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[^{3}H]Morphine binding is enhanced by IL-1-stimulated thymocyte proliferation

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Mouse thymocytes incubated in vitro with increasing concentrations of interleukin-1 (IL-1) in the presence of phytohemagglutinin (PHA) exhibited a dose-dependent increase in cell proliferation, as measured by [^{3}H]thymidine incorporation. Under these conditions, there was a parallel dose-dependent increase in specific [^{3}H]morphine binding, with a maximum increase of approximately 5-fold over basal levels. The binding sites differ from classical opioid receptors in that they are not stereo-selective. Interleukin-2 was ineffective in promoting either cell proliferation or enhanced opioid binding, but the effects of IL-1 could be mimicked by phorbol myristate acetate (PMA), suggesting the involvement of tyrosine phosphorylation. These results indicate that morphine-binding sites on immune cells can be regulated by cytokine activation.

Immune; Interleukin; Opioid binding; Cell proliferation; Up-regulation; Phorbol ester

1. INTRODUCTION

Increasing evidence indicates that the opioid and immune systems are closely associated. Opioid agonists can alter a variety of immune functions in vivo and in vitro [1–4], and several studies suggest that some immune cells contain opioid receptors [5,6] and/or opioid peptides [7,8]. However, direct demonstration of these receptors by in vitro binding assays has been difficult, and many studies suggest that these receptors are quite different from those mediating analgesia and other in vivo pharmacological effects of opioids. Indeed, one recent review concluded that no study has conclusively demonstrated the existence of specific opioid binding sites on immune cells [9].

One factor that could be of importance in determining the presence of detectable opioid receptors on immune cells is the cell's state of activation. Many types of immune cells are activated by cytokines, growth factors that bind to specific receptors on the cell surface and initiate a cascade of metabolic events culminating in cell proliferation. This activation frequently includes secretion of additional cytokines and expression of their appropriate receptors. For example, activation of T-cells by interleukin-1 (IL-1) results in secretion of interleukin-2 (IL-2) as well as expression of IL-2 receptors [10]. The action of IL-2 on these cells results in further proliferative events.

Abbreviations: IL-1, interleukin-1; IL-2, interleukin-2; PHA, phytohemagglutinin; PMA, phorbol myristate acetate

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Considerable evidence now indicates that opioids can play a major role in this activation process, including modulation of the release and/or activity of cytokines [9,11–16]. Furthermore, some studies suggest the existence of a direct interaction at the receptor level between opioids and cytokines. For example, naloxone has been reported to antagonize the actions of IL-2 on human natural killer cells, while β-endorphin inhibited binding of IL-2 to phytohemagglutinin (PHA)-stimulated lymphocytes [17]. Another group observed that IL-1 inhibited binding of several opioids to brain membranes [18]. Conversely, Wiedermann [19] found that IL-1 enhanced opioid peptide binding to frozen brain tissue.

Given this close relationship between opioids and cytokines at the level of both binding and function, it is plausible to suggest that opioid receptors, like those for cytokines, may be regulated on the immune cell by the cell's state of activation. Since we recently observed that morphine could specifically inhibit IL-1-induced proliferation of thymocytes (manuscript in preparation), it was of interest to determine whether this proliferation, in turn, altered binding of [^{3}H]morphine to the thymocytes. Here we report that IL-1 activation of thymocytes results in a dramatic increase in specific binding of [^{3}H]morphine. This effect appears to depend on the induction of proliferation in intact cells, and not simply the presence of IL-1 or binding to its receptor.

2. MATERIALS AND METHODS

2.1. *Materials*

Morphine sulfate was purchased from Malinkrodt (St. Louis, MO),

and [^{3}H]morphine from New England Nuclear (Boston, MA). PHA and phorbol myristate acetate (PMA) were obtained from Sigma Chemical Corp. (St. Louis, MO). Recombinant mouse IL-1 and IL-2 were purchased from Upstate Biotechnology, Inc. (Lake Placid, NY) and Genzyme (Boston, MA), respectively.

2.2. *Animals*

Harlan ICR mice were used in all studies.

