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Convulxin Binding to Platelet Receptor GPVI: Competition with Collagen Related Peptides

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Convulxin (CVX), a potent platelet aggregating protein from the venom of the snake *Crotalus durissus terrificus,* is known to bind to the platelet collagen receptor, glycoprotein VI (GPVI). CVX binding to human platelets was investigated by flow cytometry, using fluorescein labeled convulxin (FITC-CVX). Scatchard analysis indicated high and low affinity binding sites with Kd values of 0.6 and 4 nM and Bmax values of 1200 and 2000 binding sites per platelet. FITC-CVX binding was inhibited by collagen related peptides (CRPs) comprising a repeated GPO sequence, namely $GCO(GPO)_{10}GCOGNH_2$ and $GKO(GPO)_{10}GKOGNH_2$, which also bind to receptor GPVI. These peptides (monomeric or cross-linked forms) gave a high affinity inhibition of 10–20% for concentrations between 10 ng/ml and 5 μg/ml, followed by a second phase of inhibition at concentrations greater than 5 μg/ml. It was shown also that the inhibition of FITC-CVX binding by CRPs was independent on the time of preincubation of platelets with CRPs, and the same percentage of inhibition was seen with various concentrations of convulxin. Confocal microscopy of the distribution of FITC-CVX binding sites on platelets showed an homogeneous distribution of FITC-CVX bound to GPVI, although some limited clustering may exist. © 2000 Academic Press

Convulxin (CVX), a potent platelet activator from the venom of the snake *Crotalus durissus terrificus*, is composed of two subunits, CVXα and CVXβ covalently associated in a tridimeric structure $(\alpha\beta)_3$ (1–5). Both subunits have been cloned and sequenced, revealing an homology with the carbohydrate recognition domain of C-type lectin family of protein although they are devoid of the consensus residues responsible for the binding of sugars and Ca^{2+} (6). In agreement, the binding of CVX to platelets is Ca^{2+}-independent and is not inhibited by galactose or mannose (7). The comparison of amino acid sequences of both CVX subunits with those of botrocetin and rattlesnake lectin, two other heterodimer C-type lectins from snake venoms in which disulphide bridges were chemically localized (8, 9), revealed that the position of cysteine residues in these three proteins is similar (6). Moreover, the presence of an extra cysteine residue in each CVX subunit implies that an interchain disulphide bridge exist to stabilize the tridimeric structure $(\alpha\beta)_3$ of CVX, as reported (6).

In human platelets, collagen mainly binds to glycoproteins GPIa-IIa and GPVI (10–14). The complex interaction between collagen and its receptors has been studied recently using collagen-related peptides (CRPs). These synthetic peptides comprise a repeated GPO sequence (O for hydroxyproline), a typical triplet in collagen. CRPs spontaneously adopt its triple-helical structure which, together with quaternary structure introduced and stabilized by crosslinking, is essential for their capacity to activate platelets (13). These peptides specifically bind to platelet GPVI and their activity is independent on other collagen receptors, such as integrin GPIa-IIa or GPIV (alias CD36) (13, 14). Indeed, GPVI deficient platelets do not aggregate and do not show any detectable tyrosine phosphorylation with crosslinked CRPs (11). Moreover, platelet activation by CRP can be blocked by the Fab fragment of an anti-GPVI specific IgG (15). Platelet activation by CVX also involves the participation of GPVI (7). ^{125}I-CVX was shown to bind to an unique protein band of 62 kDa molecular weight in human platelet lysate and competition experiments either with anti-GPVI IgG or with

Abbreviations used: CVX, convulxin; FITC, fluorescein isothiocyanate; FITC-CVX; FITC labeled convulxin; CRP, collagen related peptide; PBS, phosphate buffered saline.

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unlabeled CVX gave the same degree of inhibition of protein band labeling (7), showing that on human platelets CVX binds only to GPVI.

