Expression of the transcription factor NFAT2 in human neutrophils: IgE-dependent, Ca^{2+}- and calcineurin-mediated NFAT2 activation

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Accepted 11 May 2007
Journal of Cell Science 120, 2328-2337 Published by The Company of Biologists 2007
doi:10.1242/jcs.000331

Summary

NFAT (nuclear factors of activated T cells) proteins constitute a family of transcription factors involved in mediating signal transduction. The presence of NFAT isoforms has been described in all cell types of the immune system, with the exception of neutrophils. In the present work we report for the first time the expression in human neutrophils of NFAT2 mRNA and protein. We also report that specific antigens were able to promote NFAT2 protein translocation to the nucleus, an effect that was mimicked by the treatment of neutrophils with anti-immunoglobulin E (anti-IgE) or anti-Fcε-receptor antibodies. Antigens, anti-IgE and anti-FcεRs also increased Ca^{2+} release and the intracellular activity of calcineurin, which was able to interact physically with NFAT2, in parallel to eliciting an enhanced NFAT2 DNA-binding activity. In addition, specific chemical inhibitors of the NFAT pathway, such as cyclosporin A and VIVIT peptide, abolished antigen and anti-IgE-induced cyclooxygenase-2 (COX2) gene upregulation and prostaglandin (PGE_2) release, suggesting that this process is through NFAT. Our results provide evidence that NFAT2 is constitutively expressed in human neutrophils, and after IgE-dependent activation operates as a transcription factor in the modulation of genes, such as *COX2*, during allergic inflammation.

Supplementary material available online at
http://jcs.biologists.org/cgi/content/full/120/14/2328/DC1

Key words: Neutrophils, NFAT, Calcineurin, Allergy

Introduction

Transcription factors of the 'nuclear factor of activated T cells' (NFAT) family are thought to play an important role in immune responses by regulating the expression of a variety of inducible genes such as those encoding interleukin (IL)-2, interferon-γ, tumour necrosis factor α (TNFα), and cyclooxygenase 2 (COX2) (Hogan et al., 2003; Im and Rao, 2004). Five members of the NFAT family have been identified to date. Four of them, including NFAT1 (also called NFATp or NFATc2), NFAT2 (NFATc or NFATc1), NFAT3 (NFATc4) and NFAT4 (NFATx or NFATc3), share a similar DNA-binding domain, and are modulated by the Ca^{2+}-dependent phosphatase, calcineurin (CaN) (Hogan et al., 2003; Im and Rao, 2004). Another member, NFAT5 (also known as TonEBP or NFATz), is Ca^{2+}- and CaN-independent and differs significantly in its structure from the other family members (Aramburu et al., 2006).

It is well known that NFAT members show restricted patterns of expression in a variety of organs, tissues and cell types (Rao et al., 1997). Regarding the immune system, particular NFAT protein isoforms are expressed in its different cell types; thus, NFAT1 is expressed in unstimulated monocytes and macrophages (Wang et al., 1995; Shaw et al., 1995), mast cells (Shaw et al., 1995), eosinophils (Jinquan et al., 1999), B cells (Timmerman et al., 1997), T cells (Lyakh et al., 1997) and NK cells (Shaw et al., 1995); NFAT2 is found in RAW 264.7 only after treatment with soluble RANKL (Takayanagi et al., 2002), basophils (Schroeder et al., 2002), mast cells (Hock and Brown, 2003), B cells (Timmerman et al., 1997), T cells (Lyakh et al., 1997), and NK cells (Aramburu et al., 1995); NFAT4 has been reported in eosinophils (Jinquan et al., 1999), B cells (Timmerman et al., 1997) and T cells (Lyakh et al., 1997). Finally, the presence of NFAT5 has been reported only in T cells (Trama et al., 2000).

NFAT mRNA levels often do not show a direct relationship with the intracellular amounts of their protein products (Schroeder et al., 2002; Trama et al., 2000), and most studies report separate analyses of the different NFAT protein isoforms and their transcripts. NFAT1 constitutive expression at the mRNA and protein levels has been reported in many cell types (Shaw et al., 1995; Aramburu et al., 1995; Wang et al., 1995; Jinquan et al., 1999; Lyakh et al., 1997). NFAT2 mRNA and protein are expressed in a more-restricted fashion, and in most cases their synthesis is induced only upon cell stimulation with immune complexes or with ionomycin plus phorbol ester (Lyakh et al., 1997). The NFAT3 protein has never been detected in immune-system cells, although its mRNA transcript is expressed, albeit scarcely, in peripheral blood lymphocytes (PBLs) (Hoey et al., 1995). As for NFAT4, both

the mRNA and the protein are constitutively expressed in several organs and immune-system cells (Lyakh et al., 1997; Hoey et al., 1995; Ho et al., 1995). NFAT5 mRNA is constitutively expressed at significant levels in the thymus and in peripheral lymphocytes, and the protein has been detected in splenocytes and peripheral T lymphocytes after treatment with ionomycin plus phorbol ester (Trama et al., 2000).

In addition to the restricted patterns of expression shown by NFATs, their activity is controlled through modulation of their subcellular localisation, and of their DNA-binding and transcriptional transactivation capacities.

To date, the expression of NFAT proteins or of their encoding mRNAs has not been evaluated in neutrophils (Wang et al., 1995; Carballo et al., 1999; Yang et al., 2003). In these cells, NF-κB is the only transcription factor reported to be involved in CaN-mediated processes with its transcriptional regulatory capacity being modulated through the activation of the I-κB kinase (Frantz et al., 1994). Since it is unknown whether NFATs are expressed in neutrophils, in the present study we have investigated the potential presence of the five NFAT protein isoforms and their corresponding mRNAs in human peripheral neutrophils, as well as the mechanisms mediating NFAT activation upon antigen, anti-IgE or anti-FcεR challenge in neutrophils from allergic patients. The potential modulatory roles of Ca^{2+} and/or CaN as signal-transducing intermediaries between IgE receptors and the NFAT transcription factor, and the functional role of this transcription factor, were also addressed.

Results

Expression of NFAT mRNAs in human neutrophils

We carried out conventional RT-PCR analysis using specific primers for the five known NFAT isoforms in order to evaluate their expression at the mRNA level in highly purified human neutrophils. RNA extracted from neutrophils from healthy donors and allergic patients was analysed in parallel with RNA from activated PBLs or activated Jurkat T cells. As shown in Fig. 1A, mRNAs of NFAT1, NFAT2, NFAT4 and NFAT5 were readily detected in unstimulated human neutrophils. Conversely, NFAT3 mRNA expression was not detected in neutrophils, whereas it was found in PBLs, as previously described (Hoey et al., 1995). This finding was used as an internal control of no contamination with PBLs in our neutrophil population. mRNA amplification of the Charcot-Leyden crystal protein was also assayed in order to exclude eosinophil and basophil contamination (Fig. 1B) (Gomolin et al., 1993). In addition, the amounts of NFAT1, NFAT2, NFAT4 and NFAT5 mRNAs (compared with human PBLs) were measured by real-time PCR (Fig. 1C). We found that NFAT2 was the predominant NFAT isoform expressed by neutrophils. In these cells, NFAT2 mRNA expression was ~23% that of PBLs (ratio NFAT2:β_2-microglobulin). Unlike NFAT2 mRNA, those of NFAT1, NFAT4 and NFAT5 were detected only in very small amounts. In all cases, no statistically significant differences were found between samples from allergic and healthy subjects.

