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Pain inhibition by blocking leukocytic and neuronal opioid peptidases in peripheral inflamed tissue

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ABSTRACT **Inflammatory pain can be controlled by endogenous opioid peptides. Here we blocked the degradation of opioids in peripheral injured tissue to locally augment this physiological system. In rats with hindpaw inflammation, inhibitors of aminopeptidase N (APN; bestatin) or neutral endopeptidase (NEP; thiorphan), and a dual inhibitor, NH_2-CH-Ph-P(O)(OH)CH_2-CH-CH_2Ph(p-Ph)-CONH-CH-CH_3-$COOH^-$ (P8B), were applied to injured paws. Combined bestatin (1.25–5 mg)/thiorphan (0.2–0.8 mg) or P8B (0.0625–1 mg) alone elevated mechanical nociceptive thresholds to 307 and 227% of vehicle-treated controls, respectively. This analgesia was abolished by antibodies to methionine-enkephalin, leucine-enkephalin, and dynorphin A 1–17, by peripherally restricted and by selective μ-, δ-, and κ-opioid receptor antagonists. Flow cytometry and photospectrometry revealed expression and metabolic activity of APN and NEP on macrophages, granulocytes, and sciatic nerves from inflamed tissue. Radioimmunoassays showed that inhibition of leukocytic APN and NEP by bestatin (5–500 μM)/thiorphan (1–100 μM) combinations or by P8B (1–100 μM) prevented the degradation of enkephalins. Blockade of neuronal peptidases by bestatin (0.5–10 mM)/thiorphan (0.1–5 mM) or by P8B (0.1–10 mM) additionally hindered dynorphin A 1–17 catabolism. Thus, leukocytes and peripheral nerves are important sources of APN and NEP in inflamed tissue, and their blockade promotes peripheral opioid analgesia.—Schreiter, A., Gore, C., Labuz, D., Fournie-Zaluski, M.-C., Roques, B. P., Stein, C., Machelska, H. Pain inhibition by blocking leukocytic and neuronal opioid peptidases in peripheral inflamed tissue. *FASEB J.* 26, 000–000 (2012). www.fasebj.org**

Key Words: immune cells · sensory nerves · analgesia

Inflammatory pain is often refractory to conventional treatments. In addition, current opioid analgesics produce nausea, respiratory depression, paradoxical hyperalgesia, tolerance, and addiction, while steroidal and nonsteroidal anti-inflammatory drugs (NSAIDs) induce gastrointestinal ulcers, bleeding, and serious cardiovascular complications (1–3). However, the activation of opioid receptors on peripheral sensory neurons can inhibit pain without central or systemic adverse effects. This can be achieved by exogenous synthetic opioids or by endogenous opioid peptides, such as β-endorphin (END), enkephalins (ENKs), and dynorphin A 1–17 (DYN) derived from immune cells or peripheral sensory neurons (4). Notably, the concentrations of endogenous opioids are elevated in inflamed tissue, and the number of opioid-producing leukocytes, their opioid content, and their analgesic effects become more pronounced with the progression of inflammation (5–10). These findings are of clinical relevance, since human postoperative pain is exacerbated by interrupting the interaction between endogenous opioids and their peripheral receptors with naloxone (11), and is diminished by stimulating opioid secretion (12). Advantages of targeting endogenous agonists include reduced tolerance, receptor down-regulation, desensitization, and offsite or paradoxical excitatory effects due to unphysiologically high exogenous agonist concentrations at the receptor (4, 13, 14).

However, endogenous opioid peptides are susceptible to rapid enzymatic inactivation. The best-characterized enzymes are aminopeptidase N (APN or CD13; EC 3.4.11.2) and neutral endopeptidase (NEP; neprilysin

Abbreviations: Ab, antibody; Ala-βNA, L-Ala-β-naphthylamine; APN, aminopeptidase N; βNA, β-naphthylamine; CGRP, calcitonin gene-related peptide; CNS, central nervous system; CTOP, D-Phe-Cys-Tyr-D-Trp-Orn-Thr-Pen-Thr-NH_2; DMSO, dimethyl sulfoxide; DYN, dynorphin A 1–17; END, β-endorphin; ENK, enkephalin; ICI 174,864, *N,N*-diallyl-Tyr-Aib-Aib-Phe-Leu; i.pl., intraplantar; Leu-ENK, leucine-enkephalin; Met-ENK, methionine-enkephalin; NEP, neutral endopeptidase; NLXM, naloxone methiodide; norBNI, norbinaltorphimine dihydrochloride; NSAID, nonsteroidal anti-inflammatory drug; P8B, NH_2-CH-Ph-P(O)(OH)CH_2-CH-CH_2Ph(p-Ph)-CONH-CH-CH_3-$COOH^-$; PBS, phosphate-buffered saline; pNA, p-nitroaniline; RIA, radioimmunoassay; RM, repeated measurements; Suc-Ala-Ala-Phe-pNA, succinyl-alanin-alanin-phenylalanin-p-nitroaniline

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or CD10; EC 3.4.24.11) (13–15). Both are expressed in the central nervous system (CNS), peripheral nerves, and leukocytes (16–20); among opioid peptides, ENKs are considered their preferred substrates, as shown in brain and kidney preparations *in vitro* (15, 21, 22). The actions of APN and NEP are complementary, and their concomitant blockade is most efficient. Thus, the central or systemic administration of combined individual or of dual inhibitors simultaneously inactivating both peptidases alleviated pain involving ENKs (23–31). Although cleavage of END and DYN by APN and/or NEP was shown *in vitro* (32–34), these peptides have not been directly examined *in vivo*. Peripheral analgesic actions of peptidase inhibitors were also suggested (31, 35, 36), but the contributing peptides and the cellular sources of the relevant enzymes remain enigmatic.

Here we aimed to exploit the prevention of opioid peptide degradation in inflamed tissue for the local control of pain. To inactivate peptidases, we used selective inhibitors blocking either APN (bestatin; K_i=4 μM) or NEP (thiorphan; K_i=4.7 nM) (15, 37), and a dual inhibitor (P8B) that contains phosphinic moieties providing high affinity and selectivity for APN (K_i=4.8 nM) and NEP (K_i=2 nM) compared to related peptidases (*e.g.*, angiotensin-converting enzyme; K_i=2.5 μM; refs. 38, 39). In a clinically relevant model of inflammatory pain, we examined the relative contribution of methionine (Met)-ENK, leucine (Leu)-ENK, DYN, and END, and of different types of peripheral opioid receptors (μ, δ, κ) to the inhibitor-induced analgesic effects. We also sought to identify the cellular sources of peptidases and their catabolic preferences toward the different opioid peptides. We hypothesized that APN and NEP are expressed and metabolically active on immune cells and peripheral nerves in inflamed tissue, and that blockade of these peptidases can locally suppress pain.

MATERIALS AND METHODS

Animals

Experiments were approved by the State Animal Care Committee (Landesamt für Gesundheit und Soziales, Berlin). Male Wistar rats (250–300 g; Charité-Universitätsmedizin, Berlin) received intraplantar (i.pl.) complete Freund's adjuvant (150 μl; Calbiochem, La Jolla, CA, USA) in the right hindpaw under isoflurane (Abbott, Wiesbaden, Germany) anesthesia. Animals were kept on a 12-h light-dark schedule with food and water *ad libitum*. Room temperature was 22 ± 0.5°C and humidity 60–65%. All experiments were performed 4 d after induction of inflammation. Animals were killed with isoflurane.

Behavioral experiments

Nociceptive thresholds were assessed using the paw pressure algesiometer (modified Randall-Selitto test; Ugo Basile, Comerio, Italy), as described previously (9). Briefly, the hindpaw pressure threshold (cutoff at 250 g) required to elicit paw withdrawal was determined by averaging three consecutive trials separated by 15-s intervals. Paw volume (edema) was assessed with a plethysmometer (Ugo Basile) by averaging two consecutive trials measuring the displacement volume (in milliliters; ref. 9). The sequence of paws was alternated between animals to avoid "order" effects.

