Journal of Physiology (1992), **450**, *pp.* 673–685
With 8 figures
Printed in Great Britain

PRESYNAPTIC INHIBITORY ACTION OF ENKEPHALIN ON EXCITATORY TRANSMISSION IN SUPERFICIAL DORSAL HORN OF RAT SPINAL CORD

BY YUUICHI HORI*, KATSUAKI ENDO AND TOMOYUKI TAKAHASHI†

From the Department of Physiology, Kyoto University Faculty of Medicine, Kyoto 606, Japan

(*Received 26 June 1991*)

SUMMARY

1. Tight-seal whole-cell recordings were made from marginal neurones visually identified in thin slices of 1- to 2-week-old rat lumbar spinal cord. Excitatory postsynaptic currents (EPSCs), either evoked by extracellular stimulation or those arising spontaneously in tetrodotoxin, i.e. miniature EPSCs (mEPSCs), were recorded after blocking inhibitory synaptic inputs with strychnine and bicuculline.

2. The EPSCs were abolished reversibly by kynurenic acid or 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) but not affected by (+)-2-amino-5-phosphonovalerate (APV), suggesting that they were mediated by non-NMDA (*N*-methyl-D-aspartate) glutamate receptors. Micromolar concentrations of methionine [Met^5]enkephalin reversibly reduced the magnitude of evoked EPSCs and the frequency of mEPSCs.

3. The enkephalin action on the mEPSC frequency was blocked by naloxone. A specific agonist of μ-opiate receptor, [D-Ala^2, *N*-Me-Phe^4, Gly^5]enkephalin-ol (DAGO) suppressed the mEPSC frequency. In contrast, neither a δ-opiate receptor agonist, [D-Pen^2, L-Pen^5]enkephalin (DPLPE) nor a κ-opiate receptor agonist, (5α, 7α, 8β)-(−)-*N*-methyl-*N*-[7-(1-pyrrolidinyl)-1-oxaspiro(4,5)-dec-8-yl]benzeneacetamide (U-69,593) significantly affected the mEPSC frequency.

4. The amplitude of mEPSCs or of currents induced by exogenous L-glutamate, was not affected by [Met^5]enkephalin. It is suggested that [Met^5]enkephalin presynaptically inhibits glutamatergic EPSCs by activating the μ-opiate receptor.

5. The frequency of mEPSC was reduced by about 50% by replacement of external Ca^{2+} with Mg^{2+} or by addition of Cd^{2+}. In Ca^{2+}-free–Mg^{2+} solution, [Met^5]enkephalin did not reduce the remaining mEPSCs' frequency any further.

6. It is concluded that the opiates may suppress presynaptic Ca^{2+} entry, thereby inhibiting synaptic transmission.

* Present address: Department of Physiology, Kyorin University, School of Medicine, Mitakashi, Tokyo 181, Japan.

† To whom all correspondence should be addressed.

MS 9505

INTRODUCTION

Enkephalins are the endogenous opiates concentrated in the superficial dorsal horn of the spinal cord (Hunt, Kelly & Emson, 1980; Glazer & Basbaum, 1981), where the opiate receptors are also found (Pert, Kuhar & Snyder, 1975) in association with primary afferent fibres (LaMotte, Pert & Snyder, 1976; Jessell, Tsunoo, Kanazawa & Otsuka, 1979; Fields, Emson, Leigh, Gilbert & Iversen, 1980). Most nociceptive primary afferent fibres terminate in this region (Christensen & Perl, 1970; Woolf & Fitzgerald, 1983; Sugiura, Lee & Perl, 1986). The enkephalins are assumed to play an analgesic role by inhibiting nociceptive synaptic transmission mediated presumably by substance P (Jessell & Iversen, 1977; Pearson, Brandeis & Cuello, 1982) and also by L-glutamate (Schneider & Perl, 1988; Yoshimura & Jessell, 1990).

Both the presynaptic and postsynaptic opiate actions have been reported (for review see Miller, 1984). Presynaptically, opiates suppress the release of substance P from primary sensory neurones in slice (Jessell & Iversen, 1977) and in culture (Mudge, Leeman & Fischbach, 1979). Postsynaptically, opiates produce a hyperpolarization in neurones of the superficial dorsal horn (Yoshimura & North, 1983) as well as in other central neurones (North, 1989). The postsynaptic opiate action has been shown to be induced by activation of potassium conductances (Williams, Egan & North, 1982; Miyake, Christie & North, 1989). It was then hypothesized that a similar ionic mechanism might be involved also in the presynaptic opiate action (North & Williams, 1983; Miller, 1984). However, in the central nervous system, the suppression of synaptic responses by opiates has not yet been demonstrated. The main aim of the present study was to examine whether the excitatory postsynaptic currents (EPSCs) can be suppressed by enkephalin and if so, to investigate the underlying mechanism. For this purpose we have utilized the thin slice patch-clamp techniques (Edwards, Konnerth, Sakmann & Takahashi, 1989; Takahashi, 1990) and recorded the EPSCs from neurones in the marginal zone of the superficial dorsal horn. We examined primarily the opiate action on spontaneous miniature EPSCs to identify the site of opiate action. In neuromuscular junctions, the frequency of miniature endplate potentials is known to be controlled entirely by the condition of presynaptic membrane, whereas changes in their amplitude reflect those in the properties of the postsynaptic element (Katz, 1962).

A preliminary report of this work has appeared in an abstract form (Hori, Endo & Takahashi, 1989).

