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

(stress/analgesia/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 antiidiotypic 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).

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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 plantar 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% H_2O_2 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 (0.0125%) (Sigma), including 0.003% H_2O_2 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 at 5 μg/ml, and anti-[Met]EK (Peninsula Laboratories) at 1:1000. Cross-reactivities were described elsewhere (5). To demonstrate specificity of staining, the following controls were included: (*i*) preadsorption of primary antisera with an excess of homologous antigen (1 μM β-EP, 10 nM [Met]EK, 10 nM DYN) for 24 hr at room temperature (21); (*ii*) omission of either the primary antisera, the secondary antibody, or the avidin-biotin complex.

Visualization of opioid receptors was accomplished using the modified immunogold silver staining (IGSS) technique (22, 23) with Lugol's iodine and sodium thiosulfate pretreatment (24). Tissues from inflamed and noninflamed paws were fixed and processed for Paraplast embedding as described above. The rehydrated sections were immersed in Lugol's iodine for 5 min, treated with 2.5% aqueous solution of sodium thiosulfate until they became colorless, washed in tap water, and immersed in IGSS-buffer I (0.05 M Tris-buffered saline, pH 7.4, containing 2.5% sodium chloride, 0.2% Triton X-100, 0.2% gelatin, 0.1% bovine serum albumin, and 0.01% sodium azide). Sections were then incubated for 20 min with neat goat serum, drained off, and directly overlaid with biotinylated anti-id-14 [mouse monoclonal antibody against μ- and δ-opioid receptors (14, 19) at 25 μg/ml in PBS] at 4°C (overnight) or at room temperature (2–4 hr). After washing in IGSS-buffer I (three times, 5 min each) and in IGSS-buffer II (similar to IGSS-buffer I, but pH 8.2) (three times, 5 min each), sections were covered with neat goat serum and incubated (60 min, room temperature) with AuroProbe LM streptavidin ImmunoGold reagent (Janssen Pharmaceutica) diluted at 1:250 in IGSS-buffer II containing 0.8% bovine serum albumin. After several washes in IGSS-buffer II, followed by (five times, 5 min each) washes in deionized double-distilled water (DDD H_2O) under constant gentle agitation, sections were postfixed in 1% glutaraldehyde for 20 min at room temperature, washed briefly in DDD H_2O, and then covered with few drops of IntenSE M silver enhancement mixture (Janssen Pharmaceutica) for 10–15 min at room temperature. The sections (without counterstaining) were then dehydrated, cleared, and mounted. The following controls were included to demonstrate specificity: (*i*) preincubation of anti-Id-14 with 0.6 μM 3-E7 (the antigen against which anti-id-14 was raised); (*ii*) substitution of anti-id-14 by biotinylated normal mouse IgM or omission of the ImmunoGold reagent.

Algesiometry. Nociceptive thresholds were evaluated by use of a modified Randall–Sellito paw pressure test (4, 16) with the observer blind to the experimental condition employed. Animals (n = 5 or 6 per group) were gently restrained and incremental pressure was applied onto the hind paw. The pressure required to elicit paw withdrawal, the paw pressure threshold (PPT), was determined. After baseline measurements, rats were subjected to CWS at 1–2°C for 1 min as described (5), and PPTs were reevaluated repeatedly thereafter.

In separate experiments rats received (under brief halothane anesthesia) the following i.pl. (right hind paw; injection volume, 0.1 ml) or i.v. (tail vein; injection volume, 0.2 ml) injections after baseline PPT measurements: purified anti-id-14 (2.5–50 μg i.pl.; 50 μg i.v.), control mouse IgM (MOPC 104E, Sigma) (60 μg i.pl.), purified 3-E7 (0.25–1 μg i.pl.), anti-β-EP (0.25–1 μg i.pl.), anti-[Met]EK (1–8 μg i.pl.), anti-DYN (1–8 μg i.pl.), or normal rabbit IgG (1 μg i.pl.). 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 Pharmaceutica) (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.

Immunocytochemical Localization of Opioid Peptides. Numerous inflammatory cells stained strongly with the monoclonal antibody (3-E7) and the polyclonal antibody to β-EP (Fig. 1*B*) 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. 1*B*). Opioid staining was almost entirely absent in noninflamed paws (Fig. 1*A*). Complete extinction of opioid-like immunoreactivity was attained in the specificity control experiments (Fig. 1*C*).

Immunocytochemical 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. 2*C*).

Algesiometry. 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. 3*A*). 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. 3*B*). 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. 3*B*). 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).

