Identification of sites in the cysteine-rich domain of the class P-III snake venom metalloproteinases responsible for inhibition of platelet function

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Abstract Atrolysin A and jararhagin are class P-III snake venom metalloproteinases (SVMPs) with three distinct domains: a metalloproteinase, a disintegrin-like and a cysteine-rich. The metalloproteinase and the disintegrin-like domains of atrolysin A and jararhagin contain peptide sequences that interact with α2β1 integrin and inhibit the platelet responses to collagen. Recently, the recombinant cysteine-rich domain of atrolysin A was shown to have similar effects, but the sequence(s) responsible for this is unknown. In this report, we demonstrate two complete peptide sequences from the homologous cysteine-rich domains of atrolysin A and jararhagin that inhibit both platelet aggregation by collagen and adhesion of α2-expressing K562 cells to this protein. In addition, the peptide effects on platelets do not seem to involve an inhibition of GPVI. These results identify, for the first time, sites in the cysteine-rich domain of SVMPs that inhibit cell responses to collagen and reveal the complexity of the potential biological effects of these enzymes with multifunctional domains.

Key words: Snake venom metalloproteinase; Cysteine-rich domain; Platelet aggregation; Protein tyrosine phophorylation; α2β1 integrin

1. Introduction

The engagement of platelet surface glycoproteins (GP) through adhesion to exposed subendothelium and through plasma protein-mediated aggregation is central for platelet hemostatic plug formation. At the site of vascular damage, platelets adhere through specific membrane receptors to collagen fibres and collagen-bound von Willebrand factor and become activated by signals generated by these receptors. Although many candidates have been proposed as platelet collagen receptors, α2β1 integrin and GPVI play a pivotal role [1].

Venoms from viperid snakes contain potent inhibitors of platelet aggregation that specifically target cell receptors for adhesive protein ligands. The best characterised of these inhibitors are the RGD-containing disintegrins. These are non-enzymatic cysteine-rich peptides which have a high affinity for the platelet αIIbβ3 integrin and inhibit platelet aggregation by blocking the binding of fibrinogen to this receptor [2,3]. Another group of platelet inhibitors comprise the class P-III snake venom metalloproteinases (SVMPs). These proteins are soluble high molecular weight enzymes possessing an N-terminal Zn^{++}-dependent catalytic domain, a non-RGD-containing disintegrin-like domain and a C-terminal cysteine-rich region [4,5]. The metalloproteinase domain is responsible for the degradation of matrix proteins resulting in disturbance of endothelial cell integrity and subsequent haemorrhage which is exacerbated by a disregulation of platelet function. Indeed, atrolysin A [6] and catrocollastatin [7] from the venom of *Crotalus atrox* and jararhagin [8] from the venom of *Bothrops jararaca* are P-III venom metalloproteinases that have all been demonstrated to inhibit platelet/collagen interactions [9–11]. Jararhagin recognises the platelet collagen receptor α2β1 integrin [12] and cleaves its β1 subunit, resulting in loss of platelet responses to collagen [10,13]. This effect is restricted to α2β1 integrin, since we have previously shown that jararhagin does not affect the platelet receptor GPVI [14,15].

The specificity of SVMPs for integrins is likely to be determined by the structure of the disintegrin-like and the cysteine-rich domains of P-III metalloproteinases. This is supported by the reports that both native and recombinant proteins containing such domains effectively inhibit collagen-induced platelet aggregation [16–19]. We, and others, have recently shown that synthetic peptides based on the ECD-containing sequences of disintegrin-like domain and also on RKKH-containing sequences of enzymatic domain both effectively inhibit platelet/collagen interactions [11,20,21]. Moreover, Jia et al. [19] have reported that the recombinant cysteine-rich domain of atrolysin A also inhibits collagen-induced platelet aggregation. However, it is not yet known which site(s) of the domain is (are) responsible for this effect.

Here, we investigated the effects on cellular responses to collagen of a series of synthetic peptide based on the cysteine-rich domain of both atrolysin A and jararhagin. These two proteins were chosen because they represent the archetypal P-III metalloproteinases found in most viperid venoms.

