

Monomeric immunoglobulin E stabilizes FcεRIα from the human basophil cell line KU812 by protecting it from natural turnover

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Summary

Background The high affinity IgE receptor (FcεRI) on mast cells and basophils is up-regulated by its own ligand IgE; however, the mechanism is unknown.

Objective To study the IgE-mediated effect on FcεRI on basophils by using the human basophilic cell line KU812.

Methods Expression of cell surface FcεRI was assessed by flow cytometry. Western blot technique was used to illustrate tyrosine-phosphorylation and the Ca²⁺ level in KU812 was measured by fluorescence of Fura-2. Soluble specimens of the α-chain from FcεRI (FcεRIα) were obtained by lysing 10⁷ KU812 pr. mL. FcεRIα was detected by a sandwich immunoradiometric assay employing the IgE-binding capacity of FcεRIα in conjunction with a monoclonal antibody. Polyclonal rabbit anti-FcεRIα was used for detection of FcεRIα by Western blotting.

Results We found that monomeric IgE did not induce tyrosine-phosphorylation in KU812, which was the case when stimulating with IgE cross-linked by anti-IgE binding. Further, only cross-linking of IgE, but not monomeric IgE, increased the Ca²⁺ level. Using the immunoradiometric assay, we found a temperature dependent reduction in the amount of FcεRIα. Samples incubated at 37 °C for 5 h displayed a 16-fold decrease in the FcεRIα level compared with samples incubated at 4 °C. In the presence of IgE the reduction at 37 °C was only threefold.

Conclusion These results indicate that IgE does not induce intracellular signals in KU812, i.e., tyrosine-phosphorylation or Ca²⁺ release. Instead it appears that FcεRIα is an unstable protein that IgE stabilizes and thereby protects from a temperature dependent turnover.

Keywords basophil, high affinity IgE receptor, IgE

Submitted 21 June 2002; revised 6 January 2003; accepted 20 January 2003

Introduction

The human basophil leucocyte is important in the allergic reaction [1]. Cross-binding of the high affinity IgE receptor (FcεRI) activates the cell, which results in release of histamine, lipid mediators and cytokines. These products of activated basophils are all involved in the symptoms related to allergy. The correlation between the level of IgE in serum and the surface-expression level of FcεRI, on basophils is well known [2–4], and was further confirmed *in vivo* by the advent of the humanized anti-IgE for clinical use [5]. Although the mechanism behind this IgE-induced up-regulation of FcεRI has been studied by several groups, it is still unknown. The studies have been hampered by the lack of long-living cells; the cell death of purified basophils during culture is high, and a reduction of about 50% in the amount is seen during the first week [3]. To account for the high cell death, studies have been performed using a rat basophilic leukaemia cell line, RBL-2H3 [6, 7], or

FcεRI-transfectants [8]. Another basophil model is the human basophil leukaemia cell line KU812, which was established from a patient with chronic myelogenous leukaemia in blast crisis. The cell line has phenotypic properties conforming to those of normal human basophil precursor cells and it can be differentiated into a more mature basophil [9, 10]. It is known that KU812 does not have granules and therefore cannot release histamine after cross-binding FcεRI, but histamine release is, however, possible after IL-4 treatment [11].

To study the mechanism behind the IgE-induced up-regulation of FcεRI, we used KU812 as a model of a human basophil. The cell surface-expression of FcεRI was determined with or without IgE-stimulation and intracellular signalling was characterized after ligand interaction with FcεRI. Using KU812 we found that binding of monomeric IgE to the FcεRI could neither initiate tyrosine-phosphorylation nor release of intracellular Ca²⁺. Thus, an IgE-induced FcεRI-protein synthesis seems not to be the mechanism behind FcεRI up-regulation. However, by using an immunoradiometric assay or Western blot analysis measuring the α-chain from FcεRI (FcεRIα), we found that the FcεRIα-protein is unstable. Further we showed that IgE protects FcεRIα against a temperature dependent turnover.

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Materials and methods

Reagents

Mouse anti-human FcεRI chain monoclonal antibody CRA 1 was obtained from Cosmo Bio Co. Ltd, (Tokyo, Japan). Mouse IgG₁ antibody used for negative control, mouse anti-human IgE and goat anti-mouse IgG FITC conjugated antibody were bought from Dako, Glostrup, Denmark. IgE(PS) was a kind gift from K. Ishizaka (La Jolla, CA, USA). Human IgG1 was from Hybridomus (Cytotech, Noto Bene Scientific, Hellebaek, Denmark). Rabbit anti-human FcεRIα (raised against the extracellular portion of the α-chain) was from Upstate Biotechnology Inc. Lake placid, NY, USA. Mouse anti-Syk (4D10) was from Santa Cruz Biotechnology, Santa Cruz, CA, USA. The protease inhibitors Phenylmethylsulphonylfluoride, aprotinin, leupeptin, pepstatin A, Na₃VO₄ and NaF were from Sigma (St Louis, MO, USA). NP-40 was obtained from Calbiochem, Bad Soden, Germany. PP1 was from BIOMOL Research Laboratories Inc. (Plymouth Meeting, PA, USA).

