

Differential Role for Mitogen-Activated Protein Kinases in IgE-Dependent Signaling in Human Peripheral Blood Basophils: In Contrast to p38 MAPK, c-Jun N-Terminal Kinase Is Poorly Expressed and Does Not Appear to Control Mediator Release

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Key Words

Basophils · Signal transduction · Inflammatory mediators · IgE

Abstract

Background: Exposure of human basophils to allergens results in a rapid secretion of histamine, LTC₄, IL-4 and IL-13, which dominate both the symptomology of allergic diseases and support the underlying Th2/IgE predominance associated with these reactions. The IgE-dependent release of these mediators in basophils crucially involves PI 3-kinase and the subsequent activation of p38 MAPK and ERK1&2. Here, we investigated the role of the third major member of the mitogen activated kinase family, namely the c-Jun amino terminal kinase (JNK), which is rapidly activated following IgE receptor cross-linking in murine mast cells. **Methods:** Human basophils were highly purified by magnetic cell sorting. The activities of various intracellular signaling components, in basophils that had been stimulated under various conditions, were assessed by Western blotting. Mediator secretions were also determined using either spectrofluorometric analysis (histamine) or ELISA (LTC₄, IL-4 and IL-13). **Results:** Our results show that while JNK

is moderately expressed in human basophils, it is not consistently phosphorylated upon anti-IgE stimulation. Phosphorylation of the transcription factor c-Jun, a downstream target of JNK, was also undetected in contrast to p38 MAPK and ERK1&2, which were clearly activated following anti-IgE stimulation of the cells. Additionally, inhibitors of the JNK pathway failed to prevent basophil mediator release and had no effect on the phosphorylation of p38 MAPK or ERK1&2 at concentrations which were specific for JNK blockade. **Conclusions:** These data suggest major differences in utilizing various members of the mitogen-activated kinase family in the signal transduction cascade of IgE-receptor-bearing cells.

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Introduction

There is growing evidence suggesting a major role for basophils in the pathogenesis of allergic responses where they have been demonstrated to invade tissues of the lung, nose and skin in patients with allergic asthma [1–3], rhinitis [4] and atopic dermatitis [5], respectively. This is supported further by their capacity to release histamine and

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LTC₄, which contribute to the clinical symptoms of allergy, and by the generation of the Th2-type cytokines IL-4 and IL-13 [reviewed in ref. 6, 7]. These cytokines are crucial for the development of late-phase allergic responses since they increase VCAM-1 expression [8, 9] leading to further granulocyte infiltration into affected tissues. Additionally, the relatively early generation of IL-4 (within 4 h) from basophils may facilitate differentiation of Th cells, which comigrate into tissues during allergic reactions, to a Th2 phenotype, resulting in promotion of greater IgE synthesis in B cells [reviewed in ref. 10]. It has also been shown that basophil-derived IL-4 and IL-13 can directly modulate switching of B cell immunoglobulin synthesis to IgE [11] thus underlining their potential as immunomodulators as well as effector cells of allergic responses.

The above basophil mediators are rapidly secreted after allergen-induced cross-linking of IgE, bound to high-affinity IgE receptors (FcεRI) on the cells, resulting in a complex signaling cascade culminating in degranulation, lipid metabolism and de novo synthesis of cytokines. The early events following FcεRI aggregation are known to involve the successive activation of Lyn and Syk tyrosine kinases, phospholipase C and PI 3-kinase as well as the mobilization of intracellular calcium and influx of the ion into the cells. By contrast, far less is known regarding the role of downstream intermediary signals of the mitogen-activated protein kinase (MAPK) family, which subsequently control the activation of transcription factors, cytoskeletal rearrangement and lipid metabolism necessary for mediator synthesis and secretion.

Together with others, we have recently shown that the classical MAPK pathway, resulting in ERK1&2 phosphorylation only governs LTC₄ release from basophils whereas p38 MAPK activation is necessary for the release of all major basophil mediator types [12–15]. However, the role of the third main member of the MAPK family, namely the c-Jun amino terminal kinase (JNK) – sometimes also called stress-activated protein kinase (SAPK) –, and its activation of the transcription factor c-Jun, has not yet been characterized in human basophils. Reasons to believe that the JNK pathway is involved in controlling IgE-dependent signaling have so far been found in mast cells, where JNK is phosphorylated within minutes after FcεRI stimulation and regulates TNF-α synthesis [16–18]. However, while mast cells may, in some respects, be considered as tissue-fixed counterparts of basophils, they display many differences in function compared with basophils.

In this report we have characterized the differences in ERK1&2, p38 MAPK and JNK phosphorylation in human basophils following IgE-dependent stimulation and studied the effects of specific inhibitors of these pathways on mediator release from these cells. Our results show that neither JNK nor its downstream target, c-Jun, are measurably phosphorylated in activated basophils, in stark contrast to ERK1&2 and p38 MAPK. By comparison, JNK and c-Jun were strongly expressed and phosphorylated after IgE-dependent activation in the RBL-2H3 mast cell line. These results emphasize major differences in the expression and activation of various members of the MAPK family between human basophils and mast cell lines.