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Abbreviations: SVMP, snake venom metalloproteinase; GPVI, glycoprotein VI; CMFDA, 5-chloromethyl fluorescein diacetate; HBSS, Hank's balanced salt solution; PRP, platelet-rich plasma; PGE1, prostaglandin E1; RIPA, radioimmunoprecipitation assay

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The aim of the study was to determine whether the homologous structures in both proteins have the same activities and therefore similar functions for all members of the P-III class of SVMPs. Our results showed that the cysteine-rich domain of both enzymes contains two sequences that inhibit the interaction of α2β1 integrin-expressing K562 cells and platelets with collagen. This is the first report of the identification of specific sites in the cysteine-rich domain of P-III venom metalloproteinases that inhibit cell/collagen interaction. This confirms a novel role for this domain in the interaction of the P-III SVMPs with the function of the α2β1 integrin. Furthermore, these studies underscore the complex and manifold biological activities of these toxins and the pathological effects associated with envenoming.

2. Materials and methods

2.1. Peptide synthesis

The peptides for this study were synthesised on a Rainin Symphony Peptide Synthesiser using FMOC-chemistry according to the manufacturer's recommendation. Peptides were purified to homogeneity by RP-HPLC (reversed phase high performance liquid chromatography) and desalted by gel filtration chromatography. Purified peptides were analysed by MALDI-TOF mass spectrometry on a Perkin-Elmer DE Pro mass spectrometer to confirm the correct mass of the peptide. Purified peptides were cyclised by air oxidation of cysteinyl residues as previously described [11]. Lyophilised peptides were dissolved in DMSO (dimethyl sulphoxide) to a final concentration of 50 mM immediately prior to use.

2.2. Reagents

Type I collagen fibres from equine tendon were obtained from Horm Chemie (Munich, Germany). Prostaglandin E1 (PGE1), ADP, thrombin, human fibrinogen and proteinase inhibitors were purchased from Sigma Chemical Co. (Dorset, UK). Ristocetin was obtained from Lundbeck Ltd, UK, and purified human von Willebrand factor (vWF) was from ICN Flow, UK. Sepharose 2B was obtained from Pharmacia Biotechnology (Uppsala, Sweden). Immobilon-P membrane from Millipore (Bedford, MA, USA), enhanced chemiluminescence (ECL) reagent and Hyperfilm™, from Amersham (Buckinghamshire, UK) were also used. Anti-phosphotyrosine (PY20 mAb) labelled with horseradish peroxidase (PY20-HRP) was obtained from Transduction Laboratories (Lexington, KY, USA). Polyclonal anti-Syk antibody was purchased from Santa Cruz (Temecula, CA, USA). Convulxin was a generous gift from Dr. C. Bon (Institut Pasteur, France). All other reagents and chemicals were of analytical grade.

2.3. Platelet isolation and activation

Venous blood from healthy donors was mixed with 3.8% trisodium citrate (9:1 v/v) and centrifuged at $125\times g$ for 10 min at room temperature. The supernatant platelet-rich plasma (PRP) was separated. PRP was also incubated with 1 μg/ml PGE1 for 10 min and platelets were separated by centrifugation for 30 min at $1{,}500\times g$ on a 25/34% albumin density gradient at room temperature and harvested from between the two albumin layers. The platelets were then gel-filtered on a Sepharose 2B column as described before [22]. Platelets either in PRP, or as washed suspensions, were diluted to the final concentration of 2.5×10^8 cells/ml. Platelet aggregation was initiated by adding the aggregating agent and all aggregation patterns were recorded at 37°C over 4 min, under constant stirring in an aggregometer at 1000 rpm.

2.4. Platelet protein tyrosine phosphorylation and Western blotting

To analyse protein phosphorylated on tyrosine, unstimulated or stimulated platelets were pelleted and lysed with cold radioimmunoprecipitation assay (RIPA) buffer (158 mM NaCl, 1 mM EGTA, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, in 10 mM Tris–HCl pH 7.2, containing 1 mM sodium vanadate, 1 mM phenylmethanesulfonyl fluoride, 10 μg/ml leupeptin and 100 units/ml aprotinin) for 30 min at 4°C. Lysates (25 μl) were mixed with the same volume of sample buffer, boiled for 3 min and electrophoresed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) on a 10% polyacrylamide gel under reducing conditions. After electrophoresis, the proteins were transferred to Immobilon-P membranes for Western blotting and blots visualised using ECL reagent.