METHODS

Wistar rats, 6–12 days old, were killed instantly by decapitation with scissors. Cross-sectional slices 120 μm in thickness were prepared from the lumbar spinal cord as described previously (Takahashi, 1978, 1990). After 1 h incubation at 37 °C, slices were mounted in a recording chamber on a microscope stage and continuously perfused with standard Krebs solution having the following ionic composition (mM): NaCl, 113; KCl, 3; $NaHCO_3$, 25; NaH_2PO_4, 1; $CaCl_2$, 2; $MgCl_2$, 1; D-glucose, 11; pH 7·3 after bubbling with 95% O_2 and 5% CO_2. The perfusates routinely contained strychnine (Sigma; 2–5 μM) and bicuculline (Sigma; 10 μM), to block both glycinergic and GABAergic inhibitory synaptic responses. Tetrodotoxin (TTX; Sankyo; 0·3–0·5 μM) was also included in the perfusate to isolate mEPSCs from spontaneous EPSCs arising from firings of internuncial neurones. Other chemicals were applied manually by switching the 3-way perfusion lines. Perfusion speed was about 3 ml/min. In experiments raising the K^+ concentration, the

perfusion line was switched from one to another at the inlet of the recording chamber with magnetic valves. The dead-space time was about 30 s and 5 s respectively, for the former and latter methods.

Marginal neurones in dorsal horn lamina I (Rexed, 1952) were visually identified under Nomarski optics (Fig. 1*A*). Connective tissues covering the cell membrane were removed mechanically by applying a jet of Krebs solution through a pipette with an orifice of about 10 μm (Fig. 1*A*,*). The

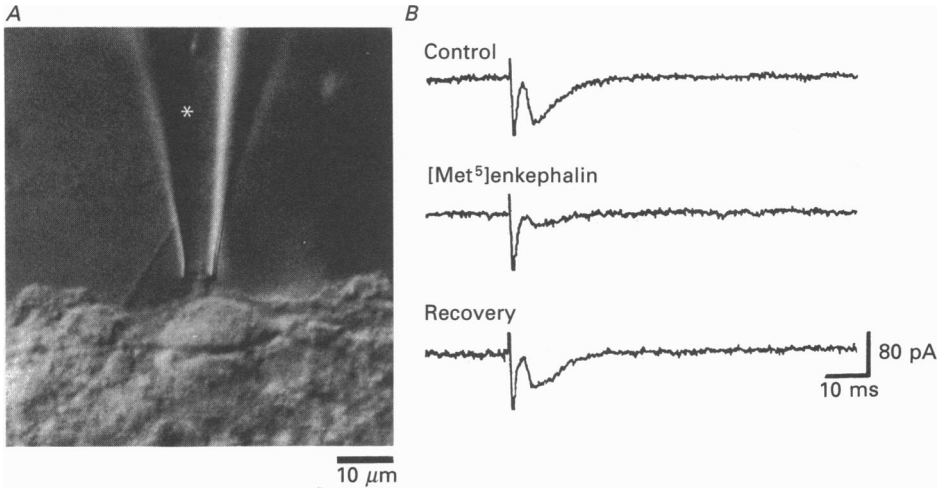

Fig. 1. *A*, a marginal neurone viewed under Nomarski optics in the dorsal border of spinal cord slice, just after cleaning by a pipette, shown above (*). *B*, the EPSCs evoked in a marginal neurone by stimulating the dorsolateral funiculus of the spinal cord with a Krebs-filled pipette (orifice about 4 μm). Stimulation at suprathreshold intensity with 200 μs duration pulses applied at 0·3 Hz. Ten consecutive records were averaged. Control (top), after application of 10 μM-[Met^5]enkephalin (middle) and after washing out enkephalin (bottom). Holding potential was at −70 mV in this and the following figures (2, 4–9). Stimulus artifacts were retouched.

debris was removed by suction with the same pipette (Edwards *et al.* 1989; Takahashi, 1990). Patch pipettes were made from thin-walled borosilicate glass capillaries, and filled with an internal solution having the following ionic composition (mM): potassium gluconate, 123; KCl, 14; sodium gluconate, 2; EGTA, 1; HEPES, 10; pH neutralized to 7·4 with KOH. For measurement of the reversal potential, potassium gluconate was replaced by caesium acetate to reduce noise arising from voltage-gated potassium conductances. The DC resistance of the pipettes filled with the standard internal solution was 5–10 MΩ. The liquid junction potential between the internal and external solutions was 9 mV for both the internal solutions. The potential values given in the text are those after correction. All experiments were carried out at room temperature (20–23 °C). The records were stored on a PCM tape-recorder (10 kHz), filtered at 3 kHz and digitized at 5–10 kHz for analysis by computer. To evaluate the statistical difference, either the two-way analysis of variance or the *t* test was used. $P < 0.05$ were taken as significantly different.

Other chemicals were obtained as follows: APV and naloxone from Sigma; [Met^5]enkephalin, DAGO and DPLPE from the Protein Research Foundation; kynurenic acid from Nakarai; CNQX from Tocris Neuramin; and U-69,593 from Upjohn.

RESULTS

Effect of [Met^5]enkephalin on EPSCs evoked in marginal neurones

In whole-cell recording from a marginal neurone, a stimulating electrode was placed in the dorsolateral funiculus region of the slice, and synaptic currents were evoked under strychnine and bicuculline (Fig. 1*B*). The synaptic currents were

identified as EPSCs because of the reversible blockage by kynurenic acid (2 mM). Bath application of [Met^5]enkephalin (10 μM) markedly suppressed the magnitude of the EPSCs (to 32 $\pm$ 14% of control, mean $\pm$ S.D. of four neurones) in a reversible manner (Fig. 1*B*). Two possible mechanisms may be postulated to account for this