Cell culture and stimulation

KU812 cells were a kind gift from Gunnar Nilsson (Uppsala, Sweden) and were maintained in RPMI 1640 (Invitrogen Life Technologies A/S, Taastrup, Denmark), supplemented with 10% foetal calf serum (Invitrogen) 100 U/mL penicillin, 100 U/mL streptomycin, 2 mM L-glutamine (Invitrogen) and 50 μM 2-mercaptoethanol (Sigma). KU812 cells were cultured at 37 °C in a humidified atmosphere with 5% CO₂. For stimulation with IgE(PS), KU812 cells were resuspended in RPMI1640 medium added 1 μg/mL IgE(PS) and cultured under normal conditions. The cells were stimulated for 24 h or more as indicated. When sensitized with 1 μg/mL IgE the incubation lasted 2 h. Stimulations were done at 37 °C.

Flow cytometry analysis of the cell surface-expression of FcεRIα

The cell surface expression of FcεRI was assessed by flow cytometry. In brief, cells were incubated with 10 μg/mL CRA 1 antibody or mouse anti-human IgE antibody for 60 min on ice. The cells were washed twice in phosphate-buffered saline (PBS, pH 7.4) added 0.5% bovine serum albumin (BSA) (Sigma) and then incubated with FITC-conjugated F(ab')₂ goat anti-mouse immunoglobulins (25 μg/mL) for 20 min on ice. The cells were washed once in PBS added 0.5% BSA and analysed by flow cytometry (Coulter EPICS[®]XL-MCL, Ramcon A/S, Miami, FL, USA). As a negative control, mouse IgG₁ antibody was used. The extent of FcεRI expression is represented as the geometric mean fluorescence intensity (geoMFI) of CRA 1. The results are corrected for the isotype control. To compare the expression of FcεRI, the ratio between stimulated and unstimulated cells is calculated.

Detection of tyrosine-phosphorylated proteins in KU812

2×10^6 KU812 cells were incubated with IgE(PS) (3 μg/mL), human IgG (3 μg/mL) or medium at 37 °C for 5, 10, 15, 30 and 60 min. The reaction was stopped by the addition of four volumes of ice-cold 1.25 × lysisbuffer (62.5 mM Tris HCL pH 7.4; 1.25% NP-40; 313 mM NaCl; 1.25 mM EDTA; 0.625 mM phenylmethylsulphonylfluoride; 1.25 μg/mL aprotinin; 1.25 μg/mL leupeptin; 1.25 μg/mL pepstatin; 1.25 mM

Na₃VO₄; 1.25 mM NaF) and immediate transfer to ice. After incubation on ice for 20 min, the detergent-insoluble materials were sedimented by centrifugation (15 000 g, 10 min, 4 °C).

For a positive control IgE sensitized cells were incubated with 10 μg/mL rabbit anti-human IgE for 5 min and lysed as described above.

The supernatants were boiled for 3 min with equal volumes of 2 × Laemmli buffer and run on 10% SDS-polyacrylamide gels, prepared according to the Laemmli protocol [12]. The gels were blotted onto PVDF-membranes (Millipore A/S, Hedehusene, Denmark). Excess binding sites were blocked by incubation with 5% BSA in TBST (50 mM Tris base, 150 mM NaCl, 0.5% v/v Tween 20, 5 mM NaN₃, pH 7.4) for 2 h at room temperature (RT). For detection of tyrosine phosphorylated proteins the membranes were then incubated with 0.1 μg/mL of the monoclonal antiphosphotyrosine antibody, clone 4G10 (Upstate Biotechnology, Inc.) for 2 h at RT. The membranes were washed three times in TBST and once in PBS, incubated with horseradish peroxidase-conjugated secondary antibody (1 : 10 000 dilution of anti-mouse immunoglobulin from Sigma) for 20 min and washed three times with TBST and once with PBS. The blots were visualized by the enhanced chemiluminescence system (Amersham Biosciences, Hillerød, Denmark) according to the manufacturer's instructions.

Measurements of intracellular Ca²⁺ in KU812

Intracellular Ca²⁺ measurements were performed using fura 2/AM as described previously [13]. Briefly, the intracellular free Ca²⁺ concentration ([Ca²⁺]_i) in KU812 cells was measured by using a digital imaging system consisting of Zeiss Axiovert 135 microscope equipped with a Zeiss Achroastigmat 40 × objective with a 1.3 numeric aperture, a CCD 72 video camera and a GenIIsys image intensifier from Dage-MTI (Michigan City, IN, USA) and MetaFluor software from Universal Imaging Corporation (West Chester, PA, USA). Cells were loaded with 5 μM fura 2/AM in PIPES buffer with 0.3 mg/mL human serum albumin (Behringwerke AG, Marburg, Germany) for 30 min at 37 °C. After washing with PIPES buffer with 0.3 mg/mL human serum albumin, the cells were transferred to a chamber slide and placed on a microscope stage. [Ca²⁺]_i was measured according to the equation $[Ca^{2+}]_i = K_d \times (R - R_{min}) / (R_{max} - R) \times (S_{f2} / S_{b2})$, where the dissociation constant $K_d = 224$ nM, $S_{f2} / S_{b2} = 1.45$, $R_{max} = 2.8$ and $R_{min} = 0.1$. Stimulation was done by adding IgE(PS) or rabbit anti-human IgE to the cell chamber, using a final concentration of 3 μg/mL IgE(PS) or 10 μg/mL anti-IgE.