Materials and Methods

Reagents and Materials

The following reagents were purchased: anti-mouse Ig horseradish peroxidase, ECL+plus (Amersham, UK); PD098059 (Alexis, Grünberg, Germany); IL-4 ELISA (BioSource, Camarillo, Calif., USA); Anthra[1,9-*cd*]pyrazol-6(2H)-one 1,9-pyrazoloanthrone (JNK inhibitor-II), DNP-BSA, H-Gly-Arg-Lys-Lys-Arg-Agr-Gln-Arg-Arg-Arg-Pro-Pro-Arg-Pro-Lys-Arg-Pro-Thr-Thr-Leu-Asn-Leu-Phe-Pro-Gln-Val-Pro-Arg-Ser-Gln-Asp-Thr-NH₂ (JNK inhibitor-I), rabbit anti-phospho SAPK/JNK, SB203580 (Calbiochem, Bad Soden, Germany); IL-13 ELISA (Boehringer Ingelheim Bioproducts, Heidelberg, Germany); LTC₄ ELISA (Immunotech, Hamburg, Germany); MACS basophil isolation kit (Miltenyi Biotec, Bergisch Gladbach, Germany); anti-rabbit Ig horseradish peroxidase, mouse anti-phospho JNK (monoclonal antibody), rabbit anti-c-Jun, rabbit anti-ERK1&2, rabbit anti-JNK, rabbit anti-p38 MAPK, phosphorylated and non-phosphorylated JNK control cell extracts from human embryonic kidney 293 cells, rabbit anti-phospho c-Jun, rabbit anti-phospho p38 MAPK (New England Biolabs GmbH, Frankfurt am Main, Germany); Ficoll-Paque (Pharmacia Biotech, Uppsala, Sweden), anti-human IgE, mouse anti-diphosphorylated ERK1&2, calcium ionophore A23187, fMLP, 2× concentrated Laemmli sample buffer, mouse monoclonal dinitrophenyl (DNP)-specific IgE, rhIL-3 (Sigma, St. Louis, Mo., USA). All other standard reagents were purchased at the highest grade obtainable.

Basophil Purification

Human basophils were obtained from buffy coats (leukocyte concentrate of 450 ml venous blood) which were freshly prepared from routine blood donations from healthy blood donors. Basophils were purified using a three-step procedure involving Ficoll density centrifugation followed by elutriation and negative selection using magnetic cell sorting (MACS basophil isolation kit; Miltenyi Biotec). We have previously reported this technique for basophil enrichment and observed that there are no functional differences between purified and unpurified basophils using this negative selection procedure [19]. Basophils obtained were 95.6 ± 0.6% pure, as determined by Alcian blue staining, with a mean yield of 5.2 ± 0.4 × 10⁶ basophils per donor and more than 96% viable cells, as judged by Trypan blue exclusion.

RBL-2H3 Cells

RBL-2H3 cells were kindly provided by Prof. Ulrich Amon (Pso-
risol Therapiezentrum, Hersbruck) and maintained in Eagle's mini-
mum essential medium (EMEM) containing 10% fetal calf serum,
glutamine and 1% penicillin/streptomycin. Trypsinized cells were
plated onto 12-well plastic plates at 1.5×10^5 cells per well and incu-
bated overnight with dinitrophenyl (DNP)-specific IgE (1 μ g/ml).
Following three washes with medium, cells were then incubated for
15 min in medium with or without various concentrations of JNK
inhibitor (JNKi-I or JNKi-II) followed by stimulation with DNP-
BSA (100 ng/ml). After stimulation (15 or 30 min), the supernatants
were discarded and the cells lysed (reagents described below) for
SDS-PAGE and Western Blot analysis.

Kinase Inhibitors

SB203580, PD098059 and JNKi-II were dissolved in DMSO, all
at 0.1 M, and subsequently diluted in HEPES-buffered-Tyrodé's
solution shortly before use. JNKi-I was dissolved in distilled water
and diluted in HEPES-buffered-Tyrodé's solution. At working dilu-
tions of the compounds (10 μ M or less) no effects were observed due
to DMSO in the absence of the drugs.

Cell Stimulation and Mediator Assay

Purified basophils (generally $1-2 \times 10^5$ nucleated cells per tube,
in three experiments $1-2 \times 10^6$ cells) were resuspended in HEPES-
buffered-Tyrodé's solution (400 μ l per tube), supplemented with
1 mM CaCl₂ and, following a short warming period (5 min), cells
were preincubated with various concentrations of the kinase antago-
nists for 15 min before stimulation with anti-IgE (1/30,000 dilution).
Controls consisted of cells incubated with the stimulus in the absence
of the inhibitors and cells incubated in buffer alone. In mediator
release studies, basophils were stimulated for either 30 min, for hista-
mine and LTC₄ release, or 5 h, for the secretion of IL-4 and IL-13.
Cells stimulated for 30 min were then centrifuged and a portion of
the supernatants (100 μ l) was assayed for LTC₄ content by ELISA
(minimum sensitivity 10.3 pg/ml), according to the manufacturer's
instructions. Histamine contents in the remaining supernatants to-
gether with the cell pellets, which were diluted accordingly and lysed
with perchloric acid (4%), were measured using a spectrofluoromet-
ric autoanalyser. Percentage histamine releases were determined
from the total histamine content in the sum of pellet and supernatant
tubes. Basophils incubated for 5 h were centrifuged and the respec-
tive release of IL-4 and IL-13 in the supernatants was assayed by
ELISA (according to the manufacturer's instructions). The minimum
sensitivity of the ELISAs were 3 pg/ml for IL-4 and 1 pg/ml for
IL-13. Statistical significances were assessed using a paired Student's
t test.

Western Blot Analysis

Purified basophils (>95% pure, 2×10^5 /sample) were treated as
indicated and incubated at 37°C for varying periods of time. After
these times, reactions were terminated rapidly by placing tubes in ice
after which all tubes were immediately centrifuged, supernatants
removed for histamine analysis (in order to gauge the response) and
cell pellets lysed by vigorous mixing in 15 μ l lysis buffer (50 mM
Tris-HCl, pH 7.5, 5 mM EDTA, 10 mM EGTA, 5 mM DTT, 1%
Nonidet P-40, 1 mM PMSF, 100 μ g/ml aprotinin, 20 μ g/ml leupep-
tin and 10 mM benzamidine). The samples were then stored at
-80°C prior to Western blotting. An equal volume of 2 $\times$ concen-
trated Laemmli sample buffer was added to the lysed cell pellets

