

Pharmacologic regulation of histamine release by the human recombinant histamine-releasing factor

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Background: The recently cloned human recombinant IgE-dependent histamine releasing factor (HrHRF) was initially thought to stimulate histamine release from human basophils from a subpopulation of allergic donors by interacting with the IgE molecules on the surface of these cells. Additional data suggest that HrHRF exerts its biologic effects by binding to a distinct cell surface structure and not to IgE.

Objective: To address the hypothesis that the HrHRF signaling pathway is distinct from the classical high-affinity IgE receptor (FcεRI) pathway, we used pharmacologic agents known to affect basophil histamine release.

Methods: In this report we compared the effect of staurosporine, Bis II, Gö 6976, rottlerin, and pertussis toxin on histamine release from human basophils mediated by the following stimuli: HrHRF, polyclonal human anti-IgE antibody, and antigen, as well as the IgE-independent stimulus, FMLP.

Results: None of these modulators, except rottlerin, could differentiate histamine release induced by anti-IgE or antigen from that induced by HrHRF. Rottlerin enhanced HrHRF-mediated histamine release and dose dependently blocked FMLP-mediated release without affecting basophil activation by either anti-IgE or antigen.

Conclusion: These data suggest a unique signaling pathway for HrHRF and thus strengthen the hypothesis that HrHRF binds to a specific receptor other than IgE. (*J Allergy Clin Immunol* 1999;103:937-43.)

Key words: Histamine-releasing factor, human basophils, IgE heterogeneity, pharmacologic regulation

Histamine-releasing factors (HRFs) comprise a heterogeneous group of proteins that are capable of mediating histamine release from human basophils. These molecules were first discovered in the supernatants from cultured PBMCs stimulated by mitogen or antigen.¹ Several groups of molecules with histamine-releasing activity have been discovered to date, including interleukins, chemokines,²⁻⁶ and a recently subcloned molecule, the

Abbreviations used

DMSO:	Dimethyl sulfoxide
FcεRI:	High-affinity IgE receptor
FMLP:	N-formyl-methionyl-leucyl-phenylalanine
HrHRF:	Human recombinant IgE-dependent histamine-releasing factor
IC ₅₀ :	Inhibitory concentration of 50%
PAG:	PIPES-albumin-glucose
PIPES:	Piperazine N,N'-bis 2 ethane sulphonic acid
PKC:	Protein kinase C
PMA:	Phorbol myristate acetate

IgE-dependent HRF.⁷ This HRF was originally detected in late-phase skin blister fluids⁸ and in nasal lavage fluid during the late-phase reaction.⁹ Furthermore, mononuclear cells from patients with atopic dermatitis who are allergic to foods spontaneously produce large amounts of HRF, and the production of HRF correlated with the severity of their disease.¹⁰

Only 50% of allergic donor basophils released histamine in response to stimulation with the IgE-dependent HRF. Furthermore, using techniques to remove IgE from the surface of the basophils with lactic acid, we have previously demonstrated that it was the IgE from certain allergic donors that conferred sensitivity to this HRF.¹¹ This led to the concept of functional heterogeneity among human polyclonal IgE molecules. We defined the IgE from donors who respond to HRF as *IgE*⁺,¹¹ and therefore all other IgE molecules are termed *IgE*⁻. The human recombinant IgE-dependent HRF (HrHRF) was found to be a complete secretagogue for histamine and IL-4 protein secretion from basophils expressing *IgE*⁺. Moreover, in *IgE*⁺ basophils HrHRF was capable of inducing IL-4 secretion with similar kinetics to anti-IgE.¹²

Several attempts were made to determine whether HrHRF specifically bound to *IgE*⁺. First, affinity chromatography and use of ELISAs failed to show direct binding of HrHRF to *IgE*⁺. Second, the rat basophilic leukemia cell line transfected with the α-chain of the high-affinity receptor for IgE demonstrated histamine release to polyclonal anti-IgE after passive sensitization with *IgE*⁺ but no

From The Johns Hopkins Asthma and Allergy Center, Baltimore.
Supported in part by National Institutes of Health grants Ro-1 AI 32651 and AI 20253 and by Bristol-Myers Squibb Pharmaceutical Research Institute.
Received for publication Jan 22, 1998; revised Nov 17, 1998; accepted for publication Nov 17, 1998.
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0091-6749/99 \$8.00 + 0 1/1/97739

histamine release when HrHRF was the stimulus (unpublished data). Third, we have recently shown that nonresponding basophils, which by definition are those basophils possessing IgE⁻, display an increased release of histamine and IL-4 when preincubated with HrHRF and then challenged with anti-IgE.¹³ These basophils also released IL-13 when primed with HrHRF and then subsequently stimulated with anti-IgE. This is noteworthy because in humans both IL-4 and IL-13 are important in isotype switching (IgM to IgE) in B cells.¹⁴ Taken together, these data suggest that one possible explanation is that HrHRF activates basophils by interaction with a specific receptor on their surface. One approach to address this would be to examine a variety of signal transduction events thought to be important in promoting secretion in human basophils or modulating secretion from human basophils. Unfortunately, for human basophils, there are many quantitative issues to be resolved. We believed that a better approach would be to determine whether pharmacologic agents could discriminate between the histamine release by HrHRF versus release mediated by other known basophil secretagogues. Under the proper conditions, this type of data might more clearly demonstrate the difference in signaling caused by HrHRF.

