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METABOLISM OF SUBSTANCE P AND BRADYKININ BY HUMAN NEUTROPHILS*

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Abstract—The catabolism of substance P and bradykinin, two peptides involved in inflammation, by human neutrophils was investigated. Substance P was cleaved by unstimulated neutrophils, but the rate of hydrolysis increased greatly (about 4-fold) when the cells were lysed by freezing and thawing or stimulated to release with fMet-Leu-Phe and cytochalasin B. The enzyme responsible for cleaving substance P was cathepsin G, hydrolyzing the Phe^7-Phe^8 bond. Neutral endopeptidase 24.11 (enkephalinase) became the main inactivating enzyme only when neutrophil cytoplasts (containing plasma membrane but no subcellular particles) or washed plasma membrane enriched high speed sediments were tested. Subcellular fractionation showed the highest substance P degrading activity to be in the granules. Purified cathepsin G readily cleaved substance P with a K_m of 1.13 mM, a k_{cat} of 6.35 sec^{-1} and a k_{cat}/K_m of 5639 M^{-1} sec^{-1}, similar to kinetic constants previously reported for the best peptide substrates of cathepsin G. Despite the high K_m, purified cathepsin G did hydrolyze SP at a much lower substrate concentration (down to 1 nM) as determined by radioimmunoassay. Bradykinin was also hydrolyzed by intact neutrophils but, in contrast, was not inactivated by cathepsin G, but by neutral endopeptidase at the Pro^7-Phe^8 bond. The inactivation of bradykinin by intact neutrophils was decreased by phorbol 12-myristate 13-acetate, probably due to down-regulation by endocytosis of the neutral endopeptidase on the plasma membrane. Thus, both bradykinin and substance P are inactivated by human neutrophils, although by different enzymes. In spite of the less favorable kinetics *in vitro* than with neutral endopeptidase, cathepsin G is the main inactivator of substance P in neutrophils. This may be due to the estimated 300 to 3600-fold higher concentration of cathepsin G in neutrophils than that of the neutral endopeptidase.

Substance P (SP) and bradykinin are important mediators in many pathological processes [1]. SP, for example, is reported to be involved in inflammatory and immunological responses of the body, including neurogenic inflammation [1–3], immediate and delayed hypersensitivity [4], arthritis [3, 5, 6] and others [1–6]. SP may contribute to inflammatory syndromes in various ways [1–6]: as a pain transmitter, as a vasodilator, and by the release of histamine from mast cells [7]. SP also stimulates lymphocyte proliferation [4, 5], neutrophil chemokinesis and chemotaxis [2–5, 8, 9], phagocytosis [2–5, 10], and oxidative metabolism [11–13] and sensitizes neutrophils to other stimulants [14]. Many actions of SP (pain, vasodilation, smooth muscle contraction) are shared with the structurally unrelated kinins. Bradykinin was named after the slow contraction it causes on the guinea pig ileum, while SP belongs to the structurally related family of tachykinin peptides which, as the name indicates, cause a fast contraction.

The duration of action of both peptides is controlled by the peptidases which hydrolyze them. The hydrolysis of any bond in bradykinin renders it inactive on its most common (B_2) receptor [15, 16]. In contrast, SP can be shortened from the intact undecapeptide to the C-terminal hexapeptide and still retain activity on smooth muscle preparations [6]. Some endogenous enzymes can inactivate both peptides. A human enzyme which deamidates the C-terminal -Met^{11}-NH_2, thereby rendering SP unreactive on its receptors, can also remove the Arg^9 of bradykinin by carboxypeptidase action [17]. Angiotensin I converting enzyme (ACE) or kininase II is a potent inactivator of kinins [15]. The same enzyme cleaves SP mainly to SP_{1-8} and SP_{9-11} *in vitro* [18–20]. Inhibition of ACE, however, can potentiate some of the activity of SP *in vivo* [19, 21, 22], indicating that ACE is an inactivator of SP. Nevertheless, SP is hydrolyzed, in many cases, primarily by the neutral endopeptidase 24.11 (enkephalinase; NEP) [23]. For example, the bronchoconstrictor activity of SP is potentiated by NEP inhibitors [22–26].

Human ACE inactivates kinins by the release of the C-terminal dipeptide Phe-Arg [15] and the same reaction is catalyzed by human NEP [27, 28]. Although the kinetics of bradykinin hydrolysis are less favorable *in vitro* with NEP than with ACE [27], NEP may act as a kininase *in vivo*, for example in the rat kidney [29]. We found that NEP is present on the cell membrane of human neutrophils [28, 30, 31]. Because of the prominent involvement of SP, bradykinin and neutrophils in inflammation, and because both peptides are substrates of NEP, we investigated the inactivation of SP and bradykinin by enzymes on

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the cell membrane and in subcellular fractions of human neutrophils. Here we report that the NEP on the cell membrane of intact neutrophils indeed hydrolyzed bradykinin but, unexpectedly, SP was inactivated primarily by the action of cathepsin G.