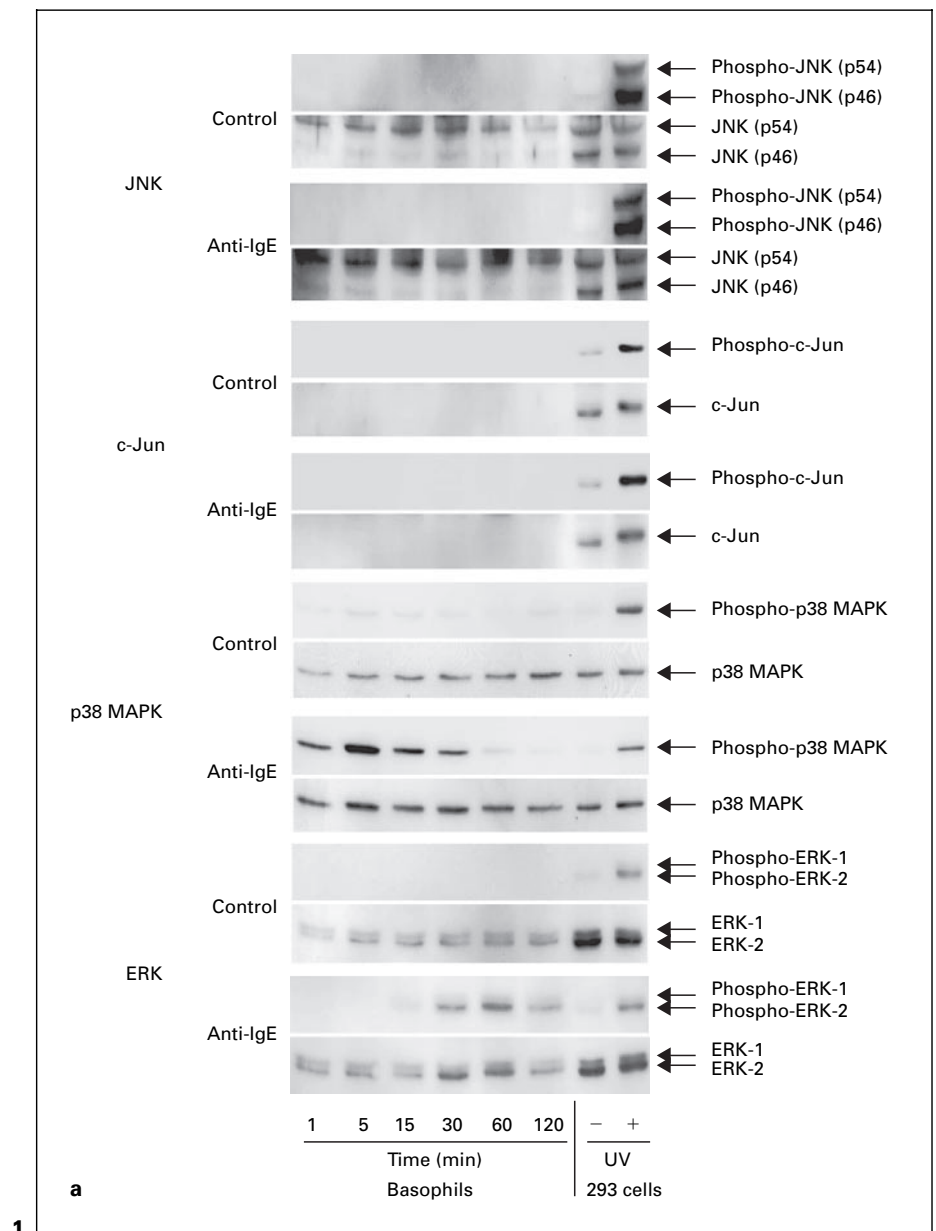
which were then boiled for 3 min. Proteins were separated by 12%
SDS-PAGE and blotted onto nitro-cellulose membranes. Commer-
cial extracts of 293 cells, that had been treated with or without UV
irradiation, were included in each SDS-PAGE as positive and nega-
tive controls for mitogen-activated protein kinase expression and
phosphorylation. The molecular weight (MW) of the resolved pro-
teins was visualized using prestained MW rainbow markers which
were also included for each SDS-PAGE run. Membranes were
blocked for 4 h in 5% skimmed milk in TBST buffer (20 mM Tris-
HCl, pH 7.5, 137 mM NaCl, 0.1% Tween 20) with gentle agitation.
Following 3×5 min washes in TBST, membranes were incubated
overnight (at 4°C) with specific mouse or rabbit antibodies to either
phosphorylated or total ERK1&2, JNK, c-Jun or p38 MAPK. Fol-
lowing incubation with the various primary antibodies, membranes
were successively washed (4×5 min, TBST) and incubated with
either anti-mouse or anti-rabbit Ig horse radish peroxidase for 2 h
with gentle agitation. After washing off unbound secondary anti-
body, proteins were visualized by autoradiography according to the
manufacturer's instructions (ECL+plus). Comparisons of phosphory-
lated (activated) signaling proteins were made not only on the basis
of equal cell numbers but by observing the even signal intensity of
total expression. Densitometric analysis was performed by dividing
the signal intensity of the phosphorylated proteins with total expres-
sions for each respective band; the results were expressed as percent-
ages of the maximum phosphorylation intensity obtained for each
antibody employed. After detection, membranes were stripped for
15-25 min using Re-blot plus reagent (Chemicon international),
washed in TBST (4×5 min) and reprobed.

Results

JNK, c-Jun, p38 MAPK and ERK1&2 Are Differentially Phosphorylated in Anti-IgE-Stimulated Human Basophils

We first wanted to determine if JNK is phosphorylated
upon anti-IgE stimulation of highly purified human baso-
phils over various periods of up to 2 h. This time frame is
sufficient for observing signaling processes governing me-
diator release, since all basophil mediator types have ei-
ther been secreted fully by then (e.g. histamine and LTC₄)
or, in the case of IL-4 and IL-13 synthesis, their secretion
from the cells has already commenced. The results shown
in figure 1a, however, demonstrate that while JNK is con-
stitutively expressed in basophils, it is not clearly phos-
phorylated by anti-IgE triggering.

