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Agonist-selective endocytosis of μ opioid receptor by neurons *in vivo*

(opioid receptors/opiate alkaloids/motility/enteric neurons/motor neurons)

CATIA STERNINI[abc], MARVIN SPANN[a], BENITO ANTON[de], DUANE E. KEITH, JR.[de], NIGEL W. BUNNETT[fg], MARK VON ZASTROW[hi], CHRISTOPHER EVANS[de], AND NICHOLAS C. BRECHA[abj]

[a]CURE Digestive Diseases Research Center, West Los Angeles Veterans Administration Medical Center, Building 115, Room 203, 11301 Wilshire Boulevard, Los Angeles, CA 90073; Departments of [b]Medicine, [j]Neurobiology, and [d]Psychiatry and Biobehavioral Sciences and [c]Opioid Research Center, University of California, Los Angeles, CA 90024; and Departments of [f]Surgery, [g]Physiology, [h]Psychiatry, and [i]Cellular and Molecular Pharmacology, University of California, San Francisco, CA 94143

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ABSTRACT **Opiate alkaloids are potent analgesics that exert multiple pharmacological effects in the nervous system by activating G protein-coupled receptors. Receptor internalization upon stimulation may be important for desensitization and resensitization, which affect cellular responsiveness to ligands. Here, we investigated the agonist-induced internalization of the μ opioid receptor (MOR) *in vivo* by using the guinea pig ileum as a model system and immunohistochemistry with an affinity-purified antibody to the C terminus of rat MOR. Antibody specificity was confirmed by the positive staining of human embryonic kidney 293 cells transfected with epitope-tagged MOR cDNA, by the lack of staining of cells transfected with the δ or κ receptor cDNA, and by the abolition of staining when the MOR antibody was preadsorbed with the MOR peptide fragment. Abundant MOR immunoreactivity (MOR-IR) was localized to the cell body, dendrites, and axonal processes of myenteric neurons. Immunostaining was primarily confined to the plasma membrane of cell bodies and processes. Within 15 min of an intraperitoneal injection of the opiate agonist etorphine, intense MOR-IR was present in vesiclelike structures, which were identified as endosomes by confocal microscopy. At 30 min, MOR-IR was throughout the cytoplasm and in perinuclear vesicles. MOR-IR was still internalized at 120 min. Agonist-induced endocytosis was completely inhibited by the opiate antagonist naloxone. Interestingly, morphine, a high-affinity MOR agonist, did not cause detectable internalization, but it partially inhibited the etorphine-induced MOR endocytosis. These results demonstrate the occurrence of agonist-selective MOR endocytosis in neurons naturally expressing this receptor *in vivo* and suggest the existence of different mechanisms regulating cellular responsiveness to ligands.**

Opioid peptides and alkaloids influence a variety of processes, including pain, ingestive behavior, motor activity, and gastrointestinal motility (1–4). These effects are mediated by the activation of multiple cell surface receptors, the δ, κ, and μ opioid receptors (DOR, KOR, and MOR) (5–7), which are members of the G protein-coupled receptor family (8). There are differences in the distribution, pharmacology, and functions of these receptors, which display a certain degree of selectivity for the three families of opioid peptides, the enkephalins, endorphins, and dynorphins. However, there is overlap in binding affinity, distribution, and function (3, 9, 10). Indeed, the MOR is activated by endogenous opioid peptides from all three peptide families (1, 7). MOR is of particular interest and of clinical importance because it is the preferred receptor for potent analgesics with high potential for abuse, such as morphine and other opioid alkaloids (6). In addition, MOR is of critical importance in the development of tolerance and drug addiction (11, 12). These phenomena, together with other side effects such as respiratory depression and inhibition of gastrointestinal propulsion, represent a major limitation in the use of these analgesics.

