Presence of the \( \mu_3 \) Opiate Receptor in Endothelial Cells

COUPLING TO NITRIC OXIDE PRODUCTION AND VASODILATION

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Initial confinement of opiate receptors to the nervous system has recently been broadened to several other cell types. Based on the well established hypotensive effects of morphine, we hypothesized that endothelial cells may represent a target for this opiate substance. Endothelial cells (human arterial and rat microvascular) contain a high affinity, saturable opiate binding site presumed to mediate the morphine effects that is stereoselectively and characteristically antagonized by naloxone. This opiate alkaloid-specific binding site is insensitive to opioid peptides. It is, therefore, considered to be the same subtype of opiate receptor (designated \( \mu_3 \)) used in the mediation of morphine in other cell types exhibiting the same binding profile. Experiments with endothelial cultures and the aortic ring of rats cultured in vitro demonstrate that morphine exerts direct modulatory control over the activities of endothelial cells, which leads to vasodilation. It induces the production of nitric oxide, a process that is sensitive to naloxone antagonism and nitric oxide synthase inhibition. In contrast with that of opiates, the administration of opioid peptides does not induce nitric oxide production by endothelial cells. In conclusion, the data presented above reveal a novel site of morphine action, endothelial cells, where a \( \mu_3 \) receptor is coupled to nitric oxide release and vasodilation.

The vast majority of pharmacological studies of the properties of opiate substances have long been almost exclusively concerned with their effects on analgesic and antinociceptive phenomena. More recently, a number of experiments have demonstrated that morphine modulates the activity of varieties of cell types, among them the immunocytes of several mammalian and invertebrate species (1, 2). In addition, this largely down-regulating effect was found to be mediated by a highly specific, opiate alkaloid-sensitive receptor used selectively by opiates (1, 2). In the present case, the receptor (\( \mu_3 \)) accomplishes this by counteracting the cellular responsiveness to a number of immunomodulatory molecules, e.g. lipopolysaccharides and some cytokines (see Ref. 3).

In this context, morphine was found to be quite potent in lowering or terminating the activation of human granulocytes and monocytes exposed to the stimulatory activity of plasma obtained from cardipulmonary bypass patients (4–7). From these observations, we surmised that a proportion of these cells may have been derived from intravascular immune cells whose adhesiveness to the vascular lining may have been altered by the presence of morphine in this tissue.

The present study was aimed at the exploration of the possibility that endothelial cells may be under the direct control of the opiates. It provided evidence for the specific binding of morphine to endothelial cells, resulting in stimulation of nitric oxide (NO)\(^1\) production in a naloxone-reversible manner and relaxation of blood vessels. It also demonstrated that these activities are mediated by the special opiate receptor \( \mu_3 \) present in the endothelial cells.

MATERIALS AND METHODS

Cell Cultures—Human arterial endothelial cells were obtained from a commercial laboratory (Cell Systems, Kirkland, WA) for binding analysis as a prefrozen pellet (10\(^7\) cells). In addition, microvascular endothelial cells (MVE) were established in our laboratory (8, 9) by SV40 transfection of endothelial cells from microdissected rat renal resistance arteries and dosed by limiting dilution. The cells were characterized as endothelial in origin based on the following criteria: distinct cobblestone-like morphology, tendency for capillary tube formation, positive identification of the factor VIII immunoreactivity, uptake of acetylated low density lipoprotein, and absent immunoreactivity of smooth muscle-specific actin. MVEs were grown in M199 medium (Mediatech, Washington, D.C.) supplemented by 5% fetal bovine serum (HyClone Laboratories, Logan, UT).

Opiate Binding Analysis—The endothelial cells (human arterial and rat microvascular were processed separately) were homogenized in 50 volumes of 0.32\(\times\) sucrose, pH 7.4, at 4°C by the use of a Brinkmann Instruments Polytron (30 s, setting no. 5). The crude homogenate were centrifuged at 900\(\times\)g for 10 min. The resulting supernatants were centrifuged at 900\(\times\)g for 10 min. The extraction procedure was repeated one more time, and the combined supernatants were centrifuged at 900\(\times\)g for 10 min. The resulting supernatants (S1') were used immediately.

