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A new FcεRI receptor-mimetic peptide (PepE) that blocks IgE binding to its high affinity receptor and prevents mediator release from RBL 2H3 cells

Annamaria Sandomenico,* Simona M. Monti, Rosanna Palumbo and Menotti Ruvo

We have recently reported on a class of IgE-binding peptides designed based on the crystallographic structure of the high affinity FcεRI. Peptides contain receptor key residues located within the two distinct binding sites for IgE and selectively bind IgE with an affinity ranging between 6 and 60 μM. We have here designed and characterized a new molecule containing the receptor loops C′–E and B–C and an optimized linker for joining them made of a Lys side chain and a β-Ala. This new peptide shows an increased affinity (around 30 times) compared to the parent loop C′–E + B–C previously described, while retaining the same two-site mechanism of binding and the same selectivity. It also blocks the binding of IgE to the cell-anchored receptor and efficiently prevents histamine release from mast cells. These properties make the peptide a useful scaffold for the development of new anti-allergic drugs. Copyright © 2011 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: IgE; allergy; FcεRI; SPR; histamine release

Introduction

A broad spectrum of pathologies listed as allergic or atopic diseases are associated with the inappropriate activation of the immune system by environmental factors and affect nearly 20% of the world population [1]. In the past two decades, an increase in cases has been recorded, predominantly in young people, which are correlated with the Western population lifestyle [2].

Type E immunoglobulins play a central role in allergic processes such as anaphylaxis, allergic rhinitis, asthma and atopic dermatitis. Their function is also associated with other different immune responses [3]. The high affinity receptor FcεRI, found on the surface of mast cells and basophiles, is the 'docking station' for IgE. It is well established that binding of antigens to receptor-bound IgE leads to their cross-linking and to receptor activation, finally inducing propagation of the cellular signaling and triggering of the allergic reaction.

The FcεRI is a tetrameric receptor constituted by an α subunit, a β subunit and a disulfide linked homodimeric γ subunit [3,4]. The extracellular part of its α subunit binds with high affinity ($K_D = 10^{-9}$–10^{-10} M) the Fc fragment of IgE in 1 : 1 molar ratio by two distinct binding sites [5–9]. The other subunits are responsible for transduction of the initial cross-linking signal into the cell. Elucidation of the structural basis of the interaction between FcεRIα and IgE has clarified the molecular details of the recognition and has provided new opportunities for the development of IgE or receptor antagonists to treat allergic diseases. It has been indeed shown that molecules able to prevent the binding of IgE to its high affinity receptor (FcεRI), that can therefore control the receptor intracellular signaling activation, have an enormous therapeutic potential for the treatment of asthma and allergic diseases; notably, most cases of moderate–severe asthma are currently cured with a monoclonal anti-IgE antibody (generic name, omalizumab; trade name, Xolair) [10,11] which binds IgE and prevents binding to the receptor. It has been reported that small peptides can also modulate the activity of IgE receptor by binding either the FcεRI or the IgE itself with high selectivity and potency [12–23], although binding to IgE should be the strategy of choice for blocking the IgE–receptor interaction, as receptor ligands have the potential to act as agonists instead than antagonists. We have recently described a set of small peptides which mimic receptor loops and bind to IgE with a moderate affinity (the K_D is in the low micromolar range) but with a remarkable selectivity, showing negligible or no binding for other immunoglobulins, such as IgG and IgA [24]. These peptides have been designed based on the crystallographic structure of the FcεRI–IgE complex [7] and contain the receptor regions corresponding to the two distinct binding sites involved in IgE recognition. FcεRI is indeed characterized by the presence of two independent binding sites, one located around Tyr131 and involving the adjacent residues from Tyr129

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Abbreviations used: *Boc-Gly-OH, N-t-butyloxycarbonylglycine; DMEM, Dulbecco's modified eagle's medium; FBS, fetal bovine serum; FITC, fluorescein isothiocyanate; IgG, polyclonal human immunoglobulin G; IgA, polyclonal human immunoglobulin A; mIgE, monoclonal anti-2,4-dinitrophenol immunoglobulin E produced in mouse; IgE, human immunoglobulin E; MBHA, 4-methylbenzhydrylamine; NHS, N-hydroxysuccinimide; TIS, tri-isopropylsilane; PBS, phosphate buffer saline.*

