Opioid enhancement of evoked [Met\(^5\)]enkephalin release requires activation of cholinergic receptors: Possible involvement of intracellular calcium

(myenteric plexus/opiate receptor/cholinergic receptor/calcium/inositol trisphosphate)

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ABSTRACT Previous work from this laboratory has shown that the electrically evoked release of enkephalin from the guinea pig myenteric plexus is regulated by an opiate receptor-mediated, concentration-dependent mechanism. Low concentrations (nanomolar) of opioids enhance release, whereas higher concentrations (10–100 nM) inhibit release. Each opioid effect is mediated by a different guanine nucleotide-binding protein. We now demonstrate that activation of cholinergic receptors in the myenteric plexus is a prerequisite for opioid excitatory effects, but not inhibitory effects, on enkephalin release. Pretreatment with the muscarinic cholinergic receptor antagonist atropine abolishes the opioid facilitation of stimulated enkephalin release but does not alter the inhibition of release that is observed with higher concentrations of opioid agonist. Exposure to the calcium ionophore A23187 overcomes the abolishment of opioid enhancement of enkephalin release produced by cholinergic receptor blockade. In tissue treated with both atropine and A23187, the magnitude of the opioid enhancement of release is indistinguishable from that observed in untreated preparations. This suggests that the lack of stimulation-induced generation of elevated cytosolic calcium is responsible for the abolishment of facilitory opioid effects when cholinergic receptors are blocked. The known coupling of muscarinic receptors to phospholipase C activation and the generation of inositol trisphosphate (which elevates cytosolic calcium) could suggest that this second messenger is critical for the manifestation of opioid facilitation of enkephalin release.

Previous work from this laboratory has shown that the electrically evoked release of [Met\(^5\)]enkephalin from the myenteric plexus can be modulated by \(\mu\), \(\delta\), and \(\kappa\) types of opiate receptor. This modulation is bimodal. Low concentrations (nanomolar) enhance the magnitude of stimulated release, whereas higher concentrations (10–100 nM) inhibit it (1).

Both the opioid enhancement and inhibition of [Met\(^5\)]enkephalin release are antagonized by the opiate-receptor antagonist naltrexone (0.1–1 \(\mu\)M) (1). However, each effect appears to be mediated by one or more different signal transducers. Recent observations suggest the association of a guanine nucleotide-binding protein (G protein) structurally related to the stimulatory (G\(_s\)) and inhibitory (G\(_i\)) G proteins (or to the regulatory G protein, G\(_r\)) with opiate excitatory and inhibitory modulation of [Met\(^5\)]enkephalin release, respectively (2).

The relative responsiveness of the [Met\(^5\)]enkephalin release process to the excitatory or inhibitory effects of opioids is not fixed and can be altered. Chronic, long-term exposure in vivo to morphine alters the balance of the excitatory and inhibitory responses to opioids (3) as does increasing the intracellular level of cAMP or stimulating cAMP-dependent processes (1). In all of these cases, opioid inhibition of [Met\(^5\)]enkephalin release is attenuated and opioid enhancement of release predominates. Thus, the direction of the opioid modulation of the evoked release of [Met\(^5\)]enkephalin (enhancement or inhibition) manifests considerable plasticity and appears to depend upon the physiological state of [Met\(^5\)]enkephalin-containing neurons and/or other neurons in the myenteric plexus.

The parameters of electrical stimulation (40 Hz, 0.2-msec duration) used to evoke the release of enteric [Met\(^5\)]enkephalin have been shown to stimulate also the release of acetylcholine from cholinergic neurons that are present in the myenteric plexus. Cholinergic function in the preparation of longitudinal muscle with adherent myenteric plexus (LMMP) is known to be sensitive to opioids (4). Therefore, an interaction with this neuronal system could potentially mediate, at least in part, the opioid modulation of [Met\(^5\)]enkephalin release. The experiments described below were performed to determine whether or not the increase in cholinergic neuronal activity that occurs in parallel with the stimulation of [Met\(^5\)]enkephalin release is a necessary condition for the opioid enhancement and/or inhibition of that release. This report shows that, although activation of cholinergic receptors is not required for opioid inhibition of electrically evoked [Met\(^5\)]enkephalin release, it is a prerequisite for the manifestation of opioid enhancement of release. It is suggested that the muscarinic receptor-coupled generation of a phosphotylinositol-derived second messenger(s) is crucial for opioid enhancing effects.