Preparation of FcεRIα-standard

KU812 cells were lysed using a 1.25 × lysisbuffer (62.5 mM Tris HCL pH 7.4; 1.25% NP-40; 313 mM NaCl; 1.25 mM EDTA; 0.625 mM phenylmethylsulphonylfluoride; 1.25 μg/mL aprotinin; 1.25 μg/mL leupeptin; 1.25 μg/mL pepstatin; 1.25 mM Na₃VO₄; 1.25 mM NaF). After adding lysis buffer, the cells were kept on ice for 20 min and then centrifuged (2000 g, 10 min, 4 °C). The supernatant was kept at -80 °C until further use. The amount of FcεRIα protein in the standard is validated in arbitrary 'α-chain units'. The standard used was assigned a value of 10,000 α-chain units and could be diluted up to 1000 times in the immunoradiometric assay.

Detection of FcεRIα, using an immunoradiometric assay

Maxisorp tubes (Biotech Line A/S, Slangerup, Denmark) were coated with 100 µl CRA 1 (2 µg/mL) in PBS and incubated at RT overnight. The tubes were washed three times with washing buffer (PBS with 0.1% tween 20) and incubated 1 h at 37 °C with 150 µl of PBS added 0.1% tween 20 including 10% FCS and 1% normal rabbit serum (Dako). After washing three times, 100 µl sample diluted in dilutions buffer (RPMI 1640, 10% FCS, 0.2% tween 20, 3 µg/mL IgE (PS)) was added and incubated overnight at RT. All samples were adjusted to an IgE concentration of 3 µg/mL before dilution. One hundred µl of anti-IgE-I¹²⁵ (Pharmacia Diagnostics AB, Uppsala, Sweden) diluted 1 : 7 in washing buffer was added to washed tubes, which were incubated overnight at RT. After washing, gamma radiation was measured on a 1471 Wizard counter (Perkin Elmer, Allerød, Denmark) for 3 min. All samples were tested in duplicate. The mean value of 10 buffer measurements plus 2 × SD was used as cut-off. Concentrations of unknown samples were calculated by measuring the vertical distance between parallel curves of sample and the FcεRIα-standard. Results are given in fold obtained by using α-chain units (defined in 'Preparation of FcεRIα-standard').

Western blot of FcεRIα

FcεRIα standard was boiled for 3 min with equal volumes of 2 × unreduced sample buffer and run on 16%

SDS-polyacrylamide gels, prepared according to the Laemmli protocol [12]. The Western blotting procedure was performed as described under 'Detection of tyrosine phosphorylated proteins in KU812'. 0.14 µg/mL of rabbit anti-human FcεRIα antibody was used as primary antibody and 1 : 7000 of goat anti-rabbit HRPO conjugated (Santa Cruz Biotechnology) was used as secondary antibody.

Immunoprecipitation of FcεRIα

FcεRIα from the FcεRIα standard was immunoprecipitated with CRA 1 coupled to protein A agarose (Kem-En-Tech A/S, Copenhagen, Denmark). FcεRIα was eluted with 100 mM glycine pH 2.8 and the elute was neutralized immediately by 1 M Tris/HCL buffer pH 8.8. The purified FcεRIα was analysed by using an immunoradiometric assay or boiled for 3 min with equal volumes of 2 × non-reducing sample buffer and run on 16% SDS-polyacrylamide gels, prepared according to the Laemmli protocol [12]. The Western blotting procedure was performed as described under 'Western blot of FcεRIα'.

Results

Expression of FcεRIα in KU812

Surface-expression To detect the basal level of FcεRI-expression on KU812, cells were sensitized for 2 h with 1 µg/mL IgE and then analysed by flow cytometry using the monoclonal