Since both CVX and CRPs bind to GPVI and activate platelets, we further examined whether they would bind to the same site on GPVI. In such a study we used fluorescein isothiocyanate labeled CVX (FITC-CVX) and flow cytometry to measure the CVX binding in the presence or in the absence of CRPs. The data reported here show two phases of inhibition, suggesting that CRPs and CVX might differentiate two conformational states of GPVI. Moreover, since CVX has a tridimeric structure each dimer might be able to bind one GPVI molecule and therefore induce clustering of GPVI receptor on platelets. We thus examined the surface distribution of GPVI on platelets and its possible clustering by CVX.

MATERIALS AND METHODS

Materials. Fluorescein isothiocyanate (FITC) was purchased from Molecular Probes (Eugene, OR). Bovine serum albumin (fraction V), apyrase (Grade V) and prostaglandin E1 were from Sigma (St. Louis, MO). CVX was obtained as previously described (16).

FITC-labeled CVX. CVX was labeled with fluorescein isothiocyanate (FITC) according to the procedure described by Moya *et al.* (17) with some modifications. To 300 μg of CVX in 100 μl of water was added 2 μl of FITC (10 mg/ml in 1 M $NaHCO_3/Na_2CO_3$, pH 9.25). The mixture was incubated for 1 h at room temperature, in the dark. Unreacted CVX and FITC-CVX were separated from free FITC by gel filtration on a Sephadex G25 column equilibrated in phosphate-buffered saline (PBS). The FITC to CVX ratio was determined by measuring the absorbance at 280 nm and 496 nm (18), knowing that an absorbance of 2.6 at 280 nm corresponds to a concentration of 1 mg/ml of CVX, as determined independently. A molar ratio FITC/CVX of 1.0 $\pm$ 0.1 was commonly obtained with this procedure. It was controlled that the FITC-CVX, labeled at this molar ratio of 1, has the same potency than native CVX to activate platelets.

Collagen-related peptides. Peptides were synthesized as C-terminal amides on TentaGel R RAM resin in a Perseptive Systems 9050 Plus PepSynthesiser, as described in detail (19). Monomer peptides used in this study were: CRP1 *i.e.* $GCO(GPO)_{10}GCOGNH_2$; CRP2 *i.e.* $GKO(GPO)_{10}GKOGNH_2$; GPO_{10} *i.e.* $(GPO)_{10}NH_2$; and GPP_{10} *i.e.* $(GPP)_{10}NH_2$. Cross-linked peptides CRP1-XL, CRP2-XL and GPP_{10}-XL were produced from the relevant peptides using SPDP, as described (14).

Cells. Human platelets were isolated from platelet concentrates provided by the *Centre de Transfusion Sanguine* of the *Hôpital Broussais* (Paris, France). To 45 ml of concentrate was added 5 ml of anticoagulation citrate-dextrose (20), containing 25 μg/ml apyrase and 100 nM prostaglandin E1. Platelets were sedimented at 1,100*g* during 15 min and resuspended in 36 mM citric acid buffered at pH 6.5 and containing 103 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$ 5 mM glucose, 3.5 mg/ml bovine serum albumin, 25 μg/ml apyrase and 100 nM prostaglandin E1. After a second sedimentation, the platelets were resuspended at 4×10^8 cells/ml in the reaction buffer (10 mM Hepes pH 7.4 containing NaCl 134 mM, $NaHCO_3$ 12 mM, KCl 2.9 mM, Na_2HPO_4 0.34 mM, $MgCl_2$ 1 mM, glucose 5 mM, bovine serum albumin 3 g/L (18). Formaldehyde-fixed platelets were prepared according to Brinkhous and Read (21).

Antibodies. Rabbit serum antibodies were raised against CVX. The IgG of the immune serum were precipitated by addition of 35% ammonium sulfate (w/v) and dialyzed against PBS. Then, specific anti-CVX IgG were purified by immunochomatography in a CVX-Sepharose 4B column, elution being achieved with 0.1 M glycine-HCl pH 2. The anti-CVX IgG were concentrated by filtration through a Diaflo YM 30 membrane (Amicon, Beverly, MA) to 4 mg/ml. Their affinity ($K_d = 4 \times 10^{-11}$ M) was determined according to Ternynck and Avrameas (22). Anti-(human integrin α_2-subunit) monoclonal antibody 6F1 (23) from the "Fifth International workshop in human leucocytes differentiation antigens" was a gift of Dr. B. S. Coller, New York.