It is generally accepted that the signal transduction pathway triggered by IgE-dependent stimuli involves activation of a number of kinases, including the high-affinity IgE receptor (FcεRI)-associated kinases Lyn and Syk.¹⁵ At the inception of these studies, protein kinase C (PKC) was also thought to play a proactive role in IgE-mediated histamine release. Therefore we compared the effect of agents thought to inhibit PKC on histamine release from human basophils mediated by HrHRF, IgE-dependent stimuli such as anti-IgE antibody or antigen, and the IgE-independent stimulus FMLP. Because the latter is known to signal in basophils through a G protein-coupled receptor,¹⁶ we also compared the ability of pertussis toxin, an inhibitor of G proteins bearing a Gαi subunit,¹⁷ to modulate histamine release by these stimuli.

METHODS

Reagents

Piperazine N,N'-bis 2 ethane sulphonic acid (PIPES) 10× contained 250 mmol/L PIPES, 1.10 mol/L NaCl, and 50 mmol/L KCl (pH 7.4). A PIPES-albumin-glucose buffer (PAG) contained one tenth 10× PIPES in addition to 0.003% human serum albumin (Calbiochem-Bering Corp, La Jolla, Calif) and 0.1% D-glucose. PAG supplemented with 4 mmol/L EDTA is designated PAG-EDTA. Isotonic Percoll (referred to as 100% Percoll) consisted of 1 part 10× PIPES and 9 parts Percoll (Pharmacia, Piscataway, NJ). Percoll solutions of 55% (density, 1.072) and 63% (density, 1.082) were made by mixing the appropriate amounts of 1× PIPES and 100% Percoll. Rotlerin (5,7-dihydroxy-2,2-dimethyl-6[2,4,6-trihydroxy-3-methyl-5-acetylbenzyl]-8-cinnamoyl-1,2-chromene)¹⁸ Bis II, a compound derivatized from tetrahydropyrido[1,2-α]indole to bisindolyl-maleimide¹⁹; and Gö 6976, a methyl- and cyanoalkyl-substituted nonglycosidic indolocarbazole²⁰ were purchased from Calbiochem Bering Corporation. A stock dilution of these compounds was prepared in dimethyl sulfoxide (DMSO) so that the final dilution of

DMSO during the histamine release assay was at least 1/34,000. FMLP, phorbol myristate acetate (PMA), and pertussis toxin and its β-oligomer were purchased from Sigma Chemical Co (St Louis, Mo). Polyclonal human IgE antibody was made in a goat and affinity purified.

Preparation of HrHRF

HrHRF was shuttled from the PGEX-2T vector, which is used in *Escherichia coli* production,⁷ into the baculovirus vector, pBlueBac III, which coexpresses β-galactosidase for color selection of successful recombination. As per MAXBAC baculovirus system manufacturer's specifications (Invitrogen, San Diego, Calif), plasmid DNA was transfected into Sf 9 cells for viral isolation and amplification. Subsequently, High Five insect cells were used for protein production, and pBlueBac III-produced HrHRF protein was detected by SDS-PAGE and Western blotting with a polyclonal anti-HrHRF antibody generated against recombinant material produced in *E. coli*.

Enriched basophil preparations

Venous blood from consenting donors was anticoagulated with 10 mmol/L EDTA. Whole blood was centrifuged at 300g, and the leukocyte interface (buffy coat) was carefully aspirated and transferred to PAG-EDTA buffer (1:1 vol/vol). Basophil-enriched suspensions were prepared by Percoll density centrifugation, as previously described.¹² Briefly, the diluted buffy-coat suspension was layered onto gradients consisting of 12 mL of 55% Percoll layered onto 12 mL of 63% Percoll in 50 mL of polypropylene centrifuge tubes. Percoll gradients were centrifuged at 700g for 20 minutes at room temperature. Basophils were in the fraction consisting of the lower half of the 55% Percoll, 63% interface, and upper half of this layer. Cells in this fraction were washed twice in PAG-EDTA and underwent a final wash in cold PAG at 4°C. Basophils were counted in Spiers-Levy chambers by using Alcian blue staining.²¹ The purity of the basophils in the cell suspensions used for these studies ranged from 5% to 40%.

