increases NR4A2 protein abundance

AR-induced NR4As are transcriptionally active

In view of the strong induction of NR4A2 and NR4A3, we performed a functional assay based on an exogenous reporter gene to determine how the activity of these factors is affected by AR activation. NR4A receptors bind to the octanucleotide 5'-A/TAAAGGTCA (NGFI-B response element, NBRE) and therefore we employed an NBRE-luciferase reporter plasmid (tk-NBREx3-luc) and quantified luciferase induction upon AR activation. Figure 5 shows

Fig. 2 Time dependent induction of NR4A2 (a), NR4A3 (b), NR4A1 (c) and CREM (d). Cells were treated with 10 μ M NECA, 1 μ M CGS-21680 (CGS) or 10 μ M NECA plus 1 μ M SCH-58261 (SCH) and RNA was collected at the indicated time points and analyzed by RT-PCR. Fold changes are calculated against untreated controls. Values represent the mean of three independent experiments $\pm$ SEM

Fig. 3 Effect of A_{2A}AR silencing on NR4A2 induction. **a** Transfection of HMC-1 cells with A_{2A}AR-siRNA (A_{2A}AR si) specifically downregulates this receptor subtype of A_{2A}AR. Scramble siRNA was included as a control **b** A_{2A}AR silenced cells were treated with for 2 h with 10 μ M of NECA or 10 μ M of NECA plus 1 μ M SCH-58261 (SCH). Values represent the mean of three independent experiments $\pm$ SEM

time-dependent induction of luciferase activity upon treatment with NECA, CGS-21680 or NECA in combination with SCH-58261. Both NECA and NECA plus SCH-58261 induced a robust response, which peaked between 12 h and 24 h with nearly a 30- and 40-fold reporter induction as compared to untreated controls respectively. NBRE reporter activity decreased sharply by 48 h and returned to background values 72 h after treatment. On the contrary, CGS-21680, did not induce luciferase expression significantly. Interestingly, reporter induction by NECA plus SCH-58261 was not statistically significantly higher than by NECA, despite the remarkable difference in induction of these transcription factors. This discrepancy can be explained by the intrinsic limitations of the artificial reporter gene system employed. The luciferase construct includes a minimal promoter region in the 5'-regulatory

Fig. 4 Western blot analysis of NR4A2. HMC-1 cells were treated with 10 μ M NECA, or the combination of 10 μ M NECA and 1 μ M SCH-58261 (SCH) for the indicated time points. GAPDH is included as an internal control. Fold change of NR4A2 protein (FC) are calculated from the ratio to GAPDH and compared to baseline expression in untreated cells (lane 1)

Fig. 5 Time-dependent induction of NBRE by AR engagement. The results are shown as ratio against the respective untreated control. For each measurement the ratio between the reporter gene (firefly) between and the internal Renilla standard was calculated (three independent determinations). Cells were treated with 10 μ M NECA, 1 μ M CGS-21680 (CGS) or 10 μ M NECA plus 1 μ M SCH-58261 (SCH)

region of the NBRE repeats. Therefore, this assay may not allow the full response (gene expression) that could be expected in the context of endogenous chromatin to take place, thereby limiting the amplitude of the responses measured and of the differences between treatments. Nevertheless, these results confirm that AR activation induces transcriptionally active NR4As that could have profound biological implications.

Fig. 6 Effect of kinase inhibitors on AR-mediated NR4A induction and ERK1/2 activation **a** Pretreatment with MEK and PKC inhibitors partially reversed NR4A2 and NR4A3 induction by 10 μ M NECA and 1 μ M SCH-58261. 0.5 μ M SB203580 (SB), 1 μ M H89, 0.1 μ M wortmannin (Wt), 100 μ M PD98059 (PD) or 10 μ M GF109203X (GF) were employed for preincubation. **b** A_{2A} AR-blockage exacerbates NECA-induced phosphorylation of ERK1/2 (N+SCH). This effect is blocked by preincubation with PD98059 (PD) and GF109203X (GF) in a concentration dependent way

AR-mediated NR4A2 and NR4A3 upregulation involves PKC and MEK kinases, and correlates with A_{2A} AR-, PKC- and MEK-sensitive ERK1/2 phosphorylation