MATERIALS AND METHODS

Materials. The following reagents were obtained from Sigma Chemicals, St. Louis, MO: Histopaque 1077, phorbol 12-myristate 13-acetate (PMA), cytochalasin B, chymostatin, *N*-formyl-Met-Leu-Phe, *N*-tosyl-Phe-chloromethylketone (TPCK), *o*-phenanthroline, *p*-chloromercuriphenylsulfonate (PCMS), bradykinin, SP and diisopropylfluorophosphate (DFP). The elastase inhibitor methoxy-Suc-Ala-Ala-Pro-Val-CH_2Cl and the cathepsin G inhibitor Z-Gly-Leu-Phe-CH_2Cl were from Enzyme Systems Products, Livermore, CA. Phosphoramidon was from Peninsula Laboratories, Belmont, CA, and Ficoll from Pharmacia, Piscataway, NJ. Eglin was a gift from CIBA AG. Pure human neutrophil cathepsin G was obtained from Athens Research and Technology, Inc., Athens, GA. Oxidized SP was prepared by incubating 1 mM SP in 0.1 M sodium acetate, pH 4.0, with 0.35% H_2O_2 for 30 min at room temperature. Antiserum to SP was provided by Dr. Michael Vasko of Indiana University and Dr. Hsiu-Ying Yang of the National Institute of Mental Health. The other chemicals used were of reagent grade obtained commercially.

Preparation and fractionation of human neutrophils. Neutrophils were isolated from 40 to 60 mL of fresh heparinized human blood by centrifugation over Histopaque-1077 cushions as described [28, 31]. Neutrophils were disrupted by nitrogen cavitation in 100 mM KCl, 3 mM NaCl, 1 mM ATP, 3.5 mM $MgCl_2$ and 10 mM piperazine-*N*,*N*′-bis(2-ethanesulfonic acid), pH 7.3, and the cavitate was collected into [ethylenebis(oxyethylenenitrilo)]tetraacetic acid (EGTA; 1.25 mM final concentration) and diluted with an equal volume of 0.78 M sucrose. Subcellular fractions were obtained by differential centrifugation (4°) of the crude homogenate at 800 *g* for 15 min to remove cellular debris and unbroken cells (P_1), 15,000 *g* for 20 min to obtain a granular pellet (P_2), and finally at 100,000 *g* for 1 hr to yield the crude membrane fraction (P_3) and the soluble fraction. The P_3 fraction was resuspended in 0.34 M sucrose, 0.1 M 2-(*N*-morpholino)ethane sulfonic acid (MES) buffer, pH 6.5. To lyse contaminating granules in the P_3 membrane fraction and to remove any granular enzymes, the P_3 fraction was diluted with an equal volume of 10 mM Tris–HCl, pH 7.4, and centrifuged for 1 hr at 100,000 *g* (this and the following steps were carried out at 4°). The pellet was resuspended in 10 mM Tris, pH 7.4, allowed to sit for 15–30 min and then sonicated. NaCl (10 M) was added to a final concentration of 1.0 M and the solution sonicated and allowed to sit for another 30 min followed by freezing. The mixture was thawed and then centrifuged at 100,000 *g* to give the washed P_3' pellet which was resuspended in 10 mM Tris–HCl, pH 7.4, containing 0.9% NaCl.

Neutrophil cytoplasts were prepared by treating neutrophils with 5 μg/mL cytochalasin B followed by centrifugation on a discontinuous Ficoll gradient as reported [32]. This causes nuclei and intact granules to sediment to the bottom of the tube while the neutrophil cytoplasts (essentially resealed plasma membranes containing cytoplasm) remain at the first interface. The top band (cytoplasts) was collected and centrifuged at 1300 *g* for 20 min at room temperature and then washed by resuspension in phosphate-buffered saline followed by recentrifugation. The pellet (granules and nuclei) was resuspended in phosphate-buffered saline, centrifuged at 15,000 *g* for 15 min, and then resuspended in 0.1 M MES buffer, pH 6.5, containing 1% Triton X-100 to solubilize granular enzymes.

Neutrophils were stimulated to release their granular contents by incubation for 10 min at 37° with cytochalasin B (3 μg/mL) followed by incubation for 15–25 min with 10^{-6} M fMet-Leu-Phe.

Assay of peptide hydrolysis. Neutrophils, cytoplasts or subcellular fractions of neutrophils were incubated with either SP or bradykinin (100 μM) in 10 mM Tris–HCl, pH 7.4, with 0.9% NaCl (final volume = 100–200 μL) at 37° for 0–120 min. Most inhibitors were preincubated with neutrophils or cell fractions for 10 min at room temperature prior to addition of substrate. The specific cathepsin G inhibitor, Z-Gly-Leu-Phe-CH_2Cl, was preincubated for 2 hr at room temperature. Control cells were kept under the same conditions [in buffer only or with added 0.1 or 1% dimethyl sulfoxide (DMSO), depending on the solvent used to dissolve the inhibitor]. Reaction mixtures were centrifuged for 1 min at 15,000 *g*, the supernatants were removed, and trifluoroacetic acid was added (to 0.5% final concentration). The samples were filtered and aliquots of 40–100 μL analyzed by HPLC. Separation and quantitation of peptide fragments were carried out on a Waters automated gradient system with a μBondapak C18 column (3.9 mm × 30 cm) as described [18, 28]. The unattenuated output from a Waters 490 detector was connected to a computerized Waters 840 data/chromatography control station and peak areas were integrated utilizing Waters Expert chromatography software. Peptide hydrolysis was assessed by measuring either the decrease in peak area of the substrate or the increase in peak area of total peptide products. Quantitation was achieved by comparing peak areas to those of authentic standards injected 2–3 times during the analysis. The limit of sensitivity was about 20 pmol (based on the SP_{1-7} peak) corresponding to about 0.5% hydrolysis of SP (at 100 μM). For kinetic studies, duplicate reactions with pure cathepsin G (50 ng) were run in 0.2 M *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES), pH 7.0, 1.0 M NaCl, at each of seven to eight substrate concentrations ranging from 50 to 1500 μM. The rate of SP_{1-7} formation was measured at each substrate concentration, and kinetic constants were determined by Lineweaver–Burk analysis.

