Complement-activation fragment C4a mediates effector functions by binding as untethered agonist to protease-activated receptors 1 and 4

HongBin Wang[a], Daniel Ricklin[a,b,1], and John D. Lambris[a,1]

[a]Department of Pathology & Laboratory Medicine, Perelman School of Medicine, University of Pennsylvania, Philadelphia, PA 19104; and [b]Department of Pharmaceutical Sciences, University of Basel, 4056 Basel, Switzerland

C4a is a small protein released from complement component C4 upon activation of the complement system's classical and lectin pathways, which are important constituents of innate immune surveillance. Despite the structural similarity between C4a and well-described anaphylatoxins C3a and C5a, the binding partner and biological function of C4a have remained elusive. Using a cell-based reporter assay, we screened C4a against a panel of both known and orphan G protein-coupled receptors and now provide evidence that C4a is a ligand for protease-activated receptor (PAR)1 and PAR4. Whereas C4a showed no activity toward known anaphylatoxin receptors, it acted as an agonist for both PAR1 and PAR4 with nanomolar activity. In human endothelial cells, ERK activation by C4a was mediated through both PAR1 and PAR4 in a $G\alpha_i$-independent signaling pathway. Like other PAR1 activators, C4a induced calcium mobilization through the PAR1/$G\alpha_q$/PLCβ signaling axis. Moreover, C4a increased stress fiber formation and enhanced endothelial permeability, both of which were reduced by PAR1 antagonists. In sum, our study identifies C4a as an untethered agonist for PAR1 and PAR4 with effects on cellular activation and endothelial permeability, thereby revealing another instance of cross-talk between the complement system and other host defense pathways.

complement | C4a | PAR1 | PAR4 | endothelial cells

The host defense relies on a tight interplay and extensive cross-talk between innate immune pathways such as the complement system, the contact and coagulation cascades, and cellular components, including platelets and endothelial cells (ECs). Although typically conferring protection from microbial intruders and accumulating debris, host defense systems can contribute to various thromboinflammatory, acute-phase, and age-related disorders if inappropriately triggered (1). Complement plays a key role in these processes by sensing pathogen- and damage-associated molecular patterns and initiating a cascade that facilitates the elimination of invading cells via phagocytosis and lytic damage and releasing inflammatory mediators to propagate danger signaling (1, 2). The anaphylatoxins C3a and C5a, which are released upon activation of complement components C3 and C5, respectively, are among the most potent complement-derived effectors that exert a number of well-described functions through binding to the anaphylatoxin receptors C3aR, C5aR1, and/or C5aR2 (3).

Although complement activation through the classical and lectin pathways leads to the release of an ~10-kDa protein from component C4, termed C4a, that shares high homology with the anaphylatoxins, the receptor and biological functions of this activation fragment have thus far remained elusive (4). Previously, C4a had been reported to inhibit C3a-induced $O_2^{\cdot-}$ generation in guinea pig macrophages, induce contraction of guinea pig ileum, and produce immediate erythema/edema when injected into human skin (5, 6). Whereas its ability to desensitize the action of C3a-induced contraction of guinea pig ileum suggested a function for C4a closely related to C3a (5), it was later shown that human C4a may act as a potent agonist of the guinea pig C3aR

but not the human receptor (7). It has to be noted that these early studies were typically performed with plasma-purified C4a, which may have contained small but physiologically relevant amounts of other anaphylatoxins. Recent studies using recombinant human C4a have shown that the protein can inhibit C3a- or C5a-mediated chemoattractant and secretagogue functions in mast cells, impair C5a-induced neointima formation, and protect against hyperoxic lung injury via a macrophage-dependent signaling pathway (8–10). Whereas these results may indicate a potential competition of C4a for the anaphylatoxin receptors, direct binding could not be confirmed to any of the receptors. Several studies have suggested that the receptor for C4a is distinct from C3aR, C5aR1, or C5aR2 (6, 7, 11) but no suitable candidate has been identified.

Nevertheless, the strong homology of C4a to C3a and C5a, which both bind G protein (C3aR, C5aR1)- or β-arrestin (C5aR2)-coupled seven transmembrane receptors (11, 12), renders this class of receptors a likely target for C4a. Indeed, a recent study has demonstrated that C4a can decrease intracellular cAMP levels by acting on G_i protein-coupled pathways (9). Thus, we hypothesized that the biological function of C4a is not mediated through anaphylatoxin receptors, but instead through a hitherto-unidentified G protein-coupled receptor (GPCR). To test this hypothesis, we used a GPCR reporter assay, which revealed that C4a is a specific ligand for protease-activated receptors 1 and 4 (PAR1 and PAR4, respectively). These receptors exert various functions on platelets and ECs, among others, and are traditionally activated by cleavage of a tethered ligand by proteases such as thrombin (13). In the case of C4a, however, PAR1/PAR4 activation appeared to be mediated by

Significance

How C4a transduces signaling and generates various biological functions has long been unresolved. Using a cell-based reporter assay, we screened C4a against a panel of 168 known and 73 orphan G protein-coupled receptors and now provide evidence that C4a is a ligand for protease-activated receptor (PAR)1 and PAR4. In human endothelial cells, our assessment of ERK activation and calcium mobilization in the presence of various antagonists and inhibitors suggests that C4a-mediated cell activation is mediated through PAR1 and PAR4. Our results demonstrate that C4a is a nontraditional agonist for PAR1 and PAR4 whose direct functional effects indicate a potentially significant direct linkage between the complement, coagulation, and endothelial barrier systems.

direct binding to the receptor. These unexpected findings were corroborated in various cell-based assays that allowed us to deduce the signaling pathways involved and describe C4a's functional effects on EC permeability. Taken together, our results demonstrate that C4a is a nontraditional agonist for PAR1 and PAR4 with direct functional effects and potential implications as a direct link between the complement, coagulation, and endothelial barrier systems.

