

Original article

Calreticulin is an interleukin-3-sensitive calcium-binding protein in human basophil leukocytes

Background: IL-3 enhances basophil histamine release upon stimulation with any known secretagogue. The molecular mechanism behind this regulation is not known, although some observations suggest that IL-3 modulates the calcium part of the signal transduction mechanism. The inhibitory action of glucocorticoids on basophils can be reversed by stimulation with IL-3.

Methods: Calcium-binding proteins in the basophil cell line KU812 were identified by two-dimensional gel electrophoresis, Calcium-overlay assay, N-terminal sequence analysis, and mass spectrometry. The presence of the same proteins in purified human basophil leukocytes was established by comigration of KU812 and human basophil proteins on the two-dimensional gels. The expression of the calcium-binding proteins in the absence and presence of IL-3 and/or anti-IgE was determined by densitometric measurement of the spots on the two-dimensional gels.

Results: Calreticulin was identified on the two-dimensional gel of KU812 proteins. A protein with exactly the same migration pattern was found on the gels of proteins from purified human basophils. Immunoblotting with a specific anti-human calreticulin antibody confirmed that this protein was calreticulin. Subsequent analysis showed that the expression of calreticulin in the basophils is upregulated twofold upon stimulation with rhIL-3, even in doses below those needed for enhancement of histamine release.

Conclusions: The expression of calreticulin in human basophil leukocytes is regulated by IL-3. Calreticulin is known to modulate IP_3 -dependent Ca^{2+} influx in different cell systems, and calreticulin overexpression inhibits steroid-induced transcriptional activation. Therefore, modulation of calreticulin expression may be one mechanism by which IL-3 exerts its effects on human basophils.

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Basophils are effector cells in the late-phase reaction of allergy. They invade the tissue hours after the initial allergy response (1–3).

In vitro experiments have shown that IL-3 enhances the secretion of histamine (4) IL-4 (5), and leukotrienes (6) from these cells. IL-3 enhances histamine secretion after anti-IgE, FMLP, A23187, and C5a stimulation, although these stimulators use different signal transduction mechanisms (4, 6–8). Therefore, it is thought that IL-3 regulates the secretion on central points in the signal transduction pathways (9).

When stimulated with anti-IgE antibodies, human basophils show a biphasic intracellular calcium response. The initial response is independent of extracellular calcium and results from release of internal calcium in the endoplasmic reticulum mediated by inositol 1,4,5-triphosphate (IP_3). The second response is due to influx from the extracellular space and is blocked by EDTA, resulting in obliteration of the histamine release (10).

Calreticulin is an intracellular calcium-binding protein primarily located in the endoplasmic reticulum. It is codistributed with the IP_3 receptor in RBL-2H3 rat basophilic leukemia cells (11), and modulates the calcium signal by interacting with the IP_3 receptor (11). Therefore, calreticulin may be a mediator protein of IL-3 action. In this study, we demonstrated that IL-3 upregulates the expression of calreticulin in basophil leukocytes.

Material and methods

Materials

Dynabeads were obtained from Dynal, Oslo, Norway. The monoclonal antihuman antibodies CD3, CD4, CD8, CD14, CD16, CD19, monoclonal anti-IgE, and horseradish peroxidase-conjugated goat antirabbit antibody were from Dakopatts, Glostrup, Denmark. Polyclonal goat antihuman anti-IgE was from Behringwerke, Marburg, Germany. Polyclonal rabbit antihuman calreticulin antibody was from ABR, Golden, USA. Recombinant human IL-3 was

obtained from Genzyme, USA. ^{35}S -Cys/Met (promixTM) was supplied by Amersham, UK. Percoll, Ficoll-Hypaque, and Ampholines pH 8–10, pH 7–9, and pH 3.5–10 were acquired from Pharmacia Biotech, Uppsala, Sweden, and ampholines, pH 5–7, from Serva, Heidelberg, Germany. ^{45}Ca was from ICN, Irvine, CA, USA. Films for autoradiography were Kodac X-omatic. PVDF membranes were from MSI, Westboro, MA, USA. ELC Western blotting detection reagents were from Amersham, UK. All other reagents used were of analytic grade.

