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Activation of Mast Cells by Trimeric G Protein Gi3; Coupling to the A3 Adenosine Receptor Directly and upon T Cell Contact

Dana Baram,* Ornit Dekel,* Yoseph A. Mekori,[†] and Ronit Sagi-Eisenberg*

Mast cells are key players in mediating and amplifying allergic and inflammatory reactions. Previously, we identified the G-protein, Gi3, as the cellular target of receptor mimetic basic secretagogues that activate mast cell independently of IgE. In this study, we demonstrate that Gi3 is the cellular target of the adenosine A3 receptor (A3R), a G-protein coupled receptor involved in inflammation and the pathophysiology of asthma. By using a cell permeable peptide comprising the C-terminal end of G α i3 fused to an importation sequence (ALL1) as a selective inhibitor of Gi3 signaling, we show that by coupling to Gi3, the A3R stimulates multiple signaling pathways in human mast cells, leading to upregulation of cytokines, chemokines, and growth factors. We further show that after contact with activated T cell membranes, endogenous adenosine binds to and activates the A3R, resulting in Gi3-mediated signaling. Specifically, the majority of ERK1/2 signaling initiated by contact with activated T cell membranes, is mediated by Gi3, giving rise to ALL1-inhibitable cellular responses. These results unveil the physiological G-protein coupled receptor that couples to Gi3 and establish the important role played by this G-protein in inflammatory conditions that involve adenosine-activated mast cells. *The Journal of Immunology*, 2010, 184: 000–000.

Basic secretagogues were recognized as early as 1951 as potent IgE-independent activators of mast cells. The sensitivity of basic secretagogues-triggered exocytosis to pertussis toxin (Ptx) has implicated Gi protein(s) as essential mediators of their triggered exocytosis. However, their mechanism of action remained largely unresolved. The large repertoire of molecules, including neuropeptides, opiates and the synthetic polyamine compound 48/80 (c48/80), which constitute this family of stimuli (1), their common structural moieties and the fact that micromolar concentrations are required to evoke their biological activity, have suggested that members of this family trigger mast cell activation in a receptor-independent manner (2, 3). Consistent with this notion, in vitro studies have demonstrated the ability of members of this family to activate directly Gi proteins (3–6). We have previously shown that rat peritoneal mast cells (RPMCs) express only two Ptx-sensitive G-proteins, Gi₂ and Gi₃ (7). Introduction of a peptide that comprised the 10 C-terminal amino acids of G α i₃ (KNNLKECGLY) into permeabilized RPMCs inhibited c48/80-induced histamine release, thus pointing out Gi3 as the principal mediator of basic secretagogues-induced exocytosis

(7). However, the identity of the physiological G-protein coupled receptor(s) (GPCRs) that couple(s) to Gi3 has remained elusive.

Recently, we demonstrated that a cell permeable version of the G α i₃ C-terminal peptide, designated ALL1, which comprised the G α i₃ C-terminal peptide fused with an importation sequence derived from the signal sequence of the Kaposi fibroblast growth factor (AAVALLPAVLLALLAP), efficiently penetrates into intact RPMCs and blocks c48/80 and substance P induced histamine secretion, protein tyrosine phosphorylation, and release of PGD₂ in a dose-dependent fashion (8). These results have indicated a therapeutic potential of this transducible peptide. Thus, it is of vast importance to elucidate the cellular pathways that are mediated by Gi3. To this end, ALL1 could serve as an efficient tool for dissecting Gi3-mediated pathways and identifying the physiological receptor(s), which couples to Gi3. In the current study, using ALL1 as our experimental tool, we identified the adenosine A3 receptor (A3R) as a GPCR that couples to Gi3 in rat and human mast cells (HMCs). Moreover, we show that A3R and Gi3 are activated in HMCs undergoing inflammatory processes. Specifically, we show that A3R/Gi3 play important roles in HMCs responses initiated on contact with activated T cells.

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Abbreviations used in this paper: A3R, adenosine A3 receptor; ADA, adenosine deaminase; AR, adenosine receptor; Cl-IBMECA, 2-chloro-N⁶-(3-iodobenzyl)-adenosine-5'-N-methyluronamide; GPCR, G-protein coupled receptor; HMC, human mast cell; NECA, 5'-N-ethylcarboxamidoadenosine; Ptx, pertussis toxin; RPMC, rat peritoneal mast cell; T^m, activated T membranes.

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Materials and Methods

Materials

The peptide ALL1 (AAVALLPAVLLALLAPKNNLKECGLY) was synthesized by PolyPeptide Laboratories (Torrance, CA). The pan-adenosine receptor (AR) agonist 5'-N-ethylcarboxamidoadenosine (NECA), A3R agonist 2-chloro-N⁶-(3-iodobenzyl)-adenosine-5'-N-methyluronamide (Cl-IBMECA), A2a receptor antagonist 7-(2-phenylethyl)-5-amino-2-(2-furyl)-pyrazolo-[4,3-e]-1,2,4-triazolo[1,5-c] pyrimidine (SCH58261), A2b receptor antagonist 8-[4-(((4-Cyanophenyl) carbamoylmethyl) oxy]phenyl]-1,3-di(*n*-propyl)xanthine hydrate (MRS1754), the A3R antagonist 9-chloro-2-(2-furanyl)-5-((phenylacetyl)amino)-[1,2,4]triazolo[1,5-c] quinoxaline (MRS1220), and adenosine deaminase (ADA) were all purchased from Sigma-Aldrich (St. Louis, MO). Ptx was purchased from List Biological Laboratories. A3R targeted siRNA (ADORA3HSS175292) and its corresponding control siRNA (negative control duplex–low GC duplex) were purchased from Invitrogen (San Diego, CA).

Abs used in this study

Antiphosphotyrosine 4G10 (Upstate Biotechnology, Lake Placid, NY), antiactive MAPK ERK1/2 (Sigma-Aldrich), antitotal ERK2 and antitubulin (Santa-Cruz Biotechnology, Santa Cruz, CA), antiphospho-MEK1/2 and antitotal MEK1/2 (Cell Signaling Technology, Beverly, MA), HRP-conjugated goat anti-mouse and anti-rabbit IgG (Jackson ImmunoResearch Laboratories, West Grove, PA). ELISA kit for IL-8 was purchased from R&D Systems (Minneapolis, MN).

Cells

HMC-1 cells and the Jurkat T cell lymphoma were maintained in suspension, in RPMI supplemented with 10% FCS (Invitrogen-Life Technologies), 2 mM L-glutamine, sodium pyruvate, and 1% of a penicillin-streptomycin-nystatin mixture (Biological Industries, Bet-Haemek, Israel). RPMCs were obtained from Wistar rats by a peritoneal lavage, and purified as previously described (7). Briefly, a suspension of washed peritoneal cells was layered over a cushion of 30% Ficoll 400 (Pharmacia Biotech, Uppsala, Sweden) in buffered saline and 0.1% BSA, and centrifuged at $150 \times g$ for 15 min. The purity of mast cells recovered from the bottom of the tube was >90%, as assessed by toluidine blue staining.

Preparation of T cell membranes

Jurkat T cells were activated with 75 ng/ml PMA (Calbiochem Merck) for 1 h at 37°C . Activated and resting Jurkat membranes were isolated as described previously (9). Aliquots of the isolated membranes were stored at -70°C .

Activation of mast cells

HMC-1. After serum-starvation for 18–20 h, HMC-1 cells were washed twice with Tyrode's buffer (137 mM NaCl, 2.7 mM KCl, 1 mM MgCl_2 , 1.0 mM CaCl_2 , 5.6 mM glucose, 1 mg/ml BSA, 20 mM HEPES, pH 7.4) and resuspended to 2×10^6 cells/ml with the same buffer, containing 0.1 mM Na_3VO_4 . In experiments, which included direct activation of ARs, 1.5 U/ml ADA was added to the cells. The cells were preincubated with ALL1 for 1 h at 37°C , prior to stimulation with AR agonists or activated Jurkat membranes. Adenosine antagonists were administered 15 min before stimulation of the cells. As the AR agonists and antagonists used were dissolved in DMSO, the same DMSO concentrations were used in vehicle controls (final DMSO concentration not exceeding 0.1%). Reactions were terminated by placing the tubes on ice, followed by a brief spin ($14,000 \times g$, 20 s) at 4°C . Cell pellets were lysed by the addition of a lysis buffer (buffer A comprising: 50 mM HEPES pH 7.5, 150 mM NaCl, 1% Triton X-100, 0.1% SDS, 50 mM NaF, 10 mM sodium pyrophosphate, 2 mM sodium orthovanadate, 10 mM EDTA, 2 mM EGTA, 1 mM PMSF, protease inhibitor mixture [Boehringer Mannheim, Indianapolis, IN], phosphatase inhibitor mixture-1 [Sigma-Aldrich]) and incubation for 20 min on ice. The lysates were then centrifuged for 15 min at $14,000 \times g$. Cell lysates were mixed with $\times 5$ concentrated Laemmli sample buffer, boiled, and subjected to SDS-PAGE and Western blotting.

RPMCs. Purified RPMCs (2×10^5 cells/ml) were incubated in Tyrode's buffer with vehicle or with the desired stimuli for 20 min. Reactions were terminated by placing the tubes on ice, followed by a brief spin ($12,000 \times g$, ~20 s) at 4°C . Supernatants were collected and used to determine the amount of histamine released. Cell pellets were lysed and used to determine ERK1/2 phosphorylation.

