Brief Definitive Report

EFFECTS OF THE NEUTROPHIL-ACTIVATING PEPTIDE NAP-2, PLATELET BASIC PROTEIN, CONNECTIVE TISSUE-ACTIVATING PEPTIDE III, AND PLATELET FACTOR 4 ON HUMAN NEUTROPHILS

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We have recently characterized a new neutrophil-activating peptide, called NAP-2, which is formed in platelet-containing cultures of stimulated human blood mononuclear cells (1). NAP-2 consists of 70 amino acids and has, therefore, about the same size as NAF/NAP-1 (72 amino acids) (2). NAF/NAP-1 and NAP-2 are structurally homologous and have similar effects on neutrophils (1). As shown in Fig. 1, NAP-2 corresponds to part of the sequence of platelet basic protein (PBP) (3) and connective tissue-activating peptide III (CTAP-III) (4), which is also known as low affinity platelet factor 4 (3). It is structurally homologous to platelet factor 4 (PF-4) (5), another α-granule protein and the murine inflammatory protein, MIP-2 (6).

We have compared the effects of NAF/NAP-1, NAP-2, and the α-granule peptides on human neutrophils, and show here that NAP-2 behaves like a typical chemotactic receptor agonist inducing cytosolic free calcium changes, chemotaxis, and exocytosis, while PBP, CTAP-III, and PF-4 have little if any such activity.

Materials and Methods

Materials. Most of the special reagents were described previously (7). Fura-2/AM was obtained from Fluka AG, Buchs, Switzerland; α-thrombin was from Hoffmann-La Roche Ltd., Basel, Switzerland; and human recombinant NAF/NAP-1 was from the Sandoz Research Institute, Vienna, Austria.

Cell Preparations. Human neutrophils were isolated and used as described previously (7). Platelets were isolated from platelet-rich supernatants prepared from buffy coats (8). Suspensions containing $\sim 10^{11}$ platelets were washed three times in 0.15 M NaCl, 0.015 M sodium citrate, 10 mM EDTA, pH 7.4, resuspended in 20 ml of the same buffer, warmed to 37°C, and then stimulated for 10 min with 5 U/ml human α-thrombin. The reaction was stopped by cooling to 4°C and the cell-free release supernatant was obtained at 30,000 *g* for 30 min and used for peptide purification.

Peptides. Platelet release supernatant was loaded at a flow rate of 25 ml/h onto a heparin-sepharose column (10 × 600 mm) equilibrated in 50 mM Tris-HCl buffer, pH 7.4, containing 50 mM NaCl. After washing with equilibration buffer, PBP and CTAP III were eluted with a linear NaCl concentration gradient (0.05–1 M) in the same buffer. PF-4 was eluted subsequently with 2 M NaCl. Fractions were tested for the presence of PBP, CTAP-III, and PF-4

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by SDS-PAGE and further purified by reversed-phase HPLC on an analytical CN-propyl column (4.6 × 250 mm, 5 μm, wide-pore; Baker Research Products, Phillipsburg, NJ) eluted (0.5 ml/min) with a gradient of 0–20% acetonitrile in 0.1% trifluoroacetic acid with an increment of 0.33% per minute. NAP-2 was purified as described (1). The purity was confirmed by NH_2-terminal sequencing of the first 12 residues.

Biological Assays. Neutrophil chemotaxis (9), cytosolic free calcium changes (7), and elastase release (7) were determined according to established methods.

Results

Peptide Purification. PBP and CTAP-III were resolved from each other, and completely separated from PF-4 by heparin-Sepharose chromatography (data not shown). Upon reversed-phase HPLC, NAP-2, PBP, CTAP-III, and PF-4 eluted with different retention times (Fig. 2). Electrophoretically pure NAP-2, eluting as a single peak from a C4-column (1), was recovered as a doublet from the CN-propyl column (Fig. 2), which was used to separate NAP-2 from the other species. HPLC-purified peptides migrated as single bands in SDS-PAGE (not shown). They were identified by NH_2-terminal sequencing of the first 12 residues. A single sequence was obtained for each species, indicating that they were indeed analytically pure.