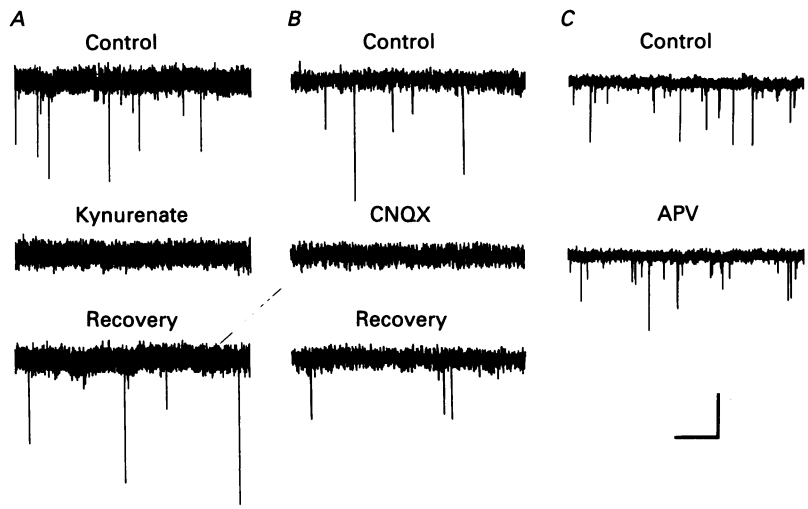

Fig. 2. Spontaneous mEPSCs recorded under TTX, strychnine and bicuculline. Control (top traces), after applying antagonists (middle traces) and after washing out the antagonists (bottom traces). Concentration of the antagonists: kynurenate (2 mM, *A*), CNQX (5 μM, *B*) and APV (50 μM, *C*). Calibration: 14 pA, 2 s (*A*), 24 pA, 4 s (*B*), 50 pA, 1 s (*C*).

enkephalin action. First, the peptide might presynaptically inhibit transmitter release. Secondly, it might suppress the sensitivity of the postsynaptic receptor. These alternative possibilities were examined by recording spontaneous mEPSCs.

With conventional intracellular recording it has been reported that [Met^5]enkephalin hyperpolarizes the superficial dorsal horn neurones (Yoshimura & North, 1983). However, we could not observe the postsynaptic effect of [Met^5]enkephalin in marginal neurones. This may be presumably due to the washing out of GTP in the whole-cell recording condition (Tatsumi, Costa, Schimerlik & North, 1990) or to the different cell type.

Spontaneous mEPSCs

After action potentials and inhibitory synaptic transmission were blocked by TTX, strychnine and bicuculline, spontaneous miniature synaptic currents were recorded (Fig. 2, top traces). The mean frequency of these spontaneous events was 0·67 $\pm$ 0·55 Hz (S.D., twenty-six neurones). By adding the glutamate-receptor antagonists, kynurenic acid (2 mM, Fig. 2*A*) or CNQX (5 μM, Fig. 2*B*) to the perfusate, the spontaneous activity was abolished. After washing out the antagonists, the miniatures recovered (Fig. 2*A* and *B*, bottom traces). The miniature events were not appreciably affected by the NMDA-receptor antagonist APV (50 μM, Fig. 2*C*). Thus, the mEPSCs appear to be mediated by the non-NMDA glutamate receptors.

The mean amplitude of mEPSCs was 25·5 pA at a holding potential of -79 mV (range: 9·7–47 pA, twenty neurones). The mean amplitudes of mEPSCs were

measured at different holding potentials (Fig. 3). The reversal potential of mEPSCs was $-7{\cdot}5 \pm 6{\cdot}5$ mV (S.D., eight neurones, Fig. 3). Since the Cl^- equilibrium potential is estimated to be -55 mV in the present experimental conditions, it is suggested that the mEPSCs are carried by cations. The mean quantal size estimated from the

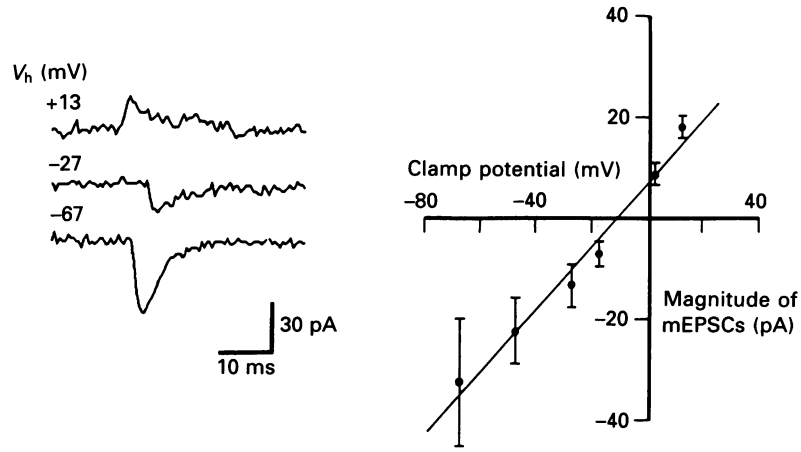

Fig. 3. Dependence of the mEPSCs' amplitude on the membrane potential. Sample records on the left, three different holding potentials as indicated. Ten mEPSCs were averaged after aligning individual mEPSCs at their peaks. Mean amplitudes and S.D.s of ten mEPSCs are indicated by symbols and bars in this and next figures. A straight line was drawn by the least-squares method.

driving force and the mean amplitude of mEPSCs was 0·36 nS. This figure is comparable to those reported for the mEPSCs in hippocampal neurones in slice and culture (0·21–0·81 nS; Bekkers, Richerson & Stevens, 1990).

Effects of [Met^5]enkephalin on mEPSC frequency

Bath application of [Met^5]enkephalin (1–10 μM) clearly reduced the mEPSC frequency (forty-six neurones). After applying [Met^5]enkephalin, the mEPSC frequency gradually declined and approached a minimum level in several minutes (Fig. 4*A*). After washing out the peptide, the mEPSC frequency slowly recovered. This inhibitory effect on the mEPSC frequency was observed with micromolar concentrations of [Met^5]enkephalin in a dose-dependent manner (Fig. 4*B*). At above 7·5 μM, the effect of [Met^5]enkephalin was apparently saturated, the mEPSC frequency being reduced to about 30% of control.