NR4As can be induced by a variety of stimuli, which are signaled through diverse intracellular regulators in a cell type and stimulus-dependent fashion (Martinez-Gonzalez and Badimon 2005). Therefore, we wanted to determine the intracellular signaling pathways participating in NR4A2 and NR4A3 induction by ARs. To address this issue we interrogated the role of the mitogen-activated kinase p38, protein kinase A (PKA), phosphoinositide 3 kinase (PI3K), protein kinase C (PKC) and MEK kinase with selective inhibitors, by assessing the AR-mediated induction of NR4A2 and NR4A3 (NECA plus SCH-58261). While 30 min pretreatment with SB203580 (p38 inhibitor), H89 (PKA inhibitor) and wortmannin (PI3K inhibitor) did not affect NR4A induction, blockage of either PKC or MEK with GF109203X or PD98059 respectively partially reverted AR-mediated induction of these factors (Fig. 6a). Based on previous reports that showed that ERK phosphorylation is involved in AR inflammatory signaling in HMC-1 cells (Feoktistov et al. 1999), and because ERK1/2 is a downstream target of PKC, we wanted to determine whether activation of A_{2A} AR correlates with ERK phos-

phorylation. NECA readily induced phosphorylation of ERK1/2 while CGS-21680 did not (Fig. 6b, lanes 2 and 3). Interestingly, NECA plus SCH-58261 resulted in exaggerated ERK1/2 phosphorylation (lane 4), demonstrating an inverse relationship between A_{2A} AR activation status and ERK1/2 phosphorylation. Furthermore, this pattern of ERK phosphorylation could be reverted by preincubation with GF109203X or with PD98059 in a concentration dependent manner (lanes 5–8). Taken together, these results indicate that PKC and MEK kinases are required for AR-dependent ERK1/2 phosphorylation and NR4A2 and NR4A3 upregulation, and that activation of A_{2A} AR opposes ERK1/2 activation by other ARs.

A_{2A} AR activation modulates PMA-induced ERK phosphorylation, NR4A2 and NR4A3 induction and NBRE transcriptional activity

Next, we wanted to assess whether A_{2A} AR activation is able to influence NR4A induction by stimuli other than adenosine. Therefore, we selected PMA, a diester of phorbol that acts as a PKC activator, which is a central regulatory molecule with a role in cytokine production, arachidonic acid release and mast cell degranulation (Chang et al. 1997; Cho et al. 2004). Stimulation of HMC-1 cells with PMA resulted in robust ERK1/2 phosphorylation that could be reverted with increasing concentrations CGS-21680 (Fig. 7a). In addition, 1 μ M CGS-21680 significantly decreased PMA-mediated NR4A2 and NR4A3 induction (by 35 and 53% respectively, Fig. 7b) and, moreover, levels of PMA-induced NR4A2 protein were also reduced (Fig. 7c). In light of these findings, we tested whether A_{2A} AR activation could affect the transcriptional activity of PMA-induced NR4As, by means of the NBRE-LUC reporter assay. Remarkably, preincubation with 1 μ M CGS-21680 caused a significant reduction in PMA-induced NBRE-LUC activity (35%, Fig. 7d).

Altogether, these results show that the inhibitory effect of A_{2A} AR on nuclear orphan receptor 4A stimulation is conserved beyond adenosinergic inflammatory signaling and that activation of this receptor can regulate intracellular signaling by other inflammatory stimuli.

Discussion

Mast cells have been traditionally associated to immediate-type hypersensitivity reactions through the release of preformed inflammatory mediators. However, in recent years it has become clear that these cells can regulate immune responses through de novo synthesis of cytokines, chemokines, and eicosanoids without degranulation (differential release inflammatory mediators), supporting a role of

