Relationship Between Inhibitory Effect of Endogenous Opioid via Mu-Receptors and Muscarinic Autoinhibition in Acetylcholine Release From Myenteric Plexus of Guinea Pig Ileum

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ABSTRACT—Relationship between activation of opioid receptors and muscarinic autoinhibition in acetylcholine (ACh) release from the myenteric plexus was studied in longitudinal muscle myenteric plexus (LMMP) preparations of guinea pig ileum. A mu-receptor agonist, [D-Ala², N-Me-Phe⁴, Gly⁵-ol] enkephalin (DAMGO), at a concentration of 1 μ M inhibited the ACh release evoked by electrical field stimulation (EFS) at 1 Hz but not at 10 Hz. After the muscarinic autoreceptors were blocked with atropine (1 μ M), DAMGO inhibited EFS-evoked ACh release also at 10 Hz. After the autoreceptors were potently activated with muscarine (200 μ M), the inhibitory effect of DAMGO at 1 Hz was abolished. A kappa-receptor agonist, U-50,488, at 1 μ M inhibited the EFS-evoked ACh release both at 1 and 10 Hz. U-50,488 inhibited ACh release regardless of the presence of atropine or muscarine. A delta-agonist, enkephalin [D-PEN^{2,5}] (PDPDE), did not show any significant effect. On the other hand, a selective mu-receptor antagonist, cyprodime, increased ACh release evoked by EFS at 1 Hz, but not at 10 Hz. After the autoreceptors were blocked, cyprodime increased EFS-evoked ACh release also at 10 Hz. The selective kappa-receptor antagonist, nor-binaltorphimine, did not affect ACh release in the absence or presence of atropine. The results suggest that endogenous opioid(s) inhibits ACh release by activating mu-, but not kappa- and delta-receptors in the LMMP of guinea pig ileum and that the inhibitory effect of endogenous opioid(s) in the ACh release is important when muscarinic autoinhibition mechanism does not fully work.

Keywords: mu-Receptor, Muscarinic autoinhibition, Acetylcholine release, Myenteric plexus, Ileum (guinea pig)

It is well known that morphine depresses the motility of gastrointestinal tract by inhibiting acetylcholine (ACh) release (see for review, Refs. 1 and 2). There are three major classes of opioid receptors, mu-, delta- and kappa-receptors (3, 4). All three types of opioid receptors were found in enteric neurons of the intestine (5–8). It was suggested that activation of mu- and kappa-receptors, which were present on the plasma membrane of cholinergic nerve terminals in longitudinal muscle myenteric plexus (LMMP) preparations of guinea pig ileum, resulted in inhibition of ACh release (9–11). In contrast, delta-receptors did not have any role in ACh release in the LMMP preparations (10, 11), although prejunctional inhibitory delta-receptors were suggested in cat distal

colon (12). Electrophysiological studies suggested that morphine and enkephalin increased resting potassium conductance of myenteric neurons and hyperpolarized the cell membrane and then in turn inhibited ACh release (13, 14). Subsequent studies suggested that activation of mu- (15) or delta- (16) receptors resulted in an increase in potassium conductance of the cell membrane, while activation of kappa-receptors resulted in direct inhibition in calcium entry to the cells (16).

The release of ACh is subject to muscarinic autoinhibition (see for review, Ref. 17). Interestingly, the inhibitory effects of opioids on ACh release in LMMP preparations of guinea pig ileum were reported to be dependent on the frequency of electrical pulses used for the stimulation: ACh release evoked by high frequency stimulation was less sensitive to morphine than that evoked by low

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frequency stimulation (9, 11, 18–21). Vizi et al. (21) suggested the association of the inhibitory effect of Met-enkephalin on ACh release with the muscarinic autoinhibition in LMMP preparations of guinea pig ileum. Our recent study also suggested that the dependency of inhibitory effects of morphine on the frequency for the electrical field stimulation (EFS) was associated with the extent of muscarinic autoinhibition induced by released ACh. It was also suggested from the study using selective opioid antagonists that an endogenous opioid(s) released from myenteric neurons by EFS inhibited ACh release, and the inhibitory effect was also associated with the extent of the autoinhibition (22). Therefore, the aim of this study is to define the receptor type on which endogenous opioid acts to modulate ACh release and the relationship between activation of receptor type of opioid and the muscarinic autoinhibition. To clarify these points, we examined the effect of selective agonists and antagonists for each opioid receptor type on ACh release from guinea pig LMMP preparations under the conditions of different extent of muscarinic autoinhibition.