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to His134 on the receptor D2 domain; the second one contains a patch of four tryptophans including W87, W110, W113 and W156 located again on D2 and within the linker between the D1 and D2 domains. Among the peptides we have designed and tested, the best performing one, named loop (C′–E + B–C), showed an overall affinity constant (defined as the affinity resulting from the combined effect of recognition on the two distinct sites) of 19 μM, and a detailed kinetic analysis by surface plasmon resonance (SPR) suggested that the relatively low affinity was due to a poor accessibility of site 1 to IgE. According to this hypothesis, we have now designed a new peptide containing the same loops but with a longer linker between them. The new peptide exhibits an overall 30-fold increased affinity for IgE compared to the parent molecule, is able to displace the binding of IgE to the membrane-anchored receptors and – most importantly – can inhibit β-hexosaminidase release from rat basophiles leukemia (RBL2H3) cells, suggesting a potential applications in the modulation of IgE-mediated allergic diseases and as scaffold for the development of more potent and effective anti-allergic lead compounds.

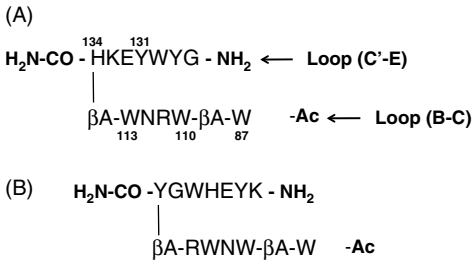

Figure 1. (A) Schematic representation of the PepE. The loop (C′–E) and the loop (B–C) corresponds to the binding Site 1 and Site 2, respectively. The histidine 134 is at the *C*-terminus of the peptide (prepared as an amide); Gly is at the *N*-terminus of loop (C′–E) and is not acetylated. A β-Ala is at the *C*-terminus of loop (B–C). The *N*-terminus of this loop (W87) is acetylated. Key residues involved in the binding with IgE are numbered. In (B), the scrambled version of the peptide used as negative control is reported.

Experimental Procedures

Materials

The BIAcore 3000 SPR system for Real-time kinetic analysis and related reagents were from GE Healthcare (Milano, Italy). All other reagents and chemicals were commercially available by Sigma–Aldrich (Steinheim, Germany) or Fluka (Steinheim, Germany). Reagents for peptide synthesis (Fmoc-protected amino acids and resins, activation and deprotection reagents) were from Novabiochem (Laufelfingen, Switzerland) and InBios (Pozzuoli, Italy). Solvents for peptide synthesis and HPLC analyses were from Romil (Dublin, Ireland); RP columns for peptide analysis and the LC–MS system were from ThermoFisher (Milan, Italy). Human IgE were from Chemicon International, while mIgE, DNP-BSA conjugate, *p*-nitrophenyl-*N*-acetyl-β-D-glucosaminide were obtained from Sigma–Aldrich (St Louis, MO, USA). IgG and IgA were a kind gift of Dr A. Verdoliva of Tecnogen SpA (Piana di Monte Verna, Italy). Solid-phase peptide synthesis was performed on a fully automated multichannel peptide synthesizer Syro I (Multisynthech, Germany). Preparative RP-HPLC was carried out on a Shimadzu LC-8A system, equipped with a SPD-M10 AV detector and with a ONYX monolithic C18 column (100 × 10 mm ID, Phenomenex, Torrance, CA, USA). DMEM and FBS were from BioWhittaker (Verseviers, Belgium); L-glutamine was from Gibco (Milano, Italy). FITC-avidin was from Santa Cruz Biotechnology (CA, USA). Data were fitted using the software GraphPad Prism 4, *versus* 4.02 (GraphPad Software, San Diego, CA, USA).