Purified basophil preparations

Purified basophils were obtained from peripheral blood by mixing with 0.9% saline, layering over a single Percoll gradient with a density of 1.082, and spinning at 1200 rpm for 15 minutes at room temperature. The interface was removed, diluted in PAG with 4 mmol/L EDTA (PAG-EDTA), and spun at 800 rpm for 8 minutes at room temperature. Two subsequent washes with PAG-EDTA at 4°C were performed. The cells were stained with Alcian Blue and counted on a Spiers-Levy Chamber to determine total cell number and percent basophil purity.²¹

Cells were suspended in PIPES buffer with 1% BSA and 2 mmol/L EDTA (PIPES/BSA/EDTA) to a density of 10⁸ cells per 100 mL of buffer. Subsequently, the Basophil Isolation Kit from Miltenyi Biotec GmbH (Auburn, Calif) was used, with slight modifications to the manufacturer's specifications. Specifically, 50 mL of FcR-blocking reagent was added per 10⁸ cells and incubated on ice for 30 to 45 minutes. Next, 100 mL of hapten-antibody cocktail per 10⁸ cells was added and incubated for 30 minutes on ice, gently mixing every 10 minutes. PIPES/BSA/EDTA buffer (650 mL) was added per 10⁸ cells in the presence of 100 mL of anti-hapten microbeads and incubated for 30 minutes on ice, again mixing every 10 minutes. The cell suspension was placed on a MidiMACS LS⁺ Separation Column (Miltenyi Biotec GmbH) and washed with 12 to 14 mL of PIPES/BSA/EDTA buffer in a 15-mL conical tube on ice. The cell suspension was pelleted by spinning at 1500 rpm for 10 minutes at 4°C. The pellet was resuspended in 1 mL of PAG with 0.03% human serum albumin (P[10×A]G), stained with Alcian Blue, and

counted in the Speirs-Levy Chamber.²¹ Basophil purity in these experiments ranged from 64% to 82%.

For histamine release experiments, cells were resuspended in P(10×A)G at approximately 0.36×10^6 basophils per milliliter. The cells were preincubated in the absence or presence of rottlerin at the indicated concentrations in a 96-well flat-bottom plate for 15 minutes at 37° in an atmosphere of 5% CO₂. Prewarmed buffer, P(10×A)G, or stimulus (HrHRF, 3.5×10^{-7} mol/L) were added to each well and incubated for an additional 45 minutes at 37° in an atmosphere of 5% CO₂ in the presence of 5 mmol/L calcium. The plate was spun at 1000 rpm for 3 minutes to bring cells to the bottom of each well, and then 100 μ L of supernatant from each condition was removed to assay for histamine. Specifically, 100 μ L of supernatant was added to 200 μ L of 8% HClO₄ and 700 μ L of PAG, and the acid was precipitated overnight at room temperature. The tubes were spun, and supernatants were decanted and assayed for histamine content.

Histamine results are expressed as a percentage of total histamine from lysed cells, and each determination is done in duplicate. Enhancement was determined by subtracting the percent histamine release from HrHRF-stimulated wells from those with both HrHRF and rottlerin and then dividing by percent histamine release from the wells stimulated with HrHRF alone.

Incubation with kinase inhibitors

Basophils (1×10^6 cells/mL) were resuspended in warm PAG and preincubated for 15 minutes with various concentrations of the kinase inhibitors. Previous studies with these inhibitors determined that a 10-minute preincubation period was adequate to observe a full effect. After this preincubation period, aliquots of cells were added in duplicate to tubes containing the prewarmed (37°C) stimuli in the presence of Ca⁺⁺ (5 mmol/L). The total reaction volume was 100 μ L. After 45 minutes of incubation at 37°C, 900 μ L of PAG was added to each tube, cells were pelleted for 2 minutes at 300g, and the supernatants were harvested.

Incubation with pertussis toxin

Basophils were resuspended at 2×10^6 cells/mL in RPMI 2% FCS containing either pertussis toxin or its inactive β -subunit (2 μ g/mL) for 2 hours at 37°C. Cells were then extensively washed and challenged.

Histamine release assay

Histamine was measured in supernatants from stimulated basophils by using the automated fluorometric method of Siraganian.²² Total histamine was calculated by using cells lysed with 8% HClO₄. Spontaneous release was subtracted from all experimental values to yield net release. The *n* values represent discrete experiments, and according to our previously published methodology, histamine measurements are performed in duplicate for each stimulus.

Statistical methods

Results are expressed throughout as means $\pm$ SEM. Treated and nontreated cells were compared by using either the Wilcoxon signed-rank test or Student's *t* test.

