Opioids from immunocytes interact with receptors on sensory nerves to inhibit nociception in inflammation

(stress/analgésia/immunocytochemistry/pain)

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ABSTRACT Exogenous opioids can produce localized opioid receptor-mediated antinociception in peripheral inflamed tissue. Previous studies show that activation of endogenous opioids by a cold water swim in rats with hind paw inflammation results in a similar local antinociceptive effect but suggest that pituitary-adrenal opioid pools are not directly involved in producing this effect. Here we show increased amounts of opioid peptides in immune cells infiltrating the inflamed tissue. Furthermore, we demonstrate immunoreactive opioid receptors on peripheral terminals of sensory neurons. The local administration of antibodies against opioid peptides or receptors or systemic pretreatment with the immunosuppressant cyclosporine blocks cold water swim-induced antinociception. These findings suggest that antinociception in inflammation can be brought about by endogenous opioids from immune cells interacting with opioid receptors on peripheral sensory nerves.

Recent evidence indicates that exogenous opioids can produce pronounced antinociceptive effects by interacting with local opioid receptors in peripheral inflamed tissue (1–4). Activation of endogenous opioid systems by environmental stimuli (cold water swim; CWS) in rats with unilateral hind paw inflammation similarly elicits localized opioid receptor-mediated antinociception in the inflamed paw (5). The pituitary-adrenal axis, the classical source for circulating peripheral opioid peptides (6), is apparently not directly involved in producing this effect (7). Another conceivable locus of origin for endogenous opioids in inflamed tissue is the immune system (8). Immune cells have been shown to contain and release opioid peptides in vitro (9–11) and various types of such cells accumulate at sites of inflammation in vivo (12). In the present study, we have examined tissue from normal and inflamed rat paws by radioimmunoassay (RIA) and immunocytochemistry for β-endorphin (β-EP), [Met]enkephalin ([Met]EK), and dynorphin (DYN), the major representatives of the three endogenous opioid peptide families (13). We report here the presence of significant amounts of immunoreactive β-EP and [Met]EK in inflamed tissue, apparently located in immunocytes. By use of a monoclonal anti-idiotypic antibody (anti-id 14) (14) we demonstrate opioid receptors immunocytochemically on small-diameter cutaneous nerves of the paw. Finally, we show in vivo that antinociception induced by CWS in the inflamed paw is abolished by prior intraplantar (i.pl.) administration of anti-id 14, which is a functional antagonist at opioid receptors (14), or antibodies to β-EP or by pretreatment with the immunosuppressive agent cyclosporine A (CsA).

MATERIALS AND METHODS

Subjects. Male Wistar rats (200–220 g) were housed individually in cages lined with sawdust. All testing was conducted in the light phase of a 12 hr/12 hr (8 a.m.–8 p.m.) light/dark cycle. The guidelines on ethical standards for investigations of experimental pain in animals (15) were followed.

Induction of Inflammation. Rats received an i.pl. injection of 0.15 ml of Freund’s complete adjuvant (Calbiochem) into the right hind foot under brief ether anesthesia. A detailed description of the time course and magnitude of the inflammatory reaction is given elsewhere (16). The inflammation remained confined to the inoculated paw. All experiments were performed 4 days after inoculation.

RIAs. Rats were decapitated and skin and adjacent subcutaneous tissue were dissected from the planter surface of both hind paws. The material was weighed and then incubated in 0.1 M HCl (six times the volume of the tissue) at 95°C for 10 min. Following homogenization and centrifugation at 10,000 × g for 20 min, supernatants were removed and filtered through YM 30 membranes (Amicon). Samples were then aliquoted, lyophilized, redissolved in RIA buffer, and assayed as described (17). Antisera from rabbit against β-EP (1–31) and DYN (1–17) were raised and characterized in our laboratory (5); anti-[Met]EK, a gift from K. H. Voigt (Marburg, F.R.G.), was characterized in ref. 18.

Immunocytochemistry. Rats were anesthetized and perfused as described (19). The skin and adjacent subcutaneous tissue were dissected from both hind paws, cut into small specimens, postfixed for 2–3 hr at room temperature, and washed overnight at 4°C in phosphate-buffered saline (PBS) (0.05 M, pH 7.3). The specimens were then dehydrated in a graded series of ethanol, cleared in methyl salicylate, and embedded in Paraplast Plus (Merck). Sections (5–7 μm thick) were cut, dewaxed in xylene, rehydrated in a graded series of ethanol, washed briefly in PBS, and then immunostained using avidin-biotin peroxidase complex (Vector Laboratories). Unless otherwise stated, all incubations were done at room temperature. PBS was used for washing after each step. Endogenous peroxidase was quenched with 0.3% H2O2 in PBS for 30 min. Slides were then incubated with normal goat serum (Vector Laboratories) for 30 min, blotted (without

Abbreviations: CWS, cold water swim; β-EP, β-endorphin; [Met]EK, [Met]enkephalin; DYN, dynorphin; CsA, cyclosporine A; PPT, paw pressure threshold; i.pl., intraplantar; ANOVA, analysis of variance.