Effects of opiate receptor antagonist and agonists on mEPSC frequency

The opiate receptor antagonist, naloxone (0·1 μM) abolished the inhibitory effect of [Met^5]enkephalin on mEPSC frequency (Fig. 4*C*), while the antagonist by itself did not show any appreciable effect on the mEPSC frequency. A specific μ-opiate receptor agonist, [D-Ala^2, *N*-Me-Phe^4, Gly^5]enkephalin-ol (DAGO) suppressed the mEPSC frequency to 51 ± 20% of control (six neurones, Fig. 4*D*). DAGO was comparable in potency to the same concentration of [Met^5]enkephalin (Fig. 4*B*). In contrast, a specific δ-opiate receptor agonist, [D-Pen^2, L-Pen^5] enkephalin (DPLPE)

did not significantly reduce the mEPSC frequency (85±19% of control, four neurones, $P > 0{\cdot}1$, t test). Also, a specific κ-opiate receptor agonist, (5α, 7α, 8β)-(−)-*N*-methyl-*N*-[7-(1-pyrroldinyl)-1-oxaspiro(4,5)-dec-8-yl]benzeneacetamide (U-69,593, 5 μM) did not affect the mEPSC frequency (98±14%, six neurones). It is

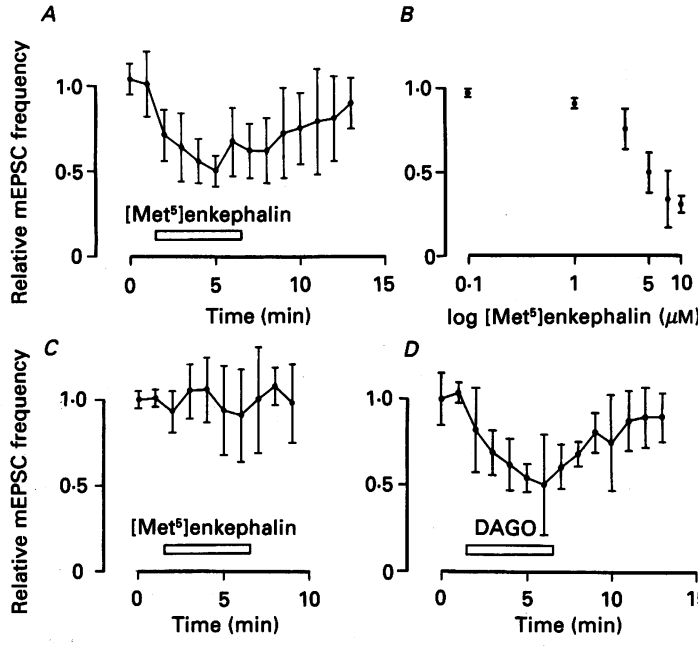

Fig. 4. *A*, the time course of reduction and recovery in mEPSC frequency following the application of [Met⁵]enkephalin (5 μM). Ordinates, the mean mEPSC frequencies measured from every 30 s records were normalized relative to the control frequency. Data from ten neurones were averaged and their mean values and S.D.s are plotted. *B*, dose dependence of the [Met⁵]enkephalin action on mEPSC frequency. Each datum point derived from three to twelve neurones. *C*, abolition of the [Met⁵]enkephalin action by naloxone (100 nM). [Met⁵]enkephalin concentration was 10 μM. Averaged data derived from six neurones. *D*, suppression of mEPSC frequency by DAGO (5 μM). Data averaged from six neurones.

suggested that the opiate inhibition of the mEPSC frequency is mediated by the μ-type receptor.

Effect of [Met⁵]enkephalin on the amplitude of mEPSCs

It is possible that an apparent reduction in the mEPSC frequency might be due to merging of small events in noise as a result of reduced amplitude of the mEPSC. Figure 5 illustrates two examples of mEPSC amplitude histograms in control (upper panels) and 5 min after application of [Met⁵]enkephalin (lower panels). The overall profile of the amplitude histograms remained unchanged after [Met⁵]enkephalin application. The mean amplitude of mEPSCs after enkephalin application was 92±15% relative to control (eight neurones). Therefore, a reduction in the mEPSC frequency by [Met⁵]enkephalin cannot be attributed to a decrease in the postsynaptic receptor sensitivity.

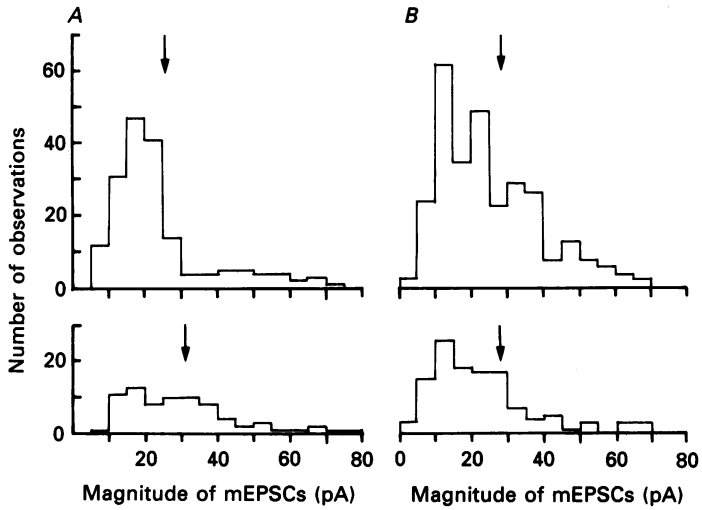

Fig. 5. Amplitude histograms of mEPSCs in two marginal neurones (*A* and *B*) before (top panels) and 5 min after application of [Met^5]enkephalin (10 μM, bottom panels). mEPSCs were sampled from 3 min stretches of records before and 5 min after enkephalin application. The mean mEPSC frequencies were 1·0 Hz (*A*, top), 0·4 Hz (*A*, bottom), 1·6 Hz (*B*, top) and 0·68 Hz (*B*, bottom), respectively. The arrows indicate the mean amplitude of mEPSCs.