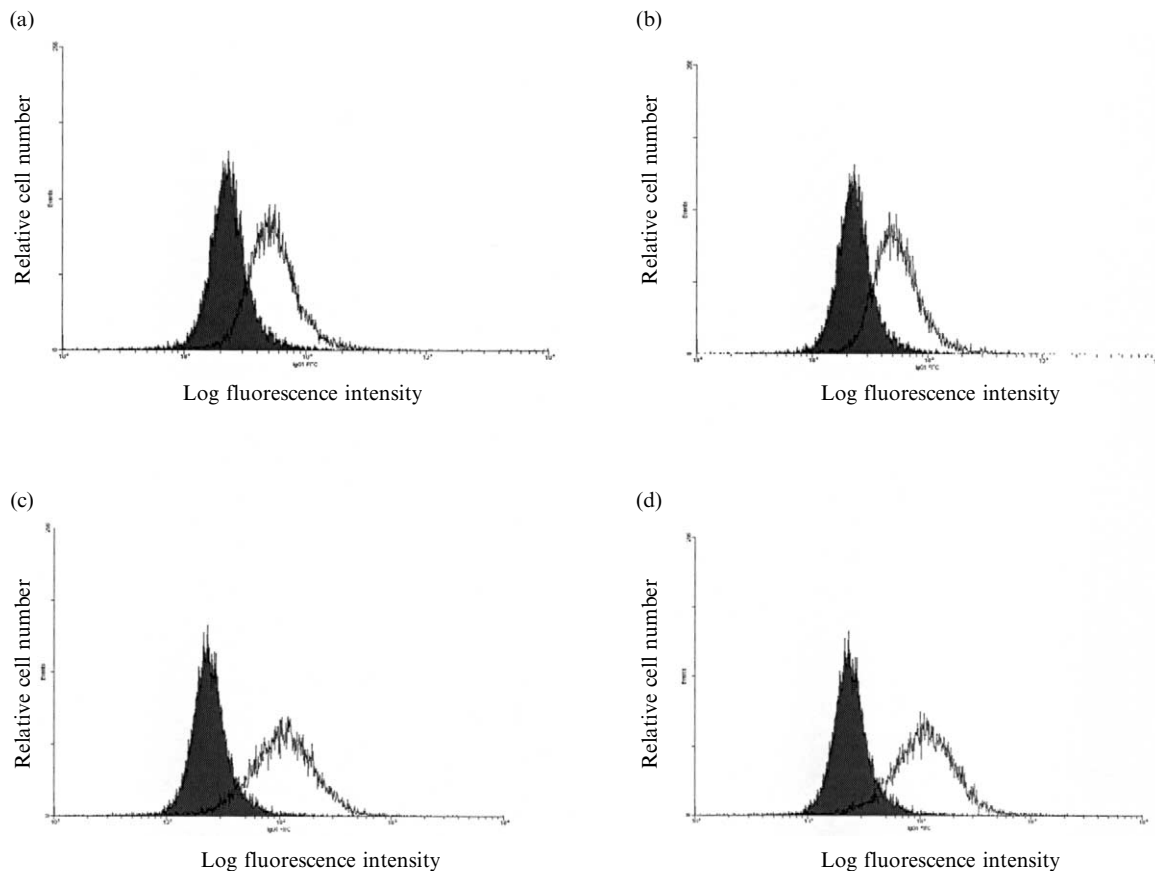


Fig. 1. The expression of FcεRIα on KU812 cells, visualized by CRA 1 or anti-IgE. Filled histogram indicates isotype control, open histogram staining with CRA 1 or anti-IgE. The FcεRI-expression on IgE-sensitized KU812 cells stained with CRA 1 (a) or anti-IgE (b). KU812 stimulated with 1 µg/mL IgE(PS) for 24 h. The FcεRI-expression is detected by CRA 1 (c) or anti-IgE (d).

Table 1. FcεRIα-protein level in KU812 with or without IgE-stimulation determined by flow cytometry (surface level) or immunoradiometric assay (total protein level). All values are the means of three experiments

Day	Surface-expression ^a (fold increase)	Total protein level ^b (fold increase)
1	2, 12 ± 0, 33	0, 98 ± 0, 21
2	2, 41 ± 0, 30	1, 16 ± 0, 03
3	2, 03 ± 0, 36	1, 16 ± 0, 07

^aThe surface-expression is calculated from the geoMFI between IgE-stimulated and unstimulated samples. ^bThe total protein level is calculated by using the α-chain units obtained in the immunoassay.

anti-FcεRIα antibody CRA 1 or anti-IgE. Figure 1(a and b) shows the flow cytometric profiles for KU812 cells sensitized with IgE for 2 h. The geometric mean fluorescence intensity (geoMFI), when using either CRA 1 or anti-IgE, was in both cases 48 ± 2 ($n = 4$).

To examine the IgE-induced effect on the expression of FcεRI on the cell surface of KU812, the cells were treated with 1 µg/mL IgE(PS) for 24 h and then analysed by flow cytometry. As illustrated in Fig. 1(c and d) this led to an increase in the fluorescence, which resulted in a geoMFI of 100 ± 5 ($n = 4$) using either CRA 1 or anti-IgE. Twenty-four hours of stimulation with IgE increased the FcεRI-expression by 2.1-fold.

An unspecific antibody effect was examined by incubating KU812 cells with 1 µg/mL human IgG for 24 h. The IgG-stimulation did not effect the FcεRI-expression (results not shown).

Total cellular FcεRIα-protein level Increase in FcεRIα-protein could also be detected, when measuring the total FcεRIα-protein level, using an immunoradiometric assay. These results show a slight increase of 1.16-fold in FcεRIα-protein, in cells stimulated with IgE for 2 or 3 days, but no increase in the protein level when stimulated for 1 day (Table 1). Together these results show that FcεRI is up-regulated by IgE-stimulation, but the changes in the surface level as determined semiquantitatively by FACS seem higher than the change in total FcεRIα-protein level.

