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Evoked release of methionine enkephalin from tolerant/dependent enteric ganglia: Paradoxical dependence on morphine

(myenteric plexus/opiate receptor)

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ABSTRACT **Experiments were performed in order to determine whether the state of tolerance to and dependence upon opiates is associated with changes in one or more of the characteristics of the electrically induced release of methionine enkephalin from enteric ganglia. Acute morphine pretreatment substantially reduces the magnitude of the evoked release of this peptide from opiate-naive ilea. However, the rate of the evoked release of enkephalin from morphine-pretreated, tolerant/dependent preparations is indistinguishable from that observed for untreated, naive ilea. Paradoxically, 15 min after acute *in vitro* withdrawal of morphine from such preparations, the presence of morphine appears to be a prerequisite for the manifestation of electrically evoked release of methionine enkephalin. The evoked release of this peptide from ilea 60 min after withdrawal is no longer dependent upon morphine. Moreover, the magnitude of the increase in the rate of enkephalin release from these preparations is almost double that observed for opiate-naive ilea. These data indicate that the manifestation of opiate tolerance/dependence for the release of methionine enkephalin from enteric ganglia comprises several adaptive processes, the consequences of which can be observed at different stages of withdrawal.**

In a recent report from this laboratory (1), the electrically induced release of methionine enkephalin ([Met^5]enkephalin) from the isolated guinea pig myenteric plexus *in vitro* was directly demonstrated and characterized. A striking characteristic of the resting and evoked release of this opioid peptide from enteric ganglia is its modulation by opiate receptors. The opiate antagonist (−)-naloxone (1 μM) causes a sustained increase (≈2-fold) in the basal rate of release of [Met^5]enkephalin from opiate-naive myenteric plexus. In contrast, the inactive stereoisomer (+)-naloxone, in identical concentrations, is devoid of any effect, indicating that the ability of the (−) isomer to enhance release is due to a specific interaction with enteric opiate receptors. The evoked (40-Hz stimulation) release of myenteric [Met^5]enkephalin is also modulated by opiate receptors, since exogenously administered morphine (1 μM) produces a substantial (82%) and reversible reduction in its release. These observations prompted us to investigate whether the state of tolerance to and dependence upon opiates is associated with changes in one or more of the characteristics of the stimulation-induced release of [Met^5]enkephalin from enteric ganglia. This report shows that evoked enkephalin release from tolerant/dependent ilea is no longer negatively modulated by morphine. On the contrary, shortly after acute morphine withdrawal *in vitro*, the presence of morphine in the ileal superfusate appears to be a prerequisite for the manifestation of electrically evoked release of [Met^5]enkephalin from such preparations.

METHODS

Tolerance and Dependence. Male albino guinea pigs (350–400 g) were made tolerant/dependent on opiates by the subcutaneous implantation of four morphine pellets, each containing 75 mg of morphine base, under light ether anesthesia. Animals were killed 72 hr after pellet implantation, and the ileum was removed and stored in Krebs' buffer containing morphine (1 μM) to prevent withdrawal. Strips of longitudinal muscle with adherent myenteric plexus (LMMP) were prepared and mounted in a stimulating superfusion chamber as described (1–4). The Krebs' solution used to superfuse the tissue contained captopril (10 mM), thiorphan (0.3 mM), bestatin (10 mM), and L-leucyl-L-leucine (2 mM) to protect against the action of proteases (5). Under these conditions, 74% of standard ^{3}H-labeled [Met^5]enkephalin that was added to the Krebs' solution and then passed over the tissue during electrical stimulation was recovered in the form of either authentic [Met^5]enkephalin or its sulfoxide derivative (1). All data have been corrected for recovery. Superfusion rate was maintained at 1 ml/min. Fractions (0.5 ml) were collected on ice and stored at −85°C. Aliquots were assayed directly without extraction (1).