Analysis of CVX binding by flow cytometry. Fresh or formaldehyde fixed platelets (200 μl; 10^7 cells/ml) were incubated with FITC-CVX (0 to 150 nM) during 30 min at room temperature). Then they were washed three times with 500 μl of Tyrode's buffer and then resuspended in 500 μl of Tyrode's buffer or PBS. The total FITC-CVX binding to platelets was determined by flow cytometry in a FACScan (Becton Dickinson, San Jose, CA) equipped with a 488 nm argon-ion laser and a 530 nm band pass filter for FITC emission. Light scatter and fluorescence signals from 10,000 cells were recorded using linear and logarithmic amplifications (24). Results were expressed in arbitrary units as the median cell fluorescence intensity. The non-specific binding, measured in a similar manner in the presence of a 100-fold excess of unlabeled CVX, was subtracted from the values of total binding to determine the specific binding. The quantitation of the number of molecules of FITC-CVX bound per platelet was determined according to Faraday *et al.* (25) using FITC calibration beads (Quantum 24 premixed, Dako, Denmark). Scatchard analysis was performed by computer using P Software from Biosoft (Cambridge, UK).

For detection of unlabeled CVX binding, 200 μl of unfixed or formaldehyde-fixed platelets (10^7 cells per ml) were incubated 30 min at room temperature with 5 or 10 nM native CVX and washed 3 times, as previously described. Then, the cells were incubated again for 30 min at 0°C with specific anti-CVX IgG 0.4 μg/ml, washed 3 times and finally incubated 30 min at 4°C with BODIPY-labeled goat anti-rabbit IgG (Molecular Probes, Eugene, OR). Cells were then washed 3 times and analyzed by flow cytometry, as before.

Confocal microscopy. Immunofluorescence staining was performed as previously described (26). Briefly, unfixed platelets were labeled with FITC-CVX or with CVX and anti-CVX, detected using BODIPY-labelled second antibody and then mounted on microscope slides as described (27). Samples were examined with a Zeiss LSM 510 confocal microscope equipped with 488 nm Argon laser and a 505–550 nm band pass filter to detect green fluorescence emission. A z-series of optical sections were recorded at 0.8 μm intervals using a 63 $\times$ lens. No fluorescence was ever observed when first and second antibodies or only second antibody were used in the absence of CVX.

RESULTS AND DISCUSSION

Binding of FITC-Labeled CVX to Human Platelets and Its Inhibition by CRPs

It has been reported previously that iodinated CVX binds specifically and with high affinity to GPVI on human platelets (7). To take advantage of flow cytometry techniques, we developed an assay to measure the binding of FITC-labeled CVX to platelets. Using the standard procedure described here CVX was labeled at a ratio of one mole of FITC per mole of CVX, without significant loss of biological activity, similar effective doses (ED_{50}) of 0.2 $\pm$ 0.05 and 0.08 $\pm$ 0.04 nM being measured for native and FITC-labeled CVX respectively. However the FITC labeling of CVX at a higher molar ratio of 3 significantly reduced the biological activity of FITC-CVX to an ED_{50} of 2 $\pm$ 1 nM. The FITC

