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EFFECTS OF MET-ENKEPHALIN ON SLOW SYNAPTIC INHIBITION IN FROG SYMPATHETIC GANGLION

W. WOUTERS and J. VAN DEN BERCKEN
Institute of Veterinary Pharmacology and Toxicology, University of Utrecht, Biltstraat 172, 3572 BP Utrecht, The Netherlands

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Summary—The influence of met-enkephalin on slow inhibitory postsynaptic potentials (IPSP) in the frog sympathetic ganglion was studied with the help of a sucrose gap technique. Application of 1 μM met-enkephalin caused a hyperpolarization of the ganglionic neurones, probably via a postsynaptic mechanism. In addition, met-enkephalin depressed the amplitude of the slow IPSP by 40%. Both effects were completely antagonized by 1 μM naloxone. D-ala-met-enkephalinamide (1 μM) and morphine (5 μM) also produced a hyperpolarization together with a depression of the slow IPSP. By varying the external potassium concentration it was shown that the depression of the slow IPSP was not due to the hyperpolarization of the ganglionic neurones by met-enkephalin. Further experimentation revealed that the sensitivity of the ganglion to exogenous dopamine, the putative transmitter for the slow IPSP, was only slightly suppressed by met-enkephalin. It is concluded that the depression of the slow IPSP by met-enkephalin is presynaptic in origin and may be the result of a decrease in the amount of transmitter released from the nerve terminals. The possibility that the reduction in transmitter release is brought about by a hyperpolarization of the nerve terminals is discussed. A hyperpolarization of the preganglionic nerve terminals was actually observed after application of 5 μM morphine.

The pentapeptides, met-enkephalin and leu-enkephalin, which are the endogenous ligands for the opiate receptors, are unevenly distributed throughout the central and peripheral vertebrate nervous system and their regional distribution often closely parallels the opiate receptor distribution (Elde, Hökfelt, Johansson and Terenius, 1976; Simantov, Kuhar, Pasternak and Snyder, 1976; Hökfelt, Elde, Johansson, Terenius and Stein, 1977; Hong, Yang, Fratta and Costa, 1977; Snyder and Simantov, 1977). Recently, enkephalins have been demonstrated in the mammalian sympathetic ganglion (Di Giulio, Yang, Lutold, Fratta, Hong and Costa, 1978). Enkephalins have been shown to be localized in nerve terminals (Elde *et al.*, 1976; Hökfelt *et al.*, 1977; Simantov, Kuhar, Uhl and Snyder, 1977). Calcium-dependent release of enkephalins induced by high potassium or veratridine has been demonstrated in brain slices and in brain synaptosome preparations (for references see McKnight, Sosa, Hughes and Kosterlitz, 1978). Schulz, Wüster, Simantov, Snyder and Herz, 1977, were able to demonstrate the release of enkephalins from guinea pig ileum after electrical stimulation. These data suggest that the enkephalins represent a hitherto unknown class of neurotransmitters (Frederickson, 1977; Iversen, Nicoll and Vale, 1978; Walker, 1978).

Enkephalins cause primarily inhibition of spontaneous and chemically induced neuronal firing in the central and peripheral nervous system (Frederickson, 1977; Iversen *et al.*, 1978; Zieglgänsberger, Siggins, French and Bloom, 1978). Excitatory effects of enkephalins have, however, also been reported (Davies and Dray, 1976). Enkephalins depress the potassium-induced release of acetylcholine (Jhamandas, Sawynok and Sutak, 1977; Subramanian, Mitznegg, Sprügel, Domschke, Domschke and Wünsch, 1977), noradrenaline (Taube, Borowski, Endo and Starke, 1976), dopamine (Subramanian *et al.*, 1977) and substance P (Jessel and Iversen, 1977) from nervous tissue. It has been suggested that this inhibition of neurotransmitter release is mediated by opiate receptors located in the presynaptic nerve terminals (Lamotte, Pert and Snyder, 1976; Pollard, Llorens, Bonnet, Costentin and Schwartz, 1977; Pollard, Llorens-Cortes and Schwartz, 1977). Glutamate-induced depolarizations in spinal neurones of the cat (Zieglgänsberger and Bayerl, 1976) and in cultured spinal neurones (Barker, Gruol, Huang, Neale and Smith, 1978) were depressed by opiates and enkephalins, indicating a postsynaptic action. In myenteric neurones, enkephalins caused a hyperpolarization of the membrane, which is probably directly responsible for the enkephalin-induced decrease in spike frequency (North and Williams, 1976, 1977). A small, enkephalin-induced, hyperpolarization was also observed in cultured spinal neurones and was found to suppress the generation of action potentials (Barker *et al.*, 1978). In this preparation enkephalin also caused transient membrane depolarizations which sometimes elicited bursts of action potentials.