Electrical impulses were applied to the tissue via a Grass S48 stimulator, which was connected to a Crown DC 300A amplifier. Maximum current applied to the tissue was approximately 60 mA per cm^2 of tissue (3). The protease inhibitors mentioned above were present during the 30- to 75-min equilibration period before the start of each experiment and thereafter for its duration. Tissue superfusate was collected before, during, and after a 30-sec period of electrical stimulation (40 Hz, 0.2-msec pulse duration) in the presence or absence of morphine (1 μM, 3 min). Enkephalin-like immunoreactivity was measured by radioimmunoassay (RIA) as described (1, 6, 7). Synthetic [Met^5]enkephalin (Peninsula Laboratories, Belmont, CA) was dissolved in 50 mM Tris·HCl (pH 7.4) containing 8 mM 2-mercaptoethanol, to yield a stock solution of 5 ng/ml, and was further diluted before each assay. Duplicate assay mixtures in 12 × 75-mm polypropylene tubes (total incubation volume 0.5 ml) were incubated at 4°C for 24 hr. Bound and free labeled ligand were separated by filtration under reduced pressure, using 0.45-μm mixed cellulose acetate/cellulose nitrate filters (Millipore HAWP 02400). Antibody-bound radioactivity was quantitated by liquid scintillation spectroscopy using a Beckman LS-250 counter (40% efficiency). A standard curve (50, 100, 250, 500, and 1000 pg per tube) in which the percentage of inhibition of binding was plotted against the logarithm of the concentration of unlabeled [Met^5]enkephalin in the reaction tube was generated in each experiment. The minimum detectable concentration was 50 pg, at which an 8% inhibition of binding was observed. Previous studies using a number of

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Abbreviation: LMMP, longitudinal muscle with adherent myenteric plexus.

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enkephalin analogs and fragments indicated that the antibodies are C-terminal specific. Crossreactivity with either [Leu^5]enkephalin or β-endorphin was <0.2% (6). The concentration of salt contained in each unknown sample (75 μl) did not affect antibody binding. All of the experiments were performed on pieces of ileum superfused with oxygenated (95% O_2/5% CO_2) Krebs' solution (118 mM NaCl/4.7 mM KCl/2.5 mM $CaCl_2$/25 mM $NaHCO_3$/11 mM glucose/1.2 mM $MgCl_2$/1.2 mM NaH_2PO_4). Statistical analyses were performed using a group t test. $P > 0.05$ is considered nonsignificant.

RESULTS

Fig. 1 demonstrates the release of [Met^5]enkephalin from opiate-naive (bar A) and opiate-tolerant/dependent (bars B–D) myenteric plexus. Previous work in this laboratory, utilizing reverse-phase high-pressure liquid chromatography fractionation in combination with RIA, has shown that 90–100% of the enkephalin-like immunoreactivity measured in LMMP extract or its superfusate is either authentic [Met^5]enkephalin or its sulfoxide derivative (1, 7). Bar A illustrates the percent increase in the rate of [Met^5]enkephalin release from opiate-naive ilea in response to electrical stimulation (40 Hz; 1.9 ng per min). The same magnitude of increase in the rate of enkephalin release (≈2-fold) was observed in 11 out of 11 experiments when LMMP strips obtained from guinea pigs chronically exposed to morphine and equilibrated (30 min) in its presence were stimulated electrically (40 Hz; bar B). Thus, the evoked release of [Met^5]enkephalin from tolerant/dependent myenteric plexus apparently was no longer susceptible to inhibition by morphine. In contrast, tolerant/dependent LMMP strips stimulated following a 15-min wash in morphine-free Krebs' solution did not manifest evoked (40 Hz) release (Fig. 1, bar C-1). In eight out of nine experiments, the magnitude of the release observed during stimulation was indistinguishable from the basal release observed in the period preceding stimulation. In the one remaining experiment, electrical stimulation produced only a 34% increase in the release rate, less than half the expected magnitude (see Fig. 1, bars A and B). Paradoxically, in all nine of these experiments the magnitude of the evoked release of [Met^5]enkephalin was restored to previously observed levels after reequilibration with morphine (1 μM) for just 3 min (bar C-2; $P < 0.001$ for C-1 vs. C-2).