Signal transduction induced by ligand–receptor interaction in many receptors is followed by a cascade of events including endocytosis and intracellular sorting into recycling or degradative pathways (13–16). Receptor endocytosis may contribute to receptor desensitization and resensitization, thereby affecting the capacity of the cells to respond to agonist stimulation by depleting the receptor from the cell surface, rendering it inaccessible to the ligands. The purpose of this study was to examine the internalization of MOR in response to opiate alkaloids in the nervous system *in vivo*. To address this issue, we used as a model the enteric nervous system of the guinea pig ileum, which has been widely utilized for functional studies to characterize the effects of opioid peptides and alkaloids (1, 17). Enteric neurons naturally express MOR, as we recently demonstrated (18). In addition, the derivatives of proenkephalins are also expressed in enteric neurons of the myenteric plexus (19). The enteric nervous system plays a key role in controlling gastrointestinal functions such as secretion and motility (20–22).

Here we identified the cellular sites of expression of MOR immunoreactivity (MOR-IR) in the enteric nervous system of the guinea pig with light microscopy, and we examined whether different opioid agonists injected systemically trigger MOR internalization *in vivo*.

MATERIALS AND METHODS

Drug Injections. Adult albino guinea pigs (300–350 g) were injected intraperitoneally with etorphine, a nonselective opioid receptor agonist (0.1 mg/kg) and killed 15, 30, or 120 min after drug injection, or with morphine, a high-affinity MOR agonist (5 and 20 mg/kg) and killed 30 min later. Controls included injection of saline or naloxone, an opioid receptor antagonist with high affinity for MOR (10 mg/kg). To determine whether antagonists blocked agonist-induced MOR internalization, some animals were injected with naloxone and etorphine and killed 30 min later, or with etorphine followed by naloxone 30 min later and killed at 10, 30, or 90 min after naloxone injection. To determine whether morphine inhibited the effect of etorphine on MOR-IR, some animals were injected with morphine 5 min prior to etorphine and killed 30 min later. Drugs were dissolved in saline.

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Abbreviations: MOR, μ opioid receptor; DOR, δ opioid receptor; KOR, κ opioid receptor; MOR-IR, MOR immunoreactivity/immunoreactive.

[c]To whom reprint requests should be addressed.

Tissue Preparation. Guinea pigs were anesthetized with sodium pentobarbital (50 mg/kg of body weight) and killed by cardiac incision. The distal ileum was dissected, washed with ice-cold saline, pinned flat, and fixed in ice-cold 4% paraformaldehyde in 0.1 M sodium phosphate buffer, pH 7.4 (PB), at 4°C overnight. Tissue was then either cryoprotected and sectioned flat with a sliding microtome at 30-μm thickness or prepared for layer separation, in which case the longitudinal muscle layer with the attached myenteric plexus and some circular muscle was separated from the rest of the gut wall (23, 24). In some experiments, tissue collected for whole mount was treated with dimethyl sulfoxide (three times, 15 min each) after fixation to increase antibody penetration.

Immunocytochemical Localization of MOR. The MOR antibody was raised in rabbits against a synthetic fragment (LENLEAETAPLP) corresponding to the intracellular C terminus of rat MOR ($MOR_{387-398}$). The crude antisera were purified on an epoxy-Sepharose column to which the peptide was coupled (25). To assess antiserum specificity, we used human embryonic kidney 293 cells transfected with epitope-tagged MOR, DOR, and KOR cDNAs. Cells were grown on glass coverslips, fixed with 4% paraformaldeyde/PB, permeabilized with 0.1% Triton X-100 in PB, and incubated with a mouse monoclonal antibody recognizing the N-terminal extracellular domain present on all three mutant receptors (anti-FLAG M1, 3 μg/ml, Kodak IBI) and with rabbit MOR antibody (1:1000) in 3% nonfat dry milk/0.1% Triton X-100/50 mM Tris·HCl buffer (pH 7.5) for 45 min at room temperature. Cells were then washed with Tris buffer, incubated with a mixture of fluorescein-labeled goat anti-mouse IgG and Texas red-coupled goat anti-rabbit IgG (1:500; Jackson ImmunoResearch) for 15 min and washed. Dual localization was visualized with fluorescein and Texas red epifluorescence filter sets. Free-floating tissue specimens were incubated in 10% normal goat serum for 30 min at room temperature, followed by MOR antiserum (1:50–1:200) for 2–4 days at 4°C and then by fluorescein or tetramethylrhodamine isothiocyanate-coupled anti-rabbit IgG (American Qualex; 1:50–1:100) or biotinylated goat anti-rabbit IgG (Vector Laboratories; 1:100) for 2 hr at room temperature or overnight at 4°C (23, 24). Samples processed for the avidin-biotin method were then incubated in avidin-biotin complex (Vectastain Elite ABC kit) and developed with 3,3′-diaminobenzidine. Antibodies were diluted in 0.5% Triton X-100 in PB with 10% normal goat serum. Tissues not treated with dimethyl sulfoxide were permeabilized with 0.5% Triton X-100. MOR-IR distribution was examined with light microscopy, and its intracellular location was determined by an MRC 1000 laser scanning confocal microscope (Bio-Rad) with a krypton/argon laser and attached to a Zeiss Axiovert 100 microscope. Images were collected at a magnification zoom of 1–3×. Typically, 10–20 optical sections were taken at 0.5-μm intervals through the cells. The resolution of the confocal microscope in the *x–y* axis was 170–200 nm, and in the *z* axis was 230–400 nm. Images were processed and labeled by using Adobe Photoshop 3.0 (Adobe Systems, Mountain View, CA).