2.5. Immunoprecipitation

Platelet lysates prepared as described above were precleared with protein G-Sepharose beads for 1 h; antibody (1–5 μg/ml) was added and the mixtures incubated overnight at 4°C. The immune complexes

CYSTEINE-RICH REGION SEQUENCES

ATROLYSIN A: (298 - 419)

KPCLHNFGYCYNGNCPIMYHQCYALWGSNVTVAPDACFDINQSGNNSFYCRKENGVNIPCAQEDVK
CGRLFCNVNDFLCRHKYSDDGMVDHGTKCADGKVCKNRQCVDVTTAYKSTSGFQI

Designed peptides:

PCLHNFGYCYNG NCPIMYHQCY HQCYALWGSNVTVAPDAC ALWGSNVTVA PDACFDINQS
GNNSFYCRKE NGVNIPCAQE DVKCGRLFCN VKCGRLFCNVNDFLA VNDFLCRHKY
SDDGMVDHGT KCADGKVCKN RQCVDVTTAY KSTSGFSQI

JARARHAGIN: (298-421)

QPCLDNYGYCYNGNCPIMYHQCYALFGADVYEAEDSCFQDNQKGNYYGYCRKENGKKIPCAPEDV
KCGRLYCKDNSPGQNNPCKMFYSNDDEHKGMVLPKTKCADGKVCSMGHCVDVATAY

Designed peptides:

PCLDNYGYC YNGNCPIMYH QCYALFGADV HQCYALFGADVYEAEDSC YEAEDSCFKD
NQKGNYYGYC RKENGKKIPC APEDVKCGRL VKCGRLYCKDNSPGQ YCKDNSPGQN
NPCKMFYSND DEHKGMVLPG TKCADGKVCS NGHCVDVATAY

Fig. 1. Peptides based on the cysteine-rich region of atrolysin A and jararhagin. The full sequences of the cysteine-rich regions of atrolysin A [6] and jararhagin [8] are shown together with the designed peptides derived from these sequences. The shaded areas depict the active sequences. Peptides were screened for their ability to inhibit platelet aggregation with collagen. Scrambled peptides (HQCALNTVNTWGVAYSDPAVC and VKCNFADCRVLFNLG) were constructed based on the active sequences only.

were subsequently precipitated on protein G-Sepharose beads for 1 h at 4°C. Beads were washed four times in 1×RIPA buffer and the samples electrophoresed in 10% polyacrylamide gels before Western blotting as described above.

2.6. Cell adhesion studies

Adhesion studies of cultured cells labelled with 5-chloromethyl fluorescein diacetate (CMFDA) were performed as described previously [23]. Briefly, collagen type I (Chemicon Inc.) was immobilised on a 96-well microtitre plate (Falcon, Pittsburgh, PA) in 0.02 N CH_3COOH solution, overnight at 4°C. Wells were blocked with 1% BSA in Hank's balanced salt solution (HBSS) containing 3 mM $MgCl_2$. The cells were labelled with fluorescein by incubation at 37°C for 15 min with 12.5 μM CMFDA in HBSS containing 3 mM $MgCl_2$. Cells were freed from unbound ligand by washing with the same buffer. Labelled cells (1×10^5/sample) were added to the wells in the presence or absence of inhibitors and incubated at 37°C for 30 min. Unbound cells were removed by aspiration, the wells were washed, and bound cells were lysed by adding 0.5% Triton X-100. The standard curve was prepared simultaneously on the same plate using known concentrations of labelled cells. The plates were read using a Cytofluor 2350 fluorescence plate reader (Millipore, Bedford, MA, USA) at an excitation wavelength of 485 nm using a 530 nm emission filter.