Nociceptive threshold and paw volume were measured before and between 5 min and 24 h after i.pl. coinjection of bestatin (5 mg) and thiorphan (0.8 mg) (Sigma-Aldrich, Taufkirchen, Germany) or after i.pl. P8B (1 mg; Université Paris Descartes, Paris, France) applied to inflamed or noninflamed hindpaws. Dose dependency of analgesic effects of bestatin (1.25–5 mg) and thiorphan (0.2–0.8 mg) combinations or of P8B (0.0625–1 mg), and their blockade by opioid peptide antibodies (Abs; Bachem, Weil am Rhein, Germany) or opioid receptor antagonists (Sigma-Aldrich) were assessed 5 min after injection into inflamed paws. The following antagonists were used: D-Phe-Cys-Tyr-D-Trp-Orn-Thr-Pen-Thr-NH_2 (CTOP; μ receptors), *N,N*-diallyl-Tyr-Aib-Aib-Phe-Leu (ICI 174,864; δ receptors), nor-binaltorphimine dihydrochloride (norBNI; κ receptors) and naloxone methiodide (NLXM), a peripherally restricted opioid receptor antagonist. Bestatin (5 mg) and thiorphan (0.8 mg) or P8B (1 mg) were coinjected either with control IgG (2 μg), anti-Met-ENK (T-4293; 0.5–2 μg), anti-Leu-ENK (T-4289; 0.125–0.5 μg), anti-DYN (T-4267; 0.125–1 μg), anti-END (T-4044; 0.5–2 μg), CTOP (0.125–1 μg), ICI 174,864 (0.625–2.5 μg), norBNI (6.25–50 μg), or NLXM (25–100 μg). Nociceptive thresholds were also tested 5 min following i.pl. anti-Met-ENK (2 μg), anti-Leu-ENK (0.5 μg), anti-DYN (1 μg), anti-END (1 μg), or NLXM (100 μg) alone. Bestatin and thiorphan were dissolved in dimethyl sulfoxide (DMSO; maximum concentration 5%; Sigma-Aldrich) with water and were diluted with 0.9% NaCl. All other substances were dissolved in water and diluted with 0.9% NaCl. Control groups were treated with the respective vehicles. Injection volume was 100–150 μl. Six to 8 animals/group were used. The experimenters were blinded to the treatments.

Tissue preparation

To obtain single-leukocyte suspensions, the plantar subcutaneous tissue was dissected from inflamed paws, cut into 1- to 2-mm pieces, and digested for 1 h at 37°C in RPMI buffer (Life Technologies, Paisley, Scotland) containing collagenase, hyaluronidase and HEPES (all from Sigma-Aldrich). The digested fragments were pressed through a 70-μm nylon filter (BD, Heidelberg, Germany), as described previously (7). To obtain leukocyte membranes, the cells were homogenized and centrifuged (15,000 *g*, 15 min at 4°C) to obtain a granular pellet and the supernatant containing membranes. The supernatant was centrifuged (48,000 *g*, 30 min at 4°C) to obtain a solid membrane fraction (40). The membranes were reconstituted in RPMI buffer (pH 7.4). Rat kidney membrane suspensions [from one kidney in phosphate buffered saline (PBS); 2 ml, 0.1 M, pH 7.4] were prepared in a similar way and used as positive controls for measuring peptidase activities (see below).

To obtain peripheral nerve suspensions, the terminal sciatic nerve branches (tibial, sural, and common peroneal nerves) innervating the plantar surface of inflamed or noninflamed paws were dissected and cut into small pieces, which were homogenized in RPMI buffer (300 μl) using ×10 power 10% pulsed ultrasound. The isolated nerves were ~2 cm long, measured from the middle of the plantar surface toward the ankle.

Flow cytometry

Single-cell suspensions prepared from inflamed paws (see above) were stained with mouse anti-rat CD45 phytoerythrin-

cyanine dye 5-conjugated mAb (12 μg/ml; BD) to label hematopoietic cells. To investigate peptidase expression, the cells were stained with fluorescein isothiocyanate-conjugated mouse anti-human mAbs crossreacting with rat CD13 (10 μg/ml) to identify APN, or with CD10 (10 μg/ml) to identify NEP (both Abs from Santa Cruz, Heidelberg, Germany), and with phytoerythrin-conjugated mouse anti-rat mAbs recognizing T lymphocytes (CD3; 12 μg/ml; Bachem) or macrophages (CD163; 5 μl/10^6 cells; Serotec, Oxford, UK). For intracellular stains, cells were permeabilized (7) and incubated with phytoerythrin-conjugated mouse anti-rat mAb recognizing granulocytes (RP-1; 6 μg/ml; BD). The staining specificity was verified by replacing primary Abs with appropriate isotype-matched control Abs. Absolute cell numbers were calculated using Tru-Count tubes (BD) with known numbers of fluorescent beads. Data were acquired using a FACS Calibur and analyzed using the CellQuest software (all from BD) (7).

Peptidase activity assays

APN activity was determined using L-Ala-β-naphthylamine (Ala-βNA; 200 μM dissolved in DMSO and diluted with PBS, 1:100; Sigma-Aldrich) as a substrate (41). The metabolite β-naphthylamine (βNA) was visualized using fast blue B salt (200 μl; 1.5 mg/ml of PBS with 10% TWEEN-20, pH 4.2; Sigma) and measured photospectrometrically at 530 nm according to the standard curve of βNA (Sigma-Aldrich). Suspensions of leukocyte membranes (from 1.5×10^6 cells) or of nerves (50 μl) (see above) were incubated with Ala-βNA and vehicles, bestatin (0.5 μM to 15 mM) or P8B (0.001 μM to 10 mM) for 4 h.

NEP activity was measured using succinyl-alanin-alanin-phenylalanin-p-nitroaniline (Suc-Ala-Ala-Phe-pNA; 100 μM in PBS; Bachem) as a substrate (40). Its metabolite p-nitroaniline (pNA) was assessed photospectrometrically at 405 nm according to the standard curve of pNA (Sigma-Aldrich). Suspensions of leukocyte membranes (from 2.5×10^6 cells) or nerves (50 μl) were incubated with Suc-Ala-Ala-Phe-pNA and vehicles, thiorphan (0.001 μM to 8 mM) or P8B (0.001 μM to 10 mM) for 4 h.

Leukocyte membrane or nerve suspensions were preincubated with vehicles or inhibitors for 15 min at room temperature before adding peptidase substrates, and the incubation was continued (for 4 h) at 37°C. Metabolites were measured in the supernatants (500 μl), and their amounts are expressed in micromoles per 10^6 leukocyte membranes per 4 h or in millimoles per gram of wet nerve tissue per 4 h. Supernatants from kidney membrane suspensions (100 μl) and PBS were used as positive and negative controls, respectively. To assess whether peptidase inhibitors interfered with photospectrometric measurements, bestatin (50 μM to 15 mM) and thiorphan (1 μM to 8 mM), or P8B (1 μM to 10 mM), without APN and NEP substrates, were tested accordingly. The selectivity of bestatin and thiorphan was examined by switching peptidase substrates. Leukocytes (1.5×10^6) were incubated with Ala-βNA and thiorphan (10 mM) or with Suc-Ala-Ala-Phe-pNA and bestatin (10 mM). All measurements were done in duplicates. Each experiment was performed 4–11 times.