RESULTS

Antibody Specificity. Epitope-tagged MOR receptors in transfected cells were immunolabeled by both the anti-FLAG antibody recognizing the common epitope tag and the MOR antiserum (Fig. 1 *A* and *B*), whereas cells expressing epitope-tagged DOR (Fig. 1 *C* and *D*) or KOR (Fig. 1 *E* and *F*) were stained only with anti-FLAG antibody, not with the MOR antiserum. Furthermore, immunostaining in tissue specimens was prevented by preadsorption of the MOR antiserum with 10 μM synthetic peptide fragment (Fig. 2*D*).

Cellular Distribution of MOR-IR in the Guinea Pig Ileum. MOR-IR was expressed in numerous neurons of the myenteric plexus of the ileum, in the interconnecting strands among plexuses, and in processes of the muscle layer (Fig. 2 *A* and *C*). Immunostaining was predominantly localized at the cell surface membrane (Figs. 2 *A* and *B*, 3*A*, and 4*A*), with foci of high immunoreactivity giving a punctate appearance (Figs. 2*B* and 4*A*). MOR-IR myenteric neurons had the morphological characteristics of Dogiel type I with an ovoid cell body, several broad dendrites protruding from the cell body, and a long axonal process (Fig. 2*B*), which could be followed within the plexus and between plexuses in the interconnecting strands. Dogiel type I neurons comprise motor neurons that control smooth muscle activity and interneurons (18–20). An estimate of the density of MOR-IR myenteric neurons was obtained by counting the positive neurons in three different areas, approximately 4 mm^2 each, of a whole-mount preparation. This analysis revealed that 25.4 ± 9.8 cells per mm^2 (mean ± SD) have MOR-IR in the myenteric plexus of the ileum. MOR-IR was not detected in submucosal ganglion cells.