RESULTS

Histamine release by HrHRF is inhibited by staurosporine

Staurosporine, a nonspecific PKC inhibitor, is known to inhibit IgE-mediated histamine release from human basophils. On the basis of its characteristics, it is likely that this drug inhibits tyrosine kinases critical to IgE-

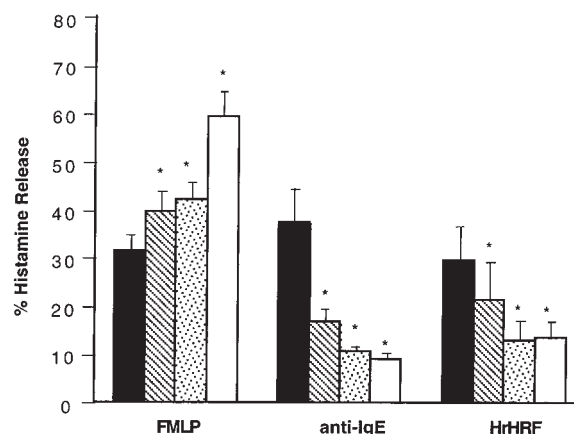


FIG 1. Human basophils were incubated in the absence of staurosporine (STSP, filled bars) or in the presence of varying concentrations of staurosporine (50 nmol/L [hatched bars], 100 nmol/L [stippled bars], or 500 nmol/L [open bars]) for 10 minutes and then stimulated with FMLP (1 μ mol/L), anti-IgE (100 ng/mL), or HrHRF (1 μ mol/L). There was a dose-dependent inhibition ($P < .05$) of both anti-IgE- and HrHRF-induced histamine release. As has been previously reported, staurosporine enhanced FMLP-induced release.²² Probability (*P*) value ($*P < .05$) is indicated for secretagogue-induced histamine release with or without staurosporine, as calculated by the Wilcoxon signed-rank test (*n* = 5).

mediated secretion. Therefore we used staurosporine as a first step to determine whether it would discriminate the signal transduction pathway between HrHRF and the IgE-mediated stimuli: anti-IgE and antigen and the non-IgE-mediated stimulus FMLP. As previously observed by others,²³ staurosporine slightly enhanced release by FMLP while it blocked anti-IgE-mediated histamine release in a dose-dependent manner from 50 to 500 nmol/L (Fig 1). HrHRF-mediated release was inhibited by staurosporine in a manner similar to that of anti-IgE (*n* = 5, $P < .05$, Wilcoxon signed-rank test).

The kinase inhibitors Bis II and Gö 6976 do not discriminate between HrHRF- and anti-IgE-mediated histamine release

We investigated whether more selective kinase inhibitors that target specific PKC isozymes are capable of differentiating between IgE-dependent and IgE-independent stimuli. Although there are no completely specific inhibitors of the PKC isozymes, we tested compounds belonging to 2 different families of staurosporine derivatives that display an enhanced selectivity for various PKC isozymes, the bisindolylmaleimides (Bis II) and the indolocarbazoles (Gö 6976). In previous experiments, Gö 6976, at a concentration of 250 nmol/L, significantly inhibited anti-IgE-mediated release, whereas Bis II at 390 nmol/L inhibited PMA-induced release (MacGlashan, unpublished data). Because the intent of our investigations was to find a difference between IgE-mediated and HrHRF stimulation, we chose concentrations that would ensure optimal inhibition. We used con-

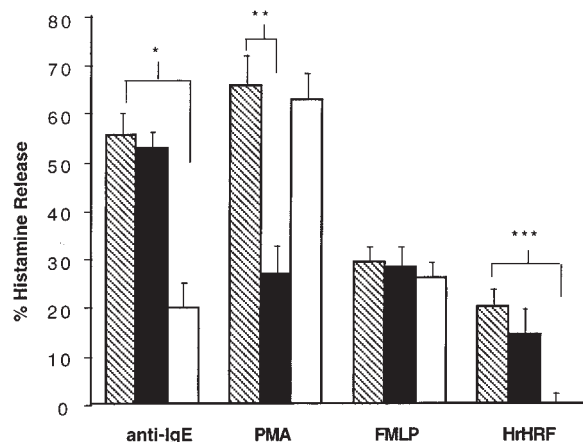


FIG 2. Histamine release by basophils preincubated with the kinase inhibitors Bis II, Gö 6976, or medium is shown. Gö 6976 (open bars) significantly inhibited anti-IgE-mediated release ($n = 8$, $*P < .05$) and completely abolished release induced by 1 $\mu\text{mol/L}$ HrHRF ($n = 8$, $***P < .01$). Bis II (filled bars) did not have any effect on anti-IgE- or HrHRF-mediated release. In contrast, it significantly inhibited release induced by 30 ng/mL PMA ($n = 8$, $**P < .05$). Release caused by FMLP (1 $\mu\text{mol/L}$) was not affected by either inhibitor ($n = 6$). Each n value is representative of separate experiments, with duplicate histamine determinations for each stimulus. All experiments are $n = 8$, with the exception of the stimulus FMLP for which the experiment was $n = 6$. All P values are calculated by the Wilcoxon signed-rank test.

