Attenuation of Very Late Antigen-5-Mediated Adhesion of Bone Marrow-Derived Mast Cells to Fibronectin by Peptides with Inverted Hydropathy to EF-Hands

René Houtman, Robert Ten Broeke, J. Edwin Blalock, Matteo Villain, Andries S. Koster and Frans P. Nijkamp

*J Immunol* 2001; 166:861-867; doi: 10.4049/jimmunol.166.2.861
http://www.jimmunol.org/content/166/2/861

---

**References**
This article cites 38 articles, 14 of which you can access for free at: http://www.jimmunol.org/content/166/2/861.full#ref-list-1

**Subscription**
Information about subscribing to *The Journal of Immunology* is online at: http://jimmunol.org/subscription

**Permissions**
Submit copyright permission requests at: http://www.aai.org/About/Publications/JI/copyright.html

**Email Alerts**
Receive free email-alerts when new articles cite this article. Sign up at: http://jimmunol.org/alerts
Attenuation of Very Late Antigen-5-Mediated Adhesion of Bone Marrow-Derived Mast Cells to Fibronectin by Peptides with Inverted Hydropathy to EF-Hands

René Houtman,* Robert Ten Broeke,* J. Edwin Blalock,† Matteo Villain,† Andries S. Koster,2* and Frans P. Nijkamp*

Release of allergic mediators from mast cells is enhanced by very late Ag (VLA)-5-mediated interaction of these cells with fibronectin. In this report, we show that VLA-5-mediated adhesion of bone marrow-derived mast cells to fibronectin can be induced by two different pathways: first, FcεRI clustering, which depends on calmodulin activation and extracellular Ca2+, and, second, by Mn2+ stimulation, which is independent of calmodulin activation and antagonized by Ca2+. Previous studies have shown the presence of several cation-binding domains in VLA-5 that are homologous to the calcium-binding EF-hands of calmodulin. To show a role for EF-hands of different proteins in VLA-5-mediated adhesion, we used calcium-like peptides (CALP), CALP1 and CALP2, designed to bind to EF-hands based on inverted hydrophathy. CALP1 and, more potently, CALP2 inhibited FcεRI-induced adhesion to fibronectin via different mechanisms. The target for the effects of CALP1 and 2 on FcεRI-induced adhesion and degranulation was intracellular and likely involved calmodulin. Interestingly only CALP2 was able to inhibit Mn2+-induced calmodulin-independent adhesion by interfering with an extracellular target, which is probably VLA-5. We conclude that CALP1 and 2 can inhibit VLA-5-mediated adhesion of mast cells to fibronectin through binding to EF-hands of multiple proteins, and that these peptides can be used as lead compounds for the development of future therapy against allergy. The Journal of Immunology, 2001, 166: 861–867.

1 Abbreviations used in this paper: VLA-5, very late Ag-5; BMMC, bone marrow-derived mast cell; CALP, calcium-like peptide; CaM, calmodulin; SA, streptavidin; EDTA, calcium release-induced calcium current; HSA, human serum albumin.

Copyright © 2001 by The American Association of Immunologists

0022-1767/01/$02.00
influx through binding to the EF-hands of Ca\(^{2+}\) channels or indirectly via CALP-CaM interaction with such channels (23). The second complementary peptide, CALP2, was designed by computer-assisted optimal inversion of the hydropathy pattern of the EF-hand four-amino acid sequence of CaM (24–26). Due to increased length and optimal hydrophathy inversion, the affinity of CALP2 for the EF-hand compared with CALP1 was increased 11-fold as determined by surface plasmon resonance detection. Interestingly, enhanced affinity by increased reciprocity of the pattern of hydrophathy and increased length of the peptide resulted in a change of functional activity. Whereas CALP1 shows biological effects similar to Ca\(^{2+}\), CALP2 acts as an antagonist for CaM (26).

