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Inhibitory Effects of Spinorphin, a Novel Endogenous Regulator, on Chemotaxis, O_2^- Generation, and Exocytosis by *N*-Formylmethionyl-leucyl-phenylalanine (FMLP)-Stimulated Neutrophils

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ABSTRACT. To characterize the inflammatory effect of spinorphin, an endogenous peptide purified from bovine spinal cord, its effects on chemotaxis, O_2^- generation, and exocytosis by *N*-formylmethionyl-leucyl-phenylalanine (FMLP)-stimulated human neutrophils (PMNs) *in vitro* were examined. At 10 μM, spinorphin significantly inhibited chemotaxis by FMLP-stimulated PMNs. Spinorphin at 100 μM also inhibited both O_2^- generation and exocytosis of β-glucuronidase and collagenase by FMLP-stimulated PMNs. The mechanisms by which spinorphin inhibits these PMN functions were examined further. Spinorphin markedly suppressed the binding of FML[^{3}H]P to its receptor on PMNs, as observed in a binding assay. However, other neuropeptides that were examined (angiotensin III and substance P) had no effect on FML[^{3}H]P binding, suggesting the possibility that spinorphin plays a specific role in the inhibition of the binding between FMLP and its receptor. The suppression of FMLP binding also caused a decrease of the FMLP-induced intracellular calcium concentration $[Ca^{2+}]_i$, which acts as a second messenger leading to PMN functions. These results suggest that spinorphin may be a new endogenous inflammation-regulatory peptide that modulates the interaction of FMLP with its receptor. BIOCHEM PHARMACOL **54**;6:695–701, 1997. © 1997 Elsevier Science Inc.

KEY WORDS. heptapeptide; neutrophil; chemotaxis; O_2^- generation; exocytosis; FMLP receptor

It is possible that enkephalins are involved in the pain-modulating mechanism in the spinal cord [1, 2]. Enkephalins, however, are short-lived, being degraded rapidly by various endogenous enzymes. Many substances that inhibit enkephalin degradation have been investigated, and some inhibitors have been reported [3, 4]. We found an endogenous factor that modulates enkephalin-degrading activity and purified it from bovine spinal cord based on its inhibitory activity toward enkephalin-degrading enzymes. Structural analysis revealed the factor to be Leu-Val-Val-Tyr-Pro-Trp-Thr, and it was named spinorphin [5]. We further analyzed the inhibitory activity of spinorphin toward various enkephalin-degrading enzymes and found that it inhibits an NEP§ (EC 3.4.24.11) as well as other enzymes, such as APN (EC 3.4.11.2) and DPPIV (EC 3.4.14.5), from monkey brain [6]. In mice, intraventricularly injected spinorphin exhibits antinociceptive activity that dose dependently prevents pharmacological stimulation [7].

It has been reported that NEP may regulate inflammatory responses by degrading neuropeptides such as enkephalins, substance P, and bradykinin, which induce some inflammatory responses [8–10]. It also has been reported that enkephalins induce O_2^- generation and exocytosis by PMNs [11, 12]. PMNs possess several kinds of enzymes on their surfaces, including NEP/CD10, APN/CD13, and DPPIV/CD26, and an inhibitor(s) of these enzymes may play an important role in the regulation of PMN-mediated inflammation. Phosphoramidon, an NEP inhibitor, enhances substance P-induced superoxide generation and chemotaxis [9], while bestatin, an APN inhibitor, prevents the inhibition of chemotaxis by interleukin-8-stimulated PMNs [13]. If spinorphin modulates these enzymes on PMNs, it is possible that it also regulates PMN functions and PMN-induced inflammation *in vivo*.

It is known that FMLP stimulation induces chemotaxis, O_2^- generation, and exocytosis by PMNs [14, 15]. First, these responses are elicited through the binding of FMLP to its receptors on their cell surface, which then causes an increase in the $[Ca^{2+}]_i$ associated with the formation of inositol 1,4,5-triphosphate (IP_3). This ligand–receptor interaction is specific, but FMLP analogues and antagonists

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§ *Abbreviations:* APN, aminopeptidase N; $[Ca^{2+}]_i$, intracellular free calcium concentration; DPPIV, dipeptidylpeptidase; FMLP, *N*-formyl-methionyl-leucyl-phenylalanine; NEP, neutral endopeptidase; and PMNs, human neutrophils.