Opioid peptide degradation

Freshly prepared suspensions of membranes from 5×10^6 leukocytes (250 μl) or peripheral nerves (50 μl) were incubated with vehicles, bestatin (5 μM to 10 mM) and thiorphan (1 μM to 5 mM), or P8B (1 μM to 10 mM) for 15 min at 37°C. Then, 100 μl of either Met-ENK (22.3 nM), Leu-ENK (2.3 nM), DYN (0.6 nM), or END (0.4 nM) [from the corresponding radioimmunoassay (RIA) kits; see below] was added to obtain a total volume of 500 μl for leukocyte membranes, or 300 μl for nerves. The samples were centrifuged (760 *g*, 5 min at 4°C) immediately after addition of opioid peptides (0 h) or after 4 h of incubation with peptidase inhibitors. Subsequently, the supernatants were collected and stored at −20°C until RIA measurements. Opioid peptide immunoreactivities were determined in the supernatants (100 μl) using RIA kits [Met-ENK, Leu-ENK, and DYN (Bachem); and END (Phoenix, Belmont, CA, USA)], analogously to the procedure described for END (6). The samples were measured in duplicates, and each experiment was performed 3–8 times. In pilot experiments, we established amounts of leukocytes and nerve suspensions required for reliable measurements of opioid peptide degradation and peptidase activities.

To confirm the specificity of opioid peptide Abs, each Ab was tested with RIA using samples containing either Met-ENK (22.3 nM), Leu-ENK (2.3 nM), DYN (0.6 nM), or END (0.4 nM) to examine whether Abs specifically detect their respective opioid peptides; samples containing opioid peptide metabolite analogs H-Gly-Gly-Phe-Leu-OH, H-Gly-Gly-Phe-Met-OH, and H-Tyr-Gly-Gly-OH (10 ng/ml for Met-ENK or 1 ng/ml for Leu-ENK, DYN, and END; Bachem) to assess whether opioid peptide Abs recognize their metabolites; and samples containing bestatin (5 μM to 10 mM) and thiorphan (1 μM to 5 mM) or P8B (1 μM to 10 mM) to test whether opioid peptide Abs recognize peptidase inhibitors.

Statistical analysis

Data are expressed as means ± SE. Two-sample comparisons were made using the *t* test for independent normally distributed data, Mann-Whitney test for independent non-normally distributed data, paired *t* test for dependent normally distributed data, and Wilcoxon test for dependent non-normally distributed data. Multiple comparisons at one time point were evaluated with 1-way ANOVA for normally distributed data or with Kruskal-Wallis 1-way ANOVA on ranks for non-normally distributed data. Multiple comparisons in repeated measurements (RMs) were assessed with 1-way RM ANOVA for normally distributed data or with Friedman RM ANOVA on ranks for non-normally distributed data. *Post hoc* comparisons were performed using the Bonferroni *t* test, Dunn test, or Newman-Keuls test. Effects on paw volume were determined by the area under the curve followed by 2-sample comparisons, as described above. Dose dependency was evaluated with linear regression. Differences were considered significant at values of $P < 0.05$.

RESULTS

APN and NEP inhibitors produce analgesia mediated by ENKs and dynorphin *via* peripheral opioid receptors in inflamed tissue

At 4 d after induction of unilateral hindpaw inflammation, rats developed ipsilateral hyperalgesia manifested by decreased mechanical thresholds, as compared to contralateral noninflamed paws ($P<0.05$; **Fig. 1*A*, *B***). We have previously shown that contralateral paw thresholds are comparable to thresholds before induction of inflammation (42). At 5 min after i.pl. application of Abs neutralizing either Met-ENK (2 μg), Leu-ENK (0.5 μg), DYN (1 μg), or END (1 μg), the