3. Results and discussion

3.1. Identification of specific sequences in the cysteine-rich domains of atrolysin A and jararhagin that inhibit platelet aggregation by collagen

In previous studies we demonstrated that the cysteine-rich domain of a P-III venom metalloproteinase is capable of inhibiting collagen-induced platelet aggregation [19]. In order to localise a region in the P-III metalloproteinase cysteine-rich domain supporting that activity, we designed and synthesised a panel of peptides spanning the complete cysteine-rich domains of atrolysin A and jararhagin. The peptides with biological activity identified in this study are shown in Fig. 1. Two regions of the cysteine-rich domain of both proteins showed inhibitory activity on platelet aggregation with collagen (Fig. 2), while scrambled sequences of these peptides were inactive and therefore used as controls. Furthermore, the active peptides did not inhibit platelet aggregation with either 0.05 units/ml thrombin, 12.5 mg/ml ristocetin in the presence of 5 μg/ml vWF, or 2 μM ADP in the presence of 200 μg/ml fibrinogen. This corroborated earlier observations of the selective inhibition of collagen-induced platelet aggregation by the recombinant cysteine-rich domain of atrolysin A [19] and, for the first time, defined the active sequences in this domain of P-III venom metalloproteinases.

3.2. Cysteine-rich peptides do not affect platelet aggregation via the GPVI platelet receptor

As mentioned above, platelet stimulation by collagen for induction of aggregation requires participation of the α2β1 integrin and GPVI. Several reports indicate that convulxin, a C-type lectin from the venom of *Crotalus durissus*, induces platelet aggregation via GPVI [24,25]. We therefore used convulxin to examine whether GPVI still functions in peptide-treated platelets that are unresponsive to collagen. Our results show that the peptides HQC-atrox and VKC-atrox and HQC-jararaca and VKC-jararaca, based on the cysteine-rich domains of atrolysin A and jararhagin, respectively, had no inhibitory effect on platelet aggregation induced by 100 ng/ml convulxin (data not shown). These findings agree with our previous investigation in which jararhagin-treated platelets were responsive to convulxin [14], indicating a functional

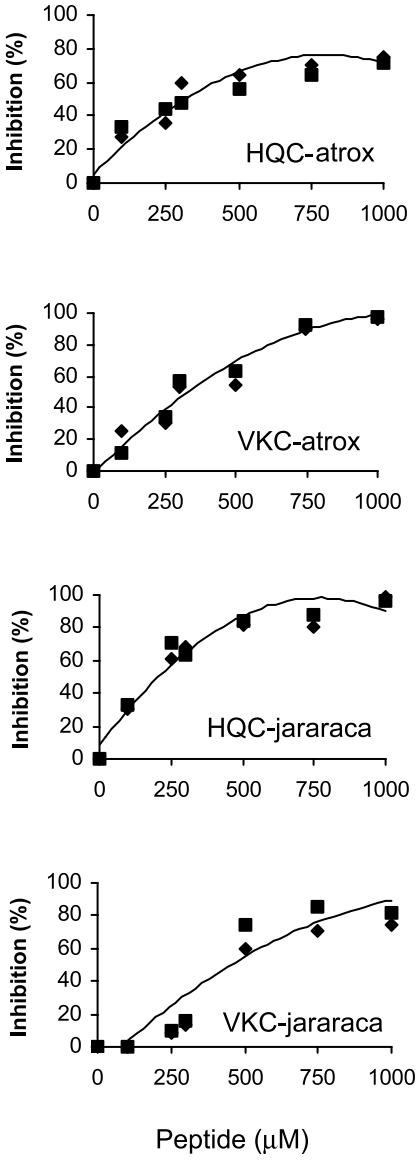

Fig. 2. Effect of active peptides based on the cysteine-rich domains of atrolysin A and jararhagin on platelet aggregation by collagen. Washed platelets (2.5×10^8/ml) were incubated with different concentrations of peptides for 2 min with constant stirring. Collagen (2 μg/ml) was then added and platelet aggregation recorded for 4 min. Controls with equivalent amounts of scrambled peptides were carried out. The maximum aggregation in the presence of active peptide was recorded for comparison with controls considered as 100%. Results from two independent experiments with platelets from two different donors are shown.

GPVI. These results therefore indicate that the most likely platelet receptor involved in the binding of these inhibitory peptides was α2β1 integrin. We already hypothesised this based on our studies with the recombinant cysteine-rich domain from atrolysin A [19].