Intracellular signalling in KU812

Tyrosine-phosphorylation To investigate whether the IgE-mediated up-regulation of FcεRI was accompanied by cellular signalling mediated by FcεRI, we studied the tyrosine-phosphorylation in KU812 after stimulation with monomeric IgE. The histamine-releasing properties of the monomeric IgE-solution were tested on human basophils. No histamine was detectable after adding IgE to the basophils, suggesting that the IgE is in fact monomeric. Figure 2(a) shows the tyrosine-phosphorylation in KU812 when stimulated with 3 µg/mL IgE(PS) for 5 to 60 min (lanes 2 to 6). Compared with the positive control (lane 7, 66 kDa protein) made by stimulating IgE-sensitized cells with anti-IgE for 5 min, IgE alone did not induce any tyrosine-phosphorylation. The 66 kDa was recognized to be Syk by using the monoclonal antibody anti-Syk (4D10) (results not shown). Tyrosine-phosphorylation found in the area around 50 kDa and 97 kDa was also found when stimulating with human IgG or cell culture medium, and was considered to be an unspecific cellular reaction and thereby not specific for the IgE-receptor (results not shown). These results

indicate that monomeric IgE does not induce tyrosine-phosphorylation in KU812.

Changes in the intracellular free Ca^{2+} concentration In order to further characterize intracellular signals mediated by FcεRI, we measured the intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$) in KU812 when stimulated with IgE. The results showed that cross-binding FcεRI on IgE-sensitized KU812 cells by anti-IgE antibody caused a rise in $[Ca^{2+}]_i$, but when adding monomeric IgE to non-sensitized KU812 cells no changes in $[Ca^{2+}]_i$ were detected (Fig. 2b). Buffer or anti-IgE added to non-sensitized KU812 cells were used as negative control and did not cause any changes in $[Ca^{2+}]_i$ (results not shown).

Considering the results from the detection of tyrosine-phosphorylation and $[Ca^{2+}]_i$ measurements, it seems unlikely that monomeric IgE induces cellular activation leading to an induced protein synthesis of FcεRI. This was further confirmed by competitive RT-PCR measurements, where we were unable to demonstrate changes at the transcriptional level for the three subunits of FcεRI, when KU812 cells were stimulated with IgE (unpublished data).

Dynamics of soluble FcεRIα

Analysis of FcεRIα-protein level in KU812 lysate, using an immunoradiometric assay As the lack of signalling and the stable expression of mRNA did not support the hypothesis that monomeric IgE could induce a protein synthesis, we turned to another point of view, i.e., the turnover of the FcεRIα-protein. The stability of FcεRIα was tested in an immunoradiometric assay, where FcεRIα was obtained by lysing KU812 cells (the FcεRIα-standard). Samples were generated by incubating the FcεRIα-standard at 4 °C or 37 °C for 5 h with or without 3 µg/mL monomeric IgE. The samples were then analysed in the immunoradiometric assay and as shown in Fig. 3 (illustrating a single experiment) the amount of FcεRIα in the sample incubated at 37 °C showed a reduction of nearly 16-fold of FcεRIα, compared with the standard curve. In the presence of IgE the reduction was only threefold. The amount of FcεRIα in samples incubated at 4 °C with or without IgE were nearly unchanged. The insert in Fig. 3 illustrates the mean fold decrease in the amount of FcεRIα from six experiments. We repeated the experiments with purified FcεRIα obtained by immunoprecipitation using CRA 1. These results showed no difference in the amount of receptors when comparing lysate and purified FcεRIα (data not shown). In summary our data demonstrate that IgE alone protects FcεRIα against a temperature dependent turnover.

Analysis of FcεRIα-protein level, using Western blot technique The protective effect of IgE on FcεRIα in the FcεRIα-standard was further confirmed by Western blot analysis (Fig. 4). We found that the intensity of the FcεRIα-band decreased in the sample incubated at 37 °C (lane 2) but that IgE could inhibit this reduction (lane 3). The amount of FcεRIα in the samples incubated at 4 °C with or without IgE, were not discernible (lane 4 and 5). No degradation products after the 37 °C incubation were detectable on the Western blot. Lane 1 shows a sample revealing the total amount of FcεRIα-protein in the standard.

