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FcεRI -INDUCED ACTIVATION BY LOW ANTIGEN CONCENTRATIONS RESULTS IN NUCLEAR SIGNALS IN THE ABSENCE OF DEGRANULATION

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Abstract

High affinity IgE receptor (FcεRI)-induced activation of mast cells results in degranulation and generation of leukotrienes and cytokines. FcεRI-induced mast cell activation was analyzed at a single cell basis using a rat basophilic leukemia (RBL-2H3) cell line transfected with a reporter plasmid containing three tandem NFAT (nuclear factor of activated T cells) binding sites fused to enhanced green fluorescent protein (GFP). Surprisingly, with this sensitive detection system, there is activation of IgE sensitized cells at concentrations of antigen as low as 10pg/ml, which was 10-fold lower than was detected by degranulation. There were differences in signaling pathways leading to degranulation compared to NFAT-mediated gene activation. Both signaling to NFAT activation and degranulation required Syk and calcineurin. However inhibitors of the phosphatidylinositol 3-kinase pathway blocked degranulation but did not NFAT activation. The results also indicate that NFAT was activated at lower intracellular signals compared to degranulation. Therefore, FcεRI activation can result in nuclear signals in the absence of the release of mediators.

Keywords

Mast cells; FcεRI; Signal transduction; NFAT signaling; Degranulation

INTRODUCTION

Mast cells play an important role in many immunological and inflammatory reactions. A role for these cells has been described in defense against bacteria, virus or parasites, the induction of immune responses and several autoimmune diseases (Marshall, 2004; Galli *et al.*, 2005). They are, however, best known as the critical cell in allergic inflammation during which they release an array of inflammatory mediators. These mediators include granular content and newly formed leukotrienes which are released in minutes and cytokines that are synthesized and released over several hours. Production of these cytokines depends on signals that are generated and transmitted to the nucleus that turn on their synthesis (Siraganian, 2003b; Gilfillan and Tkaczyk, 2006; Benhamou and Siraganian, 1992).

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The activation of mast cells for release of these mediators in allergic reactions depends on IgE and its high affinity receptor FcεRI present on the surface of these cells. FcεRIs are members of the immune receptor family that are present on many cells of the immune system. These receptors lack any intrinsic enzymatic activity, but consist of three subunits two of which have Immunoreceptor Tyrosine-based Activation Motifs (ITAM) in their cytoplasmic domain. Activation of these cells is initiated through FcεRI crosslinking and aggregation that results from binding of antigen to receptor bound IgE. This in turn results in tyrosine kinase Lyn phosphorylating tyrosine residues in the immunoreceptor tyrosine-based activation motif of the β and γ subunits of the receptor (Pribluda *et al.*, 1994; Jouvin *et al.*, 1994). Tyrosine phosphorylated receptor recruits the cytoplasmic protein tyrosine kinase Syk to the membrane which results in a conformational change in Syk, with an increase in its enzymatic activity (Zhang *et al.*, 1996; Kimura *et al.*, 1996). This leads to downstream propagation of signals such as tyrosine phosphorylation of phospholipase C-γ, LAT and SLP-76. The activated phospholipase catalyzes generation of inositol triphosphate, which releases Ca²⁺ from intracellular stores and activates store operated calcium channels for the influx of Ca²⁺ (Wang *et al.*, 2000; Saitoh *et al.*, 2000). This increase in intracellular calcium concentration not only leads to exocytosis of granules but also plays a critical role in signals for *de novo* synthesis of cytokines (Siraganian, 2003a). Increase in intracellular calcium activates the Ca²⁺/calmodulin dependent serine phosphatase calcineurin, which then dephosphorylates the nuclear factor of activated T cells (NFAT) (Hutchinson and McCloskey, 1995; Shaw *et al.*, 1995; Luo *et al.*, 1996). Dephosphorylation of these serine residues leads to the exposure of the NFAT nuclear-localization signal and consequent nuclear import of this molecule. Once in the nucleus the REL-homology domain in NFAT proteins bind to specific DNA binding sites and cooperate with other transcription partners to induce expression of various cytokine genes (Crabtree, 2001; Okamura *et al.*, 2000; Macian, 2005). In mast cells, NFAT is essential for the generation of TNF-α and IL-13 but not IL-6 (Klein *et al.*, 2006). Therefore, NFAT plays a crucial role in transmitting signals to the nucleus in many immune cells including T and B cells.

In this study NFAT-mediated GFP expression was used as an indicator to analyze single cell responses after FcεRI-induced stimulation. RBL-2H3 rat mast cells were transfected with a plasmid containing a cDNA with three tandem NFAT binding sites fused to enhanced green fluorescent protein (GFP). These cells were cloned to obtain a cell line that became GFP positive after FcεRI stimulation. Surprisingly in these cells there was NFAT activation with IgE-antigen stimulation at concentrations of antigen that were 10-fold lower than was detected by degranulation. Signaling to NFAT activation appeared to involve the same molecules as for degranulation; however there were some differences such as the requirement for phosphatidylinositol 3-kinase (PI 3-kinase) activation and levels of calcium influx. In addition, the results suggest that NFAT nuclear activation required lower levels of intracellular signals compared to degranulation. These findings could explain some biological functions of mast cells that do not correlate or require release of inflammatory mediators but depend on nuclear signals resulting in mast cell survival, growth and differentiation.

MATERIALS AND METHODS

Materials

PI 3-kinase inhibitors, wortmannin and LY294002, Src family kinase inhibitor PP2 and calcineurin inhibitor cyclosporin A (CsA) were from Tocris Bioscience (Tocris Cookson Ltd., Ellisville, MO). Syk inhibitor II (2-(2-Aminoethylamino)-4-(3-trifluoromethylanilino)-pyrimidine-5-carboxamide, dihydrochloride, dihydrate) was from Calbiochem (EMD Bioscience, La Jolla, CA). Hapten (DNP-EACA; N-2,4-DNP-epsilon-amino-n-caproic acid), EDTA (ethylenediaminetetraacetic acid) and NCTC 109 medium were from Sigma (Sigma, St. Louis, MO). Anti-phospho Erk was from Cell Signaling (Cell Signaling Technology Inc.,

Danvers, MA), anti-Orai1 was from LifeSpan Biosciences (LifeSpan Biosciences Inc., Seattle, WA). Mouse IL-3 and stem cell factor were from BioSource International (Camarillo, CA).