A possible role for Ca\(^{2+}\) influx and CaM activation in FceRI-induced affinity modulation of VLA-5 for fibronectin combined with the assumption that, based on the pattern of hydropathy, the EF-hand-like domains of VLA-5 are potential binding sites for CALPs suggest several targets for these peptides to interfere with mast cell adhesion. In this report, we investigate the ability of CALP1 and CALP2 to inhibit VLA-5-mediated adhesion of BMMC to fibronectin induced either by cationic stimulation or FceRI clustering.

Materials and Methods

Cells

BMMC were obtained as described previously (27). Briefly, bone marrow from femurs of BALB/c mice was flushed and cells were cultured at a density of 2 \times 10^5/ml in complete RPMI (RPMI 1640 medium supplemented with 4 m\(\text{g}\)/l-glutamine, 5 \times 10^{-5} M 2-ME, 1 m\(\text{M}\) sodium pyruvate, 100 U/ml penicillin, 100 mg/ml streptomycin, and 0.1 m\(\text{M}\) nonessential amino acids) supplemented with 20\% (v/v) supernatant from BALB/c splenocytes which were stimulated for 7 days with pokeweed mitogen (Sigma-Aldrich, Axel, The Netherlands) in complete RPMI. Medium was replenished once per week. For all experiments, 4- to 6-wk-old BMMC were used.

RBL-2H3 cells were cultured in 75- or 162-cm\(^2\) flasks at 37°C under humidified air, containing 5% \(\text{CO}_2\) in K-medium (RPMI 1640 supplemented with 10\% (v/v) heat-inactivated FCS, 1 mg/ml gentamicin, 100 U/ml penicillin/streptomycin, 40 m\(\text{g}\)/l-glutamine, and 0.2 M HEPES). Cells were recultured two to three times per week by treatment with 0.05\% trypsin/0.53 mM EDTA (Life Technologies, Breda, The Netherlands) and transferred to new flasks.

Design and synthesis of the hydrophilically complementary peptides

The design of the eight residue complementary peptide CALP1 (VAITY LVK) was based on a primordial CaM EF-hand motif. Selection of the complementary peptide CALP2 (VKFGVGFKVMVF) was conducted using the computer program AMINOMAT (Tecnogen ScpA, Piana di Monte Verna, Italy), with an averaging window \(\alpha\) of each of the complementary peptides generated by the program, \(b\) represents the moving averaged hydropathy assigned to every amino acid region. The program generated 1,417,176 possible sequences and chose for the one with the lowest \(Q\) value (0.0068). This value is defined by the formula: \(Q = \sum (a_i + b_i)/\alpha_n - 2\sigma x_{11}\), where \(a_i\) represents the moving averaged hydropathy assigned to every amino acid of the target peptide, \(b_i\) represents the moving averaged hydropathy assigned to every amino acid of each of the complementary peptides generated by the program, \(s\) is \((r - 1)/2\) (where \(r\) is the number of amino acids considered in the moving window), and \(n\) is the number of residues in the target peptide.

The peptides were synthesized using continuous flow solid-phase peptide synthesis with F-moc chemistry on a PerSeptive Biosystems 9050 Peptide synthesizer (PerSeptive Diagnostics, Cambridge, MA). Precipitated Fmoc amino acids with 1-hydroxy-7-azabenzotriazole and preloaded polyethylene glycol-phosphatidylserine resin were used. The peptides were purified by reversed-phase HPLC on a Delta Pack C18 300 A (300 \times 39 mm inside diameter; Waters, Milford, MA). The purity of the product was checked by reversed-phase HPLC on a Dynamax C18 (300 \times 4.8 mm inside diameter; Vydac, Hesperia, CA) column equilibrated at a flow rate of 1 ml/min and eluted with a linear gradient from 5 to 80% CH\(_3\)CN containing 0.1% trifluoroacetic acid in 40 min. MilliQ water (Millipore, Austin, TX) previously treated with Chelex 100 to remove any Ca\(^{2+}\) was used in the purification. The identity of the peptides was confirmed by time-of-flight-mass-assisted laser desorption ionization mass spectrometry (University of Birmingham Core Facility).