Immediately prior to the binding experiment, the S1' supernatant was centrifuged at 30,000\(\times\)g for 15 min, and the resulting pellet (P2) was washed once by centrifugation in 50 volumes of the sucrose/Tris-HCl buffer, pH 7.4, and centrifuged at 900\(\times\)g for 10 min. The extraction procedure was repeated one more time, and the combined supernatants were centrifuged at 900\(\times\)g for 10 min. The resulting supernatants (S1') were used immediately.

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Aliquots of membrane suspensions from these cells were incubated with nonradioactive compounds at six concentrations for 10 min at 4°C. One hundred percent binding is defined as bound 3DHM in the presence of \( 10^{-8}\)M dextrorphan minus bound 3DHM in the presence of \( 10^{-8}\)M levorphanol. \( K_i \) is defined as the concentration of drug that elicits half-maximal inhibition of specific binding. The mean ± S.E. for three experiments is given. The displacement analysis data indicate the potency of various opioid (Met-enkephalin and \( \alpha\)-Ala\(^{3}\)Met-enkephalin, \( \beta\)-Ala\(^{3}\)MePhe\(^{5}\)Gly(\( \alpha\)Me)\(^{5}\)enkephalin, DAGO, and Porvad) in the presence of morphine.
Site with Chard analysis showed a single, relatively high affinity binding ligand specification (Fig. 1 and Table I). Saturation and Scatchard analysis of 3DHM binding to human arterial endothelial cells revealed opiate binding sites and their tissue bath (Kent Scientific Corp.) containing aerated (95% O2, 5% CO2) and excess connective tissue was removed. The preparation was cut into isometric tension. The procedure was performed as described elsewhere followed by removal of the thoracic aorta for evaluation of developed amidon (100 μM) and opiate substances in displacing 3DHM (58 Ci/mM, DuPont Sigma) and opiate substances in displacing 3DHM (58 Ci/mM, DuPont Sigma).

RESULTS

Membrane homogenates of human arterial and rat microvascular endothelial cells revealed opiate binding sites and their ligand specification (Fig. 1 and Table I). Saturation and Scatchard analysis showed a single, relatively high affinity binding site with Kd values of 38 and 19 nM, with Bmax values of 1,167 and 1,098 fmol/mg membrane protein for human and rat-derived endothelial cells, respectively (Fig. 1 and Table I). Non-specific binding increased linearly with respect to the concentration of the binding ligand (Fig. 1, inset). Furthermore, a variety of opioid peptides was found to be ineffective in displacing specifically bound 3DHM (Table I). By contrast, the opiate alkaloid ligands were the most potent, and κ ligands dynorphin 1-17 and ethylketocyclazocine were weak. Interestingly, fentanyl was quite poor in this regard. Naloxone was found to be less potent than naltrexone in counteracting 3DHM binding. Of interest was the finding that the μ opioid peptide DAGO was ineffective in displacing 3DHM (Table I). Of equal interest was that the displacement profile for all ligands in both types of endothelial cells was identical. This profile demonstrating opiate alkaloid sensitivity and opioid peptide insensitivity is characteristic of the presence of the μ3 opiate receptor (1).

Fig. 1. Scatchard analysis of 3DHM binding to human arterial endothelial cells. Inset, circle represents saturation of specific sites and box represents nonspecific binding, which continues in a linear fashion.

Sigma) and opiate substances in displacing 3DHM (58 Ci/mM, DuPont NEN) and may give specific information on different receptor populations. The incubation medium for Met-enkephalin contained phosphoramid (100 μM) and bestatin (100 μM) to inhibit enzyme degradation.

Monitoring of NO Release—The cells were incubated in 2 ml of Krebs-Henseleit buffer containing (in mM) 120 NaCl, 4.6 KCl, 1.5 CaCl2, 0.5 MgCl2, 1.5 NaH2PO4, 0.7 Na2HPO4, 10 HEPES, and 10 glucose, pH 7.4.

NO release was monitored with an NO-selective microprobe manufactured by Inter Medical Co. (Nagoya, Japan). The working electrode made of platinum/rhodium alloy was coated with a film containing KCl, NO-selective nitrocellulose resin (pyroxylin lacquer), and a gas-permeable silicon membrane (10). A counter electrode was made of carbon yarn. The redox current was detected by a current-voltage converter circuit and continuously recorded. Tip diameter of the probe (25 μm) permitted the use of a micromanipulator (Zeiss-Dependorff) attached to the stage of an inverted microscope (Nikon Diaphot) to position the sensor, which was enclosed in a Faraday’s chamber, 3–5 mm above the cell surface. Calibration of the electrochemical sensor was performed by use of different concentrations of a nitrosothiol donor S-nitroso-N-acetyl-dl-penicillamine, as previously detailed (10).