Methods

Purification of the basophil leukocytes. The basophil leukocytes were purified by Dynabeads in a negative selection procedure (12). After negative selection, the cells were stained with Alcian blue, counted with a hemacytometer, and washed in serum-free RPMI 1640. Only samples with more than 80% basophil leukocytes were used for experiments. The average yield of these samples was 1.3×10^6 basophils. Spontaneous histamine release in these basophils was below 5%. KU812 cells were grown in RPMI 1640 (10% HSA). They were washed in serum-free RPMI 1640 before application to the gels.

Histamine measurement. Histamine was measured in the pellet and the supernatant by the method previously described (13). Histamine release was expressed as percentage of the total content of histamine. All measurements were made in triplicate.

Purification of mononuclear cells. For purification of mononuclear cells, blood was layered on Ficoll-Hypaque according to the instructions of the manufacturer.

Two-dimensional gel electrophoresis. The cell pellet was dissolved in lysis buffer (9.8 M urea, 2% (w/v) NP40, 2% ampholine pH 7–9, 100 mM DTT) and applied to the 2-D gels (two-dimensional gels). 2-D-gel electrophoresis was performed as described by Celis et al. (14), except that we used the Protean xi 2-D Cell from Bio-Rad. MW and pI were assigned according to comigration with 2-D-gel protein standards.

Autoradiography. Gels were fixed in 50% methanol, 10% acetic acid, for 4 h and washed several times in 30% ethanol. Subsequently, they were dried in a Bio-Rad gel dryer and exposed to radiograph film in a cassette at room temperature. The exposure time varied from 5 to 14 days.

Immunoblotting. For immunoblotting, proteins in the 2-D gel were transferred to PVDF membranes in a Tris (50 mM)-boric acid (50 mM) buffer at 130 mA overnight. After transfer of the proteins, the blot was blocked in a PBS-T (0.1% Tween) buffer with skim milk (5%) for 1 h, followed by incubation overnight at room temperature with the antibody. The HRP-conjugated secondary rabbit-anti-mouse antibody was used in a dilution of 1:1000 for 1 h at room temperature. The HRP-conjugated secondary antibody was detected by a chemiluminescence system (Super-signal).

Calcium overlay assay. Calcium overlay assay was performed as previously described (15). In brief, proteins were transferred from the gel to PVDF membranes as described above. The membranes were then washed three times in buffer (60 mM KCl, 5 mM MgCl_2 , 10 mM imidazol-HCl, pH 6.8), followed by probing with $3.5 \mu\text{l}$ $^{45}\text{CaCl}_2$ (specific activity: 26 mCi/ml) in 35 ml buffer for 35 min. After probing, the membranes were washed in 67% ethanol, and subjected to autoradiography and staining with amido black.

Amino-acid sequencing. The proteins in the gels were transferred to PVDF membranes, as described above, and stained with amido black (0.1% amido black, 45% MeOH, 7% acetic acid). Before the sequence analysis was carried out, the membrane piece was washed twice in 50% methanol including a few minutes of sonication during the first wash. The membrane piece was then transferred to the blot cartridge of the "Procise" protein sequencer (model 494A, Perkin-Elmer ABD, Foster City, CA, USA) and analyzed by essentially the preprogrammed "blot" cycles. All solvents and reagents were from Perkin-Elmer ABD, except methanol and solvent B for the HPLC separation of the PTH amino-acid derivatives.