Results

Human C4a Is a Ligand for PAR1 and PAR4. Previous studies have suggested that human C4a transduces signaling through a hitherto unknown receptor, likely of the GPCR superfamily, to exert its proposed biological functions (6, 7, 9, 11). In an attempt to identify potential C4a-reactive GPCR proteins, we screened plasma-derived human C4a in a cell-based assay comprising a panel of 168 known (gpcrMAX) and 73 orphan (orphanMAX) human GPCRs. This assay employs engineered GPCR-expressing cell lines and a reporter system based on β-arrestin recruitment and enzyme fragment complementation to monitor agonistic or antagonistic activities (14) (Fig. S1A). In agonist mode, screening of the gpcrMAX panel revealed two GPCRs that met the selectivity criteria as putative targets for C4a (Fig. 1 and Table S1): PAR1 (F2R) and PAR4 (F2RL3). We chose a screening concentration of 600 nM C4a, which corresponds to an activation of ~20% of the total plasma pool of complement component C4. At this concentration, C4a was able to induce 53% and 97% of the maximal control ligand response for PAR1 and PAR4, respectively. Importantly, C4a did not elicit any activation of the anaphylatoxin receptors (C3aR, C5aR1, and C5aR2) or other members of the PAR family (PAR2 and PAR3), confirming that it is a specific ligand for PAR1 and PAR4. No antagonistic activity (Fig. S1B and Table S2) or orphan GPCR activation (Fig. S1C and Table S3) for C4a was detected.

To confirm and quantitate the agonist activity of C4a, we subjected CHO-K1 cells expressing either human PAR1 or PAR4 to a concentration series of C4a and other anaphylatoxins, using the same β-arrestin reporter function as used for our screening. In the PAR1 assay, C4a dose-dependently activated cells with an EC_{50} of 0.8 μM, well below the analogous value for the known PAR1-activating peptide TFLLR-NH$_2$ (EC_{50} = 5 μM). Neither human C3a or C5a nor the PAR4 activator AY-NH$_2$ had an effect on PAR1 activation (Fig. 2A). Interestingly, the signal intensity of PAR1 activation induced by C4a became saturated at significantly lower levels than those induced by PAR1-activating TFLLR-NH$_2$, suggesting that C4a may act as a partial agonist for PAR1. Furthermore, the potent PAR1 antagonist RWJ56110 dose-dependently decreased C4a-induced PAR1 activation, showing a noncompetitive pattern (Fig. 2B). These results confirm that C4a can activate
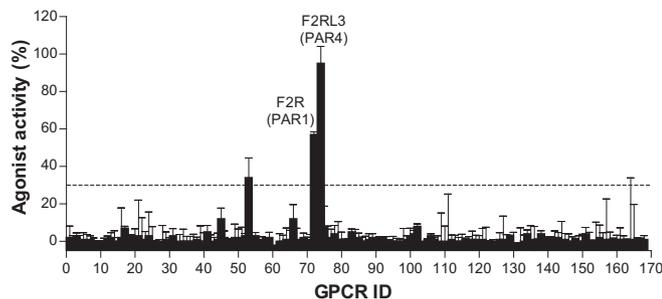
Fig. 1. C4a is a putative agonist for protease-activated receptors (PAR)1 and PAR4. Screening of the gpcrMAX panel in agonist mode reveals that PAR1 and PAR4 meet the selective criteria as putative targets for C4a. Dash line represents 30% of activation. The data represent the mean ± SD of duplicate samples.
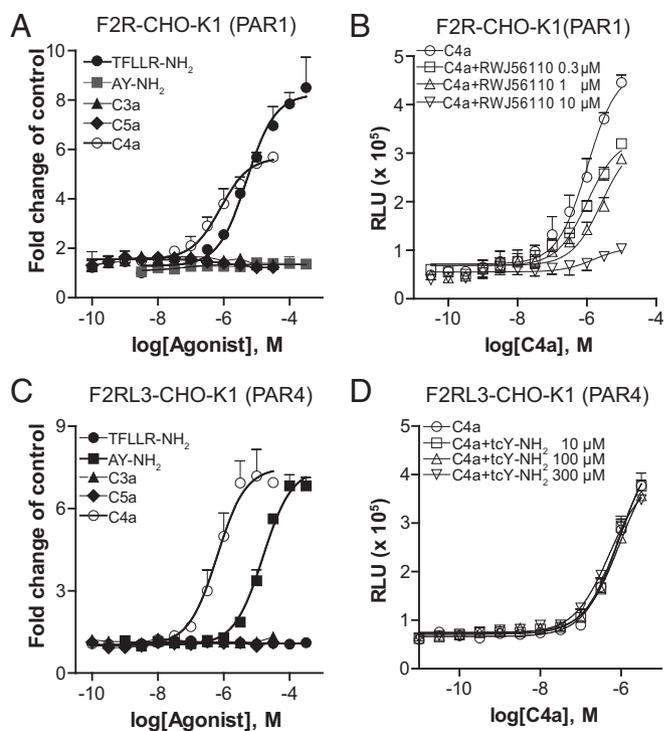
Fig. 2. C4a acts as an agonist for PAR1 and PAR4. CHO-K1 cells expressing either PAR1 or PAR4 were seeded in a 96-well plate and stimulated with PAR1 agonist TFLLR-NH$_2$, PAR4 agonist AY-NH$_2$, human C4a, C3a, or C5a. After stimulation, the chemiluminescent signal was detected. Culture medium alone was used as a control. (A) C4a and PAR1 agonist TFLLR-NH$_2$ dose-dependently activate PAR1. (B) PAR1 antagonist RWJ56110 dose-dependently inhibits C4a-induced PAR1 activation. (C) C4a and PAR4 agonist AY-NH$_2$ dose-dependently activate PAR4. (D) PAR4 antagonist tcY-NH$_2$ has no effect on C4a-induced PAR4 activation. The data are expressed as fold change compared with control or relative light unit (RLU) and represent the mean ± SE of three independent experiments.

PAR1 and that it may bind to a region in PAR1 distinct from that of TFLLR-NH$_2$.