Vascular Relaxation Experiments—Male Sprague-Dawley rats, 6–8 weeks of age, were anesthetized with 1 cc of sodium pentobarbital, followed by removal of the thoracic aorta for evaluation of developed isometric tension. The procedure was performed as described elsewhere in detail (11). The vessel was placed in physiological salt solution (PSS), and excess connective tissue was removed. The preparation was cut into 3-mm rings, mounted on metal tissue holders, and placed in a 5-ml tissue bath (Kent Scientific Corp.) containing aerated (95% O2, 5% CO2) PSS buffer maintained at 37 °C. Relaxation of rings precontracted with 1 μM phenoxyphrine was detected by a computer-interfaced force transducer (Kent Scientific Corp.) set at a sampling rate of 6/min. Data are expressed as percent maximal relaxation in response to 1 μM morphine. Opiate receptor specificity was evaluated by measurement of vascular relaxation in response to treatment with 1 μM morphine rings pretreated for 10 min with 5 μM naloxone or, for μ3 identity, with 10 μM (d-Ala2,MePhe4,Gly5-ol)2enkephalin (DAGO).

Rings of thoracic aorta (3 mm) were placed in PSS, and NO release in response to 1 μM morphine was evaluated by use of a computer-interfaced NO-specific amperometric probe as described above.

DISCUSSION

The present report demonstrates the following. 1) Endothelial cells (human arterial and rat microvascular) contain a high affinity saturable opiate binding site that is stereoslectively and characteristically antagonized by naloxone. 2) This binding site is opiate alkaloid-specific and opioid peptide-insensitive. 3) The binding to this site as well as to other cell types exhibiting this novel binding profile (Table I) is designated to be of the μ3 opiate receptor subtype. 4) Morphine can induce the production of NO from MVE cells and rat aortic ring endothelial cells in vitro, a phenomenon that is sensitive to naloxone antagonism. 5) Aortic rings respond to morphine by relaxation which was endothelial dependent. 6) Opioid peptides do not induce in vitro NO production or relaxation of the aortic ring. 6) Endothelial NO production, therefore, appears to be mediated by the μ3 opiate alkaloid-specific receptor.

With regard to the interaction of NO and opiate substances mentioned earlier, recent studies suggest a definite link. Nitric oxide has been associated with antinociception (13–15) as well as the states of tolerance and dependence (16). Peripheral morphine analgesia involves NO-stimulating cGMP (17). Morphine-depressed concanavalin A stimulated macrophage NO production (18). Morphine and NO have been linked in gastrointestinal regulation (19) and in food intake (20, 21). Thus, the present report further documents this interaction, for the first...
time, in endothelial cells mediated by way of the $\mu_3$ receptor.

The specificity of the opiate receptor subtype ($\mu_3$) in mediating endothelial NO production substantiates the strict involvement of opiate alkaloids or similar substances in this process and simultaneously excludes the involvement of opioid peptides. The $\mu_3$ opiate-selective receptor has now been found on human monocytes and granulocytes (1, 2), invertebrate and vertebrate microglia (22, 23), neuronal cell lines (24), and, in a preliminary study, on specific invertebrate neurons (25) (see Table I). Clearly, the presence of such a highly selective opiate receptor strongly suggests that endogenous substances exist that make use of this receptor. In this regard, naturally occurring morphine appears to be a strong candidate (3).