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that competed for its receptor have also been reported [16, 17]. However, whether or not endogenous antagonists for the FMLP receptor are present has scarcely been reported.

In this paper, we report the inhibitory effects of spinorphin on human PMN functions such as chemotaxis, O_2^- generation, and exocytosis, and suggest that the inhibition is due to specific suppression of the binding of FMLP to its receptor on PMNs by spinorphin.

MATERIALS AND METHODS

Reagents

FMLP, cytochrome *c*, and *p*-nitrophenyl (PNP)-β-glucuronide were from the Sigma Chemical Co., St. Louis, MO, U.S.A. Nuclepore membranes were from Coster/Nuclepore, Cambridge, MA, U.S.A. [^{3}H]Acetylated rat tail tendon type I collagen and FML[^{3}H]P were from Dupont/New England Nuclear Research Products, Boston, MA, U.S.A. 4-Aminophenyl mercuric acetate (APMA) was from the Aldrich Chemical Co. Inc., Milwaukee, WI, U.S.A.

Spinorphin

Spinorphin has been purified from bovine spinal cord based on its inhibitory activity toward enkephalin-degrading enzymes [5]. Its effects on PMN functions were examined using a synthetic peptide that had been purified more than 95% by HPLC. It did not exhibit any cytotoxicity toward neutrophils, as judged by measuring a released enzyme, lactate dehydrogenase.

Preparation of PMNs

Human peripheral PMNs were isolated from heparinized blood of healthy volunteers according to the method of Böyum [18]. PMNs were separated from the leukocyte-rich supernatant by dextran sedimentation, hypotonic lysis of erythrocytes, and discontinuous density gradient centrifugation through a layer of Ficoll-Paque®. The yield of the cell preparation was >95% PMNs, as judged from the morphology on Giemsa staining.

Chemotaxis

The chemotactic response of PMNs to an FMLP gradient was assayed in a blind-well Boyden chamber with Nuclepore membrane filters (pore size, 3 μm) as previously described [19]. Cells (5×10^5) were placed in the upper well and exposed to 10^{-7} M FMLP in the lower well across a membrane filter. Incubation was carried out for 90 min at 37°. The number of PMNs responding to the chemotactic stimulus and appearing on the opposite side of the filter was determined after fixation and Giemsa staining. At least ten microscopic fields (40 × objective; cell number per 6.25×10^{-4} mm^2) were examined on each filter.

O_2^- Generation

The superoxide dismutase-inhibitable reduction of ferricytochrome *c* by PMNs was measured spectrophotometrically by means of an endpoint assay after incubation of the cells with FMLP (10^{-7} M) at 37° for 15 min [20].

Exocytosis

PMNs were treated, in the presence of spinorphin, with FMLP (10^{-7} M) at 37° for 15 min with shaking. The cell suspensions were then centrifuged at 5000 *g* for 10 min, and the supernatants were collected. The amount of β-glucuronidase, a marker of primary granules, was determined at 37° for 15 min using PNP-β-glucuronide as a substrate. After centrifugation, the $O.D._{405}$ of the supernatants was measured [21]. Collagenase activity, a marker of secondary granules, was measured at 37° overnight using soluble [^{3}H]acetylated rat tail tendon type I collagen after activation with 4-aminophenyl mercuric acetate [22].

Enzymatic Activity

The NEP activity on PMNs was determined as the substrate-degrading activity defined as the difference between the activities in the presence and absence of an NEP inhibitor [23]. Briefly, PMNs (2.5×10^5 cells) were preincubated in 0.1 M 2-[*N*-morpholino]ethanesulfonic acid (MES) buffer containing captopril in the presence or absence of an NEP inhibitor, phosphoramidon, at 37° for 3 min. After preincubation, the substrate (2 μM; Suc-Ala-Ala-Phe-AMC) was added and then the mixture was incubated at 37° for 30 min. The reaction was terminated with 50% acetic acid. The reaction mixtures were centrifuged, and the fluorescence of the supernatants was measured (Em: 440 nm; Ex: 340 nm). The aminopeptidase activity on PMNs was determined using *l*-alanine-*p*-nitroanilide as a substrate [24]. PMNs (5×10^5 cells) were incubated with 6 mM substrate in 50 mM sodium phosphate buffer, pH 7, at 37° for 15 min. After incubation, the reaction mixtures were centrifuged, and the $O.D._{405}$ of the supernatants was measured.