NFAT2 protein is expressed in human neutrophils

The expression of NFAT1, NFAT2, NFAT4 and NFAT5 protein isoforms in human neutrophils was characterised by western blotting analysis, using specific, non-cross-reactive antibodies (Abs). Whole-cell extracts from unstimulated neutrophils were analysed in parallel with extracts from activated PBLs and Jurkat T cells. Fig. 2A shows that NFAT1 protein was not detected in neutrophils, as previously described (Wang et al., 1995), whereas PBLs expressed one major NFAT1 isoform of ~130 kDa, as previously described (Lyakh et al., 1997). The NFAT2 isoform was found as a single band of ~85 kDa, with no differences of expression between neutrophils from healthy donors and allergic patients. PBLs and Jurkat T cells exhibited one additional NFAT2 isoform of ~140 kDa, as previously described (Lyakh et al., 1997). The NFAT4 isoform was not detected in neutrophils, but it was detected in Jurkat T cells as a band of ~155 kDa, as previously described (Lyakh et al., 1997). The NFAT5 isoform was not detected in neutrophils, but it was detected in PBLs and Jurkat T cells as a band of ~170 kDa, as previously described (Trama et al., 2000). Further analysis, performed using two-colour flow cytometry staining (Fig. 2B) showed that NFAT2 was expressed in 87-90% of $CD66^+$ cells, which are neutrophils (Borregaard and Couland, 1997).

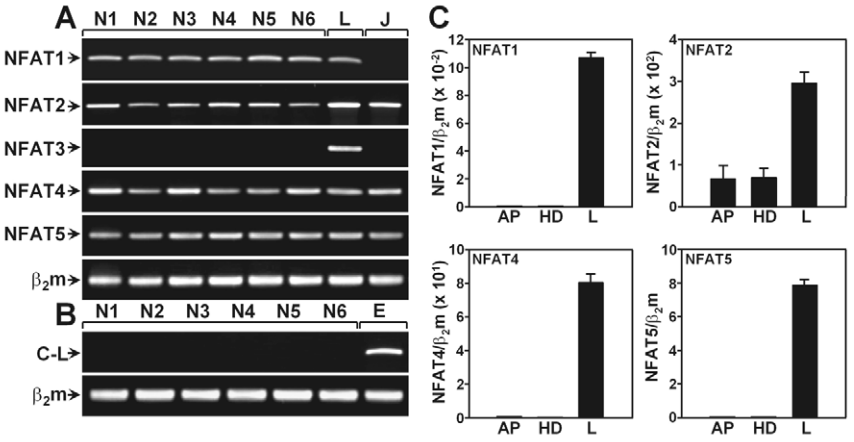


Fig. 1. Analysis of NFAT mRNA expression in human neutrophils. Total RNA was extracted from highly purified unstimulated neutrophils from both human healthy donors (N1-N3) and human allergic patients (N4-N6), as well as from PBLs (L) and Jurkat T cells (J) stimulated with 100 nM PMA plus 500 nM ionomycin, and from highly purified eosinophils (E) used as positive and negative controls. (A,B) RNA samples were analysed by conventional RT-PCR, using specific primers for the amplification of the indicated NFAT isoform transcripts (A), or of the Charcot-Leyden crystal protein (C-L) transcripts (B). Results from amplification of the β_2-microglobulin (β_2m) housekeeping gene transcript are shown in both panels. (C) RNA was isolated from neutrophils from allergic patients (AP) or healthy donors (HD) or from PBLs (L). The levels of NFAT1, NFAT2, NFAT4 and NFAT5 mRNAs were analysed by real-time PCR analysis as indicated in the Materials and Methods section. The levels of mRNAs were standardised against the level of β_2-microglobulin (β_2m) mRNA. Values are mean ± s.e.m. from eight individuals in each group.

Since some NFAT isoforms exhibit inducible synthesis (Rao et al., 1997; Aramburu et al., 1995; Takayanagi et al., 2002; Lyakh et al., 1997; Trama et al., 2000), we next examined the expression of NFAT1, NFAT2, NFAT4 and NFAT5 protein after treatment of neutrophils with NFAT inducers of transcription, such as ionomycin and/or phorbol ester, with negative results (data not shown). NFAT3 mRNA was not detected after stimulation of neutrophils with anti-IgE Abs, or the aforementioned classic NFAT inducers (data not shown).

Antigens, anti-IgE and anti-FcεRs elicit NFAT2 nuclear translocation

We next investigated the effects of the classic activators of NFAT (ionomycin and phorbol ester) and the cytokines IL4 and IL5 upon NFAT2 nuclear translocation. No activation of NFAT2 was found in neutrophils, as its was absent from nuclear extracts in all cases (data not shown). Negative results were similarly obtained when we assessed the effects of other agonists eliciting diverse neutrophil functions, such as IL10, *E. coli* lipopolysaccharide, TNFα, granulocyte macrophage-colony stimulating factor (GM-CSF), and platelet-activating factor (PAF), by themselves and in different combinations – conditions under which no NFAT2 nuclear translocation was found to occur (data not shown). Since there are reports showing the involvement of an IgE-dependent mechanism in NFAT2 translocation to the nucleus in basophils and mast cells (Schroeder et al., 2002; Hock and Brown 2003), we set out to examine whether the challenge of human neutrophils from allergic patients with anti-IgE Abs or specific antigens was able to promote nuclear translocation of NFAT2. Fig. 3A illustrates the occurrence of NFAT2 nuclear accumulation in neutrophils from allergic donors, with a concomitant disappearance of this protein from the cytosolic fraction upon anti-IgE Ab treatment. No effect was observed, however, when neutrophils were treated with non-specific goat IgG. The results observed with anti-IgE Abs cannot be ascribed to an induction of NFAT2 protein synthesis, because the treatment with stimulus along the course of the experiments did not affect the protein levels in whole-cell lysates (Fig. 3B).

Fig. 4 shows the response to specific antigens. Neutrophils from three patients who were allergic-sensitised to either *Olea*

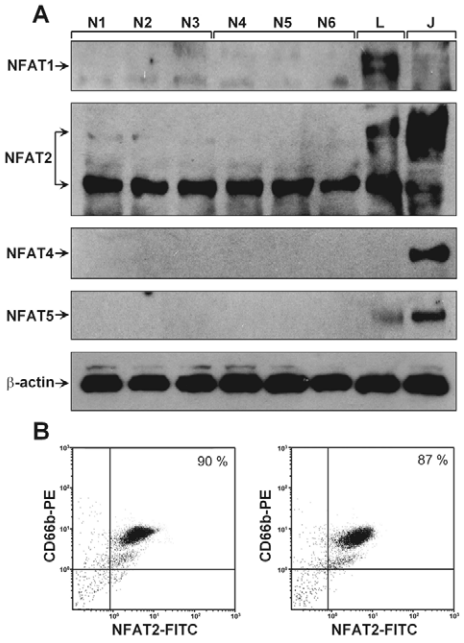


Fig. 2. Expression of the NFAT2 protein isoform in human neutrophils. (A) The potential presence of the five NFAT isoforms was analysed by western blotting of whole-cell extracts from unstimulated neutrophils obtained immediately after their isolation from both human healthy donors (N1-N3) and human allergic patients (N4-N6), as well as from PBLs (L) and Jurkat T cells (J) stimulated with 100 nM plus 500 nM ionomycin used as positive controls. β-actin was used as an internal control to verify that equal amounts of protein were loaded per lane. All blots shown are representative of at least three independent experiments in each group of individuals, with similar results. (B) Flow cytometry analysis of NFAT2 expression in neutrophils isolated from an allergic patient (left panel) and a healthy donor (right panel). After isolation, two-colour flow cytometry analysis of CD66b and NFAT2 expression was performed. Results obtained are expressed as the percentage of double-positive ($CD66b^+$ and $NFAT2^+$) cells. Results shown are representative of at least three independent experiments in each different group of individuals.