The present study demonstrates that opiate alkaloids have the potential to mediate vasodilation by way of regulation of NO production. Many reports suggest that opiates, in this regard, mediate their effects by way of the central nervous system. Effects of intravenously injected morphine in the rat include depressor response and bradycardia (12). These investigators have demonstrated that the specific $\mu$ opioid receptor agonist, DAGO, reproduced hemodynamic effects of morphine. Effects of either agonist were attenuated by the $\mu_3$-specific antagonist $\beta$-funalfexamine, whereas naloxonazine, the $\mu_1$-specific antagonist, inhibited the effects of DAGO but not those induced by morphine (12). In conscious chronically instrumented pigs, morphine at a high dose (1 mg/kg bolus intravenously) induced tachycardia, elevation in mean systemic and pulmonary arterial pressure, but did not change stroke volume or peripheral vascular resistance (26). These data indicate that in pigs, the species that shows an excitatory response to morphine, hemodynamic changes were largely induced by tachycardia. Apart from its well established hemodynamic effects, morphine has been shown to attenuate vasopressor responses to angiotensin II or substance P (27). Furthermore, the pressor effect of social deprivation during 1-15 days of isolation in rats was inhibited by administration of morphine; however, 7 days after morphine withdrawal elevation of blood pressure occurred in these rats (28). In a study on morphine-induced hypotension, Calignano et al. (29) suggested that it was mediated by adenosine. Based on the results of the present study, endothelial cells are capable of mediating a direct action of morphine or morphine-like molecules. Thus, future studies must be concerned with this phenomenon and its involvement in the regulation of vasomotor responsiveness.

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### Table I

| Ligand | K_i (nM ± S.E.) | Monocytes | Mytilus immunocytes, c | Mytilus microglia, d | Granulocytes | Arterial endothelial cells | Microvascular endothelial cells |
|--------|----------------|-----------|------------------------|---------------------|--------------|---------------------------|-------------------------------|
| DAMA   | >1000          | >1000     |                        |                     |              |                           |                               |
| Deltorphin | >1000        | >1000     | >1000                  | >1000               | >1000        | >1000                     |                               |
| Metenkephalin$^b$ | >1000        | >1000     | >1000                  | >1000               | >1000        | >1000                     |                               |
| DADLE  | >1000          | >1000     | >1000                  | >1000               | >1000        | >1000                     |                               |
| DPPE   | >1000          | >1000     | >1000                  | >1000               | >1000        | >1000                     |                               |
| DAGO   | >1000          | >1000     | >1000                  | >1000               | >1000        | >1000                     |                               |
| DHM    | 45 ± 9.1       | 62 ± 8.1  | 57 ± 8.9               | 16 ± 3.1            | 38 ± 9.4     | 39 ± 6.7                  |                               |
| Morphine | 43 ± 9.4     | 54 ± 7.3  | 51 ± 7.7               | 17 ± 3.8            | 37 ± 8.6     | 39 ± 8.1                  |                               |
| Etorphine | 67 ± 7.3     | 60 ± 10.6 |                      |                     |              |                           |                               |
| Fentanyl |                 |           |                       |                     |              |                           |                               |
| Dynorphin 1-17 | >1000       | >1000     | >1000                  | >1000               | >1000        | >1000                     |                               |
| EKC    | 200 ± 18.4     | 175 ± 24.3| 111 ± 10               | 123 ± 16.3          | 135 ± 14.5   | 135 ± 14.5                |                               |
| Naltrexone | 420 ± 47     | 545 ± 38.4| 310 ± 54               | 156 ± 15            | 145 ± 18.4   | 132 ± 16.3                |                               |

$^a$ DAMA, [d-Ala$^2$,Met$^5$]enkephalinamide; DADLE, [d-Ala$^2$,Leu$^5$]enkephalin; DPPE, [d-Pen$^2$,d-Pen$^3$]enkephalin; EKC, ethylketocyclazocine.

$^b$ Ref. 1.

$^c$ Ref. 22.

$^d$ Ref. 25.

$^e$ Ref. 2.

Fig. 2. In vitro stimulation of NO production by morphine. Representative recordings of NO in the incubation medium prior to and following opiate and opioid exposure are shown (arrow, M, morphine). a, 50 nM morphine; b, 100 nM morphine; c, 200 nM morphine; d, 200 nM naloxone + 200 nM morphine. ME, 100 nM Met-enkephalin; DAME, 100 nM [d-Ala$^2$,Met$^5$]enkephalin. Bradykinin (BK, 10$^{-6}$ M) was added for comparison.

Fig. 3. Morphine-induced relaxation of rat aorta. Morphine (1 $\mu$M) was added to aortic rings in the absence (boldface line) or presence (narrow line) of naloxone (5 $\mu$M). The data shown are representative of n = 4 experiments, which did not vary. Inset, stimulation of aortic rings with morphine (1 $\mu$M) resulted in a marked increase in NO release (boldface line) that was abrogated by pretreatment with naloxone (5 $\mu$M, narrow line). The data shown are representative of n = 4 experiments, which did not vary.
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