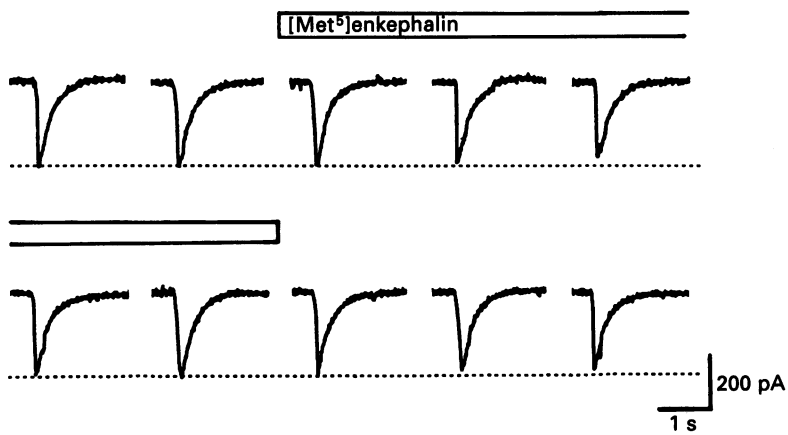

Fig. 6. Glutamate-induced current responses in a marginal neurone. L-Glutamate (200 μM) was applied at intervals of 60 s by a constant pressure from a pipette (orifice, 2 μm) positioned about 20 μm away from the soma of a marginal neurone. [Met^5]enkephalin (10 μM) was bath-applied during the period indicated by the column.

Effect of [Met^5]enkephalin on glutamate-induced current responses

The possible opiate action on postsynaptic receptor sensitivity was further tested for the glutamate-induced current in marginal neurones (Fig. 6). Local pressure application of a given dose of L-glutamate induced approximately constant current responses. Bath application of [Met^5]enkephalin (10 μM) for 5 min (open column) did not affect the magnitude of glutamate-induced currents. Thus, [Met^5]enkephalin

suppressed the magnitude of evoked EPSCs and the frequency of mEPSCs without affecting the magnitude of glutamatergic mEPSCs or the glutamate-induced current. Therefore, we conclude that [Met^5]enkephalin presynaptically inhibits the glutamatergic excitatory transmission.

Effects of [Met^5]enkephalin on potassium-induced mEPSC frequency

The mEPSC frequency was raised by increasing the external K^+ concentration (Fig. 7). A 6-fold elevation in external K^+ for 30 s caused a marked increase in the mEPSC frequency (Fig. 7*A* and *B*). As in other synapses (Fatt & Katz, 1952; Kojima & Takahashi, 1985), this potassium effect was observed only when external Ca^{2+} was present (Fig. 7*A*), suggesting the involvement of activation of voltage-gated Ca^{2+} channels in the presynaptic nerve terminals in K^+-induced mEPSCs (Katz & Miledi, 1969; Landau, 1969; Kojima & Takahashi, 1985). In this respect, the potassium-induced mEPSCs are similar to the nerve-evoked EPSCs. [Met^5]enkephalin (10 μM) reduced the frequency of mEPSCs induced by potassium (Fig. 7*B*) to 31 ± 12% of control (fourteen neurones).

Effects of [Met^5]enkephalin on the mEPSC frequency in the nominal absence of Ca^{2+}

When external Ca^{2+} was replaced by Mg^{2+} (5 mM), the frequency of mEPSCs was reduced to 46 ± 17% of control in a reversible manner (eight neurones). Similarly, Cd^{2+} (50 μM) added to the perfusate reduced the mEPSC frequency to 47 ± 15% of control (five neurones). Conversely, a 4-fold increase in external Ca^{2+} (8 mM) raised the mEPSC frequency by 3·8-fold on average (five neurones). This is similar to the results reported at the mammalian neuromuscular junction (Boyd & Martin, 1956; Hubbard, 1961) and at the inhibitory synapses of rat spinal cord (Kojima & Takahashi, 1985). It is suggested that the resting Ca^{2+} conductance is relatively high in presynaptic terminals.

In Ca^{2+}-free–Mg^{2+} (5 mM) solution, [Met^5]enkephalin no longer affected the remaining mEPSC frequency (Fig. 8). The mEPSC frequency 5 min after the application of [Met^5]enkephalin (5 μM) was 98 ± 27% of control (eight neurones). Since the mEPSC frequency in Ca^{2+}-free–Mg^{2+} solution ($0{\cdot}37 \pm 0{\cdot}25$ Hz, eight neurones) is comparable to those observed after the addition of [Met^5]enkephalin to the standard solution (7·5–10 μM, $0{\cdot}37 \pm 0{\cdot}19$ Hz, eight neurones), it is suggested that [Met^5]enkephalin may exclusively suppress the frequency of mEPSCs maintained by Ca^{2+} entry from the outside without affecting the mEPSCs which are independent of external Ca^{2+}.

Effect of Cs^+ and Ba^{2+} on the presynaptic opiate action

It has been reported that the potassium conductance mediating the postsynaptic opiate action was blocked by both external Cs^+ and Ba^{2+} (Williams, North & Tokimasa, 1988). We tested whether the presynaptic opiate action can be blocked by these metal cations. In the presence of Cs^+ (2–10 mM), the mEPSC frequency was reduced by [Met^5]enkephalin only to 69% of control, whereas in the absence of Cs^+, it was reduced by the opiate to 31% of control (significant difference, two-way analysis of variance). The effect of Cs^+ on the opiate action was reversible. Cs^+ by itself did not appreciably affect the resting frequency of mEPSC (111 ± 40% of