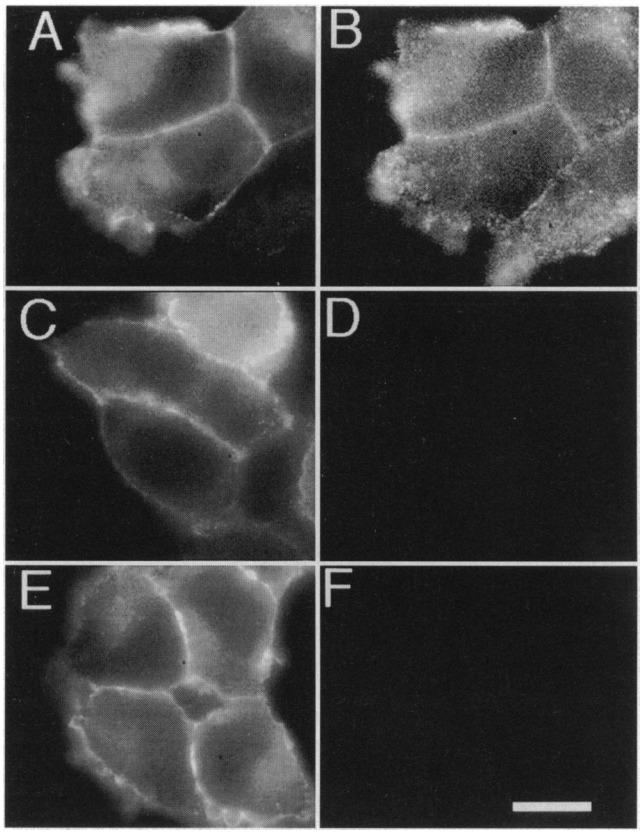

FIG. 1. Double-label immunofluorescence. Human embryonic kidney cells transfected with epitope-tagged MOR display positive immunostaining with both anti-FLAG M1 (*A*) and rabbit MOR antiserum (*B*). Immunoreactivity is predominantly located at the plasma membrane. Cells expressing epitope-tagged DOR and KOR are immunostained only with the anti-FLAG M1 (*C* and *E*), not with the MOR antibody (*D* and *F*). (Bar = 10 μm.)

Etorphine-Induced Internalization of MOR-IR in Neurons *in Vivo*. At 30 min after etorphine injection, MOR-IR appeared to be concentrated in intracellular granules (Fig. 2*E*). Immunoreactive granules were confirmed as intracellular endosomes by confocal microscopy (Fig. 3 *B* and *C* and Fig. 4 *B* and *C*); they were observed immediately beneath the cell membrane (Fig. 4*B*), throughout the cytoplasm, and in perinuclear positions (Fig. 4*C*). Immunostaining could not be detected on the cell surface, and often dendrites could not be visualized. In those neurons where dendrites could be identified, they appeared much smaller and the immunostaining was more punctate compared with normal neurons (Fig. 2 *B* and