Binding Assay

FML[^{3}H]P (100 nM) and PMN cells (2×10^6/100 μL) were incubated in 150 μL of phosphate buffer, pH 7.4, for 15 min at 37° with gentle shaking. Incubations were terminated rapidly by adding ice-cold buffer. After washing five times with phosphate buffer, Triton X-100 (1%) was added, followed by solubilization for 1 hr at 4°. Radioactivity was measured with a liquid scintillation counter. Nonspecific binding was defined as the amount of binding not inhibited by 10 μM unlabeled FMLP. Specific binding was defined as the total amount of FML[^{3}H]P bound minus the nonspecific binding.

$[Ca^{2+}]_i$

PMNs (5×10^7/mL) were washed three times with 10 mM HEPES, pH 7.4, containing 140 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 5 mM glucose, and 1 mM EDTA (HEPES buffer). The cells were resuspended in 1 mL HEPES buffer and loaded with 3 mM Fura-2AM. After incubation at room temperature for 30 min, the cells were washed twice with HEPES buffer containing 1 mM $CaCl_2$. PMNs (5×10^6 cells/1.5 mL HEPES) were preincubated at 37° for 3 min, and then a stimulant was added. Fluorescence was measured under a fluorospectrometer (Type F-2000; Hitachi Co., Tokyo, Japan) equipped with a thermostatted cuvette holder and a stirring apparatus at dual excitation wavelengths of 340 and 380 nm and a fluorescence wavelength of 510 nm. $[Ca^{2+}]_i$ levels were determined from the following equation: $[Ca^{2+}]_i = K_d \cdot \beta (R - R_{min})/(R_{max} - R)$, where R_{max} represents the maximum ratio of both fluorescence values (F_{340}/F_{380}) after lysis of the cells with Triton X-100, and R_{min} represents the minimum ratio of both fluorescence values after the addition of 10 mM EGTA; β represents the scaling factor defined as the ratio of the fluorescence at 380 nm with 0 Ca^{2+} ($F_{380\ min}$) to saturating Ca^{2+} ($F_{380\ max}$) conditions; K_d is the dissociation constant for Fura-2AM-Ca^{2+}, 224 nM [25].

Statistical Analysis

Data were analyzed by means of Student's *t*-test. A difference level of $P < 0.05$ was regarded as significant.

RESULTS

Effects of Spinorphin on PMN Functions

We reported previously that spinorphin, an endogenous regulator of enkephalin, inhibits an NEP, APN, and DPPIV from monkey brain [6]. It has also been reported that NEP regulates the inflammatory response to degrading neuropeptides such as enkephalin, substance P, and bradykinin [8–10]. Thus, we examined whether or not spinorphin *in vitro* affects inflammatory responses such as chemotaxis, O_2^- generation, and exocytosis by human PMNs, that express NEP, APN, and DPPIV on their surfaces. It has been reported that FMLP (10^{-7} M) elicits a PMN inflammatory response *in vitro*, and therefore we used it as a positive control. In the chemotactic assay, FMLP induced maximal activity after a 90-min incubation in a blind-well chamber. In this system, spinorphin (10 μM) significantly inhibited FMLP-induced chemotaxis by PMNs (85% inhibition of control, N = 3, $P < 0.001$) after 90 min of incubation, and higher concentrations of spinorphin (100 μM) inhibited the chemotactic activity completely (Fig. 1). We also examined the effects of spinorphin on O_2^- generation and exocytosis, other inflammatory response markers. Spinorphin (100 μM) inhibited O_2^- generation by 48% (24.2 ± 2.7 vs 12.6 ± 4.1 nmol/2 × 10^6 cells/15 min, control vs spinorphin-treated cells, N = 3, $P < 0.05$), and at the same concentration markedly inhibited the exocytosis of β-glucuronidase, a marker of primary granules (14.9 ± 2.4 vs 6.2 ± 4.7% of total enzyme, control vs spinorphin-treated cells, N = 3, $P < 0.05$), and collagenase, a marker of secondary granules (11.3 ± 0.6 vs 8.1 ± 0.04% of total enzyme, control vs spinorphin-treated cells, N = 3, $P < 0.005$), by FMLP-stimulated PMNs, as shown in Fig. 2 and Table 1.