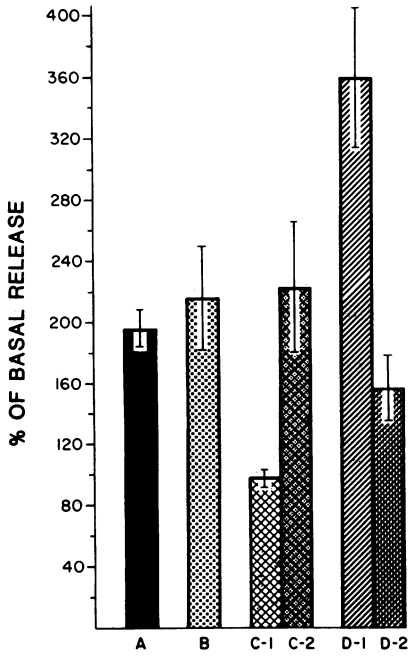

FIG. 1. Release of [Met^5]enkephalin, in response to 40-Hz stimulation, from LMMP strips obtained from opiate-naive guinea pigs (bar A) and guinea pigs chronically exposed to morphine for 72 hr before death (bars B–D). Bar A: 2 LMMP strips were equilibrated in normal Krebs' buffer for 30 min, after which they were stimulated electrically as described in *Methods* (n = 28). Bar B: ilea from tolerant/dependent guinea pigs were equilibrated for 30 min in Krebs' buffer containing morphine (1 μM), after which they were stimulated while still in its presence (n = 9). Bar C-1: tolerant/dependent ilea were equilibrated in Krebs' solution with morphine (1 μM) for 15 min, followed by a 15-min equilibration in morphine-free Krebs' solution, after which they were electrically stimulated (n = 9). Bar C-2: the same preparation as C-1, following a 3-min reequilibration in Krebs' with morphine (1 μM, n = 9); $P < 0.001$ for C-1 vs. C-2. Bar D-1: tolerant/dependent ilea were stimulated after a 60-min equilibration in morphine-free Krebs' solution (n = 11); $P < 0.02$ for D-1 vs. A. Bar D-2: the same preparation as D-1 was stimulated after a 3-min pretreatment with morphine (1 μM, n = 9); $P < 0.001$ for D-2 vs. D-1. Results are plotted as the percent (mean ± SEM) of the basal release observed in the period immediately preceding stimulation (three to four collection periods).

Dependence of the release of [Met^5]enkephalin on the presence of morphine was no longer observed following a 60-min wash in morphine-free Krebs' solution (11 out of 11 experiments, bar D-1). At this time the percent increase above basal release produced by stimulation (40 Hz) was ≈80% greater than that observed in opiate-naive ilea ($P <$ 0.02 for D-1 vs. A). Following a 3-min pretreatment with morphine (1 μM), this increment was abolished and the magnitude of the evoked release was similar to that observed for untreated, naive ilea in response to stimulation ($P > 0.05$ for D-2 vs. A).

DISCUSSION

Previous studies (4, 8–13) have shown that the ileum taken from a guinea pig that has been chronically exposed to an opiate displays the phenomenon of tolerance/dependence in a manner that has many similarities to that seen for the central nervous system. Therefore, this preparation is an excellent model for study of the physiological correlates of opiate tolerance and dependence. This report demonstrates that, in contrast to opiate-naive ilea (1), electrically evoked release of [Met^5]enkephalin from myenteric plexus rendered *in vivo* tolerant to and dependent upon opiates is no longer negatively modulated by exogenous morphine. This suggests that opiate receptor-mediated mechanisms by which the release of enteric enkephalin might be regulated *in vivo* are not operative as a consequence of chronic exposure to morphine. In addition, shortly (15 min) after acute *in vitro* withdrawal of morphine from such preparations electrical stimulation (40 Hz) no longer evokes the release of this opioid peptide. One possible explanation is that during acute *in vitro* morphine withdrawal, the rate of enkephalin release is enhanced to compensate for the removal of morphine and as a consequence the pool of releasable enkephalin is depleted during the 15-min wash in morphine-free Krebs' solution. However, in each of the nine experiments conducted, evoked release of enkephalin was restored to the expected magnitude following reequilibration (3 min) in Krebs' solution containing morphine (1 μM). Thus, the pool of releasable enteric enkephalin is clearly not depleted. Instead, it would appear that after acute removal of morphine from tolerant/dependent ilea, the presence of morphine is a prerequisite for the manifestation of increased enkephalin release in response to stimulation. Evoked release of enkephalin from such preparations has become dependent upon morphine.