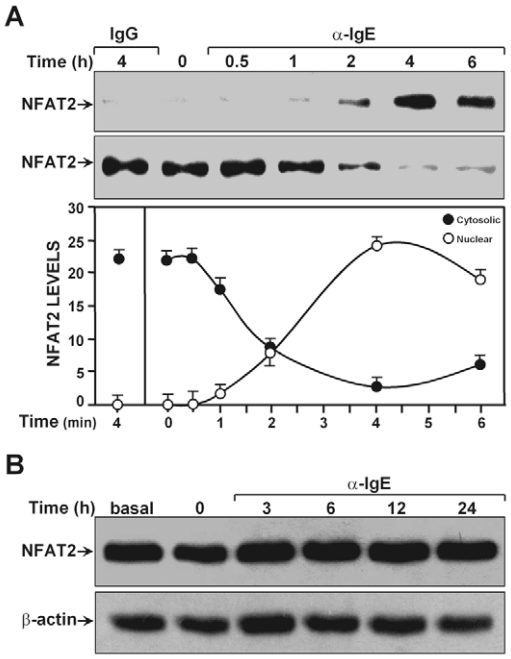


Fig. 3. Anti-IgE-elicited NFAT2 nuclear translocation in human neutrophils. Constitutive NFAT2 expression in human neutrophils. (A) Neutrophils isolated from an allergic patient were stimulated with 10 μg/ml anti-IgE (α-IgE) for the indicated times (up to 6 hours), or incubated with 10 μg/ml non-specific goat IgG (IgG) for 4 hours. Then, nuclear and cytosolic extracts were obtained. (B) Whole-cell extracts were obtained immediately after neutrophil isolation from an allergic patient (basal), or neutrophils were cultured in the absence or presence of 10 μg/ml anti-IgE (α-IgE) for the indicated times (up to 24 hours), and then whole-cell extracts were obtained. Cellular, cytosolic and nuclear NFAT2 levels were analysed by western blotting, using anti-human NFAT2-specific Abs. β-actin was used as an internal control. The blot shown is representative of three independent experiments with similar results. Histograms above each lane show the mean (± s.e.m.) values quantified from the blots from three separate experiments.

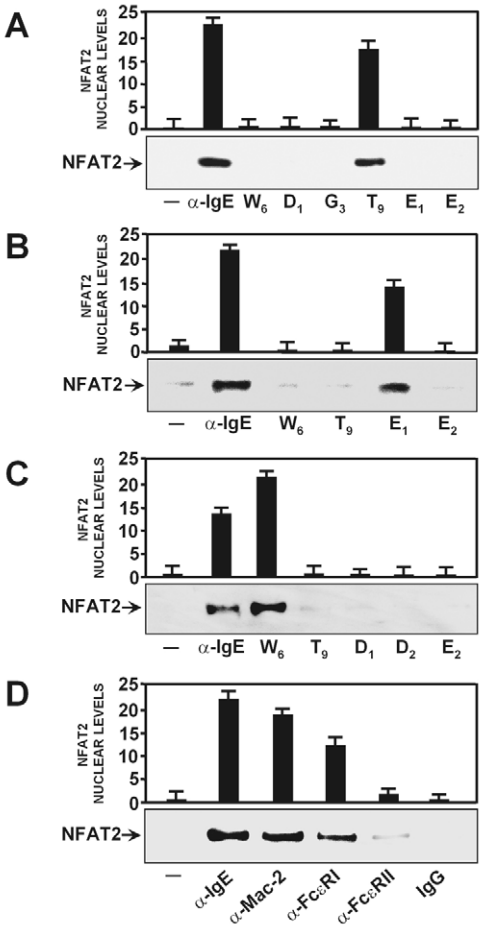


Fig. 4. Anti-FcεRs- and antigen-elicited NFAT2 nuclear translocation in human neutrophils. Neutrophils from three allergic patients sensitised to either T_9 (A), E_1 (B) or W_6 (C) were stimulated for 4 hours with 10 μg/ml anti-IgE (α-IgE) or with the indicated specific antigens (W_6, D_1, D_2, G_3, T_9, E_1 or E_2) at 10 μg/ml. (D) Neutrophils from an allergic patient were incubated with 5 μg/ml anti-FcεRII (α-FcεRII), anti-Mac-2 (α-Mac-2), or anti-FcεRI (α-FcεRI) Abs or mouse IgG_1 for 4 hours at 37°C. Then, nuclear extracts were obtained, and NFAT2 levels were analysed by western blotting, using anti-human NFAT2-specific Abs. Equal amounts of protein were loaded per lane. Histograms above each lane show the mean (± s.e.m.) values quantified from the blots from three separate experiments.

europaea (T_9; Fig. 4A), cat epithelium (E_1; Fig. 4B) or *Artemisia vulgaris* (W_6; Fig. 4C) showed a clear translocation of NFAT2 into the nucleus upon incubation with the specific antigens to which the patients were sensitised (i.e. T_9 in Fig. 4A, E_1 in Fig. 4B, and W_6 in Fig. 4C). However, when neutrophils were cultured with antigens other than those eliciting an allergic response by the corresponding donor, the NFAT2 protein did not translocate to the nucleus (Fig. 4). The specificity of this response to antigens was further assessed in neutrophils from subjects without any allergic pathology. In this case, none of the antigens tested was found to elicit NFAT2 nuclear translocation (data not shown).

Since previous studies have demonstrated the presence of the three forms of the IgE receptor in human neutrophils (Gounni et al., 2001), the next experiments were performed in order to identify the main IgE receptor(s) involved in antigen-induced or anti-IgE-induced NFAT2 nuclear translocation. Fig. 4D illustrates that NFAT2 nuclear translocation was mainly observed when neutrophils from an allergic patient were treated with Abs directed against galectin-3 (also known as Mac-2; clone A3A12) and FcεRI (clone CRA1). Conversely, the addition of the Ab directed against FcεRII (also known as CD23; clone 9P.25) had only a weak effect. We used mouse IgG_1 as isotype control (Fig. 4D).

Ca^{2+} and CaN modulate NFAT2 nuclear translocation

It is well known that elevated Ca^{2+} levels are a requirement for NFAT nuclear translocation (Clipstone and Crabtree, 1992; Liu et al., 1991; Crabtree, 1999). In this regard, we next examined the effects of the IgE-dependent neutrophil activation on Ca^{2+} mobilisation in human neutrophils from allergic patients. As we previously reported (Monteseirín et al., 1996), acute application of anti-IgE Abs or G_3 to neutrophils from an allergic patient sensitised to this antigen elicited a rapid and strong cytosolic Ca^{2+} accumulation (Fig. 5A, 1 and 2), in a magnitude lower than that obtained with the classic agent *N*-formyl-methionyl leucyl-phenylalanine (fMLP) (Fig. 5A, 3). Furthermore, when neutrophils were challenged with Abs against galectin-3 (Fig. 5A, 4) and FcεRI (Fig. 5A, 5), an increase of intracellular Ca^{2+} was detected, which was well-correlated with the effect of these Abs upon NFAT2 nuclear translocation. The same correlation was observed with the Ab against FcεRII, which had only a weak effect on Ca^{2+} mobilisation (Fig. 5A, 6). We used mouse IgG_1 as isotype control, without effect (data not shown).