To rule out any conceivable problems with our detec-
tion system, we first compared the expression and activa-
tion of JNK with human embryonic kidney 293 cells (293
cells) which had been stimulated with UV irradiation.
These control cell extracts showed marked phosphoryla-
tion of JNK in UV-treated 293 cells and a strong signal of
total JNK expression in both UV-treated and untreated
293 cells. A comparison of several different polyclonal



and monoclonal anti-phospho-JNK antibodies, from various commercial sources, confirmed the lack of anti-IgE-induced JNK activation in basophils. A weak basal phosphorylation of JNK was seen in 4 out of 12 experiments, where we doubled the recommended antibody concentration, but this resulted in multiple nonspecific banding and, in any case, did not clearly increase in intensity in stimulated basophils. Further evidence that JNK does not participate in IgE-dependent basophil signaling was given by the lack of c-Jun phosphorylation in basophils. Addi-

tionally, c-Jun, which is a major downstream target for JNK, was not usually expressed in sufficient amounts to be observed in basophils, except for 3 out of the 12 experiments (with very weak signal intensity), in contrast to 293 cell controls.

Other secretagogues for basophil activation, such as IL-3, calcium ionophore A23187 or the bacterial peptide fMLP, also failed to give rise to any marked JNK phosphorylation within a 2-hour stimulation period (results not shown). We also explored the possibility that JNK

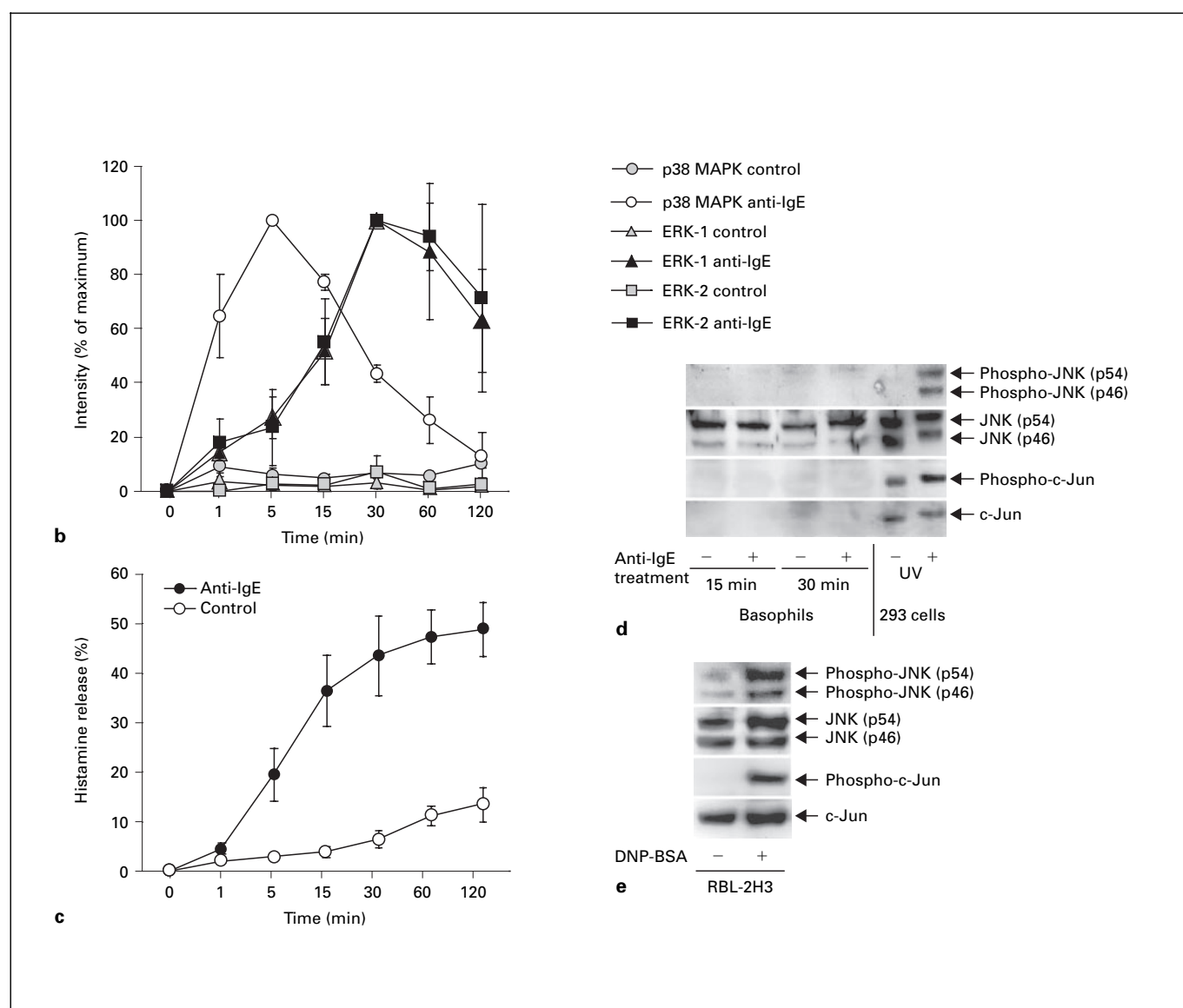


Fig. 1. **a** Kinetics of JNK, c-JUN, p38 MAPK and ERK1&2 activity in purified human basophils stimulated with anti-IgE or buffer (control). Basophils were stimulated at 37°C for the indicated times followed by cell lysis and separation of cellular proteins by SDS-PAGE for Western blotting using various phospho-antibodies against the respective proteins. After ECL detection, membranes were stripped and reprobed for the respective proteins using antibodies detecting total expression. These steps were repeated successively so that all the proteins (both phosphorylated and total expression) could be analyzed for the same blot. Human embryonic kidney 293 cells, that had been either treated (+) or untreated (-) with UV irradiation, were used as controls and the results are shown on the right-hand side of each blot for comparison. The blot shows a representative experiment out of three, where basophils had been stimulated for the time points up to 2 h (six other experiments were performed for shorter intervals; data not shown). **b** Densitometric analysis of mean intensities $\pm$ SEM from three separate experiments. Results are expressed as percentage intensities for phospho-p38 MAPK and phospho-