Cell culture

RBL-2H3 and Syk negative C4A2 cells have been described previously (Zhang *et al.*, 2007). These cell lines and transfected cells were cultured as monolayer in Minimum Essential Media (MEM) supplemented with 15% heat-inactivated FBS (fetal bovine serum), penicillin, streptomycin, amphotericin, and glutamine. Mouse MC9 cells were grown in suspension in DMEM supplemented with 20% heat-inactivated FBS, 4 mM L-glutamine, 5×10^{-5} M 2-ME, 10% NCTC 109 medium, 0.1 mM nonessential amino acids, 1 mM sodium pyruvate, antibiotics, 25 ng/ml IL-3, and 25 ng/ml stem cell factor.

For the selection of NFAT reporter cells, RBL-2H3 and C4A2 cells were cotransfected with linearized plasmid containing three tandem NFAT binding sites fused to eGFP (Ohtsuka *et al.*, 2004) and pSV-Neo plasmid at a ratio of 10:1 using Amaxa Cell Line Nucleofector Kit T (Biosystems, Middletown, CT). Transfected cells were selected by geneticin (G-418) and then cloned to select cells that had maximum GFP fluorescence response after stimulation with FcεRI-antigen (RBL-2H3 cells) or calcium ionophore (C4A2 cells).

For transient transfection of Syk in Syk deficient C4A2 reporter cells, transfection was with the cDNA of Syk cloned into pEAK12 plasmid or with the same plasmid with no insert using Amaxa Cell Line Nucleofector Kit T. Transfected cells were sensitized and activated as described below.

Activation and measurement of β-hexosaminidase release

Cells were grown in 24 well plates (2×10^5 /well) and sensitized by overnight culture with antigen-specific mAb anti-trinitrophenyl IgE 142 (IgE-TNP) at 0.16 ug/ml. Cell monolayers were washed 2 times with MEM containing 2% fetal calf serum and activated with several concentrations of antigen (DNP-HSA, 31 molecules of dinitrophenyl coupled per molecule of human serum albumin) for 1 h or overnight at 37°C in a humidified atmosphere with 5% CO₂. Supernatants from each well were removed after 1 h for the measurement of β-hexosaminidase. After 16–20 h of culture cells were harvested using Cellstripper (Mediatech, Inc, Herndon, VA), washed twice in PBS and GFP expression analyzed with a Fluorescence Activated Cell Sorter (FacsCalibur; BD Biosciences). In some experiments (supplemental Fig S1B) cells were sensitized with mAb anti-DNP48 (anti-DNP-IgE). To study the effect of inhibitors or hapten (DNP-EACA; N-2,4-DNP-epsilon-amino-n-caproic acid), cells were sensitized as above with IgE-TNP and inhibitors or hapten were added at the indicated times. In some experiments cells were stimulated for 1 h, washed with MEM containing 2% fetal calf serum, before adding inhibitors or hapten in the same medium and analyzed after 20 h of culture. For microscopy, cells were grown in 24 well plates and after 16 to 20 h activation images were acquired using a 10X objective and a sensiCam PCO CCD camera on an Olympus IX50 microscope, using Till Photonics Imaging System Software v 4.00. Parameters were kept constant throughout, phase contrast and fluorescence images were merged.

Degranulation was determined by measuring release of the granule marker, β-hexosaminidase. Briefly, duplicate aliquots from supernatants of the activated cells (50ul) were mixed with an equal amount (50ul) of substrate (1.3 mg/ml *p*-nitrophenyl-*N*-acetyl-β-D-glucosaminide), in 0.1 M citrate, pH 4.5. After incubation for 60 min at 37°C, 50 μl of 0.4 M glycine buffer (0.4M glycine, 0.4M NaOH, 0.4M NaCl), pH 10.5, was added to stop the reaction, and absorbance was measured at 405 nm. Experiments were independently repeated at least three times, and the results were comparable in all cases. Values were expressed as percentage of intracellular β-hexosaminidase that was released into the medium. Release of TNF-α was determined by

ELISA assay (rat TNF- α , R&D Systems, Minneapolis, MN) using recombinant rat TNF- α as a standard and control. The assay was performed according to the instructions of the manufacturer.

Measurement of single cell Ca²⁺ response

Cells (2×10^5 /well) were cultured overnight with IgE in 24-well culture plates. Monolayers were then washed twice with loading medium Medium 199 (Biofluid, Inc, Rockville, Md), supplemented with 2 mM CaCl₂, 0.1% BSA and 250 μ M sulfinpyrazone, and loaded with 2 μ M Fura-2 AM for 1 h at 37°C. After loading, cells were transferred to room temperature for 30 min and then washed and stimulated in working medium (containing 2 mM CaCl₂, 10 mM Tris pH 7.4, 0.01% BSA and 250 μ M sulfinpyrazone). Fura-2 fluorescence was measured in single cells, as described previously (Zhang *et al.*, 2002), using a Tillvision imaging system (Till Photononic GmbH, Grafelfing, Germany) attached to an inverted Olympus microscope with a Zeiss Fluor 10 $\times$ objective.

Immunoblotting

Cells were washed twice with cold PBS and immediately boiled with sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer. For Erk phosphorylation the cells were stimulated for 3 min before the washes. Proteins were separated by SDS-PAGE and electrotransferred to polyvinylidene difluoride membranes. The blots were probed with anti-phospho-Erk, Orai1 and Fc ϵ RI β subunit as a loading control. Proteins were visualized by SuperSignal West Pico Luminol/Enhancer Solution (Pierce, Rockford, IL).

siRNA Orai1 assay

Cells were transfected with small interference RNA ON-TARGETplus set of 4 target sequences for ORAI1 (GCCAUAAGACGGACCGACA, UCAAGAGGCAGGCGGGACA, CAACAGCAAUCCGGAGCUU and CCUGUGGCCUGGUGUUUUAU) (Dharmacon, Lafayette, CO) using Amaxa Cell Line 96-well Nucleofector Kit SF. Transfected cells were sensitized and activated as described above.

Statistics

Results were expressed as mean $\pm$ SE. Statistical analysis was by non-parametric t-test using GraphPad Prism 5 software.

