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Functional analysis of opioid receptor subtypes in the ventromedial hypothalamic nucleus of the rat

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Abstract

Effects of [Met^5]enkephalin and agonists selective for μ-, δ- and κ-opioid receptors were tested in vitro on neurons of the hypothalamic ventromedial nucleus of ovariectomized, estrogen-primed rats. Brain slices were perfused with artificial cerebrospinal fluid and opioid drugs were applied by bolus injection into the perfusion line. Single unit activity was recorded extracellularly. The majority of ventromedial hypothalamic nucleus neurons tested exhibited marked inhibitory responses to [Met^5]enkephalin. The inhibition was blocked by naloxone, by the selective δ-opioid receptor antagonist naltrindole and, to a lesser extent, by the μ-opioid receptor antagonist β-funaltrexamine. The κ-opioid receptor antagonist nor-binalmorphimine had virtually no effect on [Met^5]enkephalin inhibition. Agonists selective for δ-([D-Pen^2,D-Pen^5]enkephalin, DPDPE) and for μ-([D-Ala^2,MePhe^4,Gly-ol^5]enkephalin, DAGO) opioid receptors also potently inhibited the ventromedial hypothalamic nucleus neurons while the κ-opioid receptor agonist U50,488 only produced a small inhibition in a smaller number of units. These results provide functional evidence that [Met^5]enkephalin, a potential opioid transmitter in the ventromedial hypothalamic nucleus, can exert an inhibitory effect by acting on δ- and μ-opioid receptors.

Keywords: Opioid receptor; δ-Opioid receptor; μ-Opioid receptor; κ-Opioid receptor; [Met^5]Enkephalin; Hypothalamus; Ventromedial nucleus; Electrophysiology; (Rat)

1. Introduction

Endogenous opioid peptides derived from the three precursors, pro-opio-melanocortin, proenkephalin and prodynorphin, are widely distributed in the central and peripheral nervous systems. These opioids regulate a number of physiological processes by acting on opioid receptors (Akil et al., 1984). At least three subtypes of opioid receptors, designated δ, μ and κ, have been well characterized by pharmacological approaches (Kosterlitz, 1985). The heterogeneity of opioid receptors has been documented by binding studies (see review by Mansour et al., 1988) and recently with molecular biological methods (see review by Mansour et al., 1995). However, the functional significance of any given set of opioid receptor remains to be studied in certain central nervous system nuclei using, for example, electrophysiological or behavioral methods. In the locus coeruleus, the existence of a μ type of opioid receptor has been correlated with control of neuronal excitability (North and Williams, 1985; Williams et al., 1982). The study of opioid receptors has been greatly facilitated by the development of subtype-selective receptor agonists and antagonists useful in identifying receptor subtypes at different locations within central and peripheral tissues. In neurons, activation of opioid receptors results in increased K^+ conductance and/or reduced Ca^{2+} conductance (North, 1986). As a result, neuronal excitability or transmitter release is reduced.

Previous studies from this laboratory have shown that cells in the ventromedial nucleus of the rat hypothalamus express the gene for preproenkephalin (Harlan et al., 1987; Romano et al., 1988), a precursor whose major end product is [Met^5]enkephalin (Gubler et al., 1982). The ventromedial hypothalamic nucleus has been implicated in the control of female sexual behavior, lordosis (Pfaff, 1980; Pfaff et al., 1994) as well as other physiological processes, such as food intake and sympathetic functions. In ovariectomized rats, the expression of the preproenkephalin gene in the ventromedial hypothalamic nucleus was up-regulated by estrogen (Romano et al., 1988), a major facilitator of female sexual behaviors. Given the fact that many

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enkephalinergic neurons are local neurons and that within the ventromedial hypothalamic nucleus most synaptic connections come from neurons within the basomedial hypothalamus (Nishizuka and Pfaff, 1989), it has been reasonable to postulate that [Met^5]enkephalin produced in the ventromedial hypothalamic nucleus might have effects on the neurons in this nucleus. Since the nature of possible electrophysiological effects of [Met^5]enkephalin on ventromedial hypothalamic nucleus neurons had not been determined, we tested agonists and antagonists selective for the δ-, μ- and κ-opioid receptors during single unit recording from ventromedial hypothalamic nucleus neurons. The results show that [Met^5]enkephalin exerts an inhibitory effect on ventromedial hypothalamic nucleus neurons mainly through activation of δ- but also through μ-opioid receptors.