The release supernatants of fresh thrombin-stimulated platelets contained PBP, CTAP-III, and PF-4, but no β-thromboglobulin (βTG) or NAP-2.

Elastase Release. Elastase release from cytochalasin B–pretreated neutrophils was the biological assay used for NAF/NAP-1 (7) and NAP-2 (1). Both were now compared with the peptides from the α-granules. As shown in Fig. 3 *a*, NAP-2 induced significant release of elastase at concentrations between 0.3 and 100 nM, while only minimal activity (~2% of that of NAP-2) was obtained with PBP at 100 nM, the highest concentration used. CTAP-III, PF-4, and the lower concentrations of PBP were inactive. NAP-2 was effective in the same molarity range as NAF/NAP-1, but was about half as potent.

Cytosolic Free Calcium Changes. Stimulus-dependent fura-2 fluorescence changes were recorded, and the rate of fluorescence increase was determined. A similar, progressive increase in the rate of the calcium rise was observed after stimulation with 0.1 to 100 nM NAP-2 and NAF/NAP-1 (Fig. 3 *b*). PBP, CTAP-III, and PF-4 were virtually inactive up to a concentration of 10 nM, but showed some effect at 100 nM. PBP was slightly more active than CTAP-III and PF-4.

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NAP-2                                        AELRCMC....
CTAP-III                          NLAKGKEESLDSDLYAELRCMC....
PBP       SSTKGQTKRNLAKGKEESLDSDLYAELRCMCIKTTS-GIHPKNIQSLE
PF-4                                   EAEEDGDLQCLCVKTTS-QVRPRHITSLE
NAP-1                                     SAKELRCQCIKTYSKPFHPKFIKELR
MIP-2                                   AVVASELRCQCLKTLP-RVDFKNIQSLS

PBP/NAP-2   VIGKGTHCNQVEVIATLKDGRKICLDPDAPRIKKIVQKKLAGDESAD
PF-4          VIKAGPHCPTAQLIATLKNGRKICLDLQAPLYKKIIKKLLES
NAP-1         VIESGPHCANTEIIVKLSDGRELCLDPKENWVQRVVEKFLKRAENS
MIP-2         VTPPG.....................................
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FIGURE 1. Amino acid sequences of NAP-2 and related peptides aligned according to their four cysteine residues. Residues common to all sequences are bold face. For NAP-2 and CTAP-III, which are truncations of PBP, only a few NH_2-terminal residues are indicated (top).

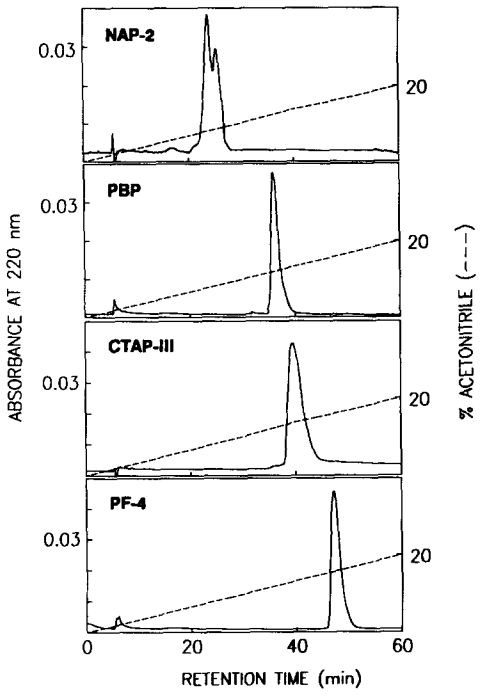

FIGURE 2. Reversed-phase HPLC of purified NAP-2, PBP, CTAP-III, and PF-4 on an analytical CN-propyl column. The retention times were 23.9 min for NAP-2, 36.0 min for PBP, 39.6 min for CTAP-III, and 47.1 min for PF-4.

