Neuropeptides (1998) 32 (4), 301–306

Immunomodulatory action of class μ-, δ- and κ-opioid receptor agonists in mice

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Summary Endogenous opioids exert a variety of functions outwith the central nervous system, including modulation of some murine lymphocyte functions. The results of this study indicate that μ-, δ- and κ-receptor selective agonists are potent in vitro stimulators of mitogen-induced proliferation of murine T-lymphocytes. Moreover, the observed enhancement of mitogen-induced proliferation was reversed by μ-, δ- and κ-receptor class selective antagonists, β-funaltrexamine, ICI 174,864 and nor-binaltorphimine, respectively. An additional study has revealed that repeated administration (four injections) of the opioid receptor selective agonists DAGO, DPDPE and U-50488 also enhanced the concanavalin A-induced proliferation of lymphocytes. These results suggest that there are three classes of opioid receptors on T-lymphocytes and that all these receptor classes are involved in the stimulation of concanavalin A-induced proliferation.

INTRODUCTION

The continuous interplay between the central nervous system and numerous physiological processes is essential to maintain physical and mental homeostasis. An example of such an interaction is the bi-directional communication between the neuroendocrine and immune system. There is a large body of evidence showing that exogenous opioids produce pleotrophic effects on in vivo and in vitro immune responses.[1] The direct in vitro immunoregulatory action of opioid peptides can be divided into two categories, depending on the reversibility of their immunoregulatory actions by the non-class-selective antagonist naloxone. The reversing effect of naloxone on the opioid-mediated biological activities indicates that the opioid receptor–ligand interactions occur in a 'classic' manner. The opioid-mediated immunoregulatory effects that are reversed by naloxone include mitogen- and antigen-induced lymphocyte proliferation[2–4] and modulation of both natural killer cell- and T-cell-mediated cytotoxicities.[5,6] However, less attention has been paid to μ-, δ- and κ-opioid receptors involved in the immunomodulatory processes.

In the present study, the abilities of opioid class selective agonists to regulate T-cell proliferative responses were studied.

Received 22 January 1998
Accepted 9 March 1998

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MATERIALS AND METHODS

Animals

The experiment was performed on male C57B1/6 mice (aged 4–6 weeks and weighing 20 ± 1 g) obtained from the Animal Farm of the Silesian Medical University. They were kept eight per cage at room temperature and under standard light conditions. They received a standard mouse chow and water ad libitum.

Agents

[D-Ala^2, N-Me-Phe^4, Gly^5-ol]enkephalin (DAGO), [D-Pen^{2,5}]-enkephalin (DPDPE) were purchased from Sigma, USA. U-50488, β-funaltrexamine (β-FNA) and nor-binaltorphimine (nor-BNI) were obtained from RBI, USA. ICI 174,864 was purchased from Cambridge Research Biochemicals, UK. FITC anti-mouse CD45R/B220 and CD90 (Thy-1.2) were purchased from Phar-Mingen, USA.

Culture medium

Culture medium RPMI 1640 (Gibco, Grand Island, NY, USA) supplemented with 10% heat-inactivated fetal calf serum (Gibco), 2 mM glutamine, 100 μg/ml streptomycin, 100 IU/ml penicillin designated below as complete medium was used.