2. Materials and methods

2.1. Slice preparation

Adult female Sprague-Dawley rats, weighing 200–225 g, were used. After receipt from the supplier, animals were housed in a room with a reversed 12:12 light-dark cycle (light: 10:00 p.m. – 10:00 a.m.; dark: 10:00 a.m. – 10:00 p.m.). Food and water were available ad lib. At least 1 week before use, the animals were ovariectomized and implanted with a silastic capsule containing 100% estradiol. On the day of the experiment, animals were decapitated under Metofane anesthesia. The brain was removed immediately and blocked in ice-cold sucrose artificial cerebrospinal fluid (ACSF) bubbled with 95% O_2 and 5% CO_2. Transverse hypothalamic slices (300–400 μm thick) containing the ventromedial hypothalamic nucleus were prepared with a Vibratome (Lancer, Series 1000). The slices were incubated for 1 h in gassed sucrose ACSF and then in regular ACSF for at least 1 h before recording single unit activity. The regular ACSF has the following composition (in mM): glucose 10, NaCl 124, $NaHCO_3$ 26, KCl 5, KH_2PO_4 1.2, $MgSO_4 \cdot 7H_2O$ 1.3 and $CaCl_2$ 2.4. The sucrose ACSF was modified ACSF, with all the NaCl substituted by sucrose (248 mM). The purpose of using sucrose ACSF was to prevent the overexcitation of neurons during slice preparation.

2.2. Recording single unit activity

A slice was placed on a nylon net submerged in a recording chamber with a volume of 2 ml. The slice was continuously perfused with gassed regular ACSF warmed by passing through the perfusion tubing submerged in a water bath kept at 37°C. The perfusion rate was adjusted to 2 ml/min. Recording electrodes were glass micropipettes filled with ACSF. The electrodes had a resistance of 5–10 mΩ. Under a dissecting microscope the electrode was guided by anatomical landmarks in the slice to aim at the ventromedial hypothalamic nucleus. The action potentials of single ventromedial hypothalamic nucleus neurons were amplified by a preamplifier and displayed in a storage oscilloscope. The histogram of firing rate of ventromedial hypothalamic nucleus neurons was generated by a histogram generator and displayed on a chart recorder for later analysis.

2.3. Experimental procedures

When a unit with stable spontaneous firing was recorded, the firing was observed for at least 5 min before testing with drugs. It was tested with [Met^5]enkephalin first. In one type of experiment, other opioid agonists, including [D-Ala^2,MePhe^4,Gly-ol^5]enkephalin (DAGO), selective for μ- (Handa et al., 1981), [D-Pen^2,D-Pen^5]enkephalin (DPDPE), selective for δ- (Mosberg et al., 1983), and U50,488, selective for κ-opioid (Von Voigtlander et al., 1982) receptors, were also applied to see if they could mimick [Met^5]enkephalin. In another type of experiment, an antagonist, such as naltrindole, selective for δ (Portoghese et al., 1988), β-funaltrexamine, selective for μ- (Jiang et al., 1990), or nor-binalmorphimine, selective for κ-opioid (Takemori et al., 1988) receptors was applied after the unit recovered from the initial [Met^5]enkephalin action to see if it could block the action of another [Met^5]enkephalin application. Application of the agonist was repeated more than 10 min after the first agonist application and 2–3 min after the administration of an antagonist. Drugs were applied by bolus injection into the perfusion line with a Hamilton syringe. All injections had the same volume of 50 μl and were performed within 1 s. Preliminary experiments showed that there were no sensitization or desensitization if injections were made at intervals of 10 min or longer. Our earlier calibration experiments showed that drugs applied this way reached peak concentration in the bath within one min of injection and were cleared in 2–5 min. In the present report the peak concentration was used to represent drug concentration at the site of action, and was calculated using the dilution factor based on these calibration experiments, which showed that drugs injected were diluted 100 times. All opioid agents were purchased from RBI (Natick, MA, USA) except for naloxone (Sigma, St. Louis, MO, USA).

2.4. Statistics

After application of a drug, a positive response to the drug was defined as a change in the spontaneous firing rate, which was greater in magnitude than 2 S.D. values of the baseline firing rate. Data reported in text and figures are mean ± S.E.M. Comparisons between means were made with Student's *t*-test or one-way ANOVA test. A P value less than 0.05 was regarded as statistically significant.