RESULTS

Fc ϵ RI activation induces GFP expression at low antigen concentrations

Signaling triggered by aggregation of Fc ϵ RI receptors in mast cells is usually determined by measuring release of granular products which quantifies the response of a group of cells (Barsumian *et al.*, 1981). Although single cell responses can be determined by analysis of receptor-induced calcium influx, such measurements are transient. To develop a stable reporter system, we used an NFAT-driven GFP expression. RBL-2H3 mast cells were transfected with a plasmid containing cDNA of three tandem NFAT binding sites fused to GFP. A stable cell line (VB9) was selected that had maximum GFP fluorescence response after stimulation with Fc ϵ RI-antigen. Stimulation of IgE sensitized cells with antigen resulted in GFP expression which was detectable at 3 h and reached maximum fluorescence by 16–20 h. Similarly, addition of either calcium ionophore or thapsigargin induced GFP expression. An unexpected observation was that there was strong receptor-induced GFP expression at low antigen concentrations (Fig 1A). As seen by fluorescent microscopy there was some increased GFP expression on addition of 1 pg/ml of antigen that became more pronounced with increasing concentrations of antigen (for example at 10 and 100 pg/ml). The intensity of the GFP

fluorescence also increased with increasing concentrations of antigen (Supplemental Fig S1A). The NFAT and degranulation response of cells were compared by activating sensitized cells with antigen and sampling supernatants after 1 h for β -hexosaminidase release and after 20 h of culture measuring the expression of GFP by FACS (Fig 1B). There was clear difference in the two responses; degranulation was prominent at 1 and 10 ng/ml of antigen whereas GFP expression was prominent at concentrations that were ~10-fold lower. Removal of antigen from the culture medium after 1 h had minimal effects on subsequent GFP expression (Fig 1B). The degranulation response was analyzed after 60 min incubation with antigen whereas the NFAT induced GFP synthesis was after overnight culture. However, the difference between the NFAT and degranulation responses was not a result of the difference in the length of time that cells were exposed to antigen. The residual β -hexosaminidase remaining in cells cultured for 20 h with the different antigen concentrations gave similar results to the release measured at 1 hour. Testing of the mouse MC9 cells and another subline of RBL-2H3 cells that were stably transfected with cDNA of three tandem NFAT binding sites fused to GFP confirmed that stimulation with low antigen concentrations results in effective NFAT activation (Supplemental Fig S1B). There was also greater sensitivity of the NFAT-GFP response compared to degranulation when cells were sensitized with low concentrations of IgE (Supplemental Fig S2A). Cells sensitized with 3, 10 or 30 ng/ml of IgE and stimulated with different concentrations of antigen all had more dramatic NFAT-induced GFP expression than degranulation. Results were again similar when cells were sensitized with a different anti-DNP IgE that is less effective in activating cells for degranulation (Supplemental Fig S2B). These results indicate that Fc ϵ RI stimulation efficiently induced NFAT activation and therefore nuclear signals at IgE-antigen concentrations that did not result in detectable degranulation.

Low antigen concentration-induced NFAT activation requires Syk

A number of biochemical steps are involved in the pathway from receptor aggregation to degranulation. We therefore investigated whether there are differences between signaling for NFAT activation at low antigen concentrations and that which result in degranulation. Syk and Src family tyrosine kinases play an important role in Fc ϵ RI-mediated signaling; therefore inhibitors of these enzymes were used as a first step to investigate their function in low antigen induced NFAT activation. Inhibitors of Src kinases (PP2) and Syk (Syk inhibitor II) blocked receptor-induced GFP expression and release of β -hexosaminidase in a dose dependent manner (Fig 2A and B). However, the GFP response required 5–10-fold higher concentrations of inhibitors suggesting that NFAT nuclear activation required lower levels of intracellular signals.

Syk is essential for Fc ϵ RI-mediated signaling to degranulation (Zhang *et al.*, 1996; Zhang *et al.*, 2002). Therefore, to confirm a role for Syk in NFAT activation, we used a Syk-deficient cell line, C4A2. From these cells, a NFAT-GFP reporter cell line was prepared that had a robust GFP expression after incubation with calcium ionophore. There was no Fc ϵ RI-induced degranulation or GFP expression in these cells Syk deficient cells. Transient transfection of Syk in these cells reconstituted receptor-induced GFP expression and this was clearly observed at low antigen concentrations (Fig 2C), even though these cells were not selected and cloned for efficient Fc ϵ RI-induced stimulation. These results indicate that low antigen-induced NFAT activation was dependent on Syk.

The phosphatidylinositol-3-kinase inhibitors did not block antigen-induced NFAT activation

Inhibitors of phosphatidylinositol 3-kinase (PI 3-kinase) block Fc ϵ RI induced increase in intracellular calcium and degranulation (Marquardt *et al.*, 1996; Yano *et al.*, 1993). As has been observed previously, the PI 3-kinase inhibitor LY294002 blocked antigen induced degranulation (Fig 2D). Surprisingly there was no inhibition of NFAT-induced GFP expression at any of the concentrations tested. Results were similar with wortmannin, a different PI 3-

kinase inhibitor (data not shown). These observations indicate that NFAT activation but not degranulation is independent of PI 3-kinase activation.

Role of changes in intracellular calcium in low antigen induced NFAT activation

Activation of NFAT depends on an increase in intracellular calcium which activates the phosphatase calcineurin that dephosphorylates NFAT that then translocates to the nucleus to initiate transcription (Gwack *et al.*, 2007). PI 3-kinase is thought to play an important role in immune receptor-induced calcium influx (Scharenberg *et al.*, 1998). However, the results with the PI 3-kinase inhibitors raised questions about the role of changes in intracellular calcium in antigen induced NFAT activation. Fura-2 loaded cells were stimulated with different antigen concentrations to monitor single cell changes in intracellular calcium (Fig 3). When sensitized cells were stimulated with 100 ng/ml of antigen there was a very rapid increase in intracellular calcium, with ~45% of cells responding within the first 1 min and nearly all cells responding by 9 min. As the concentration of antigen was decreased to 10 ng/ml or 1 ng/ml, there was a longer lag period between stimulation and the onset of a response and a corresponding decrease in fraction of cells that responded during 30 min of calcium measurements. For example, at 1 ng/ml few cells responded within the first 10 min of stimulation, and by 30 min only $12 \pm 4\%$ of cells had an increase in $[Ca^{2+}]_i$. In the cells that responded with an increase in intracellular calcium, there were also oscillations in these levels of intracellular calcium. At 0.1 ng/ml of Ag stimulation there was no detectable calcium influx during 30 min of observation. After 1 h with antigen, cell monolayers used for calcium measurements were washed with culture medium and after overnight incubation analyzed by FACS for GFP expression (Fig 3 inset). Even though the calcium influx was very slow or undetectable at low antigen concentrations, there was high GFP expression observed 20 h later. Therefore, an increase in $[Ca^{2+}]_i$ was not detectable during 30 min of calcium measurements at low antigen concentrations that resulted in NFAT activation.