Immobilization of fibronectin

Nunc Maxisorp 96-well plates (Nunc, Roskilde, Denmark) were coated for 3 h at 37°C with 200 \(\mu\text{g}\)/well and 2.5 mg/ml human fibronectin (CLB, Amsterdam, The Netherlands) in PBS, followed by three washes with 200 \(\mu\text{g}\)/well PBS.

Adhesion assay

For Mn\(^{2+}\)-induced adhesion, BMMC were washed with PBS, 1 mM EDTA in PBS, and TBS (2.9 g/l Tris-Cl, 4 g/l NaCl, 0.2 g/l KCl, 0.4 g/l glucose, and 0.1% BSA) and resuspended at a density of 5 \times 10^5 cells/ml TBS.

For FceRI-induced adhesion, BMMC were sensitized by 1-h incubation in complete RPMI at 37°C with supernatant of anti-DNP IgE-producing hybridoma 26.82 (28). Next, cells were washed as described above, and cells were resuspended in TBS with or without 1.8 mM CaCl\(_2\) as indicated, at a density of 5 \times 10^5 cells/ml.

Unless otherwise indicated, adhesion was induced by stimulation of 5 \times 10^5 cells/well with 0.2 m\(\text{M}\) Mn\(^{2+}\) (cationic stimulation) or 3 mg/ml DNP-HSA (for FceRI clustering) in PBS (23). Effects of Arg-Gly-Asp-Ser (RGDS) peptide (Sigma-Aldrich), W7 (Sanventech, Heerhugowaard, The Netherlands), CALP1, CALP2, and CALP-2-biotin were determined by incubation of stimulated cells with these compounds on immobilized fibronectin. CALP2-biotin (2 \times 10^{-4} M) was preincubated with or without streptavidin (SA, 1 \times 10^{-3} M; Boehringer Mannheim, Mannheim, Germany) in TBS for 1 h at room temperature under constant rotation before addition to the cells. For functional blocking of VLA-5, cells were preincubated for 1 h at room temperature with blocking Ab BM508 (29), a generous gift from B. M. C. Chan (University of Western Ontario, London, Ontario, Canada), before stimulation.

After a 60-min incubation at 37°C on immobilized fibronectin, nonadherent cells were removed by washing three times with 200 \(\mu\text{g}\)/well PBS. Adhesion was quantified using a Cyquant proliferation assay kit (Molecular Probes, Leiden, The Netherlands) according to the manufacturer’s protocol. In short, plates were frozen overnight at \(-20°C\), thawed, and incubated for 60 min under continuous agitation at room temperature with a DNA-specific fluorescent dye and lysing reagent. Fluorescence was measured at \(\lambda_{\text{ex}}\) :485 nm/\(\lambda_{\text{em}}\) :530 nm using a Millipore Cytofluor 2350 microplate reader, and adhesion was calculated as the residual fluorescence as a percentage of input fluorescence. Values were corrected for adhesion observed for cells incubated on fibronectin and stimulated with buffer only (always <1%). In all graphs, mean values \pm SEM (n = 4) of a representative of at least three experiments are shown.