MATERIALS AND METHODS “Strips” of the guinea pig LMMP were prepared, mounted, and superfused in a stimulating chamber as described (3, 5, 6). The Krebs solution used to superfuse (pass over) the tissue contained 10 mM captopril, 0.3 mM thiorphan, 10 mM bestatin, and 2 mM L-leucyl-L-leucine to protect against the action of proteases (7). The superfusion rate was maintained at 1 ml/min. Fractions (0.5 ml) were collected on ice and oxidized immediately with 0.3% hydrogen peroxide at 4°C overnight, after which duplicate 100-\(\mu\)l aliquots of each sample were lyophilized to dryness. The protease inhibitors mentioned above were present during the 30-min equilibration period before the start of each experiment and thereafter for its duration. Sufentanil citrate (SFNC), [2-D-penicillamine, 5-D-penicillamine]enkephalin ([D-Pen\(^2,5\)]enkephalin), and 3,4-dichloro-N-methyl-N-[2-(1-pyrrroldinyl)cyclohexyl]-

Abbreviations: SFNC, sufentanil citrate; [D-Pen\(^2,5\)]enkephalin, [2-D-penicillamide, 5-D-penicillamide]enkephalin; U50,488H, 3,4-dichloro-N-methyl-N-[2-(1-pyrrroldinyl)cyclohexyl]benzeneaceta-

mide; InsP\(_3\), inositol trisphosphate; LMMP, longitudinal muscle with adherent myenteric plexus.

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benzeneacetamide (U50,488H; Upjohn) were utilized as µ-, δ-, and κ-selective opioid receptor agonists, respectively (8, 9). Enkephalin-like immunoreactivity was determined by using an RIA procedure as described (1). A standard curve (3.12, 6.25, 12.5, 25, 50, 100, 250, 500, and 1000 pg of [Met5]enkephalin per assay tube) in which the percentage of inhibition of binding was plotted against the logarithm of the concentration of unlabeled oxidized [Met5]enkephalin in the reaction tube was generated in each experiment. The minimum detectable amount was 3 pg, at which an ~20% inhibition of binding was observed. Cross-reactivity with [Leu5]enkephalin, α-endorphin, β-endorphin, and γ-endorphin was less than 0.1% (10). High-pressure liquid chromatography by fractionation in combination with RIA indicated that >90% of enkephalin-like immunoreactivity was authentic [Met5]enkephalin (6).

The electrically induced percentage increase in the rate of [Met5]enkephalin release was calculated first in the absence of opioid agonist (cycle A), 3 min after pretreatment with various concentrations of µ-, δ-, and κ-selective agonists while still in their presence (cycle B), and after a 15-min washout (cycle C). All three cycles were obtained with the same LMMP preparation. The percentage increase above basal release under each condition was calculated by subtracting the mean basal release (obtained prior to stimulation over four or five collection periods of 30 sec each) from the peak release observed during electrical stimulation and dividing the difference by the mean basal release [(stimulated − basal)/basal]. The percentage increase (mean ± SEM) above basal release observed in the presence of opioid (cycle B) is expressed relative to that observed in its absence (cycle A and cycle B). To determine the effect of cholinergic receptor blockade on opioid enhancement or inhibition of [Met5]enkephalin release, treatment with either atropine or hexamethonium was begun 15 min before the start of the first cycle and was continued for the duration of all three cycles. In experiments involving A23187, the calcium ionophore was added to the LMMP superfuse 5 min before the application of electrical stimulation in each cycle of release.