3.3. Cysteine-rich peptides interfere with adhesion of α2-K562 cells to collagen

To validate the observation that the peptides could interact with the α2β1 integrin, we studied their effects on adhesion to collagen of K562 cells displaying this integrin. As seen in Fig. 3, active peptides based on the sequences of the cysteine-rich

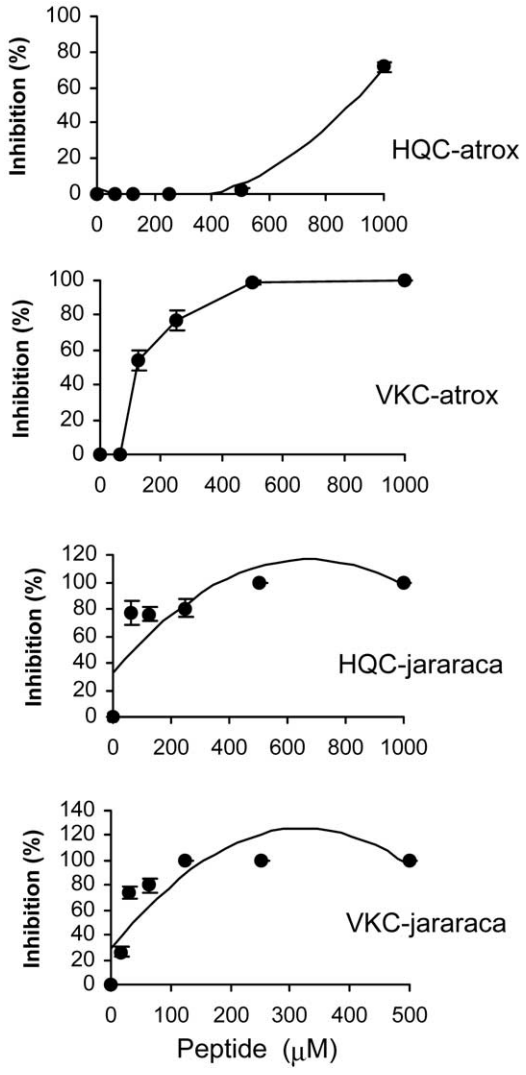

Fig. 3. Effect of peptides on the adhesion of α2-K562 cells to collagen. The effect of synthetic peptides was tested using CMFDA-labelled K562 cells transfected with α2 integrin. Different concentrations of peptides were incubated (30 min at 37°C) with the cells (1×10^5) in the 96-well plate coated with the collagen type I, in 100 μl of HBSS containing 3 mM $MgCl_2$. After washing with the same buffer the adhered cells were lysed with Triton X-100 and the plate was read using Cytofluor 2350. Percentage inhibition was calculated by comparison with fluorescence obtained from control sample with scrambled peptides. Results are means ± S.D. ($n = 3$).

regions of both atrolysin A and jararhagin displayed concentration-dependent inhibition of the adhesion of α2-K562 cells to collagen. The strongest inhibition was observed with the HQC-jararaca and VKC-jararaca peptides, followed by the VKC-atrox; the least potent inhibition was recorded with HQC-atrox (Fig. 3). These results confirm the platelet aggregation data and underscore the specificity of the peptides for α2 integrin.

3.4. *Effect of peptides on protein tyrosine phosphorylation in collagen-stimulated platelets*

In our previous studies we described the ability of jararhagin to fully abolish platelet responses to collagen, including the tyrosine phosphorylation of Syk [13]. Both HQC-atrox and VKC-atrox peptides partially inhibited protein tyrosine phosphorylation in platelets stimulated with collagen (Fig. 4). In contrast, the corresponding jararhagin-based peptides did not. Thus, although the cysteine-rich-based peptides inhibited not only platelet aggregation with collagen but also α2-K562 cell adhesion to collagen, their effects on decreasing overall protein tyrosine phosphorylation in response to this agonist were less pronounced. In addition, these peptides did not specifically affect Syk phosphorylation in response to collagen (Fig. 4). Following interaction of platelet with collagen Syk is phosphorylated and activated after being recruited to the complex with the Fc receptor γ-chain phosphorylated by Src family kinases [26]. A recent review places GPVI as the central receptor coordinating these events [27]. Therefore, the most likely explanation of the absence of an effect of these peptides on collagen-induced Syk phosphorylation is that they inhibited the binding of collagen to α2β1 integrin, but did not affect GPVI. This is strongly supported by previous observations that in platelets Syk phosphorylation by collagen depends on the engagement of GPVI and not α2β1 integrin [28,24,14]. However, despite preserved Syk phosphorylation, platelet aggregation was inhibited by these peptides (Fig. 2).