Degranulation assay

Four- to 6-wk-old BMMC were sensitized with anti-DNP IgE as described above, washed, and resuspended at 5 \times 10^5 cells/ml in Tyrode buffer (Life Technologies, Breda, The Netherlands) supplemented with 10 mM HEPES, 0.1% BSA, and 0.1% FCS (PH 7.2, 5 \times 10^5 cells/well of a 96-well plate were stimulated with CALP1, CALP2, 0.25 \(\mu\text{M}\) ionomycin, or 30 ng/ml DNP-HSA (unless otherwise indicated) alone or in combination with various concentrations of CALP or W7 for 30 min at 37°C. Supernatants were analyzed for \(\beta\)-hexosaminidase content as a parameter for mast cell degranulation as described previously (27). In short, 50 \(\mu\text{M}\) of supernatant was incubated for 60 min at 37°C with 50 \(\mu\text{M}\) of 4-methylumbelliferyl-N-acetyl-\(\beta\)-glucosaminide (Sigma-Aldrich) in citrate buffer (0.1 M, pH 4.5), the reaction was stopped by addition of 100 \(\mu\text{L}\) of glucose buffer (0.2 M glucose, 0.2 M NaCl, pH 10.7), and fluorescence was measured at \(\lambda_{\text{ex}}\) :360 nm/\(\lambda_{\text{em}}\) :460 nm using a Millipore Cytofluor 2350 microplate reader. Degranulation was calculated as the amount of \(\beta\)-hexosaminidase activity present in the supernatant as a percentage of the total \(\beta\)-hexosaminidase activity present in the cells, determined in lysates of 5 \times 10^5 cells. Degranulation values were corrected for percent \(\beta\)-hexosaminidase activity present in the supernatant of cells incubated with buffer only (always <3%). In all graphs, mean values \pm SEM (n = 4) of a representative of at least three experiments are shown.

Patch clamp analysis

Conventional whole-cell recording was performed by a List EPC-7 patch clamp amplifier (List Medical Electronics, Darmstadt, Germany). Pipettes were pulled from KG12 (Wilmad Glass, Buena, NJ) and were fire polished to produce a tip resistance of 3–5 M. The standard pipette solution contained 110 m\(\text{M}\) cesium glutamate, 10 m\(\text{M}\) CsCl, 2.9 m\(\text{M}\) MgCl\(_2\), 0.6 m\(\text{M}\) CaCl\(_2\), 10 m\(\text{M}\) EGTA-cesium, and 30 m\(\text{M}\) HEPES

Downloaded from http://www.jimmunol.org/ by guest on April 20, 2017
(pH 7.2) adjusted with CsOH, calculated free intracellular Ca2+ concentration = 10 nM. Bath solution contained 160 mM sodium glutamate, 10 mM calcium gluconate, 2 mM EGTA-sodium, 5 mM N-glucose, and 10 mM HEPES (pH 7.3) adjusted with NaOH. Solution changes in the chamber were achieved by a gravity-feed system and were completed in 1 s. The capacitative current was compensated, and a tip junction potential of +10 mV was not corrected. For continuous recording of current, RBL-2H3 cells were held at −40 mV, and the signal was digitized at 20 kHz and recorded on video cassette recorder tape. For display, the signal was filtered at 1000 Hz by an eight-pole low-pass Bessel filter (model 900; Frequency Devices, Haverhill, MA). A 320-ms voltage ramp from −90 to +90 mV was applied to the cell to obtain current-voltage relationships. The currents were sampled at 5 kHz and filtered at 1 kHz by the low-pass Bessel filter. The data were analyzed by PCLAMP software (Axon Instruments, Foster City, CA).

Statistical analysis

All data are expressed as mean values ± SEM of four incubations in at least three experiments. In some cases, error bars are covered by the symbols used. Statistically significant differences were determined using ANOVA followed by a Bonferroni post hoc or Student’s t test considering p < 0.05 to be significant. All analyses were performed using Systat for Windows (version 6.1; SPSS, Chicago, IL).