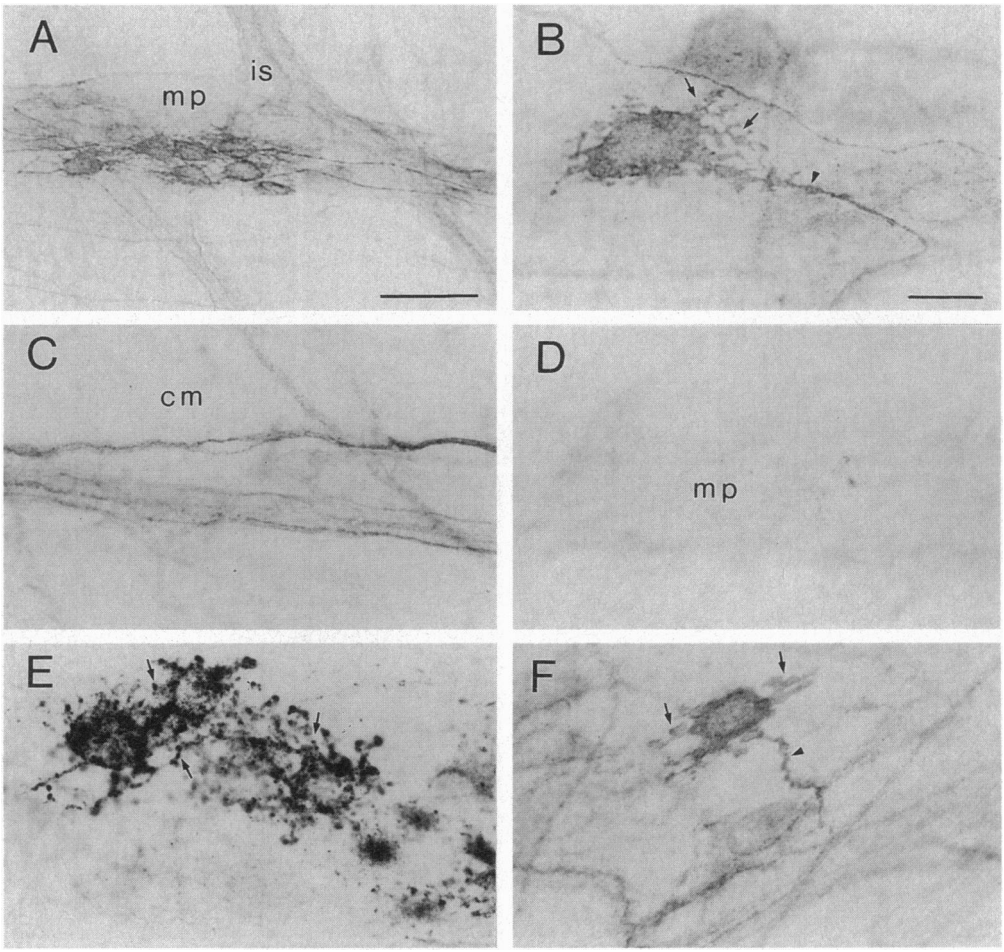

FIG. 2. Distribution of MOR-IR in the myenteric plexus (mp) and smooth circular muscle (cm). Avidin-biotin method. (*A*) Low-magnification picture illustrating the density of MOR-IR myenteric neurons and the presence of processes in the interconnecting strands (is). (*B*) High-magnification picture showing the oval shape of the cell body, thick stubby dendrites protruding from the cell body (arrows), and a long axonal process (arrowhead) of MOR enteric neurons. MOR-IR is predominantly located in the cell membrane (*A* and *B*) and has a punctate appearance (*B*). (*C*) Network of MOR-IR processes in the circular muscle. (*D*) Absence of MOR-IR in tissue incubated with MOR antiserum preadsorbed with the fragment peptide. (*E*) Translocation of MOR-IR from the outer plasma membrane to cytoplasmic vesicles (arrows) 30 min after etorphine injection. (*F*) Enteric neuron from an animal injected with etorphine and naloxone. MOR-IR is predominantly located at the cell surface; dendrites (arrows) and the axonal process (arrowhead) can be clearly identified, as in neurons from normal animals (*A* and *B*). (Bars = 50 μm in *A* and 15 μm in *B*, which also applies to *C–F*.)

E). Axonal processes also displayed punctate immunostaining and appeared more varicose than in the unstimulated neurons (not shown). Etorphine-induced MOR internalization occurred within 15 min from etorphine injection, persisted up to 2 hr (Fig. 3*C*) and was widespread to all MOR-IR enteric neurons.

Specificity and Agonist Selectivity of MOR Internalization. In control animals which received saline or the opioid antagonist, naloxone, MOR-IR was confined primarily to the cell membrane surface of neuronal cell bodies and processes (Fig. 3 *A* and *E*). The specificity of MOR internalization was confirmed by the lack of endocytosis following injection of etorphine and naloxone together (Fig. 2*F*). MOR-IR was confined primarily to the cell surface, and the dendrites and axonal process were clearly identifiable as in normal animals. Similarly, MOR-IR distribution appeared as in controls when naloxone was injected 10–90 min following etorphine exposure (Fig. 3*D*).

Morphine, which binds with high affinity to MOR, did not cause internalization (Fig. 3*F*) even at doses up to 200 times the etorphine dose. In addition, in animals which received morphine prior to etorphine, there was a reduction in the number of enteric neurons showing detectable MOR endocytosis. Indeed, in many neurons, MOR-IR remained concentrated at the cell body surface, even though the dendrites were not clearly identifiable as in normal animals (Fig. 3*G*). This suggests that morphine partially inhibited etorphine-induced MOR endocytosis.

DISCUSSION

This study demonstrates (*i*) that specific MOR-IR is located in somatic, dendritic, and axonal compartments of myenteric neurons and in fibers in the muscle layer and (*ii*) that MOR undergoes rapid internalization *in vivo* upon stimulation with the opioid agonist etorphine, but not morphine. The translocation of MOR from the plasma membrane to cytoplasmic vesicles occurs at all sites normally expressing the receptor, including the soma and neurites. To our knowledge, this is the first demonstration of a rapid and agonist-selective endocytosis of MOR in neurons naturally expressing this receptor in the intact animal. These findings extend our observations of agonist-regulated internalization of epitope-tagged opioid receptors in transfected fibroblasts and neuroblastoma cells in culture (26, 27).