In the PAR4 assay, both C4a and the PAR4-active peptide AY-NH$_2$ dose-dependently activated PAR4-expressing CHO-K1 cells, whereas neither C3a or C5a nor the PAR1 activator TFLLR-NH$_2$ generated an elevated signal compared with the vehicle control. With an EC_{50} of 0.6 μM, C4a showed a PAR4-activating potential 10 times stronger than that of AY-NH$_2$ (EC_{50} = 6.5 μM) (Fig. 2C). In contrast to the PAR1 results, the signal intensity of C4a in the PAR4 assay reached levels comparable to those of the control agonist, thereby indicating purely agonistic activity of C4a for PAR4. Interestingly, the PAR4 antagonist tcY-NH$_2$ did not inhibit C4a-triggered PAR4 activation (Fig. 2D), suggesting that the binding areas or activation modes of C4a and AY-NH$_2$ are distinct. Taken together, our results provide the evidence that C4a is a nontraditional, complement-derived agonist for PAR1 and PAR4.

In the case of C3a and C5a, a C-terminal region comprising some 20 amino acid residues has been reported to be essential for the activation of C3aR and C5aR1, respectively (15, 16). To determine whether the C terminus of C4a is similarly relevant for the activation of PAR1 or PAR4, we synthesized the corresponding 20-amino acid C-terminal peptide of C4a (C4a-CT20) and tested its agonistic activity. Even at extended concentration ranges (100–300 μM), C4a-CT20 did not elicit any significant PAR1 or PAR4 activation, indicating that the C terminus of C4a does not contain the major area of activity (Fig. S2). Moreover, C4a-desArg (missing the C-terminal arginine) dose-dependently activated both PAR1 and PAR4 (Fig. S2), but with less efficacy than did C4a. When
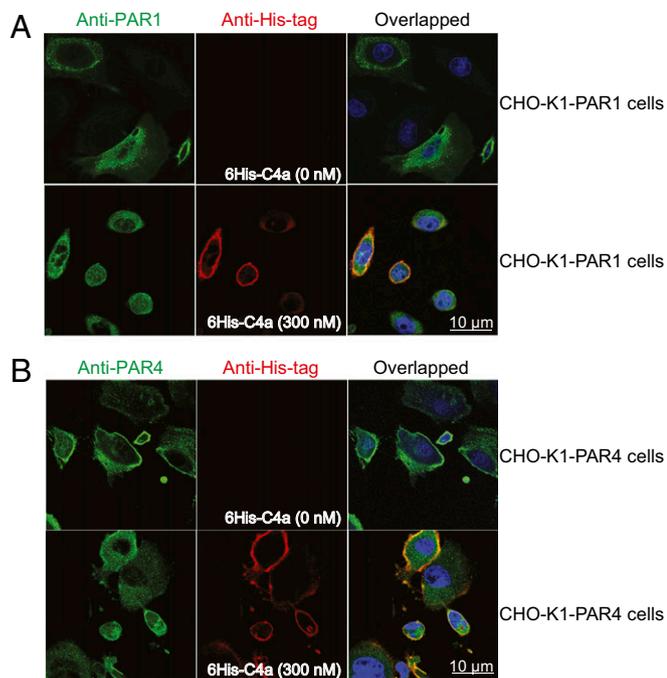
Fig. 3. On CHO-K1 cells expressing either PAR1 or PAR4, recombinant human C4a colocalizes with the corresponding receptor. His6-C4a (red, anti-6His Ab) colocalizes with PAR1 (*A*; green, anti-PAR1 Ab) or PAR4 (*B*; green, anti-PAR4 Ab) on CHO-K1 cells expressing PAR1 or PAR4, respectively, but does not bind to wild-type CHO-K1 cells (Fig. S4*A*). The experiment was performed three times with similar results, and one representative experiment is shown.

compared to the critical role of the C-terminal arginine in ana-phylatoxin activity (17, 18), the C terminus of C4a appeared to be involved in, but not essential to, C4a's effects on PAR1 and PAR4.

C4a Colocalizes with PAR1 and PAR4 in CHO-K1 Cells Expressing PAR1 or PAR4. To confirm receptor binding on cells, we looked for colocalization of C4a with PAR1 and PAR4 on CHO-K1 cells that express either receptor (Fig. S3 *A* and *B*). For this purpose, we expressed recombinant human C4a carrying a 6His-tag at its N terminus (Fig. S3 *C* and *D*). Colocalization experiments were performed using confocal microscopy to monitor the association of C4a-6His with PAR1 or PAR4 at the plasma membrane in either PAR1- or PAR4-expressing CHO-K1 cells. After incubating 300 nM 6His-C4a (red) with each cell line for 10 min, we observed colocalization of C4a with PAR1 or PAR4 (green) at the plasma membrane (Fig. 3). No binding of 6His-C4a was detected on wild-type CHO-K1 cells (Fig. S4*A*). A similar colocalization pattern was observed in the human EC line HMEC-1 (Fig. S4 *B* and *C*). Although not a proof of direct binding, our combined results strongly indicate that C4a binds to PAR1 and PAR4 and exerts partial (PAR1) or full (PAR4) ago-nistic activity. In contrast to traditional activators such as thrombin that cleave a tethered ligand at the PAR's N terminus (13), C4a appears to bind directly to the active sites of the two receptors.

C4a Induces ERK1/2 Activation in Human Endothelial Cells Through $G\alpha_i$-Independent Signaling. We then asked whether the nontypical receptor interaction of C4a invokes the same intracellular signaling pathways as do the tethered ligands. Upon their proteolytic cleavage by an activator such as thrombin and induction of G protein-mediated signaling, the activated extracellular signal-regulated kinase 1/2 (ERK1/2) translocates to the nucleus and activates nuclear transcription factors that participate in gene transcription, cell proliferation, and differentiation (19, 20). We used human EC-type cell lines to examine whether C4a-mediated stimulation of

PAR1 or PAR4 would similarly trigger ERK1/2 activation. Indeed, C4a dose- and time-dependently increased ERK1/2 phosphorylation in HMEC-1 (Fig. 4*A* and Fig. S5*A*). Pretreatment of ECs with the specific PAR1 antagonist RWJ56110 (10 μM, 30 min) significantly decreased C4a-induced ERK1/2 phosphorylation, whereas no sig-nificant effect was observed after pretreatment with pertussis toxin (PTX, 300 ng/mL for 10 h), which is known to inhibit $G\alpha_i$ protein-induced signaling (21) (Fig. 4*B*). A comparable result was obtained in the hybrid cell line EA.hy926 (i.e., a fusion of HUVEC and A549 lung adenocarcinoma cells), which is commonly used as an EC-related model (Fig. S5*B*).