Fig. 7 Effect of A_{2A} AR activation on PMA-mediated NR4A activity. **a** Increasing concentrations of CGS-21680 (CGS) antagonize PMA-induced ERK1/2 phosphorylation. β-Actin is included as an internal control, **b** Reduction of PMA-mediated NR4A2 and NR4A3 transcriptional induction by increasing concentrations of CGS-21680 (CGS). Values are relative to induction by PMA and indicate the mean of three independent experiments $\pm$ SEM. *, **: $p < 0.05$ for reduction in NR4A2 and NR4A3 induction respectively (Mann-Whitney U test), **c** Western blot analysis of NR4A2 induced by PMA alone or with the indicated concentrations of CGS-21680, **d** CGS-21680 (CGS) reduces the transcriptional activity of PMA-mediated NBRE-LUC reporter activity. For each measurement the ratio between the reporter gene (firefly) and the internal Renilla standard was calculated. Values are relative to LUC expression by PMA, and represent the mean of three independent experiments $\pm$ SEM. PMA concentration in all experiments was 50 nM. * $p < 0.05$ for NBRE-LUC activity reduction (Mann-Whitney U test)

mast cells in more persistent (chronic) inflammatory and immunological responses such as chronic bronchitis and asthma-related pulmonary inflammation (Brightling et al. 2003; Church and Levi-Schaffer 1997), renal injury (Mack and Rosenkranz 2009), and tumorigenesis (Blatner et al. 2010; Groot Kormelink et al. 2009).

The engagement of activating and inhibitory cell-surface receptors, as well as the intensity and duration of these signals, will determine the activation state of mast cells; in response to these stimuli, gene expression patterns of inflammatory mediators are altered according to the sum of positive and negative signaling events, thereby affecting the course of inflammation. Based on adenosine's ability to both positively and negatively regulate mast cell activation through the engagement of alternate AR subtypes, it has been argued that selective activation of the A_{2A}AR (anti-inflammatory) or blockage of A_{2B}AR and A₃AR (pro-inflammatory) may represent a pharmacological tool for modulating their adenosinergic inflammatory signaling. However, transcriptional effectors downstream of ARs responsible for adenosine's pro- or anti-inflammatory effects remained to be fully characterized.

In this study we present a novel link between AR activation and NR4A orphan receptors in human mast cells, and we show that selective activation of the A_{2A}AR can negatively regulate the induction of these factors. Our initial genome-wide screening revealed strong upregulation of NR4A2 and NR4A3. Recent evidence shows that NR4A2 induction represents a common point of convergence of distinct cytokine signaling pathways, suggesting an important common role for this family of transcription factors as mediating inflammatory signaling (McEvoy et al. 2002). Therefore, we reasoned that the anti-inflammatory A_{2A}AR could influence the expression of these pro-inflammatory factors. In fact, we observed that the concomitant treatment with the A_{2A}AR antagonist SCH-58261 significantly amplified NECA's effect on NR4A2 and NR4A3 induction. In view of the pleiotropic physiological roles of NR4As, adenosine's effect on this group of transcription factors could have broad biological implications.

In contrast to other members of the nuclear hormone receptor superfamily, the crystal structure and NMR data indicate that the ligand-binding pocket of NR4A receptors is covered by hydrophobic residues (Wang et al. 2003). In fact, these receptors have been shown to function as ligand-independent transcription factors that are constitutively active and whose activity is controlled at the level of protein expression and post-translational modifications (Codina et al. 2004; Fahrner et al. 1990). For this reason, adenosine's effects on the abundance of these transcription factors could have immediate biological implications. On one hand, the activation of pro-inflammatory ARs (i.e. A_{2B} and A₃ARs) in inflammatory cells could act as an amplification

signal resulting in even higher levels of NR4As and in the expression of a larger set of NBRE-responsive genes. On the other, A_{2A}AR activation could mediate inflammation resolution indirectly by limiting the expression of NR4A2 and NR4A3-dependent inflammatory genes.

NR4As also influence the function of other inflammation-associated transcription factors. For example, NR4A1 and NR4A2 form heterodimers with retinoic acid receptor and can influence retinoid signaling (Wallen-Mackenzie et al. 2003). Therefore, AR activation could affect the number of NR4A-containing complexes: AR-mediated accumulation of NR4A2 and NR4A3 would translate in a higher proportion of transcriptional complexes containing these orphan receptors. Conversely, A_{2A}AR activation would limit the availability of NR4A2 and NR4A3 for heterodimerization with other TFs. In addition, NR4A receptors can also crosstalk with other TFs and influence their activity without necessarily interacting with them. A recent study has established that NR4A receptors and the estrogen-related receptors NR3B mutually repress each others transcriptional activity (Lammi et al. 2007). Similarly, NR4A1 has been shown to negatively cross-talk with NF- κ B (Harant and Lindley 2004). As a consequence, by virtue of the induction of remarkably high levels of functional NR4A2 and NR4A3, adenosine is likely profoundly affect the expression of large sets of genes both directly (NBRE-responsive transcripts) and indirectly, by affecting the nature of transcriptional complexes and/or crosstalking with other TFs.

