Inhibitory Effect of Endomorphin-1 and -2 on Acetylcholine Release From Myenteric Plexus of Guinea Pig Ileum

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ABSTRACT—Endomorphin-1 and -2, putative endogenous ligands for the mu-opioid receptor, inhibited acetylcholine (ACh) release evoked by electrical field stimulation (EFS) at 1 Hz, which partially activates muscarinic autoreceptors, but not at 10 Hz, which fully activates muscarinic autoreceptors, in longitudinal muscle with the myenteric plexus (LMMP) preparations of guinea pig ileum. After blockade of autoinhibition by atropine, the peptides also inhibited EFS-evoked ACh release at 10 Hz. The inhibitory effects on ACh release were abolished by the mu-opioid antagonist cyprodime. These results suggest that endomorphin-1 and -2 inhibit ACh release from LMMP preparations of guinea pig ileum and that the mechanism of the inhibition must have a component in common with muscarinic autoinhibition.

Keywords: Endomorphin, Acetylcholine release, Autoinhibition

There are three major classes of opioid receptors, mu-, delta- and kappa-receptors. Enkephalins and dynorphins are candidates for endogenous ligands to activate delta- and kappa-receptors, respectively (1–3), but yet no endogenous ligand to activate mu-receptors is known. Zadina et al. recently isolated novel peptides from the bovine (4) and human (5) brain that have high affinity for mu-receptors and designated them as endomorphin-1 and -2. Both endomorphin-1 (Tyr-Pro-Trp-Phe-NH_2) and -2 (Tyr-Pro-Phe-Phe-NH_2) consist of four amino acids, and their specificities for mu-receptors were 4,000–15,000 times greater than those for delta- or kappa-receptors. Endomorphin peptides were also reported to have significant vasodilator activity in the systemic vascular bed of the rat (6) and rabbit (7) and the hindquarters vascular bed of the rat (8). In the spinal cord, the peptides were reported to have potent spinal antinociceptive effect (9).

We previously found that endogenous opioids inhibited acetylcholine (ACh) release mainly via activation of mu-receptors in longitudinal muscle with myenteric plexus (LMMP) preparations of guinea pig ileum, and the inhibitory effect was associated with muscarinic autoinhibition (10, 11). Since endomorphin-1 and -2 seem to be endogenous ligands for the mu-receptors in some tissues, we examined the effects of these peptides on ACh release in LMMP preparations of guinea pig ileum in the present study. We also examined the relationship between the effects of endomorphin peptides on ACh release and muscarinic autoinhibition.

Male guinea pigs, weighing 300–800 g, were sacrificed by bleeding. The LMMP preparations of the ileum were made as described previously (12). The preparation was mounted in an organ bath containing 3 ml of Tyrode solution. The bathing medium was kept at 37℃ and bubbled with 95% O_2 and 5% CO_2. The preparations were equilibrated for 15 min by perfusion with Tyrode solution containing physostigmine salicylate (5 μM) and choline chloride (1 μM) at a rate of 1–2 ml/min. Then, perfusion was stopped and the bathing medium was replaced by 3 ml of fresh Tyrode solution. After 1 min (in the case of 10 Hz stimulation) or 4 min (in the case of 1 Hz stimulation), the medium was collected for measurement of resting ACh release by replacing it with fresh Tyrode solution. Electric field stimulation (EFS) was carried out with a pair of platinum electrodes. For the EFS-evoked release of ACh, the stimulation was performed in trains of 20 sec at 10 Hz or 200 sec at 1 Hz, and then the bathing fluid was collected after a further 40-sec period. These experimental procedures for sample collection were repeated 2 or 3 times with 15-min intervals between each. At the end of

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the experiment, the strips were blotted and weighed for expression of ACh release as pmol per g tissue. The amount of ACh released by each stimulation was calculated by subtracting the release during the resting period (spontaneous release) immediately before stimulation from the total release during stimulation. ACh released in the medium was assayed by HPLC as described elsewhere (10). The drugs used were endomorphin-1, endomorphin-2 (Peptide Institute, Osaka), cyprodime bromide (Funakoshi, Tokyo), atropine sulfate (Sigma, St. Louis, MO, USA), physostigmine salicylate (Wako Pure Chemical, Osaka), choline chloride (Sigma). Statistical analysis of the data was carried out with a paired *t*-test and a one-way analysis of variance and Bonferroni's test. P values less than 0.05 were considered as significant. The mean±S.E.M. was used throughout the manuscript.