2.3. *Thymocyte proliferation assay*

Thymus was removed aseptically from the animal and passed through a nylon mesh to dissociate the cells. The cells were counted using the trypan blue dye exclusion method, then plated at a density of 10^6 cells per well in a 96-well plate. Thymocyte proliferation was determined by stimulating the cells with PHA (2 mg/ml) in the presence of varying concentrations of IL-1. After 48 h, the cells were pulsed for 24 h with 1 μCi of [^{3}H]thymidine, then harvested, washed and assayed for radioactivity.

2.4. *Preparation of thymocyte membranes*

Thymocytes were cultured for 72 h in the presence of different mitogens, as detailed in section 3. The cells were then harvested by centrifugation at $1000 \times g$ for 5 min and used immediately or frozen quickly in liquid nitrogen and stored at -70°C. The frozen cells were thawed slowly at 37°C, homogenized several times in a Dounce homogenizer, and centrifuged again at 1000 rpm for 5 min. The supernatant was removed and centrifuged at $10\,000 \times g$ for 45 min. The pellets were resuspended in 0.32 M sucrose and frozen at -70°C until used.

2.5. *Opioid binding assay*

Thymocyte membranes (250 mg protein) were incubated in 25 mM HEPES, pH 7.4 with [^{3}H]morphine (5 nM) in the presence or absence of unlabelled morphine (10 μM). The mixture was incubated by gentle shaking for 1 h at 0°C; preliminary experiments indicated that maximal binding occurred in this time period. The reaction was terminated by filtering the cell membranes on Whatman GF-B glass fiber filters. The filters were then washed with 3×5 ml of 25 mM HEPES, pH 7.4. Radioactivity of the filters was determined after the filters were allowed to stand in 9 ml of scintillation cocktail (Scintiverse) overnight. Specific opioid binding was defined as the difference in the average radioactivity bound to sets of replicate samples in the presence and absence of unlabelled ligand.

3. RESULTS AND DISCUSSION

In the presence of PHA, a T-cell mitogen, and increasing concentrations of IL-1, thymocytes undergo a dose-dependent increase in cell number, as determined by incorporation of [^{3}H]thymidine (Fig. 1A). As shown in Fig. 1B, there was a roughly parallel increase in [^{3}H]morphine binding (per mg of thymocyte membranes), with an IL-1 concentration of 1 ng/ml inducing an approximately 5-fold increase in binding. The [^{3}H]morphine binding sites present on the activated cells were of relatively low affinity ($K_d = 50$ nM), and showed little stereo-selectivity (Fig. 2).

In light of the reported direct interactions between IL-1 and opioids at the receptor level [17–19], it was important to determine whether the enhancement of opioid binding by IL-1 required cell proliferation, or could be accounted for simply by the physical interaction of IL-1 with opioid receptors. When membranes isolated from unactivated thymocytes were preincubated for 30 min at 37°C in the presence of IL-1