Mass spectrometry: identification of proteins. Stained bands were excised from the gel and digested with trypsin, essentially as described by Wilm et al. (16). In brief, the gel piece was cut into small cubes, and washed for 1 h in 100 mM NH_4HCO_3 (AB), and the excess liquid was removed. The proteins were then reduced for 30 min at 60°C in 100 μl 4 mM dithiotreitol in 100 mM AB, cooled, and alkylated by addition of 10 μl 100 mM iodoacetamide followed by 30-min incubation at room temperature in the dark. Excess liquid was removed, and the gel pieces were washed for 1 h in 50% acetonitrile in 100 mM AB, followed by shrinkage in acetonitrile and vacuum centrifugation. The tube with the dry gel pieces was placed in an ice bath and allowed to swell in 25 mM AB including 2 $\mu\text{g}/100 \mu\text{l}$ of modified trypsin (Promega). After 45 min, the remaining supernatant was removed, and buffer was added to cover the gel pieces during overnight incubation at 37°C. Next day, the supernatant was removed and combined with two consecutive extractions with 50 μl 0.1% trifluoroacetic acid in 60% acetonitrile. The extract was dried in a vacuum centrifuge to near dryness, followed by addition of 5 μl 0.1% trifluoroacetic acid in 30% acetonitrile. The extracted peptides were analyzed in a matrix assisted laser desorption/ionization time-of-flight mass spectrometer with reflector and delayed extraction option (Biflex, Bruker-Franzen, Bremen, Germany). For analysis, 0.4 μl of the sample was mixed with 0.1 μl internal standard mixture (angiotensin II and dynorphin, 0.06 and 1 pmol/ μl , respectively) and 0.5 μl matrix solution (α -cyano-4 hydroxycinnamic acid in acetonitrile/methanol [Hewlett Packard]). Then 0.5 μl of the mixture was applied to the probe and allowed to dry before introduction into the mass spectrometer. The peptide molecular mass fingerprint was used to search the SWISS-PROT database with the "PeptIdent" tool on the ExPASy Molecular Biology Server of the Swiss Institute of Bioinformatics (17).

Measurement of the expression of calreticulin in basophils. The expression of calreticulin in human basophils upon stimulation with IL-3 and/or anti-IgE was measured against controls in a database of 2-D gels of proteins from purified human basophils. Details of the database have been described previously (18). In brief, human basophils were purified and shortly after used for experiments. The cells were incubated at a concentration of $5 \times 10^6/\text{ml}$ in serum-free RPMI 1640 deficient in methionine, cysteine, and cystine with 0.3 mCi/100 μl ^{35}S -met/cys (specific activity: 1000 Ci/mM). One stimulation experiment consisted of paired samples subjected to different stimulation. The total number of cells in an experiment varied from 0.7×10^6 to 1.8×10^6 . Within an experiment, each sample consisted of equal amounts of basophil leukocytes. Anti-IgE and rIL-3 were added as indicated. The cells were incubated for 6 h at 37°C, 5% CO_2 . The viability of the cells was assessed after incubation with trypan blue exclusion and consistently exceeded 95%. After incubation, the cells were pelleted (400 g, 4 min), dissolved in lysis buffer, and applied to the 2-D gels. The amount of anti-IgE used was the optimal for histamine secretion from the donors as determined by earlier experiments. Buffer was added as control. Parallel samples were run for the histamine measurements.

Gels were analyzed both visually and with a table-top scanner (Mustek MFS-6000CX). The proteins were quantified with a 2-D-gel densitometry software program from Phoretix, UK. The spot volume was defined as the summation of the value of the intensities of each pixel in the spot. To correct for differences in applied material to the gel, the total gel volume was used as internal standard. Thus, the result of the densitometric measurements was presented as follows:

(spot % of total gel volume) =

(spot volume) / (total gel volume) $\times$ 100%.

Statistics. Data were analyzed by Wilcoxon's signed rank test for matched pairs.

Results

Identification of calreticulin in basophils

As a first approach, calcium-binding proteins were identified in the basophilic cell line KU812. Fig. 1 shows the autoradiography of $^{45}\text{Ca}^{2+}$ -binding proteins from the KU812 cells. All of these spots were cut out and subjected to N-terminal sequence analysis. Of the 58 proteins that were analyzed, an N-terminal sequence of nine was obtained. The arrow points at the spot with a N-terminal sequence identical to calreticulin. The same protein was shown by trypsin digestion and MALDI-TOF to contain peptides with molecular masses identical to those expected from a tryptic digest of human calreticulin (results not shown).

Calreticulin in human basophils was identified by a comigration experiment in which a surplus (2×10^6) of unlabeled KU812 cells were mixed with ^{35}S -Cys/Met-labeled 0.25×10^6 purified human basophils. After 2-D gel electrophoresis, the gel was silver-stained, dried, and subjected to autoradiography. The basophil calreticulin was found by identifying the ^{35}S -Cys/met-labeled protein with exactly the same migration as the KU812 calreticulin found by N-terminal sequencing (Fig. 2).