Peptide Synthesis and Purification

Peptides were prepared by solid-phase synthesis as *C*-terminally amidated and *N*-terminally acetylated derivatives following standard Fmoc chemistry protocols [25]. A Rink-amide MBHA resin (substitution 0.53 mmol/g) and amino acid derivatives with standard protections were used in the synthesis. Lys with a 4-methyltrityl (Mtt) protection was used to link the side chain of Lys133 to the *C*-terminus of a β-Ala needed to increase the length of the spacer between the two loops (Figure 1(A)); a β-Ala was also used to connect Trp110 and Trp87. The synthesis of PepE was performed starting from His134 and completing, in a first step, the loop C′–E (129–134). A Boc-Gly-OH residue was inserted at the *N*-terminus. Upon cleavage, a free amino group was provided on this position. Selective deprotection of Lys(Mtt) was achieved by repeated treatments with a DCM/TFA/TIS (95 : 4 : 1 v/v/v) mixture for 2 min at room temperature. Then Trp87, β-Ala, Trp110, Arg111, Asn112 and Trp113 were incorporated under canonical conditions of peptide synthesis (HBTU/HOBt/DIEA pre-activation, fivefold excess of Fmoc-protected amino acids). Finally, acetylation was performed using acetic anhydride at 0.5 M in DMF with 5% DIEA, 20 min at room temperature. The scrambled peptide (Figure 1(B)) was prepared following a similar strategy. The cleavage of peptides from the solid support was performed by treatment with a TFA/TIS/water (90 : 5 : 5, v/v/v) mixture for 90 min at room temperature, affording the crude peptides after precipitation in cold diethyl-ether. The precipitates were dissolved in water and were finally lyophilized. Products were purified to homogeneity by RP-HPLC using an ONYX monolithic C18 column (100 × 10 mm ID) applying a linear gradient of 0.05% TFA in acetonitrile from 5 to 70% over 10 min (flow rate 20 ml/min). Peptide purity and integrity were estimated by LC–MS mass analyses.

SPR Analysis

Real-time binding assays were performed on a BIAcore 3000 SPR instrument (GE healthcare). Three human immunoglobulins (IgE, IgG and IgA) and mouse IgE immobilization were achieved on CM5 Biacore sensor chips using EDC/NHS chemistry at pH 5.0 in 10 mM acetate buffer (flow rate 5 μl/min) according to the manufacturer's instructions [26]. Residual reactive groups were deactivated by treatment with 1 M ethanolamine hydrochloride, pH 8.5. Reference channels were prepared for each biosensor by activating with EDC/NHS and deactivating with ethanolamine. All binding assays were carried out in HBS buffer (10 mM HEPES, 150 mM NaCl, 3 mM EDTA, pH 7.4, surfactant P20 0.005%) at a flow rate of 30 μl/min. Analyte injections of 130 μl were performed at the indicated concentrations. Data were manipulated to obtain kinetic and thermodynamic parameters using the BIAevaluation analysis package (version 4.1, GE Healthcare). Data fitting was carried out using a biphasic model with a 1 : 1 stoichiometry and evaluating the goodness of fitting by analysis of residuals [27,28]. Non-specific binding from the reference channel was subtracted from the working channels before analysis. Binding to human IgG and human IgA were performed at the concentration of 800 nM of PepE.

IgE Biotinylation

An mIgE (0.5 mg) was dissolved in 1 ml of PBS (10 mM Na_2HPO_4, 2 mM KH_2PO_4, 137 mM NaCl and 2.7 mM KCl pH 7.4), and treated with 10 mM of succinimidyl-6-(biotinamido) hexanoate (Pierce, Italy) in DMSO. After 1 h of incubation on ice, 1 ml of Tris 50 mM pH 8 was added to deactivate residual reactive groups. The biotinylated-IgE were extensively dialyzed at 4 °C against PBS, pH 7.4, recovered and stored frozen until use.

Peptide Stability in Serum

To test the peptide susceptibility to proteolytic degradation, PepE (dissolved in H_2O at 1 mg/ml) was serially diluted in PBS pH 7.4 buffer containing 10% FCS in order to obtain 100 μg/ml solutions. The serum used for this experiment was not heat-inactivated. Samples were incubated at 37 °C for 24 h. Ten microliters aliquots (1.0 μg total peptide) were removed at 0, 4, 8, 12, 16 and 20 h. After centrifugation, the recovered samples were analyzed by RP-HPLC applying a linear gradient of 0.1% TFA/CH_3CN in 0.1% TFA/H_2O from 5 to 70% over 50 min using a 50 × 2 mm ID Biobasic C18 column (Thermo Fisher, Milan, Italy) at a flow rate of 0.20 ml/min. A reference curve was obtained by analyzing different amounts of the pure compounds under the same conditions. This curve was used to exclude effects of sample subtraction by non-specific binding to albumin or other serum proteins that were pelleted during centrifugation. Experiments were carried out twice and results were reported as a plot of peak area (%) *versus* time (data not shown).