Key words: frog sympathetic ganglion, sucrose gap technique, met-enkephalin, morphine, naloxone, hyperpolarization, depression of slow IPSP, dopamine.

Thus it appears that the enkephalins have powerful effects on the nervous system, involving presynaptic as well as postsynaptic mechanisms.

The present study is on the influence of met-enkephalin on slow synaptic inhibition in the sympathetic ganglion of the frog. The results show that met-enkephalin induced a hyperpolarization of the ganglionic neurones, together with a depression of the slow inhibitory postsynaptic potential (IPSP). Both effects were antagonized by naloxone. Further experimentation revealed that the depression of the slow IPSP is presynaptic in origin and may be due to a decrease in the amount of transmitter released from the nerve terminals by met-enkephalin. It is tentatively suggested that the inhibition of transmitter release by met-enkephalin is brought about by a hyperpolarization of the presynaptic nerve terminals. A preliminary report on some of this material has been published (Wouters and van den Bercken, 1979a).

METHODS

Preparation

The experiments were carried out on the paravertebral sympathetic ganglion of the frog (*Rana esculenta* and *R. temporaria*). The frogs were purchased from a local supplier and kept in the laboratory until required. The animals were decapitated and pithed, and the caudal portion of the paravertebral chain including the ganglia six to ten was dissected together with the rami communicantes and parts of the spinal nerves. The ninth and tenth sympathetic ganglion, which have the same functional anatomy (Nishi, 1976), were used throughout. The ganglion under study and its ramus were carefully freed of excess connective tissue to permit better penetration of the perfusates. The experiments were performed at a room temperature of 21–23°C.

Recording and stimulation

Ganglionic potentials were recorded by means of a sucrose gap technique (Nishi and Koketsu, 1968; Wallis, Lees and Kosterlitz, 1975) which was the same as described previously (Wouters and van den Bercken, 1979b). Great care was taken to obtain a stable recording. In most experiments d.c. stability better than 1 mV/hr was achieved by appropriate silicone-sealing between the adjacent compartments of the experimental chamber and by carefully keeping the level of the bathing solutions constant by means of adjustable inlet- and outlet-flows. The resistance across the sucrose gap was monitored throughout the experiments. Results of experiments in which the resistance or the potential across the sucross gap was not stable were discarded.

The ganglionic potentials were led off by a pair of Ag/AgCl electrodes, fed into a d.c. coupled amplifier with automatic drift compensation and displayed on an oscilloscope. The d.c. potential across the sucrose gap was recorded on a strip chart recorder. This potential, which reflects the resting membrane potential of the ganglionic neurones, remained essentially constant in control experiments. Switching the perfusate, or application of Ringer containing 100 μM acetic acid—the vehicle for the enkephalins—did not cause any change in potential across the sucrose gap.

Slow IPSP's were elicited by stimulation of the ramus communicans to the eighth ganglion via a suction electrode with short trains (0.5 sec; 10 Hz) of pulses with a duration of 0.1 msec. The simultaneously evoked fast excitatory postsynaptic potentials, which are mediated by nicotinic cholinergic synapses, were selectively blocked by the continuous presence of 30 μM nicotine sulphate in all superfusion solutions. Every 20 min 5 slow IPSP's were evoked at a rate of 1 per min and these were digitized with the aid of a transient recorder (Biomation Model 802), averaged by computer and plotted.