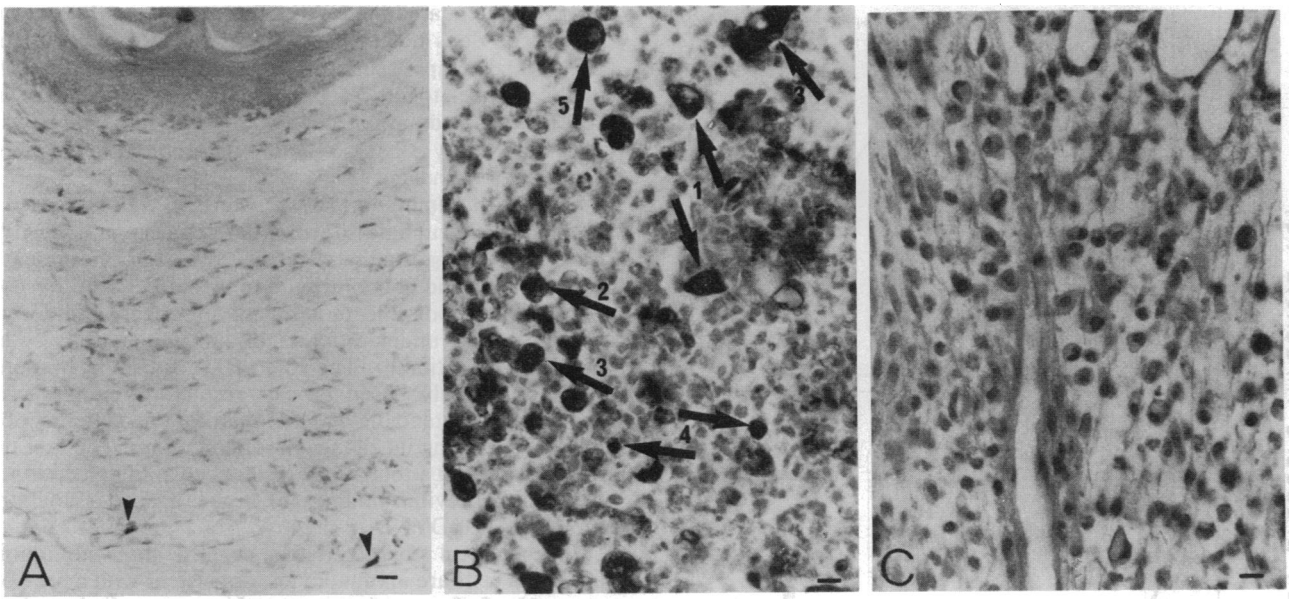

FIG. 1. Immunostaining of opioid peptides in subcutaneous tissue of noninflamed and inflamed paws. Photomicrographs demonstrate staining with polyclonal anti-β-EP (monoclonal 3-E7 yielded similar results). (*A*) Noninflamed skin and subcutaneous tissue with scattered cells staining for β-EP (▲). (*B*) Inflamed tissue. Cells staining for β-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 μm; bars in *B* and *C* = 10 μm.)

generating this antinociceptive effect, we applied antibodies to β-EP, [Met]EK, or DYN i.pl. prior to CWS. The monoclonal antibody (3-E7) and the polyclonal antibody against β-EP but not those to [Met]EK or DYN dose-dependently ($P < 0.001$, linear regression ANOVA) abolished the CWS effect (Fig. 3*C*). These findings demonstrate the functional significance of β-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. 3*D*). 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

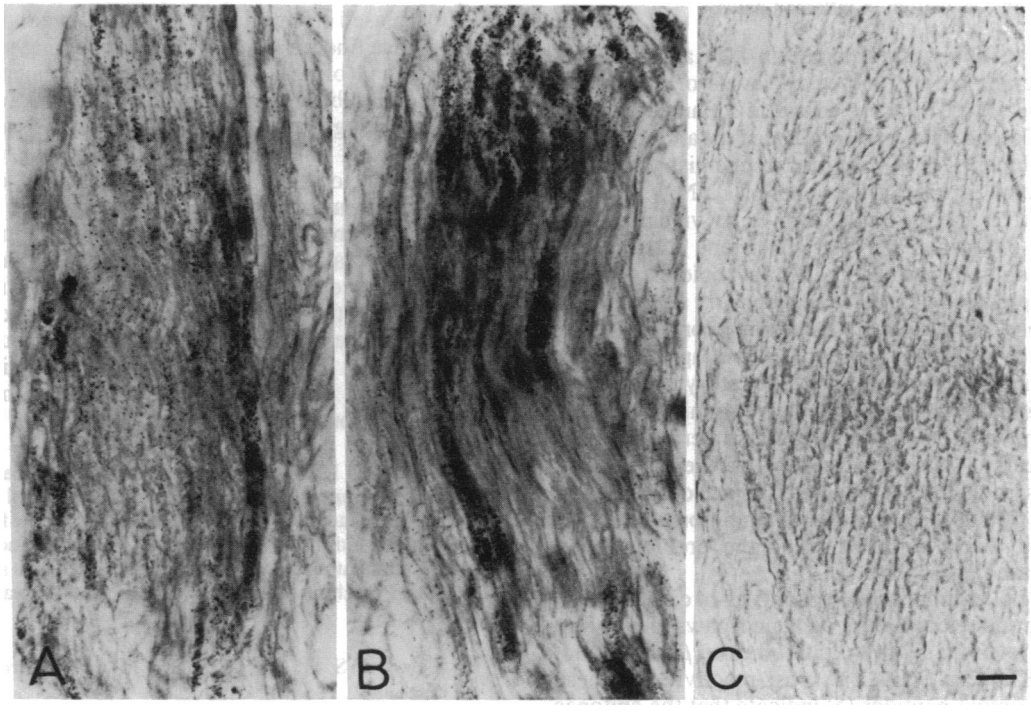

FIG. 2. Immunostaining of opioid receptors in noninflamed (*A*) and inflamed (*B*) paws. ImmunoGold silver-stained opioid receptors appear in the form of black granules associated with small-diameter unmyelinated fibers in the longitudinal section of a cutaneous nerve. (*C*) Preadsorption control experiment in a section adjacent to *B*. (Bar = 5 μm.)