Cellular Assays

Cell culture and flow cytometry

The RBL2H3 cell line was maintained in DMEM supplemented with 10% heat-inactivated FBS, containing 2 mM L-glutamine at 37 °C in a humidified atmosphere at 5% CO_2. For binding experiments, RBL2H3 cells were readily detached by gentle scraping with PBS/EDTA 0.05%, centrifuged at 100 × *g* for 5 min, washed with PBS containing 0.2% BSA and re-suspended in the same buffer to a final density of 1×10^6 cells/ml [13,14]. The positive control was defined as the fluorescence intensity of 13 nM biotinylated-mIgE bound to the cells. Increasing concentration of peptide between 1.3 and 650 μM were pre-incubated with 13 nM biotinylated-mIgE at 4 °C for 1 h. The mix was added to cell suspensions for 45 min followed by the addition of 10 μl FITC-avidin. After 30 min, the cells were washed twice with PBS/BSA and analyzed with a flow cytometer equipped with a 488-nm argon laser (FACSCalibur, Becton Dickinson, Providence, RI, USA). For each sample, 20 000 events were acquired and analyzed using the Cell Quest software. Experiments were performed three times and the results were expressed as percentage fluorescence intensity of the sample minus the fluorescence of the cells incubated with FITC-avidin alone.

Degranulation assay

Degranulation was assessed by measuring β-hexosaminidase release according to the method described by Dearman *et al*., [29] opportunely modified. RBL2H3 were allowed to adhere overnight in 96-well plates (1×10^6 cells/well). Cells were washed twice with HEPES buffer saline (10 mM HEPES pH 7.4, 135 mM NaCl, 6.2 mM D-glucose, 5 mM KCl, 1 mM $CaCl_2$, 0.5 mM $MgCl_2$) (HBS) and then exposed to mouse anti-DNP IgE (250 ng/ml) for 45 min at 37 °C in presence or absence of varying concentration of PepE ranging between 10 and 1000 μM. After two washes, cells were stimulated with 100 ng/ml DNP-BSA at 37 °C for 2 h. The spontaneous and total β-hexosaminidase release from RBL2H3 was measured by incubating 30 μl of supernatant or lysed cell with 50 μl of 4 mM *p*-nitrophenyl-*N*-acetyl-β-D-glucosaminide (in 0.1 M citrate buffer pH 4.5) for 2 h at 37 °C. Enzyme reaction was terminated by adding 100 μl of 0.2 M Gly-NaOH, pH 10.7. The absorbance at 405 nM was measured in a microplate reader (Bio-Rad 680). Cells were lysed with 100 μl 0.1% Triton in HBS. The specific release (%) was calculated subtracting to the ratio between the spontaneous and total cellular release of stimulated cells the same ratio related to unstimulated cells as reported in the following equation:

$$\text{Specific release} = [\text{Spontaneous release}/(\text{Spontaneous release} + \text{Cellular release})] - [\text{Controlspontaneous release}/(\text{Control spontaneousrelease} + \text{Control cellular release})] \times 100$$

For the inhibition experiments, values of specific release were normalized to 100% and the inhibition values were accordingly corrected.