Chemotaxis. As shown in Fig. 4 *a*, NAP-2 and NAF/NAP-1 induced concentration-dependent chemotaxis between 0.03 and 1 nM. In this range, the two curves were very similar in the six experiments performed. At higher concentrations, however, NAF/NAP-1 was consistently more active than NAP-2. PBP and CTAP-III had no

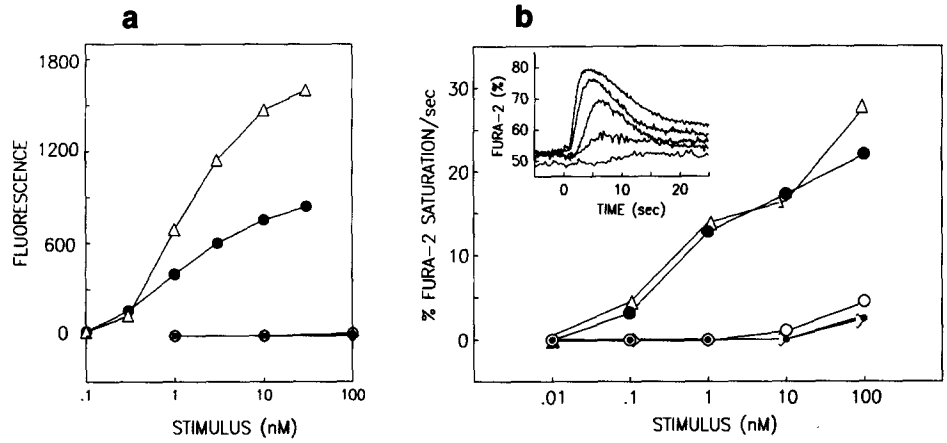

FIGURE 3. (*a*) Release of elastase from cytochalasin B-treated human neutrophils stimulated with NAF/NAP-1 (△), NAP-2 (●), PBP (○), CTAP-III (·), and PF-4 (◇). (*b*) Rate of the cytosolic free calcium rise in human neutrophils stimulated with NAF/NAP-1 (△), NAP-2 (●), PBP (○), CTAP-III (·), and PF-4 (◇). (*Inset*) Single fura-2 fluorescence recordings in neutrophils stimulated at time 0 with 100, 10, 1, 0.1, and 0.01 nM NAP-2 (top to bottom). Similar results were obtained in five (*a*) and three (*b*) additional experiments performed with different cell preparations.

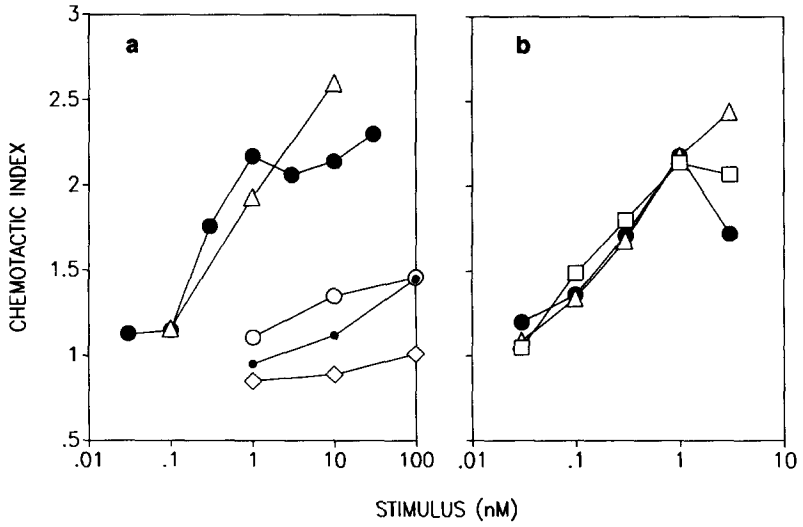

FIGURE 4. Neutrophil chemotaxis induced by (*a*) NAF/NAP-1 (Δ), NAP-2 (●), PBP (○), CTAP-III (·), and PF-4 (◇); (*b*) NAF/ NAP-1 (Δ), NAP-2 (●), and C5a (□). The highest effect, obtained with 10 nM NAF/NAP-1, corresponded to the average migration of 41.2 ± 8.6% of the cell sample applied. Similar results were obtained in five (*a*) and two (*b*) additional experiments performed with different cell preparations.

effect at 1 nM, and showed low levels of activity at 10 and 100 nM. PBP was again slightly more active than CTAP-III. Fig. 4 *b* shows that NAP-2 and NAF/NAP-1 were equipotent with C5a, the most active naturally occurring chemotactic peptide described so far.