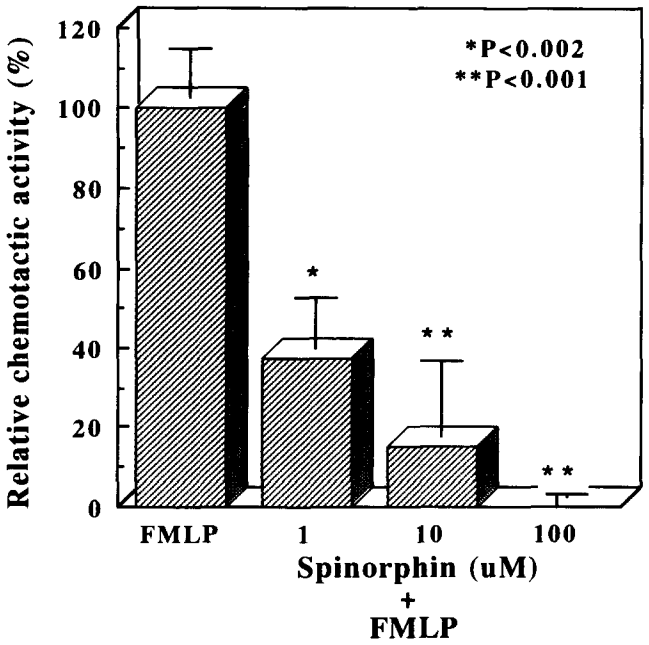

FIG. 1. Effect of spinorphin on chemotaxis by FMLP-stimulated PMNs. The chemotactic response to FMLP (10^{-7} M) was determined using a modified blind-well Boyden chamber. The chemotactic activity by FMLP-stimulated PMNs (control) was 41.2 ± 13.0 cells/field (N = 4). Each value is the mean ± SD for two determinations in three different experiments. Each concentration of spinorphin alone was not chemotactic.

Effects of Spinorphin on NEP and APN Expression, and Their Activities on PMNs

To clarify the mechanism underlying the inhibitory effect of spinorphin on inflammatory responses, the possibility that spinorphin causes increases in the expression of NEP and APN on the PMN cell surface was examined first. When PMNs were treated with spinorphin (100 μM) at 37° for 30 min, the expression of NEP (reactivity to anti-CD10 MAb) and APN (reactivity to anti-CD13 MAb) on the cells was not significantly different from that on control cells in the absence of spinorphin (data not shown). Under these conditions, spinorphin strongly inhibited the NEP activity on PMNs to the same extent as a specific NEP inhibitor, phosphoramidon (Fig. 3A), and moderately inhibited the APN activity (Fig. 3B). This suggests that the inhibitory effect of spinorphin on PMN functions is enzyme independent because the inhibition of NEP and APN on PMNs normally enhances the inflammatory responses to some neuropeptides.