Controversy exists as to whether functionally active PAR4 is expressed on ECs (22–25). To address this question, we used the selective PAR4 agonist AY-NH$_2$ to treat HMEC-1 and EA.hy926 cells, then analyzed phosphorylated ERK to determine whether functional PAR4 was expressed. We observed that AY-NH$_2$ caused significant ERK activation (Figs. S5*C* and S6*A*), sug-gesting that functional PAR4 was expressed on the EA.hy926 and HMEC-1 cell lines. Pretreatment with the PAR4 antagonist tcY-NH$_2$ (300 μM, 30 min) did not inhibit either C4a-induced PAR4 activation in CHO-K1–PAR4 cells or ERK phosphoryla-tion (Figs. 2*D* and 4*B*), suggesting that C4a binds to a motif in PAR4 distinct from that for tcY-NH$_2$. To confirm this hypothesis, we pretreated HMEC-1 cells with a blocking PAR4 antibody (26) and examined C4a-induced ERK phosphorylation. In contrast to tcY-NH$_2$, anti-PAR4 significantly decreased C4a-induced ERK phosphorylation, indicating that C4a also mediates ERK activation via PAR4 through a mode distinct from known ligands (Fig. S6*B*). To further elucidate the role of PAR1 or PAR4 in C4a-induced ERK activation, HMEC-1 cells were pretreated with the PAR1 antagonist RWJ56110, anti-PAR4, or a combination of both. The results revealed that inhibition of both PAR1 and PAR4 could completely inhibit the effects of C4a, suggesting that PAR1 and PAR4 are both involved in C4a-mediated ERK activation in ECs (Fig. S6*C*). Taken together, our data reveal that C4a activity on PAR1 and PAR4 induces ERK phosphorylation in human ECs through a $G\alpha_i$-independent signaling pathway.

C4a Triggers Calcium Mobilization Through a $G\alpha_q$-Coupled, PLCβ-Dependent Signaling Pathway. PAR1 has been reported to be activated by thrombin or the PAR1 agonist TFLLR-NH$_2$ through $G\alpha_q$ activation, which increases the concentration of intracellular calcium {i.e., [Ca^{2+}]$_i$} (27–29). Given the ability of C4a to act as a PAR1 agonist,
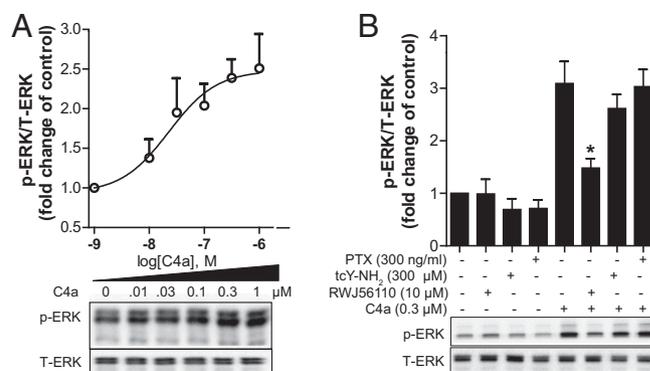

Fig. 4. In human HMEC-1 endothelial cells, C4a increases ERK phosphorylation through a $G\alpha_i$-independent signaling pathway. (*A*) C4a dose-dependently en-hances ERK phosphorylation. (*B*) PAR1 antagonist RWJ56110 (10 μM), but not pertussis toxin (PTX; 0.3 μg/mL, 10 h), inhibits ERK activation upon C4a exposure (7 min). The data are expressed as fold change in densitometry of Western blots from the control group and represent the mean ± SE of three independent experiments. (*n* = 3; *P < 0.05 vs. control; pairwise two-sided Student's *t* test.)

we used HMEC-1 cells to measure the change in $[Ca^{2+}]_i$ in response to increasing concentrations of C4a. The assay revealed that C4a-mediated stimulation induced a significant, concentration-dependent increase in $[Ca^{2+}]_i$ (Fig. 5A). Pretreatment with the PAR1 antagonist RWJ56110 (10 μM, 30 min) significantly inhibited C4a-induced $[Ca^{2+}]_i$, indicating an involvement of PAR1 activation in C4a-mediated calcium mobilization (Fig. 5B). To determine whether this increase in $[Ca^{2+}]_i$ is mediated through $G\alpha_q$-phospholipase Cβ (PLCβ)-dependent signaling, we pretreated HMEC-1 cells with the PLC inhibitor U73122 or its inactive analog U73343. As expected, only U73122, and not U73343, significantly inhibited the C4a-mediated $[Ca^{2+}]_i$ increase in these cells (Fig. 5C). In sum, these results indicate that C4a triggers an increase in $[Ca^{2+}]_i$ in human ECs through the PAR1/$G\alpha_q$/PLCβ signaling axis.

C4a Increases Endothelial Permeability Through the PAR1 Signaling Pathway. Increased endothelial permeability is the hallmark of inflammatory vascular edema, and PAR1 has been identified as a major mediator in this context (30). GPCR stimulation by inflammatory mediators affects endothelial permeability via an increase of $[Ca^{2+}]_i$, which activates signaling pathways that mediate cytoskeletal reorganization through myosin light chain-dependent contraction and disassembly of VE-cadherin at adherent junctions (31). Given our observation that C4a-mediated activation of PAR1 caused an elevation in $[Ca^{2+}]_i$, we hypothesized that C4a might increase endothelial permeability by activating PAR1. Using EA.hy926 and HMEC-1 cell lines, which are frequently used for assessing endothelial permeability (32, 33), we found that C4a dose-dependently increased permeability. Pretreatment of either cell line with the PAR1 antagonist RWJ56110 significantly inhibited this effect (Fig. 5D and Fig. S7A), indicating that PAR1 activation is indeed involved in C4a-induced endothelial permeability. Since permeability changes are linked to alterations in the endothelial actin cytoskeleton (34), we examined actin organization in EA.hy926 and HMEC-1 cells after C4a treatment (300 nM). Under basal conditions, actin was arranged in a fine reticular pattern, with some localized at the cell periphery. C4a treatment caused a significantly higher formation of actin stress fibers in human ECs, which could be significantly inhibited by pretreatment with the PAR1 antagonist RWJ56110 (10 μM, 30 min) (Fig. 5E and Fig. S7B). Taken together, our data indicate that C4a increases endothelial permeability via PAR1-mediated signaling affecting the actin cytoskeleton.