3. Results

3.1. Inhibition by opioids of spontaneous firing of ventromedial hypothalamic nucleus neurons

Spontaneous single unit activity was recorded from 72 ventromedial hypothalamic nucleus neurons. The firing rate ranged from 0.2 to 23 spikes/s. with an average of 7.4 ± 0.6 spikes/s. [Met^5]enkephalin at a concentration of 1 μM, applied by injection into the perfusion line, produced inhibition of spontaneous firing in 65 out of 72 units. Taking all units into account, the average firing rate was reduced from 7.4 ± 0.6 spikes/s to 2.2 ± 0.4 spikes/s (71 ± 4% inhibition, $P < 0.001$). The magnitude of inhibition was independent of baseline rate or pattern of the spontaneous firing. The inhibitory effect was dose-dependent (an example is shown in Fig. 1, panel A, and averaged data in Fig. 2, open circles). When tested at intervals of 10 min or longer, the inhibitory response was reproducible with a S.D. less than 15%. The time course of the inhibition produced by [Met^5]enkephalin varied considerably. In a portion of units, the inhibition lasted 1–2 min and then the firing rate recovered to control level abruptly, followed by an overshoot in firing rate, which lasted 2–5 min (see example in Fig. 5, panel D). The overshoot was within 20% of baseline firing rate. In the remaining units, the recovery was a gradual process. Overall, the average 90% recovery time was 3.3 ± 0.2 min.

The μ-selective agonist DAGO and the δ-selective agonist DPDPE also produced potent inhibition of ventromedial hypothalamic nucleus neurons (see sample records in Fig. 1B). At the concentration of 1 μM, DAGO reduced the mean firing rate from 7.2 ± 1.1 to 2.5 ± 0.5 spikes/s (67 ± 7% inhibition, $n = 23$, $P < 0.001$). DPDPE decreased the mean firing rate from 7.6 ± 0.9 to 2.6 ± 0.5 spikes/s (64 ± 7% inhibition, $n = 31$, $P < 0.001$). Inhibition produced by the κ-selective agonist U50,488, as illustrated in Fig. 1B (mean firing rate from 6.8 ± 0.8 to 5.2 ± 0.8 spikes/s, 23 ± 6% inhibition, $n = 23$, $P < 0.05$), was significantly smaller than those produced by [Met^5]enkephalin, DAGO or DPDPE ($P < 0.01$, ANOVA test). These data are summarized in Fig. 3. Fig. 2 shows the concentration-dependence of the inhibitory effect of [Met^5]enkephalin, DAGO, DPDPE and U50,488. As can be seen, the curves for [Met^5]enkephalin, DAGO and DPDPE were similar both in shape and maximum response to each other (the maximum responses for [Met^5]enkephalin, DAGO and DPDPE were 63 ± 8%, 59 ± 4% and 64 ± 6%, respectively, $P > 0.05$). The concentration-response curve for U50,488 was rather flat with a maximum (31 ± 4%) response significantly smaller than those of [Met^5]enkephalin, DAGO and DPDPE. In 20 units for which all four agonists were tested on each individual unit (for example, see Fig. 4, top trace), their inhibitory effects

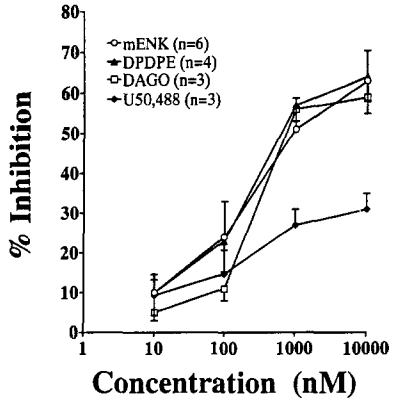

Fig. 2. Concentration-related inhibition of spontaneous firing of ventromedial hypothalamic nucleus neurons by mEnk or selective μ-, δ- and κ-opioid receptor agonists. Concentration-response curves were obtained by injecting drugs with increasing concentrations in a volume of 50 μl. Consecutive injections were made at intervals of at least 10 min, during which the firing rate had recovered from the inhibition produced by the preceding injection. Note the similarity, in both slope and maximum response of the curves for mEnk, DAGO and DPDPE, and the smaller slope and maximum response of the curve for U50,488.