Discussion

This study shows that NAP-2 is a powerful activator of human neutrophils, inducing cytosolic free calcium changes, chemotaxis, and exocytosis at concentrations between 0.3 and 10 nM, while its homologues, the platelet α-granule proteins PBP, CTAP-III, and PF-4, are practically inactive at concentrations up to 100 nM. NAP-2 is equipotent with NAF/NAP-1 and C5a as a stimulus for chemotaxis, but weaker than these and other agonists as an inducer of exocytosis.

Of the platelet α-granule proteins studied, PF-4 was the first to be characterized (5). It was reported to be chemotactic for neutrophils, monocytes (10, 11), and fibroblasts (12), and to induce neutrophil adherence and granule release (11). Except in the case of fibroblasts (12), these responses were obtained at PF-4 concentrations between 100 nM and ~20 μM (11, 12), i.e., 100–10,000-fold higher than NAP-2, NAF/NAP-1, and C5a in the present study. CTAP-III was reported to enhance mucopolysaccharide synthesis and other metabolic processes in synovial cells (4, 13) and to be mitogenic for fibroblasts (4), but not for 3T3 cells (3). Such effects were not obtained with the CTAP-III precursor PBP (3) and the CTAP-III cleavage product βTG (4). None of these peptides were reported to activate neutrophils.

Our observation that PF-4 is not chemotactic for neutrophils does not necessarily contradict the earlier findings obtained at higher concentrations (10, 11). However, in view of the high potency of NAP-2, it must be realized that a minimal contamina-

tion with this peptide could account for the neutrophil-stimulating effects previously attributed to PF-4.

Platelets have long been thought to participate in intravascular inflammatory processes through the release of vasoactive, proexudative, and phagocyte-activating products (14). Neutrophils, which are often found in association with platelet aggregates (15, 16), are presumably attracted by chemotactic mediators released as a consequence of platelet activation. Up to now, platelet-activating factor was considered the most potent of these attractants (17). The recent identification of NAP-2 as a powerful chemotactic fragment of PBP, however, suggests the existence of an alternative mechanism of neutrophil recruitment that is related to α-granule exocytosis. The recruitment of neutrophils into platelet aggregates and thrombi via platelet-derived chemotaxins may constitute a mechanism for the degradation of thrombotic deposits and the recanalization of obstructed vessels. On the other hand, activated neutrophils could aggravate the course of thrombotic diseases and their sequelae, as in atherosclerosis, by inducing inflammation and tissue damage.

Summary

Platelet basic protein (PBP), connective tissue–activating peptide III (CTAP-III), and platelet factor 4 (PF-4) were purified from human platelet release supernatants by heparin-Sepharose ion-exchange and reversed-phase HPLC, and their neutrophil-activating effects were compared with those of NAP-2, a peptide of 70 amino acids corresponding to part of the sequence of PBP (1) and with sequence homology to NAF/NAP-1. NAP-2–induced elastase release and a rise in cytosolic free Ca^{2+} at concentrations between 0.3 and 100 nM, and neutrophil chemotaxis at concentrations between 0.03 and 10 nM. It was half as potent as NAF/NAP-1 in inducing exocytosis but showed the same activity in the other responses. By contrast, only minimal if any effects were obtained with PBP, CTAP-III, and PF-4 up to 100 nM.

NAP-2 thus appears to behave like a typical chemotactic receptor agonist. It could be generated from PBP and/or CTAP-III released from activated platelets and lead to the accumulation of neutrophils in platelet aggregates.

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