Discussion

In this study, we deorphanize the complement effector protein C4a by providing direct evidence that this C4 activation fragment acts as an untethered ligand for PAR1 and PAR4. Our findings thereby resolve long-standing questions as to whether and how C4a transduces signaling and generates biological functions. The connection we have observed between C4a, PAR1/4 activation, and EC permeability adds important insights to our rapidly emerging knowledge concerning the interplay between the complement, coagulation, and endothelial barrier systems in host defense and inflammation (35).

Although it has long been speculated that C4a possesses GPCR activity, by analogy to the orthologous anaphylatoxins C3a and C5a, such an effector pathway can now be corroborated. Based on the hypothesis that C4a acts on a hitherto-unidentified GPCR, we screened C4a against 168 known and 73 orphan human GPCR in a uniform cell-based reporter assay. Despite earlier yet unconfirmed or disputed claims that C4a acts on C3aR or C5aR2 (5–7, 11, 36), our assays did not reveal any significant agonistic or antagonistic activity for these GPCRs, nor for the remaining anaphylatoxin receptor C5aR1, at a concentration of 600 nM. This therefore supports previous conclusions that C4a should not be regarded as an anaphylatoxin in the classical sense (3, 4). Importantly, however, the assay results suggest that C4a may nevertheless display effector activity, since they identified the two related receptors PAR1 and PAR4, but not the other members of the PAR family (i.e., PAR2 and PAR3), as putative targets for C4a (Fig. 1). Detailed PAR1 or PAR4 activation assays confirmed that C4a dose-dependently activates PAR1 or PAR4 (Fig. 2 A and C). Neither C3a nor C5a was able to induce any agonistic activity in these receptors in the concentration range tested, suggesting that C4a follows a distinct effector pathway among the orthologous activation fragments.

While showing an increase in efficacy of >6-fold in the PAR1 activation assay compared with the known PAR1 agonist TFLLR-NH₂ (EC₅₀ ~ 0.8 and 5 μM, respectively), the saturation of the C4a signal at ~50% of the control suggests that C4a potentially acts as a partial agonist of PAR1 (Fig. 2A). Moreover, the PAR1 antagonist RWJ56110 inhibited C4a-induced PAR1 activation in a noncompetitive manner, indicating that RWJ56110 influences, but does not block, the binding region for C4a on PAR1 (Fig. 2B). In contrast, results of our comparison of C4a with the established agonist AY-NH₂ in the PAR4 activation assay pointed to full agonistic activity of C4a, with a 10-fold stronger
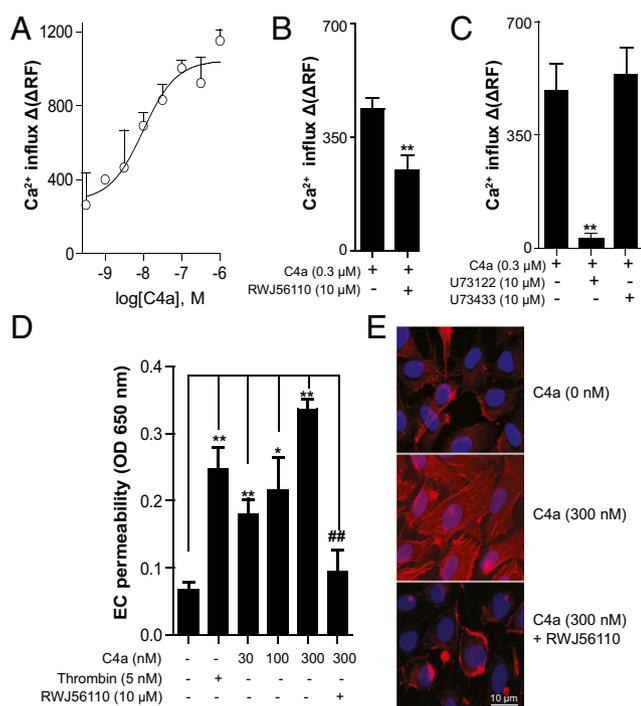

Fig. 5. C4a increases $[Ca^{2+}]_i$ and endothelial permeability via PAR1 activation. (A) C4a dose-dependently increases $[Ca^{2+}]_i$ in HMEC-1 cells. The data are expressed as relative fluorescence [Δ(ΔRFU)] and represent the mean ± SE of four to seven independent experiments. (B) PAR1 antagonist RWJ56110 (10 μM) significantly inhibits C4a-mediated elevation of $[Ca^{2+}]_i$. The data are expressed as relative fluorescence [Δ(ΔRFU)] and represent the mean ± SE of seven independent experiments. (n = 7; **P < 0.01 vs. control; pairwise two-sided Student's t test.) (C) Phospholipase C inhibitor U73122 inhibits C4a-induced elevation of $[Ca^{2+}]_i$. The data are expressed as relative fluorescence [Δ(ΔRFU)] and represent the mean ± SE of eight independent experiments. (n = 8; **P < 0.01 vs. control; pairwise two-sided Student's t test). (D) In EA.hy926 cells, C4a dose-dependently increases endothelial permeability, and the PAR1 antagonist RWJ56110 significantly inhibits C4a-induced endothelial permeability. The data are expressed as OD at 650 nm and represent the mean ± SE of four independent experiments. [n = 4; *P < 0.05 vs. control; **P < 0.01 vs. control; ##P < 0.01 vs. C4a (300 nM); pairwise two-sided Student's t test.] (E) C4a-induced stress fiber formation is significantly decreased by pretreatment with PAR1 antagonist RWJ56110. The experiment was performed three times with similar results, and one representative experiment is shown.