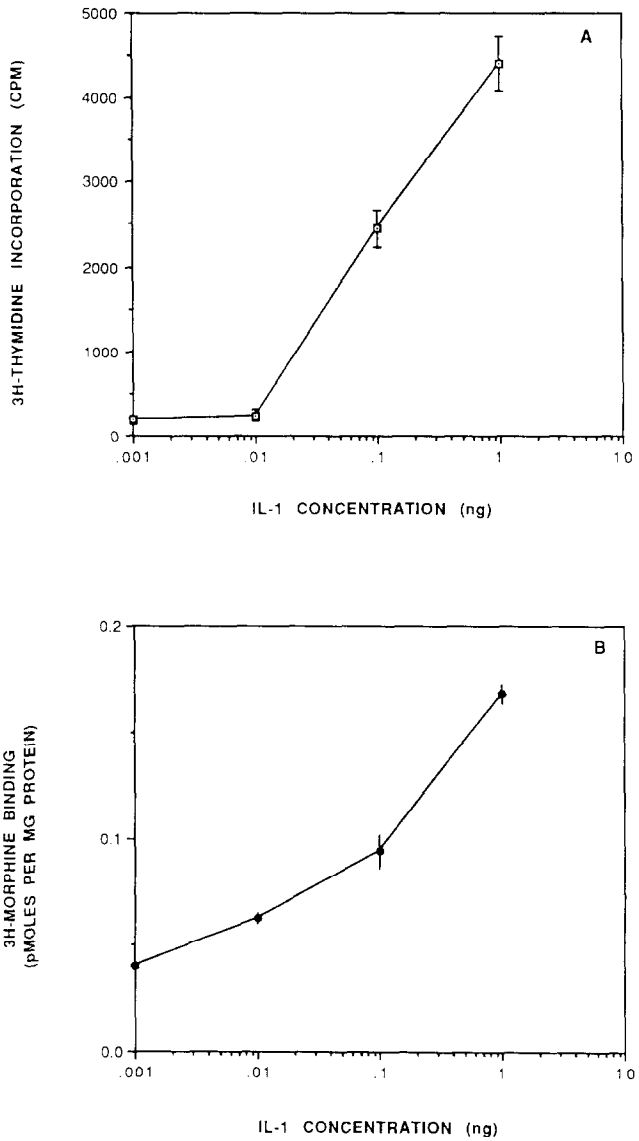

Fig. 1. Dose-dependent stimulation of proliferation and [^{3}H]morphine binding in thymocytes by IL-1. Thymocytes were cultured with PHA (2 μg/ml) in the presence of increasing concentrations of IL-1, as indicated. (A) IL-1 stimulation of proliferation. After 48 h of culture with PHA and IL-1, the cells were pulsed for 24 h with 1 μCi of [^{3}H]thymidine. Cells were then harvested, washed, and assayed for radioactivity. (B) IL-1 stimulation of specific [^{3}H]morphine binding. After 72 h of culture with PHA and IL-1, cells were harvested, membranes prepared, and assayed for [^{3}H]morphine binding. Values shown are means of 2 experiments, with ranges indicated by the vertical bars.

concentrations as high as 10 ng/ml, there was no increase of [^{3}H]morphine binding as subsequently assayed (data not shown). Furthermore, as shown in Fig. 3B, IL-1 in the absence of PHA was able to increase [^{3}H]morphine binding, but only to about half the extent mediated by the same concentration of IL-1 in the presence of PHA; again, the increase of opioid binding paralleled the increase in [^{3}H]thymidine incorporation (Fig. 3A). The cytokine IL-2 given alone was vir-

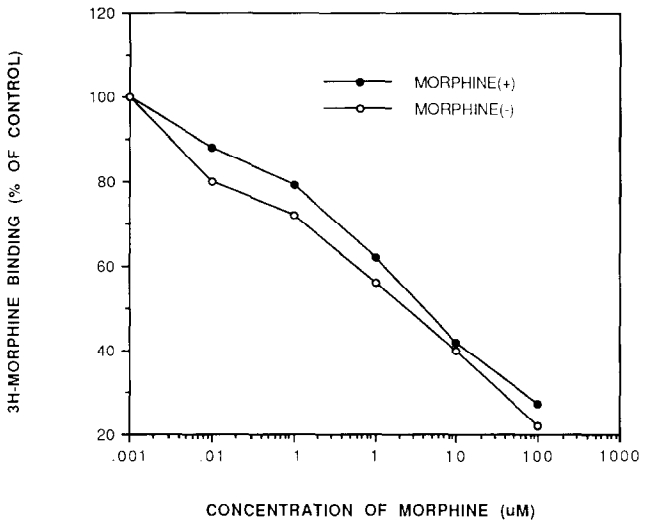

Fig. 2. Effect of (−) and (+)-morphine binding to activated thymocytes. The experiment was carried out as in the legend to Fig. 1A, except that [^{3}H]morhine binding was determined in the presence of various concentrations of (−) and (+)-morphine (unfilled and filled circles, respectively). Values are means of triplicate determinations in a single, representative experiment.

tually without effect on both cell proliferation and opioid binding (Fig. 3A,B).

PHA alone, at sufficiently high concentration, was able to induce the full increase in proliferation and binding (Fig. 3). This is consistent with the fact that this mitogen stimulates secretion of endogenous IL-1. Treatment of the cells with PMA also induced the full effect on both proliferation and [^{3}H]morphine binding (Fig. 3). This phorbol ester activates protein kinase C, a key event in cell proliferation, suggesting that IL-1 enhancement of opioid binding may also be mediated through this second messenger. However, we cannot rule out the possibility that PMA has a direct effect on opioid binding, mediated through phosphorylation and activation of opioid receptors, rather than an indirect effect mediated through phosphorylation of the same set of proteins activated by IL-1.