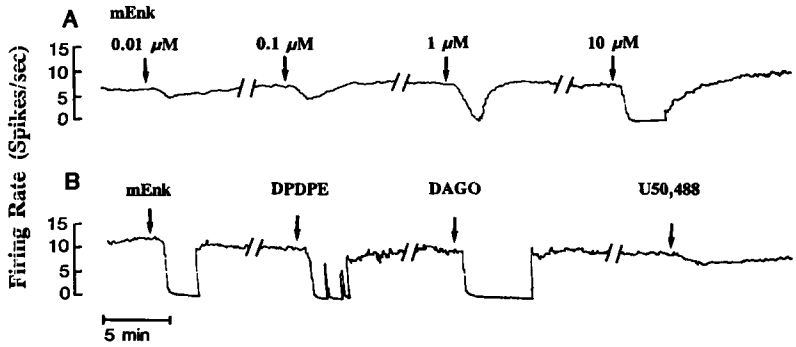

Fig. 1. A: Sample record showing dose-related inhibition of the spontaneous firing of ventromedial hypothalamic nucleus neurons by [Met^5]enkephalin (mEnk). Traces in this and following figures show firing rates of ventromedial hypothalamic nucleus neurons as a function of time. mEnk, injected into the perfusion line (indicated by arrows), at concentrations indicated, inhibited the spontaneous firing of the ventromedial hypothalamic nucleus neuron recorded. Note the progressive increase in magnitude of the inhibition with increasing mEnk concentration. B: Sample record showing the inhibitory effect of mEnk, DPDPE, DAGO and U50,488 on the same unit. All four drugs at the concentration of 1 μM, were injected at arrows. Note the complete inhibition of spontaneous firing produced by mEnk, DPDPE and DAGO, compared with a much smaller inhibition produced by U50,488 in the same unit. Breaks in this and the following figures are 2–5 min.

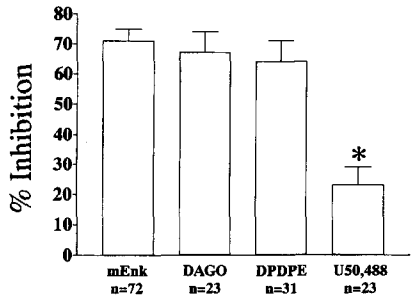

Fig. 3. Comparison of inhibitions produced by mEnk, DAGO, DPDPE and U50,488 at a concentration of 1 μM. Drugs were injected as described above. Note that mEnk, DAGO and DPDPE produced inhibition of similar magnitude. Asterisk shows that the inhibition produced by U50,488 was smaller than by the other drugs ($n = 23$, $P < 0.01$, one-way ANOVA test).

Table 1
Comparison of the inhibitory effect of [Met5]enkephalin (mEnk), DPDPE, DAGO and U50,488 on hypothalamic ventromedial nucleus neurons (all agonists at 1 μM)

Magnitude of inhibition	mEnk	DPDPE	DAGO	U50,488
< 10%	1/20	2/20	5/20	11/20
10–49%	6/20	5/20	8/20	7/20
50–89%	3/20	6/20	2/20	0/20
90–100%	10/2	7/20	5/20	2/20
P (*t*-test, two-tailed)				
vs. mEnk	—	ns	ns	< 0.001
vs. DPDPE	ns	—	ns	< 0.01
vs. DAGO	ns	ns	—	ns

* ns, not significant.

were compared. As can be seen from Table 1, 10 out of 20 units responded to [Met^5]enkephalin with a nearly complete inhibition, while at the other extreme, 11 of 20 units responded to U50,488 with an inhibition of less than 10%. The two-tailed *t*-test showed that the reponse magnitudes to [Met^5]enkephalin and DPDPE were not different ($P > 0.05$), but both were significantly greater than those to U50,488 ($P < 0.01$). The response magnitudes of DAGO were intermediate between those to [Met^5]enekphalin and DPDPE on one hand and those to U50,488 on another, but were not significantly different from either. These results suggest that [Met^5]enkephalin produces an inhibitory effect on ventromedial hypothalamic nucleus neurons by activation of δ- and/or μ-opioid receptors.