One of the targets of calcium mobilisation is CaN (phosphatase 2B), which, in a calcium-dependent manner, dephosphorylates cytosolic NFAT to translocate it to the nucleus (Loh et al., 1996). Since we have previously shown that CaN is expressed by human neutrophils (Carballo et al., 1999), we aimed our experiments at analysing whether IgE-dependent activation could modulate CaN activity in human neutrophils. To assess this possibility, CaN activity was here assayed in the presence of anti-IgE or specific antigens. It was found that this treatment actually led to a significant increase in CaN activity, with maximal levels being reached after 30 minutes of anti-IgE or antigen treatment, and remaining high for at least 4 hours (Fig. 5B). No effect was observed, however, when neutrophils were treated with non-specific goat IgG. This prompted us to study whether IgE-dependent NFAT2 nuclear translocation was dependent on CaN activity. As shown in Fig. 6A,B, in anti-IgE-activated neutrophils from an allergic patient, the CaN inhibitor, cyclosporin A (CsA), and cell-permeable VIVIT peptide, an inhibitor of CaN and NFAT interactions (Aramburu et al., 1999), prevented – in a concentration-dependent manner – the translocation of NFAT2 to the nucleus.

Given this negative effect of CsA and VIVIT peptide on IgE-dependent NFAT2 nuclear translocation, and that anti-IgE enhanced CaN activity, we set out to examine the possibility of physical interaction between CaN and NFAT2. The coimmunoprecipitation results shown in Fig. 6C illustrate that these were indeed two interacting molecules, as both NFAT2 and the two CaN subunits, A and B, could be precipitated together by using either anti-CaN or anti-NFAT2 Abs.

Antigens and anti-IgE elicit NFAT2 DNA binding: role of CaN

To assess whether neutrophil NFAT proteins were able to form competent DNA-binding complexes, we used an NFAT oligonucleotide as a probe in gel retardation assays carried out with nuclear extracts obtained from anti-IgE-stimulated neutrophils. The formation of a DNA-protein complex elicited by anti-IgE treatment was found to occur in a time-dependent manner, being detected after 0.5 hours of treatment and persisting for at least 4 hours (Fig. 7A). The formation of the radiolabelled complex was specifically competed for by a 100-fold molar excess of the cold NFAT probe. Since the NFAT2 protein is the only isoform constitutively expressed in neutrophils, its binding specificity was further verified by using a specific anti-NFAT2 monoclonal Ab. As also shown in Fig. 7A, this Ab completely supershifted the DNA-protein complex formed. Fig. 7B shows that maximal levels of NFAT DNA binding were reached at a 10 μg/ml dose of anti-IgE after 4 hours of treatment, and that no such effect was observed when the cells were treated with non-specific goat IgG for 4 hours. Therefore, present results indicate that anti-IgE treatment increases NFAT DNA-binding activity in human neutrophils, and that the NFAT2 protein is a constituent of the DNA-protein complex which forms under these conditions.

The dependence of this process on CaN activity was further evidenced by the treatment of neutrophils with CsA or cell-permeable VIVIT peptide, which were found to prevent antigen-dependent and IgE-dependent NFAT DNA-binding activity (Fig. 8C).

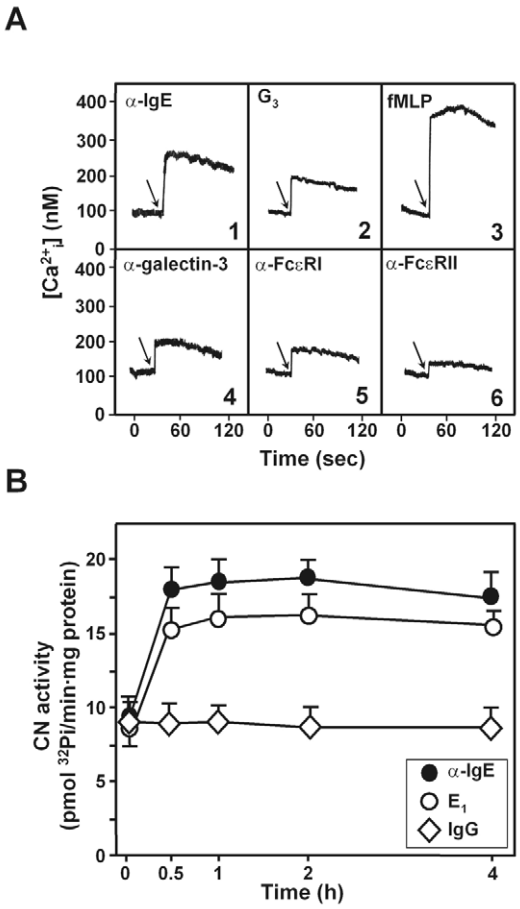


Fig. 5. Antigens, anti-FcεRs and anti-IgE increase cytosolic Ca^{2+} levels and CaN activity in human neutrophils. (A) Fura-2-AM-loaded neutrophils from an allergic patient sensitised to G_3 were treated at the times indicated with 10 μg/ml anti-IgE (α-IgE) (1), 10 μg/ml G_3 (2), 50 nM fMLP (3), 5 μg/ml anti-galectin-3 (α-galectin-3) (4), 5 μg/ml anti-FcεRI (α-FcεRI) (5), or 5 μg/ml anti-FcεRII (α-FcεRII) (6), and cytosolic Ca^{2+} was spectrofluorometrically recorded for 2 minutes. Each graph is representative of a set of three experiments that yielded similar results. (B) Neutrophils from an allergic patient sensitised to E_1 were incubated with 10 μg/ml anti-IgE (α-IgE), 10 μg/ml E_1 antigen, or unspecific goat IgG (IgG) for the indicated times. Then, the cells were lysed, and CaN activity was assayed. Values are the means ± s.e.m. from four separate experiments performed in triplicate.

Involvement of transcription factor NFAT2 on COX2 upregulation by antigens and anti-IgE Ab

The involvement of NFAT2 in the activation of COX2 gene expression, and its role in inflammatory disorders, are known (Rao et al., 1997; Iñiguez et al., 2000). We have previously shown that COX2 expression becomes upregulated in human neutrophils at both the mRNA and protein levels, with a concomitant PGE_2 release, in response to challenge with specific antigens or anti-IgE Ab (Vega et al., 2006). Thus, we next studied the involvement of this transcription factor in the IgE-promoted COX2 gene expression, using specific inhibitors of the NFAT pathway. As shown in Fig. 8A,B, the use of CsA and VIVIT peptide strongly attenuated antigen-induced and anti-IgE Ab-induced COX2 protein and mRNA expression and the concomitant PGE_2 release, indicating that this transcription factor could be involved in IgE-dependent *COX2* expression.

Discussion

NFATs constitute a family of transcription factors whose presence has been described in virtually all cell types of the immune system, with the exception of neutrophils. The results described here show, for the first time a constitutive and similar expression of the NFAT2 mRNA and protein in unstimulated human neutrophils from allergic patients or healthy donors. No expression of other NFAT isoforms was found at the protein level, and very low levels, compared with those of PBLs, of the NFAT1, NFAT4 and NFAT5 transcripts were determined by real-time PCR, which could explain the absence, or presence at too low levels to be detected, of the protein.

Despite the inducible behaviour of NFAT2 in many leukocyte cell types (Lyakh et al., 1997), we show that NFAT2 protein expression was not modified after cell treatment, in agreement with a previous report in human basophils (Schroeder et al., 2002). Conversely, the NFAT1 protein, which here was not found in neutrophils, has been shown to represent the most common NFAT protein isoform, and to display a constitutive expression pattern in a wide range of leukocyte types (Shaw et al., 1995; Aramburu et al., 1995; Wang et al., 1995; Jinquan et al., 1999; Lyakh et al., 1997).