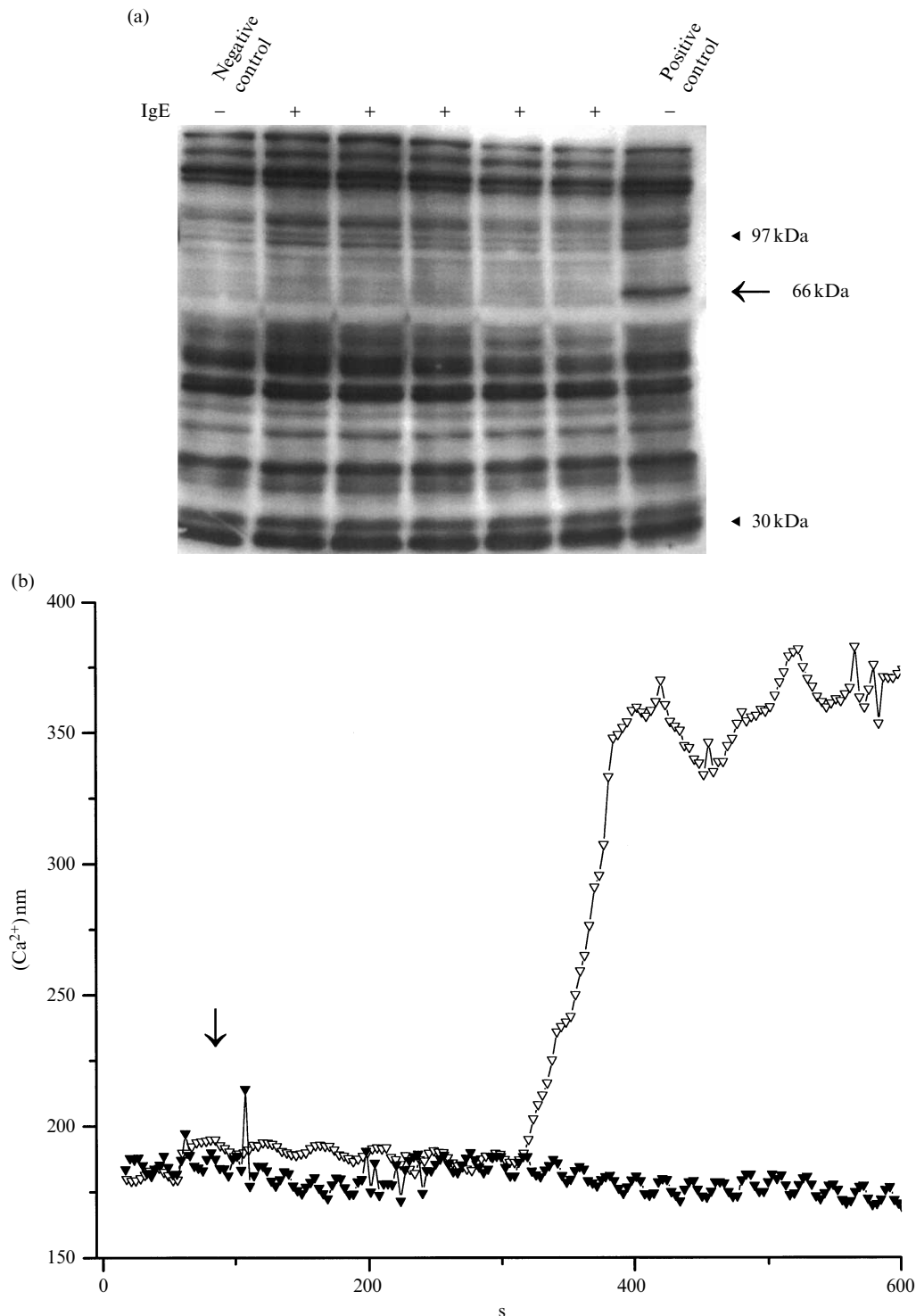


Fig. 2. (a) Detection of tyrosine-phosphorylation in KU812 stimulated with 3 $\mu\text{g/mL}$ IgE(PS). Lane 1: negative control (only medium added). Lanes 2 to 6: IgE(PS) stimulation for 5, 10, 15, 30 or 60 min. Lane 7: positive control (IgE-sensitized KU812 stimulated with 10 $\mu\text{g/mL}$ rabbit anti-human IgE for 5 min). Arrow indicates specific 66 kDa protein (Syk) tyrosine-phosphorylated by anti-IgE stimulation. (b) The level of intracellular Ca^{2+} in KU812 when stimulated with 3 $\mu\text{g/mL}$ IgE(PS) (▼) or IgE-sensitized KU812 stimulated with 10 $\mu\text{g/mL}$ rabbit anti-human IgE (▽). Arrow indicates time of stimulation.

Discussion

Studies of the FcεRI on basophils have been hampered by the low number of basophils in peripheral blood, the difficulties in obtaining large quantities of pure basophils, and the great loss

when culturing the cells. Our approach has therefore been the use of the basophil leukaemic cell line KU812 [10]. The cell line constitutively expresses FcεRI, and when sensitized, the receptor binds its ligand as demonstrated in Fig. 1(a + b). When cultured for 24 h with IgE, the receptor surface expression