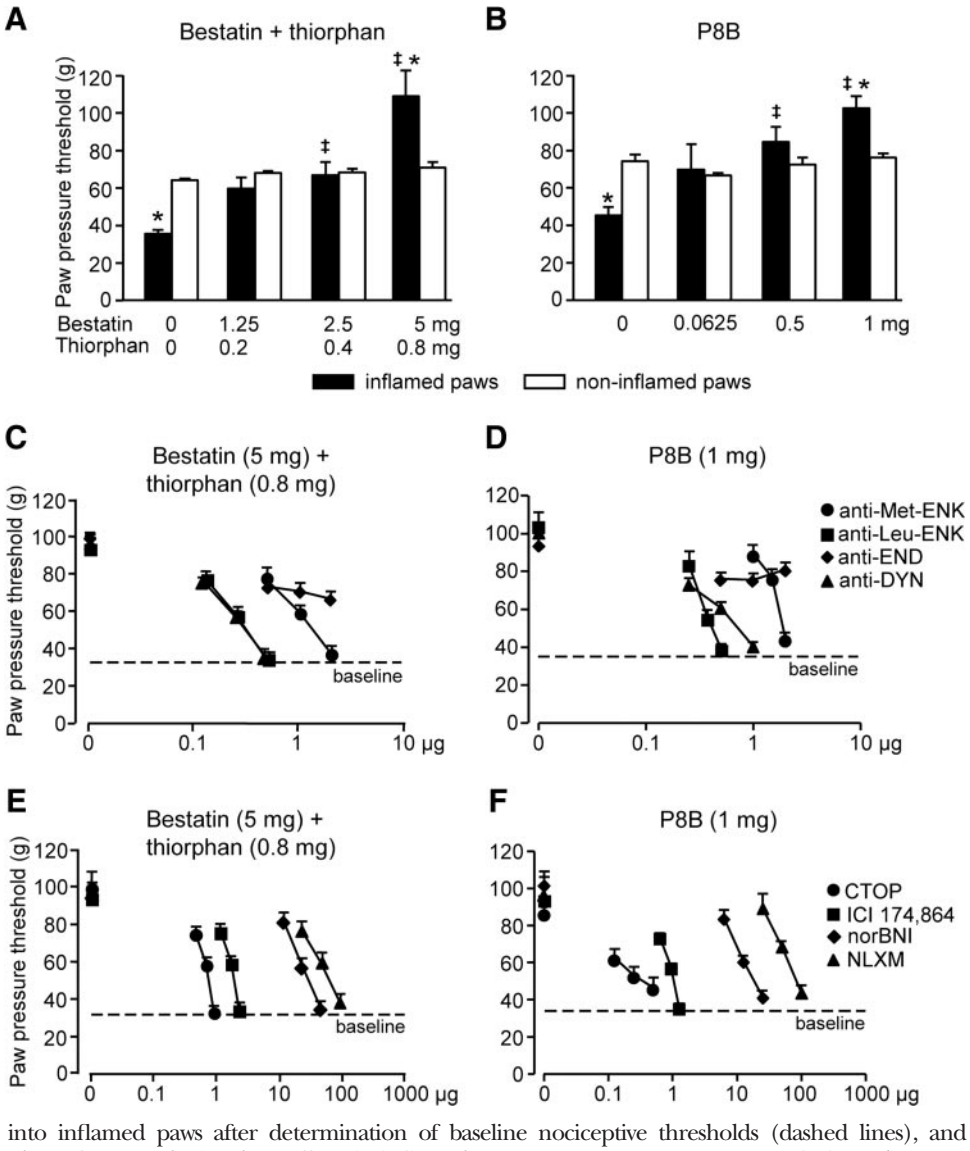

Figure 1. Contribution of opioid peptides and receptors to analgesia produced by APN and NEP inhibitors. *A*, *B*) Dose-dependent analgesic effects in inflamed paws of bestatin (1.25–5 mg) and thiorphan (0.2–0.8 mg) injected simultaneously (*A*), and of P8B (0.0625–1 mg; *B*) ($P<0.001$, linear regression). *$P < 0.05$ *vs.* corresponding noninflamed paws; paired *t* test. ‡$P < 0.05$ *vs.* inflamed paws of controls; 1-way ANOVA, Bonferroni *t* test. *C*, *D*) Analgesic effects of combined bestatin (5 mg) and thiorphan (0.8 mg) (*C*), and of P8B (1 mg; *D*) were dose-dependently and fully blocked by anti-Met-ENK (0.5–2 µg), anti-Leu-ENK (0.125–0.5 µg), and anti-DYN (0.125–1 µg) ($P<0.001$, linear regression). Anti-END (0.5–2 µg) dose-dependently attenuated the effect of bestatin and thiorphan ($P<0.001$, linear regression) and significantly ($P<0.05$, 1-way ANOVA, Bonferroni *t* test) but not dose-dependently ($P>0.05$, linear regression) attenuated the effect of P8B, although the attenuation was only partial. *E*, *F*) Analgesic effects of combined bestatin (5 mg) and thiorphan (0.8 mg) (*E*), and of P8B (1 mg; *F*) were dose-dependently blocked by selective antagonists at µ (CTOP; 0.125–1 µg)-, δ (ICI 174,864; 0.625–2.5 µg)-, and κ (norBNI; 6.25–50 µg)-opioid receptors, and by the peripherally restricted antagonist NLXM (25–100 µg) ($P<0.001$, linear regression). Antibodies or antagonists were coinjected with peptidase inhibitors into inflamed paws after determination of baseline nociceptive thresholds (dashed lines), and measurements were performed 5 min thereafter. Panels *C–F* show effects in inflamed paws. Data are means ± SE; $n = 6–8$ rats/group.

thresholds of inflamed paws were further decreased to 18 ± 1, 24 ± 2.7, 20 ± 1, and 27 ± 2.9 g, respectively, as compared to i.pl. control IgG (2 µg) (35±1.7 g; $P<0.05$). A similar effect was produced by i.pl. NLXM (100 µg), a peripherally restricted opioid receptor antagonist (18±2 *vs.* 32±1 g; NLXM *vs.* control; $P<0.05$).

In contrast, concomitant i.pl. injection of bestatin (1.25–5 mg) and thiorphan (0.2–0.8 mg) or application of P8B (0.0625–1 mg) alone into inflamed paws dose dependently elevated nociceptive thresholds ($P<0.001$; Fig. 1*A*, *B*). For the highest inhibitor doses, these effects significantly exceeded the thresholds of noninflamed paws ($P<0.05$; Fig. 1*A*, *B*). These analgesic effects peaked at 5 min (Fig. 1) and returned to baseline at 15 min following injections (data not shown). There were no changes in nociceptive thresholds of contralateral noninflamed paws ($P>0.05$; Fig. 1*A*, *B*). Paw volume of inflamed paws (1.6–1.7 ml) was not affected by i.pl. vehicles or peptidase inhibitors ($P>0.05$; data not shown). The volume of noninflamed paws (0.9–1 ml), as evaluated by the area under the curve over 24 h, was slightly increased by i.pl. vehicle (5% DMSO; 27±0.4 *vs.* 25±0.5; injected *vs.* contralateral paws; $P<0.05$) and by combined bestatin (5 mg) and thiorphan (0.8 mg) (27±0.4 *vs.* 30±0.2; vehicle *vs.* inhibitors; $P<0.05$), but not by P8B (1 mg) (25±0.4 *vs.* 24±0.8; injected *vs.* contralateral paws) or its corresponding vehicle (0.9% NaCl; 23±0.6 *vs.* 22±0.3; injected *vs.* contralateral paws; $P>0.05$).

Analgesia produced by the most effective doses of simultaneously applied bestatin (5 mg) and thiorphan (0.8 mg) or of P8B (1 mg) was dose-dependently and fully blocked by anti-Met-ENK (0.5–2 µg), anti-Leu-ENK (0.125–0.5 µg), and anti-DYN (0.125–1 µg) ($P<0.001$; Fig. 1*C*, *D*). Anti-END (0.5–2 µg) dose-dependently attenuated the effect of bestatin and thiorphan ($P<0.001$) and significantly ($P<0.05$) but not dose-dependently ($P>0.05$) reduced the effect of P8B. In both cases, anti-END only partially (by 14–33%) attenuated the inhibitor-induced analgesia (Fig. 1*C*, *D*). Analgesic effects of a combination of bestatin (5 mg)

and thiorphan (0.8 mg) or of P8B (1 mg) were dose-dependently abolished by selective antagonists at μ- (CTOP; 0.125–1 μg), δ- (ICI 174,864; 0.625–2.5 μg), and κ-opioid receptors (norBNI; 6.25–50 μg) as well as by NLXM (25–100 μg) ($P<0.001$; Fig. 1*E, F*). None of these treatments altered nociceptive thresholds of contralateral noninflamed paws ($P>0.05$; data not shown). Together, these data indicate that opioid peptides are tonically released in inflamed tissue. The local blockade of APN and NEP produces analgesia mediated predominately by Met-ENK, Leu-ENK, and DYN acting at peripheral μ-, δ- and κ-opioid receptors.

Blockade of APN and NEP on leukocytes infiltrating inflamed tissue prevents the degradation of ENKs

We then examined leukocytes as a source of peptidases degrading opioids. Single-cell suspensions from inflamed paws were stained with anti-CD45 to gate on hematopoietic cells by flow cytometry. Macrophages constituted 31% of $CD45^+$ cells, followed by granulocytes (17%) and T lymphocytes (4%). The remaining 48% of cells might represent monocytes, dendritic cells, natural killer cells, and B lymphocytes, all expressing CD45 (43). The absolute numbers of macrophages was higher than granulocytes ($P<0.05$), and T lymphocytes were less abundant than granulocytes and macrophages ($P<0.05$; **Fig. 2*A***). Among $CD45^+$ cells, 23% were APN-positive and 12% were NEP-positive. Triple-color flow cytometry showed that 24% of macrophages expressed APN, and 37% expressed NEP. Among granulocytes, 15% expressed APN, and 14% expressed NEP. Neither enzyme was detected on T lymphocytes (Fig. 2*A*). Minimal staining (0–3%) by the respective isotype-matched control Abs confirmed specificity of Abs to peptidases and leukocyte subpopulations (data not shown).

To verify enzymatic activity and specificity of leukocytic APN and NEP, we first assessed the metabolism of their specific synthetic substrates by photospectrometry in membrane suspensions from leukocytes infiltrating inflamed paws. There was no substrate hydrolysis at baseline in any experiments (data not shown). After 4 h of incubation with the APN substrate Ala-βNA, its metabolite βNA accumulated in inhibitor-free control groups. Bestatin (0.5–50 μM) or P8B (0.001–0.1 μM) concentration-dependently prevented the generation of βNA ($P<0.01$;