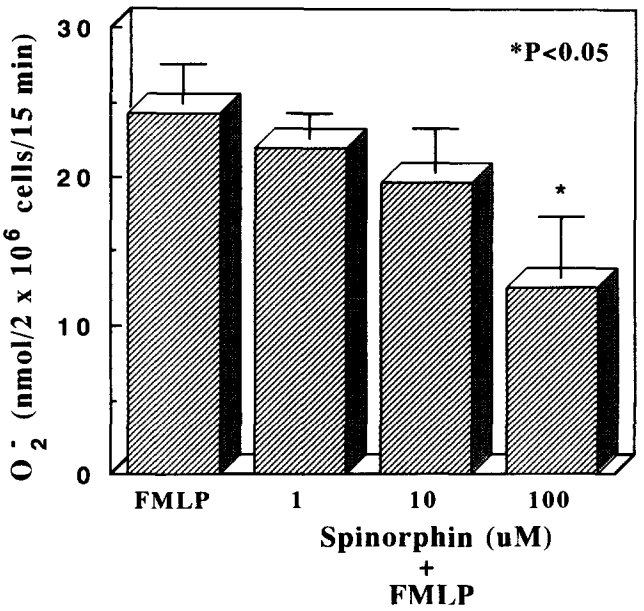

FIG. 2. Effect of spinorphin on O_2^- generation by FMLP-stimulated PMNs. PMNs were stimulated with FMLP (10^{-7} M) in the presence of spinorphin at 37° for 15 min, and then the superoxide dismutase-inhibitable reduction of ferricytochrome *c* was measured as O_2^- generation. O_2^- generation of FMLP-stimulated PMNs (control) was 24.2 ± 2.7 nmol/2 × 10^6 cells/15 min (N = 3). Each value is the mean ± SD for two determinations in three different experiments. Each concentration of spinorphin alone produced less than 15% of the O_2^- produced by FMLP-stimulated PMNs, and the activity was subtracted.

Effect of Spinorphin on the Binding of FMLP to Its Receptor

Chemoattractants such as FMLP interact specifically with the PMN cell surface. Accordingly, as the first step in the interaction of a chemoattractant with responding PMN cells, the effect of spinorphin on binding of the ligand to its receptor was examined. FML[^{3}H]P (100 nM), a potent chemotactic peptide with high specific radioactivity, and PMN cells (2 × 10^6) were incubated at 37° for 15 min in the presence of unlabeled FMLP or spinorphin. The radioactivity in the presence of 10 μM FMLP (nonspecific binding) was suppressed to 20.6 ± 3.5% (N = 4) of the total binding. As shown in Fig. 4, spinorphin (10 μM) also suppressed the radioactivity to 50.8 ± 12.0% (N = 4, $P < 0.01$) of the total binding. To determine whether or not the inhibition of the binding of FMLP to its receptor with spinorphin is the nonspecific effect, other neuropeptides were investigated. Under the same conditions, angiotensin III, a regulator of blood pressure, and substance P, a neurotransmitter, each had no effect, as shown in Fig. 5, suggesting that the suppression of ligand binding with spinorphin is not nonspecific, and that it may be involved specifically in the interactions between FMLP and its receptor.

Effect of Spinorphin on FMLP-Stimulated $[Ca^{2+}]_i$ Changes

It has been shown that FMLP activates phospholipase C, leading to increases in $[Ca^{2+}]_i$ and protein kinase C activation [14, 15]. To detect a decrease in the signal transduction after ligand binding to its receptor on the cell surface, we examined the role of spinorphin in FMLP-stimulated $[Ca^{2+}]_i$ changes. The addition of 10^{-7} M FMLP caused an immediate and transient increase in $[Ca^{2+}]_i$, which reached the peak level (227.2 ± 17.6 nM, N = 5) within 15 sec. $[Ca^{2+}]_i$ then decreased rapidly to near the basal level by 1–2 min after the stimulation, and then again increased gradually to a plateau level. In the presence of spinorphin, the transient $[Ca^{2+}]_i$ changes were suppressed concentration dependently, 62.5% of the level in an FMLP-induced sample being reached at 100 μM (N = 5, $P < 0.01$) (Table 2), suggesting that spinorphin suppressed $[Ca^{2+}]_i$ changes as a second messenger for FMLP-induced PMN functions.

DISCUSSION

In this study, we have shown that spinorphin significantly inhibits such *in vitro* FMLP-stimulated PMN functions as chemotaxis, O_2^- generation, and exocytosis. Previously, few peptides were reported to inhibit inflammatory responses. Cirino *et al.* [26] reported that an acetylated polypeptide corresponding to residues 2–26 of human lipocortin 1

TABLE 1. Effect of spinorphin on exocytosis by FMLP-stimulated PMNs

Stimulus	β-Glucuronidase (%)	Collagenase (%)
FMLP	14.9 ± 2.4	11.3 ± 0.6
FMLP + spinorphin		
1 μM	14.0 ± 5.4	9.4 ± 0.6
10 μM	14.8 ± 3.8	8.9 ± 0.05*
100 μM	6.2 ± 4.7*	8.1 ± 0.04†

