Cutting Edge: Human Anaphylatoxin C4a Is a Potent Agonist of the Guinea Pig But Not the Human C3a Receptor

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The interaction of human anaphylatoxin C4a with the guinea pig (gp) and human (hu) C3a receptors (C3aR) was analyzed using human rC4a, which exhibited C4a-specific activity on guinea pig platelets. A gpC3aR of 475 residues with a large second extracellular loop and a peptide sequence ~60% identical to the huC3aR was isolated from a genomic DNA library and found to be expressed in guinea pig heart, lung, and spleen. HEK-293 cells cotransfected with this clone, and a cDNA encoding Goe-16 specifically bound ($K_d = 1.6 \pm 0.7 \, \text{nM}$) and responded functionally to C3a with an intracellular calcium mobilization (ED$_{50} = 0.18 \pm 0.02 \, \text{nM}$). Human rC4a weakly bound to both the hu- and gpC3aR (IC$_{50} > 1 \, \mu \text{M}$). However, only HEK-293 cells expressing the gpC3aR responded functionally to rC4a (ED$_{50} = 8.7 \pm 0.52 \, \text{nM}$), while cells expressing the huC3aR did not ($c \leq 1 \, \mu \text{M}$). Thus, through an interaction with the C3aR, huC4a may elicit anaphylatoxic effects in guinea pigs but not in man. *The Journal of Immunology*, 1998, 161: 2089–2093.

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4 Abbreviations used in this paper: huC3aR, human C3a receptor; gp, guinea pig (i.e., gpC3aR); FLIPR, fluorometric imaging plate reader; c, concentration.

Various cell activation and granule secretion reactions (for an overview, see Refs. 1 and 2).

Human C4a, first described in 1979, is regarded as the third anaphylatoxin (3) due to its structural similarity to C3a and C5a, its dependence on a carboxyl-terminal arginine residue for biologic activity, and its proinflammatory properties in guinea pigs. Although less active than C3a and C5a, in guinea pigs, human C4a induces smooth muscle contraction, increases vascular permeability (3), and induces granule secretion from platelets and O$_2$ generation in macrophages (4, 5).

These biologic effects of C4a are subject to low dose desensitization (tachyphylaxis). Preincubation with substimulatory concentrations of C4a abrogates the functional response toward a subsequent 100% stimulus. In addition, cross-desensitization in guinea pig ileal contraction assays was observed between C4a and C3a, but not between C4a and C5a (3, 4). Based on these observations, C3a and C4a are thought to act on a common receptor. This view, however, has recently been questioned by Murakami and coworkers, who failed to detect desensitization of C3a-induced responses after pretreatment of guinea pig macrophages with human C4a (5). Furthermore, C4a did not inhibit binding of $^{125}$I-labeled C3a to guinea pig macrophages (5). These findings would indicate distinct and separate receptors for C3a and C4a in guinea pigs.

In man, convincing evidence for anaphylatoxic effects of human C4a is still missing. C4a has been reported to inhibit C3a-induced chemotaxis of macrophages (6), although at extremely low concentrations ($10^{-16} \, \text{M}$). Also, C4a and C4a analogue synthetic peptides lead to a dose-dependent wheal and flare generation when injected into human skin (3, 7), although no negative controls were included in these experiments. Evidence for C4a effects in species other than man or guinea pig has not been reported.

Recently, the C3aR from man (huC3aR) (8–10) and mouse (11, 12) have been cloned. Stably transfected cell lines expressing these receptors and human neutrophils have been tested for functional response towards serum-purified human C4a and found to be completely unresponsive (11, 13). This would suggest the existence of a separate C4a receptor in man and mouse, on the premise that C4a is an anaphylatoxin in these species as well. However, no...
positive control demonstrating biologic activity of the C4a preparation used in these experiments was presented.

Highly purified human C4a is difficult to prepare and, due to the high C4a concentrations required in most test systems, even trace contamination with other biologically active molecules, especially C3a and C5a, may jeopardize the experiments, as has been reported in one of the above-mentioned investigations for serum-purified C4a (13). This possible contamination may well be the cause for some of the discrepant results presented above. Using human rC4a and cells functionally expressing a cloned guinea pig (gp) or huC3αR, we provide evidence that C4a functions as an agonist of the gpC3αR but not the huC3αR.