Our data also indicate that challenge with specific antigens is an effective stimulus for NFAT2 activation by neutrophils from allergic patients. This mechanism was highly specific. Antigens other than those that produce clinical symptoms did not evoke NFAT nuclear translocation, and were ineffective on neutrophils from healthy donors. Presumably, the antigen-mediated effect occurred in an IgE-dependent manner. This

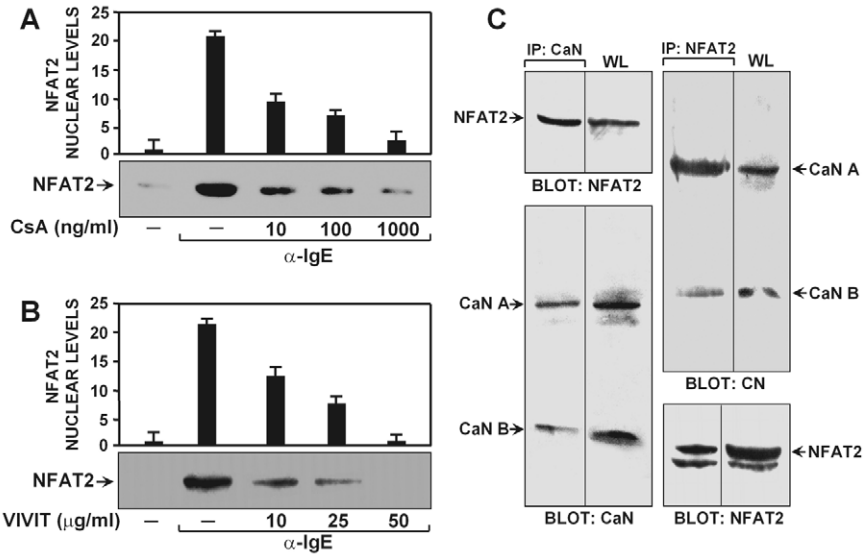
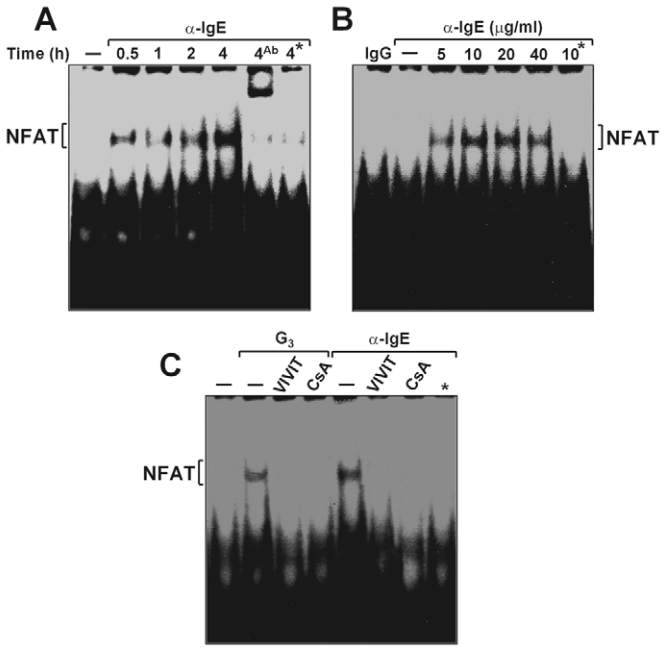


Fig. 6. Involvement of CaN in IgE-dependent NFAT2 nuclear translocation in human neutrophils. Neutrophils from an allergic patient were pretreated with CsA (A) or VIVIT peptide (B) at the indicated doses for 1 hour, and then treated with 10 μg/ml anti-IgE (α-IgE) for 4 hours. Nuclear extracts were obtained, and NFAT2 levels analysed by western blotting, using anti-human NFAT2-specific Abs. Equal amounts of protein were loaded per lane. Histograms above each lane show the mean (± s.e.m.) values quantified from the blots from three separate experiments. (C) Whole-cell lysates (1 mg of protein) from untreated neutrophils from an allergic patient were incubated at 4°C for 18 hours with anti-CaN Abs. After immunoprecipitation (IP), the pellets were analysed by western blotting, using anti-NFAT2 (left upper panel). Thereafter, the blot was stripped, and reprobed with anti-CaN (left lower panel). Immunoprecipitation was carried out as indicated in A, except that anti-NFAT2 Abs was used, and subsequent western blotting analysis was carried out using anti-CaN Abs (right upper panel). After stripping, the blot was reprobed with anti-NFAT2 Abs (right lower panel). The signals directly obtained from neutrophil whole-cell lysates (80 μg) subjected to western blotting analysis without immunoprecipitation (WL) are also shown in both panels.

hypothesis is supported by three main facts: (i) we have previously reported the presence of specific IgE on the surface of neutrophils from allergic patients (Monteseirín et al., 2003; Monteseirín et al., 2004); (ii) anti-IgE Ab challenge triggered an equivalent response to that obtained with the antigens; and (iii) ligation of IgE receptors (mainly galectin-3 and FcεRI) with specific Abs evoked NFAT2 activation. In line with our data, the liberation of IL8 by these cells following the stimulation by FcεRI has been demonstrated previously (Gounni et al., 2001), and the challenge of neutrophils by galectin-3 stimulates superoxide production (Yamaoka et al., 1995).



We believe that both the expression of NFAT2 mRNA and protein and the NFAT2 activation detected in our system cannot be ascribed to possible contamination by other leukocytes for several reasons: (1) if the effects were due to a contamination with PBLs, we would detect NFAT3 mRNA in our neutrophil preparations (Hoey et al., 1995); (2) if the effects were due to a contamination with eosinophils or basophils, we would find mRNA for Charcot-Leyden crystal protein in our neutrophil preparations (Gomolin et al., 1993); and (3) if the effects were due to a contamination with monocytes, we would detect NFAT1 protein in our neutrophil preparations (Wang et al., 1995). Furthermore, we did not detect NFAT2 mRNA in peripheral blood monocytes (data not shown), and other authors have shown that NFAT2 is expressed only by RAW 264.7 monocytes and/or macrophages after soluble RANKL stimulation (Takayanagi et al., 2002).

Another goal of this study was to explore the presumptive roles of Ca^{2+} and CaN in the IgE-dependent NFAT activation in human neutrophils. We have previously shown that intracellular Ca^{2+} increase occurs through an IgE- and antigen-dependent mechanism in these cells (Monteseirín et al., 1996),

Fig. 7. Anti-IgE-elicited NFAT2 DNA-binding activity in human neutrophils. Role of CaN. (A,B) Neutrophils from an allergic patient were treated either with 10 μg/ml anti-IgE (α-IgE) for the indicated times (A), or with the indicated doses of anti-IgE for 4 hours (B). (C) Neutrophils from an allergic patient sensitised to G_3 were pretreated with 1 μg/ml CsA or 50 μg/ml VIVIT peptide for 1 hour, and then treated with 10 μg/ml anti-IgE (α-IgE) or 10 μg/ml G_3 antigen for 4 hours. Nuclear extracts were then obtained, and binding to the ^{32}P-labelled NFAT oligonucleotide probe was assessed by electrophoretic mobility shift assays. Incubation of neutrophils with 10 μg/ml non-specific IgG for 4 hours was also carried out as a negative control (IgG). Assays performed in the presence of 1 μl of anti-NFAT2 (Ab) or carrying a 100-fold molar excess of unlabelled oligonucleotide (*) are also shown.

and that CaN is expressed by human neutrophils (Carballo et al., 1999). Moreover, CaN has been implicated in several allergic conditions, such as atopic dermatitis (Akhavan and Rudikoff, 2003) and asthma (Kon, 2000), and inhibitors of CaN are being employed for the treatment of these pathological processes (Akhavan and Rudikoff, 2003; Kon, 2000).