MOR-IR neurons represent a large population of myenteric neurons. They have the morphological appearance of Dogiel type I neurons, which comprise motor neurons that transmit information to the muscle cells and control smooth muscle

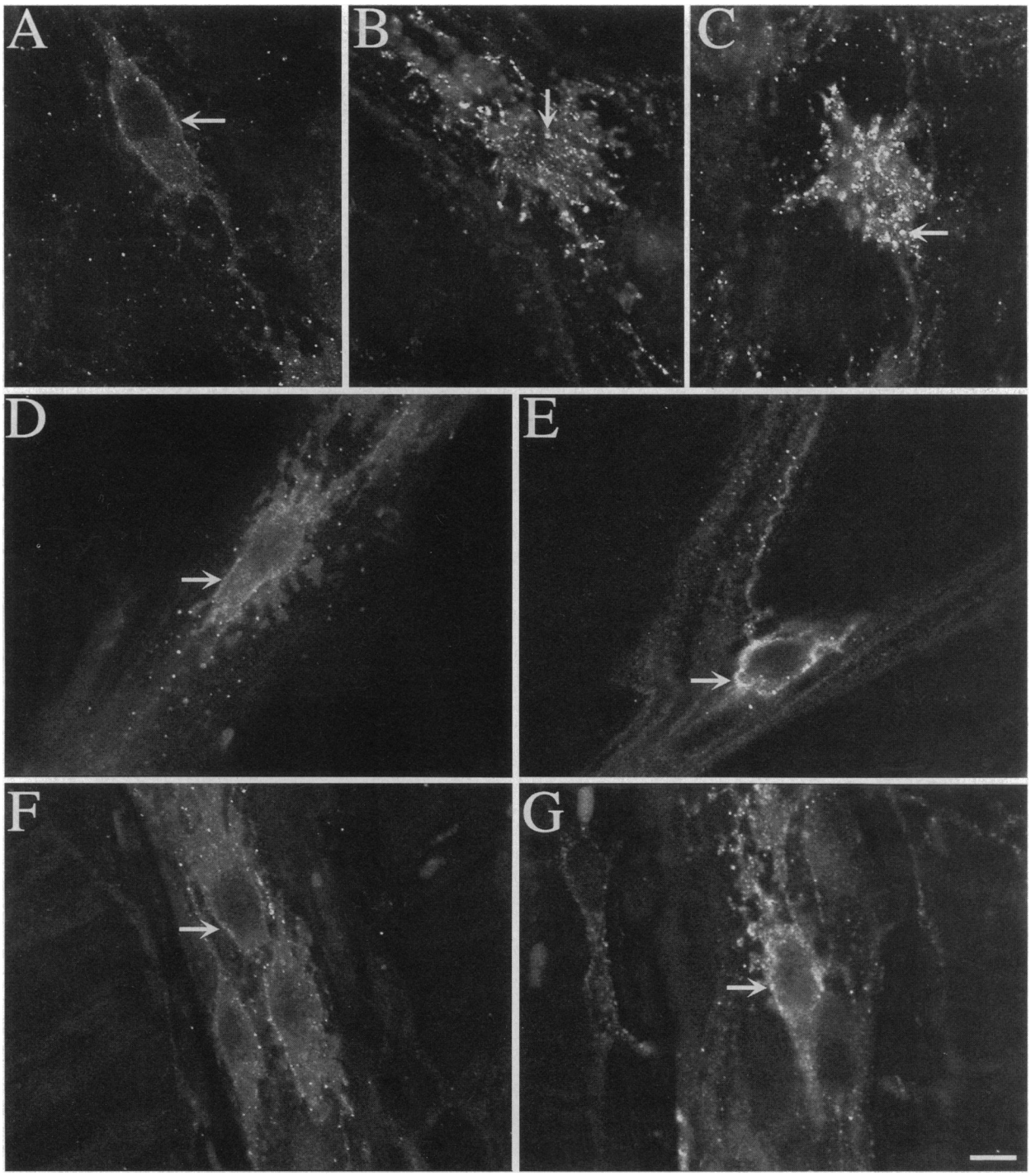

FIG. 3. Confocal microscopic sections showing the localization of MOR-IR at the cell surface membrane (arrow) in a neuron from a normal animal (*A*) and MOR-IR in cytoplasmic vesicles (arrows) in neurons from animals treated with etorphine for 30 (*B*) or 120 (*C*) min. (*D–G*) MOR-IR at the cell surface membrane (arrows) in enteric neurons from animals treated by etorphine followed by naloxone 30 min later and killed 90 min later (*D*), by naloxone alone (*E*), by morphine alone (*F*), and by morphine prior to etorphine (*G*). (Bar = 10 μm.)