3.2. Antagonism of opioid inhibition

To further characterize opioid receptors that mediate the inhibitory effect of [Met^5]enkephalin on ventromedial hypothalamic nucleus neurons, the inhibitory response to [Met^5]enkephalin was tested with the non-selective antagonist naloxone and with various selective antagonists. Naloxone completely blocked the inhibition produced by [Met^5]enkephalin (example shown in Fig. 5). Selective antagonists tested included naltrindole, selective for δ- (Portoghese et al., 1988), β-funaltrexamine, selective for μ- (Jiang et al., 1990) and nor-binalmorphimine, selective for κ-opioid (Takemori et al., 1988) receptors. The specificity of these antagonists was first examined on the inhibition produced by the corresponding selective agonists. The results showed no cross antagonism between these agonists and antagonists (data not shown). Fig. 4 illustrates inhibition produced by DPDPE which was blocked by naltrindole but not affected by nor-binalmorphimine or β-funaltrexamine. The blocking effects of these three antagonists on [Met^5]enkephalin action are summerized in Fig. 6. Sample records are shown in Fig. 5.

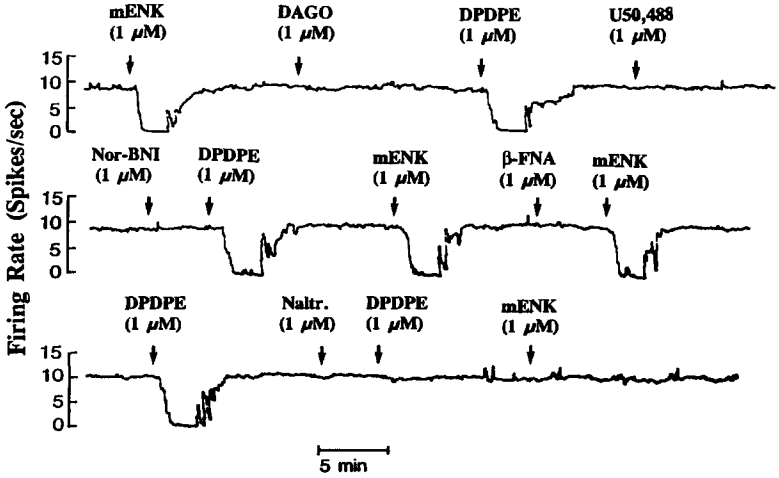

Fig. 4. Sample records from a ventromedial hypothalamic nucleus neuron. On top trace, mEnk, DAGO, DPDPE and U50,488 were injected, at arrows, into the perfusion line. Note that this unit was not responsive to DAGO or U50,488. On middle and lower traces, the mEnk- and DPDPE-induced inhibition was tested after administration of the κ antagonist nor-binalmorphimine (Nor-BNI), μ antagonist β-funaltrexamine (β-FNA) and the δ antagonist naltrindole (Naltr.). Note that the inhibition was unaffected by Nor-BNI or β-FNA, and that the inhibition was completely blocked by naltrindole. Traces in this figure are continuous.

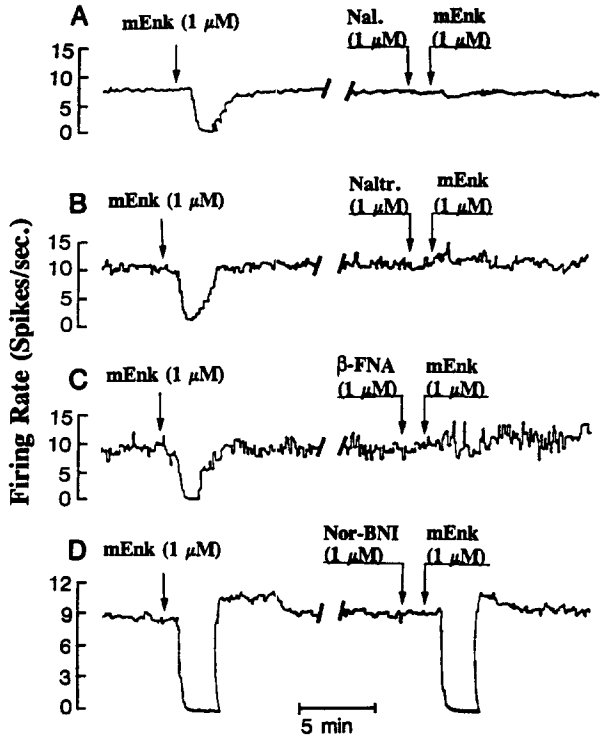

Fig. 5. Sample records of antagonism of the mEnk-induced inhibition. Records were obtained from 4 individual units. The inhibition was blocked by naloxone (Nal., A), naltrindole (Naltr., B) and β-FNA (C), but not by Nor-BNI (D).