MATERIALS AND METHODS

Male guinea pigs, weighing 300–800 g, were lightly anesthetized with diethylether and killed by bleeding. The longitudinal muscle preparations of the ileum with the myenteric plexus were made as described previously (23). The preparation was mounted in an organ bath containing 3 ml of Tyrode solution of the following composition: 136.9 mM NaCl, 2.7 mM KCl, 1.8 mM CaCl₂, 1.05 mM MgCl₂, 11.9 mM NaHCO₃, 0.4 mM NaH₂PO₄ and 5.6 mM glucose. Bathing medium was kept at 37°C and bubbled with 95% O₂ and 5% CO₂.

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 at an interval of 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. EFS was carried out with a pair of platinum electrodes, one at the top and the other at the bottom of the preparation. The parameters for EFS were as follows: supramaximal voltage (50 V), pulse duration of 0.5 msec, 200 pulses at 1 or 10 Hz. For the EFS-induced release of ACh, the stimulation was performed in trains of 20 sec at 10 Hz or 200 sec at 1 Hz, and the bathing fluid was collected after a further 40-sec period. These experimental procedures for sample collection were repeated 2 or 3 times. Our preliminary study showed that amounts of ACh released

by EFS were fairly constant up to the 3rd trial; however, it became variable after the 4th stimulation. At the end of 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. Extent of inhibition by drugs tested in increased ACh release in the presence of atropine was calculated as follows:

$$\% \text{ inhibition} = \{1 - (\text{ACh}_{\text{atropine+drug}} - \text{ACh}_{\text{control}}) / (\text{ACh}_{\text{atropine}} - \text{ACh}_{\text{control}})\} \times 100$$

where ACh_{control} and ACh_{atropine} represent the amount of ACh release in the absence and presence of atropine, respectively. ACh_{atropine+drug} represents that in the presence of both atropine and drug.

ACh released in the medium was assayed by HPLC. For analysis by HPLC, ACh in aliquots of the medium was precipitated with KI₃ in the presence of tetraethylammonium and 50 pmol ethylhomocholine as a coprecipitant and an internal standard, respectively. The quaternary ammonium compounds precipitated were dissolved in about 1 ml of acetonitrile, and then the solution was passed through a Bio Rad AG 1 × 8 anion exchange resin column (Bio-Rad Lab., Richmond, CA, USA) to trap I₃[−] in the solution. The effluents were evaporated to dryness under reduced pressure and the dried samples were stored in a refrigerator until assay. At use, the dried samples were dissolved in 100 μl water and filtered through a 0.45-μm-pore-sized filter, and 50 μl of the filtrates were injected into an HPLC assay system for ACh determination. ACh was determined by a Yanaco reversed-phase HPLC (Yanaco, Kyoto), developed first by Potter et al. (1983), using a postcolumn enzyme (acetylcholinesterase plus choline oxidase) reactor (Eicom AC-Enzympak; Eicom, Kyoto) instead of the enzyme-flow system of the original method.

The drugs used were [D-Ala², N-Me-Phe⁴, Gly⁵-ol] enkephalin (DAMGO), U-50,488 methane sulfonate, enkephalin [D-PEN^{2,5}] (PDPDE), cyprodime bromide, nor-binaltorphimine hydrochloride (Funakoshi, Tokyo) and naloxone hydrochloride, atropine sulfate, muscarine hydrochloride and physostigmine salicylate (Sigma, St. Louis, MO, USA).