We have described here that, after IgE-dependent treatment, intracellular Ca^{2+} mobilisation and increase of Ca^{2+}-dependent phosphatase CaN activity occurred in human neutrophils. Furthermore, CaN modulated IgE-dependent NFAT2 nuclear translocation and DNA-binding activity, as evidenced using the CaN-interfering drugs CsA (Lee and Park, 2006; Liu et al., 1991) or VIVIT peptide (Aramburu et al., 1999).

Prostaglandin H_2 synthase (cyclooxygenase, COX) is the rate-limiting enzyme in the conversion of arachidonic acid into eicosanoids, i.e. PGs and thromboxanes (TXs) (Smith, 1992). COX exists in two isoforms – COX1 and COX2 – of which COX1 is constitutively expressed in most tissues and appears responsible for the production of PGs under normal physiological conditions (Smith et al., 2000), and COX2 expression is associated with inflammatory processes, such as allergy (Profita et al., 2003). Adjacent NFAT-binding sites are present in the promoter region of inducible genes such as *COX2* (Iñiguez et al., 2000). We have previously shown that COX2 expression becomes upregulated in human neutrophils at both mRNA and protein levels, with a concomitant PGE_2 release, in response to challenge with specific antigens or anti-IgE Ab (Vega et al., 2006). We now describe here that this expression was dependent on the Ca^{2+}-CaN-NFAT pathway.

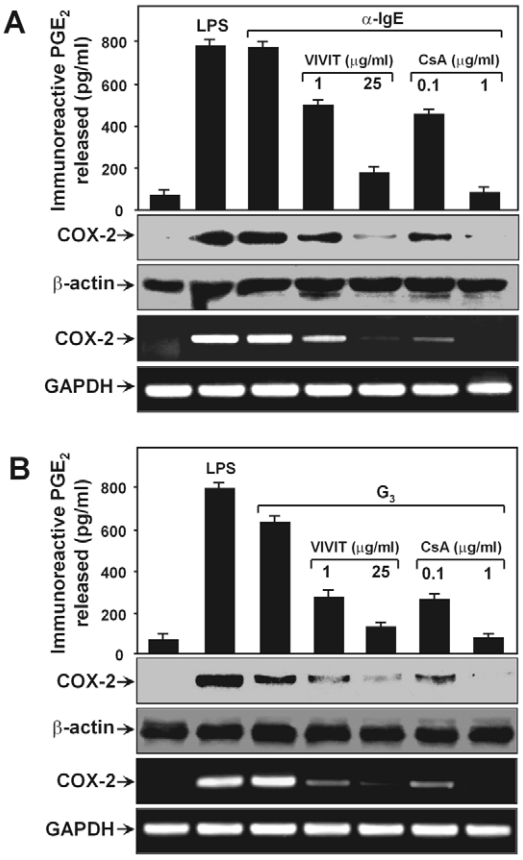


Fig. 8. Involvement of NFAT in IgE-dependent COX2 expression in human neutrophils. Neutrophils from an allergic patient sensitised to G_3 antigen were pretreated with CsA or cell-permeable VIVIT, at the indicated doses for 1 hour, and then incubated for 24 hours with 1 μg/ml LPS or 10 μg/ml anti-IgE Ab (α-IgE; A) or 10 μg/ml G_3 (B). COX2 mRNA levels were analysed by conventional RT-PCR, using COX2- and GAPDH-specific primers; COX2 and β-actin protein expression was analysed by western blotting, and the levels of PGE_2 were measured in the culture medium supernatants, using an enzyme immunoassay. The values shown are the mean ± s.e.m. from three independent assays in which each measurement was performed in triplicate. All the experiments were performed at least in triplicate.

Collectively, our results show that NFAT2 is the only member of the NFAT family of transcription factors expressed by human neutrophils, and provide evidence that Ca^{2+} and CaN both have a role in mediating the specific antigen- and anti-IgE-elicited nuclear translocation through Fcε receptors and enhancement of DNA-binding activity of NFAT2. To date, only NF-κB had been implicated as a target of CaN-mediated processes in neutrophils, its effects being exerted through the activation of I-κB kinase (Frantz et al., 1994). Recently, a new protein preferentially expressed in neutrophils, designated calcineurin-NFAT-activating and immunoreceptor tyrosine-based activation motif (ITAM)-containing protein', or CNAIP, has been suggested to act as an intermediary molecule between CaN and NFAT activation (Yang et al., 2003). Preferential activation of NFAT2 is correlated with mouse-strain susceptibility to allergenic inflammation (development of airway hyper-reactivity, pulmonary eosinophilia, mucus cell hyperplasia, high serum IgE levels, and increased IL4 and IL5 production) (Keen et al., 2001). Our data suggest a role for NFAT2 in the pathogenesis of the allergic state, and provide strong evidence that NFAT2-mediated transcriptional regulation of genes involved in allergic responses, such as *COX2*, is modulated through Ca^{2+} and CaN activation in human neutrophils.

Materials and Methods

Materials

The antigens used in this work were commercially available antigen extracts, including D_1 (*Dermatophagoides pteronisinus*), D_2 (*Dermatophagoides farinae*), G_3 (*Dactylis glomerata*), T_9 (*Olea europaea*), W_6 (*Artemisia vulgaris*), E_1 (cat epithelium) and E_2 (dog epithelium), purchased from Bial-Arístegui (Bilbao, Spain). Goat anti-human IgE Ab and the Fix and Perm cell permeabilisation kit were from Caltag Laboratories (Burlingame, CA). CsA, phorbol 12-myristate 13-acetate (PMA), goat IgG, ionomycin, LPS, Fura-2 acetoxymethyl ester (AM), digitonin, fMLP, and protein G-Sepharose beads were from Sigma-Aldrich (Madrid, Spain). Ficoll-Hypaque, phosphate-buffered saline (PBS), RPMI 1640, heat-inactivated foetal bovine serum, penicillin and streptomycin were purchased from Bio-Whittaker (Verviers, Belgium). Cell-permeable VIVIT peptide was purchased from Calbiochem (San Diego, CA). PCR primers, random primers, High Pure RNA Isolation Kit, *Taq* polymerase, and the double-stranded oligonucleotide 5′-GGAGGAAAAACTGTTTCATACAGAAGCGT-3′, encompassing the distal NFAT-binding site (underlined) of the human IL2 gene promoter, were obtained from Roche (Madrid, Spain). The synthetic phosphopeptide used as a substrate for CaN activity assays was purchased from Peninsula Laboratories (Belmont, CA). Mouse monoclonal Abs specific for human NFAT1 and NFAT2, and rabbit polyclonal Abs for human NFAT5, were obtained from Affinity Bioreagents (Madrid, Spain). Mouse monoclonal Ab specific for human COX2 was obtained from Cayman Chemical (Ann Arbor, MI). Goat anti-mouse IgG-coated micromagnetic beads, goat anti-mouse IgG (GAM), mouse monoclonal Abs specific for FcεRII (also known as CD23) clone 9P.25, CD9, CD14, CD203c, CD66b and CD16 were purchased from Immunotech-IZASA (Barcelona, Spain). Mouse monoclonal Ab specific for human NFAT4 (sc-8405) and anti-β-actin (sc-8432) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA), and the rabbit antiserum against CaN was kindly provided by C. B. Klee (National Institutes of Health, Bethesda, MD). Mouse monoclonal Ab specific for FcεRI (α chain) clone AER-37 (also known as clone CRA1) was obtained from eBioscience (San Diego,

CA). Mouse monoclonal Ab specific against galectin-3 (also known as Mac-2) clone A3A12 was purchased from Abcam (Cambridge, UK). Horseradish peroxidase-conjugated horse anti-mouse IgG and goat anti-rabbit IgG secondary Abs, interleukins, TNFα and GM-CSF were purchased from Promega (Madison, WI). Scion Image software was purchased from Scion Corporation (Frederick, MD). All culture reagents used in this work had endotoxin levels of 0.01 ng/ml, as verified by the Coatest *Limulus* lysate assay (Chromogenix, Mölndal, Sweden).