We used [^{3}H]morphine as ligand in these studies because of our previous observation that morphine inhibits thymocyte proliferation (manuscript in preparation), and because morphine is one of the most commonly used opioids clinically, where its effects on the immune system are particularly important. It remains to be determined whether binding of other opioid alkaloids, as well as opioid peptides, is similarly affected by thymocyte proliferation. However, the very low degree of stereo-selectivity of these binding sites indicates they are quite distinct from opioid receptors in the mammalian brain; in this respect, they are like other opioid binding sites detected on immune cells [9].

The physiological function, if any, of these morphine binding sites also remains to be elucidated, but one possibility is that they regulate the proliferative

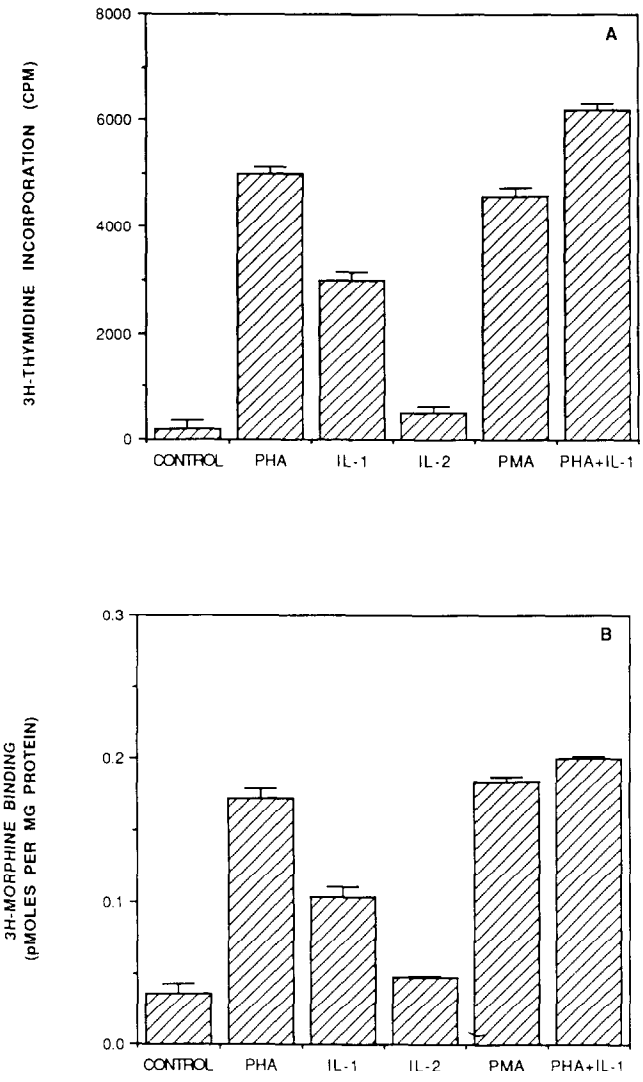

Fig. 3. Effect of various stimulating agents on T-cell proliferation. Thymocytes were cultured for proliferation experiments as given in the legend to Fig. 1A (panel A) or for specific [^{3}H]morphine binding as given in the legend to Fig. 1B (panel B). Concentrations of stimulating agents used: PHA (20 μg/ml); IL-1 (1 ng/ml); IL-2 (50 000 U/ml); PMA (10 μM); PHA (2 μg/ml) + IL-1 (1 ng/ml). Values shown are means of triplicate determinations, with standard deviations shown as vertical bars.

response. While both enhancement and inhibition of immune cell proliferation have been reported by various investigators [9], we have found that morphine inhibits IL-1-induced proliferation of T-cells in this system, in a dose-dependent manner (manuscript in preparation). If this action is mediated by the same binding sites identified in this study, the latter could act as part of a negative feedback process, by which opioids limit proliferation of the cells. This could act as a counterbalance to the positive feedback system provided by up-regulation of IL-2 receptors, activation of which increases cell proliferation.

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