Each value is the mean ± SD for two determinations in four different experiments. Each concentration of spinorphin (1, 10, and 100 μM) alone caused 2.4, 2.4, and 2.6% of the control exocytosis (5.21 ± 1.32 μmol/hr/2 × 10^6 cells) for β-glucuronidase, and 1.5, 1.6, and 2.0% of the control exocytosis (9.56 ± 1.67 ng type I collagen/hr/2 × 10^6 cells) for collagenase. The enzymatic activities in the absence of any stimulus were subtracted.

*, † Statistically significant differences between the FMLP alone and the FMLP + spinorphin groups: *$P < 0.05$, and †$P < 0.005$.

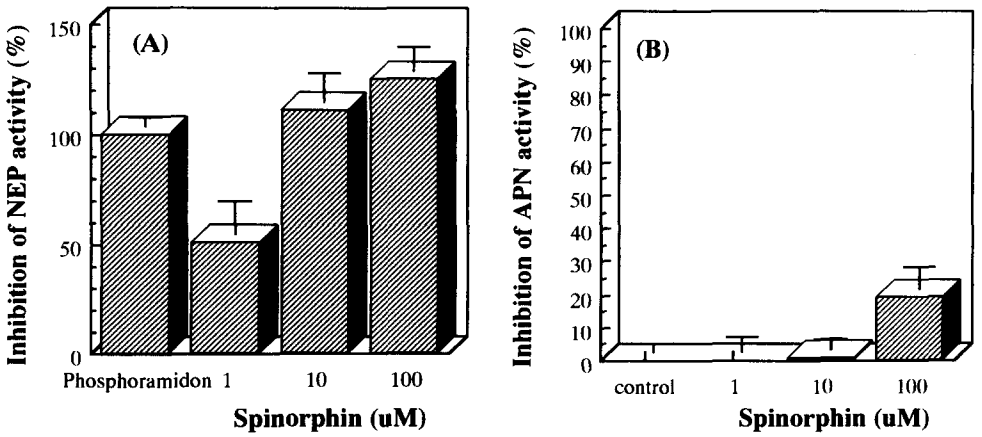

FIG. 3. Inhibitory effects of spinorphin on the enzymatic activities of NEP and APN. (A) PMNs (2.5×10^5 cells) were reacted with a fluorescent substrate, and then the degrading activity was measured with a fluorescence spectrophotometer, as described under Materials and Methods. NEP activity is expressed as a percentage of that of control cells reacted with phosphoramidon, a specific NEP inhibitor, and the level which 10 μM phosphoramidon inhibited was defined as 100%. NEP activity of control cells was 0.83 ± 0.28 pmol/min/10^6 cells (N = 5). (B) PMNs (5×10^5 cells) were reacted with *l*-alanine-*p*-nitroanilide at 37° for 15 min. After stopping the reaction at 4° and centrifugation, the amount of degraded *p*-nitroaniline was measured at $O.D._{405}$ as an APN activity. APN activity of control cells was 4.25 ± 1.75 nmol/min/10^6 cells (N = 9). Each value is the mean ± SD for two determinations in five (for NEP) and nine (for APN) different experiments.

causes a dramatic reduction in cytokine-induced leukocyte migration, with an ID_{50} of approximately 40 μg/mouse, in the mouse air-pouch model. Trotz and Said [27] reported that vasoactive intestinal peptide (VIP) inhibits phospholipase A_2 activity in a dose-dependent manner, thus reducing the release of arachidonic acid. However, little is known about the identities of these endogenous small peptides that inhibit inflammation.