centrations slightly above the previously determined inhibitory concentration of 50% (IC_{50}) values, 300 nmol/L for Gö 6976 and 400 nmol/L for Bis II. As depicted in Fig 2 and as expected, Gö 6976 at 300 nmol/L significantly inhibited anti-IgE-mediated histamine release ($n = 8$, $P < .05$, Wilcoxon signed-rank test) but also completely abolished release by HrHRF ($n = 8$, $P < .01$, Wilcoxon signed-rank test). In contrast, Bis II at 400 nmol/L did not have any effect on histamine release by either of these 2 stimuli, but it did have significantly diminished release induced by 30 ng/mL PMA ($n = 8$, $P < .05$, Wilcoxon signed-rank test). Release by FMLP was not affected by these kinase inhibitors ($n = 6$).

Rottlerin uniquely discriminates between IgE-dependent and IgE-independent stimuli

Because neither Gö 6976 nor Bis II were able to exert a specific action on HrHRF-mediated histamine release, we investigated the effects of rottlerin, a nonstaurosporine-derived kinase inhibitor isolated from *Mallothus philippinensis*, which targets the calcium-dependent PKC isozymes. To date there are no pharmacologic agents that are absolutely specific for PKC isoenzymes. However, rottlerin exhibits a certain specificity for the PKC δ isozyme (IC_{50} , 5.3 $\mu\text{mol/L}$), an enzyme found in abundance in human basophils when compared with human mast cells (MacGlashan, unpublished data). In other systems the calmodulin-dependent kinase (CaM-kinase III) is blocked at the same IC_{50} range and is therefore the only kinase other than PKC δ affected by rottlerin

at low concentrations.^{18,24} In an experiment not shown, rottlerin was titrated to determine its effect on both basophil viability and histamine release by a number of stimuli. Because at concentrations greater than 1 $\mu\text{mol/L}$ cell viability was decreased, we chose to use rottlerin at 500 nmol/L. At this concentration, rottlerin did not significantly affect release induced by a range of anti-IgE (Fig 3, A) or antigen (Fig 3, B) concentrations (0.005 to 0.1 $\mu\text{g/mL}$). In contrast, rottlerin inhibited release by FMLP (Fig 3, C). The inhibition of FMLP by rottlerin was significant only at FMLP concentrations of 10^{-6} mol/L ($P < .004$, Student's t test) and 10^{-7} mol/L ($P < .04$, Student's t test). No significant inhibition was seen at 10^{-5} mol/L FMLP, although at this concentration there was a trend toward decreased histamine release in the presence of this agent. FMLP concentrations of 10^{-8} mol/L did not induce significant histamine release either in the presence or the absence of rottlerin. Importantly, histamine release by HrHRF was significantly enhanced by preincubation of basophils with rottlerin (Fig 3, D). Because nanomolar amounts of HrHRF did not induce significant histamine release, HrHRF concentrations in these experiments were chosen in a range that induced optimal histamine release. Under these conditions, enhancement of HrHRF-mediated release by rottlerin was observed at 10^{-6} mol/L HrHRF ($P < .005$, Student's t test) and 2×10^{-6} mol/L HrHRF ($P < .05$, Student's t test). Rottlerin did not induce histamine release above controls in unstimulated cells (data not shown). Additionally, matched controls with DMSO alone did not affect basophil histamine release (data not shown).

A dose-dependent enhancement by rottlerin on HrHRF-induced histamine release in purified human basophils

To determine that this effect of rottlerin was acting specifically on the basophils and not contaminating cells, human basophils were purified from a starting purity of 4% to 82%. As shown in Fig 4, there was a dose-dependent enhancement by rottlerin of HrHRF-induced histamine release. As is evident in this figure, the enhancement is maximal at a rottlerin concentration of 500 nmol/L.