ERK1&2 relative to the maximum signal obtained by anti-IgE stimulation. (These results are normalized to the staining obtained with the respective non-phospho-antibodies by dividing the scanned values of the phospho-kinases by the total kinase expression. JNK and c-Jun are not shown since no consistent activation was observed.) **c** Corresponding histamine release measured in the supernatants of the above experiments $\pm$ SEM. Releases are expressed as percentages of the amount of the amine present in the supernatant to that in basophils lysed at the same density as used for the Western blots. **d** Effect of increasing basophil density to 1.5×10^6 lysed cells per lane for the conditions shown and in comparison to 293 cells. Results are representative of three separate experiments using high basophil densities. **e** Expression of JNK and c-Jun in RBL-2H3 cells (sensitized with DNP-specific IgE) that had been incubated for 30 min either in the presence or absence of DNP-BSA. SDS-PAGE was performed using cell lysates equivalent to 50,000 cells per lane. Results show representative data from three separate experiments.

may only be activated by cellular stress. To this purpose we exposed basophils to either 40 J/m² UV-C irradiation or 100 μ M hydrogen peroxide, both of which are known to cause JNK phosphorylation in diverse cell types. However, we did not detect any JNK phosphorylation, although unpublished data show that other members of MAPK, such as p38 MAPK and ERK1&2, are strongly activated by hydrogen peroxide using the same toxic concentration.

In comparison to the lack of JNK and c-Jun activity, both p38 MAPK and ERK1&2 were transiently phosphorylated in basophils stimulated with anti-IgE (fig. 1a). Phosphorylation of these kinases was also observed in the 293 cell control extracts at an intensity similar to that seen for activated basophils, and serves as further evidence for the comparative lack of JNK and c-Jun in basophil activation. Densitometric analysis of these Western blots displayed a stark difference in the kinetics of basophil-derived p38 MAPK and ERK1&2 activation (fig. 1b). In agreement with previous findings [14, 15], p38 MAPK was rapidly phosphorylated, reaching a peak at 5 min before eventually diminishing to basal levels at 2 h after anti-IgE stimulation. The kinetics of ERK activation was clearly slower than for p38 MAPK, where both ERK1 and ERK2 phosphorylations peaked between 30 min and 1 h before clearly receding at 2 h of incubation with anti-IgE. In some experiments ERK phosphorylation was detected even after 5 min of stimulation with anti-IgE, but generally maximum activity was seen at around 30 min. This is in agreement with our previous findings [12], but, in our hands, ERK phosphorylation in basophils was slower than that reported by another group [14]. To gauge the mediator-releasing activity of basophils used in the above experiments we also measured their ability to secrete histamine. Figure 1c shows that basophil histamine release was essentially complete within 30 min of IgE-dependent stimulation.

Since we were unable to detect JNK phosphorylation or c-Jun expression in basophils employed at a density of 2×10^5 cells per lane (fig. 1a), we increased the number of basophils to 1.5×10^6 cells per lane (fig. 1d). (Given the limited number of basophils we could purify from each donor we only looked at 15-min and 30-min stimulation periods.) Although these cells released considerable amounts of histamine to anti-IgE stimulation (not shown), we still could not detect phosphorylated JNK and failed to see any expression of c-Jun. Finally, we compared these data to the RBL-2H3 mast cell line (sensitized with DNP-specific IgE) and clearly observed both the expression of JNK and c-Jun as well as phosphorylation of

both proteins following 30-min IgE-dependent stimulation using DNP-BSA (fig. 1e). Activation of JNK and c-Jun was also observed after 15 min of stimulation (not shown).

Specific Inhibitors of JNK Do Not Abrogate IgE-Dependent Mediator Secretion in Basophils

Since we could not convincingly detect JNK activity in basophils, we next wanted to confirm whether JNK blockade, using specific JNK antagonists, also has no effect on mediator production and release from these cells. We thus compared the actions of two different compounds: the peptide-derived cell-permeable JNKi-I (which blocks downstream c-Jun activity) [20] and the ATP-competitive cell-permeable JNKi-II (which directly inhibits JNK phosphorylation) [21]. Our results showed that neither compound caused any significant decrease in anti-IgE-stimulated release of either histamine (fig. 2a), LTC₄ (fig. 2b), IL-4 (fig. 2c) or IL-13 (fig. 2d) at concentrations known for specific JNK blockade. A slight potentiation was observed regarding IL-4 production using both types of JNK inhibitor, but this was not statistically significant and was not observed for IL-13 release except for the highest concentration of JNKi-II. At 10 μ M, JNKi-II also inhibited histamine and LTC₄ release, but this is unlikely to be due to specific JNK antagonism since it also blocks other MAPK members at this high concentration. Furthermore, given an IC₅₀ of 40 nM for this compound and an IC₅₀ of 1 μ M for JNKi-I, one would have expected to see a change in mediator release at much lower concentrations of these agents. In our experiments, however, the results clearly show that blockade of JNK or its downstream actions on c-Jun have no inhibitory effect on IgE-dependent release of basophil-derived mediators.

In addition to the above, we also assessed the capacity of the above antagonists to affect cell viability using basophils that had been incubated with the drugs for a period of 5 h (which were also used in parallel for measuring cytokine release in the supernatants). However, after this relatively long period of incubation, neither JNKi-I nor JNKi-II affected basophil viability compared with either unstimulated controls or cells treated with anti-IgE alone (data not shown). As a control for the efficacy of the above inhibitors, we could demonstrate that both compounds reduced the downstream phosphorylation of c-Jun in DNP-BSA-stimulated RBL-2H2 cells (fig. 3). In two experiments we also studied the effects of the compounds on JNK phosphorylation, which was inhibited by JNKi-II (at 1 and 10 μ M) but not by JNKi-I (not shown), in keeping with their properties mentioned above.