β -hexosaminidase activity

Activity of the secretory granule-associated enzyme β -hexosaminidase was determined by incubating 20 μl aliquots of supernatants and cell lysates

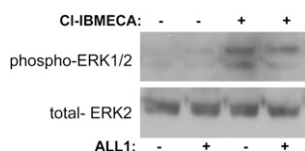


FIGURE 1. Stimulation of ERK1/2 phosphorylation by CI-IBMECA and inhibition by ALL1 in RPMC. Purified RPMCs (2×10^5 cells/ml) were preincubated for 1 h at 37°C with vehicle or with 200 μM ALL1 as indicated. Buffer or CI-IBMECA (100 nM) was subsequently added and the cells incubated for further 20 min. At the end of incubation, the cells were sedimented, washed and lysed. Cell extracts were resolved by SDS-PAGE and immunoblotted with antiphospho-ERK1/2. Blots were then stripped and reprobed with antitotal-ERK2 as indicated. A representative blot of two similar experiments is shown.

for 90 min at 37°C with 50 μl substrate solution consisting of 1.3 mg/ml p-nitrophenyl-N-acetyl- β -D-glucosaminide (Sigma-Aldrich) in 0.1 M citrate pH 4.5. Reactions were stopped by the addition of 150 μl 0.2 M glycine pH 10.7. OD was read at 405 nm. Results were expressed as percentage of total β -hexosaminidase activity present in the cells.

Determination of protein phosphorylation

ERK1/2 phosphorylation was determined as described in (8). Briefly, cell lysates prepared by the addition of lysis buffer A were centrifuged for 15 min at $12,000 \times g$. Supernatants were mixed with $5 \times$ concentrated Laemmli sample buffer. Samples were boiled, resolved by SDS-PAGE, under reducing conditions, and transferred to nitrocellulose or polyvinylidene difluoride (Immobilon-P, Millipore, Bedford, MA) membranes.

Western blotting

Blots were blocked for 1 h in TBST (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.05% Tween 20) containing 5% skim milk, followed by overnight incubation at 4°C with the desired primary Abs. Blots were washed three times and incubated for 1 h at room temperature with HRP-conjugated secondary Ab. Immunoreactive bands were visualized by ECL method according to standard procedures.

RNAi of A3R

HMC-1 cells (5×10^5 cells/well) were seeded in 24-well plates. Cells were incubated for 72 h with Stealth siRNA Duplex Oligoribonucleotides, at a final concentration of 100 nM, prepared according to the manufacturer's instructions in Opti-MEM I Reduced Serum Medium, and in the presence of Lipofectamine 2000. The sequences of siRNA used were as follows: ADORA3 HSS175292(3_RNAI): CACCUUCUAUUUCAUUGUCUCUCUA and UAGAGAGACAAUGAAAUAAGGUG and as a negative control low GC Duplex.

Detection of array of phosphorylated kinases

For analyzing the phosphorylation states of MAPKs and other serine/threonine kinases of the MAPK pathway, HMC-1 cells were preincubated with ALL1 or vehicle and activated as described previously, using 5×10^6 cells for each treatment. The analysis was performed by the Proteome Profiler Array-Human Phospho-MAPK Array Kit (R&D Systems), according to the manufacturer's instructions. A total of 300 μg protein of cell lysates were used for each array.

Expression microarray analysis

After preincubation with ALL1 and 3 h activation, total RNA was extracted from duplicate samples of 6×10^6 HMC-1 cells using the RNeasy Mini Kit

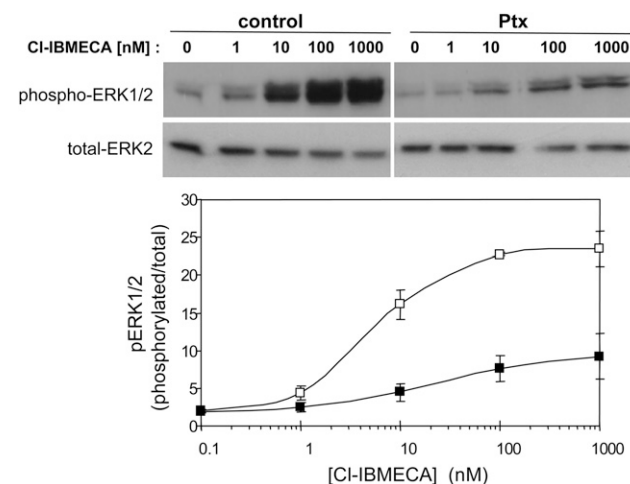


FIGURE 2. Stimulation of ERK1/2 phosphorylation by CI-IBMECA and inhibition by Ptx in HMC-1 cells. HMC-1 cells (2×10^6 cells/ml) were preincubated overnight with 100 ng/ml Ptx as indicated. Cells were washed and activated for 1 min with the indicated concentrations of CI-IBMECA. Cell lysates were resolved by SDS-PAGE and immunoblotted with antiphospho-ERK1/2, followed by reprobing with antitotal-ERK2 as indicated. A representative blot is shown. The intensities of the bands corresponding to phospho-ERK1/2 and total-ERK2 were quantified and the relative (phosphorylated/total) pixel densities were calculated. Means $\pm$ SEM are presented. Open symbols, without; solid symbols, with Ptx.

(RNeasy Mini Kit; Qiagen, Valencia, CA), according to the manufacturer's protocol. Gene expression was screened for by using the Affymetrix Human Genome U133A 2.0 array (Affymetrix, Santa Clara, CA), which contains probe set for ~23,000 genes and expressed sequence tags. This was performed by the Microarray Unit at the Weizmann Institute of Science (Rehovot, Israel). Data analysis was performed by RACE gene expression platform (Remote Analysis Computation for gene Expression data, <http://race.unil.ch/>). Lists of statistically significant ($p < 0.05$) up-regulated or downregulated genes by at least 2-fold expression (compared with the nontreated cells) were generated, and these were then further analyzed and subdivided into functional categories with the bioinformatic analysis resource DAVID (Database for Annotation, Visualization and Integrated Discovery, <http://david.abcc.ncifcrf.gov/>). Array data from these experiments are available on the Microarray Database Web site (www.ncbi.nlm.nih.gov/geo/; accession number GSE19888).

Analysis of released cytokines

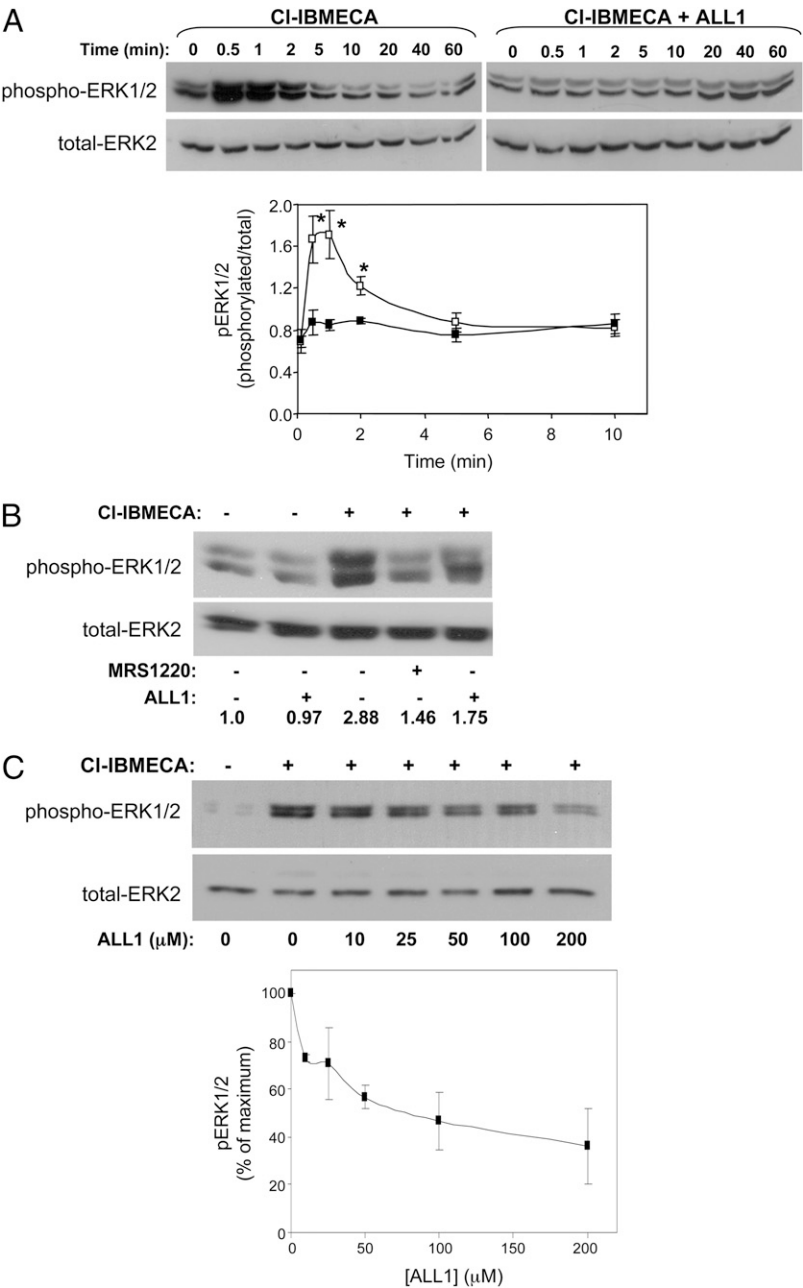
For determination of secreted cytokines, HMC-1 cells (3×10^6 cells in 1.5 ml for each sample) were washed with RPMI, resuspended in RPMI containing 0.1% BSA and preincubated with ALL1 for 1 h. The cells were then stim-

ulated with AR agonists or activated Jurkat membranes and incubated overnight. Next day, supernatants were collected, and tested for levels of secreted cytokines using the Proteome Profiler Array-Human Cytokine Array Panel A (R&D Systems), according to the manufacturer instructions. For IL-8 detection, HMC-1 cells (2×10^6 cells/ml) for each sample were seeded in 24-well plates in RPMI containing 0.1% BSA. Cells were preincubated for 1 h with vehicle or 200 μ M ALL1 and subsequently stimulated with activated Jurkat membranes overnight at 37°C. Supernatants were collected and tested for IL-8 secretion using a sandwich ELISA kit (R&D Systems), according to the manufacturer's instructions. OD was read at 450 nm.