Results and Discussion

Peptide Design and Synthesis

In a previous work [24], we have reported a set of peptide-based FcεRIα-mimetics (first generation of IgE-binding peptides) designed based on the crystallographic structure of the IgE–FcεRI complex (PDB code 1F6A [7]). These peptides recognize with a pretty low affinity (micromolar range) but with high specificity the immunoglobulins E, as verified by SPR and ELISA assays. Among these peptides, mimicking loops C′–E and B–C [loop (C′–E + B–C)] was one of the most effective, exhibiting an overall affinity constant of 19 μM. Despite the simple structure and the low size, loop (C′–E + B–C) also showed a cooperative mechanism of binding to IgE, due to the presence, as in the native receptor, of two distinct recognition sites localized on the two loops. This finding was strongly supported by the observation that the isolated synthetic loops were essentially unable to bind to IgE, thus also suggesting that the relative orientation and the linker between them could play a very important role for the recognition. Starting from these considerations, we looked again at the crystal structure of the complex, observing that the real distance between loops in the receptor (for instance, the *N*-α of Trp113 and the *N*-α of Asn133, about 11 Å) was somehow greater than that actually achievable with the Lys side chain in the synthetic loop (C′–E + B–C), which was about only 7 Å. We thus redesigned the peptide introducing a new linker made of the side chain of the Lys used in place of Asn133 and by an additional β-Ala. The new linker, in a fully extended conformation, had a size of about 12 Å, a distance very close to that needed. The corresponding peptide was prepared by the solid-phase method [24] introducing the β-amino acid before incorporating residues from Trp113 to Trp110 and the subsequent β-Ala and Trp87 (Figure 1(A)). *N*-terminal and *C*-terminal ends were acetylated and amidated to improve the polypeptide stability, whereas the Gly adjacent to Tyr129 was left with a free amino group to increase the solubility. As a control, a scrambled peptide was prepared by a similar synthetic strategy. After cleavage, the final peptides were about 60% in the crude mixture, as evaluated by LC–MS. After purification they were recovered with high purity (100%, by LC–MS analysis) and the experimental molecular weight

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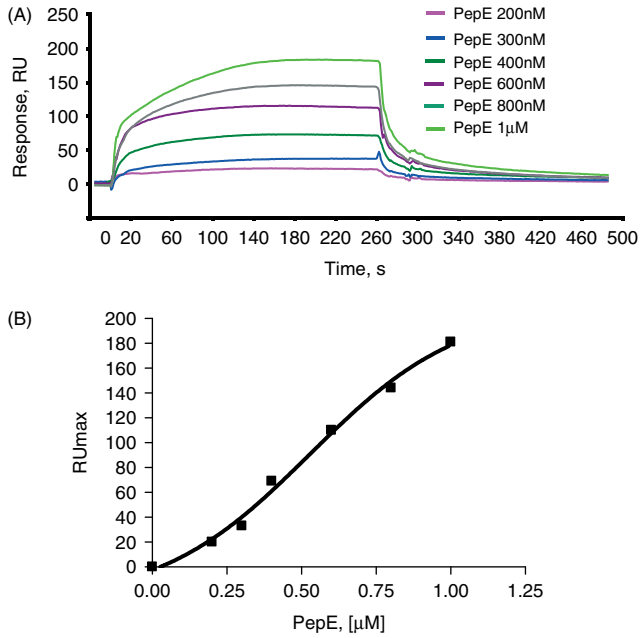

Figure 2. (A) Sensorgrams of the binding of PepE to mouse IgE immobilized on the surface of a Biacore sensor chip. Concentrations were between 200 and 1 mM. Experiments were carried out at a 25 °C, at a constant flow rate of 30 μl/min using HBS as running buffer (130 μl injected for each experiment). (B) Plot of RUmax from each single experiment *versus* concentration (μM). Data were fitted by a nonlinear regression algorithm.

was consistent with the expected value of 1994 amu for both molecules. The new peptide was named PepE.