Mitogen-induced proliferative responses

Spleen cells were obtained under sterile conditions as described by Van den Bergh et al.[7] After centrifugation (10 min, 350 × *g*), they were resuspended in 4 ml of lysis buffer (155 mM ammonium chloride, 10 mM potassium hydrocarbonate, pH 7.0) and kept on ice for 7 min to lyse erythrocytes. Afterwards, 4 ml of fetal calf serum were layered under the cell suspension and then centrifuged (20 min, 50 × *g*). The cell pellet was resuspended in culture medium. Cell viability determined with trypan blue was > 90%. Spleen cells were cultured in quadruplicate (1.0×10^5 cells/0.1 ml/well) in a 96-well flat-bottomed microtitre culture plate (Beckton Dickinson). Mitogens were added in a volume of 10 μl/well. Lipopolysaccharide (Sigma) was added at final concentrations of 0.5 and 5.0 μg/ml and concanavalin A (Sigma) at 1.0 and 5.0 μg/ml. As concanavalin A preferentially stimulates T-cell proliferation, while lipopolysaccharide stimulates B-cell proliferation, splenocytic response to these mitogens was considered to be either T- or B-cell proliferation. After incubation for 72 h, the number of cells was assessed by tetrazolium dye (thiazolyl blue) assay, in which the amount of formazan is proportional to the number of metabolically active cells.[8] Thiazolyl blue (MTT) in a volume of 50 μl (5 mg/ml RPMI 1640) was added to each culture. After incubation for 3 h (95% air and 5% CO_2 at 37°C), 50 μl of solvent (450 ml dimethylformamide, 135 g sodium dodecyl sulphate and 550 ml distilled water) were added to each culture. After overnight incubation at room temperature, optical density at 570 nm was measured. The experiment was performed in quadruplicate. The number of splenocytes was calculated by a calibration curve (extinction/splenocyte number). The number of mitogen-stimulated splenocytes obtained from opioid-treated animals was compared to the number of unstimulated splenocytes from the same animals and expressed as a percentage. In addition, the percentage of splenocytes obtained from peptide-treated animals was compared with the percentage of splenocytes from saline-treated animals.

Statistical analysis

Differences were analysed by Student's t-test. Results are expressed as the arithmetic mean of three experiments (four measurements in each experiment).

Animal treatment

The present study investigated whether selective μ-, δ- and κ-receptor agonists (DAGO, DPDPE and U-50488 respectively) given at a dose of 10 μg/mouse for 1 and 4 days affect the proliferation of immune cells. The peptides were dissolved in sterile phosphate-buffered saline and given intraperitoneally (i.p.) in a volume of 0.1 ml/mouse. In determining the percentages of T- and B-cells, the opioid dose was 10 μg/mouse.

Determination of percentages of B- and T-cells

Spleen cells were obtained according to Van den Bergh et al,[7] and then suspended in RPMI 1640 medium supplemented with 5% fetal calf serum and 0.1% sodium azide. Then the cells (1×10^6) were incubated with 10 μl of monoclonal antibodies (Mabs) conjugated with fluorescein isothiocyanate in a total volume of 100 μl for 30 min (4°C). The following antibodies were used: Thy 1.2 for T-cells and CD45R/B220 for B-lymphocytes. After incubation, the cells were washed twice with RPMI medium supplemented with 5% fetal calf serum and 0.1% sodium azide and then resuspended in 50 μl of the same medium. At least 400 cells in each smear were counted under a fluorescent microscope and the percentage of cells with green fluorescence was calculated.

RESULTS

Lymphocyte proliferation study

A single injection of DAGO at a dose of 10 μg increased concanavalin A- and lipopolysaccharide-stimulated lymphocyte proliferation. Single injections of DPDPE and U-50488 (both at 10 μg/mouse) did not change concanavalin A-, and lipopolysaccharide-stimulated proliferation. Four injections of DAGO, DPDPE and U-50488 at a dose of 10 μg enhanced the proliferation of lymphocytes induced by both mitogens (Table 1).

To examine whether selective μ-, δ- and κ-agonists affect in vitro mitogen-stimulated proliferation, splenocytes were co-cultured with mitogens and DAGO, DPDPE or U-50488. All agonists studied enhanced concanavalin A- and lipopolysaccharide-induced lymphocyte proliferation at concentrations of 10^{-6}–10^{-12} M (Table 2).

To confirm the specificity of the agonist–receptor interactions, an additional study of μ-, δ- and κ-selective antagonists was performed. Splenocytes were cultured in the presence of concanavalin A, receptor agonists (10^{-6}–10^{-12} M) and a constant concentration of receptor-selective antagonist (10^{-6} M). The first part of the study, which was conducted with DAGO and β-FUN, a μ-specific antagonist, revealed that β-FUN reversed the DAGO-stimulated proliferation of lymphocytes. On the other

Table 1 The influence of μ-, δ- and κ-opioid receptor agonists on proliferation of stimulated mouse splenocytes