Data analysis and statistical considerations

Experiments were performed 2–10 times. In Western blotting/, the intensities of the appropriate bands were quantified by densitometry, and levels of phosphorylated proteins were normalized to the corresponding levels of nonphosphorylated proteins. Means and SEM of these normalized values were calculated for each set of experiments. Where indicated, Student *t* test was also applied. *p* value of <0.05 was considered significant. Unless indicated means and SEM of three independent experiments are shown.

FIGURE 3. Stimulation of ERK1/2 phosphorylation by CI-IBMECA and inhibition by ALL1 in HMC-1 cells. **A**, HMC-1 cells (2×10^6 cells/ml) were either left untreated or preincubated for 1 h at 37°C with 200 μ M of ALL1, as indicated. Cells were then stimulated with 100 nM CI-IBMECA for the indicated periods. Cell lysates were resolved by SDS-PAGE and immunoblotted with antiphospho-ERK1/2, followed by reprobing with antitotal-ERK2 as indicated. A representative blot is shown. The intensities of the bands corresponding to phospho-ERK1/2 and total-ERK2 were quantified and the relative (phosphorylated/total) pixel densities were calculated. Open symbols, without; solid symbols, with ALL1. Means $\pm$ SEM of four independent experiments are presented. $*p < 0.02$. **B**, HMC-1 cells (2×10^6 cells/ml) were either left untreated or preincubated for 1 h at 37°C with 200 μ M of ALL1 or for 15 min with 100 nM of the A3R antagonist MRS1220, as indicated. Cell lysates were resolved by SDS-PAGE and immunoblotted with antiphospho-ERK1/2, followed by reprobing with antitotal-ERK2. A representative blot is shown. The intensities of the bands were quantified and relative (phosphorylated/total) pixel densities were calculated and are presented relative to the value of nontreated cells. **C**, HMC-1 cells (2×10^6 cells/ml) were either left untreated or preincubated for 1 h at 37°C with the indicated concentrations of ALL1. ERK1/2 phosphorylation was determined as previously described. A representative blot is shown. The intensities of the bands were quantified and relative (phosphorylated/total) pixel densities were calculated. The means $\pm$ SEM are presented.



Results

ALL1 inhibits A3R-activated ERK signaling in purified RPMCs and HMCs

When considering mast cell GPCRs that might couple to Gi3, ARs appear attractive candidates. Adenosine has long been implicated in a variety of inflammatory processes, including allergy and asthma (10–13). This metabolite is produced and released under conditions of increased energy consumption, such as stress or hypoxia, where it then functions in an autocrine as well as paracrine fashion (10, 14). The effects of adenosine are mediated through binding to four distinct GPCRs, the A1, A2a, A2b, and A3 receptors, which couple to Ptx sensitive Gi proteins (i.e., the A1 and A3 receptors), Gs (A2a and A2b receptors), or Gq/G11 (the A2b receptor) [reviewed in (15)]. Mast cells express different levels of the A2a, A2b, and A3 receptors (16), therefore marking those receptors as potential mediators of allergic and inflammatory processes. Because out of the mast cell expressed AR, only the A3R couples to Ptx-sensitive G-protein(s), we examined the effect of the Gi3-directed inhibitory peptide ALL1, on A3R signaling in purified RPMCs. Unlike basic secretagogues, the selective A3R agonist CI-IBMECA failed to trigger histamine release from purified RPMCs (not shown). However, CI-IBMECA did stimulate phosphorylation of the ERK1/2 MAP kinases and this phosphorylation was diminished completely by ALL1 (Fig. 1). These results have therefore demonstrated that the adenosine A3R provokes ERK signaling by coupling to Gi3 in primary rat mast cells, though, its signaling output does not include the entire signaling network that is elicited by the family of basic secretagogues, which do also stimulate exocytosis.

To explore the relevance of these findings to HMCs, the effects of CI-IBMECA and ALL1 on the HMC-1 cells were investigated. Indeed, similarly to its impact on RPMCs, CI-IBMECA induced phosphorylation of ERK1/2 in HMC-1 cells (Fig. 2). Phosphorylation was dose dependent and abolished completely by Ptx, consistent with the known coupling of the A3R to Ptx-sensitive G-proteins (Fig. 2). CI-IBMECA-induced phosphorylation was rapid, reaching maximal phosphorylation at 1 min after activation with CI-IBMECA (Fig. 3A). Importantly, in analogy to its profound inhibitory effect on CI-IBMECA-stimulated ERK phosphorylation in RPMCs, ALL1 totally inhibited ERK phosphorylation in the HMC-1 cells (Fig. 3A). In fact, CI-IBMECA-induced ERK1/2 phosphorylation could be equally eliminated by either MRS1220, the antagonist of the A3R, or by ALL1 thus lending further support to the notion that Gi3 is the principal mediator of the A3R (Fig. 3B). Inhibition by ALL1 was dose dependent with an IC_{50} value of 30 μ M (Fig. 3C).

CI-IBMECA also induced phosphorylation of MEK1/2 (Fig. 4). Moreover, consistent with their localization upstream of ERK1/2, MEK1/2 proteins reached their maximal phosphorylation at 30 s after activation with CI-IBMECA (Fig. 4). As with ERK1/2, CI-IBMECA-induced phosphorylation of MEK1/2 was inhibited completely by ALL1 (Fig. 4).

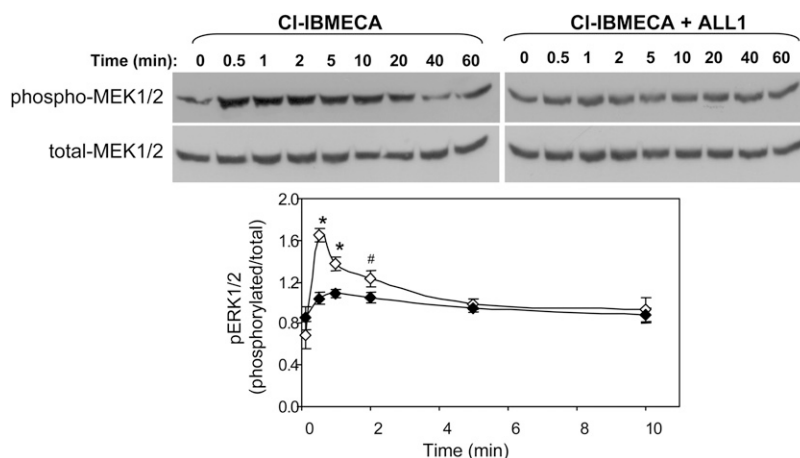
ALL1 inhibits NECA-induced ERK1/2 activation

To assess the relative contribution of Gi3-mediated pathways to the overall adenosine signaling outputs, we explored the effect of ALL1 on ERK1/2 phosphorylation, stimulated by the pan-adenosine agonist NECA. Strikingly, ALL1 inhibited by >80% NECA-enhanced ERK1/2 phosphorylation (Fig. 5A). In fact, ALL1 displayed equal potency in inhibiting ERK1/2 phosphorylation stimulated by NECA alone or in the combined presence of the A2a and A2b antagonists, SCH58261 and MRS1754, which leave only the A3R active (Fig. 5A). These results have therefore suggested the existence of a negative cross-talk between the A2a and A2b receptors, thereby leaving the A3R as the major signaling receptor in the propagation of ERK signaling. Indeed, although MRS1220, the selective A3R antagonist, inhibited by ~60% NECA-stimulated ERK1/2 phosphorylation, SCH58261 or MRS1754, the respective A2a and A2b AR antagonists, rather potentiated NECA-stimulated ERK1/2 phosphorylation, confirming their negative contribution to the overall adenosine signaling (Fig. 5B). This notion was further supported by the finding that unlike the signal elicited by either the A2a or A2b receptors, which when activated alone reflected 50% of NECA-stimulated signal, the A3R signal output was equipotent to that elicited by NECA (Fig. 5B).