When the inhibition produced by [Met^5]enkephalin (1 μM) was tested with these antagonists, the inhibition was best antagonized by naltrindole at 1 μM (84 ± 5% block of inhibition, $n = 13$, $P < 0.001$). At the same concentration, β-funaltrexamine produced a smaller, but significant block (37 ± 10% block of inhibition, $P < 0.05$, $n = 10$). Nor-binalmorphimine only produced a small, non-significant block (7 ± 2% block of inhibition, $n = 13$). These results suggest that the inhibitory effects of [Met^5]enkephalin on ventromedial neurons were mainly mediated by δ- and, to a lesser extent, by μ-opioid receptors.

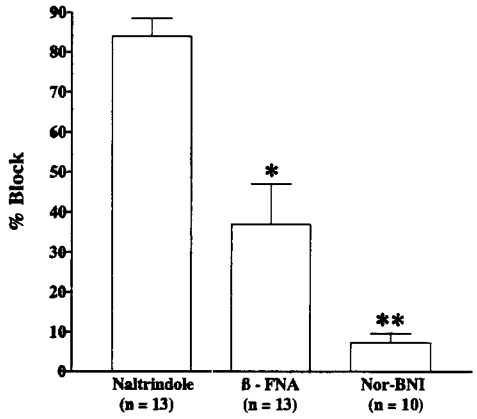

Fig. 6. Antagonism of the mEnk-induced inhibition. Bars show average percent blockade of the mEnk (1 μM)-induced inhibition by naltrindole ($n = 13$, $P < 0.001$), β-FNA ($n = 13$, $P < 0.05$) and Nor-BNI ($n = 10$, $P > 0.05$). Concentration of all antagonists was 1 μM. Asterisks show statistical significance of comparisons between the antagonisms produced by the 3 antagonists. * $P < 0.05$, ** $P < 0.01$, compared to naltrindole, t-test, two-tailed.

4. Discussion

Our understanding of the heterogeneity of many receptor types, especially opioid receptors, is based mainly on binding studies. Findings from binding studies, however, can not be assumed without convergent evidence to relate directly to opioid peptide functions. The present study demonstrates that in the ventromedial hypothalamic nucleus of female rats, ovariectomized and primed with estrogen, the inhibitory effect of [Met^5]enkephalin is mainly mediated by the δ and μ subtypes of opioid receptors. This finding seems contradictory to binding studies which showed that the ventromedial hypothalamic nucleus contains mostly κ, with only few δ- or μ-opioid receptors (e.g. Tempel and Zukin, 1987). The present results, however, by no means prove that δ- or μ-opioid receptors outnumber κ receptors in ventromedial hypothalamic nucleus, because it is not known whether the less efficient inhibitory effect of the κ agonist U50,488 reflects fewer receptor numbers or a less efficient signal transduction pathway. Alternatively, κ receptors in the ventromedial hypothalamic nucleus may have other cellular functions, such as trophic or metabolic actions, rather than the control of neuronal excitability.

To our knowledge, there has been no systematic examination of opioid actions on ventromedial hypothalamic nucleus neurons by electrophysiological approaches. Kerr et al. (1974) reported that, in intact rats, systemic application of morphine increases firing rate of ventromedial hypothalamic nucleus neurons. It was not known, however, whether the increased firing reflected a direct excitatory action of morphine on ventromedial hypothalamic nucleus neurons or indirect routes, including inputs from distant neurons. The inhibitory response of ventromedial hypothalamic nucleus neurons observed under the present in vitro conditions was not likely mediated by interneurons, since previous studies have shown that under brain slice conditions, neither excitatory nor inhibitory responses of neurons in the ventromedial hypothalamic nucleus (or in other nuclei, such as the arcuate nucleus and suprachiasmatic nucleus) to exogenous substances (including acetylcholine, histamine, norepinephrine and γ-aminobutyric acid, GABA) were affected by synaptic blockade (Kow and Pfaff, 1984, 1987; Jorgenson et al., 1989; Ogawa et al., 1991).