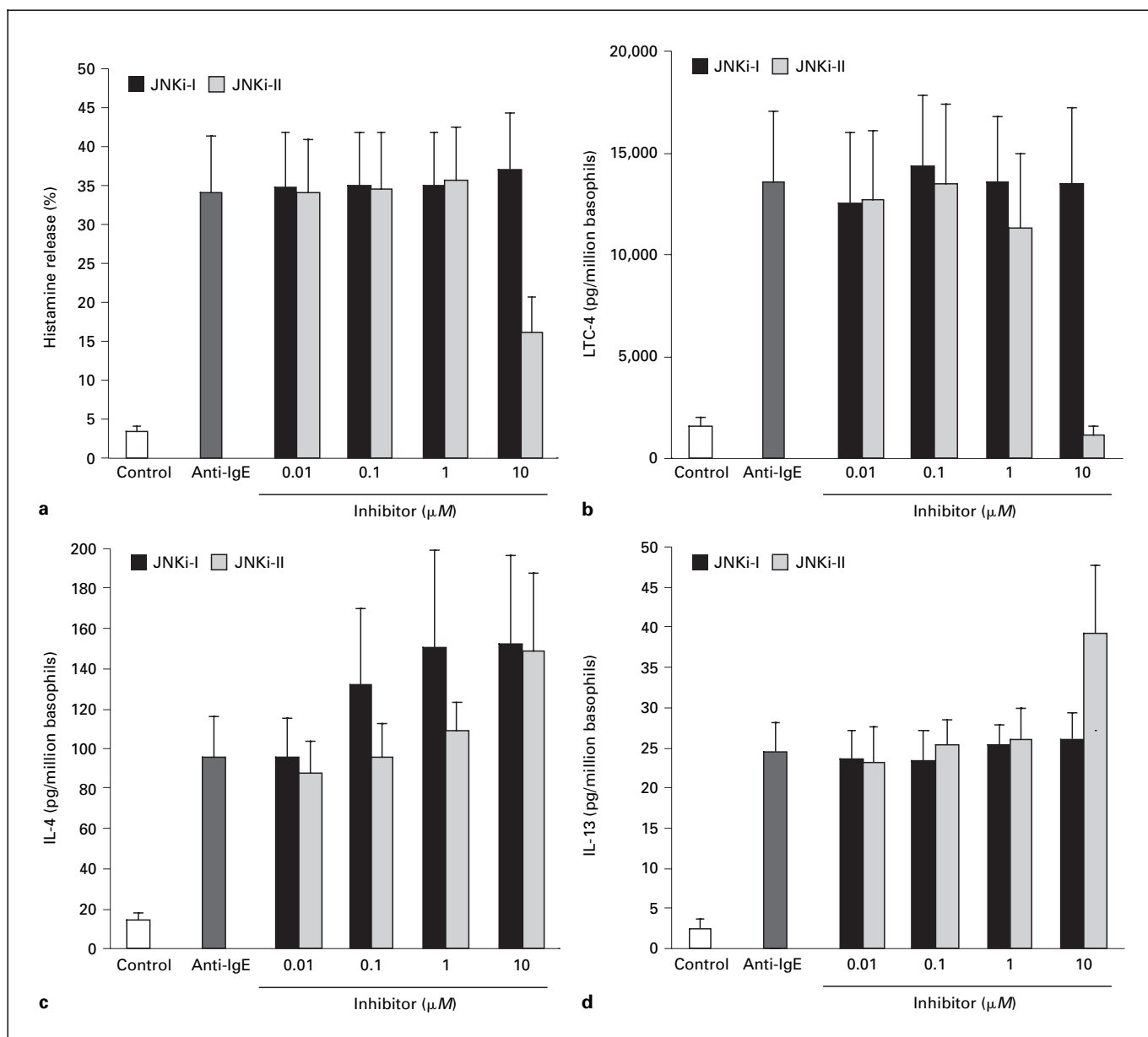


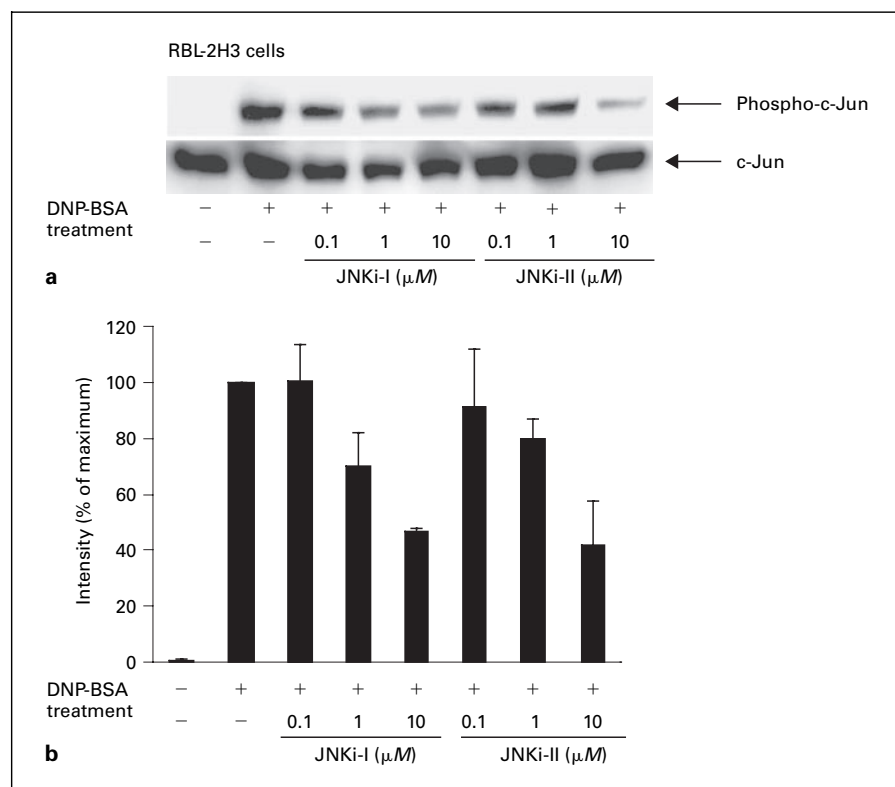
Fig. 2. Effects of the JNK-pathway antagonists (JNKi-I and JNKi-II) on anti-IgE induced histamine (a), LTC₄ (b), IL-4 (c) and IL-13 (d) release from basophils (n = 4–6). Histamine release is shown as percentage release ± SEM of the amount of the amine measured after 30 min of stimulation in the supernatant compared with total levels present in the sum of the supernatant and cell pellet. Supernatants were assayed for LTC₄, IL-4 and IL-13 releases by ELISA following either 30 min (LTC₄) or 5 h of stimulation (for cytokine secretion).