Agent	Percentage of lymphocytes stimulated by			
	Con A 1 μg/ml	Con A 5 μg/ml	LPS 0.5 μg/ml	LPS 5 μg/ml
		1 injection		
Control	578 ± 6	1050 ± 10	550 ± 3	690 ± 6
DAGO	671 ± 12*	1847 ± 15*	687 ± 19*	863 ± 15*
DPDPE	550 ± 18	950 ± 30	550 ± 10	690 ± 6
U-50488	531 ± 11	970 ± 15	514 ± 9	630 ± 9
		4 injections		
Control	612 ± 12	1130 ± 16	570 ± 12	737 ± 14
DAGO	806 ± 18*	1430 ± 25*	682 ± 37*	869 ± 15*
DPDPE	975 ± 15*	1940 ± 30*	663 ± 10	880 ± 19*
U-50488	865 ± 17*	1840 ± 20*	702 ± 35*	920 ± 14*

Mice (6/group) were injected with the studied agent at a dose of 10 μg/mouse or phosphate-buffered saline 1 day or 4 days before splenic lymphocyte proliferation was assessed (see Materials and methods). Results are expressed as the mean ± SE of two independent experiments. *Significant change in comparison with control ($P < 0.01$).

Table 2 Dose-dependent enhancement of proliferation by μ-, δ- and κ-selective opioid receptor agonists

		Percentage of lymphocytes stimulated by			
Agent	Concentration in M	Con A 1 μg/ml	Con A 5 μg/ml	LPS 0.5 μg/ml	LPS 5 μg/ml
Medium	–	600 ± 18	1110 ± 35	570 ± 11	700 ± 12
DAGO	10^{-4}	582 ± 15	1051 ± 30	575 ± 10	701 ± 11
	10^{-6}	855 ± 20[a]	1400 ± 20*	643 ± 15*	794 ± 10
	10^{-8}	924 ± 19[a]	1630 ± 30*	664 ± 14*	827 ± 20*
	10^{-10}	980 ± 16[a]	1670 ± 11*	688 ± 15*	850 ± 18*
	10^{-12}	900 ± 26[a]	1540 ± 30*	610 ± 25	837 ± 12*
	10^{-14}	615 ± 22	1090 ± 24	570 ± 20	690 ± 15
	10^{-16}	580 ± 33	1030 ± 33	530 ± 25	705 ± 13
DPDPE	10^{-4}	590 ± 20	1130 ± 22	559 ± 20	660 ± 10
	10^{-6}	730 ± 18*	1450 ± 35*	640 ± 23*	791 ± 11
	10^{-8}	810 ± 19*	1570 ± 30*	680 ± 25*	827 ± 19*
	10^{-10}	840 ± 25*	1590 ± 35*	708 ± 25*	868 ± 20*
	10^{-12}	780 ± 31*	1540 ± 45*	680 ± 26*	848 ± 10*
	10^{-14}	600 ± 18	1155 ± 37	590 ± 23	705 ± 10
	10^{-16}	565 ± 23	1110 ± 48	570 ± 24	701 ± 15
U-50488	10^{-4}	630 ± 22	1030 ± 36	590 ± 23	732 ± 28
	10^{-6}	750 ± 30*	1440 ± 30*	610 ± 25*	746 ± 20
	10^{-8}	825 ± 21*	1500 ± 40*	676 ± 28*	826 ± 25*
	10^{-10}	960 ± 25*	1630 ± 25*	690 ± 19*	840 ± 22*
	10^{-12}	850 ± 17*	1472 ± 37*	653 ± 23*	768 ± 27
	10^{-14}	622 ± 20	1161 ± 33	600 ± 24	770 ± 19
	10^{-16}	650 ± 22	1030 ± 15	580 ± 24	745 ± 18

Splenocytes were incubated with various concentrations of opioids for 1 h, followed by stimulation with mitogen. At day 3 proliferation was determined by the MTT method. The results are represented as means ± SE of two experiments (eight determinations). *Significant change in comparison with control ($P < 0.01$).

hand, the same concentration of ICI 174,864 or nor-BNI did not block the lymphocyte proliferation stimulated by DAGO, suggesting that DAGO interacted with the class μ receptor only. The second part of experiment, in which DPDPE and the δ-selective antagonist ICI 174,864 were used, revealed that DPDPE added to culture augmented the mitogen-induced proliferation of lymphocytes. ICI 174,864 was found to reverse the DPDPE-stimulated proliferation of lymphocytes. Nor-BNI and β-FUN were each found to have no effect on the stimulating effects of DPDPE on the concanavalin A response, suggesting that DPDPE interacted only with the class δ receptors. The final part of the experiment, in which the interaction of the κ-selective agonist U-50488 and antagonist nor-BNI was studied, revealed that U-50488 acted selectively on the κ-receptor, because nor-BNI added to splenocyte cultures reversed the stimulating effect of U-50488 on concanavalin A-induced proliferation, but neither β-FUN or ICI 174,864 reversed the effects of U-50488 (Table 3).