Statistical analyses of the data was carried out with a paired Student's *t*-test. *P* values less than 0.05 were considered significant. The mean ± S.E.M. was used throughout this manuscript.

RESULTS

Effects of mu-, kappa- and delta-opioid agonists on ACh release evoked by EFS from LMMP preparations of guinea pig ileum

DAMGO, a selective mu-agonist, at a concentration of 1 or 10 μM reduced EFS-evoked ACh release at 1 Hz by about 30% or 40%, respectively, but it did not significantly affect that at 10 Hz (Fig. 1). U-50,488, a selective kappa-agonist, at a concentration of 1 μM inhibited EFS-evoked ACh release both at 1 and 10 Hz. The extent of the inhibition at 1 and 10 Hz was about 60% and 50%, respectively. On the other hand, PDPDE, a selective delta-agonist, at a concentration of 1 μM did not show any significant effect on EFS-evoked ACh release at 1 or 10 Hz.

Effects of atropine and muscarine on inhibition of ACh release induced by opioid agonists

We next examined the effects of the selective opioid agonists on EFS-evoked ACh release in the presence of atropine or muscarine to evaluate the relationship between the inhibition of ACh release via each opioid

receptor type and muscarinic autoinhibition. Although DAMGO (1 μM) did not affect EFS-evoked ACh release at 10 Hz in the absence of atropine (Fig. 1), it significantly inhibited EFS-evoked ACh release at 10 Hz under the blocking of autoinhibition by atropine (Fig. 2). U-50,488 (1 μM) significantly reduced EFS-evoked ACh release at 10 Hz in the absence or presence of atropine by about 60% respectively (Figs. 1 and 2). PDPDE up to 10 μM did not show any significant effect on EFS-evoked ACh release at 10 Hz even in the presence of atropine (Fig. 3). The inhibitory effects of these opioid agonists on increased ACh release in the presence of atropine are summarized in Fig. 3. The inhibitory effects of DAMGO and U-50,488 were concentration-dependent. Interestingly, U-50,488 at the higher concentrations reduced ACh release to a level lower than that in the absence of atropine, while DAMGO did not show such a tendency.

Muscarine at 200 μM inhibited EFS-evoked ACh release at 1 Hz by $22.3 \pm 4.0\%$ ($n=7$, $P<0.05$), but did not significantly affect that at 10 Hz ($n=5$). Although DAMGO reduced ACh release evoked at 1 Hz (Fig. 1), the inhibitory effect disappeared under the conditions where the muscarinic autoreceptors were activated by

Fig. 1. Effects of DAMGO (1 and 10 μM), U-50,488 (1 μM) and PDPDE (1 μM) on ACh release evoked by EFS at 1 Hz (upper panel) or 10 Hz (lower panel) from LMMP preparations of guinea pig ileum. 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 at $P<0.05$ (Student's t -test). For further details, see the Materials and Methods.

Fig. 2. Effects of DAMGO (1 μ M) and U-50,488 (1 μ M) on ACh release evoked by EFS at 1 Hz in the presence of muscarine at 200 μ M (upper panel) or at 10 Hz in the presence of atropine at 1 μ M (lower panel) from LMMP preparations of guinea pig ileum. Values are each the mean for 5 experiments, with S.E.M. *Significantly different from the value in the absence of drugs tested at $P < 0.05$ (Student's t -test). For further details, see the Materials and Methods.

Fig. 3. Concentration-dependent effects of DAMGO, U-50,488 and PDPDE on ACh release evoked by EFS at 10 Hz in the presence of atropine. Data are expressed as a percentage of the net increase in ACh release in the presence of atropine (1 μ M). Values are each the mean for 3 to 5 experiments, with S.E.M. ○: without drug, ●: DAMGO, ▲: U-50,488, ■: PDPDE.

muscarine (Fig. 2). U-50,488 showed about 50% inhibition in EFS-evoked ACh release at 1 Hz even in the presence of muscarine (Fig. 2). These results suggest that the inhibitory effect of opioids via activation of μ -, but not κ -receptors are closely associated with the extent of muscarinic autoinhibition.