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washing), and overlaid with the primary antisera at 4°C overnight. Thereafter they were incubated with the biotinylated secondary antibody (Vector Laboratories) for 30 min and subsequently incubated with avidin-biotin-conjugated peroxidase (Vector Laboratories) for 30–45 min. Finally, the slides were stained with 3',3'-diaminobenzidine tetrahydrochloride (0.025%) (Sigma), including 0.003% H2O2 in 0.05 M Tris-buffered saline (pH 7.6) and 0.03% (wt/vol) nickel chloride for 3–5 min, counterstained with nuclear fast red, and then dehydrated and mounted. The following antibodies were used in the stated dilutions: a monoclonal antibody generated against β-EP (3-E7) (20) at 3–5 µg/ml, polyclonal rabbit anti-β-EP at 3–5 µg/ml, anti-DYN (1–8 µg/ml), and anti-[Met]EK (1–8 µg/ml). Purification procedures and specificities of these antibodies were described elsewhere (5, 14, 20). Sixty minutes (anti-id-14) or 15 min (other antibodies) later the animals were subjected to CWS and PPTs were redetermined immediately thereafter. Additional groups received intraperitoneal CsA (Sandoz Pharmaceuticals) (0.75–3 mg per injection in 1 ml) or vehicle (Cremophor EL, 39 mg/ml; ethanol, 15.8 mg/ml in NaCl) at 48, 24 and 4 hr prior to testing.

**RESULTS**

Concentrations of Immunoreactive Opioid Peptides in Skin and Subcutaneous Tissue. Immunoreactive β-EP and [Met]-EK were significantly increased in inflamed as compared to noninflamed paws (Table 1). DYN levels were below the detection limit in both cases.

Immunochemical Localization of Opioid Peptides. Numerous inflammatory cells stained strongly with the monoclonal antibody (3-E7) and the polyclonal antibody to β-EP (Fig. 1B) and somewhat less intensely for [Met]-EK (not shown). A small number of cells displayed a faint reaction for DYN (not shown). The intensity of immunoreactivity in the inflamed paw was most pronounced in the periphery of inflammatory foci within the plantar subcutaneous tissue. The opioid-containing cells had morphological appearances consistent with macrophages, mast cells, lymphocytes, and plasma cells (Fig. 1B). Opioid staining was almost entirely absent in noninflamed paws (Fig. 1A). Complete extinction of opioid-like immunoreactivity was attained in the specificity control experiments (Fig. 1C).

Immunochemical Visualization of Opioid Receptors. Intense staining of immunoreactive opioid receptors was detected on small-diameter cutaneous nerves of noninflamed and inflamed paws (Fig. 2 A and B). In specificity control experiments this immunoreactivity was completely abolished (Fig. 2C).

Aldosemetry. Immediately following CWS the PPT increased significantly on the inflamed (P < 0.01, analysis of variance (ANOVA)) but not on the noninflamed paw (P = 0.24, ANOVA) (Fig. 3A). This increase was dose-dependently (P < 0.001, linear regression ANOVA) blocked by prior local (i.pl.) but not by systemic (i.v.) administration of equivalent doses of anti-id-14 (Fig. 3B). Anti-id-14 by itself as well as vehicle (NaCl) had no effect on nociceptive thresholds in inflamed paws (data not shown). Specificity of this antagonism was shown by a lack of activity of control IgM (Fig. 3B). Since anti-id-14 is a functional antagonist at opioid receptors (14), this finding indicates that the CWS-induced antinociceptive effect is mediated by a local opioid receptor-specific mechanism in the inflamed paw, which is consistent with our previous data using the classic opioid antagonist naloxone (5). To identify the opioid peptide(s)

| Table 1. Immunoreactive β-EP, [Met]EK, and DYN in tissue extracts from noninflamed and inflamed hind paws |
|-----------------|------------------|------------------|
| **Opioid peptide** | **Noninflamed, pmol/g of wet tissue** | **Inflamed, pmol/g of wet tissue** |
| β-EP           | 0.06 ± 0.02      | 0.45* ± 0.05    |
| [Met]EK        | 0.09 ± 0.02      | 0.29* ± 0.05    |
| DYN            | ND               | ND               |

Values represent means ± SEM. (n = 6–8). ND, not detectable (detection limit, 5 fmol per tube).