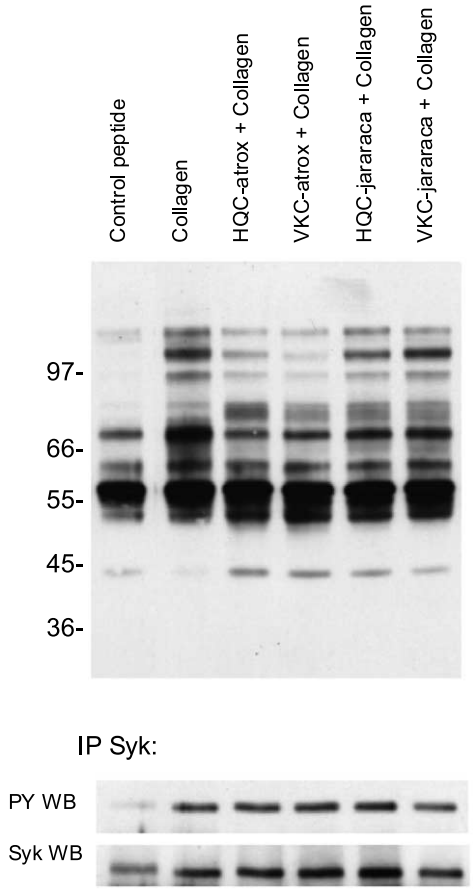

Fig. 4. Inhibition of protein tyrosine phosphorylation in collagen-stimulated platelets by atrolysin A- and jararhagin-based active peptides. Resting washed platelets were treated with cysteine-rich or control scrambled peptides (500 μM) for 2 min. Collagen (2 μg/ml) was then added and platelet aggregation recorded for 4 min. The reaction mixtures were centrifuged and the pelleted cells lysed in RIPA buffer. Proteins were separated by SDS–PAGE and Western blotted using an anti-phosphotyrosine antibody. The top panel shows phosphotyrosine blots of proteins in the whole cell lysates. The bottom panel shows tyrosine-phosphorylated and total non-phosphorylated immunoprecipitated Syk. These data are representative of two independent experiments with similar results.

This could not be attributed to a direct effect on the fibrinogen receptor αIIbβ3 integrin. This receptor requires inside-out activation for fibrinogen binding necessary for platelet aggregation [29] but has never been described to be a target of P-III venom metalloproteinases [10]. Furthermore, when platelets were preincubated with cysteine-rich peptides and then stimulated by ADP instead of collagen to activate αIIbβ3 integrin, platelet aggregation in fibrinogen-containing suspensions was fully preserved (not shown). Thus, the most likely interpretation of our results is that in spite of collagen interaction with GPVI and Syk phosphorylation, aggregation can not take place in this experimental setting without an additional signal generated by the involvement of α2β1 integrin. Taken together, these data further confirm the high specificity of the cysteine-rich region of atrolysin A for α2β1 integrin observed by Jia et al. [19] and demonstrate the same function for the cysteine-rich domain of jararhagin. Since the collagen-binding site of α2β1 integrin resides in the I domain (amino acids 140–349) of α2 subunit, it is likely that cysteine-rich peptides inhibit platelet stimulation by collagen by binding to this domain.

As regards native jararhagin, we previously observed cleavage of the platelet α2β1 integrin and complete abrogation of Syk phosphorylation by this enzyme [13] in the presence of functional GPVI, as detected by both convulxin and collagen-related peptide [14,15]. This indicates that (a) a signal requiring intact β1 subunit of the integrin may also be involved in Syk activation or (b) that jararhagin targets also other, as yet unidentified platelet receptor(s) crucial for Syk activation.

3.5. *Cys-rich peptide sequences from jararhagin can themselves stimulate platelet protein tyrosine phosphorylation*

We also investigated whether the peptides alone would cause any effect on platelets. When the response of platelets was monitored by aggregometer, HQC-atrox, VKC-atrox and HQC-jararaca had no effects, but VKC-jararaca peptide caused a weak, rapidly reversible aggregation (Fig. 5).