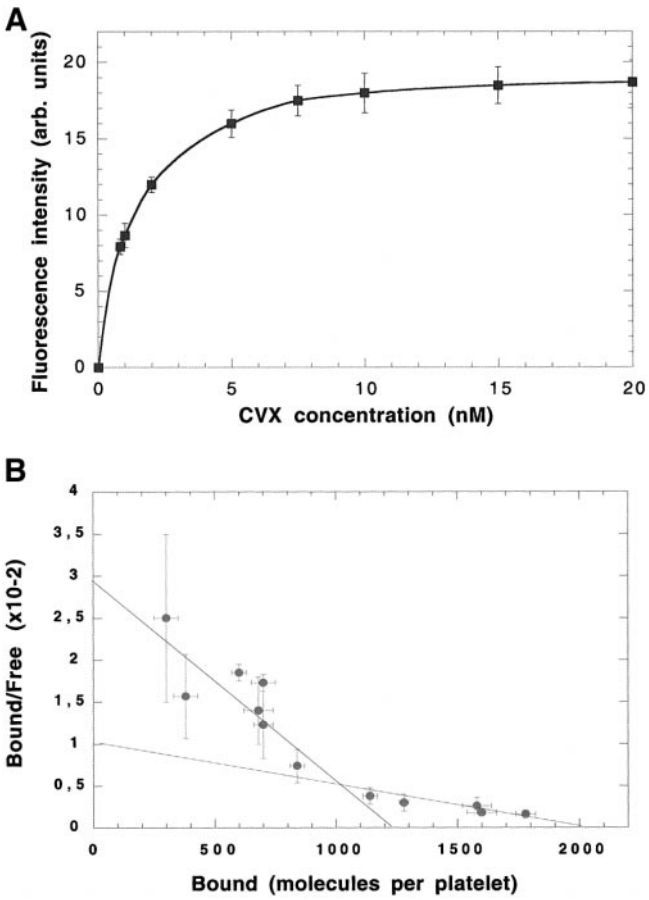

FIG. 1. Specific binding of FITC-CVX on platelets. Platelets (200 μl; 10^7 cells/ml) were incubated for 30 min at room temperature with 0 to 25 nM FITC-CVX, in the absence (total binding) or in the presence (nonspecific binding) of an excess of unlabeled CVX (50 fold the FITC-CVX tested) of unlabeled CVX. Fluorescence associated to platelets was determined by flow cytometry, then converted into molecules of FITC-CVX bound to platelets using calibrated beads as described under Materials and Methods. (A) The values corresponding to the specific binding (determined as the difference between the total and the non specific bindings) were plotted against CVX concentrations. They are the mean ± standard error of three determinations. (B) Scatchard's representation from these data.

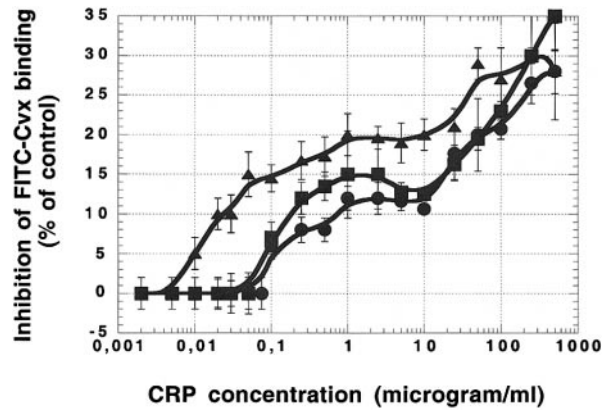

FIG. 2. Inhibition of FITC-CVX binding by CRP-peptides. Platelets (200 μl; 10^7 cells/ml) were incubated for 30 min at room temperature with 5 nM FITC-CVX in the absence (control) and in the presence of the indicated concentrations of CRPs: CRP1 (circles); CRP2 (squares); CRP1-XL (triangles). Specific FITC-CVX binding of was then determined as in Fig. 1 and binding inhibition by CRPs, expressed as percent of control, is represented in the function of peptide concentrations. The values are the mean ± standard error of three determinations.