*Significance of difference between levels in inflamed and noninflamed tissue (P < 0.05, Wilcoxon test).
FIG. 1. Immunostaining of opioid peptides in subcutaneous tissue of noninflamed and inflamed paws. Photomicrographs demonstrate staining with polyclonal anti-\(\beta\)-EP (monoclonal 3-E7 yielded similar results). (A) Noninflamed skin and subcutaneous tissue with scattered cells staining for \(\beta\)-EP (A). (B) Inflamed tissue. Cells staining for \(\beta\)-EP include macrophages (1) and monocytes (2) (large cell bodies, vacuolated cytoplasm, and irregular-shaped nuclei); mast cells (3) (large oval centrally located nuclei, granular cytoplasm); lymphocytes (4) (large nuclei, small amounts of cytoplasm); plasma cells (5) (oval shape, rounded nuclei situated at one pole of the cell). Polymorphonuclear leukocytes are abundant but only faintly immunostained. (C) Preadsorption control experiment. (Bar in A = 15 \(\mu\)m; bars in B and C = 10 \(\mu\)m.)

generating this antinociceptive effect, we applied antibodies to \(\beta\)-EP, [Met]EK, or DYN i.pl. prior to CWS. The monoclonal antibody (3-E7) and the polyclonal antibody against \(\beta\)-EP but not those to [Met]EK or DYN dose-dependently (\(P < 0.001\), linear regression ANOVA) abolished the CWS effect (Fig. 3C). These findings demonstrate the functional significance of \(\beta\)-EP. Finally, to confirm the functional involvement of immune cells, the animals were pretreated with daily injections of the immunosuppressant CsA. CsA, but not its vehicle, resulted in dose-dependent (\(P < 0.001\), linear regression ANOVA) blockade of CWS-induced antinociception in inflamed paws (Fig. 3D). In the absence of CWS, CsA had no effect on nociceptive thresholds (data not shown).

DISCUSSION
The first set of experiments demonstrates the presence of significant amounts of opioid peptides in peripheral inflamed

FIG. 2. Immunostaining of opioid receptors in noninflamed (A) and inflamed (B) paws. ImmunoGold silver-stained opioid receptors appear in the form of black granules associated with small-diameter unmyelinated fibers in the longitudinal section of a cutaneous nerve. (C) Preadsorption control experiment in a section adjacent to B. (Bar = 5 \(\mu\)m.)
anti-\beta-EP attenuated the CWS effect it appears that, even if the epitope does not include N-terminal tyrosine, an antibody–ligand complex cannot activate opioid receptors in this situation. Taken together, the present evidence suggests that the CWS-induced antinociceptive effect in inflamed paws is brought about by \beta-EP interacting with opioid receptors on peripheral terminals of primary afferent neurons.

The fact that immunoreactive [Met]EKT was increased in inflamed tissue but anti-[Met]EKT did not attenuate the CWS effect in vivo suggests that [Met]EKT was either not released or, if released, did not contribute to this effect, but this does not exclude a possible function of proenkephalin-derived opioid peptides under different circumstances (e.g., other environmental stimuli). The role of DYN cannot be determined definitively based on our results. Immunoreactive DYN was not detectable by RIA; therefore no statement can be made regarding its change in inflammation. The fact that anti-DYN did not attenuate CWS-induced antinociception, in line with our previous findings (5), argues against a functional involvement of DYN.

Fig. 3. (A) Effect of CWS on PPT of inflamed (●) and noninflamed (○) hind paws. (B) Effect of i.pl. (●) and i.v. (○) anti-id-14 and of control IgM (○) on CWS-induced PPT elevation in inflamed paws. (C) Effect of 3-E7 (●), anti-\beta-EP (●), anti-[Met]EKT (●), anti-DYN (●), and normal rabbit IgG (○) on CWS-induced PPT elevation in inflamed paws. (D) Effect of intraperitoneal CsA (●) and vehicle (○) on CWS-induced PPT elevation in inflamed paws.

Morphological characteristics suggest that the opioid-containing cells are immunocytes, consistent with a previous report (33). In fact, various types of immune cells have been shown to express the genes for proopiomelanocortin or proenkephalin and to produce opioid peptides derived from these precursors (for review, see ref. 8). Therefore we conducted a last set of behavioral experiments in animals immunosuppressed with CsA. This compound inhibits activation and proliferation of T and B lymphocytes, macrophages, and mast cells in vitro (34–39) and their effector functions in vivo (40, 41). The results clearly show that the CWS-induced effect is abolished by CsA and thus confirm the functional involvement of immune cells.

In summary, we have demonstrated by RIA and immunocytochemistry the presence of significant amounts of immunoreactive \beta-EP and [Met]EKT in inflamed paws. These opioid peptides appear to be mainly located in cells of the immune system. Opioid receptors were visualized immunocytochemically on peripheral sensory nerves. Our in vivo experiments indicate that these receptors can be activated by \beta-EP released from immune cells and mediate antinociception. These findings have several interesting implications. (i) The concept of peripheral opioid antinociception is further corroborated by the morphological demonstration of receptors and endogenous ligands in peripheral tissue. Thus, local application of small doses of conventional opiates or the development of opioid agonists unable to cross the blood–brain barrier may provide a new perspective for pain management by producing analgesia without central side effects such as respiratory depression, sedation, or dependence. (ii) Previous concepts of stress-induced analgesia may have to be extended in that opioid mechanisms outside the central nervous and endocrine systems can contribute to such effects. (iii) In addition to their immunological functions, immunocytes can be involved in intrinsic mechanisms of antinociception in inflammation.

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