Results

Cationic modulation of BMMC adhesion to fibronectin

To investigate whether the previously reported cation-mediated modulation of VLA-5 affinity for fibronectin (10) can be extrapolated to BMMC, extracellular Ca2+ was removed by EDTA treatment and cells were incubated with various concentrations of MnCl2 on immobilized fibronectin. As is shown in Fig. 1A, MnCl2 dose-dependently induces adhesion of BMMC to fibronectin. Maximal adhesion of ~75% of cells is reached at 0.2 mM MnCl2, and this concentration was used in all subsequent experiments. To investigate whether Ca2+ can antagonize Mn2+-induced adhesion, cells were treated with EDTA and incubated on immobilized fibronectin with MnCl2 and various concentrations of CaCl2. Fig. 1B shows that CaCl2 dose-dependently reduces MnCl2-induced adhesion. Previous data showing that adhesion of BMMC to fibronectin is VLA-5-mediated were confirmed by virtually complete inhibition of Mn2+-induced adhesion by soluble fibronectin ligand, RGDS peptide, and blocking Ab BMA5 as is shown in Fig. 1C. From these data we conclude that in BMMC, the regulation of adhesion to fibronectin by cations parallels earlier published data (10) reporting that Mn2+ induces a conformational change of VLA-5 to high affinity for fibronectin, via a mechanism that is antagonized by Ca2+.

A role for extracellular Ca2+ in FcεRI-induced adhesion of BMMC to fibronectin

To investigate whether the presence of extracellular Ca2+ is necessary for the induction of adhesion by FcεRI clustering, anti-DNP IgE sensitized BMMC were treated with EDTA and stimulated with human serum albumin (HSA)-conjugated DNP (DNA-HSA) on immobilized fibronectin in CaCl2-supplemented buffer (final concentration, 1.8 mM) or calcium-free buffer. As is shown in Fig. 2, stimulation of BMMC via FcεRI dose-dependently induces adhesion to fibronectin in buffer supplemented with Ca2+; however, the absence of Ca2+ completely abrogates FcεRI-induced adhesion. Significant adhesion in calcium-supplemented buffer in the absence of allergen was never observed. From these results, we conclude that in addition to FcεRI-induced degranulation, the presence of extracellular Ca2+ is critical for the FcεRI-induced adhesion of BMMC to fibronectin.

A role for CaM in FcεRI- or Mn2+-induced adhesion

To study whether CaM plays a role in antigenic or cationic modulation of VLA-5 affinity for fibronectin, we investigated the effect of the CaM inhibitor, W7, on the adhesion induced by either FcεRI clustering or Mn2+ stimulation. Anti-DNP IgE-sensitized cells were stimulated with DNP-HSA and EDTA-treated cells were stimulated with Mn2+ on immobilized fibronectin with various concentrations of W7. As is shown in Fig. 3A, W7 inhibits Ag-induced adhesion; however, it has no effect on adhesion of cells stimulated with MnCl2. To exclude nonspecific effects of W7 on FcεRI-signaling, we tested the effect of W7 on degranulation of BMMC after stimulation with ionomycin, which induces calcium influx and bypasses the FcεRI-signaling events leading to CaM activation (30). Fig. 3B shows a dose-dependent inhibition of ionomycin-induced β-hexosaminidase release by W7, thereby excluding possible effects of this inhibitor on adhesion upstream of CaM activation. From these data, we conclude that VLA-5-mediated adhesion of BMMC to fibronectin due to FcεRI clustering or Mn2+ stimulation is mediated via two distinct pathways that are CaM dependent and independent, respectively.

The effect of CALP1 and CALP2 on FcεRI and Mn2+-induced adhesion of BMMC to fibronectin

Our data indicate that FcεRI-induced adhesion is dependent on the activation of CaM and therefore an effect of both CALP1 and 2 on...
FceRI-induced adhesion might be expected. Anti-DNP IgE-sensitized BMMC were stimulated with DNP-HSA in the presence of various concentrations of either CALP1 or CALP2 on immobilized fibronectin. Indeed, as is shown in Fig. 4A, we find that both CALP1 and CALP2 are able to dose-dependently inhibit adhesion induced by FceRI clustering. We observed that CALP2 is a more potent inhibitor than CALP1 (EC_{50} \approx 3.5 \times 10^{-5} \text{ vs } 1 \times 10^{-4} \text{ M, respectively}) and that both peptides alone failed to induce adhesion of our cells to fibronectin (data not shown).