The results described above show changes caused by opioids added to culture after stimulation of lymphocytes by a mitogen for 1 h. Resting lymphocytes

Table 3 Effects of the opioid selective antagonist on opioid receptor-selective agonist-mediated stimulation of mitogen-induced proliferation by splenocytes

Agent	Concentration of agonist			
	10^{-6} M	10^{-8} M	10^{-10} M	10^{-12} M
DPDPE	1500 ± 24*	1541 ± 30*	1570 ± 15*	1467 ± 34*
DPDPE + ICI 174,864	1165 ± 28	1160 ± 24	1183 ± 31	1155 ± 30
DPDPE + β-FUN	1467 ± 65*	1522 ± 28*	1567 ± 39*	1513 ± 17*
DPDPE + nor-BNI	1507 ± 31*	1577 ± 21*	1568 ± 49*	1522 ± 28*
Medium		1205 ± 29		
DAGO	1513 ± 25*	1600 ± 28*	1689 ± 30*	1559 ± 20*
DAGO + β-FUN	1165 ± 23	1178 ± 18	1180 ± 20	1200 ± 16
DAGO + nor-BNI	1542 ± 18*	1688 ± 26*	1733 ± 15*	1600 ± 44*
DAGO + ICI 174,864	1500 ± 16*	1660 ± 23*	1620 ± 40*	1560 ± 45*
Medium		1160 ± 34		
U-50488	1600 ± 23*	1780 ± 34*	1800 ± 27*	1560 ± 30*
U-50488 + nor-BNI	1190 ± 19	1158 ± 24	1192 ± 33	1998 ± 17
U-50488 + β-FUN	1610 ± 26*	1743 ± 19*	1723 ± 38*	1542 ± 33*
U-50488 + ICI 174,864	1623 ± 19*	1742 ± 45*	1743 ± 40*	1523 ± 32*
nor-BNI		1230 ± 45		
ICI 174,864		1145 ± 31		
β-FUN		1195 ± 36		
Medium		1250 ± 27		

Splenocytes were incubated with various amounts of agonist or agonist + constant dilution of antagonist (10^{-6} M) for 1 h followed by stimulation with concanavalin A. At day 3 proliferation was determined by the MTT method. Results are represented as means ± SE of two experiments (eight determinations). Significant change in comparison with control ($P < 0.01$).

incubated for 24 h with DAGO at concentrations of 10^{-6}–10^{-12} M (cells were washed six times after incubation with DAGO) showed an enhanced mitogen-induced proliferation similar to that observed with an opioid added to cell culture after mitogen stimulation for 1 h (data not shown).

Modulation of T- and B-cell percentages by opioid receptor agonists

To test whether the observed modulation of mitogen-induced proliferation depended on the number of T- and B-cells, the expression of Th 1.2 and CD45R/B220 antigens – markers of mouse T- or B-cells respectively – was analysed in mice treated with opioid agonists. A single injection of DAGO (10 μg/mouse) increased the percentage of T-cells, but it did not alter the percentage of B-cells. DPDPE and U-50488 (both at 10 μg/mouse) did not alter the percentages of B- and T-cells (Table 4).

Table 4 Effect of μ-, δ- and κ-opioid receptor agonists on percentages of lymphocytes

Agent	Dose μg/mouse	Percentage of	
		T-lymphocytes	B-lymphocytes
Control	–	37 ± 3	39 ± 3
DAGO	10	49 ± 1*	38 ± 3
DPDPE	10	37 ± 1	39 ± 2
U-50488	10	43 ± 1	40 ± 2

Mice (6/group) were injected with agents once. The frequency of lymphocyte phenotype was determined 1 day after injection (see Materials and methods). Results are expressed as the mean ± SE of two experiments (in each experiment at least 400 cells were counted in 3 samples). *Significant increase in comparison with control ($P < 0.01$).