Effects of Specific Kinase Inhibitors on p38 MAPK and ERK1&2 Activity

We finally addressed whether the compounds used above, which inhibit JNK and c-Jun activity, have any effects on p38 MAPK or ERK1&2 phosphorylation in activated basophils (fig. 4a, b). Figure 4a shows that IgE-

dependent phosphorylations of p38 MAPK were largely unaffected by either JNKi-I or JNKi-II and that ERK1&2 activation was only reduced by JNKi-II at a concentration that was no longer specific for JNK blockade. In contrast, the p38 MAPK antagonist, SB203580, moderately inhibited the phosphorylation of both kinases, whereas the

Fig. 3. a Effects of JNKi-I and JNKi-II on c-Jun phosphorylation in RBL-2H3 cells stimulated with DNP-BSA (or medium for negative control). Protein loading is indicated by total c-Jun expressions. Lysates from approximately 50,000 cells per lane were employed. **b** Densitometric analysis of mean intensities $\pm$ SEM from three separate experiments. Results are expressed as percentage intensities for phospho-c-Jun relative to the maximum signal obtained by DNP-BSA stimulation. (These results are normalized to the staining obtained with the respective non-phospho-antibodies by dividing the scanned values of phospho-c-Jun by the total c-Jun expression.)



MEK antagonist (which specifically blocks ERK1&2 activity) affected only the phosphorylation of ERK1&2. A comparison of the densitometric analysis of the Western blots and histamine release from these experiments shows that only the blockade of p38 MAPK results in significantly reduced histamine release from basophils. In contrast, neither JNK inhibitors nor the abrogation of ERK1&2 phosphorylation by the MEK antagonist, PD098059, had any significant affect on the release of the amine.

Discussion

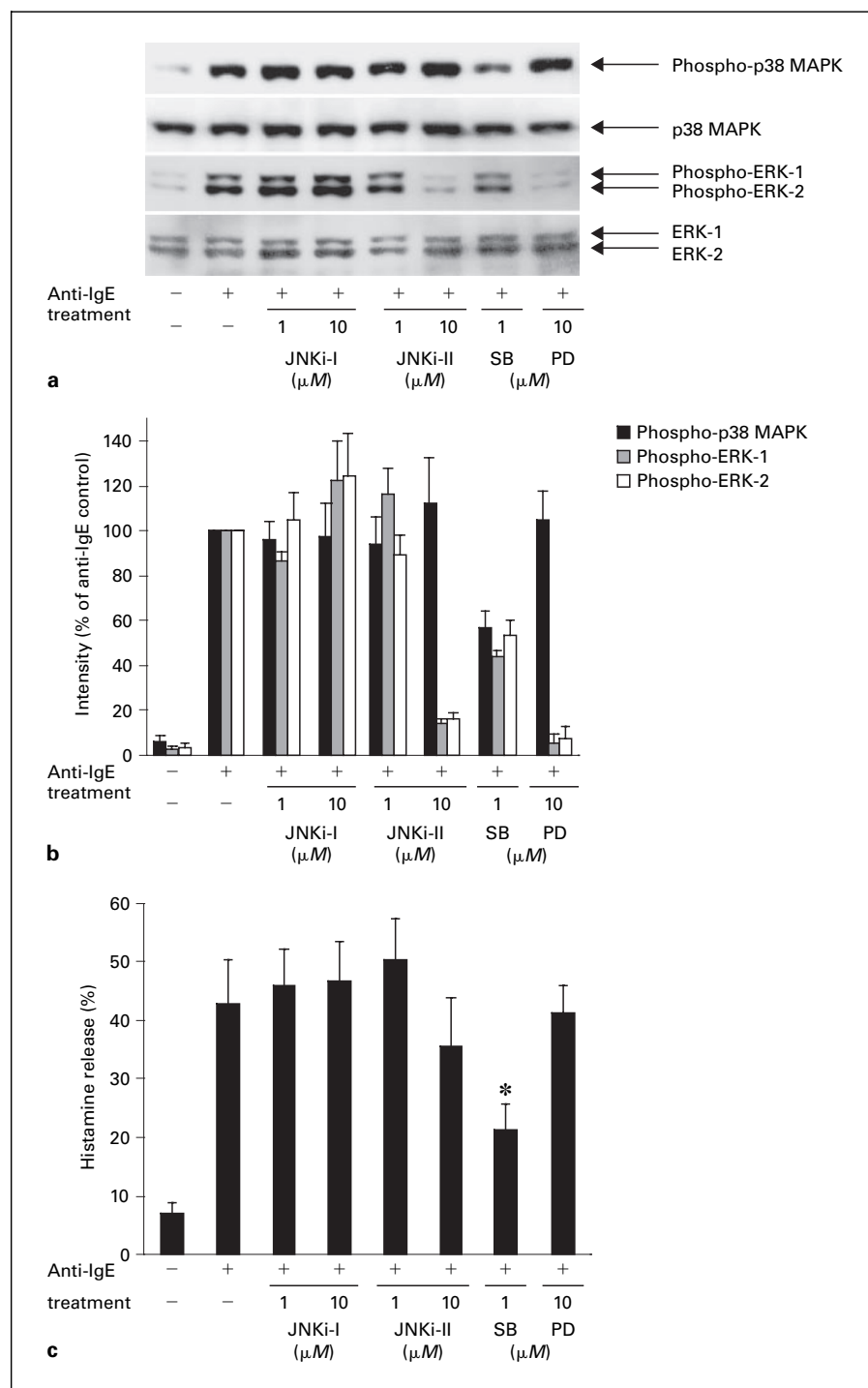
The results presented here demonstrate major differences between the three major members of the MAPK family, i.e. ERK1&2, p38 MAPK and JNK, as intermediary pathways controlling mediator release in basophils. Our data concerning JNK suggest, and to the best of our knowledge for the first time, a defunct role for the JNK-c-Jun pathway in mediator-related signaling in an FcεRI-positive cell. The evidence for this is based on three main findings: Firstly, that JNK, which was moderately expressed in basophils, was not phosphorylated above basal

levels (which were detected in only 4 out of 12 experiments and was not dependent on the cell density) over a period of 2 h by either anti-IgE or other stimuli which trigger mediator secretion from basophils. Secondly, this is supported by the lack of, or at least very weak, expression of the downstream transcription factor c-Jun, which was not seen to be phosphorylated in any experiment. Thirdly, that specific antagonists of the JNK pathway did not affect the release of various basophil mediators at concentrations where specific blockade would have occurred.