IMMUNOLOGY AND INFLAMMATION

efficacy than that of AY-NH$_2$ (EC$_{50}$ ~ 0.6 and 6.5 μM, respectively). Interestingly, C4a-triggered PAR4 activation could not be inhibited by the PAR4 antagonist tcY-NH$_2$, indicating that there are distinct binding sites for C4a and AY-NH$_2$ on PAR4 (Fig. 2D). Whereas the key residues defining the C4a-mediated activities remain to be determined, the protein again appears to deviate from classical anaphylatoxin behavior, since the C-terminal region of C4a did not seem to be dominant in defining signaling activity, in contrast to the case for both C3a and C5a (17, 18). A desarginated form of C4a exerted significant activity, whereas no stimulation of PAR1 or PAR4 could be achieved with a synthetic peptide comprising the C terminus of C4a. In summary, our assay data support the finding that C4a acts as a ligand for PAR1 and PAR4, although they indicate distinct signaling patterns for the two receptors and a binding mode deviating from known PAR agonists.

The PAR family of receptors typically follows a unique activation mechanism distinct from that of other GPCRs. Their effectors, such as thrombin and other proteases, act through cleavage of an N-terminal fragment of the respective receptor that allows the shortened terminus to bind the active site as a tethered ligand (37). Although synthetic peptides derived from the truncated N terminus can directly activate PAR1 or PAR4 without cleavage of the receptors (13, 38), proteolytic activation of PARs with in situ generation of a tethered ligand is the best-described physiological route of PAR activation. Since C4a has no known proteolytic activity, C4a-induced activation of both PAR1 and PAR4 appears to be mediated by direct binding to the GPCR, rather than by formation of a tethered ligand. Indeed, our immunofluorescence study showed that C4a colocalizes with PAR1 or PAR4 on engineered CHO-K1 cells and on human ECs (Fig. 3 and Fig. S4 *B and C*), supporting the hypothesis that C4a acts as an untethered ligand to activate PAR1 or PAR4.

Despite its different proposed mode of activation, C4a stimulates ERK1/2 phosphorylation in human ECs, as do protease-activated, tethered ligands. The PAR1 antagonist RWJ56110, but not the general Gα$_i$ inhibitor PTX, was able to inhibit C4a-mediated ERK1/2 activation, suggesting that the activation was strongly mediated by PAR1 through Gα$_i$-independent signaling (Fig. 4B). Consistent with the observation that C4a-induced PAR4 activation could not be inhibited by the PAR4 antagonist tcY-NH$_2$ in the reporter cell assay, tcY-NH$_2$ had no significant effect on C4a-induced ERK phosphorylation (Fig. 4B and Fig. S5B). However, a previously described antibody blocking PAR4 activity was able to impair C4a-mediated ERK activation in ECs, suggesting that both PAR1 and PAR4 are involved in the C4a-mediated stimulation of ECs.

As expected, C4a-induced activation of ECs has direct functional consequences, as illustrated by the results of both calcium mobilization and endothelial permeability assays. We found that C4a treatment caused a significant calcium influx that could be substantially inhibited by pretreatment with a PAR1 antagonist or the PLC inhibitor U73122 (Fig. 5 A–C), confirming that the C4a-induced increase in [Ca^{2+}]$_i$ is mediated through a PAR1/Gα$_q$/PLCβ signaling axis. Several studies have linked Ca^{2+} signaling and increased endothelial permeability, and PARs have previously been found to influence EC permeability (39–41). Along these lines, our findings revealed that C4a dose-dependently increases permeability in ECs and provides mechanistic insights into this effect by showing that C4a induces stress fiber formation. These effects could be significantly inhibited by the PAR1 antagonist RWJ56110. Although we focused on the established PAR1 model due to previous associations, a contribution of PAR4 in these effector functions cannot be excluded and warrants further investigation.

Previous studies may have been influenced by small amounts of C3a or C5a present in the plasma-purified C4a preparations (11). We therefore used several approaches to exclude possible

contaminants from the C4a samples used in this study to avoid interference with PARs or other receptors: Plasma-purified C4a used in this study was obtained from a commercial source and had an attested purity of ≥97% (by SDS/PAGE). The identity of the protein was confirmed by mass spectrometry, and the C4a preparations were tested endotoxin-free. In our screening experiments, we found that C4a did not activate anaphylatoxin receptors (C3aR, C5aR1, or C5aR2), allowing us to exclude potential contamination with C3a and/or C5a (Fig. 2 *A* and *C*). While the presence of thrombin in the C4a preparation could theoretically contribute to PAR activation, the fact that agonistic activity was only observed for PAR1 and PAR4 but not for PAR2 and PAR3 reveals an activity profile distinct from that of thrombin. To further dismiss a poorly detectable yet functionally significant contamination with thrombin, we used a thrombin-specific activity assay based on cleavage of a chromogenic substrate. Whereas purified thrombin activated the substrate at concentrations as low as 0.1 nM, the C4a preparation did not show any notable activity even at a concentration of 3,000 nM (Fig. S8). This assay essentially excludes thrombin contamination in the C4a preparation and confirms that C4a by itself does not exert thrombin-like enzymatic activity, but rather acts as a direct nontethered ligand.

Our study provides further evidence of extensive cross-talk between the complement, coagulation, and endothelial barrier systems. These systems are considered a first line of defense against infection and injury (42), and interplay between them is increasingly considered important (35, 43). For example, C5a has been reported to induce tissue factor expression on human ECs (44), and cross-reactivity between serine proteases from the complement and coagulation systems, including thrombin, has been described (45). Among these, the reported ability of mannose binding lectin-associated serine protease 1 (MASP-1) to proteolytically activate PAR4 and modulate EC functions is most relevant here (24). However, in contrast to C4a, MASP-1 appears to act as a "classical" PAR activator with a preference for PAR4 (24). Like thrombin, MASP-1 has a relatively broad substrate specificity and can cleave several proteins of the complement and coagulation systems. Among its functions in the lectin pathway of complement is the activation of MASP-2, which can subsequently cleave C4 to release C4a (46). It will therefore be interesting to see whether the MASP-1 and C4a-related mechanisms are independent or act in a coordinated manner.