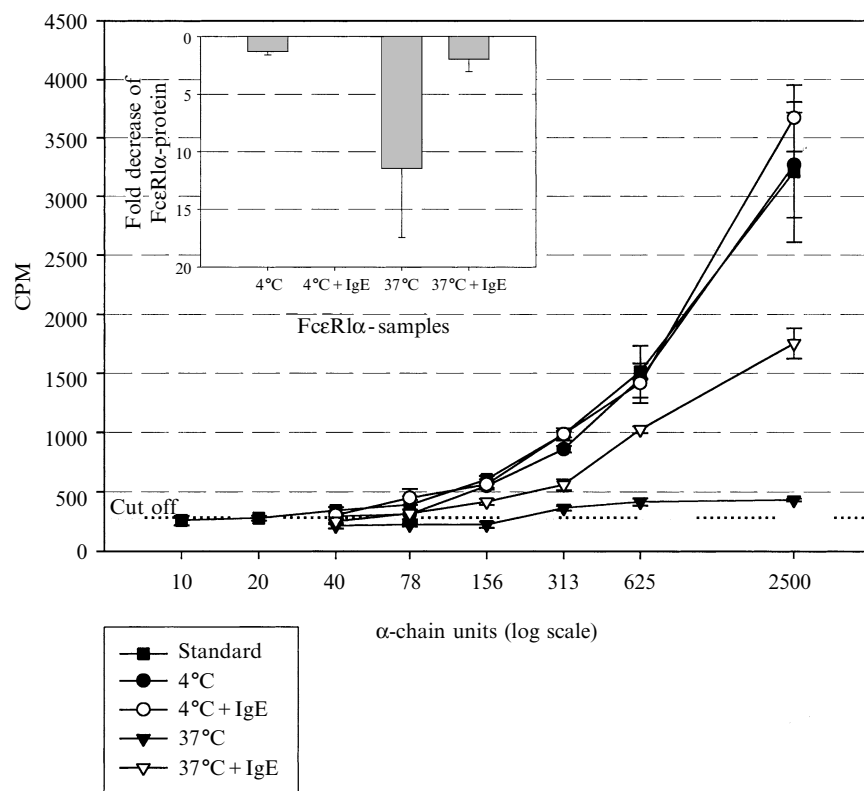


Fig. 3. Specimens of FcεRIα, obtained by using the FcεRIα-standard (lysate of 10⁷ KU812 per mL) detected in a sandwich immunoradiometric assay. Samples were incubated at 4 °C or 37 °C with or without 3 µg/mL IgE(PS) for 5 h and then measured. Inserted diagram shows the mean fold decrease of FcεRIα in samples from six experiments.

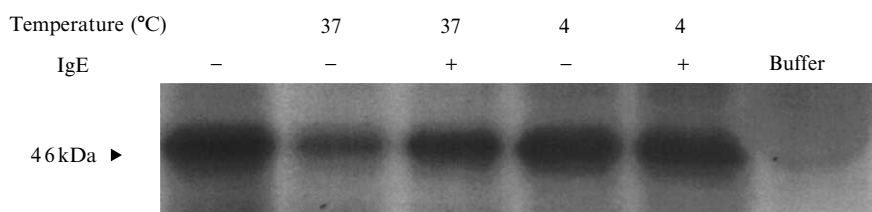


Fig. 4. Western blot of FcεRIα-protein in the FcεRIα-standard incubated at 4 °C or 37 °C with or without 3 µg/mL IgE(PS) for 5 h. Lane 1: FcεRIα-standard (no incubation). Lane 2: FcεRIα-standard incubated at 37 °C. Lane 3: FcεRIα-standard incubated at 37 °C added IgE. Lane 4: FcεRIα-standard incubated at 4 °C. Lane 5: FcεRIα-standard incubated at 4 °C added IgE. Lane 6: buffer (RPMI1640, 10% FCS, 3 µg/mL IgE).

increases, in parallel with findings on basophils [14–16], and cell lines transfected with FcεRI αβγ₂ and αγ₂ complexes [8].

The KU812 cell line can transduce signals via the FcεRI, as evidenced by changes in [Ca²⁺]_i and phosphorylation after cross-linking of the receptor. This enabled us to characterize the conditions in which monomeric IgE was present in the cell culture. Kalesnikoff et al. have reported that monomeric anti-DNP IgE (clone SPE-7) could induce intracellular phosphorylation when binding to FcεRI, which leads to cytokine production and cell survival [17]. This is, however, in conflict with the results generated by Asai et al. [18]. This latter group also reported the ability of IgE (monomeric anti-DNP IgE (H1 DNP-G-206)) to suppress induced apoptosis in BMMCs, which occur in parallel with the IgE-induced up-regulation, but they do not find any intracellular signalling as a result of the IgE-binding. In spite of an up-regulation of the receptor in the presence of monomeric IgE, we could not detect an increase in [Ca²⁺]_i or any phosphorylation which is in parallel to the findings of Asai et al. These results are further confirmed by experiments with transfected cell lines in which Borkowski et al. showed that the classical pathway involving the Src family kinase Lyn is not involved in the IgE-mediated up-regulation [8].

In a control experiment we did see a tyrosine-phosphorylation around 50 and 97 kDa that seemed to decline with time, when the cells were stimulated with IgE. This phosphorylation is also found when stimulating the cells with human IgG or normal cell culture medium, and for this reason we do not consider the phosphorylation of these proteins as specifically caused by IgE. Instead we believe that the phosphorylation of the 50 and 97 kDa proteins is caused by an unspecific reaction initiated by the gentle manipulation of the cells. The same particular phosphorylation pattern is also found when cells are mixed in the tube without any specific stimulation. Thus it seems unlikely that IgE by itself induces a signal that may increase transcription of the FcεRI α-, β- and γ-genes. This was confirmed by quantitative RT-PCR experiments, indicating an unaltered steady state of mRNA for all three subunits of the receptor whether or not IgE was present in the culture supernatant (unpublished data). These results are in accordance with data obtained in human basophils [19].

The above data confirm that the KU812 cell line shares characteristics with human basophils and is consistent with the cellular models that have been used for studies of the mechanism behind IgE-induced up-regulation of FcεRI

surface-expression. These models indicate that the protein synthesis of the FcεRI is constitutive and independent of the level of IgE in the cellular environment. Thus changes in the surface-expression must be ascribed either to modified internalization rates or to changes in the degradation of the receptor molecule. To study the possibility of degradation we took the cellular model one step further and developed an immunoassay for determination of the FcεRIα-protein in lysed cells. The sandwich immunoassay is based on the ligand-binding functionality of FcεRIα, i.e., to be detected it must maintain the integrity of the epitope for the CRA1 antibody as well as the IgE-binding domain.