ALL1 inhibits T cell membrane-induced activation of mast cells

Next, we investigated the contribution of adenosine and in particular the contribution of the A3R and its Gi3-coupled G-protein, to the overall signaling of mast cells undergoing inflammatory processes. Such conditions can be induced by exposing mast cells to activated T cells. Specifically, we have previously demonstrated that direct and prolonged contact between HMCs and PMA-activated T cells induces mast cell activation, resulting in degranulation and release of cytokines (17), metalloproteinases (9), and fibrotic factors such oncostatin-M (18). We further showed that contact with membranes derived from activated T cells suffices to achieve maximal activation (9). Fig. 6 shows that HMC-1 cells incubated with increasing concentrations of membranes derived from activated Jurkat T cells, display a dose-dependent release of β -hexosaminidase, an enzyme typically released from mast cell secretory granules during activated exocytosis. The HMC-1 cells released up to 25% of their granule content in response to contact

FIGURE 4. Analysis of ALL1 inhibition of CI-IBMECA-stimulated MEK1/2 phosphorylation. HMC-1 cells (2×10^6 cells/ml) were either left untreated or preincubated for 1 h at 37°C with 200 μ M of ALL1, as indicated. Cells were then stimulated with 100 nM CI-IBMECA for the indicated periods. Cell lysates were resolved by SDS-PAGE and immunoblotted with antiphospho-MEK1/2. Blots were subsequently stripped and reprobed with antitotal MEK1/2 Abs as indicated. A representative blot is shown. The intensities of the bands were quantified and relative (phosphorylated/total) pixel densities were calculated. Means $\pm$ SEM are presented. # $p < 0.05$; * $p < 0.02$. Open symbols, without; solid symbols, with ALL1.



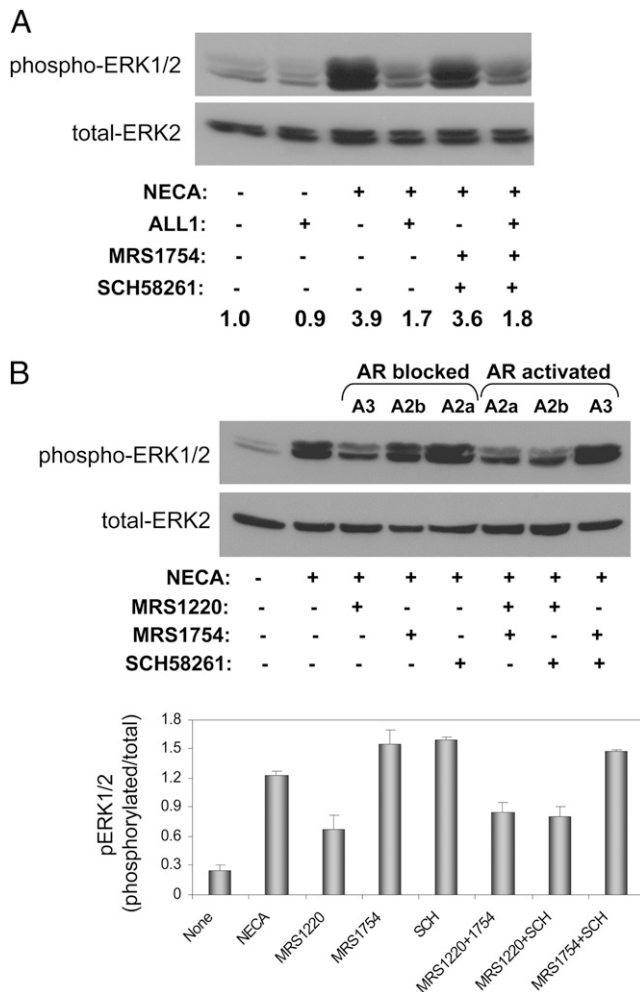


FIGURE 5. Analysis of ALL1 inhibition of NECA-stimulated ERK1/2 phosphorylation. **A**, HMC-1 cells (2×10^6 cells/ml) were either left untreated or preincubated for 1 h at 37°C with $200 \mu\text{M}$ ALL1. Where indicated 100 nM of an AR antagonist was added for the last 15 min of incubation. Cells were then left untreated or stimulated for 1 min with $10 \mu\text{M}$ NECA. Phosphorylation of ERK1/2 was determined as described previously. A representative blot is shown. The intensities of the bands were quantified and relative (phosphorylated/total) pixel densities were calculated and are presented relative to the value of nontreated cells. **B**, Cells were either left untreated or incubated for 15 min with 100 nM of each of the indicated AR antagonists. Cells were then untreated or stimulated for 1 min with $10 \mu\text{M}$ NECA. Phosphorylation of ERK1/2 was determined as described previously. A representative blot is shown. The intensities of the bands were quantified and relative (phosphorylated/total) pixel densities were calculated. Means $\pm$ SEM are presented.

with membranes of activated (Fig. 6A), but not resting T cells (inset of Fig. 6A). Contact with activated T membranes (T^*m) also elicited signaling events, including stimulation of ERK1/2 and MEK1/2 phosphorylation, which could be detected as early as 5 min after exposure to the activated membranes (Fig. 6B, 6C). To address the possibility that endogenously formed adenosine might contribute to the signaling detected under these inflammatory conditions, we included in our assay ADA, which effectively deactivates endogenous adenosine. Indeed, in its presence, T^*m stimulated MEK1/2 phosphorylation was reduced by 50%, therefore confirming the contribution of endogenously generated adenosine to T^*m signaling (Fig. 6C). Furthermore, preincubation of the cells with ALL1, prior to their exposure to T^*m produced a similar inhibitory effect, suggesting that the adenosine contribution to ERK signaling was entirely mediated by Gi3 (Fig. 6C).

ALL1 also inhibited T^*m -stimulated phosphorylation of ERK1/2 and this inhibition was dose dependent, displaying an IC_{50} value of $37 \mu\text{M}$, which was in the range of inhibition of Cl-IBMECA-induced phosphorylation (Fig. 6D). Consistent with this notion, T^*m -induced MEK1/2 phosphorylation was inhibited by $\sim 40\%$ by Ptx (Fig. 7A). Notably, inhibition by ALL1 was specific as demonstrated by the fact that stimulation of ERK1/2 phosphorylation by PMA was ALL1-resistant (Fig. 7B).

That the A3R was indeed the principal mediator of adenosine signaling in T^*m -activated HMC-1 cells was indicated further by the profound inhibition of T^*m -stimulated ERK1/2 phosphorylation exerted in cells in which the A3R was selectively down-regulated by siRNA (Fig. 8). Hence, A3R targeted siRNA, which reduced the expression level of the A3R by 60%, but not control siRNA, eliminated completely stimulation of ERK1/2 phosphorylation by either Cl-IBMECA or T^*m (Fig. 8).

Assessment of the Gi3-mediated signaling networks

We now aimed to dissect the signaling response to T cell membrane cues and A3R-mediated signaling networks. To this aim and further identification of the Gi3 mediated pathways, we used Phospho-Signaling Protein Arrays to compare the patterns of phosphorylated kinases in T^*m versus Cl-IBMECA-activated mast cells, in the absence or presence of ALL1. These arrays enabled us to explore the phosphorylation states of all three major families of MAPKs, namely, the ERK1/2, c-Jun N-terminal kinases (JNK1-3), and the α - δ isoforms of p38. In addition, we also monitored the phosphorylation states of Akt1-3, GSK-3 α/β , RSK-1 and 2, and MSK2. Altogether, these signaling arrays included 18 kinases, which have been implicated as major regulators of mast cell function (19). Fig. 9A depicts one set of such arrays, demonstrating significant enhancements in the phosphorylation of a number of kinases in response to Cl-IBMECA and their complete inhibition by ALL1. A summary of the results obtained by a number of arrays is shown in Fig. 9B. Of all tested substrates, ERK1/2, RSK1, p38 α,β,γ , JNK, and GSK3 α displayed statistically significant ($p < 0.05$) enhancements in both their phosphorylation and inhibition by ALL1 (Fig. 10A).

A similar analysis of the phosphorylation pattern of T^*m activated cells revealed a somewhat different profile. Although several arrays suggested that ERK1/2 and RSK1 display both significant enhancements in phosphorylation as well as inhibition by ALL1 ($p < 0.001$) (Figs. 9B, 10B), the T cell membranes did not stimulate phosphorylation of either the JNK family members, or of p38 isoforms β - δ , or GSK3 α ; whereas phosphorylation of p38 α was stimulated only slightly (by 1.5-fold, $p = 0.037$). Strikingly, although not enhanced by T membranes, the basal constitutive phosphorylation of GSK3 α and p38 γ was significantly ($p < 0.05$) inhibited by ALL1 (Fig. 10B).

To substantiate these results even further, we also evaluated the effect of MRS1220 on the phosphorylation profile of the ALL1-sensitive phosphoproteins in cells activated by either Cl-IBMECA or T^*m . The results obtained demonstrated that ALL1 and MRS1220 affected the same set of phosphoproteins (Fig. 10A, 10B).

Taken together, these results demonstrated that a subset of T^*m provoked signaling is mediated exclusively by adenosine through its A3R and Gi3 protein. Indeed, close inspection of the profile of protein tyrosine phosphorylation stimulated by contact with T^*m revealed that induced phosphorylation of a subset of proteins, including pp34, pp45, pp55, and pp83 was totally inhibited by ALL1 (Fig. 10C).