Activation of mast cells (for example by the high affinity IgE receptor) requires the activation of receptor-proximal tyrosine kinases, mobilization of internal Ca²⁺ and the formation of signaling complexes coordinated by adaptor proteins (Rivera and Gilfillan 2006). PI3K is a central player in mast cell activation that signals to regulatory molecules such as PKC and phospholipases C and D (PLC and PLD) among others, which ultimately regulate mast cell degranulation, arachidonic acid metabolite production and cytokine gene transcription. AR activation has traditionally been linked to stimulation or inhibition of the adenylyl cyclase; A_{2A}AR and A_{2B}AR activation lead to increased cAMP levels that in turn activate the canonical PKA pathway and the exchange protein directly activated by cAMP (Epac) (Palmer and Trevethick 2008), while A₁AR and A₃AR activation leads to cAMP decrease (Zhou et al. 1992). In addition, AR signaling in mast cells has also been linked to PLC and calcium mobilization (A_{2B}AR and A₃AR), PI3K (A₃AR), as well as PKC and MAP kinases (A₁, A_{2A} and A₃AR) (Jacobson and Gao 2006; Spicuzza et al. 2006).

Several intracellular regulators have been linked to NR4A induction (Martinez-Gonzalez and Badimon 2005). In this study we established that AR-mediated NR4A2 and NR4A3 upregulation in HMC-1 did not involve PKA nor

PI3K nor p38. Instead, PKC and MEK kinase inhibition could partially revert the induction of these factors and, moreover, the activity of these kinases correlated with ERK1/2 phosphorylation. Interestingly, some studies have also shown the involvement of ERKs in adenosine signaling (Feoktistov et al. 1999) but downstream targets of ERK upon AR activation had remained elusive. The results presented here suggest that activation of ERK1/2 kinases downstream of PKC mediates NR4As induction by AR. Remarkably, A_{2A}AR can counterbalance NECA-induced ERK1/2 phosphorylation, correlating with its modulatory effect on NR4A2 and NR4A3 induction. However, blockage of PKC and a complete inhibition of ERK phosphorylation resulted only in about a 50% decrease NR4A2 and NR4A3 induction, suggesting the involvement of at least one additional intracellular signaling pathway in AR-dependent nuclear orphan receptor upregulation in mast cells. The contribution of other intracellular signaling pathways remains to be investigated.

Finally, we examined whether the antagonistic effect of A_{2A}AR on ERK1/2 phosphorylation and NR4A2 and NR4A3 induction is preserved to other mast cell activating stimuli, by the concomitant activation of A_{2A}AR and mast cell stimulation with PMA. Strikingly, A_{2A}AR engagement resulted not only in a modulation of PMA-mediated ERK1/2 phosphorylation, but also limited NR4A2 and NR4A3 induction, and their activity as assessed by the NBRE-LUC reporter assay. These results show that A_{2A}AR modulatory's effect on the ERK1/2-NR4A signaling axis is not limited to adenosinergic signaling.

Taken together, the results presented in this study establish a novel effector signaling axis downstream of adenosine, and suggest NR4A antagonism as a mechanism mediating A_{2A}AR anti-inflammatory effects in mast cells. Thus, this data contributes to the understanding of how receptor-specific signals are integrated towards modulation of the inflammatory response, which could facilitate the development of AR-based strategies of immunomodulation.