To confirm the identification of the basophil calreticulin, immunoblotting was performed on a 2-D gel of 0.15×10^6 human basophils (purity: 98%) with the polyclonal rabbit anticalreticulin antibody that is specific to calreticulin (19). Immunoblotting with this antibody confirmed the location of calreticulin on the gels of human basophil proteins (Fig. 3).

Synthesis of calreticulin is enhanced by IL-3

The expression of calreticulin in the basophils upon IL-3 and/or anti-IgE treatment of the cells was measured as described in the Methods section. Fig. 4a–d shows a representative sequence of gels from one donor with the expression of calreticulin in basophils upon stimulation with anti-IgE (50 IU/ml), (rh)IL-3 (100 ng/ml), or both. Fig. 5a–c shows densitometric measurements of calreticulin in 12 experiments. There was a significant twofold

increase (median) in the expression of calreticulin upon stimulation with IL-3. Stimulation with both (rh)IL-3 and anti-IgE resulted in a similar increase, whereas anti-IgE alone had no significant effect. Fig. 5d shows the histamine release measured in the experiments. As the contaminating cells in the purified samples were mainly lymphocytes, control experiments were conducted in which purified mononuclear cells were obtained and stimulated like the basophils. The content of basophil leukocytes in these samples was below 2%. There was no regulatory effect of any stimulation in these experiments (Fig. 5e).

(rh)IL-3 dose-response relation

Five experiments were done to find a possible dose-response relationship between IL-3 and the expression of calreticulin. Previous experiments showed maximal (rh)IL-3-induced histamine release from purified basophils at a concentration of 100 ng/ml IL-3 (20). Therefore, the basophils were incubated with (rh)IL-3 at concentrations of 10, 100, and 1000 ng/ml. We found that the expression of calreticulin was enhanced already at a concentration of 10 ng/ml (rh)IL-3 (Fig. 6).

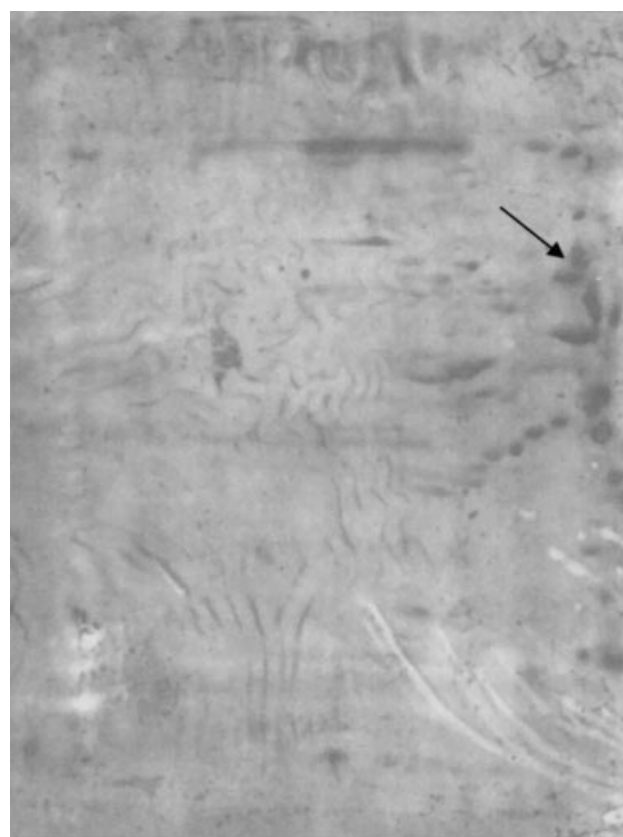


Figure 1. Calcium overlay assay of KU812 cell proteins. Proteins from KU812 cells were separated on 2-D gel and blotted onto PVDF membrane. Proteins were probed with $^{45}\text{Ca}^{2+}$ and subjected to autoradiography. Calcium-binding proteins were excised from membrane and subjected to protein analysis. Arrow points at protein identified as calreticulin.