Materials and Methods

Materials

Human C3a was obtained from Advanced Research Technologies (San Diego, CA), 125I-labeled human C3a from NEN-DuPont (Boston, MA), and rC5a from Sigma (St. Louis, MO). The C3a carboxyl-terminal analogue synthetic peptide (WWGKKYRASKLGLAR, (W63, W64)C3a(63-77)) was obtained from Bachem (King of Prussia, PA), and the C3a synthetic peptide P117 (control peptide P251) were prepared as described (9). Oligonucleotides were obtained from MWG Biotech (Ebersberg, Germany). N-terminal hexahistidine-tagged rC4a or rC4a with an N-terminal methionine residue was expressed in Escherichia coli and purified to homogeneity. The binding and functional activities of these two preparations of rC4a were equivalent.

Guinea pig platelet release assay

Functional characterization of rC4a was performed in a guinea pig ATP release assay, as described (15). Guinea pigs from strains C2BB/R+ (C3αR positive) and C2BB/R− (C3αR-negative) from our own breeding colonies were used as platelet donors (16). Desensitization was measured by pre-incubation of the platelets with the deactivating stimulus (a concentration leading to <10% ATP release, determined empirically at the beginning of each experiment) for 5 min at 37°C and subsequent addition of a 100% stimulus of either C3a (c = 10 nM), rC4a (c = 4 μM), or rC5a (c = 250 nM).

Cloning of the guinea pig C3αR

A partial DNA sequence of the gpC3αR was obtained by PCR amplification of genomic DNA using oligonucleotide primers derived from regions conserved between the human and mouse C3αR sequences (8–12). This fragment was used to screen a guinea pig genomic DNA library in AFI XII (Stratagene, La Jolla, CA). A λ clone containing a genomic insert, which encoded the gpC3αR, was identified. The open reading frame of this clone plus an extra 48 bp of genomic DNA sequence at the 3′ end was subcloned into pcDNA3 (Invitrogen, San Diego, CA) and designated pSL94.

Receptor characterization

Competitive binding assays were performed essentially as described (9). A microtiter plate-based calcium mobilization assay, utilizing a fluorometric imaging plate reader (FLIPR; Molecular Devices, Sunnyvale, CA), was used for the functional characterization of HEK-293 cells transiently expressing either the gpC3αR or huC3αR and Go16 (17). Briefly, cells were transfected using Lipofectamine Plus reagent (Life Technologies, Gaithersburg, MD), and the following day they were plated in poly-L-lysine-coated 96-well black/clear plates (Becton Dickinson, Bedford, MA). After 18 to 24 h, the medium was aspirated from each well, and the cells were loaded with Fura-3-AM (Molecular Probes, Eugene, OR). At initiation of the assay, fluorescence is read once every second for 60 s and then every 3 s for the following 60 s. Agonist was added at 10 s, and the maximal fluorescence count above background after addition was used to define maximal activity for that concentration of agonist. FLIPR software normalizes fluorescence readings to give equivalent readings for all wells at zero time.

Results and Discussion

Generation and characterization of rC4a

To avoid the problem of contamination in serum-purified human C4a preparations, we expressed rC4a. As shown in Figure 1A, human rC4a was approximately three orders of magnitude less potent in a guinea pig ATP release assay than C3a, but induced ATP release at high nanomolar concentrations (ED50 ≈ 1.3 × 10−10 M), rC4a (squares; ED50 = 4.1 ± 0.6 × 10−7 M) and the C3α analogue synthetic peptide P117 (triangles) (n = 3) (9). Desensitization of rC4a and C3a, but not rC5a, responses after preincubation of the platelets with substimulatory concentrations of rC4a or the C3α analogue synthetic peptide P117, respectively, and subsequent addition of C3a (10 nM), rC4a (4 μM), or rC5a (250 nM). RLU, relative light units; “X/Y”, deactivating stimulus/100% stimulus.

Cloning and characterization of gpC3αR

The coding regions of many G protein-coupled receptors, including the human C3αR (19), are not interrupted by introns. Therefore, we cloned the gpC3αR directly from a genomic DNA library.
Using primer combinations conserved in the C3aR sequences of man (8–10) and mouse (11, 12), a genomic DNA fragment was amplified with high homology to the huC3aR. Using this fragment as a probe to screen a guinea pig library, a genomic clone was isolated with an open reading frame of 1428 bp, which encoded a protein of 475 residues with a calculated molecular mass of 53,570 Da and four potential N-glycosylation sites (Fig. 2). The sequence encodes a G protein-coupled receptor with seven hydrophobic transmembrane domains, a large second extracellular loop of 165 amino acid residues, and a high homology to the C3aR sequences of man, mouse, and rat (8–12, 20). However, only half of the residues are conserved in all four known C3aR sequences (240/475 = 50.5%), and only 37 of the 165 residues in the second extracellular loop (22.4%) in the gpC3aR are found at the same position in the other C3aR sequences. The peptide sequence of this gpC3aR was disclosed in a recently published book (2); however, the nucleotide sequence has not been published nor does it appear in GenBank, and no functional or binding data have been presented. It is unlikely that this receptor is a pseudogene, since by RT-PCR, expression of this transcript was detected in guinea pig heart, lung, and spleen RNA (data not shown).