Peptide products of SP hydrolysis were collected after separation by HPLC, lyophilized and hydrolyzed in 6 N HCl. Amino acid analysis was carried out using the Waters' Pico-Tag system according to the instructions of the manufacturer.

The hydrolysis of SP by cathepsin G at low SP

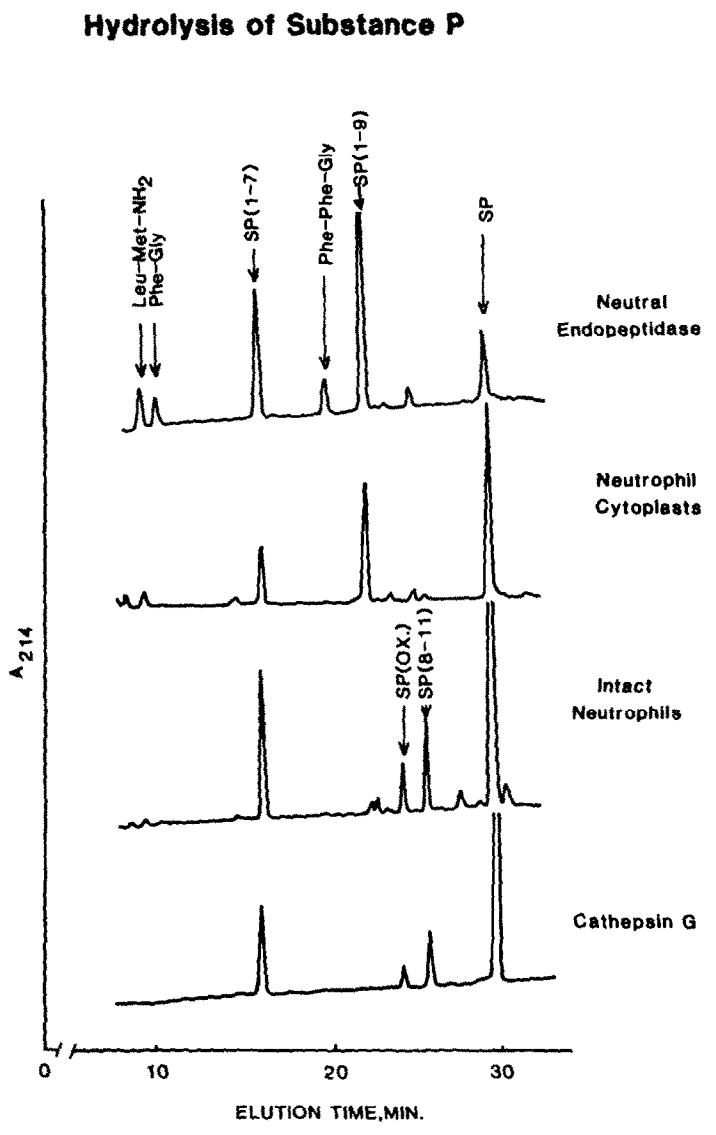

Fig. 1. Separation of metabolites of SP by HPLC. Arrows denote the elution positions of authentic standards. SP(OX.) = oxidized SP.

concentrations was measured by radioimmunoassay. SP (at concentrations of 1 μM, 50 nM and 1 nM) was incubated with 50 ng pure cathepsin G in 100 mM HEPES, pH 7.0, 0.5 M NaCl containing 0.2 mg/mL lysozyme (to prevent nonspecific loss of SP) for 10 min at 37° (final volume = 200 μL). Control reactions were carried out in the absence of enzyme to rule out nonenzymatic loss of SP. Reactions were stopped by addition of eglin (10^{-5} M final concentration) and DFP (10^{-4} M final concentration), and all subsequent steps were carried out with samples in an ice bath. Aliquots of the reaction mixture were diluted to an appropriate concentration for the radioimmunoassay in 0.05 M phosphate buffer, pH 7.0, containing 0.2% lysozyme and 0.06% dextran. The radioimmunoassay for SP was carried out using the tracer ^{125}I-(^{8}Tyr)-SP as described [33].

Protein assay. Protein concentrations were measured using the method of Bradford [34] with bovine serum albumin as standard.