labeling of CVX was shown to be covalent by polyacrylamide gel electrophoresis (result not shown). Then, we developed an assay to measure the binding of FITC-CVX to platelets. Platelets (200 μl; 10^7 cells/ml) were incubated with FITC-CVX (5 nM) and the fluorescence intensity of the platelets was measured by flow cytometry. It was observed that FITC-CVX readily stained human platelets and that the labeling was specific since it was completely displaced by a 100 fold excess of unlabeled CVX. Demonstration that the association of FITC-CVX with platelets was not via GPIa-IIa was shown by the lack of effect of the monoclonal antibody 6F1 (4 μg/ml) on the binding (result not shown) (23). The kinetics of binding of FITC-CVX to platelets were determined at room temperature and at 4°C. For both temperatures, the binding increased during 10–15 min to reach a similar value at both temperatures (data not shown). Therefore, subsequent binding experiments were carried out at room temperature and measurements were performed after an incubation time of 30 min, long enough to reach the equilibrium. Scatchard analysis of the FITC-CVX specific binding to human platelets (Fig. 1) shows two types of association: high affinity binding, with a Kd of 0.6 ± 0.2 nM and a Bmax of 1,200 ± 90 binding sites per cell, and a low affinity binding, with a Kd of 4 ± 2.5 nM and a Bmax of 2,000 ± 500 sites per platelet. These results are in agreement with those previously reported for the binding of ^{125}I-CVX to fixed human platelets (7). Finally, the dissociation of FITC-CVX from platelets was measured by adding a 80-fold excess of unlabeled CVX one hour after FITC-CVX. The fluorescence bound to the plate-

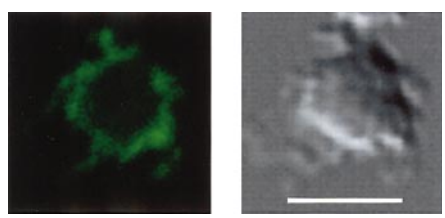

FIG. 3. Surface distribution of FITC-CVX on human platelets. Fresh human platelets were incubated for 30 minutes at room temperature with 10 nM CVX and then washed. Fluorescent labeling of bound CVX was achieved with specific anti-CVX rabbit IgG and BODIPY-labeled goat anti-rabbit IgG, as described under Materials and Methods. Samples were analyzed by confocal fluorescence microscopy. The pictures show a medial optical cut of a representative platelet (left), and the corresponding image obtained by differential interference contrast (Nomarski). The bar represents 5 μm.

TABLE I

Inhibition of Specific FITC-CVX Binding by CRPs at Various Concentration of Both Molecules

	CVX (nM)			
CRPs (μg/ml)	0.2	1	5	25
0	0.87	6.32 (0%)	14.69 (0%)	31.66 (0%)
0.5	1.14	5.64 (11%)	12.6 (15%)	26 (18%)
250	0.1 (10%)	3.57 (44%)	10.8 (25%)	24.5 (23%)

Note. Platelets (200 μl; 10^7 cells/ml) were incubated for 30 min at room temperature with the indicated concentration of FITC-CVX and CRP2. The specific FITC-CVX binding and its inhibition by CRP2 were determined as indicated in Fig. 2. Values, expressed as percent of control in the absence of CRP2, are the mean ± S.E. of three independent experiments.

lets decreased of about 25% in three hours, indicating that the dissociation of FITC-CVX from its platelet receptor was very slow (data not shown).