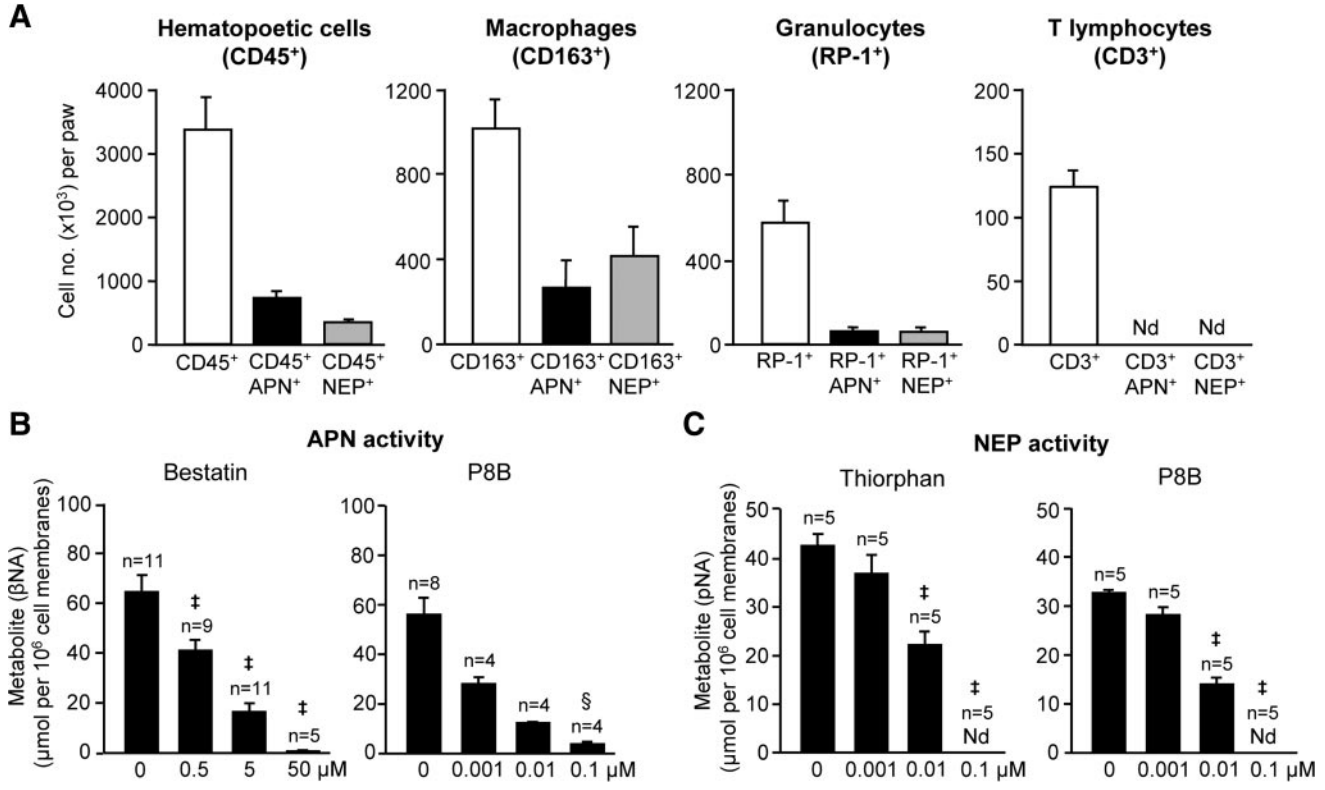

Figure 2. Leukocytic expression of APN and NEP, and blockade of their enzymatic activities by peptidase inhibitors. *A*) Flow cytometry showing expression of APN and NEP in hematopoietic ($CD45^+$) cells including macrophages ($CD163^+$) and granulocytes ($RP\text{-}1^+$), but not T lymphocytes ($CD3^+$), from inflamed paws ($n=7$ rats). *B*) Hydrolysis of APN-specific substrate Ala-βNA (200 μM) to metabolite βNA was concentration-dependently prevented by bestatin (0.05–50 μM) or P8B (0.001–0.1 μM) in leukocyte membrane suspensions ($P<0.01$, linear regression). *C*) Hydrolysis of NEP-specific substrate Suc-Ala-Ala-Phe-p-nitroaniline (100 μM) to metabolite pNA was concentration-dependently prevented by thiorphan (0.001–0.1 μM) or P8B (0.001–0.1 μM) in leukocyte membrane suspensions ($P<0.001$, linear regression). Inhibitors were incubated 15 min with the membranes, then peptidase substrates were added and photospectrometry was performed 4 h later (*B, C*). Data are means ± SE. *n*, number of experiments per group; Nd, not detected. $^{‡}P < 0.05$ *vs.* control group; 1-way RM ANOVA, Bonferroni *t* test. $^{§}P < 0.05$ *vs.* control group; Friedman RM ANOVA on ranks, Dunn test.

Fig. 2*B*). Incubation with the NEP substrate Suc-Ala-Ala-Phe-pNA led to elevated concentrations of its metabolite pNA in control groups. This pNA production was concentration-dependently blocked by thiorphan (0.001–0.1 μM) or P8B (0.001–0.1 μM) ($P<0.001$; Fig. 2*C*).

To assess the relative catabolic preferences of leukocytic APN and NEP toward different opioid peptides, we incubated leukocyte membranes with Met-ENK (22.3 nM), Leu-ENK (2.3 nM), DYN (0.6 nM), or END (0.4 nM) in the absence or presence of concomitantly applied bestatin (5–500 μM) and thiorphan (1–100 μM), or of P8B (1–100 μM). At baseline, there were no differences in opioid peptide concentrations between controls and inhibitor-treated preparations, measured by RIA ($P>0.05$; data not shown). At 4 h of incubation, decreased amounts of Met-ENK (by 41–73%; **Fig. 3*A, E***), Leu-ENK (by 77%; Fig. 3*B, F*), DYN (by 46–47%; Fig. 3*C, G*), and END (by 37–39%; Fig. 3*D, H*) ($P<0.05$) were observed in control groups. Thiorphan and bestatin concentration-dependently prevented the degradation of Met-ENK ($P<0.001$; Fig. 3*A*) and Leu-ENK ($P<0.05$; Fig. 3*B*), as did P8B ($P<0.05$; Fig. 3*E, F*). None of the inhibitors altered the levels of DYN or END ($P>0.05$; Fig. 3*C, D, G, H*). Collectively, these findings indicate that in inflamed tissue, granulocytes and macrophages express active APN and NEP, which degrade Met-ENK and Leu-ENK. Blockade of both peptidases with single and dual inhibitors prevented the catabolism of specific substrates and both ENKs, but not of DYN and END.

Blockade of APN and NEP on peripheral nerves innervating inflamed tissue prevents the degradation of ENKs and dynorphin

We next examined the catabolic activity of APN and NEP on peripheral sensory nerves innervating inflamed and noninflamed paws. We incubated suspensions of peripheral branches of the sciatic nerve with the peptidase-specific synthetic substrates. Photospectrometry showed no substrate hydrolysis at baseline in any experiment (data not shown). Following 4 h incubation of