SPR Direct Binding Assays

The interaction between PepE and IgE was assessed by Real-time kinetic analysis using the SPR technique. Assays were carried out using a BIAcore 3000 system. The immobilization of human and mouse immunoglobulins E was efficiently performed on a CM5 sensor chip in 10 mM NaAc, pH 5.5 at a flow rate of 5 μl/min for 7 min at 25 °C achieving a similar immobilization level of about 10 000 RU. After deactivation of reactive groups on both the assay and reference channels solutions at increasing concentrations of PepE, between 200 nM and 1 μM were injected at a 30 μl/min flow rate using HBS pH 7.4 as running buffer. Sensorgrams of the dose-dependent binding assays carried out using the mIgE-immobilized chip, are reported in Figure 2, while the kinetic and thermodynamic parameters are shown in Table 1. Data were repeated with the human IgE variant, obtaining very similar results (not shown). Data were analyzed by curve fitting using several models of interaction using the BIAevaluation package (version 4.1, Pharmacia Biosensor, Uppsala, Sweden). As previously reported [24], for loop (C′–E + B–C), we verified that experimental data were best fitted adopting a biphasic model. The two equilibrium constants K_{D1} ($20 \pm 1.5 \times 10^{-6}$ M) and K_{D2} ($16 \pm 1.1 \times 10^{-6}$ M) were extrapolated from the K_{a1}, K_{a2}, K_{D1} and K_{D2} values, which were in turn estimated as average of values determined from the curves at different concentrations (Table 1). Remarkably, comparing the new K_D's with those obtained with the previous peptide, it can be seen that the two sites equally contribute to the interaction with IgE, suggesting that extension of the linker between (C′–E) and (B–C) induces a much greater availability of both loops. The largest difference can be observed on the K_{D2}, presumably the one corresponding to site 1, which is considerably lower compared to that previously measured (about 12 times, 190 *vs* 16 μM). Also the K_{D1} is somehow lower, but only threefold. As shown in Table 1, these differences reflect the differences on kinetic constants, K_as and K_Ds, which indeed are in some cases quite different. In particular, the K_{a2} increases about 50 times (from 1.33×10^2 $M^{-1} \times s^{-1}$ to 4.39×10^3 $M^{-1} \times s^{-1}$) and this increase is only partially compensated by the slightly higher (<3 times) K_{D2} (2.54×10^{-2} s^{-1} *vs* 7.07×10^{-2} s^{-1}). This observation suggests that one of the two binding sites is much more readily accessed by the peptide, even though it also dissociates a little bit more rapidly. In contrast, the difference between the two K_{D1} are due to a small difference between the two dissociation rates, with that of PepE being only slightly lower than that of the shorter peptide.

In conclusion, the kinetic and thermodynamic data support the view that the two-site mechanism is retained with this new molecule. Furthermore, it turns out that increasing the distance between the two loops has a broad beneficial effect on the recognition between the peptide and IgE, especially on the K_{D2}, and that this effect is mostly due to an increased accessibility of one of the loops to the immunoglobulin, rather than to a reduced dissociation rate.

To further assess the occurrence of a cooperative effect between the two sites, we evaluated the apparent macroscopic dissociation constant by plotting the RU_{max} values associated to each single determination against the polypeptide concentration. As can be seen in Table 1 and in Figure 2(B), an overall K_D of about 500 nM was extrapolated by data fitting using a nonlinear regression analysis. This value is about 40 times lower than both the single K_Ds and about 30-fold lower compared to that of the precursor peptide (Table 1), confirming that a cooperative mechanism occurs between the two sites and that an overall gain of affinity is induced by the longer spacer.

Using SPR, we also assessed the specificity of PepE for IgE. For this purpose, polyclonal human IgG, IgA and IgE were covalently immobilized on the same CM5 sensor chips according to the previously reported procedures, achieving an immobilization level very close to 10 000 RU. Then, PepE was injected on the chips at

Table 1. Association and dissociation rates and dissociation constants for the peptides reported in this study

Peptide	Mean association rate (M^{-1} s^{-1}) K_{a1}	Mean dissociation rate (s^{-1}) K_{d1}	Mean association rate (M^{-1} s^{-1}) K_{a2}	Mean dissociation rate (s^{-1}) K_{d2}	Mean K_{D1} (M)	Mean K_{D2} (M)	Global K_D (M)
Loop (C′–E + B–C)	1.35×10^4	8.70×10^{-1}	1.33×10^2	2.54×10^{-2}	$65 (\pm 6) \times 10^{-6}$	$19 (\pm 5) \times 10^{-5}$	$19 (\pm 4) \times 10^{-6}$
PepE	1.69×10^4	3.38×10^{-1}	4.39×10^3	7.07×10^{-2}	$20 (\pm 1.5) \times 10^{-6}$	$16 (\pm 1.1) \times 10^{-6}$	$0.5 (\pm 1.3) \times 10^{-6}$

Data for the loop (C′–E + B–C) and PepE are referred to a fitting with a biphasic model of binding to IgE.