Since both CVX and collagen bind to GPVI (11, 14, 26) it is interesting to examine whether they interact with the same binding site on this molecule. To test this hypothesis we evaluated the capacity of CRPs to inhibit CVX binding on platelets. Platelets (200 μl; 10^7 cells/ml) were incubated for 30 min at room temperature with 5 nM FITC-CVX in the absence and in the presence of various concentrations of cross-linked or monomeric CRPs (20 ng/ml–500 μg/ml). As shown in Fig. 2, monomeric forms of $GCO(GPO)_{10}GCOG\text{-}NH_2$ (called CRP1) and $GKO(GPO)_{10}GKOG\text{-}NH_2$ (called CRP2), as well as a cross-linked form of CRP1 called CRP1-XL (14) were examined. Competition curves indicate a biphasic displacement of FITC-CVX by CRPs. A first inhibition, limited to about 10–20% of the FITC-CVX binding, can be seen for low CRP concentrations. The IC_{50} determined for this displacement (0.1 μg/ml for the monomeric forms of CRP1 and CRP2- and below 0.01 μg/ml for the cross-linked form CRP1-XL) are much lower than the CRP concentrations that activate human platelets (14, 28). On the other hand, the second type of inhibition, that might reach 100%, is observed for higher CRP concentrations, in the range as that required for platelet activation (14, 28). The same experiments performed by preincubating platelets with CRPs before adding FITC-CVX gave similar results. The competition between CRPs and CVX was further analyzed using different concentrations of FITC-CVX and of CRPs. As shown in Table I, the percentage of inhibition determined at low (0.5 μg/ml) and high (250 μg/ml) levels of CRP2 appeared independent of FITC-CVX concentrations. Also, GPO_{10}, which only contains the triple helical core of CRP1 and CRP2, shows the same biphasic effect (data not shown). The same pattern of inhibition was obtained as with CRP1 or CRP2. Interestingly, the peptides GPP_{10} and GPP_{10}-XL which do not contain hydroxyproline and do not bind GPVI (25) were unable to inhibit FITC-CVX binding (data not shown). The simplest hypothesis to explain these observations is to postulate that CRPs bind to physiologically ineffective high affinity (0.01–0.1 μg/ml) binding sites and to low affinity (0.1–1 mg/ml) binding sites which are responsible for platelet activation, both types of CRP binding sites being recognized by CVX. The fact that CRPs and CVX recognize GPVI suggests that GPVI might exist in two conformational states which can be activated, or not, by CRPs. The two types of binding sites observed for FITC-CVX and ^{125}I-CVX also suggest that CVX can differentiate these two conformational states of GPVI. However, the difference in affinity for the two forms of GPVI is much smaller for CVX (Kd values differ by a factor of 5–10) than for CRPs (IC_{50} values differ by factors 100–1,000). Another question arises concerning the structures of CVX and CRPs since their sequences do not show any similarity (6, 15, 28). Repeated GPO sequences allow CRPs to adopt a triple helical structure essential for GPVI binding as well as for platelet activation (13, 26, 27). Such a sequence does not exist in CVX. Tertiary and quaternary structures of CVX established by molecular modeling and/or X-ray crystallography will help to elucidate how such different molecules bind to GPVI.

Distribution of CVX Bound to the Surface of Human Platelets

Recently GPVI has been purified, cloned and sequenced (30), showing that it belongs to the immunoglobulin superfamily, having an extracellular domain with two Ig C2-domain loops which are proposed to be the binding sites for CRPs and for CVX. Cell surface redistribution and/or multivalent association of such receptors appears to be important for their physiological response in a variety of cell types. For instance, histamine release by mast cells is known to result from the multivalent clustering of Fc receptors for IgE on the cell surface (29). Moreover, the stimulating activity of lectins and of antibodies depends on their multivalent binding to receptors on the cell surface causing them to aggregate into patches or caps (30). Since CVX is a homotridimer $(\alpha\beta)_3$, it has been assumed that each $\alpha\beta$ dimer can bind to a separate GPVI molecule (10) and therefore that $(\alpha\beta)_3$ might induce extensive receptor clustering at the platelet surface (12). Therefore confocal microscopy was used to examine the distribution of CVX bound to fresh human platelets, incubated either with FITC-CVX (10 nM) or with unlabeled CVX (10 nM) which was detected with rabbit anti-CVX IgG and BODIPY-labeled anti-rabbit IgG. The fluorescence was homogeneously distributed on the surface of the platelets (Fig. 3). A similar distribution of fluorescence was also seen when the cells were incubated with CVX prior to fixation (data not shown). Although we cannot

rule out the existence of clusters of receptors below the resolution of the confocal microscope, our data indicate that GPVI is homogeneously distributed on the surface of platelets. On the other hand, the two Ig C2-domain loops could be each bound to one $\alpha\beta$ dimer of the same CVX molecule, in agreement with the observation that CVX cannot form a massive crosslinking between GPVI molecules.

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