RESULTS

Metabolism of SP by neutrophils. Intact, isolated neutrophils inactivated SP at a rate of 372 ± 69 pmol/min/10^6 cells (± SEM, N = 11). The two major products of the reaction detected by HPLC coeluted with SP_{1-7} and the C-terminal SP_{8-11} (Fig. 1). The peaks were positively identified by collection from the HPLC followed by amino acid analysis. The other major product peak coeluted with SP having oxidized Met^{11}-NH_2 (Fig. 1). Thus, the intact cells cleave SP primarily at the Phe^7-Phe^8 bond. Because NEP is on the surface of neutrophils and cleaves peptides such as SP at the N-terminus of hydrophobic amino acids

Table 1. Inhibition of the hydrolysis of SP by intact human neutrophils and by isolated subcellular fractions*

Inhibitor	Concn (M)	Inhibition (%)			
		Intact neutrophils	P_2 fraction	P_3 fraction (crude)	P_3' fraction (washed)
Z-Gly-Leu-Phe-CH_2Cl	10^{-5}	43	76	76	29
	10^{-4}	77	92	ND	39
Phosphoramidon	10^{-6}	10	6	7	64
Chymostatin	10^{-4}	98	95	91	54
DFP	10^{-3}	48	91	76	18
Eglin	10^{-5}	68	93	89	24
TPCK	10^{-4}	11	37	ND	34
o-Phenanthroline	10^{-3}	12	7	ND	80
PCMS	10^{-3}	0	ND	ND	ND

* For details and abbreviations, see Materials and Methods. Results are mean values from 2–8 experiments. ND = not determined. Inhibitors were used at concentrations high enough to inhibit the corresponding purified enzymes as found in many previous experiments. Inhibitor characteristics are as follows: Z-Gly-Leu-Phe-CH_2Cl is specific for cathepsin G; phosphoramidon is specific for NEP; chymostatin inhibits many chymotrypsin-like enzymes including cathepsin G; DFP is a general serine protease inhibitor; eglin is specific for elastase and cathepsin G; TPCK is a chymotrypsin inhibitor which only partially inhibits cathepsin G; *o*-phenanthroline is a general metalloprotease inhibitor and thus inhibits NEP; and PCMS is a general sulfhydryl protease inhibitor.

[23], we used the specific NEP inhibitor, phosphoramidon, to characterize the inactivating enzyme(s) further. Unexpectedly, phosphoramidon (1 μM; Table 1) inhibited only 10% of the activity. In addition, although SP_{1-7} is one of the NEP hydrolysis products [18], the pattern of products produced by intact cells was much different than that produced by purified human NEP ([18]; Fig. 1). The inactivation of SP by intact neutrophils was inhibited almost completely by 100 μM chymostatin (Table 1), an inhibitor of chymotrypsin-like enzymes [35]. The specific cathepsin G inhibitor, Z-Gly-Leu-Phe-CH_2Cl [36], inhibited 43% of SP hydrolysis at a 10 μM concentration and 77% at 100 μM, while eglin, another effective cathepsin G inhibitor [37], inhibited 68%. DFP, the nonspecific serine protease inhibitor, blocked about half the activity (48%). Other compounds, including the sulfhydryl protease inhibitor PCMS (1 mM), the metalloenzyme inhibitor *o*-phenanthroline (1 mM), and TPCK (0.1 mM), a chymotrypsin inhibitor which does not inhibit cathepsin G significantly [38], were ineffective (Table 1).

These results indicate that, in spite of the high rate of SP hydrolysis by NEP *in vitro* [18, 39], another enzyme, probably the granular cathepsin G, is the major inactivator of SP in intact neutrophils. If this is the case, conditions which release granules should increase SP hydrolysis. Indeed, when cells were freeze-thawed, the rate of SP degradation increased to 441% of the control cells (average of two experiments; Fig. 2) and when neutrophils were stimulated to release their granular contents by adding a combination of fMet-Leu-Phe and cytochalasin B, SP hydrolysis increased to 393 ± 60% (N = 4; Fig. 3). At the concentration used in the incubation mixture (100 μM), SP itself did not cause significant release of granular enzymes.

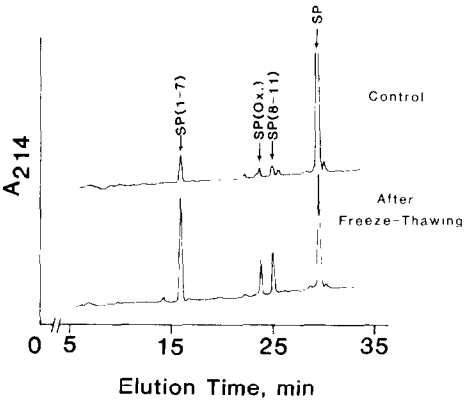

Fig. 2. Hydrolysis of SP by intact human neutrophils or neutrophils after freeze–thawing. Equivalent numbers of intact cells (top; control) or freeze–thawed cells (bottom) were incubated with 100 μM SP for 60 min at 37°, and products were separated by HPLC as described. Note the increased degradation of SP by freeze–thawed neutrophils. For further details see legend to Fig. 1 and text.