Contribution of Gi3 signaling to cytokine expression and release

To gain insight into the physiological importance of A3R/Gi3 signaling, we assessed their contribution to the cellular processes stimulated by

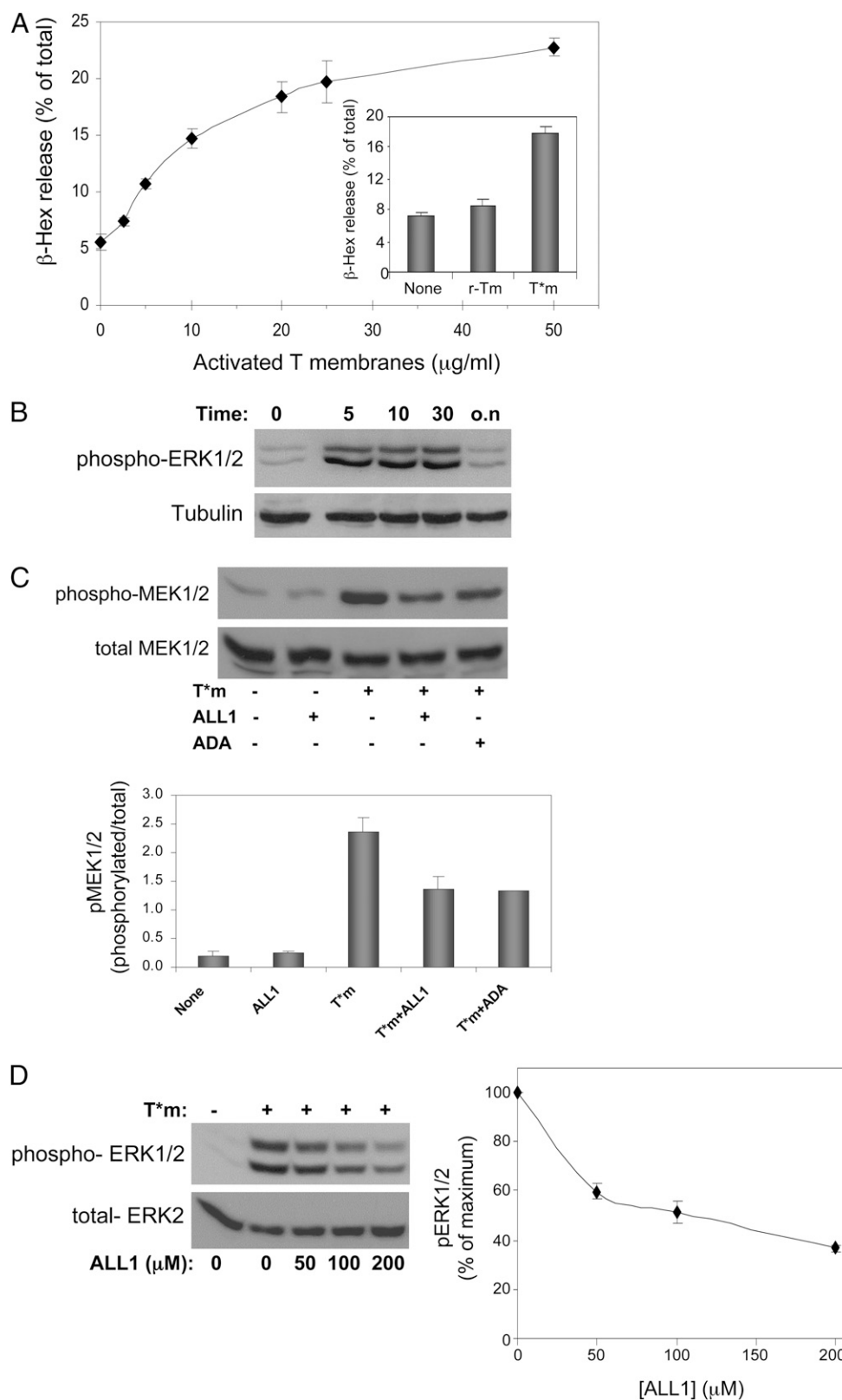


FIGURE 6. Activation of HMC-1 cells by T cell-derived membranes. **A**, HMC-1 cells (2×10^6 cells/ml) were incubated for 20 h with increasing concentrations of membranes isolated from PMA-activated Jurkat T cells or with 20 μg/ml of membranes isolated from resting or activated Jurkat T cells (*Inset*). Supernatants were collected for measurement of β-hexosaminidase release. The extent of release is presented as percentage of total β-hexosaminidase activity. The data points presented are means $\pm$ SEM of 20 determinations, derived from 10 independent experiments. **B**, HMC-1 cells (2×10^6 cells/ml) were incubated with 20 μg/ml of activated T cell membranes for the indicated periods or overnight. Cell lysates were resolved by SDS-PAGE and immunoblotted with antiphospho-ERK1/2 Abs, as indicated. Blots were subsequently stripped and reprobed with antitubulin Abs. Representative blots are shown. **C**, HMC-1 cells (2×10^6 cells/ml) were either left untreated or were preincubated for 1 h at 37°C with 200 μM ALL1 as indicated. Cells were subsequently left untreated or stimulated for 5 min with activated T cell membranes in the absence, or the presence of 1.5 U/ml ADA as indicated. Cell lysates were resolved by SDS-PAGE and MEK1/2 phosphorylation was analyzed as previously described. A representative blot is shown. The intensities of the bands corresponding to

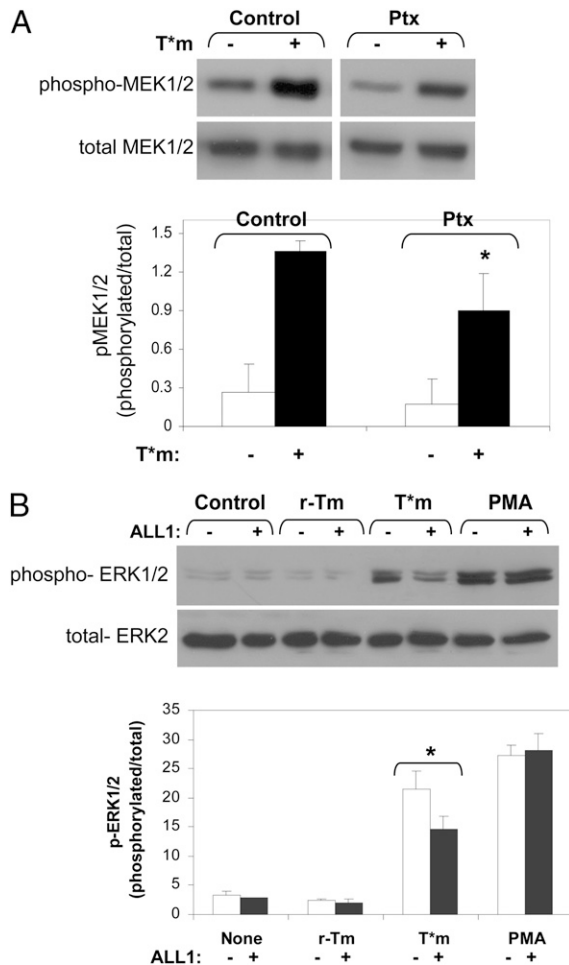


FIGURE 7. Effect of Ptx and ALL1 on MEK/ERK activation by T cell-derived membranes. *A*, HMC-1 cells (2×10^6 cells/ml) were preincubated for 2 h at 37°C with or without 300 ng/ml Ptx, as indicated. Cells were washed and either left untreated or activated for 5 min with 20 μ g/ml activated T*m. Cell lysates were resolved by SDS-PAGE and immunoblotted with antiphospho-MEK1/2, followed by stripping and reprobing with antitotal MEK1/2 Abs as indicated. A representative blot is shown. The intensities of the bands were quantified and relative (phosphorylated/total) pixel densities were calculated and plotted. * $p = 0.0243$. *B*, HMC-1 cells (2×10^6 cells/ml) were preincubated for 1 h at 37°C with 200 μ M ALL1, as indicated. Cells were the left untreated or stimulated for 5 min with 20 μ g/ml resting or T*m, or with 50 ng/ml PMA. Blots were processed with antiphospho-ERK1/2 and antitotal ERK2 Abs and quantified as previously described. * $p = 0.0186$.

activated T cells. ALL1 had no effect on β -hexosaminidase release (not shown) therefore excluding A3R from contributing to the secretory process and the releases of preformed mediators. However, because mast cell activation is tightly linked with the production and release of cytokines and chemokines, we focused on the profile of their expression and release consequently to contact with T*m or exposure to CI-IBMECA. The contribution of Gi3 was explored by evaluating the impact of ALL1 on these processes.

Using human gene arrays and setting our score as at least a 2-fold change at $p < 0.05$, we found the expression of 15 chemokines

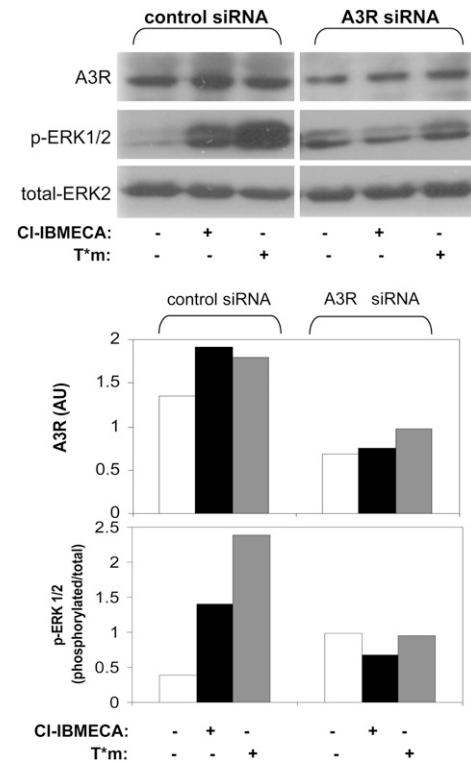


FIGURE 8. Inhibition of ERK1/2 phosphorylation by A3R RNAi. HMC-1 cells (5×10^5 cells/sample) were incubated for 72 h with 100 nM of A3R directed siRNA (Duplex 292, Invitrogen) or with its negative control siRNA (negative 292, Invitrogen), as described in *Materials and Methods*. Cells were then left untreated or stimulated for 1 min with 100 nM of CI-IBMECA or for 5 min with 20 μ g/ml of T*m as indicated. Cells lysates were resolved by SDS-PAGE and immunoblotted with anti-A3R Abs (upper panel) or with antiphospho-ERK1/2, followed by antitotal-ERK2 as indicated. A representative blot is shown. The intensities of the bands were quantified and relative (phosphorylated/total) pixel densities were calculated and plotted. White columns correspond ERK1/2 phosphorylation of untreated cells, black columns to CI-IBMECA-stimulated cells, and gray columns to T*m-stimulated cells.

and cytokines to be upregulated by 2- to 15-fold after exposure to CI-IBMECA (100 nM) (Fig. 11A). These included the CC chemokines MCP-1, CCL1, MIP1 α , MIP1 β , RANTES, CCL7, and CCL8; the CXC chemokines IL-8 and CXCL3; the cytokines IL-3, IL-24, vascular endothelial growth factor A, CSF 1 and 2, and osteopontin. Preincubation with ALL1 markedly inhibited the expression of all of the above signals (Fig. 11A). IL-7 was downregulated by 2-folds, and this effect too was partially reversed by ALL1. Therefore, these results indicated that Gi3 mediated A3R-induced modulation of cytokine/chemokine expression.