The observation that Mn^{2+}-induced adhesion of BMMC to fibronectin is mediated via a CaM-independent pathway provides us with the opportunity to investigate whether CALP1 and CALP2 are able to interfere with adhesion through binding to a target other than the CaM EF-hand. EDTA-treated BMMC were stimulated with Mn^{2+} and various concentrations of either CALP1 or CALP2 on immobilized fibronectin. As is shown in Fig. 4B, CALP1 does not have an effect on Mn^{2+}-induced adhesion, but CALP2 dose-dependently inhibits Mn^{2+}-induced adhesion of our cells to fibronectin. This suggests a target for CALP2 other than CaM because this pathway is CaM independent.

**Effect of CALP1 and CALP2 on mast cell degranulation**

Our data show that both CALP1 and 2 inhibit FceRI-induced adhesion of BMMC to fibronectin probably by interference of these peptides with CaM activation in one way or another. To more accurately define a possible mechanism of action, we tested the effects of these peptides on FceRI degranulation, a mast cell parameter that has been shown to be dependent on Ca^{2+} influx and CaM activation. Anti-DNP IgE-sensitized BMMC were stimulated with DNP-HSA in the presence of various concentrations of CALP1 or CALP2 and the release of granular \( \beta \)-hexosaminidase, as a measure for degranulation, was determined. As is shown in Fig. 5A, CALP1 and, more potently, CALP2 dose-dependently inhibited Mn^{2+}-induced degranulation (Fig. 5A). This excludes the possibility that CaM inhibitor CALP2 (26) interferes with signals unique to Ag (e.g., recruitment of kinases, activation of phospholipase C, etc.) because ionomycin stimulation bypasses these signals. From these data, we conclude that CALP1 and 2 both attenuate FceRI-induced degranulation by interfering with the same targets as those in FceRI-induced adhesion, thereby disturbing activation of CaM.

**Effect of CALP1 on calcium release-induced calcium current (I_{Ca}

Although CALP1 was previously shown to activate CaM (26), this peptide fails to induce degranulation. This suggests a different mechanism for this peptide to interfere with CaM activation as compared with CALP2. Previous data showed the ability of CALP1 to interfere with the influx of extracellular calcium in T...
cells and neurons by blocking calcium channels (23). To address this issue in mast cells, we performed patch clamp experiments to test the ability of CALP1 to inhibit ICRAC, which plays an important role in mast cell degranulation (31–33). As is shown in Fig. 6, CALP1 is able to inhibit ICRAC in RBL-2H3 cells, a widely used mast cell model. These cells were used because they are prototypical for ICRAC. Compared with the typical total inhibition induced by lanthanum (34), the inhibition of ICRAC by CALP1 is partial (~30%), which could be attributed to the presence of EGTA in the pipette solution. From these data, we conclude that CALP1 reduces influx of extracellular Ca\(^{2+}\) through the inhibition of ICRAC.

**Effect of prevention of CALP2 cell entry on its ability to inhibit adhesion**

Our data show that CALP1 and 2 are able to inhibit Ca\(^{2+}/\)CaM-dependent adhesion, suggesting an intracellular target for these peptides. In contrast, only CALP2 is able to attenuate Ca\(^{2+}\)- and CaM-independent adhesion, and we propose that possible targets for CALP2 are located in the extracellular portion of the VLA-5 dimer.