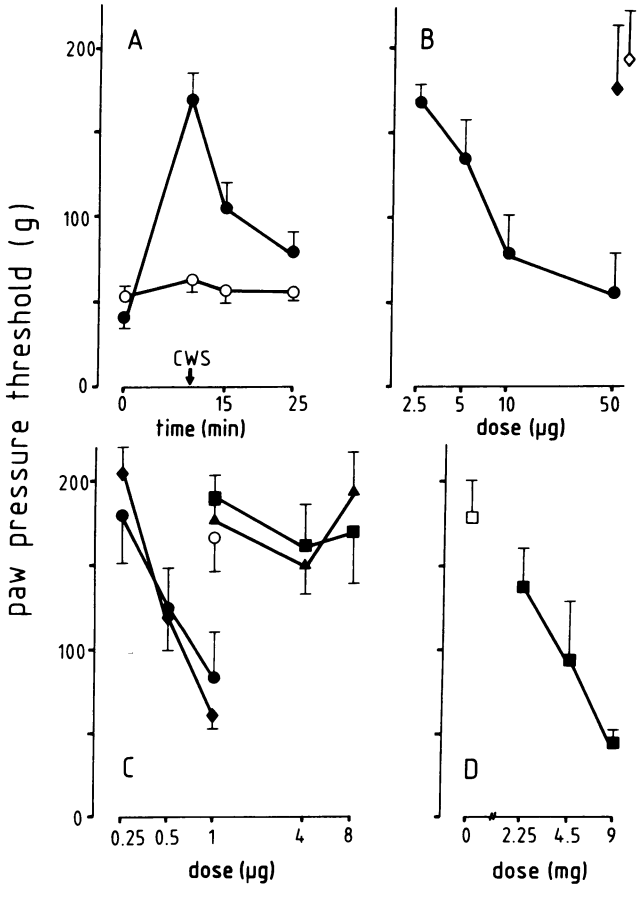

FIG. 3. (*A*) Effect of CWS on PPT of inflamed (●) and noninflamed (○) hind paws. (*B*) Effect of i.pl. (●) and i.v. (◆) anti-id-14 and of control IgM (◇) on CWS-induced PPT elevation in inflamed paws. (*C*) Effect of 3-E7 (◆), anti-β-EP (●), anti-[Met]EK (■), anti-DYN (▲), and normal rabbit IgG (○) on CWS-induced PPT elevation in inflamed paws. (*D*) Effect of intraperitoneal CsA (■) and vehicle (□) on CWS-induced PPT elevation in inflamed paws.

tissue. Parallel results were obtained in RIA and immunohistochemical experiments. Increased levels of immunoreactive β-EP and [Met]EK were found in inflamed as compared to noninflamed tissue, accompanied by an increase in staining for both peptides in immunocytochemistry. Neither technique detected significant amounts of DYN.

Immunoreactive opioid receptors were visualized on small-diameter cutaneous nerves, consistent with studies demonstrating opioid binding (25–27) and opioid receptor-specific electrophysiological effects on sensory neurons (28–30). Conceivably, activation of these receptors can cause attenuation of the excitability of nociceptive input terminals (28–30) and/or inhibition of release of excitatory transmitters (31, 32) and ultimately result in antinociception. Since exogenous opioids have been shown to produce peripheral antinociceptive effects (1–4) it seemed likely that the endogenous opioid peptides seen in the inflamed tissue could activate these receptors directly to exert a local inhibition of nociception. We tested this hypothesis using the previously described CWS paradigm (5).

Indeed, local i.pl. administration of anti-id-14 could abolish CWS-induced antinociception in inflamed paws. Likewise, this effect was reversed by both antibodies to β-EP but not by anti-[Met]EK or anti-DYN. The cross-reactivities of these antibodies with opioid peptides (5) indicate that the epitopes for 3-E7 and anti-[Met]EK but not for anti-β-EP or anti-DYN include N-terminal tyrosine, which is essential for a peptide's ability to bind to opioid receptors (13). Since 3-E7 and anti-β-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 β-EP interacting with opioid receptors on peripheral terminals of primary afferent neurons.

The fact that immunoreactive [Met]EK was increased in inflamed tissue but anti-[Met]EK did not attenuate the CWS effect *in vivo* suggests that [Met]EK 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.

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 β-EP and [Met]EK 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 β-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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