The activation of calcineurin requires a rise in intracellular calcium; however the fura-2 studies did not detect such an increase with low antigen concentrations where there was NFAT activation. Cyclosporin A (CsA), an inhibitor that blocks calcineurin mediated dephosphorylation of NFAT, was used to clarify the role of calcineurin in this pathway (Martinez-Martinez and Redondo, 2004). Previous experiments have shown that CsA blocks mast cell responses (Trenn *et al.*, 1989; Hultsch *et al.*, 1990; Hultsch *et al.*, 1991; Cirillo *et al.*, 1990). There was no NFAT activation by any antigen concentration when cells were stimulated in the presence of CsA (Fig 4A). There was also complete inhibition of degranulation by addition of CsA. However, addition of CsA (1 μ M) after 1 h of antigen stimulation only partially decreased NFAT induced GFP expression with a decrease in both the percent positive cells and intensity of fluorescence. To further define the role of Ca^{2+} in antigen-induced NFAT activation, cells were stimulated for 1 h in medium lacking calcium or magnesium but containing 0.05 mM EDTA and then replaced with regular culture medium containing Ca^{2+} with or without CsA for 20 h (Fig 4B). In the absence of extracellular calcium, antigen-induced increase of $[Ca^{2+}]_i$ during this first hour would only be due to the release of Ca^{2+} from intracellular stores. There was some decrease in GFP expression in these cells stimulated with antigen in absence of calcium for 1 h and then cultured overnight in medium containing calcium. However, when CsA was added to the media to inhibit calcineurin during the overnight culture in calcium containing media, there was complete inhibition of GFP expression. These results suggest that the pathway to NFAT activation due to the intracellular calcium release does not proceed beyond calcineurin. It could also indicate that receptor-induced signals are generated throughout 20 h of culture. The inhibition of GFP expression by CsA indicates that activation of calcineurin is essential for NFAT activation at all antigen concentrations and that optimal NFAT responses require this activation even after the first hour of stimulation.

Persistence of FcεRI-mediated signaling during 20h culture period

The partial decrease in NFAT activation when CsA was added 1 h after antigen exposure suggested that there could be continued receptor-mediated signaling during the following 20 h of culture. In contrast, washing cells after 1 h of antigen exposure (albeit probably not completely removing antigen) did not reduce NFAT activated GFP expression (Fig 1A and 4A). The following experiments tried to resolve the contribution of receptor-mediated signaling after the first 1 h of incubation to NFAT induced GFP expression.

Sensitized cells were incubated with antigen for 1 h then washed and further cultured with or without 50 μM hapten (Fig 4C). Addition of hapten decreased NFAT-induced GFP expression observed at 20 h. This decrease by hapten was similar to what was observed by the addition of CsA after 1 h of antigen. As expected, the NFAT response was completely blocked when hapten was added before antigen and left throughout the period of culture. These results indicate that the predominant signals for NFAT activation occur during the first hour of antigen exposure, however there is continued signaling during subsequent 20 h of culture.

Orai1 has been suggested to be the store-operated Ca^{2+} release-activated Ca^{2+} (CRAC) channel for the influx of extracellular calcium after cell activation. We therefore tested the possible role of Orai1 (CRAM1) by using siRNA to reduce its expression. Orai1 protein levels decreased ~50% after transfection with a pool of siRNAs and/or two individual sequences from this pool (Supplemental Fig S3)). Both degranulation and NFAT activation were decreased by this reduced Orai1 expression, however inhibition of degranulation was greater, (~40%) than the decrease in GFP expression (~5% to 10% decrease). These results again suggest that the intracellular signals that results in degranulation are more sensitive to inhibition than are those that result in NFAT activation.

FcεRI activation at low antigen concentrations induces Erk phosphorylation and TNFα release

Degranulation as well as cytokine production induced by FcεRI stimulation are mediated downstream of calcium influx by activation of pathways that include MAP kinases such as ERK and p38. There was phosphorylation of Erk at low antigen concentrations (Fig 5A). This was clearly apparent by the densitometry of the immunoblots (Fig 5B); although the major increase in Erk phosphorylation was with the higher antigen concentrations. By gene array analysis there was ≥ two fold change in 93 transcripts including those for IL3 and IL16 at low antigen levels (data not shown). The NFAT transcription factor collaborates with other transcription factors to induce the generation of cytokines such as TNFα (16–18). Mast cells release both preformed and newly synthesized TNFα in a relatively short period of time after stimulation. There were detectable TNFα released 2 hours after antigen stimulation (Fig 5C), however the amount of TNFα released after 0.001 and 0.01 ng/ml of antigen although greater than background was not statistically different than the controls. The results suggest that cytokine release requires cooperation of multiple transcription factors and parallels more with the optimal Erk phosphorylation.

DISCUSSION

NFAT-induced GFP expression was used in this study as an indicator of single cell response to FcεRI stimulation. Unexpectedly, there was NFAT activation in the absence of detectable degranulation such as at low antigen concentrations. This signaling to NFAT activation utilized some of the same signaling molecules, such as Syk and calcineurin, involved in degranulation but did not require phosphatidylinositol-3 kinase activation (PI 3-kinase). However, NFAT activation and therefore nuclear signals appeared to require lower levels of intracellular signals.

The NFAT transcription factors are highly phosphorylated proteins localized in the cytoplasm of resting cells. Once cells are stimulated the increase in intracellular calcium activates calcineurin, a calmodulin-dependent serine/threonine phosphatase, resulting in dephosphorylation of NFAT proteins (Macian, 2005; Hogan *et al.*, 2003). The ensuing conformational change of NFAT allows nuclear transport of these molecules resulting in gene transcription (Okamura *et al.*, 2000). An increase in intracellular calcium is essential for dephosphorylation of NFAT. In the present experiments high antigen concentrations induced an increase in intracellular calcium as measured by fura-2 fluorescence, while changes were not detectable at low antigen concentrations which induced GFP expression. However, we assume that there were still increases in intracellular calcium. As antigen concentration was decreased there was an increase in the lag time between addition of antigen and onset of changes in $[Ca^{2+}]_i$. Therefore, at low antigen concentrations changes in $[Ca^{2+}]_i$ could have occurred at a slow rate or over a longer period of time than the calcium measurements. Also such small changes in $[Ca^{2+}]_i$ could be buffered by fura-2. Prolonged low increases in $[Ca^{2+}]_i$ is sufficient for NFAT activation in B cells (Dolmetsch *et al.*, 1997). In the present experiments, there was prominent oscillations in $[Ca^{2+}]_i$ after Fc ϵ RI stimulation, such oscillations are known to activate NFAT and appear to enhance signaling efficiency at low levels of stimulation in other immune cells (Dolmetsch *et al.*, 1998; Li *et al.*, 1998). The present results suggest that there are differences in the rate or amplitude of intracellular calcium signals generated by low antigen compared to that by higher antigen concentrations which could explain the capacity of only higher level stimulation to result in degranulation.