Patients and controls

The groups studied included adult atopic patients with bronchial asthma, and healthy non-atopic volunteer controls. The asthmatic patients had given positive results in skin-prick (Bial-Arístegui, Bilbao, Spain) and specific-IgE (HYTEC 288, Hycor Biomedical-IZASA, Barcelona, Spain) tests to at least one common allergenic antigen. These subjects had not received any treatment or specific hyposensitisation, and had not experienced episodes of asthma for at least 3 months, or respiratory-tract infections in the 4 weeks before blood sampling. The healthy group had no history of allergy or bronchial symptoms, and were negative for the skin-prick and specific-IgE tests toward a battery of inhalant allergenic antigens (house-dust mites, pollens, moulds and animal danders). This study was previously approved by the Virgen Macarena Hospital Ethics Committee, and each subject had given prior informed consent.

Cell isolation and culture

Human neutrophils were isolated as previously described (Böyum, 1968). Neutrophil preparations were further purified, depleting $CD9^+$ cells [eosinophils (Saito et al., 1986)], $CD203c^+$ cells [basophils (Buhring et al., 2001)], and $CD14^+$ cells [monocytes (Wright et al., 1991)] using a magnetic cell-sorting system (MACS) by incubation with mouse anti-human CD9, anti-human CD203c, and anti-human CD14 Abs, and then with anti-mouse IgG micromagnetic beads. The purity of neutrophils was on average >99%. To prepare mRNA for RT-PCR, neutrophils were purified even further by repeating the above procedure three more times, using anti-CD9, anti-human CD203c, and anti-human CD14 Abs each time, which reduced contaminating eosinophils to 0.001-0.004% of the final cell population. Monocytes, basophils and lymphocytes were not detected in the neutrophil preparations (Fig. S1 in supplementary material). Human eosinophils and PBLs were analogously purified by MACS. Jurkat T cells (clone E6-1) were obtained from the American Type Culture Collection. Neutrophils were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated foetal bovine serum, 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin, and maintained at 37°C in an atmosphere of 95% O_2 and 5% CO_2. Where used, CsA and VIVIT were added 1 hour before cell-activating compounds. None of the reagents affected the viability of the cells at the concentrations used in this work, as confirmed by the Trypan Blue dye-exclusion test.

Nucleic acid extraction and conventional RT-PCR analysis

Total RNA was isolated from highly purified neutrophils, PBLs and Jurkat T cells using the High Pure RNA Isolation Kit according to the manufacturer's instructions. Total RNA (1 μg) was reverse-transcribed to first-strand cDNA using random primers. The first-strand cDNA was amplified with primer sets for human NFAT1 (accession number: NM_173091): 5′-AAACTCGGCTCCAGAATCCA-3′ and 5′-TGGACTCTGGGATGTGAACT-3′; NFAT2 (accession number: NM_172390): 5′-GCTATGCATCCTCCAACGTC-3′ and 5′-AGTTGGACTCGTAGGAGGAG-3′; NFAT3 (accession number: NM_004554): 5′-ACACAGCCCTATCTTCAGGA-3′ and 5′-ATCTTGCCTGTGATACGGTG-3′; NFAT4 (accession number: NM_173165): 5′-ACCCTTTACCTGGAGCAAAC-3′ and 5′-CTTGCAGTAGCG-ACTGTCTT-3′; NFAT5 (accession number: NM_173214): 5′-CGTGTGTGTG-GCTTCTATGT-3′ and 5′-TGCCTCTCAATCAGAGAGAG-3′; Charcot-Leyden crystal protein (accession number: NM_001828): 5′-AGGAGACAACAATGTCC-CTG-3′ and 5′-TCACAGCCTCAGGCTTGATT-3′; glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (accession number: J04038): 5′-CCACCCATGGC-AAATTCCATGGCA-3′ and 5′-TCTAGACGGCAGGTCAGGTCCACC-3′; β_2-microglobulin (β_2m) (accession number: NM_004048.2): 5′-CCAGCAGAGAA-TGGAAAGTC-3′ and 5′-GATGCTGCTTACATGTCTCG-3′; COX2 (accession number: M90100): 5′-TCAAATGAGATTGTGGGAAAATTGCT-3′ and 5′-AGA-TCATCTCTGCCTGAGTATCTT-3′. Aliquots (20 μl) taken after 35 cycles were electrophoresed on agarose gels and visualised on ethidium bromide-stained agarose gels, and photographed under UV light.

Real-time PCR

After total RNA extraction, cDNA was synthesised as described above. Real-time PCR was performed on a LightCycler detection system (Roche) using SYBR Green I and the thermocycler conditions recommended by the manufacturer. PCR reactions were carried out in duplicate in a total volume of 20 μl containing 3 μl of cDNA template, 3 mM $MgCl_2$, and 0.5 μM primers. Each sample was analysed for β_2-microglobulin to normalise for RNA input amounts and to perform relative quantifications. Several 10-fold dilutions (10^{-1}-10^{-5}) were checked for optimal cycling on the LightCycler, and three of them were selected for standard curves. The same primers described above were used for NFAT1, NFAT 2, NFAT4, NFAT5 and β_2-microglobulin. Melting curve analysis showed a single sharp peak with the expected melting temperature (T_m) for all samples.

Preparation of whole-cell extracts

For the analysis of NFAT1, NFAT2, NFAT4 and NFAT5 expression, neutrophils (10^7 cells), PBLs (5×10^6 cells), or Jurkat T cells (5×10^6 cells) were resuspended in 50 μl of ice-cold lysis buffer (50 mM Tris pH 7.4, 10 mM EGTA, 50 mM NaCl, 1% Triton X-100, 2 mM sodium orthovanadate, 100 μM phenylarsine oxide, and the following protease-inhibitor mixture: 10 μg/ml aprotinin, 10 μg/ml leupeptin, 10 μg/ml soybean trypsin inhibitor, 10 μg/ml *N*-tosyl-L-phenylalanyl-chloromethyl ketone, 10 μg/ml captopril, 1 mM phenylmethylsulphonyl fluoride, 1 mM benzamidine, and 10 mM iodoacetamide), and vortexed for 10 seconds following sonication for 10 seconds. For the analysis of COX2 expression, neutrophils (10^7 cells) were resuspended in 50 μl of ice-cold lysis buffer (20 mM Hepes pH 7.9, 5 mM KCl, 0.1% Nonidet P-40, 1 mM EDTA, 1 mM DTT and 10 mM NaF, supplemented with the same protease mixture indicated above) and vortexed for 10 seconds following sonication for 10 seconds. After centrifugation at 12,000 ***g*** for 2 minutes at 4°C, the supernatant obtained constituted the whole-cell extract. Protein concentration was determined by the Bradford procedure.

Preparation of nuclear and cytosolic extracts

Neutrophils (10^7 cells) were resuspended in 100 μl of buffer A (20 mM Hepes pH 7.9, 10 mM KCl, 10% glycerol, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol (DTT), 20 mM NaF, 2 mM sodium orthovanadate, 100 μM phenylarsineoxide, 2 mM diisopropylfluorophosphate and the above protease inhibitor mixture) and incubated for 30 minutes on ice, followed by vigorous vortexing for 15 seconds. Then, Nonidet P-40 was added to a final concentration of 0.5% and, after centrifugation at 12,000 ***g*** for 10 minutes at 4°C, the supernatant obtained constituted the cytosolic extract. The pellet was resuspended in high-salt buffer (buffer A supplemented with 20% glycerol and 420 mM NaCl). Following incubation for 30 minutes on ice, sonication for 5 seconds, and re-centrifugation as above, the supernatant obtained constituted the nuclear extract. Protein concentration was determined by the Bradford procedure.