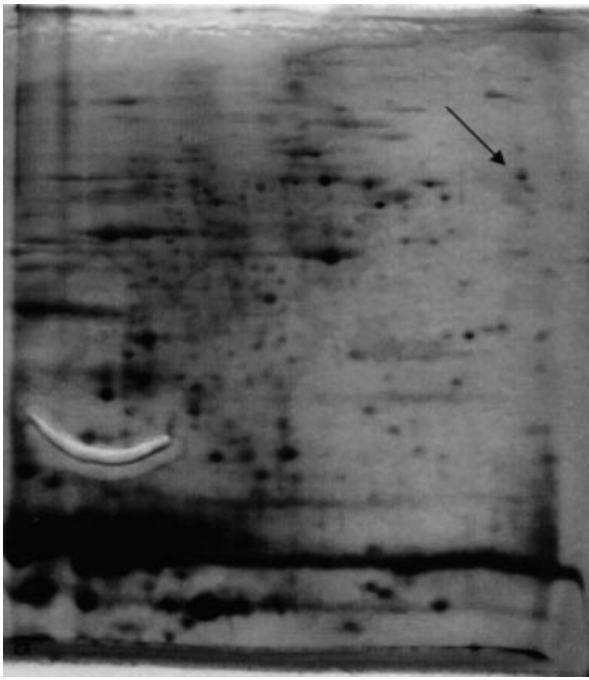


Figure 2. Comigration of calreticulin from KU812 cells and human basophils. 0.25×10^6 purified human basophils were labeled with ^{35}S -Cys/Met overnight and mixed with 2×10^6 KU812 cells. Proteins in mixture were separated by 2-D-gel electrophoresis. Gel was silver-stained (a) and dried, and autoradiogram (b) was obtained. Arrow points in (a) at KU812 calreticulin and in (b) at corresponding basophil labeled calreticulin.

Discussion

In this study, we identified calreticulin on 2-D gels of proteins from purified human basophils. Subsequent

analysis at different experimental conditions showed that IL-3 upregulates the expression of calreticulin in these cells. IL-3 enhanced the synthesis of calreticulin at a dose lower than that needed for stimulation of

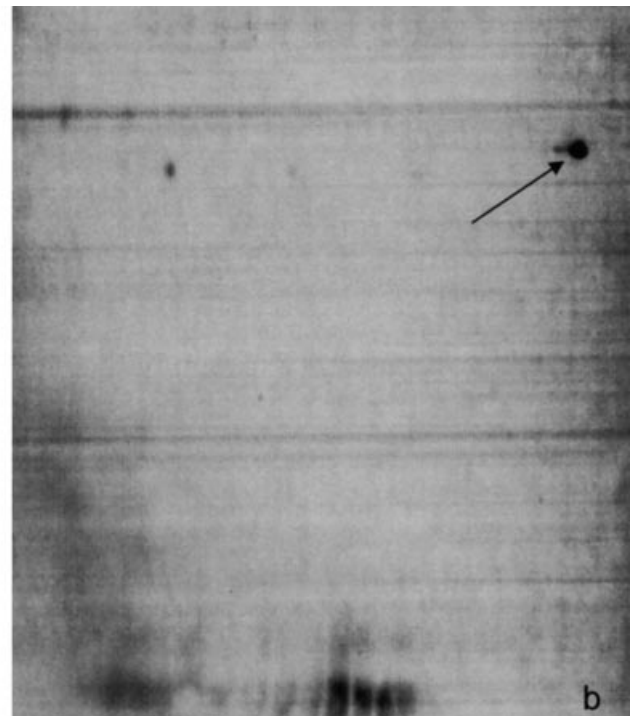
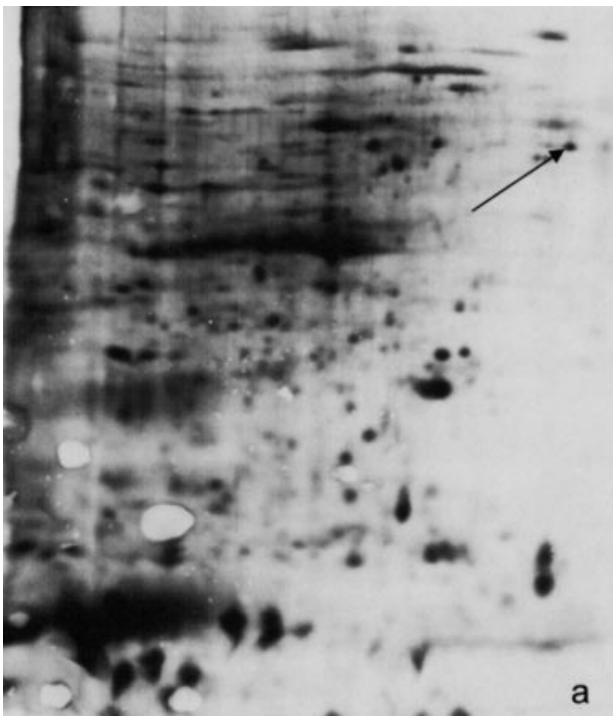