activity (20). In addition to several stubby dendrites protruding from the cell body, they have a clearly distinguishable long axonal process that projects outside the ganglion. MOR-IR axonal terminals are distributed to the circular muscle layer and form a dense network in the inner portion of this layer. This distribution closely matches that of the opioid peptide enkephalin (19), which is expressed in myenteric neurons and processes that run to the circular muscle and deep muscular plexus. The presence of MOR-IR in myenteric motor neurons and processes is consistent with the functional evidence that enkephalin and opiate agonists inhibit the electrically evoked release of acetylcholine by acting on enteric neurons (1, 22) primarily via MORs. This has been regarded as the mechanism of opioid inhibition of the peristaltic reflex, which is responsible for the delayed gastrointestinal transit and severe constipation induced by opiates in some species. Opioid receptors and peptides might also modulate the acetylcholine effect on peristaltic activity, as suggested by the hypersensitivity of the peristaltic reflex to acetylcholine in the morphine-tolerant state (1). Indeed, peristaltic waves are increased in opioid withdrawal, which is probably the mechanism behind withdrawal diarrhea. MOR expression in somatodendritic and

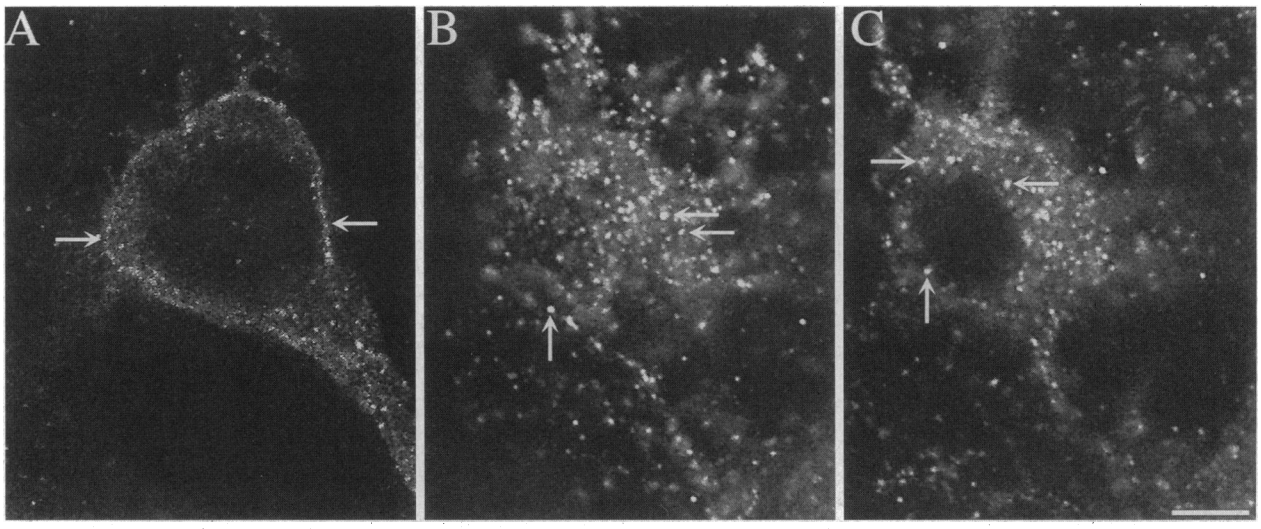

FIG. 4. Confocal microscopic sections showing localization of MOR-IR at the cell surface membrane (arrows) of an enteric neuron in resting conditions (*A*) and cytoplasmic (*B*) and perinuclear (*C*) location of MOR-IR vesicles (arrows) 30 min after etorphine exposure. (Bar = 5 μm.)

axonal domains of enteric neurons and the occurrence of agonist-induced MOR internalization at both the soma and neurites suggest that this receptor influences gastrointestinal motility by pre- and postsynaptic mechanisms. The localization of MOR in neuronal elements of the gut correlates with the distribution of opioid-binding sites in the enteric nervous system (1), but it is uncertain whether the cloned MOR can be classified as a μ1 or a μ2 receptor, receptors that can be distinguished on the basis of their pharmacological properties (28). However, it is possible that the reported differences in μ receptor binding sites are due to posttranslational processing of MOR (7).