Fractionated cells. To localize the major SP inactivator, we disrupted the cells by nitrogen cavitation and separated subcellular fractions by differential centrifugation. Both the granule fraction (P_2) and the crude membrane fraction (P_3) rapidly degraded SP (P_2 = 137 nmol/min/mg protein; P_3 = 95.4 nmol/min/mg protein). Table 1 shows the inhibition pattern of SP inactivation by the P_2 and P_3 fractions and

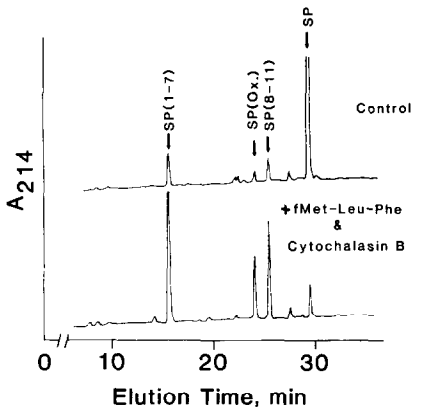

Fig. 3. Increased hydrolysis of SP by activated neutrophils. Equivalent numbers of intact neutrophils (top; control) or neutrophils stimulated to release their granular contents with 10^{-6} M fMet-Leu-Phe and 3 μg/mL cytochalasin B (bottom) were incubated with SP for 60 min at 37° and the products separated by HPLC. For further details see legend to Fig. 1 and text.

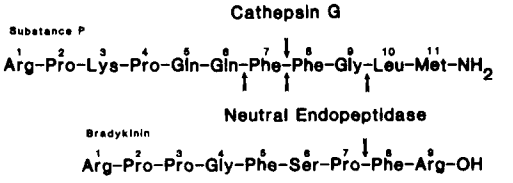

Fig. 4. Hydrolysis of SP and bradykinin by neutral endopeptidase and cathepsin G. Arrows show the primary sites of cleavage.

by the washed P_3' fraction. The hydrolysis of SP by the granules (P_2 fraction) was due to cathepsin G, as its inhibitors, Z-Gly-Leu-Phe-CH_2Cl (10^{-4} M), chymostatin, eglin or DFP, inhibited more than 90% of the activity (Table 1). The same azurophil granules which contain cathepsin G also contain elastase [40, 41]. Elastase, however, did not participate in SP inactivation, as its specific inhibitor methoxy-Suc-Ala-Ala-Pro-Val-CH_2Cl (10 μM) was inactive (not shown). Phosphoramidon and *o*-phenanthroline were not effective, and TPCK inhibited only 37% of the activity in the P_2 fraction.

It is obvious from the inhibition pattern that the activity in the crude P_3 membrane fraction was due primarily to contaminating cathepsin G as its inhibitors were 76–91% effective, while the action of the NEP inhibitor, phosphoramidon, was negligible (Table 1).

Although it was not apparent with SP as substrate, NEP was active in the crude P_3 membrane fraction since another of its well known substrates, bradykinin [23, 27, 42], was readily cleaved by this preparation and the reaction was inhibited completely by 1 μM phosphoramidon as we reported before [28]. To remove contaminating granular enzymes, the P_3 fraction was treated with hypotonic buffer, sonication and a high salt concentration, followed by resedimentation at 100,000 *g* to yield a washed P_3' membrane pellet (see Materials and Methods). These procedures markedly reduced the overall SP hydrolysis rate (by 68%) and changed the inhibition pattern to one which reflected a predominant role for NEP, as its inhibitors (phosphoramidon and *o*-phenanthroline) inhibited SP hydrolysis by 64–80% (Table 1). Nevertheless, reactions in the presence of cathepsin G inhibitors (Z-Gly-Leu-Phe-CH_2Cl, DFP, eglin) indicated that about 20–40% of the activity was still due to the presence of cathepsin G in the membrane fraction (Table 1). (Although chymostatin, an effective cathepsin G inhibitor, blocked 54% of the activity in the washed P_3' fraction, we found in control experiments with pure NEP that chymostatin was also a weak inhibitor of NEP—about 40–50% inhibition at the concentration used.) The results suggest that either the washed P_3' fraction still contained some trapped granules or lysed granular membranes or granules that had fused with plasma membranes contained some membrane-bound cathepsin G.

Neutrophil cytoplasts. To investigate the distribution of SP degrading activity further, neutrophil cytoplasts were isolated by treating neutrophils with cytochalasin B followed by high speed centrifugation over a Ficoll gradient [32]. Under these conditions, nuclei and granules sediment at the bottom of the tube, leaving neutrophil cytoplasts at the gradient interface devoid of intracellular organelles as shown by electron microscopy [23, 32]. Cytoplasts cleaved SP at a rate of 5.1 nmol/min/mg protein, mainly due to NEP activity on the cell membrane, since 1 μM phosphoramidon inhibited 76% of the activity while serine protease inhibitors were ineffective (not shown). In addition, the pattern of products produced (Leu-Met-NH_2, Phe-Gly, SP_{1-7}, SP_{1-9}) was similar to that obtained with purified NEP ([18]; Fig. 1). The granular material collected from the bottom of the tube was much more active, cleaving SP at a rate of 310 nmol/min/mg protein. The activity in this fraction had the same profile of inhibition as that in the P_2 granular fraction (not shown), again indicating the involvement of cathepsin G.