Western blotting analyses

For the analysis of NFAT1, NFAT2, NFAT4, NFAT5, β-actin and COX2 expression, whole-cell extracts were separated by SDS-PAGE (80 μg/lane) and electrophoretically transferred to polyvinylidene difluoride membranes. The membranes were probed, without need of prior blocking (Vega et al., 2004) for 2 hours at room temperature with specific Abs against the four human NFAT isoforms above, or with Abs against human COX2 or β-actin, at a 1:2000 dilution in PBS containing 1% bovine serum albumin and 0.2% Tween 20. Then, after washing with PBS, they were incubated for 30 minutes with horseradish peroxidase-conjugated horse anti-mouse IgG or goat anti-rabbit IgG secondary Abs at a 1:5000 dilution. Immunoreactive bands were visualised using an enhanced-chemiluminescence assay, as described previously (Carballo et al., 1999). For the analysis of NFAT2 nuclear translocation, neutrophil nuclear or cytosolic fractions were subjected to SDS-PAGE (60 μg/lane) and western blotting using anti-human NFAT2 Abs, as indicated above. Protein levels were determined by scanning densitometry analysis using the Scion Image software. The densitometric intensities of signals from NFAT2-immunoreactive bands are presented in arbitrary units.

NFAT2 expression analysis by flow cytometry

For intracellular detection of NFAT2 by flow cytometry, human neutrophils (2×10^6 cells) were incubated with Abs against a neutrophil marker, i.e. phycoerythrin (PE)-conjugated CD66b, or the IgG_1-PE isotype control Ab. Neutrophils were then fixed and permeabilised using the Fix and Perm cell permeabilisation kit (Caltag Laboratories), following the manufacturer's instructions. Mouse IgG_1 against human NFAT2 was added and samples were incubated for 30 minutes. Then, samples were washed with PBS, and incubated for 30 minutes with fluorescein isothiocyanate (FITC)-conjugated GAM. After washing with PBS, cells were resuspended in PBS and analysed by flow cytometry (Coulter-IZASA, Barcelona, Spain). GAM-FITC was used as isotype control in cells when NFAT2 Ab was omitted.

Coimmunoprecipitation analysis

Neutrophil whole-cell extracts (1 mg of protein, prepared from 10^8 cells as described above) were separately incubated with 2 μg of anti-NFAT2 or of anti-CaN Abs in 1 ml of lysis buffer for 18 hours at 4°C, and then with protein G-Sepharose beads for 4 hours. The pellets were washed three times with 20 mM sodium phosphate buffer, pH 7.0, containing the previous described protease-inhibitor mixture, and then boiled in Laemmli sample buffer. Eluted immunoprecipitated proteins were separated by SDS-PAGE and analysed by western blotting, using anti-NFAT2 and anti-CaN Abs.

Intracellular Ca^{2+} levels

Neutrophils (1×10^7 cells/ml) were loaded with 2.5 μM Fura-2 AM for 30 minutes at 37°C in loading buffer (20 mM Hepes, 37 mM NaCl, 2.7 mM KCl, 1 mM $MgCl_2$,

1 mM $CaCl_2$, pH 7.5). Excess Fura-2 AM was removed by washing the cells twice with loading buffer supplemented with 0.5% (w/v) BSA. Calcium measurements were carried out with a Shimadzu (Kyoto, Japan) RF-5301 spectrofluorophotometer equipped with a magnetic stirrer and a thermostatically controlled cuvette holder, with an excitation wavelength of 340 nm and an emission wavelength of 510 nm. Maximal and minimal fluorescence (F_{max} and F_{min}, respectively) were determined in the presence of digitonin and EGTA-Tris-HCl, respectively. Intracellular calcium concentrations were calculated using the following equation:

$$[Ca^{2+}]_i = K_d(F–F_{min}) / (F_{max}–F) ,$$

where the K_d for Fura-2 equals 224 nmol/l.

CaN phosphatase assay

CaN phosphatase activity was measured as previously described (Carballo et al., 1999). Reaction mixtures containing 2 μM ^{32}P-labelled phosphopeptide corresponding to a segment of the RII subunit of cAMP-dependent kinase (Blumenthal et al., 1986) and 80 μg of whole-cell extract were incubated in a total volume of 60 μl of assay buffer (20 mM Tris, pH 8.0, 100 mM NaCl, 6 mM $MgCl_2$, 0.5 mM DTT, 0.1 mM $CaCl_2$, 0.5 μM okadaic acid, and 0.1 mg/ml bovine serum albumin) for 15 minutes at 30°C. The reactions were stopped by the addition of 500 μl of 100 mM potassium phosphate buffer, pH 7.0, containing 5% trichloroacetic acid. The reaction mixtures were passed through columns (500 μl) of activated Dowex cation-exchange resin, and free radiolabelled inorganic phosphate (^{32}Pi) was quantified in the eluates by scintillation counting. Linearity of the assays after 15 minutes of reaction was adequately verified. Assays were performed in triplicate, and the c.p.m., measured in blank assays lacking neutrophil lysate, was subtracted. Data are expressed as the number of pmol of inorganic phosphate released per minutes per mg of protein.

NFAT DNA-binding activity

Electrophoretic mobility shift assays of NFAT DNA binding were performed as described previously (Carballo et al., 1999), using as a probe the ^{32}P-radiolabelled double-stranded NFAT oligonucleotide indicated above. The reaction was performed in 20 μl of binding buffer (10 mM Hepes, pH 7.9, 10% glycerol, 0.1 mM EDTA, 8 mM $MgCl_2$, 0.2 μg/ml poly(dI-dC), 1 mM DTT) containing 10 μg of neutrophil nuclear extract, and was allowed to proceed for 20 minutes at room temperature. The protein-DNA complexes were separated on 4% polyacrylamide gels, and autoradiographs were obtained. For DNA competition assays, a 100-fold molar excess of unlabelled oligonucleotide was added before addition of the radiolabelled probe. For supershift assays, monoclonal Abs against NFAT2 (1 μl) were added to the nuclear extracts at 4°C, 20 minutes before starting the binding reaction.

Prostaglandin E_2 release

Neutrophils (10^7 cells) were incubated in 1.5 ml of RPMI medium on 24-well plates in the presence of stimuli at the doses indicated in each case. The concentrations of PGE_2 released were measured in the culture supernatants, using the Prostaglandin E_2 Enzyme Immunoassay Kit (Cayman Chemical) according to the manufacturer's protocol.

Statistical analysis

Data are expressed as means ± s.e.m. Comparisons between groups were made using one-way ANOVA. A P value of <0.05 was considered significant.

A.V. and P.C. were supported by fellowships from the Ministerio de Ciencia y Tecnología, and Fundación Alergol, Spain. R.E. is a recipient of a postdoctoral grant (Juan de la Cierva) (SAF2003-00200) from the Ministerio de Educación y Ciencia, Spain. This work was funded by grants from the Consejería de Salud, Junta de Andalucía (SAS-55/04 and SAS-74/04). A.V. and P.C. contributed equally to this work.

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JCS000331 Supplementary Material

Files in this Data Supplement:

- **Supplemental Figure S1** -

  Fig. S1. Light microscopy of neutrophils. After isolation as described in the Materials and methods section, cells were smeared onto poly-L-lysine-treated glass slides. Slides were stained with Quick Panoptic hematological dye (Química Clínica Aplicada S.A., Amposta, Spain) and photographed under visible light microscopy. Neutrophils were identified from their standard morphology and distinctive Quick Panoptic staining.