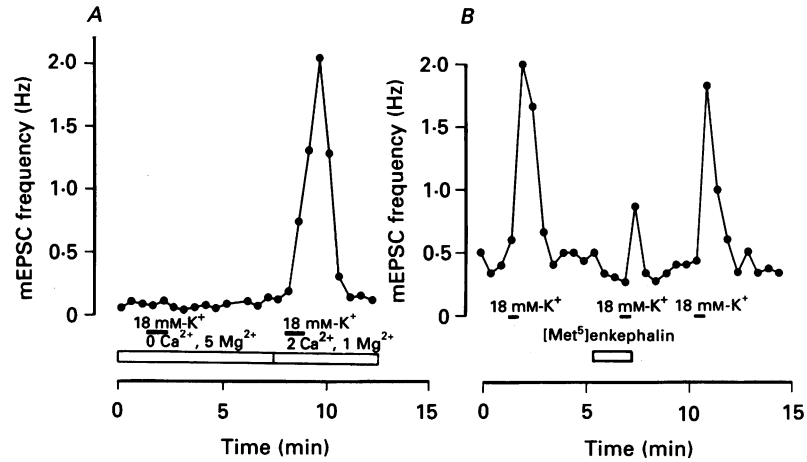

Fig. 7. Potassium-induced mEPSCs. The potassium concentration of the perfusate was raised from 3 to 18 mM for 30 s at the bars. *A*, in nominally Ca^{2+}-free–Mg^{2+} (5 mM) solution (0 Ca^{2+}, 5 Mg^{2+}) and in standard Krebs solution (2 Ca^{2+}, 1 Mg^{2+}). *B*, [Met^5]enkephalin (10 μM) was applied at the period indicated by the column. In 2 mM-Ca^{2+}, 1 mM-Mg^{2+} solution.

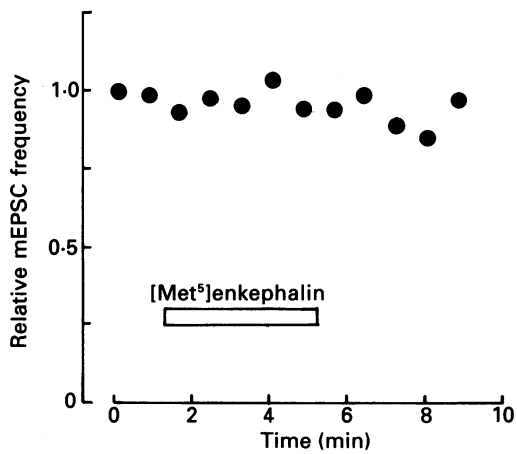

Fig. 8. The absence of [Met^5]enkephalin action on the mEPSC frequency in a nominally Ca^{2+}-free–Mg^{2+} (5 mM) solution. Each datum point derived from the mean mEPSC frequency of eight neurones normalized to control frequency before applying [Met^5]enkephalin (5 μM).

control, six neurones) nor did it affect those elevated by potassium (18 mM); 4·0 ± 1·2-fold increase (four neurones) in the presence of Cs^+ compared with 3·7 ± 2·2-fold increase (six neurones) without Cs^+.

In contrast to Cs^+, external Ba^{2+} did not appreciably affect the opiate action on mEPSC frequency. The potassium-induced mEPSCs were reduced in frequency by [Met^5]enkephalin to 31 and 26 % respectively in the absence and presence of external Ba^{2+} (1–5 mM).

DISCUSSION

The main finding of the present study was that [Met^5]enkephalin suppressed glutamatergic excitatory transmission in marginal neurones of the spinal cord. The presynaptic site of opiate action is evidenced by two facts: (i) marked reduction in the frequency of mEPSCs without changes in their amplitude and (ii) suppression in the magnitude of nerve-evoked EPSCs without affecting the postsynaptic sensitivity to L-glutamate.

In the central nervous system, presynaptic opiate action has been supported by the findings that opiates suppressed the release of substance P from the trigeminal nucleus in slice (Jessell & Iversen, 1977) and from sensory neurones in culture (Mudge *et al.* 1979). It was hypothesized that opiate presynaptically inhibits nociception mediated by substance P (Jessell & Iversen, 1977). Recently, Schneider & Perl (1988) and Yoshimura & Jessell (1990) have reported that synaptic transmission mediated by Aδ and C fibres is blocked by kynurenate or CNQX. This suggests that nocuous sensation is also mediated by glutamate receptors. Thus, our present results support the view that opiates may presynaptically inhibit nociception mediated by L-glutamate or its analogues.

A nerve impulse causes Ca^{2+} entry in the nerve terminals by gating voltage-dependent Ca^{2+} channels (Katz & Miledi, 1969). A transient increase in intracellular Ca^{2+} concentration induces simultaneous release of multiple quantal packets (del Castillo & Katz, 1954*a*; Katz, 1962). Nerve terminals at rest spontaneously release individual packets that are recorded as miniature endplate potentials (mEPPs) at the neuromuscular junction (Fatt & Katz, 1952; del Castillo & Katz, 1954*b*). Similar spontaneous events were recorded after the synaptic transmission was abolished by TTX or replacement of external Ca^{2+} by Mn^{2+} or Mg^{2+} in central neurones (Shapovalov, Shiriaev & Tamarova, 1979; Brown, Wong & Prince, 1979; Takahashi, 1984; Kojima & Takahashi, 1985). It has been shown that the spontaneous miniature inhibitory postsynaptic potentials (mIPSPs) share many characteristics in common with the mEPPs (Kojima & Takahashi, 1985). In the present study, we have shown that the frequency of mEPSCs is highly dependent on the external K^+ and Ca^{2+} concentrations as are the mEPPs and mIPSPs in mammals. Furthermore, the mEPSC frequency was reduced by addition of low concentrations of Cd^{2+}. Thus, it is suggested that presynaptic Ca^{2+} channels are partially open at rest, allowing Ca^{2+} to enter from the outside. [Met^5]enkephalin suppressed the frequency of mEPSCs in the presence of external Ca^{2+} but not in its absence. This excludes the possibility that the exocytotic process of transmitter is directly affected by opiates. Therefore, we suggest that opiates may suppress Ca^{2+} entry into nerve terminals.