Purified cathepsin G. These results were further supported by experiments using purified human cathepsin G. The enzyme cleaved SP primarily at Phe^7-Phe^8 to release SP_{1-7} and SP_{8-11} (Figs. 1 and 4). A minor product peak eluting between the two major peaks was not positively identified, but it is likely to be SP_{1-8}, as its elution time was different from the standards for SP free acid, SP_{1-10} and SP_{1-9} (SP_{1-8} standard was not available). In addition, a small peak eluting at the same time as SP_{9-11} was seen when high SP concentrations were used for kinetic experiments. Based on the peak area, this product represented, at most, 21–27% of the SP_{1-7} produced at substrate concentrations ranging from 50 to 1500 μM. In kinetic studies, the K_m of SP for cathepsin G was 1.13 ± 0.17 mM, with a k_{cat} of $6.35 \pm 1.7\,sec^{-1}$ (N = 4). The specificity constant (k_{cat}/K_m) was $5639\,M^{-1}sec^{-1}$.

Because the K_m value of SP is rather high, thus making the physiological relevance of this reaction questionable, we incubated SP with purified cathepsin G (50 ng) for 10 min at much lower substrate

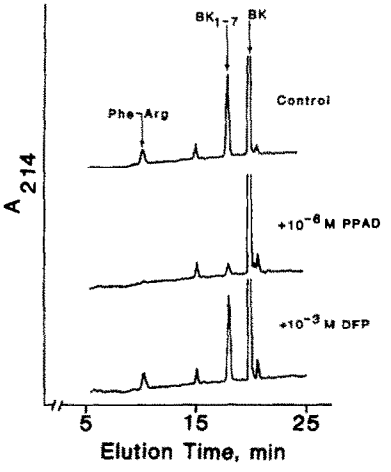

Fig. 5. Metabolism of bradykinin by human neutrophils. Bradykinin (BK; 100 μM) was incubated with 9×10^5 neutrophils for 30 min at 37° in the absence or presence of 10^{-6} M phosphoramidon (PPAD) or 10^{-3} M diisopropylfluorophosphate (DFP). Products were separated by HPLC and the arrows denote the elution of authentic standards.

concentrations (down to 1 nM) and measured remaining SP by radioimmunoassay. Based on amounts of intact SP recovered after incubations in the absence of enzyme, cathepsin G hydrolyzed in 10 min 25% of the peptide at 1 μM, 32% at 50 nM and 38% at 1 nM SP concentration (mean values from two to three separate experiments). These data indicate that neutrophil cathepsin G can hydrolyze SP at concentrations approaching physiological relevance.

Hydrolysis of bradykinin. Intact neutrophils cleaved bradykinin to produce bradykinin$_{1-7}$ and Phe^8-Arg^9-OH (Fig. 5) at a rate of 101 ± 19 pmol/min/10^6 cells (SEM, N = 6) (Fig. 6). No other significant products were detected; thus, the cells cleave bradykinin at the Pro^7-Phe^8 bond (Fig. 2). Both ACE and NEP hydrolyze this bond [15, 23, 27], but neutrophils contain only NEP on the plasma membrane [28, 30, 31] and not ACE. Phosphoramidon (1 μM) inhibited the reaction by 74%, whereas the inhibitor of serine proteases, 1 mM DFP, did not block bradykinin hydrolysis (Figs. 5 and 6). When cells were lysed by freeze–thawing, the rate of bradykinin hydrolysis did not increase, indicating that intracellular enzymes did not contribute to bradykinin degradation. Furthermore, neutrophil cytoplasts cleaved bradykinin in the same fashion as intact cells and 1 μM phosphoramidon markedly inhibited the reaction (Fig. 7). In addition, under conditions which resulted in significant SP hydrolysis, the P_2 granular fraction or purified cathepsin G did not cleave bradykinin.

Modulation of bradykinin inactivation. We reported recently that PMA down-regulates NEP activity on intact neutrophils by inducing endocytosis, followed by the intracellular inactivation of NEP, presumably by proteolytic enzymes in the endocytic vesicles [31]. When PMA was added to the cell suspension 5 min prior to the addition of bradykinin, the hydrolysis of bradykinin, depending on the concentration of PMA, dropped by 21% at