Effects of μ - and κ -opioid antagonists on EFS-evoked ACh release

We further examined the effects of cyprodimine, a selective μ -antagonist, and nor-binaltorphimine, a selective κ -antagonist, on ACh release to determine the receptor subtype on which endogenous opioids act. Cyprodimine at 0.1 μ M increased EFS-evoked ACh release at 1 Hz, but not at 10 Hz (Fig. 4). The concentration of cyprodimine completely antagonized the inhibitory effect of 1 μ M DAMGO on the ACh release, but not that of 0.1 μ M U-50,488 (not shown). Under the autoinhibition-blocked condition by atropine, cyprodimine also increased EFS-evoked ACh release at 10 Hz (Fig. 5). On the other hand, nor-binaltorphimine at 20 nM did not affect EFS-evoked ACh release at 1 or 10 Hz in the absence or presence of atropine (Figs. 4 and 5). The concentration of

Fig. 4. Effects of cyprodime (100 nM) and nor-binaltorphimine (nor-BNI: 20 nM) on ACh release evoked by EFS at 1 Hz (upper panel) or 10 Hz (lower panel) from LMMP preparations of guinea pig ileum. 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 at $P < 0.05$ (Student's *t*-test).

nor-binaltorphimine was reported to increase IC_{50} value of the kappa-agonist about 150-fold, but that of mu-agonist only two-fold in the guinea pig ileum (24). These results suggest that endogenous opioids inhibit ACh release mainly via mu-receptors in the guinea pig ileum.

DISCUSSION

DAMGO, a mu-selective agonist, inhibited EFS-evoked ACh release at 1 Hz, but not at 10 Hz in LMMP preparations of guinea pig ileum. U-50,488, a selective kappa-agonist, inhibited EFS-evoked ACh release both at 1 and 10 Hz. However, PDPDE, a selective delta-agonist, did not affect ACh release. The result showing the inability of PDPDE in inhibiting ACh release is consistent with the previous studies (9, 11). It was also reported that DAMGO and U-50,488, but not PDPDE, inhibited ACh release induced by nicotine, serotonin and tachykinin (10). Therefore, it is likely that activation of mu- and

Fig. 5. Effects of cyprodime (100 nM) and nor-binaltorphimine (nor-BNI: 20 nM) on ACh release evoked by EFS at 10 Hz in the presence of atropine (1 μ M). 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 at $P < 0.05$ (Student's *t*-test).

kappa-receptors results in decrease in ACh release from the LMMP preparations of guinea pig ileum. Participation of delta-receptors in ACh release inhibition seems scarce, if any.

Previous studies reported that morphine exhibited different effects in the different frequencies of EFS used for ACh release from ileal myenteric plexus (18–21) and that DAMGO, but not U-50,488, also exhibited the different effects in ACh release from the plexus (9, 11) and in norepinephrine release from mouse vas deferens (25). Vizi et al. (21) first suggested by using Met-enkephalin that the extent of the inhibitory effect of opioids were associated with the extent of muscarinic autoinhibition. We also recently found that the inhibitory effect of exogenously added morphine and endogenous opioid on ACh release from guinea pig LMMP preparations were associated with the extent of muscarinic autoinhibition (22). From the study, it was suggested that since released ACh during 10-Hz EFS activated the muscarinic autoreceptors maximally, the inhibitory effect of morphine was masked by the autoinhibition and that released ACh during 1-Hz EFS only partially activated the autoreceptors; therefore, morphine further inhibited ACh release. Indeed, morphine did inhibit EFS-evoked ACh release at 10 Hz after blocking the autoinhibition by atropine, and the inhibitory effects of morphine at 1-Hz EFS disappeared after activating the autoreceptors by muscarine. Therefore, the relationship between the inhibitory effects of receptor type-selective agonists and antagonists on ACh release and autoinhibition was examined in the present study.