Histamine release by HrHRF is insensitive to pertussis toxin

Because rottlerin affected both HrHRF- and FMLP-mediated release, we investigated whether reagents modulating FMLP-mediated release would affect histamine release mediated by HrHRF. FMLP is known to signal through a receptor coupled to a G protein bearing an α_i subunit.¹⁶ This subunit can be adenosine diphosphate-ribosylated by pertussis toxin, which greatly reduces the capacity of G α_i proteins to bind guanine nucleotides and to activate downstream signaling events.¹⁷ We preincubated human basophils for 2 hours with a saturating concentration of pertussis toxin (2 $\mu\text{g/mL}$) and exposed the cells to various stimuli. The β -subunit of pertussis toxin,

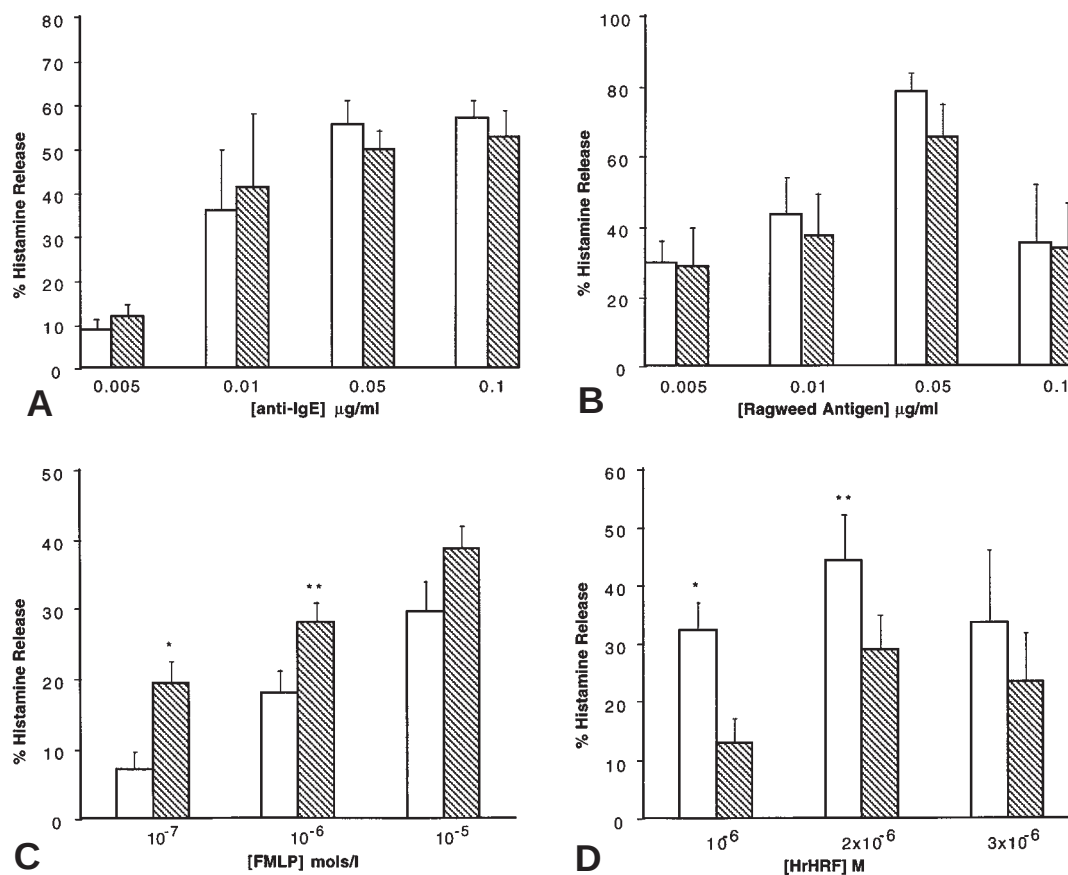


FIG 3. Effect of rottlerin on histamine release induced by IgE-dependent and IgE-independent stimuli. Basophils from donors allergic to ragweed were preincubated in the presence (*open bars*) or absence (*hatched bars*) of rottlerin and then stimulated with varying concentrations of anti-IgE (**A**), antigen E (**B**), FMLP (**C**), and HrHRF (**D**). **A**, Anti-IgE concentrations were 0.005 µg/mL (n = 4), 0.01 µg/mL (n = 3), 0.05 µg/mL (n = 5), and 0.1 µg/mL (n = 5). **B**, Antigen E concentrations were 0.005 µg/mL (n = 3), 0.01 µg/mL (n = 5), 0.05 µg/mL (n = 4), and 0.1 µg/mL (n = 2). **C**, FMLP concentrations were 10⁻⁷ mol/L (n = 2, *P < .04), 10⁻⁶ mol/L (n = 7, **P < .004), and 10⁻⁵ mol/L (n = 2). **D**, HrHRF concentrations were 10⁻⁶ mol/L (n = 10, *P < .005), 2 × 10⁻⁶ mol/L (n = 6, **P < .05), and 3 × 10⁻⁶ mol/L (n = 3). Probability (P) values were calculated by using Student's *t* test.

which is known to intercalate into membranes but does not have the bioactivity of pertussis toxin, was used as a control. As shown in Fig 5, the effect of pertussis toxin on histamine release by HrHRF (n = 4) or anti-IgE (n = 3) did not differ from that of the β-subunit alone. As expected, pertussis toxin, but not its β-subunit alone, completely abolished histamine release induced by FMLP (n = 4, *P* < .002, Student's *t* test). This would suggest that HrHRF is not stimulating histamine release from human basophils by interacting with a receptor coupled to a pertussis toxin-sensitive Gαi protein.