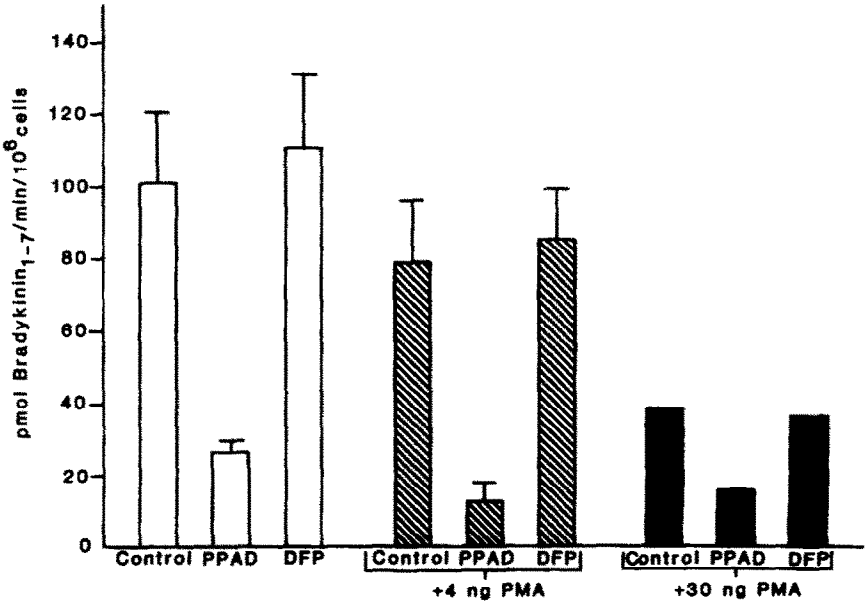

Fig. 6. Hydrolysis of bradykinin by human neutrophils in the presence and absence of added phorbol 12-myristate 13-acetate (PMA). Ordinate: activity expressed as bradykinin$_{1-7}$ released by 10^6 cells per min. Error bars denote the SEM for the reactions without PMA (N = 6) and in the presence of 4 ng PMA (N = 4). Results with 30 ng PMA are the averages of two experiments. PPAD = 10^{-6} M phosphoramidon; DFP = 10^{-3} M diisopropylfluorophosphate.

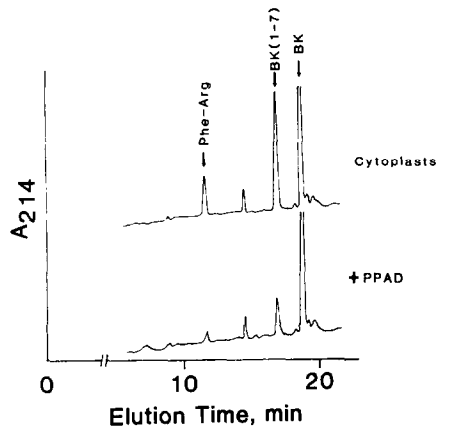

Fig. 7. Hydrolysis of bradykinin by human neutrophil cytoplasts. Neutrophil cytoplasts were prepared as described in the text and incubated for 60 min at 37° with 100 μM bradykinin (BK) in the absence (top) or presence (bottom) of 1 μM phosphoramidon (PPAD). The pattern of cleavage and inhibition by phosphoramidon indicate the primary involvement of NEP, as seen in the intact neutrophils (see Fig. 5).

40 ng/mL and 62% at 300 ng/mL (Fig. 6). This was taken as an indication that the activity of NEP on the cell surface was decreased by PMA-stimulated endocytosis. Enzymes released from neutrophils stimulated by PMA did not contribute significantly to the inactivation of bradykinin, because the pattern of enzymatic hydrolysis of the peptide, determined in HPLC, did not change (not shown). Moreover, the inactivation of bradykinin in the presence of PMA was still inhibited by phosphoramidon, but not by DFP (Fig. 6).

DISCUSSION

These experiments show that SP and bradykinin are both hydrolyzed by neutrophils, but by different enzymes. SP is a good substrate of NEP with quite favorable kinetics [18, 39]; in addition, in experiments *in vivo*, NEP effectively inactivates SP [22–26]. Although neutrophils contain NEP, this enzyme is not the major inactivator of SP in these cells. Most of the SP inactivator was found in a granular fraction, isolated from the neutrophils by centrifugation and identified as cathepsin G of the azurophil granules. These granules release cathepsin G but may also contain the enzyme in a form bound to membranes (or to other macromolecules which are membrane-associated), as stringent washing conditions did not remove all cathepsin G activity from the membrane fraction. This is consistent with previous studies where cathepsin G was resistant to solubilization [43–45], possibly as a result of binding to membranes or membrane-associated macromolecules. NEP became the major inactivator of SP only after the cells were depleted of their cathepsin G content by removal of the granules to form cytoplasts or when a membrane-enriched washed fraction isolated from lysed cells was used. Elastase, which is also present in azurophil granules [40, 41, 44, 45], does not play a role in the inactivation process.

The apparent inactivity of neutrophil NEP with SP is somewhat puzzling, especially considering the more favorable kinetics of SP hydrolysis [39] when compared with those of cathepsin G (K_m = 1.13 mM, k_{cat} = 6.35 sec^{-1}). Detailed kinetic studies have shown cathepsin G, in comparison with other enzymes, to have a generally low reactivity [46]. In fact, the k_{cat}/K_m for SP (5639 M^{-1} sec^{-1}) is similar to that of the best 4-nitroanilide (NA) substrate for cathepsin G (Suc-Val-Pro-Phe-NA; k_{cat}/K_m = 6900 M^{-1} sec^{-1}) and higher than that for the best extended peptide substrate (Ac-Ala-Ala-Pro-Phe-Ala-Ala-NH_2; k_{cat}/K_m = 1000 M^{-1} sec^{-1}) or angiotensin I (k_{cat}/K_m = 2070 M^{-1} sec^{-1}) [46]. In addition, cathepsin G cleaved SP at a significant rate at physiologically relevant SP concentrations (down to 1 nM). Moreover, cathepsin G may be much more active in its native environment or when bound to membranes or other macromolecules than in its purified, soluble form. Another factor which would favor cathepsin G over NEP in cleaving SP is the much higher concentration of cathepsin G in neutrophils. Calculations based on these and other studies [28] indicate that neutrophils contain about 2.0–2.8 × 10^4 molecules of NEP per cell, while the reported cathepsin G concentration ranges from about 300 to 3600 times higher, 8 × 10^6 to 7 × 10^7 molecules/cell [41, 47]. Thus, even partial release of cathepsin G from the cells would yield concentrations of the enzyme much higher than that of NEP and consequently lead to the predominant hydrolysis of SP by cathepsin G. This may have happened in our experiments. Even though the cells were not stimulated to release cathepsin G, neutrophils were isolated by a standard procedure [28, 31] which takes 2–4 hr and involves hypotonic shock to lyse contaminating red cells. Whether this would be the case *in vivo* is unknown, but at the sites of inflammation cathepsin G would undoubtedly be released.