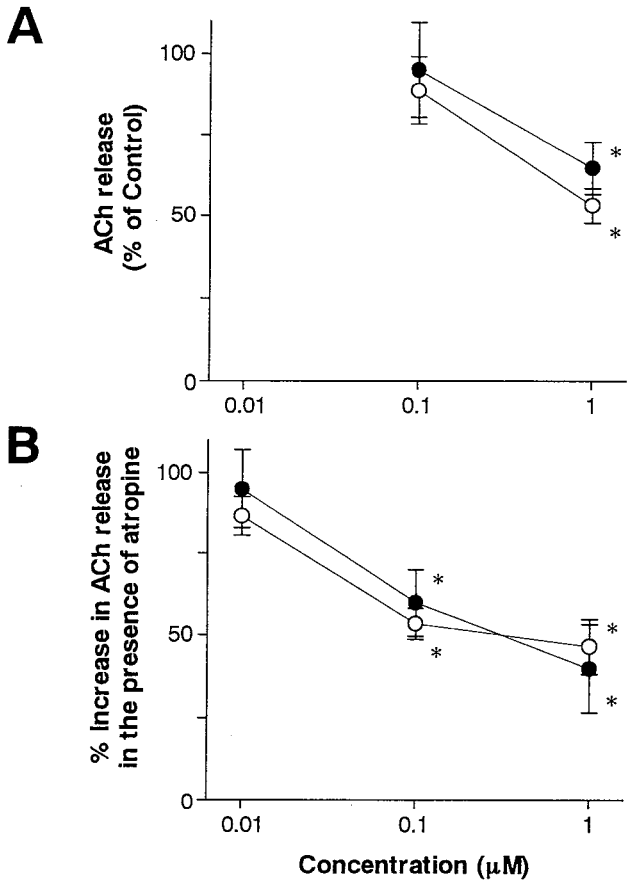

Fig. 1. Effects of endomorphin-1 (○) and -2 (●) on EFS-evoked ACh release at 1 Hz (A) or 10 Hz in the presence of 1 μM atropine (B) from LMMP preparations of guinea pig ileum. EFS-evoked ACh release was calculated by subtracting the spontaneous release from the total release during stimulation. A: The first or second stimulation was carried out in the absence or presence of the indicated concentrations of endomorphin-1 or -2, respectively. Data are expressed as a percentage of the ACh release in the first stimulation. B: The first stimulation was carried out in the absence of test drug, and the second or third was in the presence of 1 μM atropine without or with the indicated concentrations of endomorphine-1 or -2, respectively. Data are expressed as a percentage of the net increase of ACh release in the presence of atropine. Values are each the mean for 4 to 6 experiments, with S.E.M. *Significantly different from the value in the absence of drugs tested (A) or the value in the presence of atropine alone (B) at $P<0.05$ (paired *t*-test). For further details, see Methods.

EFS at 1 Hz or 10 Hz evoked significant ACh release from LMMP preparations of guinea pig ileum to about 1.6- or 2.9-fold the spontaneous release as shown in the previous study (10). Endomorphin-1 and -2 at 1 μM significantly inhibited ACh release evoked by EFS at 1 Hz (Fig. 1A), but the peptides did not significantly affect EFS-evoked ACh release at 10 Hz even at 10 μM (89.6±6.4%, n=4 or 109.6%, n=2 of the corresponding control release, respectively, for endomorphin-1 or -2). Blockade of muscarinic autoreceptors by atropine (1 μM) resulted in a significant increase in EFS-evoked ACh release at 10 Hz as reported elsewhere (13–15). In the presence of atropine, endomorphin-1 and -2 concentration dependently inhibited EFS-evoked ACh release at 10 Hz (Fig. 1B). A mu-selective antagonist, cyprodime, abolished the inhibitory effects of endomorphin-1 and -2 on EFS-evoked ACh release at 1 Hz (Fig. 2A) and those at 10 Hz in the presence of atropine (Fig. 2B). Therefore, it is likely that both endomorphin-1 and -2 inhibit ACh release via activating mu-receptors.

We previously showed that released ACh during EFS at 10 and 1 Hz activates muscarinic autoreceptors fully or partially, respectively (10). Since endomorphin-1 and -2 inhibited EFS-evoked ACh release at 1 Hz but not at 10 Hz and both the peptides inhibited EFS-evoked ACh release at 10 Hz in the presence of atropine (autoreceptor-blocked condition), it was suggested that the peptides exhibited their inhibitory effects on ACh release when muscarinic autoreceptors were not fully activated. These results of endomorphins are consistent with those of [D-Ala^2, *N*-Me-Phe^4, Gly^5-ol] enkephalin (DAMGO), a mu-selective agonist, in our previous study (11). Endomorphin-1 and -2 were reported to have similar potencies to the mu-receptor determined by receptor binding studies; i.e., K_i values were 0.36 and 0.69 nM, respectively (4). Endomorphin-1 and -2 also showed similar potencies in the inhibitory effects on ACh release in LMMP preparatons of guinea pig ileum in the present study.