DISCUSSION

Although HrHRF was originally thought to induce histamine release from certain donors' basophils by an IgE-dependent mechanism,¹¹ as mentioned above, further investigations have revealed that HrHRF is capable of modulating release of histamine and IL-4 and IL-13 from all basophils.¹³ In this article we investigated

whether HrHRF- and IgE-mediated signals can be pharmacologically differentiated from one another. To accomplish this, we used a series of pharmacologic modulators affecting signal transduction events. The purpose of these studies was to determine whether there was a pharmacologic agent that discriminated HrHRF-induced histamine release from anti-IgE-induced release. Using these types of experiments, we did not intend to investigate exact signal transduction events.

The general kinase inhibitor, staurosporine, and a somewhat more specific derivative, Gö 6976, did not discriminate between HrHRF and the IgE-dependent stimulus anti-IgE, whereas they did differentiate anti-IgE stimulation from the IgE-independent stimulus FMLP. Bis II did not exert any significant modulation in basophils stimulated with HrHRF, anti-IgE, and FMLP, whereas it downregulated PMA-induced release. In a cell-free system, nanomolar concentrations of the indolocarbazole Gö 6976 have been described to inhibit the Ca(++)-dependent PKC isozymes α and β1, while showing no

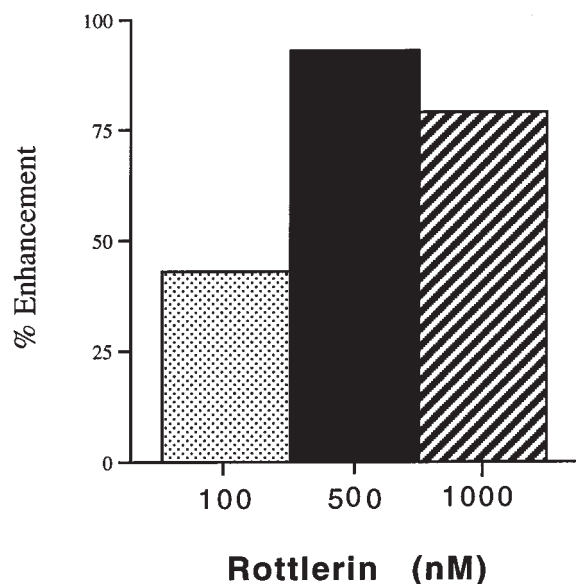


FIG 4. A dose-response curve of rottlerin enhancing HrHRF-induced histamine release from purified human basophils is shown. Basophils from peripheral blood were purified by negative selection as described in the Methods section. Starting basophil purity was 4%, which was increased to 82% by this technique. Percent histamine release to HrHRF (3.5×10^{-7} mol/L) was 14%. The graph demonstrates percent enhancement of varying doses of rottlerin (from 100 to 1000 nmol/L) on HrHRF-induced histamine release. This is representative of 3 separate experiments with purified basophils.

effect, even at micromolar concentrations, on the kinase activity of the Ca^{++} -independent PKC subtypes δ , ϵ , and ζ .²⁰ Bis II, on the other hand, inhibits PKC in vitro with an IC_{50} value of approximately 13 nmol/L.¹⁹ Despite the body of information on these inhibitors, the exact specificity of these compounds has not been fully defined in a cellular system.

In contrast to the above inhibitors, rottlerin did differentiate between HrHRF and other IgE-dependent stimuli (anti-IgE and antigen). The main difference between the staurosporine-related inhibitors and rottlerin is that they discriminate, to an extent, between calcium-dependent and calcium-independent PKC isozymes, respectively.^{18,24} In a cell-free system, rottlerin has been described to have an IC_{50} of 6 $\mu\text{mol/L}$ for the recombinant form of PKC δ , which is one order of magnitude lower than that for the other PKC isoforms.¹⁸ It is likely that the IC_{50} obtained in vitro differs from that calculated in a cellular system because it has been described for other kinase inhibitors, such as Gö 6976.^{20,26} However, the only observable effect of rottlerin on histamine release occurred at concentrations ranging from 100 to 500 nmol/L (data not shown). At 500 nmol/L, a concentration below its IC_{50} in vitro,¹⁸ rottlerin could significantly enhance release by HrHRF, although it did not affect the anti-IgE or antigen response. The cellular substrates involved in the upregulation of HrHRF-mediated release by rottlerin are largely unknown. Involvement of other