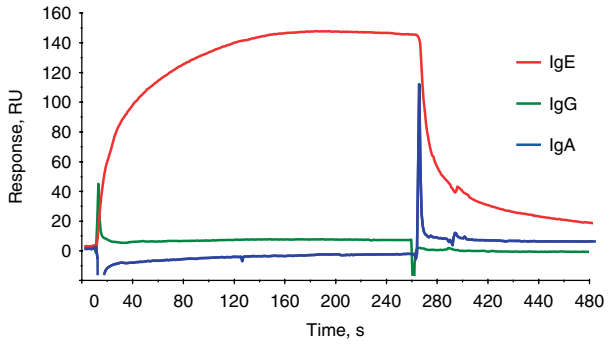

Figure 3. Comparative sensorgrams for the binding of PepE to immobilized IgE, IgG and IgA. The peptide, analyzed at a concentration of 800 nM with a constant flow rate of 30 µl/min, only binds to IgE.

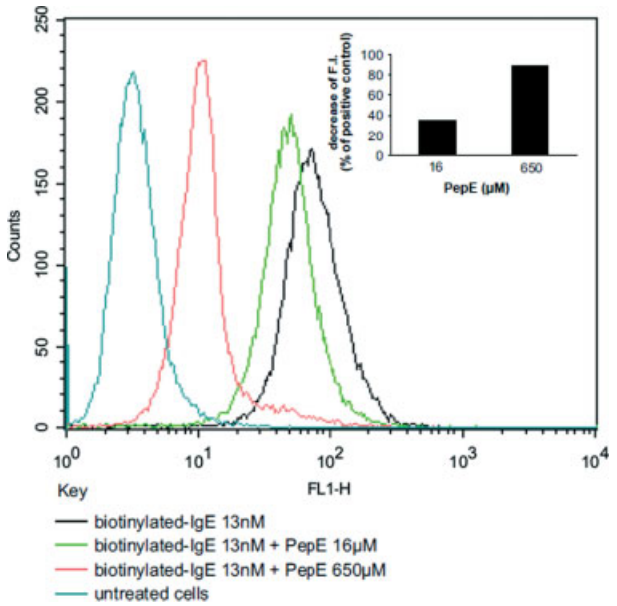

Figure 4. Flow-cytometric analysis of inhibition of labeled-IgE binding to RBL2H3 cells by increasing concentrations of PepE. RBL2H3 cells at a density of 1×10^6/ ml were incubated with 13 nM IgE and increasing concentration of peptide for 1 h at 4 °C, then the fluorescence intensity was measured upon addition of FITC-avidin. The mean fluorescence intensity for each concentration of peptide is given in the top right corner. Values of fluorescence intensity were obtained from histogram statistic of CellQuest software.

a fixed concentration (800 nM). Data in Figure 3, clearly show that the new PepE retains a strong selectivity for the IgE and has no affinity for the other immunoglobulin classes. Remarkably, the control scrambled peptide did not bind IgE nor the control immunoglobulins (not shown), further confirming the selectivity of PepE and the accuracy of the design.

Cellular Competitive Assays

Inhibition of biotinylated-IgE binding to RBL2H3 cells by PepE

To assess the ability of the new peptide to displace the binding of IgE to its high affinity receptor, competitive binding experiments were performed with increasing concentration of PepE at a fixed concentration of biotinylated-IgE (see the section of Methods). As reported in Figure 4, in agreement with its increased affinity, the peptide afforded a 50% inhibition (IC_{50}) at a concentration of about