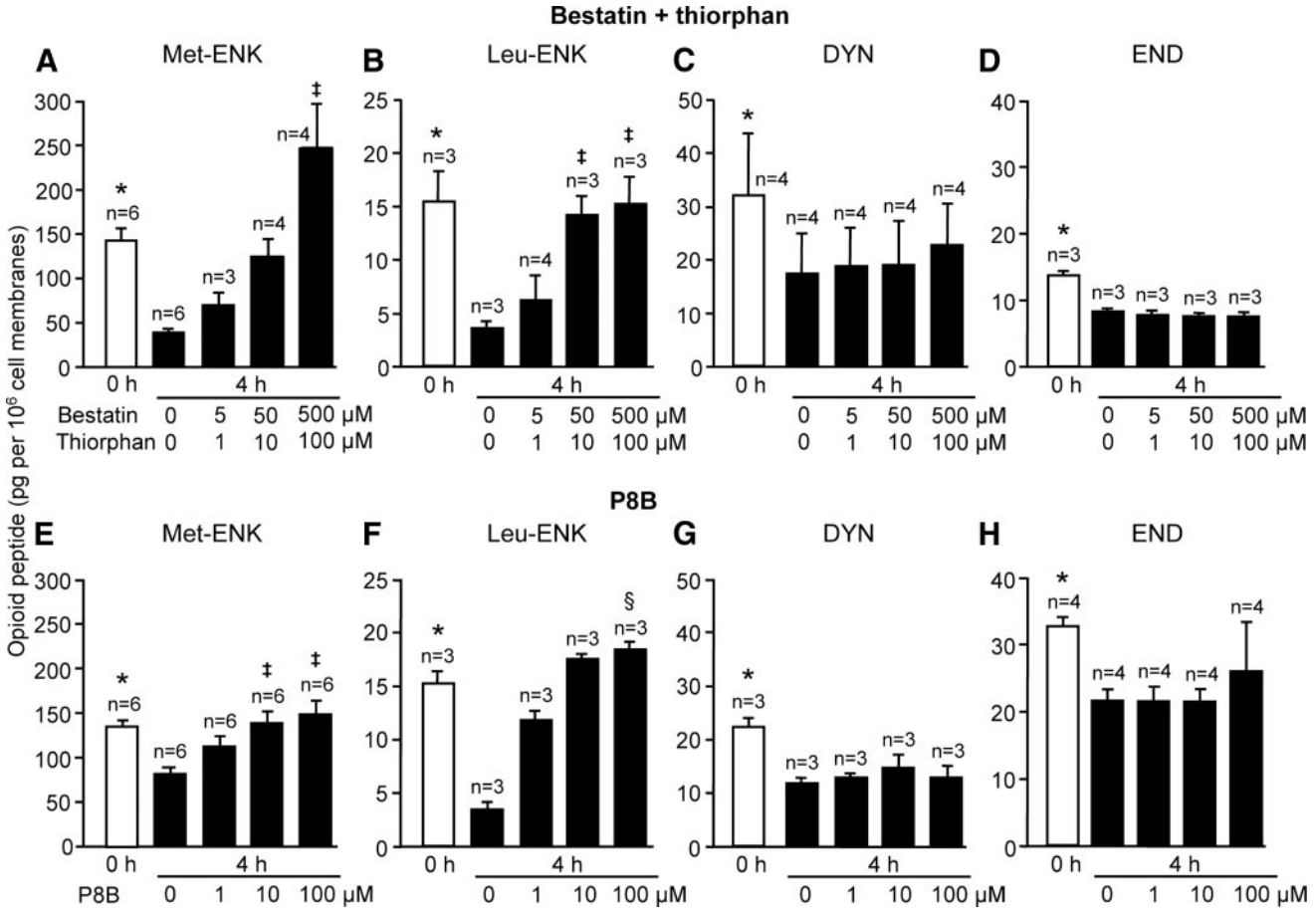

Figure 3. Blockade of APN and NEP on leukocytes from inflamed paws prevents the degradation of ENKs. Combined bestatin (5–500 μM) and thiorphan (1–100 μM) (*A–D*), and P8B (1–100 μM) (*E–H*), concentration-dependently prevented the degradation of Met-ENK and Leu-ENK ($P<0.001$, *A*; $P<0.05$, *B, E, F*; linear regression) but not of DYN and END ($P>0.05$, 1-way RM ANOVA; *C, D, G, H*). Leukocyte membranes were incubated for 15 min with inhibitors before addition of opioid peptides (0.4–22.3 nM). Opioid concentrations were measured immediately thereafter (0 h) and 4 h following coincubation with the membranes. Data are means ± SE. *n*, number of experiments per group. $^{*}P < 0.05$ *vs.* control group at 4 h; paired *t* test. $^{‡}P < 0.05$ *vs.* control group at 4 h; 1-way RM ANOVA, Bonferroni *t* test. $^{§}P < 0.05$ *vs.* control group at 4 h; Friedman RM ANOVA on ranks, Dunn test.

nerve suspensions with the APN substrate Ala-βNA, its metabolite βNA accumulated in inhibitor-free control groups (**Fig. 4*A, B***). There was no significant difference between nerves from inflamed and noninflamed paws ($P>0.05$; Fig. 4*A*). After incubation of nerve suspensions with the NEP-specific substrate Suc-Ala-Ala-Phe-pNA, its metabolite pNA level increased in control groups (Fig. 4*A*, *C*). Significantly higher amounts of pNA (by 42%) accumulated in nerve suspensions from inflamed than from noninflamed paws ($P<0.05$; Fig. 4*A*). In suspensions from inflamed paws, βNA accumulation was decreased by 95% by bestatin (15 mM) or by 61% by P8B (10 mM) ($P<0.05$; Fig. 4*B*). pNA accumulation was fully blocked by thiorphan (8 mM) and decreased by 92% by P8B (10 mM) ($P<0.05$; Fig. 4*C*). Bestatin (50 μM to 15 mM), thiorphan (1 μM to 8 mM), or P8B (1 μM to 10 mM) alone produced no signals (data not shown), indicating no interference of the inhibitors with photospectrometric measurements. The degradation of the APN substrate was not affected by thiorphan (10 mM), and that of the NEP substrate was not affected by bestatin (10 mM) ($P<0.05$; data not shown), confirming NEP selectivity of thiorphan and APN selectivity of bestatin.

We then assessed the metabolic activity of APN and NEP on peripheral nerves toward opioid peptides. At baseline, incubation of nerve suspensions from inflamed paws with Met-ENK (22.3 nM), Leu-ENK (2.3 nM), DYN (0.6 nM), or END (0.4 nM) showed no significant differences in opioid peptide concentrations among groups ($P>0.05$; data not shown). At 4 h of incubation, reduced levels of Met-ENK (by 78–85%; **Fig. 5*A, E***), Leu-ENK (by 79–88%; Fig. 5*B*, *F*), DYN (by 39–55%; Fig. 5*C*, *G*), and END (by 77–83%; Fig. 5*D*, *H*) ($P<0.05$) were observed in control groups. Bestatin (0.5–10 mM) with thiorphan (0.1–5 mM) or P8B (0.1–10 mM) concentration-dependently prevented the degradation of Met-ENK ($P<0.001$; Fig. 5*A*, *E*), Leu-ENK ($P<0.001$, Fig. 5*B*; $P<0.01$, Fig. 5*F*), and DYN ($P<0.01$, Fig. 5*C*; $P<0.001$, Fig. 5*G*). The amount of DYN significantly increased over baseline levels after treatment with bestatin (5–10 mM) and thiorphan (1–5 mM) or with P8B (10 mM) ($P<0.05$; Fig. 5*C*, *G*). END concentrations were not altered by inhibitors ($P>0.05$; Fig. 5*D*, *H*). Together, it appears that the enzymatic activity of NEP, but not of APN, expressed on peripheral nerves is enhanced in inflamed tissue. Besides Met-ENK and Leu-ENK, DYN emerges as an additional substrate of neuronal APN and NEP. Both ENKs and DYN were prevented from degradation by single and dual peptidase inhibitors.

Control RIA experiments by switching antigens showed that each opioid peptide Ab specifically recognized its respective opioid peptide. This also applies to the *in vivo* application of Abs, since they were obtained from the same antisera. Also, none of the Abs recognized the opioid peptide metabolites Tyr-Gly-Gly-OH, Gly-Gly-Phe-Met-OH, and Gly-Gly-Phe-Leu-OH. In some Leu-ENK RIAs, bestatin (500 μM) with thiorphan (100 μM) produced a small (nonsignificant) signal that was subtracted