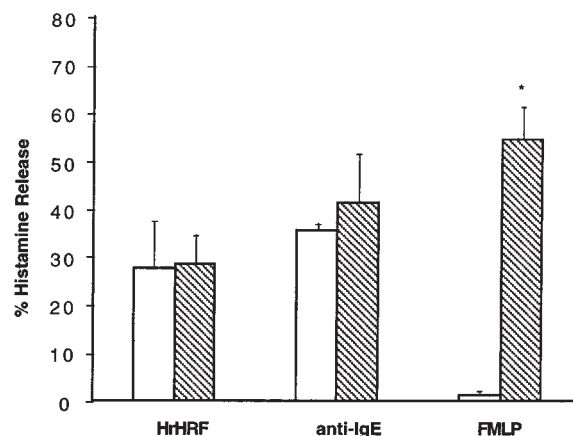


FIG 5. Pertussis toxin differentially regulates histamine release by various stimuli. Basophils were preincubated with pertussis toxin (open bars) or its inactive β -subunit (hatched bars), washed, and then challenged with HrHRF (0.5 $\mu\text{mol/L}$), anti-IgE (100 ng/mL), or FMLP (1 $\mu\text{mol/L}$). Histamine release induced by both HrHRF or anti-IgE was unaffected, whereas FMLP-induced release was completely abrogated by pertussis toxin ($n = 4$, $*P < .002$). Probability (P) values were calculated by using Student's t test.

kinases, such as casein kinase II and *src* kinases in the enhancement seems unlikely on the basis of the described IC_{50} of rottlerin for these enzymes (from 30 to $>100 \mu\text{mol/L}$, respectively).¹⁸ On the other hand, calmodulin-dependent kinase III has been shown to be blocked by rottlerin at the same IC_{50} as PKC δ .¹⁸ Calmodulin-dependent kinase III catalyzes the phosphorylation of elongation factor-2,²⁴ a protein apparently unrelated to any basophil activation event leading to histamine release.

Although the role of PKC δ in regulating histamine release is not defined, it seems to be quantitatively the most abundant PKC isozyme in human basophils (MacGlashan, unpublished observations). Therefore it is tempting to speculate that PKC δ is involved in basophil activation by HrHRF and that its activity can be regulated by rottlerin. That the anti-IgE, antigen, and HrHRF responses are differentially regulated by rottlerin is of importance because it suggests the existence of 2 independent basophil-activation pathways by these stimuli. On the other hand, the commonality between the signal transduction pathways of IgE and HRF unveiled by staurosporine, Bis II, and Gö 6976 would suggest that HRF acts as a modulator of a signal that is primarily generated by the Fc ϵ RI. Further experiments to determine the precise signal transduction events caused by HrHRF and anti-IgE antibody are planned by using cell lines.

FMLP was the only stimulus other than HrHRF with its effects modulated by rottlerin. On the basis of this observation, we investigated whether signal-transducing components of the FMLP pathway, such as G α i proteins, are also involved in the HrHRF pathway. We have demonstrated that histamine release from basophils stimulated by HrHRF is not altered by preincubation of cells with pertussis toxin, which suggests that a G α i protein is

not involved in the release process mediated by HrHRF. This response is not altered by the contribution of other leukocytes present in the incubation mixture.¹⁶ Because individual cells contain 4 to 5 types of α -subunits that are not inhibited by pertussis toxin,¹⁷ the lack of inhibition of the HrHRF-mediated histamine release by pertussis toxin does not totally exclude the involvement of other G proteins. However, it can be concluded that the HrHRF pathway does differ in this respect from the pathway used by most C-C chemokines, which are dependent on a G protein-associated receptor sensitive to pertussis toxin.²⁷

A recent report from our laboratory has shown that kinetics of IL-4 release from responsive basophils (ie, basophils possessing IgE⁺) stimulated with HrHRF are comparable with those of anti-IgE-stimulated basophils.¹² These results suggested the existence of a direct interaction between IgE and HrHRF. However, in additional experiments with nonresponsive IgE donors, HrHRF was capable of enhancing anti-IgE-induced mediator release.¹³ This supports the possibility of a distinct receptor for HrHRF. It is feasible that IgE⁺ (as opposed to IgE⁻) binding to FcεRI provides a signal that lowers the activation threshold of the basophil. Furthermore, the fact that IL-3 and D₂O are complete secretagogues for basophils bearing IgE⁺ supports this hypothesis.^{5,28} Alternatively, HrHRF might stimulate IgE⁺ basophils by binding to both its putative receptor and IgE, which would lead to direct mediator release, whereas HrHRF/HrHRF-receptor interaction in IgE⁻ basophils would trigger an "incomplete" or priming signal. The independent signal transduction pathway for HrHRF uncovered by rottlerin suggests the existence of such a receptor. Studies designed to demonstrate whether there is specific binding of radiolabeled HrHRF are in progress to determine the actual existence of this receptor.

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