Profound cross-talk among complement, coagulation, and ECs is also involved in a variety of pathophysiological processes contributing to disease (35). Transplantation and atypical hemolytic uremic syndrome are among the most prominent examples (1), but thrombosis occurs in other complement-related diseases such as systemic lupus erythematosus or rheumatoid arthritis (47). In many of these conditions, complement activation occurs close to the EC surface and can generate high local concentrations of anaphylatoxins. Given the high plasma concentration of C4 (~3 μM), the 0.6 μM C4a used for the initial GPCR screening corresponds to an activation of 20% or less of the C4 pool, and significant cell activation was observed at much lower concentrations in the EC assays. It is therefore reasonable to assume that PAR1 and/or PAR4 stimulation can occur at physiologically or pathologically relevant concentrations of C4a, especially in disorders driven by the classical and/or lectin pathways. It will be interesting to explore whether there is additional cross-talk between C4a and the anaphylatoxins C3a and C5a, which are typically liberated alongside C4a during complement activation and exert functional effects in the low nanomolar range. Even between the different assays used in this study, the C4a effector concentrations varied notably, indicating that factors such as receptor density/distribution play a critical role and warrant further investigation. In this context, it should be noted that PARs are not only expressed on ECs but also on platelets and leukocytes, among other cells (20, 48).

Our findings provide important evidence that C4a should indeed be considered a complement-derived effector protein that acts as an agonist for PAR1 and PAR4, thereby revealing another important link between the complement, coagulation, and endothelial barrier systems. When compared to previously reported PAR effectors, C4a appears to act in unique manner as it can activate PARs in a nonproteolytic, untethered mode. Although we have primarily focused on EC activation in this study, C4a-mediated PAR1/4 activation can also affect other cell types, with implications for immune regulation and thromboinflammatory disorders. Under what conditions this intriguing cross-talk mechanism plays a role and whether targeting C4a pharmacologically can confer an advantage in certain diseases both remain to be explored.

Materials and Methods

GPCR activity was assessed using a PathHunter β-arrestin reporter assay. Colocalization experiments were performed using recombinant 6His-C4a and anti-PAR1/4 antibodies in CHO-K1 cells using confocal microscopy. ERK activation and calcium mobilization in endothelial cells were measured by Western blots and fluorometry, respectively. Endothelial permeability was assessed using an established endothelial cell leakage assay. Additional information on materials and methods can be found in *SI Materials and Methods.*