SP interacts with inflammatory cells in various ways. Direct actions of SP on neutrophils, such as stimulating chemotaxis [2–5, 8], chemokinesis [9], oxidative metabolism [11–13], lysosomal enzyme release [2–5, 8, 12], and phagocytosis [10] require very high, non-physiological concentrations (10–300 μM). However, low concentrations of SP do stimulate neutrophil chemokinesis [9] and enhance their responsiveness to other mediators such as fMet-Leu-Phe and anaphylatoxin C5a [11, 14]. SP is also a potent stimulant of human T-lymphocyte proliferation *in vitro* [4, 48], and it releases interleukin 1 from a murine macrophage cell line [49] and from human blood monocytes [50]. In addition, SP is a vasodilator [6] and increases vascular permeability [1–6] and adherence of neutrophils to vessel walls [51, 52]. Some of the peripheral actions of SP were attributed to its stimulation of histamine release from mast cells [1–7, 53], including flare after intradermal injection [54].

In many organs, NEP is a major inactivator of SP. This has been especially well characterized in the lung where the NEP inhibitor, phosphoramidon,

potentiates the actions of SP in causing cough, bronchial smooth muscle contraction, and glandular mucus secretion [22, 24–26, 55, 56]. ACE can also cleave SP [18–20] and, in some model systems, ACE inhibitors clearly potentiate the actions of SP [19, 21, 22]. Although many other enzymes have been shown to cleave SP, their physiological importance is unclear at this time.

Cathepsin G cleaves peptides after an aromatic amino acid (preferably Phe) and after aliphatic amino acids with large hydrophobic side chains [46]. Consistent with this specificity, it hydrolyzes SP primarily between the two aromatic residues Phe^7-Phe^8 (Fig. 2). Cathepsin G is related to mast cell chymase and its substrate specificity somewhat resembles that of chymotrypsin [38, 46]. It was noticed early on [57] that crude SP was rapidly inactivated by chymotrypsin and a recent publication (noted after the conclusion of most of the experiments presented in this report) described that chymase from dog mastocytoma cleaves SP also at the Phe^7-Phe^8 bond [58].

Bradykinin shares many properties with SP. It is a vasodilator, enhances vascular permeability, and is a potent bronchoconstrictor [59, 60]. Bradykinin is involved in inflammatory processes, such as neurogenic inflammation of the respiratory tract, and it is nociceptive as it stimulates nerve endings in C-fibers [61]. In contrast to the results we obtained with SP, bradykinin was inactivated by the NEP on the neutrophil plasma membrane. Although bradykinin is hydrolyzed by chymotrypsin [62] and chymase of mast cells [63], cathepsin G cleaves bradykinin only very slowly [63] or not at all [64]. Under the conditions of our assays, we detected no hydrolysis of bradykinin by either cathepsin G or by the granular fraction of neutrophils. In any case, the activity reported for cathepsin G was only 0.022 μmol/min/mg with 1.49 mM bradykinin [63]. With purified human NEP [27] bradykinin has a K_m of 120 μM and a k_{cat} of 4770 min^{-1} that yields a specific activity of 49 μmol/min/mg with 1.49 mM bradykinin, over 2000-fold faster than cathepsin G. In other experiments using inhibitors *in vivo*, NEP was a major inactivator of bradykinin released intrarenally [29]. The present studies indicate that neutrophil NEP could inactivate bradykinin *in vivo*, especially at sites of inflammation.

Of the other type of blood cells, mature lymphocytes seem to be devoid of NEP, but lymphoblasts in acute lymphoblastic leukemia contain a high concentration of this enzyme where it is also called the common acute lymphoblastic leukemia antigen or CALLA [65].

The present study also shows that PMA decreased the inactivation of bradykinin by NEP of neutrophils, very likely by down-regulation of NEP on the cell surface caused by endocytosis [31]. Diacylglycerol has an effect similar to that of PMA [31]; thus it is possible that agents which stimulate phosphatidylinositol-specific phospholipase C (liberating diacylglycerol) could down-regulate NEP levels on neutrophils, thus reducing inactivation of bradykinin and possibly other peptides.

In conclusion, SP and bradykinin, two peptides frequently involved in inflammatory reactions, were readily cleaved by isolated human neutrophils. SP was inactivated primarily by the granular cathepsin G and bradykinin by NEP on the cell membrane.

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