Figure 3. Immunoblot of calreticulin in human basophils. Proteins from 0.15×10^6 radioactively labeled human basophils (purity: 98%) (a) were blotted with anticalreticulin antibody (b) as described in text. Arrows point at calreticulin.

histamine release. The donors used for these experiments all released histamine upon anti-IgE and IL-3 stimulation.

As IL-3 is a known basophilopoietin, it might be speculated that the observed upregulation of calreticulin was due to a general upregulation of protein expression. However, of the 58 proteins identified by the calcium-overlay assay (Fig. 1), only two (calreticulin and an unknown protein) showed significant upregulation by IL-3. Furthermore, we used the total amount of protein synthesis (represented by the densitometric measurement of the total gel volume) as internal standard.

The mechanism by which IL-3 upregulates histamine release in basophils is not known, but some observations indicate that IL-3 interferes with the calcium part of the signal transduction pathways. Thus, IL-3 affects the level of the Ca^{2+} peaks after long-term (24-h) (21), but not short-term (15-min) (22), exposure to the cells. During a 3-day stimulation period, IL-3 converted nonresponder basophils into responders (23), and another study showed that nonresponder basophils are unable to increase Ca^{2+} levels after stimulation with anti-IgE (24).

Calreticulin is a calcium-binding protein residing in the endoplasmic reticulum (ER). Several observations suggest that calreticulin plays a role in the regulation of Ca^{2+} homeostasis: overexpression of calreticulin in different

cellular systems results in an increased amount of intracellularly stored Ca^{2+} (25–27). Calreticulin is codistributed with an IP_3 receptor in RBL-2H3 cells (28). The inositol 3 phosphate (IP_3)-dependent calcium release may be influenced by calreticulin. Thus, calreticulin-deficient mouse embryonic fibroblasts have decreased agonist-induced IP_3 -dependent Ca^{2+} release compared to controls (29). In the neuroblastoma-glioma NG-108-15 cells, downregulation of calreticulin expression with an antisense cDNA results in the inhibition of IP_3 -dependent Ca^{2+} release from the ER (30). Overexpression of calreticulin in *Xenopus laevis* oocytes results in inhibition of the frequency of IP_3 -induced repetitive Ca^{2+} waves (11). Therefore, it is possible that the upregulation of calreticulin after IL-3 stimulation in human basophils could contribute to the modulation of intracellular calcium release, and in this manner be a factor in the regulation of the degranulation of basophils. Calreticulin also seems to be essential for integrin-mediated calcium signaling and cell adhesion (31).

Another intriguing function of calreticulin is its role in the modulation of steroid signal transduction. Transcriptional activation of androgen, glucocorticoid, retinoic acid, and vitamin D receptors is inhibited *in vivo* in cells overexpressing calreticulin (27, 32–35). Upregulation of calreticulin expression may therefore correlate with increased resistance to steroids. It is known that IL-3 and glucocorticoids modulate histamine release