At a low concentration 0.1 μM, both endomorphin-1 and -2 significantly inhibited EFS-evoked ACh release at 10 Hz in the presence of atropine, but did not affect EFS-evoked ACh release at 1 Hz. The difference in potency of the peptides may be associated with the extent of muscarinic autoinhibition: since a muscarinic agonist, bethanechol decreased EFS-evoked ACh release at 1 Hz, but not at 10 Hz (10), it was again suggested that the muscarinic autoinhibition mechanism only partially

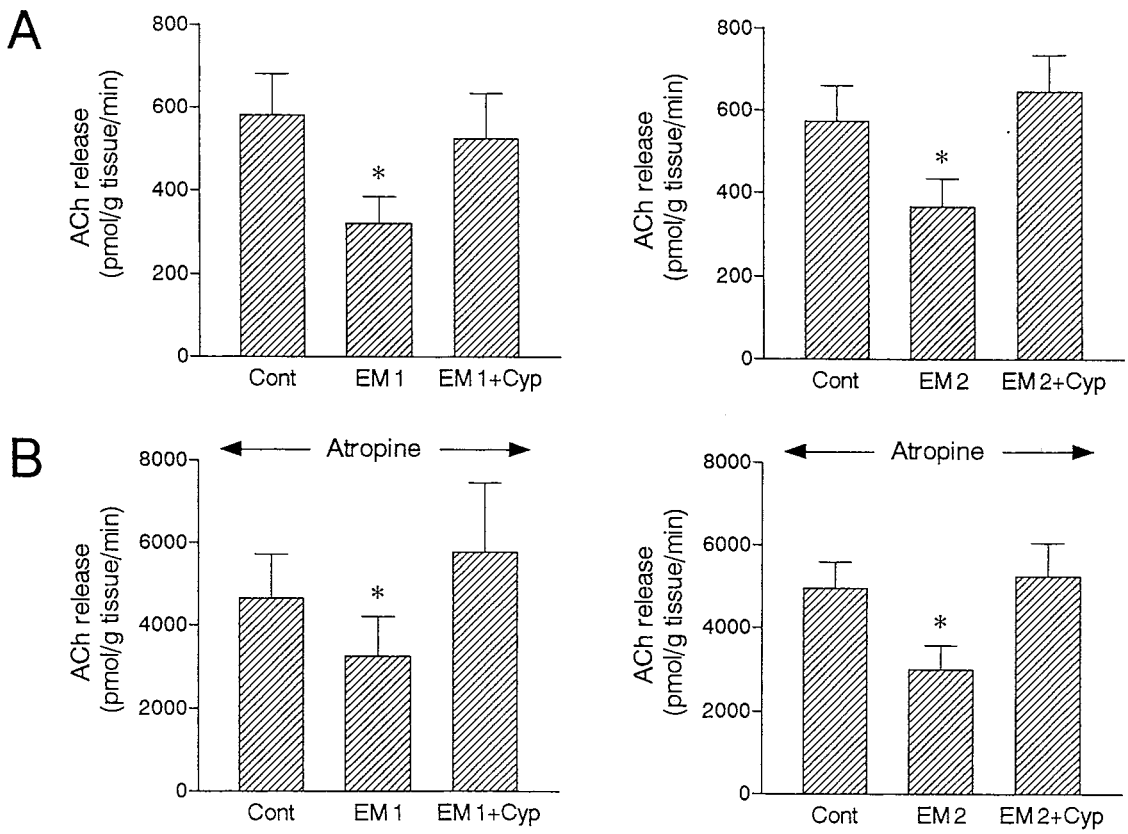

Fig. 2. Reversal effects of cyprodime on the inhibition of EFS-evoked ACh release induced by 1 μM endomorphin-1 (left panel) and endomorphin-2 (right panel). EFS-evoked ACh release was calculated by subtracting the spontaneous release from the total release during stimulation. A: ACh release was evoked by EFS at 1 Hz. The first stimulation was carried out in the absence of test drug (Cont), and the second or third was in the presence of 1 μM endomorphin (EM)-1 or -2 without or with 100 nM cyprodime (Cyp), respectively. Data are expressed as a percentage of the ACh release in the first stimulation. B: ACh release was evoked by EFS at 10 Hz in the presence of 1 μM atropine. The first stimulation was carried out in the presence of atropine alone (Cont). The second or third stimulation was in the presence of 1 μM endomorphin-1 or -2 without or with 100 nM cyprodime, respectively. Data are expressed as a percentage of the ACh release in the presence of atropine (not a net increase by atropine). Values are each the mean for 5 to 6 experiments, with S.E.M. Cont: Control, Cyp: Cyprodime. *Significantly different from the value of each control at $P<0.05$ (Bonferroni's test).

works during EFS at 1 Hz, while the mechanism fully works at 10 Hz in the absence of 1 μM atropine. Thus, it seems likely that decreasing the extent of muscarinic autoinhibition would increase the inhibitory effect of opioid via mu-receptors on ACh release. It was suggested in our previous study that the phospholipase C – protein kinase C system modulates ACh release, and the activation of muscarinic autoreceptor may negatively modulate ACh release at a point upstream of the system (15). Therefore, the inhibitory mechanism mediated through mu-receptors in the LMMP preparations may share a common pathway to that of muscarinic autoinhibition. In summary, the present study suggests that endomorphin-1 and -2 inhibit ACh release in the LMMP preparations of guinea pig ileum via activating mu-receptors, and that the potent inhibition of ACh release through activation of the muscarinic autoreceptors by ACh released only under the experimental conditions such as EFS at 10 Hz in the presence of anti-cholinesterase probably masks the inhibitory effect of opioid via mu-receptors.

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