The spinorphin concentration at which significant inhibition of *in vitro* PMN functions occurred was rather high (10–100 μM). This may be due to the stability of spinor-

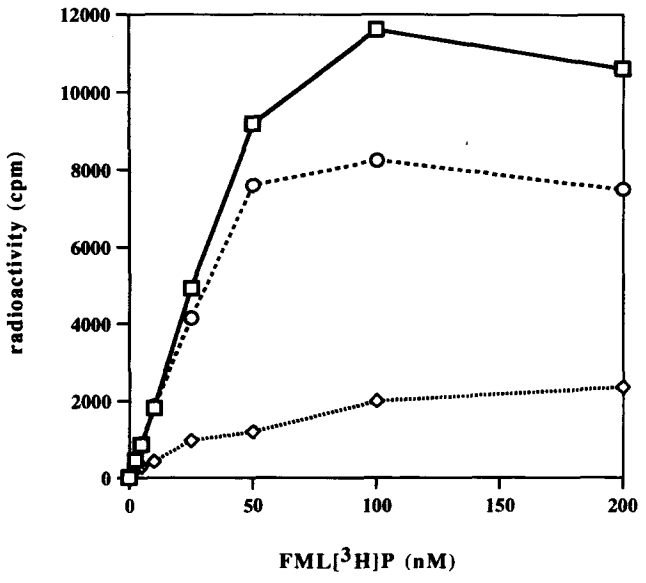

FIG. 4. Effect of spinorphin on the binding of FML[^{3}H]P to its receptor on PMNs. PMNs (2×10^6 cells) were reacted with 100 nM FML[^{3}H]P at 37° for 15 min in the presence or absence of 10 μM peptide. Nonspecific binding was defined as the amount of binding not inhibited by 10 μM unlabeled FMLP. Specific binding was defined as the total amount of FML[^{3}H]P bound minus the nonspecific binding. Representative results of four different experiments are shown. Key: (□) total binding; (◇) nonspecific binding; and (○) specific FML[^{3}H]P binding in the presence of spinorphin.

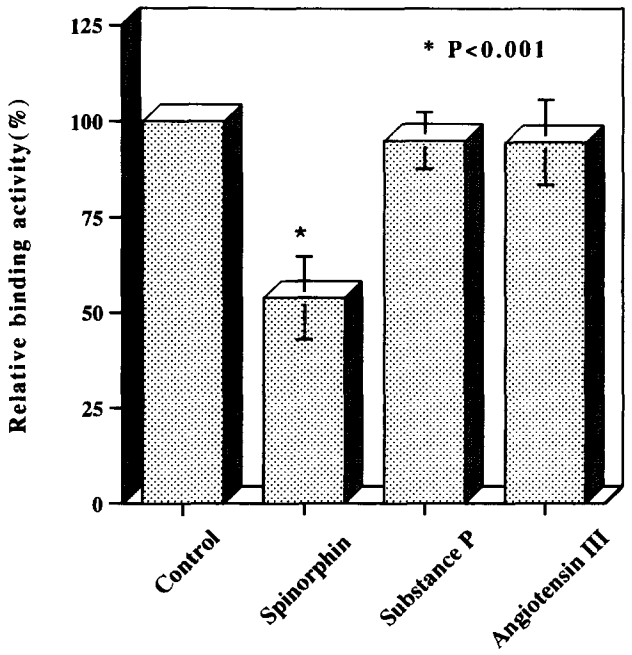

FIG. 5. Effects of various neuropeptides on the binding of FML[^{3}H]P to its receptor on PMNs. The binding assay was performed as described in Fig. 4. Each value represents the relative binding activity compared with the specific binding (control) and the mean ± SD for two determinations in four different experiments. Specific binding in the presence of spinorphin was suppressed significantly compared with that of control (2830 ± 160 vs 5360 ± 3.90 cpm, N = 4, $P < 0.001$).

TABLE 2. Effect of spinorphin on FMLP-induced $[Ca^{2+}]_i$ changes

Stimulator	$[Ca^{2+}]_i$ (nM)
Background	108.3 ± 16.4
FMLP	227.2 ± 17.6 (100)
FMLP + spinorphin	
1 μM	207.8 ± 6.8 (83.7)
10 μM	204.0 ± 6.5 (80.5)
100 μM	182.6 ± 7.2* (62.5)

Each value is the mean ± SD for two determinations in five different experiments. The values in parentheses are the relative $[Ca^{2+}]_i$ compared with that of the FMLP alone group with the background subtracted.