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References

- Blatner NR, Bonertz A, Beckhove P, Cheon EC, Krantz SB, Strouch M, Weitz J, Koch M, Halverson AL, Bentrem DJ, Khazaie K (2010) In colorectal cancer mast cells contribute to systemic regulatory T-cell dysfunction. *Proc Natl Acad Sci U S A* 107:6430–6435
- Brightling CE, Bradding P, Pavord ID, Wardlaw AJ (2003) New insights into the role of the mast cell in asthma. *Clin Exp Allergy* 33:550–556
- Chang EY, Szallasi Z, Acs P, Raizada V, Wolfe PC, Fewtrell C, Blumberg PM, Rivera J (1997) Functional effects of over-expression of protein kinase C- α , - β , - δ , - ϵ , and - η in the mast cell line RBL-2H3. *J Immunol* 159:2624–2632
- Cho SH, You HJ, Woo CH, Yoo YJ, Kim JH (2004) Rac and protein kinase C- δ regulate ERKs and cytosolic phospholipase A2 in Fc ϵ RI signaling to cysteinyl leukotriene synthesis in mast cells. *J Immunol* 173:624–631
- Church MK, Levi-Schaffer F (1997) The human mast cell. *J Allergy Clin Immunol* 99:155–160
- Codina A, Benoit G, Gooch JT, Neuhaus D, Perlmann T, Schwabe JW (2004) Identification of a novel co-regulator interaction surface on the ligand binding domain of Nurr1 using NMR footprinting. *J Biol Chem* 279:53338–53345
- Driver AG, Kukoly CA, Ali S, Mustafa SJ (1993) Adenosine in bronchoalveolar lavage fluid in asthma. *Am Rev Respir Dis* 148:91–97
- Fahrner TJ, Carroll SL, Milbrandt J (1990) The NGFI-B protein, an inducible member of the thyroid/steroid receptor family, is rapidly modified posttranslationally. *Mol Cell Biol* 10:6454–6459
- Feoktistov I, Biaggioni I (1998) Pharmacological characterization of adenosine A2B receptors: studies in human mast cells co-expressing A2A and A2B adenosine receptor subtypes. *Biochem Pharmacol* 55:627–633
- Feoktistov I, Goldstein AE, Biaggioni I (1999) Role of p38 mitogen-activated protein kinase and extracellular signal-regulated protein kinase kinase in adenosine A2B receptor-mediated interleukin-8 production in human mast cells. *Mol Pharmacol* 55:726–734
- Feoktistov I, Ryzhov S, Goldstein AE, Biaggioni I (2003) Mast cell-mediated stimulation of angiogenesis: cooperative interaction between A2B and A3 adenosine receptors. *Circ Res* 92:485–492
- Fozard JR (2003) The case for a role for adenosine in asthma: almost convincing? *Curr Opin Pharmacol* 3:264–269
- Groot Kormelink T, Abudukelimu A, Redegeld FA (2009) Mast cells as target in cancer therapy. *Curr Pharm Des* 15:1868–1878
- Harant H, Lindley IJ (2004) Negative cross-talk between the human orphan nuclear receptor Nur77/NAK-1/TR3 and nuclear factor-kappaB. *Nucleic Acids Res* 32:5280–5290
- Huszar E, Vass G, Vizi E, Csoma Z, Barat E, Molnar Vilagos G, Herjavec I, Horvath I (2002) Adenosine in exhaled breath condensate in healthy volunteers and in patients with asthma. *Eur Respir J* 20:1393–1398
- Jacobson KA, Gao ZG (2006) Adenosine receptors as therapeutic targets. *Nat Rev Drug Discov* 5:247–264
- Lammi J, Rajalin AM, Hupponen J, Aarnisalo P (2007) Cross-talk between the NR3B and NR4A families of orphan nuclear receptors. *Biochem Biophys Res Commun* 359:391–397
- Mack M, Rosenkranz AR (2009) Basophils and mast cells in renal injury. *Kidney Int* 76:1142–1147
- Manna PR, Dyson MT, Eubank DW, Clark BJ, Lalli E, Sassone-Corsi P, Zeleznik AJ, Stocco DM (2002) Regulation of steroidogenesis and the steroidogenic acute regulatory protein by a member of the cAMP response-element binding protein family. *Mol Endocrinol* 16:184–199
- Martinez-Gonzalez J, Badimon L (2005) The NR4A subfamily of nuclear receptors: new early genes regulated by growth factors in vascular cells. *Cardiovasc Res* 65:609–618
- McEvoy AN, Murphy EA, Ponnio T, Conneely OM, Bresnihan B, FitzGerald O, Murphy EP (2002) Activation of nuclear orphan receptor NURR1 transcription by NF-kappa B and cyclic adenosine 5'-monophosphate response element-binding protein in rheumatoid arthritis synovial tissue. *J Immunol* 168:2979–2987
- Murphy EP, McEvoy A, Conneely OM, Bresnihan B, FitzGerald O (2001) Involvement of the nuclear orphan receptor NURR1 in the regulation of corticotropin-releasing hormone expression and actions in human inflammatory arthritis. *Arthritis Rheum* 44:782–793