The rapid redistribution of MOR-IR in enteric neurons after exposure to etorphine is specific and agonist selective. The opioid antagonist naloxone, which competes with opioid agonists in binding to opioid receptors, does not evoke MOR endocytosis, and it prevents and completely inhibits the etorphine-induced MOR internalization. Interestingly, MOR endocytosis is not triggered by morphine, a high-affinity MOR agonist (29), which, like opioid peptides and etorphine, regulates receptor-mediated transduction by inhibition of adenylyl cyclase (30). However, morphine is less efficacious in stimulating γ-thio-GTP binding than other opioid agonists such as [D-Ala^2,*N*-MePhe^4,Gly^5-ol]enkephalin (DAMGO), suggesting that morphine has low intrinsic activity at μ receptors (31). The observation that morphine partially inhibited the etorphine effect on MOR-IR distribution in our preparation indicates that morphine was active. It appears probable that morphine acts as a partial antagonist for etorphine by occupying MOR in such a way that etorphine binding is prevented or altered. Morphine pretreatment might then reduce the number of receptors available for etorphine. The different effects of opioid agonists on MOR trafficking suggest the existence of different mechanisms through which cells regulate their responsiveness to ligands. Morphine is widely used clinically, and etorphine has also begun to be used for pain control (32). The use of these powerful analgesic drugs is often limited by their considerable side effects, including tolerance and addiction. It is likely that morphine produces tolerance by a different mechanism than etorphine.

The agonist-selective effect on MOR trafficking in neurons naturally expressing this receptor in the intact animal is comparable to the epitope-tagged opioid receptor internalization observed in transfected cells (26, 27). In transfected cells exposed to etorphine or enkephalin, MOR-IR is translocated from the plasma membrane into early endosomes (27), suggesting that MOR is internalized via an endocytic pathway similar to that of other G protein-coupled receptors, including the adrenergic and thrombin receptors, and the peptide receptor neurokinin 1 (14, 33, 34). By analogy, it is likely that a similar pathway is utilized by MOR *in vivo*. The neurokinin 1 receptor internalization in response to ligand stimulation has also been demonstrated *in vivo* in the nervous system (35–37) and in endothelial cells (38). Neurokinin 1 receptors recycle within 1–4 hours from ligand exposure (34–38). In our experiments *in vivo*, MOR internalization was still detectable and pronounced at 2 hr, the longest time examined. This long-lasting internalization was also observed in cells in culture. The definition of the time course of MOR endocytosis will be important for better understanding of the functional regulation of this receptor.

The rapid, agonist-induced internalization of MOR may contribute to the process of receptor desensitization, by depleting the cell surface of receptors, which therefore cannot be further stimulated; other mechanisms may be involved also, such as receptor phosphorylation, as has been shown for adrenergic receptors and for the δ opioid receptor (39, 40). Receptor internalization and recycling may also contribute to resensitization (41, 42). The formation and subsequent fate of receptor-binding complex after stimulation, which includes receptor internalization and intracellular sorting, are key cellular events in the regulation of cellular responsiveness to ligands. The rapidity of MOR endocytosis evoked by etorphine is consistent with the process of receptor desensitization, which occurs within minutes from the exposure to ligands and is not accompanied by a decrease in receptor density, unlike the process of receptor down-regulation, which requires longer times to occur and is characterized by a decrease in receptor density. The increase in MOR-IR in vesicular structures is accompanied by the disappearance of MOR-IR in the plasma membrane, suggesting that the overall levels of MOR are not diminished in enteric neurons after ligand stimulation. This parallels observations in transfected cells, where the rapid endocytosis of opioid receptors occurs without a decrease in receptor density as measured in cell lysates (27), suggesting that the process of internalization is not accompanied by degradation.

In summary, our study demonstrates the occurrence of a rapid, agonist-selective endocytosis of MOR-IR in neurons naturally expressing this receptor *in vivo*. Furthermore, our results support the hypothesis that the effect of opioids and alkaloids on intestinal motility is mediated by MOR, which might function both pre- and postsynaptically.

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