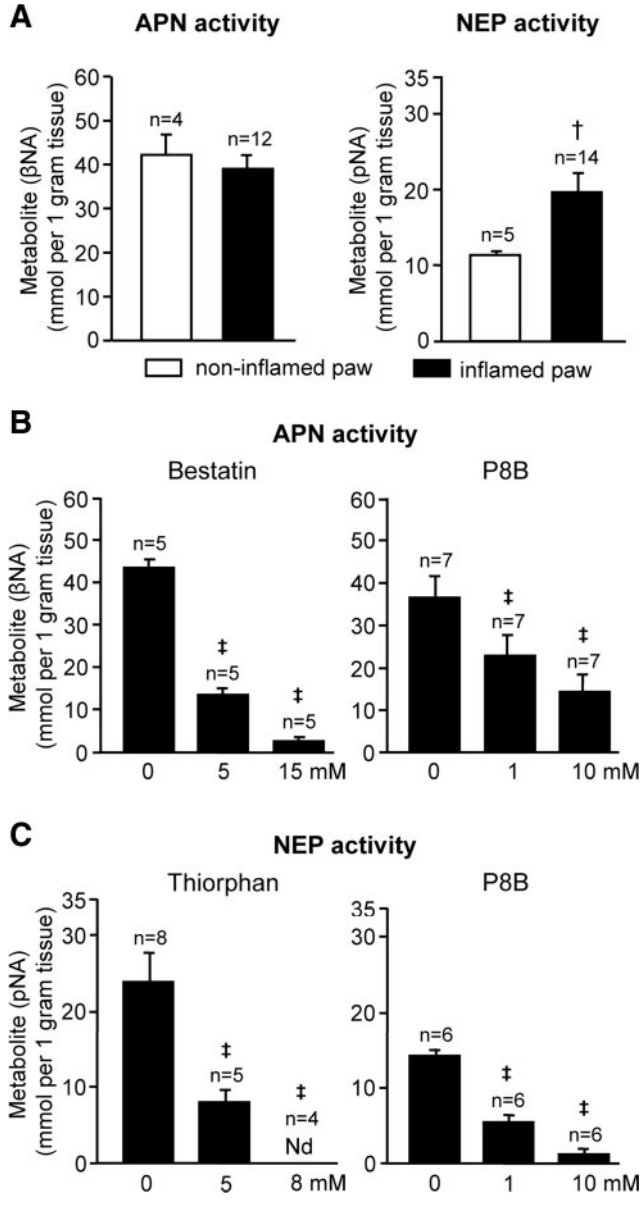

Figure 4. Blockade of APN and NEP activities on peripheral nerves by peptidase inhibitors. *A*) Degradation of APN-specific substrate Ala-βNA (200 μM) to metabolite βNA was similar in nerves from noninflamed and inflamed paws ($P>0.05$, *t* test). Degradation of NEP-specific substrate Suc-Ala-Ala-Phe-pNA (100 μM) to metabolite pNA was significantly higher in nerves from inflamed than from noninflamed paws. Number of experiments in inflamed paws represents the sum of experiments from both control groups in panels *B* and *C*. *B*) Hydrolysis of APN substrate Ala-βNA (100 μM) was blocked by bestatin (5–15 mM) and diminished by P8B (1–10 mM) in nerves from inflamed paws. *C*) Hydrolysis of NEP substrate Suc-Ala-Ala-Phe-pNA (200 μM) was blocked by thiorphan (5–8 mM) and P8B (1–10 mM) in nerves from inflamed paws. Substrates were added to sciatic nerve suspensions without inhibitors (*A*) or 15 min after incubation with inhibitors (*B*, *C*), and photospectrometry was performed 4 h later. Data are means ± SE. *n*, number of experiments per group; Nd, not detected. $^{\dagger}P < 0.05$; Mann-Whitney test. $^{\ddagger}P < 0.05$ *vs.* control group; 1-way RM ANOVA, Bonferroni *t* test.

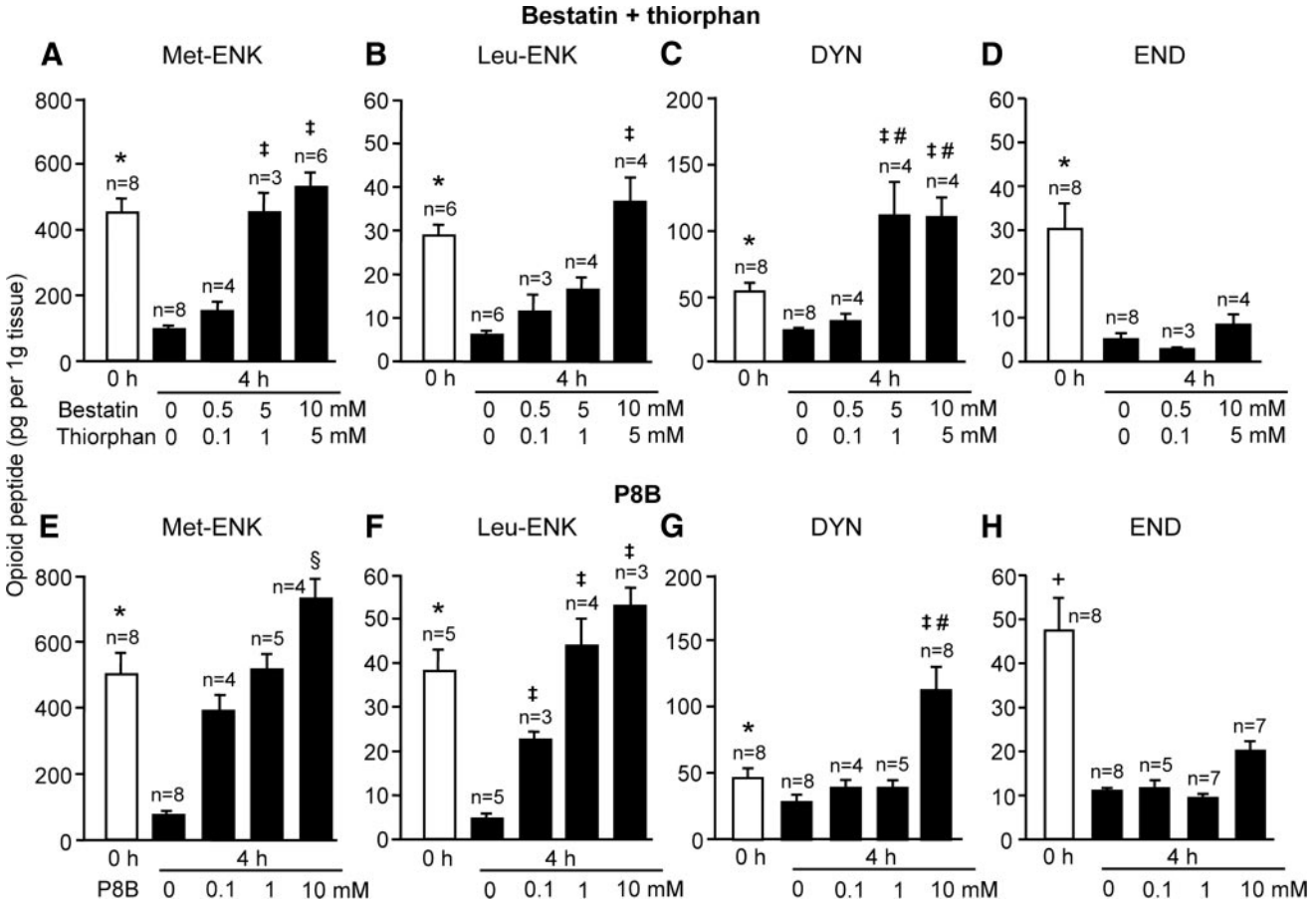

Figure 5. aBlockade of APN and NEP on peripheral nerves from inflamed paws prevents the degradation of ENKs and dynorphin. Combined bestatin (0.5–10 mM) and thiorphan (0.1–5 mM) (*A–D*), and P8B (0.1–10 mM) (*E–H*) concentration-dependently prevented the degradation of Met-ENK ($P<0.001$, *A*, *E*), Leu-ENK ($P<0.001$, *B*; $P<0.01$, *F*) and DYN ($P<0.01$, *C*; $P<0.001$, *G*; linear regression) but not of END ($P>0.05$, 1-way RM ANOVA, *D*; Friedman RM ANOVA on ranks, *H*). Sciatic nerve suspensions were incubated for 15 min with inhibitors before addition of opioid peptides (0.4–22.3 nM). Opioid concentrations were measured immediately thereafter (0 h) and 4 h following coincubation with nerves. Data are means ± SE. *n*, number of experiments per group. $^{*}P < 0.05$ *vs.* control group at 4 h; paired *t* test. $^{+}P < 0.05$ *vs.* control group at 4 h; Wilcoxon test. $^{\ddagger}P < 0.05$ *vs.* control group at 4 h; 1-way RM ANOVA, Bonferroni *t* test. $^{\S}P < 0.05$ *vs.* control group at 4 h; Friedman RM ANOVA on ranks, Dunn test. $^{\#}P < 0.05$ *vs.* 0 h; 1-way RM ANOVA, Bonferroni *t* test.

from the corresponding Leu-ENK data. The inhibitors did not interact with the other three opioid peptide Abs (data not shown).