The expression levels of 13 of the 15 chemokines and cytokines modulated by CI-IBMECA were also modulated 2- to 118-folds after exposure to the T*m (Fig. 11B). Similar to the CI-IBMECA treatment, expression of IL-7 was downregulated by 5-folds (Fig. 11B). ALL1 affected only a subset of these genes, including IL-3, CSF2, IL-8, and IL-24 (Fig. 11B).

phospho-MEK1/2 and total-MEK2 were quantified and the average relative (phosphorylated/total) pixel densities were calculated and plotted. *D*, HMC-1 cells (2×10^6 cells/ml) were either left untreated, or preincubated for 1 h at 37°C with increasing concentrations of ALL1. Cells were subsequently either left untreated or stimulated for 5 min with 20 μ g/ml activated T*m as indicated. Cell lysates were resolved by SDS-PAGE and ERK1/2 phosphorylation was analyzed as previously described. A representative blot is shown. The intensities of the bands corresponding to phospho-ERK1/2 and total-ERK2 were quantified and the relative (phosphorylated/total) pixel densities were calculated.

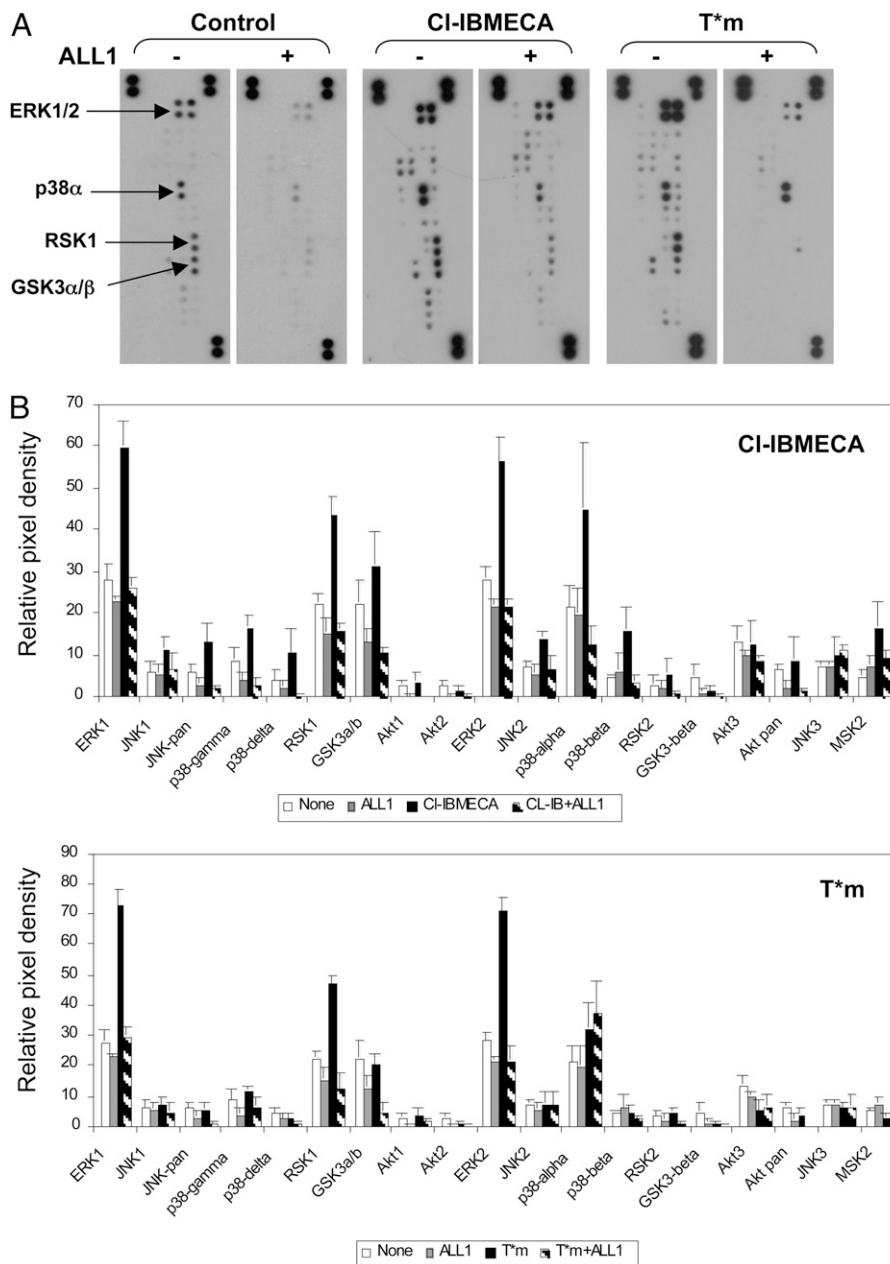


FIGURE 9. Phospho-signaling arrays after cell stimulation by CI-IBMECA or activated T cell membranes; Effect of ALL1. **A**, HMC-1 cells (5×10^6 cells per treatment) were left untreated or preincubated for 1 h at 37°C with $200 \mu\text{M}$ ALL1 as indicated. Cells were then stimulated for 1 min with 100 nM CI-IBMECA or for 5 min with $20 \mu\text{g/ml}$ activated T cell membranes as indicated. Cell lysates were analyzed by Human Phospho-MAPK Arrays (R&D Systems). A total of $300 \mu\text{g}$ protein of each lysate were used. A representative array is presented. **B**, Array signals from scanned films were quantified and normalized accordingly to positive controls levels. The data obtained from two arrays run in duplicates are presented.

Although CI-IBMECA upregulated the expression of 15 chemokines/cytokines, it failed to trigger any detectable secretion. Using cytokine arrays and monitoring 36 cytokines and chemokines did not detect any increase in chemokines/cytokines levels above the basal constitutive secretion in supernatants derived from CI-IBMECA-triggered cells. In marked contrast, T*m enhanced the release of the constitutively released cytokines and induced the release of others, including MIP1 α , MIP1 β , IL-8, serpin E1, CSF2, TNF- α , RANTES, and GRO- α (Fig. 12A). Remarkably, ALL1 had no effect on the constitutively released cytokines, but it inhibited release of most of the induced chemokines/cytokines, with inhibition ranging between 23–70% (Fig. 12A). For example, by this analysis, ALL1 inhibited by 70% T*m-stimulated release of IL-8 (Fig. 12A). These results were analyzed further by measuring the actual amounts of IL-8 released by ELISA. No IL-8 was detected in supernatants derived of nontreated cells, whereas in supernatants of T*m-stimulated cells 1900 pg/ml IL-8 were detected. This amount was reduced by 40% in the presence of ALL1 (Fig. 12B).

Discussion

The pivotal role of GTP binding proteins in the activation process of mast cells was first demonstrated by the elegant studies of Gomperts, who showed that introduction of nonhydrolysable GTP analogs, into the cytosol of mast cells, induced exocytosis (20). Shortly after, Ptx-sensitive G-proteins were identified as major mediators of mast cells activation induced by the family of basic secretagogues (21). A decade later, we identified the trimeric G-protein Gi3 as an essential element in the trigger of exocytosis by basic secretagogues (7). However, basic secretagogues are believed to function as receptor mimetic agents that directly activate Gi proteins (3, 5, 6), whereas the physiological GPCR that couples to Gi3 and its role in the pathophysiology of mast cells have remained obscure. Our recent development of a cell permeable peptide (ALL1) that selectively inhibits Gi3 function in intact cells (8), provided us with a powerful tool to begin dissecting signaling pathways initiated by various mast cell stimuli, and identifying those that are mediated by Gi3. In this study, we have used ALL1 to investigate the possible role of Gi3 in HMC signaling conveyed by contact with activated