The opioid responsiveness of untreated preparations and preparations treated with cholinergic receptor antagonists was analyzed by using a three-way analysis of variance. Experiments involving the effect of atropine on excitatory opioid responsiveness in forskolin- or naltroxone-treated preparations was analyzed by using a one-way analysis of variance. Planned comparisons were tested by using Student’s t test; P > 0.05 is considered insignificant. The effect of A23187 on excitatory opioid responsiveness in atropine-treated and untreated preparations was analyzed by using Student’s t test to avoid the potential confound of performing multiple t tests, P > 0.01 is considered insignificant (Bonferroni method).

RESULTS

Effect of Cholinergic Receptor Blockade on the Opioid Enhancement and Inhibition of Evoked [Met5]Enkephalin Release. Muscarinic cholinergic receptor blockade altered the ability of opiate receptor type-selective agonists to regulate the evoked release of [Met5]enkephalin. However, a three-way analysis of variance indicated that there is a significant interaction between the concentration of opioid agonist and atropine (F1,57 = 29.94, where F1,57 is the F ratio obtained at the indicated degrees of freedom; P < 0.05). The effect of atropine on opioid responsiveness was dependent upon the concentration of agonist used. This interaction was the same regardless of the receptor selectivity of the opioid agonist.

Fig. 1 illustrates the effect of muscarinic or nicotinic cholinergic receptor blockade on the ability of excitatory concentrations of SFNC (1 nM), [d-Pen2,5]-enkephalin (5 nM), or U50,488H (1 nM) to enhance the evoked release of [Met5]enkephalin from the myenteric plexus. After pretreatment with atropine, the ability of opioids to facilitate stimulated [Met5]enkephalin release was no longer observed. In contrast, inhibitory opioid responses remained unaltered (Fig. 2). Since all three cycles of release were obtained in atropine-treated preparations, the inhibition of release produced by atropine alone (=32%; see below) was not a confounding factor. Thus, the opioid enhancement of [Met5]enkephalin release appears to be mediated via a muscarinic-sensitive process(es) that is not required for opioid inhibitory action.

In contrast with atropine, the nicotinic receptor antagonist hexamethonium was devoid of any effect on the ability of 1 nM SFNC or U50,488H to enhance [Met5]enkephalin release. However, hexamethonium did block the enhancement of [Met5]enkephalin release produced by the δ-selective opioid agonist [d-Pen2,5]-enkephalin (see Fig. 1).

Forskolin-Induced Reversal of Opioid Inhibition to Enhancement of Evoked Enkephalin Release Requires Activation of Cholinergic Receptors. Elevation of enteric intracellular levels of cAMP via pretreatment with forskolin increased the magnitude of the stimulated release of [Met5]enkephalin (1). In forskolin-treated preparations, inhibitory concentrations of opioid no longer produced an inhibition of stimulated [Met5]enkephalin release (F2,13 = 17.45, P = 0.002 and F2,15 = 7.95, P = 0.004 for SFNC and [d-Pen2,5]-enkephalin, respectively). In these preparations, a previously inhibitory concentration of opioid now produces an enhancement of the magnitude of evoked (40 Hz) [Met5]enkephalin release (1). Since all three cycles of release were obtained in forskolin-
treated myenteric plexus, the stimulatory effect of forskolin on evoked enkephalin release is not a confounding factor. Experiments in atropinized preparations now indicate that a component of this forskolin effect is sensitive to cholinergic receptor blockade. Fig. 3 illustrates that after pretreatment with 0.5 \( \mu \)M forskolin and 1 \( \mu \)M atropine, inhibitory responses to 10 nM SFNC or [\( \text{D}-\text{Pen}^{2,5} \text{L}-\text{enkephalin} \) remained completely attenuated. However, the reversal of the opioid-induced inhibition to enhancement of [\( \text{Met}^5 \text{enkephalin} \) release, previously observed in LMMP preparations treated with forskolin alone, was no longer observed. In the presence of forskolin and atropine, an inhibitory concentration of SFNC or [\( \text{D}-\text{Pen}^{2,5} \text{L}-\text{enkephalin} \) was devoid of any influence on the magnitude of evoked [\( \text{Met}^5 \text{enkephalin} \) release.