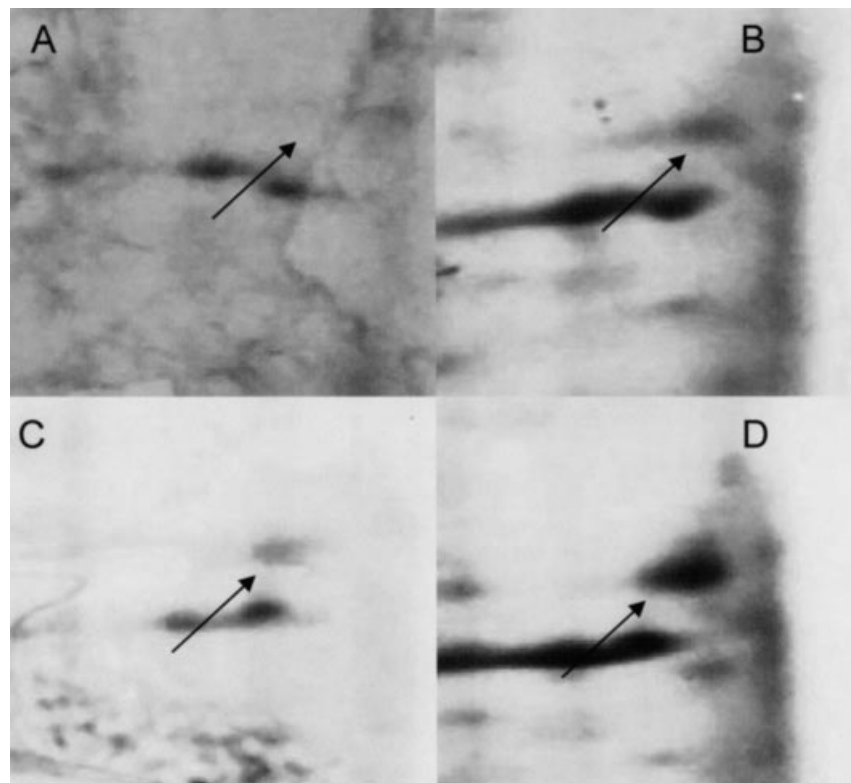


Figure 4. Expression of calreticulin in purified human basophils. Panels A–D show 2-D-gel electrophoretic patterns from one experiment in which basophils were incubated with media and buffer (control) (A), rhIL-3 100 ng/ml (B), anti-IgE 50 IU/ml (C), or anti-IgE 50 IU/ml plus rhIL-3 100 ng/ml (D) for 6 h. Arrow points at calreticulin.