1. Ricklin D, Reis ES, Lambris JD (2016) Complement in disease: A defence system turning offensive. *Nat Rev Nephrol* 12:383–401.
2. Ricklin D, Hajishengallis G, Yang K, Lambris JD (2010) Complement: A key system for immune surveillance and homeostasis. *Nat Immunol* 11:785–797.
3. Klos A, Wende E, Wareham KJ, Monk PN (2013) International union of basic and clinical pharmacology. [corrected]. LXXXVII. Complement peptide C5a, C4a, and C3a receptors. *Pharmacol Rev* 65:500–543.
4. Barnum SR (2015) C4a: An anaphylatoxin in name only. *J Innate Immun* 7:333–339.
5. Gorski JP, Hugli TE, Müller-Eberhard HJ (1979) C4a: The third anaphylatoxin of the human complement system. *Proc Natl Acad Sci USA* 76:5299–5302.
6. Murakami Y, Yamamoto T, Imamichi T, Nagasawa S (1993) Cellular responses of guinea-pig macrophages to C4a; inhibition of C3a-induced O2- generation by C4a. *Immunol Lett* 36:301–304.
7. Lienenklaus S, et al. (1998) Human anaphylatoxin C4a is a potent agonist of the guinea pig but not the human C3a receptor. *J Immunol* 161:2089–2093.
8. Jiang X, Ma Y, Yu J, Li H, Xie F (2016) Protective effect of C4a against hyperoxic lung injury via a macrophage-dependent but not a neutrophil/lymphocyte-dependent signaling pathway. *Mol Med Rep* 13:1250–1256.
9. Xie P, et al. (2012) Inhibitory effects of C4a on chemoattractant and secretagogue functions of the other anaphylatoxins via Gi protein-adenylyl cyclase inhibition pathway in mast cells. *Int Immunopharmacol* 12:158–168.
10. Zhao Y, Xu H, Yu W, Xie BD (2014) Complement anaphylatoxin C4a inhibits C5a-induced neointima formation following arterial injury. *Mol Med Rep* 10:45–52.
11. Ames RS, Tornetta MA, Foley JJ, Hugli TE, Sarau HM (1997) Evidence that the receptor for C4a is distinct from the C3a receptor. *Immunopharmacology* 38:87–92.
12. Gerard NP, Gerard C (1991) The chemotactic receptor for human C5a anaphylatoxin. *Nature* 349:614–617.
13. Vu TK, Hung DT, Wheaton VI, Coughlin SR (1991) Molecular cloning of a functional thrombin receptor reveals a novel proteolytic mechanism of receptor activation. *Cell* 64:1057–1068.
14. Yin H, et al. (2009) Lipid G protein-coupled receptor ligand identification using beta-arrestin PathHunter assay. *J Biol Chem* 284:12328–12338.
15. Hugli TE, Erickson BW (1977) Synthetic peptides with the biological activities and specificity of human C3a anaphylatoxin. *Proc Natl Acad Sci USA* 74:1826–1830.
16. Hugli TE (1986) Biochemistry and biology of anaphylatoxins. *Complement* 3:111–127.
17. Gerard C, Hugli TE (1981) Identification of classical anaphylatoxin as the des-Arg form of the C5a molecule: Evidence of a modulator role for the oligosaccharide unit in human des-Arg74-C5a. *Proc Natl Acad Sci USA* 78:1833–1837.
18. Wilken HC, Götze O, Werfel T, Zwirner J (1999) C3a(desArg) does not bind to and signal through the human C3a receptor. *Immunol Lett* 67:141–145.
19. Eishingdrelo H, Kongsamut S (2013) Minireview: Targeting GPCR activated ERK pathways for drug discovery. *Curr Chem Genomics Transl Med* 7:9–15.
20. Coughlin SR (2000) Thrombin signalling and protease-activated receptors. *Nature* 407:258–264.
21. Mangmool S, Kurose H (2011) G(i/o) protein-dependent and -independent actions of pertussis toxin (PTX). *Toxins (Basel)* 3:884–899.
22. Fujiwara M, Jin E, Ghazizadeh M, Kawanami O (2004) Differential expression of protease-activated receptors 1, 2, and 4 on human endothelial cells from different vascular sites. *Pathobiology* 71:52–58.
23. Kataoka H, et al. (2003) Protease-activated receptors 1 and 4 mediate thrombin signaling in endothelial cells. *Blood* 102:3224–3231.
24. Megyeri M, et al. (2009) Complement protease MASP-1 activates human endothelial cells: PAR4 activation is a link between complement and endothelial function. *J Immunol* 183:3409–3416.
25. O'Brien PJ, et al. (2000) Thrombin responses in human endothelial cells. Contributions from receptors other than PAR1 include the transactivation of PAR2 by thrombin-cleaved PAR1. *J Biol Chem* 275:13502–13509.
26. Madhusudhan T, et al. (2012) Cytoprotective signaling by activated protein C requires protease-activated receptor-3 in podocytes. *Blood* 119:874–883.
27. Heemskerk JW, Feijge MA, Henneman L, Rosing J, Hemker HC (1997) The Ca2+-mobilizing potency of alpha-thrombin and thrombin-receptor-activating peptide on human platelets: Concentration and time effects of thrombin-induced Ca2+ signaling. *Eur J Biochem* 249:547–555.
28. Offermanns S, Toombs CF, Hu YH, Simon MI (1997) Defective platelet activation in G alpha(q)-deficient mice. *Nature* 389:183–186.
29. Yang X, Sun L, Ghosh S, Rao AK (1996) Human platelet signaling defect characterized by impaired production of inositol-1,4,5-triphosphate and phosphatidic acid and diminished pleckstrin phosphorylation: Evidence for defective phospholipase C activation. *Blood* 88:1676–1683.
30. McLaughlin JN, et al. (2005) Functional selectivity of G protein signaling by agonist peptides and thrombin for the protease-activated receptor-1. *J Biol Chem* 280:25048–25059.
31. Parnell E, et al. (2012) Regulation of the inflammatory response of vascular endothelial cells by EPAC1. *Br J Pharmacol* 166:434–446.
32. Bae JS, Yang L, Manithody C, Rezaie AR (2007) The ligand occupancy of endothelial protein C receptor switches the protease-activated receptor 1-dependent signaling specificity of thrombin from a permeability-enhancing to a barrier-protective response in endothelial cells. *Blood* 110:3909–3916.
33. Soh UJ, Trejo J (2011) Activated protein C promotes protease-activated receptor-1 cytoprotective signaling through β-arrestin and dishevelled-2 scaffolds. *Proc Natl Acad Sci USA* 108:E1372–E1380.
34. Prasain N, Stevens T (2009) The actin cytoskeleton in endothelial cell phenotypes. *Microvasc Res* 77:53–63.
35. Roumenina LT, Rayes J, Frimat M, Fremeaux-Bacchi V (2016) Endothelial cells: Source, barrier, and target of defensive mediators. *Immunol Rev* 274:307–329.
36. Hugli TE, Kawahara MS, Unson CG, Molinar-Rode R, Erickson BW (1983) The active site of human C4a anaphylatoxin. *Mol Immunol* 20:637–645.
37. Vu TK, Wheaton VI, Hung DT, Charo I, Coughlin SR (1991) Domains specifying thrombin-receptor interaction. *Nature* 353:674–677.
38. Scarborough RM, et al. (1992) Tethered ligand agonist peptides. Structural requirements for thrombin receptor activation reveal mechanism of proteolytic unmasking of agonist function. *J Biol Chem* 267:13146–13149.
39. Moore TM, et al. (1998) Store-operated calcium entry promotes shape change in pulmonary endothelial cells expressing Trp1. *Am J Physiol* 275:L574–L582.
40. Norwood N, et al. (2000) Store-operated calcium entry and increased endothelial cell permeability. *Am J Physiol Lung Cell Mol Physiol* 279:L815–L824.
41. Tiruppathi C, et al. (2000) G protein-coupled receptor kinase-5 regulates thrombin-activated signaling in endothelial cells. *Proc Natl Acad Sci USA* 97:7440–7445.
42. Amara U, et al. (2008) Interaction between the coagulation and complement system. *Adv Exp Med Biol* 632:71–79.
43. Markiewski MM, Nilsson B, Ekdahl KN, Mollnes TE, Lambris JD (2007) Complement and coagulation: Strangers or partners in crime? *Trends Immunol* 28:184–192.
44. Ikeda K, et al. (1997) C5a induces tissue factor activity on endothelial cells. *Thromb Haemost* 77:394–398.
45. Huber-Lang M, et al. (2006) Generation of C5a in the absence of C3: A new complement activation pathway. *Nat Med* 12:682–687.
46. Dobó J, et al. (2014) Multiple roles of complement MASP-1 at the interface of innate immune response and coagulation. *Mol Immunol* 61:69–78.
47. Macedo AC, Isaac L (2016) Systemic lupus erythematosus and deficiencies of early components of the complement classical pathway. *Front Immunol* 7:55.
48. Ossovskaya VS, Bunnett NW (2004) Protease-activated receptors: Contribution to physiology and disease. *Physiol Rev* 84:579–621.

IMMUNOLOGY AND INFLAMMATION