Pretreatment with hexamethonium also prevented the forskolin reversal of [\( \text{D}-\text{Pen}^{2,5} \text{L}-\text{enkephalin} \)-induced inhibition to enhancement but had no effect on the ability of forskolin to reverse the inhibition of [\( \text{Met}^5 \text{enkephalin} \) release produced by SFNC. These results parallel the effect of nicotinic receptor blockade on the enhancement of release produced by these compounds in the absence of forskolin (see Fig. 1).

**Effect of Cholinergic Receptor Blockade on the Naloxone Reversal of Opioid Inhibition to Enhancement of [\( \text{Met}^5 \text{enkephalin} \) Release.** The opioid inhibition of [\( \text{Met}^5 \text{enkephalin} \) release was quantitated in untreated preparations, preparations pretreated with naloxone, and in preparations pretreated with both naloxone and atropine (Fig. 4). In the absence of atropine, naloxone pretreatment abolished opioid inhibition of [\( \text{Met}^5 \text{enkephalin} \) release; in the presence of this narcotic antagonist, inhibitory concentrations of opioid agonist produced an enhancement of the magnitude of evoked [\( \text{Met}^5 \text{enkephalin} \) release. This interaction was altered by atropine (\( F_{2,12} = 62.13, P < 0.001 \) and \( F_{2,13} = 24.24, P < 0.001 \) and \( F_{2,13} = 9.92, P < 0.003 \) for SFNC and \( \text{[D-Pen}^{2,5}\)enkephalin and U50,488H, respectively). In LMMP preparations pretreated with both naloxone and atropine, the reversal of the opioid inhibition to enhancement of release was no longer observed. After muscarinic receptor blockade, naloxone still blocked the opioid inhibition but did not unmask opioid enhancement of [\( \text{Met}^5 \text{enkephalin} \) release.

**Effect of Atropine Alone on the Electrically Evoked Release of [\( \text{Met}^5 \text{enkephalin} \).** The effect of blocking muscarinic cholinergic receptors on the evoked release of [\( \text{Met}^5 \text{enkephalin} \) was determined by comparing the magnitude of the increase in release produced by electrical stimulation before and during treatment with 1 \( \mu \)M atropine in the same preparation. Atropine pretreatment reduced the magnitude of evoked release by 32.2 \( \pm 5\% \) (\( n = 6; P < 0.05 \)). Thus, activation of cholinergic receptors is required for full responsivity of the [\( \text{Met}^5 \text{enkephalin} \) release process to electrical stimulation.

**Effect of Cholinergic Receptor Activation on the Magnitude of the Basal Release of [\( \text{Met}^5 \text{enkephalin} \).** The release of [\( \text{Met}^5 \text{enkephalin} \) in the absence of electrical stimulation was determined before, during, and after cholinergic receptor activation to determine whether or not stimulation of these receptors was sufficient to enhance [\( \text{Met}^5 \text{enkephalin} \) release. Carbachol was used to stimulate cholinergic receptors. The release of [\( \text{Met}^5 \text{enkephalin} \) observed while in the pres-
Blockade of trisphosphate calcium from the of atropine began for its duration. Treatment with atropine began 15 min before the start of the first cycle and was continued for the duration of the experiment. The percent enhancement or inhibition of evoked release was calculated as described, $*$, $P < 0.05$ for C vs. B or A; DPDPE, [d-Pen$^{2,5}$]-enkephalin.