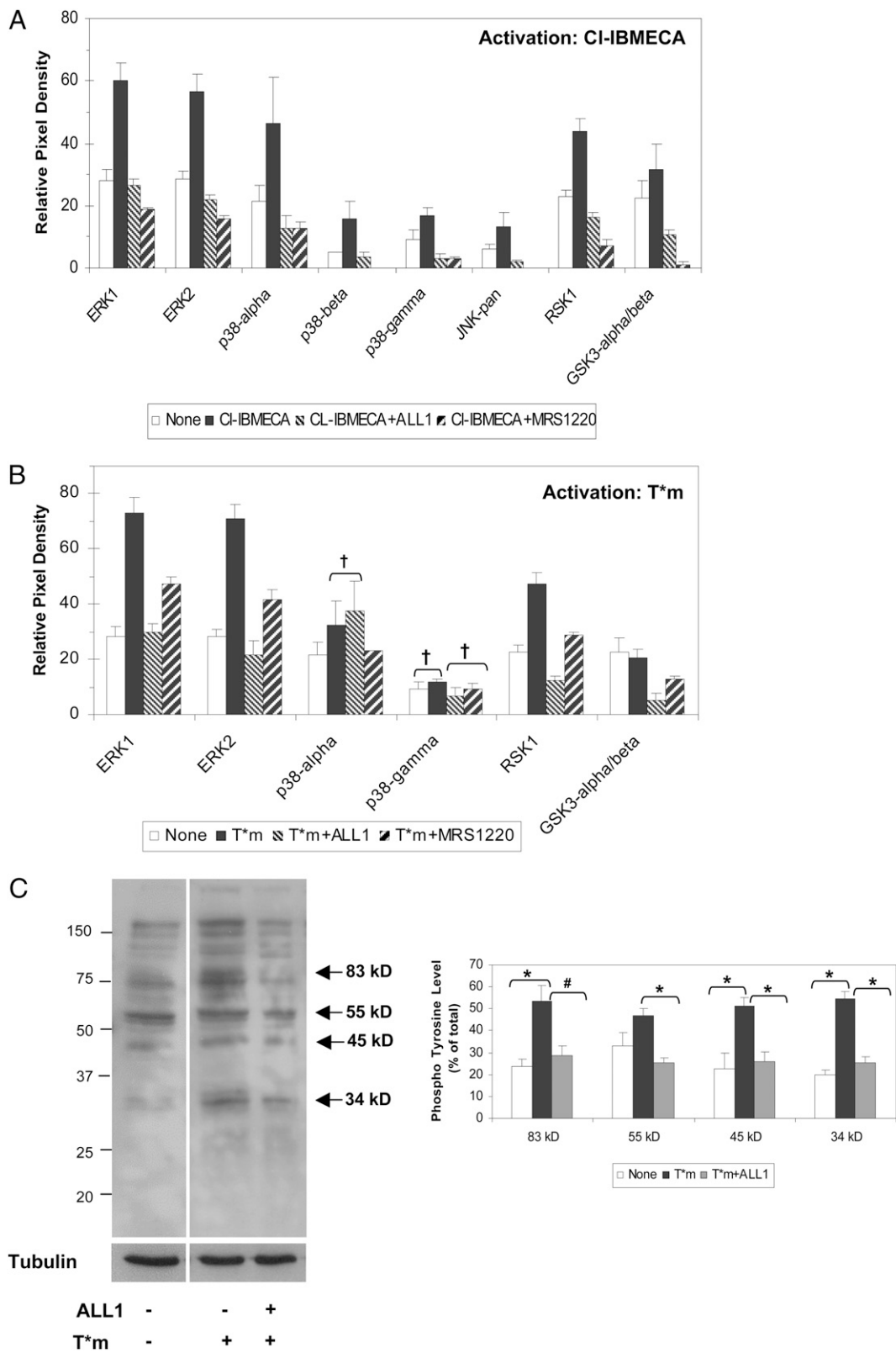


FIGURE 10. Phospho-signaling arrays after cell stimulation by CI-IBMECA or activated T cell membranes; effect of ALL1 and MRS1220. **A**, Cells were left untreated or preincubated for 1 h at 37°C with 200 μ M ALL1 as indicated. Cells were then stimulated with for 1 min with 100 nM CI-IBMECA alone or in the presence of 100 nM of the A3R antagonist MRS1220. Cell lysates were analyzed as previously described. **B**, Cells were preincubated as previously described and stimulated for 5 min with 20 μ g/ml activated T cell membranes (T*m) alone or in the presence of 100 nM of the A3R antagonist MRS1220. Cell lysates were analyzed as previously described. Except for the columns marked ($\dagger$), all the data were statistically significant ($p < 0.05$). **C**, HMC-1 cells (2×10^6 cells/ml) were either left untreated or preincubated for 1 h at 37°C with 200 μ M ALL1 as indicated. Cells were then either left untreated or stimulated for 5 min with 20 μ g/ml activated T cell membranes (T*m). Cell lysates were resolved by SDS-PAGE and immunoblotted with antiphosphotyrosine Abs. Blots were subsequently stripped and reprobed with antitubulin. A representative blot is shown. The intensities of total protein tyrosine phosphorylation of each lane and of bands corresponding to proteins of 83, 55, 45, and 34 kDa were quantified and relative (phosphorylated/total) pixel densities were calculated. The means $\pm$ SEM of four independent experiments are presented. # $p < 0.05$; * $p < 0.02$.

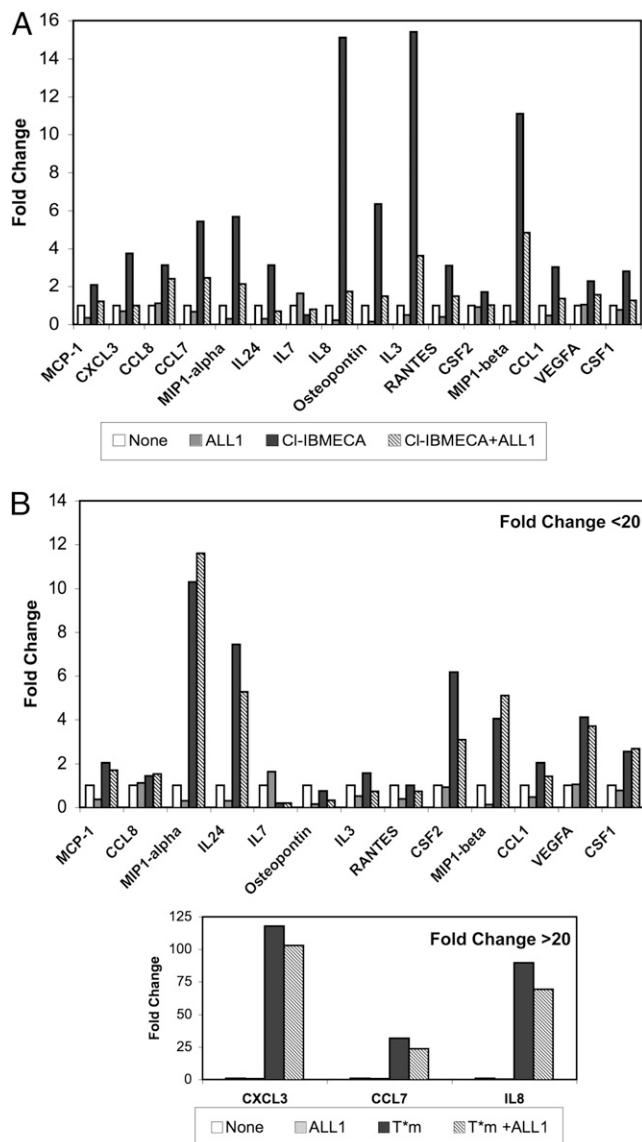


FIGURE 11. Expression of cytokines/chemokines and growth factors; effect of ALL1. **A**, HMC-1 cells (6×10^6 cells per treatment) were either left untreated or preincubated for 1 h at 37°C with $200 \mu\text{M}$ ALL1 as indicated. Cells were then stimulated for 3 h with 100 nM CI-IBMECA. Total RNA was isolated and subjected to microarray analysis, as described in *Materials and Methods*. The overexpressed or downregulated genes, filtered according to fold change of at least ± 2 as compared with untreated cells (None) and significance of $p < 0.05$, were analyzed using the functional annotation tool in <http://david.abcc.ncifcrf.gov>. **B**, Cells were treated as previously described, but stimulated with $20 \mu\text{g/ml}$ activated T cell membranes. *Upper panel* fold change <20 ; *lower panel* fold change >20 .

T cells. Such conditions mimic activation of mast cells within inflamed tissues, hence the physiological relevance of this experimental paradigm. In the light of recent new findings, strongly supporting a profound role for GPCRs in modulating mast cell responses, reassessment of the role and contribution of Gi3 to mast cell activation by stimuli other than the family of basic secretagogues is highly timely.