To test for an extra- vs intracellular non-CaM target, CALP2 was labeled with biotin at the N terminus. This peptide was incubated and complexed with SA to prevent it from entering the cells and thereby specifically target extracellular sites. BMMC were stimulated on immobilized fibronectin either via Fc\(\varepsilon\)RI or with Mn\(^{2+}\) along with free or complexed CALP2-biotin. Prevention of cell entry largely abrogates the inhibiting effect of CALP2 on Fc\(\varepsilon\)RI-induced adhesion (Fig. 7A, IgE) as well as the inhibiting effect on Fc\(\varepsilon\)RI-induced degranulation (Fig. 7B), which was used as a control CaM-mediated mast cell parameter. The inhibiting effect of exclusively extracellular CALP2 on Mn\(^{2+}\)-induced adhesion of BMMC to fibronectin is largely retained (Fig. 7A, Mn\(^{2+}\)). These data show that beside the ability of CALP2 to inhibit adhesion through binding to intracellular targets such as CaM, this peptide can also attenuate adhesion of BMMC to fibronectin by way of binding to extracellular targets.

**Discussion**

The observation that VLA-5 can modulate mast cell degranulation makes this integrin a potential target for future therapeutic strategies against allergy. Our data show that a high-affinity state of VLA-5 and subsequent adhesion of BMMC to fibronectin can be induced via two separate pathways: first, by clustering of Fc\(\varepsilon\)RI that is dependent on activation of CaM and the presence of extracellular Ca\(^{2+}\) and, second, by Mn\(^{2+}\) stimulation independent of CaM and antagonized by Ca\(^{2+}\).

Because Fc\(\varepsilon\)RI-induced adhesion as well as degranulation are both dependent on extracellular Ca\(^{2+}\) and activation of CaM, it is plausible to assume that both cell functions are induced by the same mechanism and therefore affected similarly by CALP1 and 2. Inhibition by CALP2 seemed predictable as this peptide was shown to be an antagonist for CaM activation (26) and should...
therefore have the same effect as the CaM inhibitor W7. Inhibition of adhesion and degranulation by CALP1 cannot be explained by inhibition of CaM, since it was shown that this peptide mimics Ca\(^{2+}\) by activating CaM (22, 26). Importantly, it was shown recently that CALP1 not only mimics Ca\(^{2+}\) in its binding and activating characteristics toward CaM, but is also able to block non-selective cation channels of Jurkat cells (23). This channel was shown to play a role in calcium mobilization in mast cells (35) and could serve as target for CALP1 in our cells. Additionally, we show that CALP1 reduces calcium release-activated calcium current (I\(_{\text{Ca,LO}}\)), which has been shown to play an important role in FcεRI-induced mast cell degranulation (31–33). The closure of cation channels also explains the lack of the intrinsic ability of CALP1 to induce degranulation, which is characteristic of Ca\(^{2+}\) ionophores (36, 37). Comparing CALP1 and CALP2, the latter was the most potent inhibitor of both adhesion and degranulation, which can be explained by the fact that this peptide has the higher affinity for the CaM EF-hand. The EC\(_{50}\) of both peptides for inhibition of adhesion was ~5-fold higher than that for inhibition of degranulation. Furthermore, in our experiments we find that the amount of allergen needed for optimal degranulation is 10-fold higher than that for optimal adhesion (3 vs 30 ng/ml; data not shown). From these results, we conclude that the signaling events located downstream of CaM activation leading to either degranulation or adhesion need different levels of CaM activation for an optimal response. Another explanation might be that the differences in optimal doses are related to the strength of synergistic signals other than those operating through CaM.

Mn\(^{2+}\)-induced adhesion is independent of CaM activation and an inhibiting effect of any CALP on adhesion is therefore mediated via other targets than those described for the FcεRI pathway. We find that CALP2 but not CALP1 is able to inhibit Mn\(^{2+}\)-induced adhesion of BMMC to fibronectin. This could be explained by the higher affinity of CALP2 for EF-hands. Limited solubility of CALP-1 prevents us from testing the inhibiting ability of this peptide at a higher concentration. The EC\(_{50}\) of CALP2-inhibition of Mn\(^{2+}\)-induced adhesion is in the same range as that for inhibition of FcεRI-induced adhesion. It is tempting to speculate that the target for CALP2 in the inhibition of Mn\(^{2+}\)-induced adhesion is one or more of the EF-hand-like domains of VLA-5. Because these motifs show hydropathy profiles identical to the CaM EF-hand, they should therefore bind CALP2 with comparable affinity.