Two possible mechanisms may be conceived for the mechanism of presynaptic opiate action: (i) voltage-gated Ca^{2+} channels in presynaptic nerve terminals may be blocked by opiates through activation of intracellular messengers and (ii) the channels may be deactivated as a result of hyperpolarization due to activation of potassium channels. The first possibility may be supported by the observations that the high voltage-activated Ca^{2+} channels are blocked by the opiates in the dorsal root ganglia (MacDonald & Werz, 1986) and in the neuroblastoma–glioma hybrid cells (Tsunoo, Yoshii & Narahashi, 1986; Shimahara & Icard-Liepkalns, 1987; Hescheler,

Rosenthal, Trautwein & Schultz, 1987). The second possibility is consistent with the observations that opiates hyperpolarize locus coeruleus neurones and substantia gelatinosa neurones by increasing the potassium conductance (Yoshimura & North, 1983; Williams *et al.* 1988; Miyake *et al.* 1989). The opiate receptors responsible for Ca^{2+} channel block are κ-receptors, whereas those responsible for activation of K^+ channels are μ-receptors. However, it was recently reported that transient-type Ca^{2+} channel currents recorded from sensory neurones in culture are suppressed by activation of μ-type opiate receptors (Schroeder, Fischbach, Zheng & McCleskey, 1991).

In the present study, external Cs^+ but not Ba^{2+} antagonized the inhibitory opiate action on the mEPSC frequency. These two metal ions have been reported to effectively suppress the opiate-induced potassium currents in locus coeruleus neurones (Williams *et al.* 1988). Thus, the ionic channels involved in presynaptic opiate action may not be identical with those reported for the postsynaptic opiate actions. Interestingly, a novel type of opiate-activated potassium current that was resistant to external Ba^{2+} has recently been found in hippocampal cells (Wimpey & Chavkin, 1991).

We are grateful to Dr Motoy Kuno for critical reading of the manuscript. This study was supported by a grant-in-aid for Scientific Research from the Ministry of Education, Science and Culture in Japan (no. 014801240) to T.T.

REFERENCES

BEKKERS, J. M., RICHERSON, G. B. & STEVENS, C. F. (1990). Origin of variability in quantal size in cultured hippocampal neurones and hippocampal slices. *Proceedings of the National Academy of Sciences of the USA* **87**, 5359–5362.

BOYD, I. A. & MARTIN, A. R. (1956). Spontaneous subthreshold activity at mammalian neuromuscular junctions. *Journal of Physiology* **132**, 61–73.

BROWN, T. H., WONG, R. K. S. & PRINCE, D. A. (1979). Spontaneous miniature synaptic potentials in hippocampal neurons. *Brain Research* **177**, 194–199.

CHRISTENSEN, B. N. & PERL, E. R. (1970). Spinal neurons specifically excited by noxious or thermal stimuli: Marginal zone of the dorsal horn. *Journal of Neurophysiology* **33**, 293–307.

DEL CASTILLO, J. & KATZ, B. (1954*a*). Changes in end-plate activity produced by pre-synaptic polarization. *Journal of Physiology* **124**, 586–604.

DEL CASTILLO, J. & KATZ, B. (1954*b*). Quantal components of the end-plate potential. *Journal of Physiology* **124**, 560–573.

EDWARDS, F. A., KONNERTH, A., SAKMANN, B. & TAKAHASHI, T. (1989). A thin slice preparation for patch clamp recordings from neurones of the mammalian central nervous system. *Pflügers Archiv* **414**, 600–612.

FATT, P. & KATZ, B. (1952). Spontaneous subthreshold activity at motor nerve endings. *Journal of Physiology* **117**, 109–128.

FIELDS, H. L., EMSON, P. C., LEIGH, B. K., GILBERT, R. F. T. & IVERSEN, L. L. (1980). Multiple opiate receptor sites on primary afferent fibres. *Nature* **284**, 351–353.

GLAZER, E. J. & BASBAUM, A. I. (1981). Immunohistochemical localization of leucine-enkephalin in the spinal cord of the cat; enkephalin containing marginal neurons and pain modulation. *Journal of Comparative Neurology* **196**, 377–389.

HESCHELER, J., ROSENTHAL, W., TRAUTWEIN, W. & SCHULTZ, G. (1987). The GTP-binding protein, G_o, regulates neuronal calcium channels. *Nature* **325**, 445–447.

HORI, Y., ENDO, K. & TAKAHASHI, T. (1989). Enkephalin presynaptically inhibits miniature excitatory postsynaptic currents in marginal neurons of rat spinal cord. *Japanese Journal of Physiology* **39**, 216.

HUBBARD, J. I. (1961). The effect of calcium and magnesium on the spontaneous release of transmitter from mammalian motor nerve endings. *Journal of Physiology* **159**, 507–517.

HUNT, S. P., KELLY, J. S. & EMSON, P. C. (1980). The electron microscopic localization of methionine-enkephalin within the superficial layers (I and II) of the spinal cord. *Neuroscience* **5**, 1871–1890.

JESSELL, T. M. & IVERSEN, L. L. (1977). Opiate analgesics inhibit substance P release from rat trigeminal nucleus. *Nature* **268**, 549–551.

JESSELL, T. M., TSUNOO, A., KANAZAWA, I. & OTSUKA, M. (1979). Substance P: Depletion in the dorsal horn of rat spinal cord after section of the peripheral processes of primary sensory neurons. *Brain Research* **168**, 247–259.

KATZ, B. (1962). The transmission of impulses from nerve to muscle, and the subcellular unit of synaptic action. *Proceedings of the Royal Society* B **155**, 455–477.