A number of important observations emerged from this study. First, we provide unequivocal evidence for the coupling of Gi3 with the A3 AR. In the past, our findings have demonstrated that in connective tissue type RPMCs, Gi3 can be directly activated by the receptor mimetic basic secretagogues, such as the synthetic c48/80 (7) or substance P (8). In this study, we show that the A3R is at

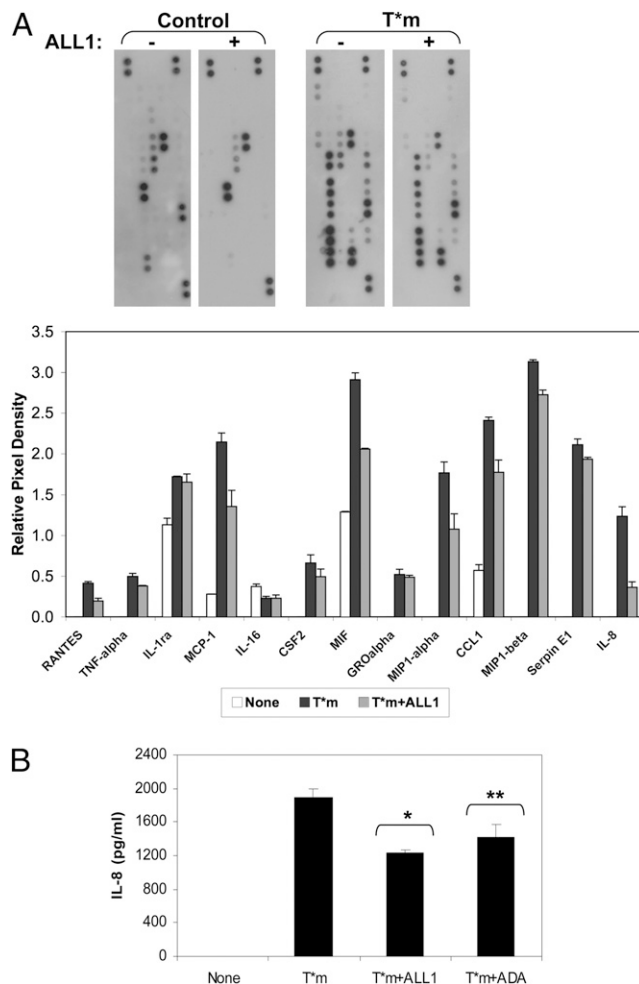


FIGURE 12. Effect of ALL1 on cytokine/chemokine secretion. **A**, HMC-1 cells (2×10^6 cells/ml) were either left untreated or preincubated for 1 h at 37°C with $200 \mu\text{M}$ ALL1 as indicated. Cells were then activated for 20 h with T cell membranes (T*m). Each supernatant ($1 \mu\text{m}$) was used to analyze released chemokines and cytokines by human cytokines arrays (R&D Systems). Representative array images are shown on the *upper panel*. Profiles created by quantifying dot densities, background-subtracted, and normalized to positive controls, are presented in the *lower panel*. **B**, HMC-1 cells (2×10^6 cells/ml) were either left untreated or preincubated for 1 h at 37°C with $200 \mu\text{M}$ ALL1 as indicated. Cells were then activated for 20 h with T*m or T*m in the presence of ADA (1.45 U/ml). Supernatants were collected and the amount secreted of IL-8 was determined by ELISA (R&D Systems). OD was read at 450 nm . The means $\pm$ SEM of two experiments are presented. * $p = 0.00395$; ** $p = 0.09$.

least one GPCR that couples selectively with Gi3. Moreover, the A3R couples with Gi3 not only in RPMCs, but also in HMCs, exemplified in this study by the HMC line HMC-1, therefore strongly suggesting a key physiological role for Gi3 as a central player in the activation process of HMCs. Although we cannot exclude the possibility that additional GPCRs couple to Gi3, our findings clearly identified the A3R as an upstream physiological partner interacting with Gi3 in HMCs. Indeed, ALL1 inhibited all signaling outputs initiated by the selective A3R agonist CI-IBMECA, including activation of the three MAPK families, ERK1/2, JNK1-3, and p38 α - δ . One outcome of these multiple signaling outputs is the upregulation of 15 genes, including the chemokines MCP-1, CCL1, 7, and 8, MIP1 α and β , RANTES, IL-8, CXCL3, the cytokines IL-3 and IL-24 and the growth factors, vascular endothelial growth factor A, CSF1/2, and osteopontin and down-regulation of IL-7. Modulation of all these genes was prevented by

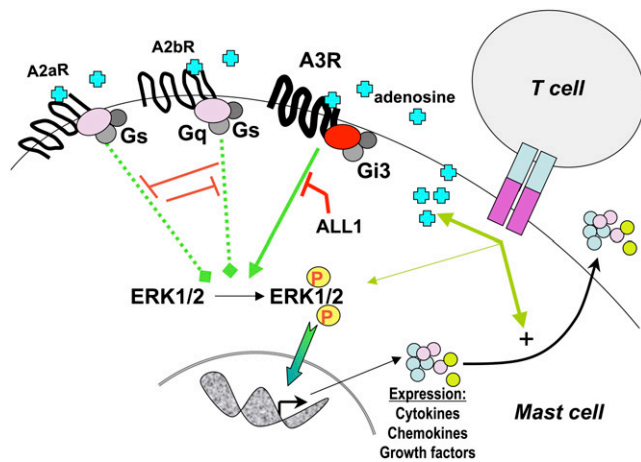


FIGURE 13. A model depicting the signaling pathways associated with cytokines/chemokines/growth factors formation in T cell activated mast cells. According to this model, contact with membranes, derived from activated T cells, stimulates ERK1/2 activation, causing the expression of cytokines, chemokines, and growth factors. Contact with the activated T cell membranes also provides signals for the release of these mediators. In addition, on contact with T cell membranes, adenosine is formed and released. By binding to its specific A2a, A2b, and A3 receptors, adenosine initiates a complex signaling cross-talk, whereby the A3R, by coupling to the G-protein Gi3, eventually remains the only AR that contributes to ERK1/2 signaling. This gives rise to ALL1 sensitive responses.

ALL1, confirming their mediation by Gi3. Notably, although binding to the A3R upregulated the genes listed previously, it failed to trigger any detectable release of their expressed proteins. Therefore, additional signals, which the A3R is incapable of eliciting, are most likely needed to accomplish the release process.

Using ALL1 also enabled us to identify the involvement of Gi3 in mediating mast cell activation by contact with T cell membranes. In particular, dissection of the signaling networks that are activated in mast cells on their contact with activated T cell-derived membranes has firmly established a role for Gi3 in T cell-induced ERK1/2 signaling. Furthermore, ALL1 sensitive signaling pathways were also prone to inhibition by either the A3R antagonist MRS1220 or A3R targeted siRNA, therefore identifying the A3R as the Gi3 upstream GPCR that plays a central role in mediating ERK1/2 signaling. This pathway evokes major regulatory functions, including control of mast cell proliferation and production of chemokines and cytokines. For example, RSK1, a downstream effector of ERK1/2 that is activated by the A3R and Gi3, regulates the transcription factor MITE, which is known to regulate growth and function of mast cells (22). In this study, we focused on the production and secretion of cytokines and chemokines. Clearly, not all signaling pathways that are initiated by contact with the T cell membranes are mediated by the A3R (see model, Fig. 13). However, we obtained evidence for the involvement of Gi3 signaling in the upregulation of genes, including cytokines, chemokines, and growth factors. Though the relative contribution of A3R/Gi3 signaling varied indicating the involvement of complex and unique networks in the regulation of gene transcription in mast cells undergoing inflammatory processes, the contribution of the A3R to a subset of processes (i.e., upregulation of IL3, CSF2, and osteopontin or secretion of MIF and IL-8) was significant. Therefore, our results provide unequivocal evidence for a role of the A3R in mediating signaling provoked in HMCs undergoing inflammatory processes. Notably, although studies in rodent mast cells and rodent animal models have firmly established the central role played by the A3R in mediating inflammatory processes,

including bronchoconstriction, hypotension, and cutaneous allergic reactions (23–26), the role of the A3R in humans remained controversial. The literature split between reports revealing proinflammatory functions of the A3R, as opposed to evidence demonstrating anti-inflammatory and protective outcomes have led Gessi et al. to coin the A3R as the “Dr. Jekyll and Mr. Hyde” of the inflammatory reaction (27). Indeed, unlike most GPCRs, the rodent and human A3R share only 74% homology, therefore giving rise to the possibility that these receptors may couple to different G proteins and accordingly yield differential signaling. However, we clearly demonstrate that both the rodent and human A3R couple to Gi3. Moreover, we show that similarly to the rodent A3R, the human receptor stimulates MAP kinases and provokes proinflammatory signals, including upregulation of inflammatory genes. Moreover, we show that the A3R is activated and functional in HMCs undergoing inflammatory processes. These results are in agreement with previous results, which demonstrated that activation of the A3R in HMC-1 cells induces the expression, though not secretion, of angiopoietin-2 (16). Activation of the A2bR could enhance secretion of the angiogenic factor, but a maximal response depended on the simultaneous activation of both the A2b and A3 receptors, indicating that A2b and A3 receptors function cooperatively (16). Consistent with this notion, the A3R is upregulated in mast cells of chronic obstructive pulmonary disease patients (28). Interestingly, in mast cells derived from human cord blood only an inhibitory A2aR-mediated function could be detected (29). Hence, although the reasons for these discrepancies are presently unknown, these data clearly indicate that the HMC-1 cells are more physiologically relevant for adenosine studies than the cord blood-derived mast cells.

In summary, in view of the central role played by adenosine in mediating inflammatory reactions alone, or as a modulator of activation by other stimuli including the FcεRI and T cells, our studies mark the A3R and its downstream Gi3 protein as key players in mediating mast cell-dependent inflammatory processes. In particular, our studies implicate Gi3 as playing a central role in asthma, where the mast cell A3R and adenosine hyper-responsiveness are a major concern (24). Studies aimed at identification of additional upstream targets affected by Gi3, as well as searching after additional signaling outputs downstream of Gi3, are currently underway. Finally, we show that all three ARs that are expressed in mast cells are connected by a complex cross-talk. The biological activity of adenosine, acting alone or within a framework event generating its formation, is therefore mediated by a complex interaction network elicited by distinct ARs, which are most likely temporally and spatially regulated. Accordingly, A3R function may vary as a function of the concomitant signaling outputs received at a given time window. It is therefore of major importance to delineate the signaling networks elicited by the A3R under defined physiological settings.

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Disclosures

The authors have no financial conflicts of interest.

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