HEK-293 cells transiently transfected with this receptor specifically bound human C3a. As shown in Figure 3, competitive displacement studies revealed the presence of a high affinity receptor for C3a with an apparent Kᵦ of ~2 nM. As previously shown with the huC3aR, this binding was specific for C3a, because ¹²⁵I-labeled C3a could be displaced in a dose-dependent manner by the synthetic C3a analogue peptide P117, but not by the unrelated peptide P251 (9). In addition, supramicromolar concentrations of rC4a (IC₅₀ = 1 μM) were able to competitively displace radiolabeled C3a from both the guinea pig and huC3aR. Although human C4a is able to bind weakly to both the gpC3aR and huC3aR, the affinity of this interaction is three orders of magnitude lower than the affinity of C3a binding to the same receptors.

C4a mediates functional responses via the gpC3aR but not the huC3aR

To compare the functional activity of the gpC3aR and huC3aR, we cotransfected the cDNA for each receptor, together with a cDNA clone encoding Gα₁₆, into HEK-293 cells and tested for intracellular calcium mobilization in response to rC4a, rC5a, C3a, or a C3a analogue peptide. Previously, we reported that cotransfection of the huC3aR sequence with Gα₁₆ was necessary to render transfected HEK-293 cells responsive to C3a (9). As shown in Figure 4A, cells expressing the gpC3aR responded in a dose-dependent manner not only to C3a (ED₅₀ = 0.18 ± 0.02 nM) and the C3a peptide (ED₅₀ = 0.15 ± 0.01 nM), but also to rC4a (ED₅₀ =...
or G...on cells expressing either the gpC3aR or huC3aR, but only at formed on guinea pig cells (14). Minimal activity was noted withacterize this “superagonist” C3a analogue peptide were all per-

**FIGURE 3.** Competitive binding curves in HEK-293 cells transiently transfected with the gpC3aR (filled symbols) or huC3aR (open symbols), respectively. $^{125}$I-labeled C3a was competitively displaced by increasing concentrations of C3a (circles), rC4a (squares), the synthetic C3a analogue peptide P117 (triangle), or the nonrelated synthetic peptide P251 (diamonds). C3a, $K_d = 1.6 \pm 0.9 \text{nM (gpC3aR)}$; $K_d = 1.0 \pm 0.9 \text{nM (huC3aR)}$; P117, $IC_{50} = 1.9 \pm 1.0 \times 10^{-7} \text{M (gpC3aR)}$ ($n \geq 3$).

$8.7 \pm 0.52 \text{nM})$. Cells transfected with the cDNA for the gpC3aR or Gr-16 alone did not respond to any of the peptides tested (data not shown).

In marked contrast to the results obtained with rC4a on cells expressing the gpC3aR, cells transiently expressing the huC3aR did not respond to rC4a concentrations as high as 1 \text{ \mu M}, but did respond to C3a ($ED_{50} = 0.36 \pm 0.07 \text{nM}$) and the C3a peptide ($ED_{50} = 3.1 \pm 0.3 \text{nM} \text{; Fig 4B}$). Previously, comparable results were obtained with stable cell lines expressing the cloned huC3aR (13). The gpC3aR and huC3aR responded differently to human rC4a as well as to the C3a synthetic peptide, which was virtually equipotent with C3a vs the gpC3aR but approximately an order of magnitude less potent than C3a vs the huC3aR. This may not be an unexpected result, as the biological assay originally used to character-

$FIGURE 4$. Calcium mobilization dose response curves of HEK-293 cells transiently expressing Gα-16 and the gpC3aR (A) or the huC3aR (B) to a stimulus of C3a (circles), (W63,W64)C3a(63-77) (squares), rC4a (diamonds), or rC5a (triangles). Concentration response curves for each agonist were run on six individual plates using FLIPR in two individual experiments. Values presented are the mean ± SEM.

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**References**