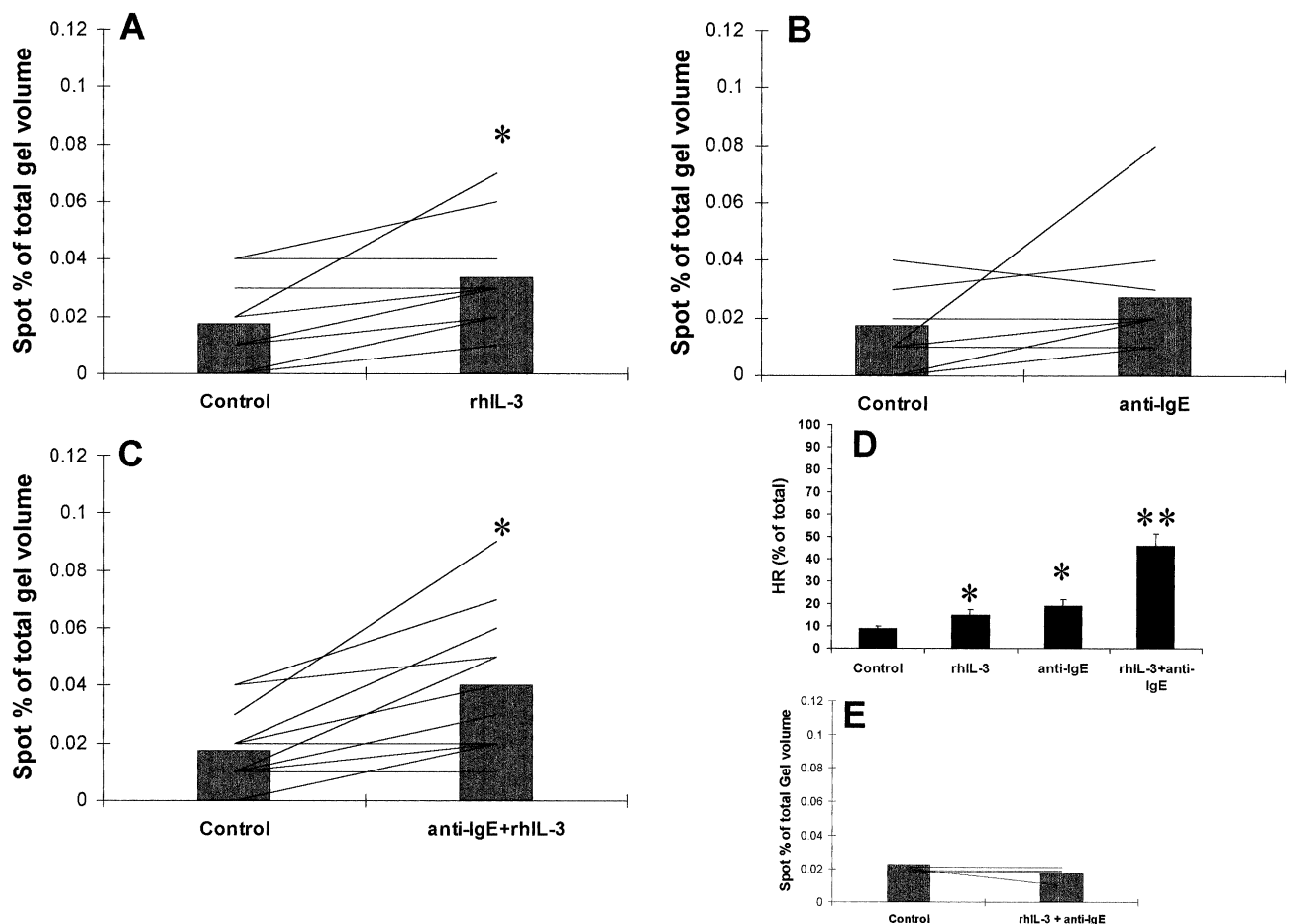


Figure 5. Experiment described in Fig. 4 was repeated 12 times with purified human basophils, and panels A–C show densitometric measurements of calreticulin at different experimental conditions compared to controls: with rhIL-3 100 ng/ml (A), with anti-IgE 50 IU/ml (B), or with rhIL-3 100 ng/ml plus anti-IgE 50 IU/ml (C). Histamine release (mean $\pm$ SEM) in samples of all 12 experiments is shown as well (D). * $P < 0.01$, ** $P < 0.001$ compared to control. Panel E represents control experiments. Diagram shows densitometric measurements of calreticulin in mononuclear cells stimulated with anti-IgE and rhIL-3 compared to control cells.

from basophils antagonistically (36). IL-3-induced calreticulin expression could be the molecular mechanism behind this phenomenon. Differences in the expression of calreticulin may also be the pathophysiologic mechanism behind differential steroid sensitivity in patients with allergic diseases. The expression of calreticulin in human basophils *in vitro* showed no modulation by dexamethason (P. M. Shah, H. V. Nielsen, unpublished observation).

In conclusion, we have found that IL-3 upregulates the expression of calreticulin in human basophil leukocytes *in vitro*. This finding is interesting because calreticulin has been shown to modulate IP_3 -receptor-dependent Ca^{2+} influx. This might explain the IL-3-induced upregulation of histamine release from basophils upon stimulation. Furthermore, some observations indicate that calreticulin modulates glucocorticoid signal transduction *in vivo*. This molecular mechanism offers a possible explanation of the antagonistic effects of IL-3 and glucocorticoids seen *in vitro* in basophil histamine release.

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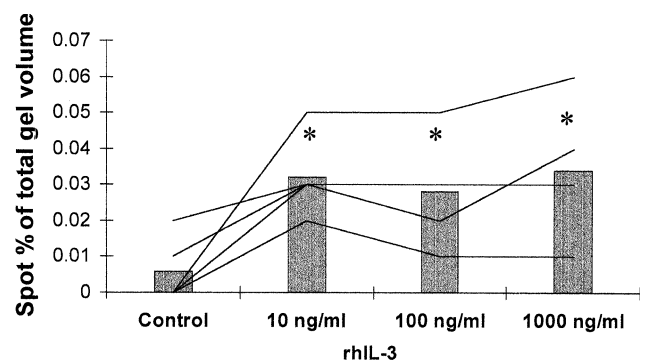


Figure 6. Densitometric measurements of calreticulin in basophil leukocytes after incubation with different concentrations of rhIL-3 for 6 h ($n = 5$). Expression of calreticulin was significantly higher for all values of rhIL-3 compared to control. * $P < 0.05$.

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