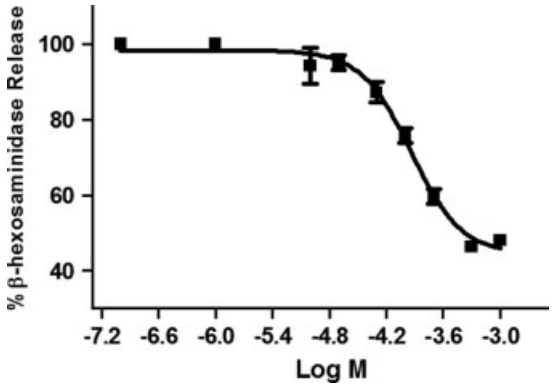

Figure 5. Inhibition of IgE-induced β-hexosaminidase release by increasing concentration of PepE (10–1000 µM) in RBL2H3 cell line. The activity of the β-hexosaminidase in the supernatant and in solubilized cell pellets was measured with *p*-nitrophenyl-*N*-acetyl-β-D-glucosaminide as the enzyme substrate and expressed as percentage of specific release. A maximum of 35% specific release was achieved under the described conditions. For the inhibition experiments, values were normalized to 100%. Results are means ± standard error of mean of three independent determinations.

32 µM which is fourfold lower than that of the progenitor peptide. Remarkably, at the highest concentration used (650 µM), PepE displaced about 90% of bound IgE, suggesting that IgE effects on mast cells can be modulated by varying the peptide concentration. The specificity of this effect was confirmed by using up to 1 mM of the scrambled peptide (Figure 1(B)), and a 100-fold excess of non-labeled-IgE (complete inhibition) in the same experiment (not shown).

Inhibition of IgE-mediated β-hexosaminidase release

To further confirm the ability of PepE to block the activity of IgE, we evaluated the inhibitory effect of the peptide on the degranulation of RBL2H3 cells [21]. The assay is based on stimulation of IgE-sensitized RBL2H3 cells with specific antigens. Antigens trigger a cascade of intracellular signals responsible for both degranulation and release of allergic mediators, including the β-hexosaminidase enzyme which coexists with histamine in secretory granules of mast cells and basophiles. To perform the experiment, we first set up the optimal pre-saturation conditions (mIgE 250 ng/ml and DNP-albumin 100 ng/ml), then the competitive experiments were performed by using PepE at increasing concentrations. Importantly, as reported elsewhere [29], we achieved a maximum spontaneous release of mediators of about 35% (not shown). When the cells were treated with the peptide, the release of β-hexosaminidase from RBL2H3 cells was significantly inhibited in a dose-dependent manner, as shown in Figure 5. As shown in the same figure, we achieved a maximum inhibition of about 50%, as also reported for other compounds [14,17], indeed increasing the peptide concentration up to 1 mM, no further inhibition was observed. This effect could be explained by a partial aggregation of the peptide at the high concentrations (1 mM) which could lead, in turn, to a re-association of IgE to the cellular receptor. However, though the inhibition was not complete, this experiment demonstrates the high specificity of the peptide effects on the antigen-dependent IgE-mediated mechanisms of mast cell degranulation.

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Conclusions

We have designed and chemically synthesized a new FcεRI-receptor mimetic named PepE. The peptide has been obtained by simply increasing the length of the linker in a peptide we have previously described [loop (C′–E + B–C)] [24], and for which we noticed that the distance between the two loops was too short to properly reach the two independent sites on the IgE. Introduction of a β-Ala within the linker, increases the distance between the two loops of about 5 Å and this very simple change effectively improves peptide binding to IgE, affording an overall K_D of about 500 nM, 30-fold lower than that observed with the shorter variant. PepE also efficiently blocks the interaction of IgE with the native receptor, as demonstrated on RBL2H3 cells and prevents the primary effects of allergy following antigen-IgE stimulation. This property has been clearly demonstrated on cells, determining the release of mediators in the presence and in the absence of peptide. We have shown that cellular degranulation can be reduced by addition of increasing amounts of peptide, thus indicating that PepE does effectively block receptor activation by binding to IgE and preventing their binding and cross-linking on the cell surface. Also, a downregulation of receptor cell-surface expression could be induced by treatment with the peptide and this could be a further mechanism of attenuation of the down-stream receptor signaling. Importantly, this peptide, as the only drug available on the market that targets the FcεRI–IgE interaction [10,11], does bind to IgE and not the receptor, therefore preventing the possibility of agonistic activities.

Although the molecule certainly requires further modification to improve the affinity and needs an *in vivo* proof-of-concept, the data so far obtained are promising and support the idea that this small peptide can efficiently mimics the two distinct receptor binding sites and can be therefore a useful starting point for the development of compounds with therapeutic applications in allergy.

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