Under the conditions that the autoinhibition was blocked by atropine, DAMGO inhibited EFS-evoked ACh release at 10 Hz, which was not affected by DAMGO in the absence of atropine. In the presence of muscarine inducing the full autoinhibition, DAMGO did not inhibit EFS-evoked ACh release at 1 Hz, which was significantly inhibited by DAMGO in the absence of muscarine. On the other hand, U-50,488 showed an inhibitory effect under every condition studied. The inhibitory effect was blocked by nor-binaltorphimine but not by cyprodime (data not shown). These results suggest that the inhibitory effect of opioids via activating mu-receptors develops only when the preparation is not subject to potent autoinhibition, whereas the effect of opioids via activating kappa-receptors develops regardless of the extent of autoinhibition. The reason why the effects of opioids via activating mu- and kappa-receptors were differently affected by the extent of muscarinic autoinhibition remained unknown. It was suggested that mu- (morphine, normorphine and DAMGO) and kappa- (dynorphin, tifluadom and U-50,488H) opioids inhibited transmitter release by different mechanisms. Activation of mu-recep-

tors resulted in an increase in potassium conductance, which in turn hyperpolarized the neuronal membrane, while activation of kappa-receptors resulted in a decrease in calcium conductance by direct action on voltage-dependent calcium channels (15, 26). Therefore, there may be a possibility that the mechanism underlying the inhibition of ACh release via opioid receptors may overlap with or be different from the autoinhibition mechanism according to the kind of opioid receptor type activated. Several possible mechanisms following the activation of presynaptic muscarinic receptors, such as blockade of action potential propagation, reduction of action potential duration and interaction with the availability of Ca^{2+} for depolarization-secretion coupling, have been discussed (see for review, Ref. 27). In LMMP preparations of guinea pig ileum, it was suggested that muscarinic autoreceptor activation primarily reduced the intraneuronal availability of calcium for the stimulus-secretion coupling (28). A recent study in the CA3–CA1 synapse of rat hippocampal slices indicated that muscarinic receptor activation inhibited synaptic transmission via inhibition of presynaptic voltage dependent Ca^{2+} channels (29). We have also reported that the phospholipase C-protein-kinase C system positively modulates ACh release from the LMMP preparations when nerve terminals are excited and that the autoinhibitory mechanism negatively modulates the ACh release at a point upstream of the phospholipase C - protein kinase C system when excess ACh was released (30). However, interaction between the intracellular mechanisms resulting from activation of mu-opioid receptors and of muscarinic autoreceptors has not been clarified at present.

The mu-antagonist cyprodime significantly increased EFS-evoked ACh release at 1 Hz, but not at 10 Hz (Fig. 5). After blocking autoinhibition by atropine, cyprodime also increased EFS-evoked ACh release at 10 Hz. This mode of action of cyprodime on ACh release was consistent with that of the non-selective opioid antagonist naloxone (22). On the other hand, nor-binaltorphimine, a kappa-antagonist, did not affect ACh release in any experimental condition of the present study. The presence of enkephalin- (31) and dynorphin- (32) immunoreactive neurons have been shown in guinea pig intestine. Costa et al. (33) reported that about 95% of the neurons that contain enkephalin also contain dynorphin. Despite the fact that dynorphin has been thought of as an endogenous ligand to kappa-receptors (34), the participation of kappa-receptors in the modulation by endogenous opioids was not appreciable. Therefore, it seems likely that endogenous opioids modulate ACh release mainly via activating mu-opioid receptors and that although kappa-receptors are present, endogenous opioid that acts on the receptors is not released in LMMP preparations of

guinea pig ileum.

In summary, the present results suggest that the inhibitory effect of opioids on ACh release via activating mu-, but not kappa- and delta-, receptors is associated with the extent of muscarinic autoinhibition, and endogenous opioid modulates ACh release mainly via activating mu-receptors in the guinea pig LMMP preparation. The inhibitory modulation by endogenous opioid(s) seems to be important when the muscarinic autoinhibition mechanism does not fully work.

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