KATZ, B. & MILEDI, R. (1969). Tetrodotoxin-resistant electric activity in presynaptic terminals. *Journal of Physiology* **203**, 459–487.

KOJIMA, H. & TAKAHASHI, T. (1985). Characterization of miniature inhibitory post-synaptic potentials in rat spinal motoneurones. *Journal of Physiology* **368**, 627–640.

LAMOTTE, C., PERT, C. B. & SNYDER, S. H. (1976). Opiate receptor binding in primate spinal cord: Distribution and changes after dorsal root section. *Brain Research* **112**, 407–412.

LANDAU, E. M. (1969). The interaction of presynaptic polarization with calcium and magnesium in modifying spontaneous transmitter release from mammalian motor nerve terminals. *Journal of Physiology* **203**, 281–299.

MACDONALD, R. L. & WERZ, M. A. (1986). Dynorphin A decreases voltage-dependent calcium conductance of mouse dorsal root ganglion neurones. *Journal of Physiology* **377**, 237–249.

MILLER, R. (1984). How do opiates act? *Trends in Neurosciences* **7**, 184–185.

MIYAKE, M., CHRISTIE, M. J. & NORTH, R. A. (1989). Single potassium channels opened by opioids in rat locus ceruleus neurons. *Proceedings of the National Academy of Sciences of the USA* **86**, 3419–3422.

MUDGE, A. W., LEEMAN, S. E. & FISCHBACH, G. D. (1979). Enkephalin inhibits release of substance P from sensory neurons in culture and decreases action potential duration. *Proceedings of the National Academy of Sciences of the USA* **76**, 526–530.

NORTH, R. A. (1989). Drug receptors and the inhibition of nerve cells. *British Journal of Pharmacology* **98**, 13–28.

NORTH, R. A. & WILLIAMS, J. T. (1983). How do opiates inhibit neurotransmitter release? *Trends in Neurosciences* **6**, 337–339.

PEARSON, J., BRANDEIS, L. & CUELLO, A. C. (1982). Depletion of substance P-containing axons in substantia gelatinosa of patients with diminished pain sensitivity. *Nature* **295**, 61–63.

PERT, C., KUHAR, M. & SNYDER, S. H. (1975). Autoradiographic localization of the opiate receptor in rat brain. *Life Sciences* **16**, 1849–1854.

REXED, B. (1952). A cytoarchitectonic atlas of the spinal cord in the cat. *Journal of Comparative Neurology* **100**, 297–379.

SCHNEIDER, S. P. & PERL, E. R. (1988). Comparison of primary afferent and glutamate excitation of neurons in the mammalian spinal dorsal horn. *Journal of Neuroscience* **8**, 2062–2073.

SCHROEDER, J. E., FISCHBACH, P. S., ZHENG, D. & MCCLESKEY, E. W. (1991). Activation of μ-opioid receptors inhibits transient high- and low-threshold Ca^{2+} currents, but spares a sustained current. *Neuron* **6**, 13–20.

SHAPOVALOV, A. I., SHIRIAEV, B. I. & TAMAROVA, Z. A. (1979). Synaptic activity in motoneurons of the immature cat spinal cord *in vitro*. Effects of manganese and tetrodotoxin. *Brain Research* **160**, 524–528.

SHIMAHARA, T. & ICARD-LIEPKALNS, C. (1987). Activation of enkephalin receptors reduces calcium conductance in neuroblastoma cells. *Brain Research* **415**, 357–361.

SUGIURA, Y., LEE, C. L. & PERL, E. R. (1986). Central projections of identified, unmyelinated (C) afferent fibers innervating mammalian skin. *Science* **234**, 358–361.

TAKAHASHI, T. (1978). Intracellular recording from visually identified motoneurons in rat spinal cord slices. *Proceedings of the Royal Society* B **202**, 417–421.

TAKAHASHI, T. (1984). Inhibitory miniature synaptic potentials in rat motoneurons. *Proceedings of the Royal Society* B **221**, 103–109.

TAKAHASHI, T. (1990). Membrane currents in visually identified motoneurones of neonatal rat spinal cord. *Journal of Physiology* **423**, 27–46.

TATSUMI, H., COSTA, M., SCHIMERLIK, M. & NORTH, R. A. (1990). Potassium conductance increased by noradrenaline, opioids, somatostatin, and G-proteins: Whole-cell recording from guinea pig submucous neurons. *Journal of Neuroscience* **10**, 1675–1682.

TSUNOO, A., YOSHII, M. & NARAHASHI, T. (1986). Block of calcium channels by enkephalin and somatostatin in neuroblastoma–glioma hybrid NG108-15 cells. *Proceedings of the National Academy of Sciences of the USA* **83**, 9832–9836.

WILLIAMS, J. T., EGAN, T. M. & NORTH, R. A. (1982). Enkephalin opens potassium channels in mammalian central neurones. *Nature* **299**, 74–76.

WILLIAMS, J. T., NORTH, R. A. & TOKIMASA, T. (1988). Inward rectification of resting and opiate-activated potassium currents in rat locus ceruleus neurons. *Journal of Neuroscience* **8**, 4299–4306.

WIMPEY, T. L. & CHAVKIN, C. (1991). Opioids activate both an inward rectifier and a novel voltage-gated potassium conductance in the hippocampal formation. *Neuron* **6**, 281–289.

WOOLF, C. J. & FITZGERALD, M. (1983). The properties of neurones recorded in the superficial dorsal horn of the rat spinal cord. *Journal of Comparative Neurology* **221**, 313–328.

YOSHIMURA, M. & JESSELL, T. M. (1990). Amino-acid mediated EPSPs at primary afferent synapses with substantia gelatinosa neurones in the rat spinal cord. *Journal of Physiology* **430**, 315–335.

YOSHIMURA, M. & NORTH, R. A. (1983). Substantia gelatinosa neurones hyperpolarized *in vitro* by enkephalin. *Nature* **305**, 529–530.