Dependence of evoked enkephalin release on morphine is transient. After a 60-min wash in morphine-free Krebs'

solution, stimulated enkephalin release no longer requires the presence of morphine. In these preparations, stimulated enkephalin release is actually greater (≈80%) than that observed from opiate-naive ilea or from tolerant/dependent ilea that have been equilibrated in Krebs' solution with morphine (30 min) prior to stimulation (Fig. 1, compare bar D-1 with bars A and B). This enhanced release from tolerant/dependent preparations after morphine withdrawal probably reflects an earlier attempt to compensate for the inhibition of release caused by the continued presence of morphine. Morphine can inhibit the evoked release from 60-min-withdrawn preparations, and as a consequence of this inhibition the magnitude of the evoked release is restored to the level observed from naive ilea in the absence of morphine (compare bar D-2 with bar A). Observations analogous to these have also been made with neuroblastoma–glioma hybrid cells. Acute removal of morphine from cells that have been grown in its continued presence unmasks an increase in the activity of adenylate cyclase which is returned to control (opiate-naive) levels following reequilibration in morphine-containing growth medium (14).

The above data indicate that the interval following the removal of morphine from tolerant/dependent myenteric plexus is an important determinant of the physiological sequelae of opiate withdrawal that are manifested. In the present study, different times during the withdrawal from morphine (15 min vs. 60 min) are associated with opposite effects on enkephalin release (lack of evoked release vs. enhanced release in response to stimulation). This could indicate that multiple adaptations are utilized to compensate for the effect(s) of the continued exposure to an opiate on enkephalin release.

Current concepts of the state of tolerance and dependence make it difficult to propose a biochemical mechanism that would account for the inverse response to morphine in opiate-naive ilea vs. ilea immediately after morphine withdrawal (15 min). One hypothetical biochemical model that is consistent with this observation involves the generation of some new component of the process(es) subserving enkephalin release (perhaps an ion channel) as a consequence of chronic exposure to morphine, the functioning of which (open state) is stabilized by morphine. Following the suppression of the original release process by the continued presence of morphine, enkephalin release would be mediated via an alternative pathway involving this putative new component. Alternatively, there could be an anatomical/physiological basis for the present findings. In 15-min-withdrawn preparations, morphine might be required to suppress an inhibitory interneuron(s) whose activity is enhanced in tolerant/dependent preparations in order to compensate for reduced opiate receptor-mediated modulation of enkephalin release. Differences in the temporal onset of withdrawal for μ, δ, or κ subtypes of opiate receptor, some of which could mediate opposing physiological processes, might also at least partially explain the paradoxical effect of morphine on enkephalin release. Both of these models are speculative but allow for a conceptual framework that could lead to a better perspective of some of the process(es) mediating the formation and manifestation of opiate tolerance and dependence.

In summary, the manifestation of opiate tolerance/dependence for the evoked release of [Met^5]enkephalin from enteric ganglia appears to comprise several adaptive processes whose consequences are manifested at different stages of withdrawal. The data presented are consistent with the occurrence of enhanced activity of the original release process and with the activation of an alternative mechanism(s) or neuronal pathway through which enkephalin release is regulated.

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