By the immunoassay it was demonstrated that the total level of cellular FcεRIα-protein was slightly increased (about 1.16-fold on 2 days, Table 1) in cell cultures that had been cultured with IgE. Although data from flow cytometry cannot be compared directly, the relatively larger increase in surface expression compared with the total protein determination indicates that FcεRIα simply remains longer on the surface. Further the changes in FcεRI level cannot be explained by an increased protein synthesis because the change in the total receptor pool is small. Moreover, whereas cycloheximide inhibited receptor expression, IgE still up-regulated receptor expression in cycloheximide-treated cells (results not shown). In conclusion, the increase in the total level of cellular FcεRIα protein is a result of stabilized surface FcεRIα by IgE-binding.

Interestingly, when applying this cell-free system of FcεRIα-protein determination, a strong time- and temperature-dependent degradation was found. Studies of the reduction of FcεRIα over 24 h illustrate a fast turnover. After 1 h more than a sixfold reduction was found and the differences in reduction of FcεRIα in 2 h and 12 h samples was minimal (results not shown). As demonstrated in Fig. 3 this turnover could be almost completely inhibited by addition of IgE. These findings were confirmed in Western blot experiments using a rabbit antibody directed against FcεRIα, a method with less requirements for functional integrity of the receptor molecule. No degradation products could be found in these Western blots. The results suggest that proteolytic activity in the lysate caused the turnover, and that IgE could protect the subunit against proteolytic cleavage. However, when protease inhibitors were tested, including those in the lysis buffer, they all failed to protect FcεRIα against degradation (results not shown). The inability of protease inhibitors to reduce the receptor turnover has also been described by others [19]. Moreover, it seems unlikely that another factor in the lysate causes the turnover as immunoprecipitated FcεRIα-protein is degraded with identical kinetics and had identical IgE-inhibition of the degradation as FcεRIα in the whole lysate.

The explanation of the turnover could be that the conformation of FcεRIα changes during the 37 °C incubation and this conformation-change (resulting in loss of selective epitopes and/or the IgE-binding capacity) is the reason why there is a difference in the results obtained by the immunoradiometric assay (detecting only IgE-binding FcεRIα-chain) and the Western blot. Using the immunoradiometric assay we saw that nearly all FcεRIα was undetectable in the 37 °C sample, but by using the Western blot still some FcεRIα was detected in the 37 °C sample. A conformational change could further result in degradation of the protein but apparently the concentration of the fraction (or fractions?) is too small to be detected by

silver-staining (result not shown) and the putative fraction(s) become unrecognizable by the polyclonal antibody used for the Western blot.

Even though the possibility of a temperature-induced conformation-change is not further investigated, we know now that FcεRIα is an unstable protein, with a temperature dependent turnover (higher at physiological temperatures compared with 4 °C) and that binding of IgE stabilizes the protein. It has been found that IgE induces a conformational change in FcεRI after ligand binding, which could confer a more stable form to the FcεRIα-protein [20, 21]. Experiments using IgE occupied or unoccupied receptors show that the loss of receptor only takes place in the group of unoccupied receptors [19]. It can be argued that the FcεRIα-protein in a lysate or in an immunoprecipitated preparation is much more vulnerable to degradation compared with the situation where it is integrated in the cell membrane, but the striking increase in stability by its binding to IgE, suggests that the degradative processes may also be working in the intact cell.

In the study of the FcεRIα-protein, recombinant forms have been used, without any reports of instability. The reason for this could be that the recombinant FcεRIα-constructs comprise less than the total native sequence or have a modified glycosylation depending on the expression systems used [22–24]. Such modifications could be essential for the stabilization of the FcεRIα-protein.

The findings that monomeric IgE also acts as an anti-apoptotic molecule in BMMCs is very interesting [18]. Considering this phenomenon and that IgE stabilizes FcεRIα, gives us a new picture of IgE in the allergic reaction. IgE not only arms the effector cells, it also stabilizes its own receptor and protects the host cell against apoptosis. These new findings provide support for the use of the anti-IgE treatment. By the removal of IgE we know that the number of FcεRI decreases and these new results also suggest it to be the case for the number of effector cells [5].

Collectively, our results indicate that the effect of IgE is not to protect the FcεRIα-protein from a proteolytic breakdown but to prevent FcεRIα from a natural turnover. FcεRIα is apparently an unstable protein that IgE stabilizes, thereby prolonging its biological lifetime. This further supports the idea that FcεRIα is the primary candidate in receptor stabilization [8]. However, this may not be the only operative mechanism *in vivo*. It has been suggested that the stabilizing effect of IgE is caused by an inhibition of internalization and degradation of FcεRI [8]. Further studies are necessary to clarify whether the IgE stabilization of FcεRIα is the only mechanism in the FcεRI up-regulation or whether other dynamic factors also play a role.

Acknowledgements

The authors would like to thank Charlotte Thygesen and Gita Kampen for valuable discussions. Financial support was obtained from the University of Copenhagen

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