In our experiments using SA-complexed CALP2, a partial inhibition of FcεRI-induced adhesion can be observed and there are several explanations for this phenomenon. First, the presence of unbound CALP-2-biotin could attenuate CaM activation. We show that an effect of CALP2 on IgE-mediated degranulation could already be observed at low concentrations. Second, there could be a direct effect of CALP2 on the EF-hand like domains of VLA-5 in FcεRI-induced adhesion, which we cannot exclude. In the same experiment we also observed a partial attenuation of the inhibiting capacity of complexed CALP2 compared with free CALP2. This could be explained by the fact that the binding of CALP2 to the EF-hand like domain of VLA-5 is partially interfered sterically by the bulky SA molecule to which it is bound.

Although we have no direct data showing interaction between VLA-5 and CALP2, several arguments support our conclusion that this integrin is targeted during inhibition of CaM-independent adhesion. First, based on hydropathy and sequence homology to CaM EF-hands the EF-hand-like domains of VLA-5 represent potential binding sites for CALP2. Second, the affinity of CALP2 and Ca\(^{2+}\) for CaM was shown to be comparable (26, 38). Accordingly, we find that the EC\(_{50}\) values of CALP2 and Ca\(^{2+}\) for inhibition of Mn\(^{2+}\)-induced adhesion are comparable. Third, we have experimentally excluded a role for CaM. Fourth, although EF-hand motifs are present in numerous proteins that play a role in various cellular processes (39), most of these are localized intracellularly. The observation that our target is located extracellularly narrows the list of possible candidates and mainly leaves us with the integrins. We and others have shown that in 4- to 6-wk-old BMMC, adhesion to fibronectin is entirely VLA-5 mediated (14, 29), which strongly suggests this integrin as the most obvious candidate for CALP2-mediated inhibition.

Previous data show the existence of at least three classes of cation-binding domains within the VLA-5 αβ dimer (10, 11). One class selectively binds Mn\(^{2+}\), which induces VLA-5 high affinity. A second class shows competitive binding of Mn\(^{2+}\), inducing VLA-5 high affinity, and Ca\(^{2+}\) acting as an antagonist. A third class selectively binds Ca\(^{2+}\), antagonizing the effect of Mn\(^{2+}\). There are several mechanisms by which CALP2 could inhibit VLA-5 binding to fibronectin. One is by competitive binding to the Mn\(^{2+}\)-specific domain. By binding of CALP2 to this domain, localized in the ligand binding site of the integrin (40), ligand binding would be physically prevented. Furthermore, it has been suggested that the inhibiting effect of Ca\(^{2+}\) can be attributed to a change of conformation upon binding of this cation to the second and/or third class of binding domain in the VLA-5 dimer (10, 11). This conformational change could lead to masking of the Mn\(^{2+}\)- and/or ligand-binding domain of VLA-5. Binding of CALP2 to the EF-hand binding to tropomin C induces a conformational change different from the one induced by Ca\(^{2+}\) (26). Binding of CALP2 to the Ca\(^{2+}\)-specific domains in VLA-5 might lead to a low-affinity conformation of this integrin.

Previous studies have shown that hydrophatic patterning can be used to tailor-make peptide ligands targeted to the EF-hand of CaM and that by increasing affinity the functional activity of the peptide can be modulated from agonistic to antagonistic (22, 26). In this report, we show that these peptides can be applied to block cell adhesion by interfering with CaM and other targets, presumably the EF-hand-like domain of VLA-5. Their ability to block adhesion and thereby modulate the activity of mast cells indicates that these peptides could serve as possible lead compounds for the development of future therapies against allergy and other pathologies in which proteins with EF-hands are involved.

References