![Graph](image)

**Fig. 4.** Effect of atropine on the ability of naloxone to block opioid inhibition and unmask enhancement of [Met$^5$]-enkephalin release. Responses to opiate receptor type-selective agonists were determined in untreated preparations (bar A) and in preparations treated with naloxone (1 $\mu$M; bar B) or naloxone and atropine (bar C). Naloxone was added to the superfusate 3 min before the onset of electrical stimulation in each cycle of release. Treatment with atropine began 15 min before the start of the first cycle and was continued for the duration of the experiment. The percent enhancement or inhibition of evoked release was calculated as described. $*$, $P < 0.05$ for C vs. B or A; DPDPE, [d-Pen$^{2,5}$]-enkephalin.

![Graph](image)

**Fig. 5.** Effect of the calcium ionophore A23187 on the atropine blockade of opioid facilitation of [Met$^5$]-enkephalin release. Treatment with atropine and A23187 (1.0 $\mu$M each) was begun 15 and 5 min, respectively, before the start of each cycle and maintained for its duration. The percent enhancement of evoked [Met$^5$]-enkephalin release produced by $\mu$-, $\delta$-, or $\kappa$-selective opioids was calculated as described. The magnitude of the opioid enhancement of [Met$^5$]-enkephalin release determined in the presence of atropine and A23187 is compared with that observed in preparations treated with atropine alone. $*$, $P < 0.002$ for B vs. A or C; DPDPE, [d-Pen$^{2,5}$]-enkephalin, and 0.20 for SFNC, [d-Pen$^{2,5}$]-enkephalin, and U50,488H, respectively.

**Effect of A23187 on Opioid Enhancement of [Met$^5$]-Enkephalin Release in the Absence of Atropine.** In the absence of atropine, treatment of the LMMP preparation with A23187 did not increase the magnitude of opioid facilitation of evoked [Met$^5$]-enkephalin release ($P > 0.10$; data not shown). This indicates that increase of intracellular calcium does not in and of itself increase excitatory opioid responsivity of the myenteric plexus.

**DISCUSSION**

This report shows that, in the myenteric plexus, stimulation of cholinergic receptors is a prerequisite for the manifestation of opioid enhancement of stimulated [Met$^5$]-enkephalin release. Under conditions in which muscarinic cholinergic receptors are blocked (via atropine), excitatory concentrations of SFNC, [d-Pen$^{2,5}$]-enkephalin, or U50,488H fail to facilitate the evoked release of [Met$^5$]-enkephalin. Muscarinic receptor blockade also produces a small but nevertheless significant reduction in the magnitude of the electrically stimulated release of [Met$^5$]-enkephalin. However, exposure of the LMMP preparation to carbachol, a cholinergic receptor agonist, does not enhance the basal release of this opioid peptide. Thus, activation of muscarinic receptors is necessary for optimum release of [Met$^5$]-enkephalin in response to electrical depolarization as well as for the enhancement of this release by opioids. Stimulation of muscarinic receptors, however, does not by itself activate the release process—i.e., cholinergic activity has a permissive effect. The ability of
opioids to enhance the magnitude of stimulated [Met\(^5\)]-enkephalin release could be due to their ability to interact with the cholinergic-sensitive component of the release mechanism.

In contrast with opioid facilitation of [Met\(^5\)]enkephalin release, the opioid inhibition of release does not require cholinergic tone; muscarinic receptor blockade does not produce a significant reduction in the inhibitory opioid effect on [Met\(^5\)]enkephalin release. This emphasizes the divergence in the biochemical process(es) that mediate each component of the bimodal opioid modulation of evoked [Met\(^5\)]enkephalin release. Additionally, these data indicate that the ability of opioids to inhibit stimulated [Met\(^5\)]enkephalin release is not secondary to its well-documented ability to reduce the release of enteric acetylcholine (4).

Blockade of nicotinic cholinergic receptors with hexamethonium does not reduce excitatory opioid responses to SFNC or USO 488H but does attenuate the enhancement of [Met\(^5\)]enkephalin release produced by [d-Pen\(^2\),d-Pen\(^4\)]enkephalin (and the forskolin reversal of [d-Pen\(^2\),d-Pen\(^4\)]enkephalin inhibition to enhancement). The basis for this difference in the \(\delta\) opioid receptor-mediated enhancement is not clear. It is possible that only the \(\delta\) type of opiate receptor is colocalized with the nicotinic cholinergic receptor. Alternatively, all types of opiate receptor may be colocalized with nicotinic receptors. However, nicotinic receptors might be segregated within the cell such that the processes to which they are coupled might be unavailable to interact with events linked to \(\mu\) or \(\kappa\) receptors.