PI 3-kinase catalyses phosphatidylinositol-3,4,-bisphosphate and phosphatidylinositol-3,4,5-trisphosphate (PtdIns(3,4,5)P₃) synthesis which then recruits pleckstrin homology domain containing molecules such as BTK, Akt and phospholipase C to the plasma membrane (Scharenberg *et al.*, 1998; Franke *et al.*, 1997). PI 3-kinase activation is linked to Fc ϵ RI stimulation as demonstrated by observations that PI 3-kinase inhibitors block degranulation, (Yano *et al.*, 1993; Yano *et al.*, 1995; Barker *et al.*, 1995) activation of mitogen-activated protein kinases and generation of some but not all cytokines (Ishizuka *et al.*, 1997; Marquardt *et al.*, 1996). Similarly gene inactivation of p110 δ PI 3-kinase impairs Fc ϵ RI-mediated degranulation and cytokine generation (Ali *et al.*, 2004). However, PI 3-kinase inhibitors do not block completely the calcium response (Barker *et al.*, 1999; Ching *et al.*, 2001; Sil *et al.*, 2007), as they appear to block only store-operated Ca²⁺ channel without inhibiting release of calcium from intracellular stores (Barker *et al.*, 1998). Our data would therefore suggest that NFAT activation is a result of low levels of an increase in intracellular calcium and clearly distinguishes calcium requirements for degranulation from those of NFAT activation.

The prominent finding in these experiments was the robust NFAT-GFP response of cells at very low antigen concentrations compared to degranulation. In these experiments the degranulation response was analyzed after 60 min incubation whereas NFAT activation that resulted in GFP synthesis required at least 8 h. However, the difference in the length of time that cells were exposed to antigen cannot fully explain the difference between the NFAT and degranulation responses. There was only a slight decrease in NFAT-GFP response when antigen was removed after 1 h of incubation although persistent cell bound antigen could still form new cross-links and activate signaling pathways even after washing cells. Similarly, GFP expression was still pronounced even after addition of hapten to block this continued signaling after the first hour of incubation. There was also only a slight decrease in NFAT-GFP response when CsA was added after 1 h of incubation to block further activity of calcineurin. All these manipulations had similar effects on the NFAT response with low and high antigen concentrations. This strongly suggests that robust NFAT-GFP responses at very low antigen concentrations are not simply a result of prolonged receptor-induced signaling. Nevertheless, the first hour of cell/antigen interaction was critical in activating NFAT induced GFP expression for both low and high antigen expression.

The present experiments suggest that there were continued receptor-initiated intracellular signals beyond the first hour of incubation. As described above, removal of antigen from cultures after 1 hour with or without addition of hapten decreased the eventual GFP response. Similarly addition of CsA after 1 h also decreased NFAT activation. NFAT-GFP responses were only slightly decreased if cells were incubated with antigen in the absence of calcium during the first 60 min. However, this response was completely inhibited by addition of CsA to block calcineurin during the rest of the incubation period. Therefore, in these cells signaling had not proceeded beyond calcineurin in the absence of calcium influx. When the first incubation was in regular medium containing calcium, CsA only partially decreased NFAT-GFP response still suggesting a role for calcineurin throughout the period of culture. In parallel experiments, washing cells after 1 h followed by addition of hapten also decreased to the same extent the eventual NFAT-GFP response. This suggests that signaling is probably related to continued disassembly-assembly by multivalent antigen interacting with receptor-bound IgE to form new cross-links and initiate intracellular signals throughout 20 h of culture. Therefore, continued receptor-antigen interactions contribute to the generation of nuclear signals.

There were similarities in the signaling pathways that results in degranulation and NFAT activation; inhibitors of Syk and Src kinases blocked both degranulation and NFAT activation. Syk is essential for FcεRI-induced increase in intracellular calcium, degranulation (Zhang *et al.*, 1996) and as shown by the present experiments was required for NFAT activation. There also was clear evidence for a requirement for an increase in intracellular calcium for the NFAT response. However, an obvious difference in the pathways was the role of PI 3-kinase which was essential for degranulation but not for NFAT activation. Nonetheless no difference in signaling pathways to NFAT could be detected at very low compared to high antigen concentrations indicating that intracellular signals that result in NFAT activation at low and high antigen concentrations are identical.

Immune-receptor induced cell activation depends on formation of IgE-Ag complexes on the cell surface. The rate at which these complexes form depends on the concentration of IgE, Ag and affinity of their interactions; higher concentrations of Ag would be more capable of formatting such complexes. Low Ag levels would result in low receptor occupancy but in these experiments still activated NFAT but not degranulation. The difference between NFAT stimulation and degranulation suggests that signals for degranulation require much larger complexes than does that for NFAT activation. This was apparent when the system was manipulated such as by lowering IgE concentration, changing the number of DNP groups on antigen or by using a lower affinity IgE. The capacity of weak and strong signals to induce lymphokine secretion has been investigated in T cells and in mast cells. In T cells, weak receptor stimulation initiates calcium responses that are sufficient to induce molecules that are different from those induced after strong receptor stimulation (Badou *et al.*, 2001). In mast cells weak FcεRI stimulation with low antigen concentrations induced the lymphokine MCP-1 (monocyte chemoattractant protein 1) whereas interleukin 10 required stronger stimulation with higher antigen levels (Gonzalez-Espinosa *et al.*, 2003). Similar differences have also been noted when the level of intracellular signals is manipulated by changing the extent of receptor occupancy. A weak stimulus may also prime the signaling machinery so that cells become more sensitive for a subsequent stimulation.