Dopamine-induced responses

The effect of met-enkephalin on the chemical sensitivity of the ganglion to exogenous dopamine, which is the putative transmitter of the slow IPSP (Libet, 1970), was tested in the following way. The tip of a No. 27 hypodermic needle was positioned just over the ganglion with the aid of a micromanipulator. The needle was connected to a 1 ml syringe which was driven by an electronically controlled step motor. By this means small, discrete, amounts (approx. 1 μl) of a 0.1 M solution of dopamine in Ringer could be ejected. Ejection of Ringer alone was without effect. The dopamine-induced responses were recorded on a strip chart recorder.

Solutions and drugs

The ganglion was continuously superfused at a rate of 0.5 ml/min (bath volume 0.2 ml) with Ringer containing in mM: NaCl 116, KCl 2.4, $CaCl_2$ 1.8 and HEPES buffer 3; pH was adjusted to 7.3. In the experiments in which the relation between amplitude of the slow IPSP and membrane potential of the ganglionic neurons was studied, the external K^+ concentration was changed by replacing NaCl with KCl on an equimolar basis. The postganglionic nerve was superfused with isotonic KCl (119 mM). Drugs were applied to the ganglion by switching from the superfusing Ringer to one which contained the desired concentration of the drugs. Nicotine-sulphate (Sigma), dopamine hydrochloride (Fluka AG), morphine hydrochloride (Diosynth) and naloxone hydrochloride (gift from ACF) were dissolved in Ringer to make stock solutions. Met-enkephalin and D-ala-met-enkephalinamide (both from Beckman) were dissolved in 100 mM acetic acid to form a 1 mM stock solution shortly before use. The stock solutions were further diluted with Ringer at pH 7.3 to obtain the final concentration of the drugs.

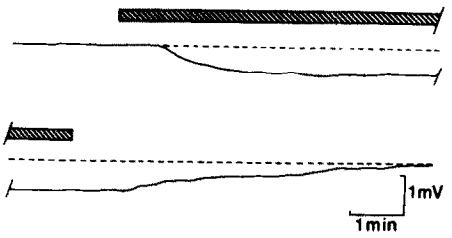

Fig. 1. Hyperpolarization of the ganglionic neurones after application of 1 μM met-enkephalin, as indicated by horizontal bar. After washing the preparation with met-enkephalin-free Ringer the potential returned to its original level. Dashed line indicates baseline voltage across sucrose gap.

RESULTS

Hyperpolarization of ganglionic neurones

Application of 1 μM met-enkephalin caused a hyperpolarization of the ganglionic neurones as illustrated in Figure 1. The hyperpolarization was small in amplitude, ranging from 0.2 to 0.7 mV in different experiments, mean value 0.37 $\pm$ 0.21 mV ($\pm$ SD, $n = 8$), but was observed in all experiments with met-enkephalin. The hyperpolarization started as soon as the peptide reached the ganglion and attained its maximum within 2 to 4 min. Thereafter the potential remained constant throughout the time met-enkephalin was applied, which in one experiment was 50 min. After the superfusion was switched back to met-enkephalin-free Ringer the potential returned to its original level (Fig. 1). Reapplication of 1 μM met-enkephalin produced again a hyperpolarization. Application of 1 μM naloxone prevented or abolished the hyperpolarization induced by met-enkephalin. Naloxone alone (1 μM) produced no change in potential. A similar hyperpolarization of the ganglionic neurones was observed after 1 μM D-ala-met-enkephalinamide or 5 μM morphine. No attempts were made to compare the relative potencies of the two enkephalins and morphine, although the latter drug seemed the least potent. These results suggest that the hyperpolarization of the ganglionic neurones is brought about by activation of specific opiate receptors on the postsynaptic neuronal membrane.

Depression of slow IPSP

In separate control experiments the amplitude and time course of the slow IPSP remained constant for a period of several hours. Superfusion with Ringer containing 100 μM acetic acid had no effect. After application of 1 μM met-enkephalin a small hyperpolarization was observed, as described above. In addition, the amplitude of the slow IPSP decreased considerably. In 11 experiments the mean decrease in amplitude amounted to 40.1 $\pm$ 5.5%.