Muscarinic cholinergic receptor blockade abolishes not only the opioid enhancement of [Met\(^5\)]enkephalin release but also the reversal of opioid inhibition to facilitation of release that is observed in forskolin- or naloxone-treated preparations. This suggests that the augmentation of [Met\(^5\)]enkephalin release that is observed under each of these conditions is mediated via a common mechanism. It should be noted that despite the abolition (via atropine) of the forskolin or naloxone reversal of opioid inhibition to enhancement, naloxone or forskolin is still able to antagonize the opioid inhibition of [Met\(^5\)]enkephalin release (negation of inhibition and reversal to enhancement are dissociable). The uncoupling of excitatory responses to inhibitory concentrations of opioid is comprised of two separable events: (i) the forskolin- or naloxone-induced neutralization of some aspect of the opioid inhibitory process that masks (ii) the opioid activation of excitatory mechanisms. Only the latter requires cholinergic tone and thus, the former continues to be observed despite the presence of atropine.

The ability of atropine to abolish opioid excitatory effects on stimulated [Met\(^5\)]enkephalin release could suggest that the intracellular concentration of a muscarinic receptor-coupled second messenger(s) is critical for this opioid effect to be manifest. In a variety of tissues, muscarinic receptors have been shown to be coupled to activation of phospholipase C and the hydrolysis of phosphatidylinositol 4,5-bisphosphate (11-15). This results in the generation of Ins\(_3\), which causes an increase in cytosolic calcium levels (14-18). It is tempting to speculate that this cholinergic receptor-coupled event may be critical for the manifestation of opioid excitatory effects.

Ins\(_3\) is highly charged and does not penetrate the cell membrane. Consequently, the exogenous application of Ins\(_3\) cannot be used to stimulate its intracellular receptor and release calcium from the endoplasmic reticulum. However, this consequence of activating Ins\(_3\) receptors can be simulated by the extracellular application of a calcium ionophore such as A23187. This treatment with A23187 overcomes the abolishment of opioid enhancement of [Met\(^5\)]enkephalin release that is produced by blockade of muscarinic receptors. However, identical treatment in the absence of atropine does not enhance excitatory opioid responsiveness. This suggests that the lack of stimulation-induced generation of increased cytosolic calcium (presumably resulting from the formation of Ins\(_3\)) is responsible for the abolishment of facilitative opioid effects when cholinergic receptors are blocked. The involvement of phosphotidylinositol-derived second messengers in the opioid facilitation of [Met\(^5\)]enkephalin release would be consistent with a previous finding in bovine adrenal chromaffin cells. In this preparation, muscarinic receptor stimulation does not by itself evoke the release of epinephrine but nevertheless enhances nicotine-induced catecholamine secretion, an effect that is paralleled (and presumably mediated) by the rapid accumulation of Ins\(_3\) (19).

In summary, cholinergic activity (muscarinic receptor activation) is a prerequisite for opioid enhancement, but not inhibition, of stimulated [Met\(^4\)]enkephalin release. The ability of A23187 to reverse the atropine blockade of the opioid enhancement of release combined with the known coupling of muscarinic receptors to phospholipase C activation and the generation of Ins\(_3\) could suggest that this second messenger is critical for the manifestation of opioid facilitation of [Met\(^5\)]enkephalin release. The divergence of the biochemical mechanism(s) that mediates opioid facilitation or inhibition indicates that in vivo each effect can be differentially and independently regulated. The pharmacological manipulation of relative responsiveness to opioid excitation or inhibition might increase the effective use of narcotics to manage pain.