Examination of protein tyrosine phosphorylation again showed little or no change when platelets were treated with the HQC-atrox and VKC-atrox (Fig. 6). In contrast, both HQC-jararaca and VKC-jararaca caused some increase in tyrosine phosphorylation of several proteins as indicated by arrows (Fig. 6), confirming limited platelet stimulation by these peptides as observed with VKC-jararaca in the aggregation studies (Fig. 5).

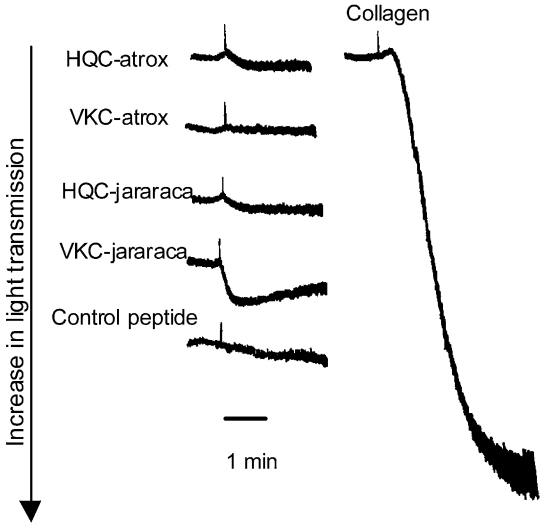

Fig. 5. Aggregation responses of platelets to peptides alone in comparison to collagen. The reactions of platelets incubated with peptides (500 μg/ml) were monitored and recorded as shown. Platelets were also stimulated with collagen (2 μg/ml) for comparison. These are representative results of triplicate determinations.

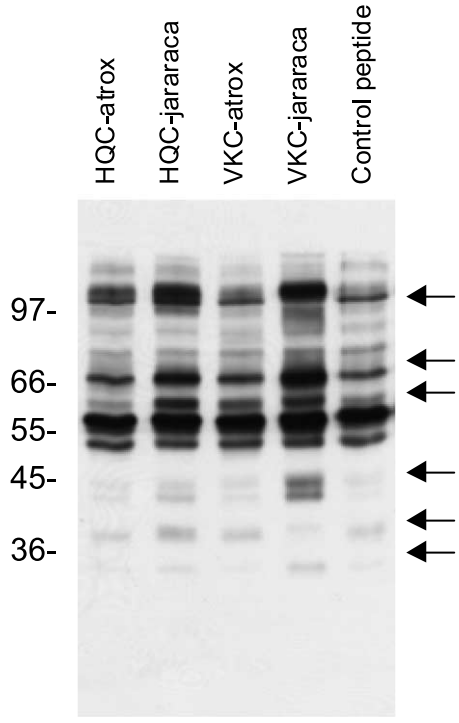

Fig. 6. Platelet protein tyrosine phosphorylation following incubation with cysteine-rich peptides based on atrolysin A and jararhagin. Platelets were stirred in the absence or presence of 500 μM peptide for 2 min. Cells were then centrifuged, lysed and analysed for protein tyrosine phosphorylation as in Fig. 4. The arrows point to bands with increased tyrosine phosphorylation, as compared to the control. This is a representative blot using samples collected from experiments carried out as described in Fig. 5.

Recently, we reported that surfaces coated with both active and inactive jararhagin have a stimulatory effect on fibroblasts similar to that of fibrillar collagen [30]. Our platelet studies suggest that this effect may originate from the interaction of fibroblast α2β1 integrin with HQC-jararaca and VKC-jararaca sites within the cysteine-rich region of jararhagin. The weak stimulatory effect that we observed with peptides in solution could have been greatly amplified in studies with fibroblasts by extensive receptor crosslinking with surface-bound ligands. Although these are interesting observations, their further follow-up was beyond the scope of the present work.

In conclusion, the present study identifies peptide sequences in the cysteine-rich regions of the P-III metalloproteinases atrolysin A and jararhagin that interfere with interaction of platelets and other cells with collagen, most likely via binding to α2β1 integrin. Our study therefore supports and extends observations made previously with the whole SVMPs proteins and the recombinant atrolysin A cysteine-rich domain. Furthermore, our study underscores the functional complexity of the P-III SVMPs that may explain multiple pathological effects associated with these toxins.

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