DISCUSSION

Our findings demonstrate that in painful inflamed tissue, APN and NEP are metabolically active on immune cells and peripheral nerves. The leukocytic enzymes preferentially degrade ENKs, while neuronal peptidases also decompose DYN. Blocking leukocytic and neuronal APN and NEP prevents the catabolism of opioids *in vitro* and amplifies their action on peripheral opioid receptors to locally inhibit inflammatory pain *in vivo*.

Previous studies using NEP-deficient mice reported contradictory results on thermal nociception, but enhanced visceral and neuropathic pain (44–46). In contrast, pharmacological blockade of APN and NEP consistently produced antinociceptive effects mediated by ENKs and opioid receptors in the CNS, in models of thermal, visceral, inflammatory, and neuropathic pain (15, 23, 24, 26–29, 31, 36, 38, 39, 45). Some reports suggested an involvement of peripheral opioid receptors (31, 35, 36) but did not delineate enzyme-expressing cells, peptides, or receptor types. We now show that in peripheral inflamed tissue μ-, δ-, and κ-opioid receptors contribute to APN and NEP inhibitor-induced analgesia, since it was reversed by their selective antagonists and by a peripherally confined antagonist. As earlier studies clearly demonstrated the respective μ, δ, and κ selectivity of CTOP, ICI 174,864, and norBNI injected i.pl. in doses and time-courses employed in our current study (47, 48), the similar degree of inhibition by each antagonist suggests interactions between opioid receptors, possibly *via* oligomerization (49). In contrast to earlier studies in the CNS, we now show that not only Met-ENK but also Leu-ENK and DYN, and to a minor degree, END, mediate the effects of inhibitors in pe-

ripheral inflamed tissue, as they were abolished by anti-Met-ENK, anti-Leu-ENK, and anti-DYN, and slightly decreased by anti-END. Because our RIA experiments excluded cross-reactivity of these Abs, the complete blockade of analgesia by either Ab suggests that each peptide alone is sufficient for the full effect, consistent with their overlapping functions (50). Clearly, the inhibitor-induced analgesia is very efficient, since it surmounted the thresholds of noninflamed paws. In contrast to longer-lasting analgesia following systemic injections of peptidase inhibitors (26–29, 31, 36), the relatively short duration of this effect (15 min) in our study is comparable to the actions of inhibitors in the CNS (23, 38, 39). This does not necessarily impair clinical relevance, as, for example, intrathecal thiorphan and bestatin produced long-lasting analgesia (up to 18 h) in human patients (25). Thus, the time course of peptidase inhibitor analgesic effects might depend on the route of administration and the species.

Notably, we found nerves, granulocytes, and macrophages as sources of active APN and NEP in inflamed tissue. This is in line with the presence of NEP on granulocytes and monocytes in synovial fluid of patients with arthritis (51). The absence of peptidases on T lymphocytes in flow cytometry is likely related to the low number of these cells in our relatively short-lasting inflammatory model, in contrast to chronic arthritis (51, 52). APN and NEP were previously detected in peripheral nerves (17, 18), but so far the peptidases' metabolic actions were only shown in healthy subjects (17, 40, 41, 53). We now demonstrate the enzymatic activity of both leukocytic and neuronal APN and NEP derived from inflamed tissue by showing that each peptidase degraded its specific synthetic substrate in a thiorphan-, bestatin-, and P8B-reversible manner. Interestingly, we observed significantly higher activity of NEP, but not of APN, in nerves from inflamed than noninflamed paws. Previously, NEP action was found unchanged in CNS homogenates of polyarthritic rats (54) but, similar to APN, was elevated in synovial tissue of patients with rheumatoid arthritis (51, 55). However, the cell types expressing peptidases were not identified in those studies, and none of them considered the context of pain or analgesia. Moreover, enzymatic activities of peptidases might depend on their cellular sources and/or disease type. Our data indicate that leukocytic and neuronal APN and NEP in painful tissue are functional and that inflammation enhances the activity of neuronal NEP.

Previous studies reported ENK catabolism in spleen and circulating leukocytes (53, 56) or its inhibition in the healthy CNS (15, 24, 29, 38, 57), while opioid degradation by APN and NEP on peripheral nerves has not been addressed so far. We now demonstrate that APN and NEP on leukocytes and peripheral nerves from injured tissue degrade Met-ENK and Leu-ENK, since their *in vitro* catabolism was prevented by bestatin and thiorphan or by P8B. DYN was catabolized in both leukocyte membrane and nerve preparations, whereas blockade of APN and NEP inhibited DYN degradation only in nerve preparations. This indicates that DYN was hydrolyzed by peptidases on nerves but not on immune cells. In previous studies, dynorphins were not degraded by APN and NEP from brain or kidney (21, 22), but in the spinal cord (34). Thus, the differences between leukocytic and neuronal APN and NEP in DYN catabolism remain to be elucidated. Our finding that DYN concentrations following incubation of nerve suspensions with inhibitors were higher than the initially added amounts of exogenous DYN suggests that endogenous DYN released from nerves (58) was also protected. A similar (nonsignificant) tendency was observed for ENKs, which are also found in nerves (4). The higher concentrations of peptidase inhibitors required to prevent catabolism of ENK, DYN, and synthetic substrates in whole-nerve suspensions might be related to a more difficult (anatomical) access of inhibitors to enzymes (*e.g.*, due to perineurium and/or Schwann cells) compared to the crude membrane preparations from leukocytes. Similar to other studies (21, 22, 34), END degradation was independent of APN and NEP, both in immune cell and nerve preparations in our experiments. END may be inactivated by γ-endorphin-generating enzyme on leukocytes (59). Nevertheless, NEP-dependent END degradation was described (32), and END degradation by APN and NEP on fibroblasts and endothelia (19) cannot be excluded. This may account for the slight attenuation of i.pl. inhibitor-induced analgesia by anti-END in our *in vivo* experiments.

While we found that APN and NEP inhibitors clearly exert pain-relieving actions *in vivo*, NEP can also cleave pronociceptive mediators, such as calcitonin gene-related peptide (CGRP), substance P and bradykinin (60, 61). Although NEP blockade increased CGRP concentrations in skin, it did not cause pain in humans (62). Also, the endogenous NEP inhibitor sialorphin prevented substance P degradation *in vitro* but decreased pain *in vivo* (61), and NEP blockade elevated Met-ENK but not substance P in cerebrospinal fluid (57). Indeed, a predominant *in vivo* degradation of substance P by peptidases insensitive to APN and NEP inhibitors has been suggested (14, 19). In addition, substance P and bradykinin are considered natural inhibitors of APN (19), and opioids attenuate the release of CGRP and substance P from neurons (4). All these mechanisms may contribute to analgesia produced by APN and NEP inhibitors in peripheral inflamed tissue.

In summary, we discovered immune cells and peripheral nerves as sources of APN and NEP, which degrade opioid peptides in painful inflamed tissue. While Met-ENK and Leu-ENK are preferred targets, DYN emerges as an additional substrate of APN and NEP on peripheral nerves. The blockade of both peptidases protects ENKs and DYN from metabolism and promotes local amelioration of inflammatory pain. Our results open an innovative strategy for the treatment of persistent inflammatory pain based on enhancing the actions of naturally occurring opioid peptides selectively in injured tissues. This will avoid overstimulation of opioid

receptors by excessive amounts of exogenous agonists and the detrimental systemic and central side effects of conventional opioids and NSAIDs. Moreover, blocking pain at the site of its origin may prevent peripheral and central sensitization and the subsequent development to chronic pain. FJ

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