DISCUSSION

Opioid peptides have been widely reported to modulate cell-mediated immune responses in vitro and in vivo.[9–11] However, less attention has been paid to the class, or classes, of opioid receptors involved in these immunomodulatory effects. The results of the present study indicate that selective μ-, δ- and κ-agonists are potent stimulators of mitogen-induced T-cell proliferation, but they are weak inducers of B-cell proliferation. Moreover, the enhancement of T-cell proliferation was reversed by μ-, δ- and κ-receptor class selective antagonists, β-FUN, ICI 174,864 and nor-BNI, respectively. These findings are in agreement with a previous study (manuscript in preparation), in which methionine-enkephalin augmented the mitogen-induced proliferation of T-cells. As methionine-enkephalin preferentially interacts with μ- and δ-receptor classes,[12,13] it is not surprising that μ- and δ-receptor selective agonists (DAGO and DPDPE) were capable of enhancing lymphocyte proliferation. Moreover, T-lymphocyte proliferation was also enhanced by the selective κ-receptor class agonist U-50488.

The mechanisms by which opioid peptides enhance lymphocyte proliferation are currently open to speculation. In

accord with earlier data,[12,13] the study of pharmacological characteristics of cloned μ-, δ- and κ-receptors has proven that methionine- and leucine-enkephalins and β-endorphin bind to μ- and δ-receptors with comparable affinity, while their affinity for κ-receptors is considerably lower. However, in a study with presumably different cloned receptors, Yasuda et al[14] reported that β-endorphin binds to δ- and κ-receptors with comparable affinity. Collectively, these studies together with the results of the present study suggest that three classes of opioid receptors are present on T-lymphocytes. All these receptors interact with their ligands in a 'classic' manner and are involved in the stimulation of T-cell proliferation.

After a single injection of selective μ-, δ- and κ-receptor agonists, only the μ-receptor ligand DAGO was capable of modulating the proliferative response, whereas prolonged administration (4 days) of all three classes of receptor agonists enhanced T- and B-cell proliferation. However, the opioid-induced B-cell response was weak (increased by about 20%) in comparison with the agonist-induced T-cell response. A similar effect (potent stimulation of T-cells and very weak stimulation of B-cells) was reported by Srisuchart et al,[15] who injected methionine-enkephalinamide for 7 days. The mechanism of opioid-stimulated lymphocyte proliferation in vivo is not clear. As a single injection of DAGO, but not DPDPE or U-50488, enhanced the number of T-cells, it is likely that the increased T-lymphocyte proliferation reflects increased percentages of T-cells present in the cell populations tested. T- and B-lymphocytes have been proven to have opioid receptors,[16] therefore the possibility that the prolonged administration of the opioids studied directly affects the activities of both cell types cannot be excluded.

Opioid peptides may also indirectly alter the activity of immune cells by affecting levels of neurohormones, such as adrenocorticotrophin and somatostatin. These two neurohormones inhibit the proliferation of lymphocytes; others, such as growth hormone, prolactin and substance P, increase the activity of immune cells. On one hand, the plasma level of growth hormone is increased by the μ-receptor agonist fentanyl, whereas it is not affected by the δ-receptor agonist ICI 154,129 and the κ-receptor agonist U-50488.[17] DAGO increases release of prolactin, while DPDPE and U-50488 do not.[18] On the other hand, DAGO may decrease somatostatin levels.[19] It seems that DAGO, in contrast to DPDPE or U-50488, may indirectly increase the activity of immune cells by upsetting the balance between the inhibiting and stimulating factors in favour of the latter. Gonzales et al[20] observed tolerance to hormones caused by prolonged treatment with μ- and δ-receptor agonists. Thus it is likely that the induction of T-cell proliferation by DAGO, DPDPE and U-50488 administered for 4 days may reflect a direct interaction between μ-, δ- and κ-receptors located on T-cells and their respective ligands.

It has become increasingly evident that opioid peptides produce a variety of effects on the cells of the immune system. These effects are mediated by at least three opioid receptor classes. Neurohormone production and potential de novo synthesis of opioid peptides by immune cells, as well as their known immunoregulatory effects, suggest that opioid peptides may be a component of an autocrine regulatory network controlling lymphocyte activation.

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