The maturation, differentiation and survival of mast cells are regulated by growth factors and other cell-surface receptor induced signals. Prolonged contact with antigen, especially low levels, is more likely to be the situation that occurs in vivo unlike the short exposures that is used for in vitro experiments. Therefore, the findings of nuclear signals in the absence of degranulation could occur in vivo and provide an important signal for maturation and survival of mast cells.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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The abbreviations used are

FcεRI	high affinity IgE Fc receptor
RBL-2H3	rat basophilic leukemia cell line
GFP	green fluorescence protein
NFAT	nuclear factor of activated T cells
ITAM	immunoreceptor tyrosine-based activation motifs
PI 3Kinase	Phosphatidylinositol 3-kinase
CsA	cyclosporin A
MCP-1	monocyte chemoattractant protein 1
GM-CSF	granulocyte-macrophage colony-stimulating factor
TNF-α	tumor necrosis factor-alpha

References

- Ali K, Bilancio A, Thomas M, Pearce W, Gilfillan AM, Tkaczyk C, Kuehn N, Gray A, Giddings J, Peskett E, Fox R, Bruce I, Walker C, Sawyer C, Okkenhaug K, Finan P, Vanhaesebroeck B. Essential role for the p110delta phosphoinositide 3-kinase in the allergic response. *Nature* 2004;431:1007–1011. [PubMed: 15496927]
- Badou A, Savignac M, Moreau M, Leclerc C, Foucras G, Cassar G, Paulet P, Lagrange D, Druet P, Guery JC, Pelletier L. Weak TCR stimulation induces a calcium signal that triggers IL-4 synthesis, stronger TCR stimulation induces MAP kinases that control IFN-gamma production. *Eur J Immunol* 2001;31:2487–2496. [PubMed: 11500833]
- Barker SA, Caldwell KK, Hall A, Martinez AM, Pfeiffer JR, Oliver JM, Wilson BS. Wortmannin blocks lipid and protein kinase activities associated with PI 3-kinase and inhibits a subset of responses induced by Fc epsilon R1 cross-linking. *Mol Biol Cell* 1995;6:1145–1158. [PubMed: 8534912]

- Barker SA, Caldwell KK, Pfeiffer JR, Wilson BS. Wortmannin-sensitive phosphorylation, translocation, and activation of PLC gamma 1, but not PLC gamma 2, in antigen- stimulated RBL-2H3 mast cells. *Mol Biol Cell* 1998;9:483–496. [PubMed: 9450969]
- Barker SA, Lujan D, Wilson BS. Multiple roles for PI 3-kinase in the regulation of PLC gamma activity and Ca²⁺ mobilization in antigen-stimulated mast cells. *J Leukoc Biol* 1999;65:321–329. [PubMed: 10080534]
- Barsumian EL, Isersky C, Petrino MG, Siraganian RP. IgE-induced histamine release from rat basophilic leukemia cell lines: isolation of releasing and nonreleasing clones. *Eur J Immunol* 1981;11:317–323. [PubMed: 6166481]
- Benhamou M, Siraganian RP. Protein-tyrosine phosphorylation: an essential component of Fc epsilon RI signaling. *Immunol Today* 1992;13:195–197. [PubMed: 1320890]
- Ching TT, Hsu AL, Johnson AJ, Chen CS. Phosphoinositide 3-kinase facilitates antigen-stimulated Ca²⁺ + influx in RBL-2H3 mast cells via a phosphatidylinositol 3,4,5-trisphosphate-sensitive Ca²⁺ entry mechanism. *J Biol Chem* 2001;276:14814–14820. [PubMed: 11278575]
- Cirillo R, Triggiani M, Siri L, Ciccarelli A, Pettit GR, Condorelli M, Marone G. Cyclosporin A rapidly inhibits mediator release from human basophils presumably by interacting with cyclophilin. *J Immunol* 1990;144:3891–3897. [PubMed: 1692065]
- Crabtree GR. Calcium, calcineurin, and the control of transcription. *J Biol Chem* 2001;276:2313–2316. [PubMed: 11096121]
- Dolmetsch RE, Lewis RS, Goodnow CC, Healy JI. Differential activation of transcription factors induced by Ca²⁺ response amplitude and duration. *Nature* 1997;386:855–858. [PubMed: 9126747]
- Dolmetsch RE, Xu K, Lewis RS. Calcium oscillations increase the efficiency and specificity of gene expression. *Nature* 1998;392:933–936. [PubMed: 9582075]
- Franke TF, Kaplan DR, Cantley LC, Toker A. Direct regulation of the Akt proto-oncogene product by phosphatidylinositol-3,4-bisphosphate [see comments]. *Science* 1997;275:665–668. [PubMed: 9005852]
- Galli SJ, Nakae S, Tsai M. Mast cells in the development of adaptive immune responses. *Nat Immunol* 2005;6:135–142. [PubMed: 15662442]
- Gilfillan AM, Tkaczyk C. Integrated signalling pathways for mast-cell activation. *Nat Rev Immunol* 2006;6:218–230. [PubMed: 16470226]
- Gonzalez-Espinosa C, Odom S, Olivera A, Hobson JP, Martinez MEC, Oliveira-dos-Santos A, Barra L, Spiegel S, Penninger JM, Rivera J. Preferential signaling and induction of allergy-promoting lymphokines upon weak stimulation of the high affinity IgE receptor on mast cells. *J Exp Med* 2003;197:1453–1465. [PubMed: 12782712]
- Gwack Y, Feske S, Srikanth S, Hogan PG, Rao A. Signalling to transcription: store-operated Ca²⁺ entry and NFAT activation in lymphocytes. *Cell Calcium* 2007;42:145–156. [PubMed: 17572487]
- Hogan PG, Chen L, Nardone J, Rao A. Transcriptional regulation by calcium, calcineurin, and NFAT. *Genes Dev* 2003;17:2205–2232. [PubMed: 12975316]
- Hultsch T, Albers MW, Schreiber SL, Hohman RJ. Immunophilin ligands demonstrate common features of signal transduction leading to exocytosis or transcription. *Proc Natl Acad Sci USA* 1991;88:6229–6233. [PubMed: 1712484]
- Hultsch T, Rodriguez JL, Kaliner MA, Hohman RJ. Cyclosporin A inhibits degranulation of rat basophilic leukemia cells and human basophils. Inhibition of mediator release without affecting PI hydrolysis or Ca²⁺ fluxes. *J Immunol* 1990;144:2659–2664. [PubMed: 1690774]
- Hutchinson LE, McCloskey MA. Fc epsilon RI-mediated induction of nuclear factor of activated T-cells. *J Biol Chem* 1995;270:16333–16338. [PubMed: 7608202]
- Ishizuka T, Terada N, Gerwins P, Hamelmann E, Oshiba A, Fanger GR, Johnson GL, Gelfand EW. Mast cell tumor necrosis factor alpha production is regulated by MEK kinases. *Proc Natl Acad Sci U S A* 1997;94:6358–6363. [PubMed: 9177222]
- Jouvin MH, Adamczewski M, Numerof R, Letourneur O, Valle A, Kinet JP. Differential control of the tyrosine kinases Lyn and Syk by the two signaling chains of the high affinity immunoglobulin E receptor. *J Biol Chem* 1994;269:5918–5925. [PubMed: 8119935]