Electrophysiological actions of [Met^5]enkephalin in the ventromedial hypothalamic necleus are particularly interesting because it is also a site of hormone-dependent transcription of preproenkephalin. That is, estradiol raises preproenkephalin mRNA levels specifically in the ventro-

medial hypothalamic nucleus (Romano et al., 1988) in females but not in males (Romano et al., 1989) by transcriptional mechanisms (Brooks et al., 1992; Priest et al., 1995). In turn, a high percentage of synapses in the basal medial hypothalamus are of local origin (Nishizuka and Pfaff, 1989) and enkephalinergic neurons frequently work through short axons. Therefore, the responses we have recorded may reflect the actions of locally produced [Met^5]enkephalin. To fit the correlations between preproenkephalin mRNA and estrogen-dependent lordosis behavior (Lauber et al., 1990) it is necessary to hypothesize that enkephalin inhibits GABAergic inhibitory inputs, analogous to several enkephalin/GABA mechanisms.

It is worth noting that most neurons recorded in the present study were responsive both to the μ agonist DAGO and the δ agonist DPDPE. This dual responsiveness can not be explained simply by non-selective agonists since experiments with selective antagonists showed no cross antagonisms between μ- and δ-opioid receptors. Therefore, the dual-responsive neurons indeed most likely possess both subtypes of opioid receptors. We also note that the inhibitory response of some neurons to [Met^5]enkephalin could be blocked by the δ antagonist naltrindole and by the μ antagonist β-funaltrexamine, suggesting that [Met^5]enkephalin produces inhibition by acting on both δ and μ receptors. In a small number of units in the ventromedial hypothalamic nucleus, either naltrindole or β-funaltrexamine could produce a complete block of the [Met^5]enkephalin effect. This entire set of facts may be explained by the concept of the 'receptor complex' as originally postulated by Rothman and Westfall (1982a,b). Based on their own experimental findings, they hypothesized that μ- and δ-opioid receptors can exist as a complex; an agonist binding to one site may affect agonist binding to the second site. The function of the second binding site is, thereby, allosterically modulated. It is likely that in the units where [Met^5]enkephalin-induced inhibition was completely blocked by either β-funaltrexamine or naltindole, the inhibitory action of [Met^5]enkephalin must depend on its interaction with both δ and μ sites; when either site was blocked by a selective antagonist, the entire effect of [Met^5]enkephalin was prevented. Indeed, the hypothesis of a μ-δ receptor complex has been supported by subsequent biochemical studies (Schoffelmeer et al., 1990). These authors identified opioid-binding proteins in the rat striatum and bovine frontal cortex. The protein isolated from the rat striatum had a molecular mass of 80 kDa. Binding of β-endorphin to this protein was reduced by selective μ as well as δ agonists. In contrast, the opioid-binding protein isolated from the bovine frontal cortex was distributed in two bands on SDS gel with a molecular mass of 65 and 53 kDa, respectively. The binding of β-endorphin to the 65 kDa protein was reduced by selective μ agonists and that to the 53 kDa protein was reduced by selective δ agonists. Thus, μ- and δ-opioid receptors may exist presumably either as separate proteins as in the case of the bovine frontal cortex or as a receptor complex as in the case of the rat striatum.

One of the reasons for ventromedial hypothalamic nucleus recording involved lordosis behavior, whose performance depends on net ventromedial hypothalamic nucleus electrical excitation (Kow and Pfaff, 1988). Since estrogen can increase electrical activity in some ventromedial hypothalamic nucleus neurons (Bueno and Pfaff, 1976) and can induce preproenkephalin gene expression (Romano et al., 1988) it was surprising that here the inhibitory effect of [Met^5]enkephalin was so uniform and convincing. One possibility to note is that we inhibited GABA neurons (Lupica, 1995), in turn disinhibiting some other neurons. A second consideration is that our ventromedial hypothalamic nucleus neurons predominantly were not the very slowly firing cells reported by Bueno and Pfaff (1976) to be activated by estrogen. Thirdly, it may be that the enkephalin produced in the ventromedial hypothamlamic nucleus actually acts elsewhere (Yamano et al., 1986; C. Priest, personal communication) and that the inhibitory responses we recorded are important for other behaviors or physiological functions.

Acknowledgements

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