- Nilsson G, Blom T, Kusche-Gullberg M, Kjellen L, Butterfield JH, Sundstrom C, Nilsson K, Hellman L (1994) Phenotypic characterization of the human mast-cell line HMC-1. *Scand J Immunol* 39:489–498
- Ohta A, Sitkovsky M (2001) Role of G-protein-coupled adenosine receptors in downregulation of inflammation and protection from tissue damage. *Nature* 414:916–920
- Palmer TM, Trevethick MA (2008) Suppression of inflammatory and immune responses by the A(2A) adenosine receptor: an introduction. *Br J Pharmacol* 153(Suppl 1):S27–S34
- Pei L, Castrillo A, Chen M, Hoffmann A, Tontonoz P (2005) Induction of NR4A orphan nuclear receptor expression in macrophages in response to inflammatory stimuli. *J Biol Chem* 280:29256–29262
- Pei L, Castrillo A, Tontonoz P (2006a) Regulation of macrophage inflammatory gene expression by the orphan nuclear receptor Nur77. *Mol Endocrinol* 20:786–794
- Pei L, Waki H, Vaitheesvaran B, Wilpitz DC, Kurland IJ, Tontonoz P (2006b) NR4A orphan nuclear receptors are transcriptional regulators of hepatic glucose metabolism. *Nat Med* 12:1048–1055
- Ralph JA, McEvoy AN, Kane D, Bresnihan B, FitzGerald O, Murphy EP (2005) Modulation of orphan nuclear receptor NURR1 expression by methotrexate in human inflammatory joint disease involves adenosine A2A receptor-mediated responses. *J Immunol* 175:555–565
- Rivera J, Gilfillan AM (2006) Molecular regulation of mast cell activation. *J Allergy Clin Immunol* 117:1214–1225, quiz 1226
- Ryzhov S, Goldstein AE, Matafonov A, Zeng D, Biaggioni I, Feoktistov I (2004) Adenosine-activated mast cells induce IgE synthesis by B lymphocytes: an A2B-mediated process involving Th2 cytokines IL-4 and IL-13 with implications for asthma. *J Immunol* 172:7726–7733
- Ryzhov S, Goldstein AE, Biaggioni I, Feoktistov I (2006) Cross-talk between G(s)- and G(q)-coupled pathways in regulation of interleukin-4 by A(2B) adenosine receptors in human mast cells. *Mol Pharmacol* 70:727–735
- Spicuzza L, Di Maria G, Polosa R (2006) Adenosine in the airways: implications and applications. *Eur J Pharmacol* 533:77–88
- Suzuki H, Takei M, Nakahata T, Fukamachi H (1998) Inhibitory effect of adenosine on degranulation of human cultured mast cells upon cross-linking of Fc epsilon RI. *Biochem Biophys Res Commun* 242:697–702
- Wallen-Mackenzie A, Mata de Urquiza A, Petersson S, Rodriguez FJ, Friling S, Wagner J, Ordentlich P, Lengqvist J, Heyman RA, Arenas E, Perlmann T (2003) Nurr1-RXR heterodimers mediate RXR ligand-induced signaling in neuronal cells. *Genes Dev* 17:3036–3047
- Wang Z, Benoit G, Liu J, Prasad S, Aarnisalo P, Liu X, Xu H, Walker NP, Perlmann T (2003) Structure and function of Nurr1 identifies a class of ligand-independent nuclear receptors. *Nature* 423:555–560
- Winoto A, Littman DR (2002) Nuclear hormone receptors in T lymphocytes. *Cell* 109(Suppl):S57–S66
- Zetterstrom RH, Solomin L, Jansson L, Hoffer BJ, Olson L, Perlmann T (1997) Dopamine neuron agenesis in Nurr1-deficient mice. *Science* 276:248–250
- Zhou QY, Li C, Olah ME, Johnson RA, Stiles GL, Civelli O (1992) Molecular cloning and characterization of an adenosine receptor: the A3 adenosine receptor. *Proc Natl Acad Sci U S A* 89:7432–7436