- Kimura T, Sakamoto H, Appella E, Siraganian RP. Conformational changes induced in the protein tyrosine kinase p72syk by tyrosine phosphorylation or by binding of phosphorylated immunoreceptor tyrosine-based activation motif peptides. *Mol Cell Biol* 1996;16:1471–1478. [PubMed: 8657120]
- Klein M, Klein-Hessling S, Palmetshofer A, Serfling E, Tertilt C, Bopp T, Heib V, Becker M, Taube C, Schild H, Schmitt E, Stassen M. Specific and redundant roles for NFAT transcription factors in the expression of mast cell-derived cytokines. *J Immunol* 2006;177:6667–6674. [PubMed: 17082579]
- Li WH, Llopis J, Whitney M, Zlokarnik G, Tsien RY. Cell-permeant caged InsP(3) ester shows that Ca²⁺ + spike frequency can optimize gene expression. *Nature* 1998;392:936–941. [PubMed: 9582076]
- Luo C, Shaw KT, Raghavan A, Aramburu J, Garcia-Cozar F, Perrino BA, Hogan PG, Rao A. Interaction of calcineurin with a domain of the transcription factor NFAT1 that controls nuclear import. *Proc Natl Acad Sci U S A* 1996;93:8907–8912. [PubMed: 8799126]
- Macian F. NFAT proteins: key regulators of T-cell development and function. *Nat Rev Immunol* 2005;5:472–484. [PubMed: 15928679]
- Marquardt DL, Alongi JL, Walker LL. The phosphatidylinositol 3-kinase inhibitor wortmannin blocks mast cell exocytosis but not IL-6 production. *J Immunol* 1996;156:1942–1945. [PubMed: 8596048]
- Marshall JS. Mast-cell responses to pathogens. *Nat Rev Immunol* 2004;4:787–799. [PubMed: 15459670]
- Martinez-Martinez S, Redondo JM. Inhibitors of the calcineurin/NFAT pathway. *Curr Med Chem* 2004;11:997–1007. [PubMed: 15078162]
- Ohtsuka M, Arase H, Takeuchi A, Yamasaki S, Shiina R, Suenaga T, Sakurai D, Yokosuka T, Arase N, Iwashima M, Kitamura T, Moriya H, Saito T. NFAM1, an immunoreceptor tyrosine-based activation motif-bearing molecule that regulates B cell development and signaling. *Proc Natl Acad Sci U S A* 2004;101:8126–8131. [PubMed: 15143214]
- Okamura H, Aramburu J, Garcia-Rodriguez C, Viola JP, Raghavan A, Tahlilani M, Zhang X, Qin J, Hogan PG, Rao A. Concerted dephosphorylation of the transcription factor NFAT1 induces a conformational switch that regulates transcriptional activity. *Mol Cell* 2000;6:539–550. [PubMed: 11030334]
- Pribluda VS, Pribluda C, Metzger H. Transphosphorylation as the mechanism by which the high-affinity receptor for IgE is phosphorylated upon aggregation. *Proc Natl Acad Sci USA* 1994;91:11246–11250. [PubMed: 7526393]
- Saitoh S, Arudchandran R, Manetz TS, Zhang WG, Sommers CL, Love PE, Rivera J, Samelson LE. LAT is essential for Fc epsilon RI-mediated mast cell activation. *Immunity* 2000;12:525–535. [PubMed: 10843385]
- Scharenberg AM, El-Hillal O, Fruman DA, Beitz LO, Li ZM, Lin SQ, Gout I, Cantley LC, Rawlings DJ, Kinet JP. Phosphatidylinositol-3,4,5-trisphosphate (PtdIns-3,4,5-P-3) Tec kinase-dependent calcium signaling pathway: a target for SHIP-mediated inhibitory signals. *EMBO J* 1998;17:1961–1972. [PubMed: 9524119]
- Shaw KT, Ho AM, Raghavan A, Kim J, Jain J, Park J, Sharma S, Rao A, Hogan PG. Immunosuppressive drugs prevent a rapid dephosphorylation of transcription factor NFAT1 in stimulated immune cells. *Proc Natl Acad Sci U S A* 1995;92:11205–11209. [PubMed: 7479966]
- Sil D, Lee JB, Luo D, Holowka D, Baird B. Trivalent ligands with rigid DNA spacers reveal structural requirements for IgE receptor signaling in RBL mast cells. *ACS Chem Biol* 2007;2:674–684. [PubMed: 18041817]
- Siraganian RP. Biochemical events in basophil/mast cell activation and mediator release. In: Adkinson, NF., Jr; Yunginger, JW.; Busse, WW.; Bochner, BS.; Holgate, ST.; Simons, FER., editors. *Middleton's Allergy Principles and Practice*. Mosby; Philadelphia, PA: 2003a. p. 243–278.
- Siraganian RP. Mast cell signal transduction from the high-affinity IgE receptor. *Curr Opin Immunol* 2003b;15:639–646. [PubMed: 14630197]
- Trenn G, Taffs R, Hohman R, Kincaid R, Shevach EM, Sitkovsky M. Biochemical characterization of the inhibitory effect of CsA on cytolytic T lymphocyte effector functions. *J Immunol* 1989;142:3796–3802. [PubMed: 2541201]
- Wang D, Feng J, Wen RR, Marine JC, Sangster MY, Parganas E, Hoffmeyer A, Jackson CW, Cleveland JL, Murray PJ, Ihle JN. Phospholipase C gamma 2 is essential in the functions of B cell and several Fc receptors. *Immunity* 2000;13:25–35. [PubMed: 10933392]

- Yano H, Agatsuma T, Nakanishi S, Saitoh Y, Fukui Y, Nonomura Y, Matsuda Y. Biochemical and pharmacological studies with KT7692 and LY294002 on the role of phosphatidylinositol 3-kinase in Fc epsilon RI- mediated signal transduction. *Biochem J* 1995;312:145–150. [PubMed: 7492304]
- Yano H, Nakanishi S, Kimura K, Hanai N, Saitoh Y, Fukui Y, Nonomura Y, Matsuda Y. Inhibition of histamine secretion by wortmannin through the blockade of phosphatidylinositol 3-kinase in RBL-2H3 cells. *J Biol Chem* 1993;268:25846–25856. [PubMed: 7503989]
- Zhang J, Berenstein E, Siraganian RP. Phosphorylation of Tyr342 in the linker region of Syk is critical for Fc epsilon RI signaling in mast cells. *Mol Cell Biol* 2002;22:8144–8154. [PubMed: 12417718]
- Zhang J, Berenstein EH, Evans RL, Siraganian RP. Transfection of Syk protein tyrosine kinase reconstitutes high affinity IgE receptor mediated degranulation in a Syk negative variant of rat basophilic leukemia RBL-2H3 cells. *J Exp Med* 1996;184:71–79. [PubMed: 8691151]
- Zhang J, Suzuki K, Hitomi T, Siraganian RP. TOM1L1 Is a Lyn Substrate Involved in Fc{epsilon}RI Signaling in Mast Cells. *J Biol Chem* 2007;282:37669–37677. [PubMed: 17977829]

Fig 1A

Fig 1B

Fig. 1.