* Statistically significant difference between the FMLP alone and the FMLP + spinorphin groups, $P < 0.01$.

phin, a 7 amino acid peptide. Spinorphin is degraded easily by enkephalin-degrading enzymes. Among these enzymes, APN is the most active. HPLC analysis demonstrated that about 30% of spinorphin is degraded at the N-terminal Leu upon reaction with whole PMNs at 37° for 30 min (data not shown). Using the mouse air-pouch assay as an acute inflammatory model, spinorphin (ID_{50} = 30 μg/mouse) in the presence of Leuhistin, an aminopeptidase N inhibitor, was found to enhance significantly the inhibition of carrageenan-induced neutrophil accumulation, compared with spinorphin alone. Therefore, we plan to produce analogs with structures protected at the N-terminus. We hope that such analogs will be more stable toward exoproteases and more effective at lower doses.

Next, we examined the inhibitory effects of spinorphin on PMN functions. We focused on the relationship between FMLP, its receptor, and peptide-modulating enzymes on PMNs. NEP and APN are key enzymes on the PMN cell surface that can modulate inflammatory responses [8, 9]. Shipp and Look [10] reported that enzymes on PMNs, such as NEP and APN, cooperate to hydrolyze FMLP after ligand binding and regulate some associated PMN inflammatory responses. Therefore, we first measured the effect of spinorphin on the enzymatic activities of NEP and APN on PMN cells. Spinorphin inhibited NEP activity strongly and APN activity moderately (Fig. 3). It is known that the inhibition of NEP and APN on PMNs suppresses the degradation of substrates, and normally increases the inflammatory responses to some neuropeptides [28]. However, spinorphin inhibits several kinds of PMN functions (Figs. 1 and 2, and Table 1), suggesting that the inhibitory effects of spinorphin do not involve the actions of enzymes such as NEP and APN.

It is also known that endogenous peptides such as N-formylated peptides like FMLP and C5a, a degradative fraction produced during complement activation, could play significant roles in the chemoattraction and activation of leukocytes at sites of inflammation. PMNs have specific receptors for such peptides [29, 30]. In the present study, as we only used FMLP as a ligand (stimulator), we next examined the process of the binding of FMLP with its receptor using radiolabeled ligand FML[^{3}H]P. As shown in Figs. 4 and 5, spinorphin significantly suppressed FML[^{3}H]P binding to 50.8 ± 12.0 (N = 4, $P < 0.01$) of the control level. Interestingly, other neuropeptides, such as angiotensin III, a regulator of blood pressure, and substance P, a neurotransmitter, had no effect on FML[^{3}H]P binding with PMNs (Fig. 5), suggesting that the role of spinorphin is specific, and that it is involved in the interaction of FMLP and its receptor. An inhibitory effect of spinorphin was observed on FMLP-stimulated PMNs, but not on 12-*O*-tetradecanoylphorbol 13-acetate (TPA)-stimulated ones (data not shown). This action is also suggested to be FMLP-specific. These results indicate that spinorphin is not a true inhibitor of FMLP-stimulated PMN responses, but rather that it may be a regulator of the interaction of FMLP with its receptor on PMNs.

$[Ca^{2+}]_i$ is a second messenger in signal transduction systems, and changes in $[Ca^{2+}]_i$ are an indicator of cell activation. The activation pathway for soluble chemoattractants such as FMLP, which causes an increase in $[Ca^{2+}]_i$, has been well established [14, 15]. As spinorphin suppressed the binding of FMLP to its receptor, as judged in a binding assay, we examined whether or not spinorphin also decreased transient $[Ca^{2+}]_i$ changes, as a second messenger, produced upon FMLP stimulation of PMNs. As shown in Table 2, spinorphin significantly decreased the transient $[Ca^{2+}]_i$ changes (38% inhibition compared with the control), indicating that it suppressed the FMLP signaling leading to PMN responses such as chemotaxis, O_2^- generation, and exocytosis.

In conclusion, spinorphin significantly inhibited inflammatory responses of human PMNs stimulated with FMLP, and therefore may be a novel endogenous anti-inflammatory peptide that modulates the interaction between FMLP and its receptor. In the future, spinorphin will become an important tool for studying neuropeptide mechanisms, such as enkephalin-mediated pain and inflammation.

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