Our initial concerns regarding the sensitivity of the Western blot assays were ruled out by both increasing the basophil density and comparing with extracts from UV-stimulated and unstimulated 293 cells. These control cell extracts showed clear banding at the correct molecular range for all the proteins studied and showed comparable signal intensities for the phosphorylation of ERK1&2 and p38 MAPK as anti-IgE stimulated basophils. Furthermore, in comparison to basophils, the mast cell line RBL-2H3 clearly gave rise to phosphorylation of both JNK and c-Jun after IgE-dependent triggering.

The apparent lack of JNK involvement in basophils is in sharp contrast to mast cells, where, in addition to our

Fig. 4. a Effect of JNK, p38 MAPK and MEK antagonists on anti-IgE induced p38 MAPK and ERK1&2 phosphorylation from highly purified human basophils. Cells were preincubated with the drugs for 15 min before stimulation with anti-IgE for 15 min. PD098059 (PD) and SB203580 (SB), which specifically inhibit MEK and p38 MAPK, respectively, were employed as a positive control for ERK and p38 MAPK blockade. Even cell loading was determined using antibodies against total ERK and p38 MAPK. **b** Densitometric analysis of pooled intensities from 4 separate experiments, expressed as percentage $\pm$ SEM of maximum intensity obtained by anti-IgE alone for the respective phospho-p38 MAPK and phospho-ERK1&2. (Results were normalized by dividing the scanned values of the phosphokinases by total expressions.) **c** Histamine releases from the same experiments are shown as mean percentage releases $\pm$ SEM. * $p < 0.05$, significant reduction of histamine release compared with basophils incubated with anti-IgE alone using a paired Student's *t* test.



own data with RBL-2H3 cells, JNK has been reported to be phosphorylated using both IgE-dependent and -independent stimuli [16–18, 22–24]. With respect to mediator production, Ishizuka et al. [16] also showed that TNF α synthesis in murine mast cells is heavily dependent on

JNK activation, compared with a much lesser involvement of p38 MAPK. In our present study, focussing on human basophils, we saw no clear evidence that JNK participates in controlling mediator production and release.

We have expanded on our earlier observations to directly comparing phosphorylation of p38 MAPK with other members of the MAPK family over longer periods of stimulation than previously reported. We found that p38 MAPK, in contrast to JNK, is clearly and rapidly expressed in anti-IgE stimulated basophils (within 5 min) and remains active above basal levels for over 1 h after FcεRI triggering. In comparison, the phosphorylations of ERK1&2 generally require at least 30 min to reach a maximum, in our hands, before slowly diminishing. Although, this can vary: ERK activation is more rapid following basophil priming with IL-3 prior to IgE-dependent stimulation, as we previously showed [12], and in one study [14] the anti-IgE induced phosphorylation of ERK1&2 was considerably faster than observed in our laboratory. An explanation for these discrepancies may be due to donor variation in the amount of priming of basophils *in vivo* with IL-3, but further studies must verify this.

With respect to p38 MAPK and ERK1&2, only the blockade of p38 MAPK resulted in the abrogation of histamine release. This is in accordance with earlier studies, including our own, showing that specific p38 MAPK antagonists prevent the IgE-dependent release of all types of basophil mediators while the blockade of ERK1&2 activation restrictively only modulates the production of LTC₄ [12–15]. Thus, the emerging portrayal concerning the role of various MAPK pathways indicates that only p38 MAPK universally regulates basophil mediator signaling as opposed to ERK1&2 (involved only in LTC₄ synthesis) and JNK, which apparently has no clear function in this setting.

Further evidence supporting the fundamental differences in utilizing various MAPK pathways between basophils and their FcεRI tissue-fixed mast cell compatriots is the different sensitivities of MAPK components to PI 3-kinase control. PI 3-kinase is an essential component of IgE-dependent signaling and is closely associated to, and rapidly activated by, FcεRI aggregation [25, 26]. In basophils, PI 3-kinase is known to crucially control p38 MAPK as well as ERK1&2 activation and specific inhibition of the enzyme has also been reported to prevent both histamine release and the synthesis of LTC₄ and IL-4/IL-13 [12, 13, 15, 27]. In antigen-stimulated murine bone marrow-derived mast cells, however, it has been shown that blockade of PI 3-kinase only partially prevents p38 MAPK activation, and has even less action on ERK1&2, whereas JNK activity is totally abrogated [22].

At the level of mediator release, PI 3-kinase inhibitors also display more differential inhibitory properties in mast cells, depending on the mast cell type used, than the

complete cessation of secretion of all mediator classes from basophils, as we have previously shown [12]. Marquardt et al. [28] reported that PI 3-kinase inhibition blocks IgE-dependent and -independent histamine release from murine bone marrow-derived mast cells without affecting either cytokine or eicosanoid production. Another study, using the rat mast cell line RBL-2H3 showed, however, that cytokine production is reduced by PI 3-kinase blockade and that it is dependent on the downstream activity of p38 MAPK and JNK [29]. Together with our current findings, the above cited reports exemplify major differences in both the utilization of various MAPK pathways and their sensitivity to upstream PI 3-kinase input in controlling the three main mediator classes from different FcεRI-positive cells.

In summary, the results presented here show a lack of involvement of JNK and c-Jun in IgE-mediated basophil activation in comparison to p38 MAPK and ERK1&2. This highlights major differences in the contribution of various MAPK family members in controlling mediator secretion between basophils and other FcεRI-positive cells, a problem which may impact the future development of therapeutic agents targeting these signaling networks for the treatment of allergic disorders.

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