FcεRI activation induces GFP expression at low antigen concentrations but not degranulation. (A) An RBL-2H3 cell line transfected with the NFAT-GFP reporter (VB9) was sensitized with IgE and then stimulated with the indicated concentrations of Ag (0.001 to 10 ng/ml). After 20 h, cells were observed in an inverted microscope and phase contrast and fluorescence images were merged. Images were acquired using a 10X objective and the sensiCam PCO CCD camera on an Olympus IX50 microscope, using Till Photonics Imaging System Software v 4.00. The results shown are representative of at least five independent experiments. Scale bar, 100μm. (B) IgE-sensitized VB9 cells were activated with the indicated concentrations of Ag for 1 h and supernatants from each well were removed for measurement of β-hexosaminidase release

and expression of GFP was determined by FACS after 20 h of culture. In one set, cells were washed twice after 1 h of antigen before further culture. The results are expressed as percent release of total cellular β -hexosaminidase or percent GFP positive cells. There was no significant difference in GFP response between cells either not washed or washed after 1 h of incubation except with 0.1 ng/ml of antigen ($P < 0.05$). Results are from five independent experiments and are presented as mean $\pm$ SE.

Fig. 2.

Low antigen-induced NFAT activation requires Src and Syk tyrosine kinases but not PI 3-kinase. Src kinase family inhibitor PP2 (A) and the inhibitor for Syk, Syk inhibitor II (B) blocked degranulation more effectively than NFAT activation. Cells (VB9) were incubated with the indicated concentrations of inhibitors for 10 min, antigen (1 or 10 ng/ml) was then added and supernatants from each well removed after 1 hr for measurement of β -hexosaminidase release. After 20 hr of culture, cells were analyzed by FACS for GFP expression. The results shown are representative of 3 independent experiments. (C) The C4A2 Syk negative cell line stably expressing NFAT-GFP reporter was transiently transfected with a plasmid containing Syk. Cells were sensitized with IgE, stimulated with the indicated

concentrations of antigen (Ag) and after 20 h analyzed by FACS for percent GFP positive cells. The results shown are representative of 2 independent experiments. EP = plasmid with no insert. (D) The VB9 cells were incubated with the indicated concentrations of the PI 3-kinase inhibitor, LY294002, for 10 min, antigen (1 or 10 ng/ml) was then added and supernatants from each well removed after 1 h for measurement of β -hexosaminidase release. After 20 h of culture, cells were analyzed by FACS for GFP expression. The results shown are the average of 5 independent experiments.

Ag	% Responded at (min)			Ca ²⁺ response	% GFP 20h
ng/ml	0-10	10-20	20-30	%	FACS
0.1	0	0	0	0	31.8±2.7
1	1±1	6±1	5±4	12±4	68.9±5.0
10	16±6	35±6	22±1	74±6	75.4±8.6
100	100±0	0	0	100	75.3±7.0

Fig. 3.

Low antigen concentrations did not induce detectable calcium influx but resulted in GFP expression. VB9 cells were loaded with Fura-2 and stimulated with the indicated concentrations of antigen at time indicated by the arrow. The response of 100 randomly chosen cells was monitored for 30 min and the plot shows the time at which there was a detectable increase in intracellular calcium. After 1 h with antigen, cell monolayers were washed and cultured overnight in regular culture medium. Expression of GFP was then evaluated by FACS analysis. Results are presented from two independent experiments. Inset table summarizes the two parts of the experiments; the FACS data at 20 h is shown as percentage of positive cells $\pm$ SE.

Fig. 4.

Calcium dependent steps and continued FcεRI signaling are required for maximal antigen induced NFAT expression of GFP. (A) The calcineurin inhibitor cyclosporin A blocked both degranulation and expression of GFP in VB9 cells. In the β-hexosaminidase experiment, 1 μM cyclosporin A (CsA) added 10 min before antigen dramatically inhibited release determined at 1 h. Addition of cyclosporin A (1 μM) after 1 h of antigen stimulation decreased NFAT induced GFP expression at 20 h (percent positive cells as determined by FACS) while addition of cyclosporin A before antigen inhibited GFP expression. The results shown are representative of 3 independent experiments. Data are shown as mean ± SE. (B) The role of Ca²⁺ in antigen-induced NFAT activation. Cells were stimulated for 1 h with the indicated concentrations of antigen in regular medium or medium without calcium or magnesium but containing 0.05 mM EDTA. Monolayers were washed twice with regular medium and then

incubated for 20 h with or without 1 μ M cyclosporin A. The results are from 2 independent experiments, data are shown as mean $\pm$ SE. (C) Continued Fc ϵ RI signaling during 20 h of incubation is required for maximal NFAT induced GFP expression. Cells (VB9) were incubated with the indicated concentrations of antigen (DNP-HSA) for 1 h, washed twice and then further cultured in medium with or without 50 μ M hapten. There was no response in cells preincubated for 10 min with hapten at the same concentrations before addition of antigen for 1 h and then washed and cultured in medium (baseline in figure). GFP expression was determined at 20 h. The results are from 6 independent experiments, data are shown as mean $\pm$ SE. The curves for the GFP response with hapten added were significantly different than the control ($P < 0.05$).

Fig 5.

Low antigen-induced NFAT activation triggers MAP Kinase Erk activation and TNF α release. (A) IgE-sensitized VB9 cells were stimulated with the indicated concentrations of Ag for 3 minutes and total lysates were immunoblotted with anti-phospho-Erk and anti-Fc ϵ RI β as a loading control. (B) Densitometry of the changes in phospho-Erk corrected for loading; these are means $\pm$ SE of three experiments as in (A). (C) Cells were stimulated with the indicated concentrations of antigen (DNP-HSA) in complete media for 2 h and supernatants from each well were removed for measurement of TNF α by ELISA. The results are from 4 independent experiments, data are shown as mean $\pm$ SE.