Experiments in which slow IPSP's were evoked at a rate of 1 per 2 min showed that the depression of the slow IPSP began as soon as the peptide had reached the ganglion. The depression attained a steady level within 5–10 min, as illustrated in Figure 2, and remained virtually constant during the time met-enkephalin was applied, which in one case was up to 50 min. After switching back to superfusion with met-enkephalin-free Ringer the amplitude of the slow IPSP returned to 99.1 $\pm$ 3.0% ($n = 7$) of its value at the beginning of the experiment (see Fig. 2). Reapplication of 1 μM met-enkephalin produced a similar depression of the slow IPSP. The time course of the

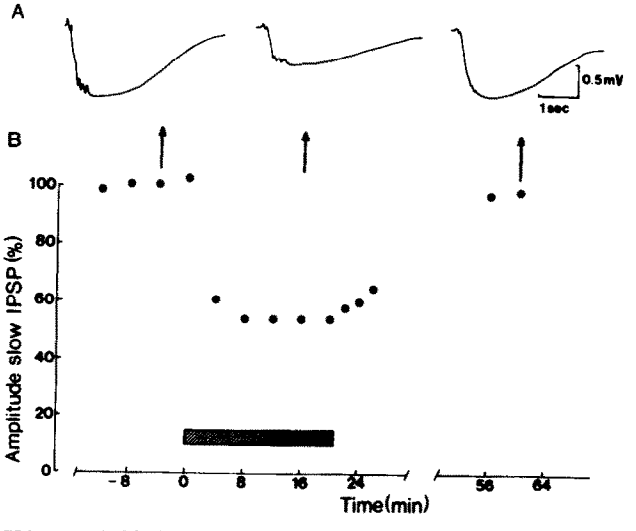

Fig. 2. (A) Slow IPSP's recorded before and during application of 1 μM met-enkephalin, and after wash out. The simultaneously evoked fast EPSP's were selectively suppressed by the continuous presence of 30 μM nicotinesulphate in the superfusion solution. Deflections during the first part of the slow IPSP are due to stimulus artefacts and to incompletely suppressed fast EPSP's. (B) Amplitude of slow IPSP expressed as percentage of amplitude at time zero, before and during application of 1 μM met-enkephalin, and after wash out. Met-enkephalin was applied for a period of 20 min as indicated by the horizontal bar. Arrows indicate the moment of recording of slow IPSP's in A. Data from one experiment.

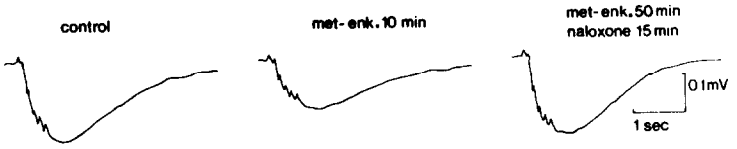

Fig. 3. Antagonism of met-enkephalin-induced depression of slow IPSP brought about by naloxone. Slow IPSP's were recorded before, during application of 1 μM met-enkephalin and during subsequent application of 1 μM naloxone, as indicated. Deflections during the first part of slow IPSP are due to stimulus artefacts and incompletely suppressed fast EPSP's.

slow IPSP, i.e. time to peak and decay time, was not affected by met-enkephalin. The met-enkephalin-induced depression of the slow IPSP was antagonized by 1 μM naloxone, as illustrated in Figure 3. Application of 1 μM naloxone prior to the application of met-enkephalin prevented the latter from depressing the slow IPSP. Naloxone alone (1 μM) had no effect on the slow IPSP. D-ala-met-enkephalinamide (1 μM) and morphine (5 μM) produced a similar decrease in amplitude of the slow IPSP. In both cases this effect was antagonized by 1 μM naloxone.

Relation between slow IPSP amplitude and membrane potential

There is evidence that the driving force for the slow IPSP in the frog sympathetic ganglion is the difference between the K^+ equilibrium potential, E_K, and the resting potential, V_m (Smith and Weight, 1977). Therefore, it is possible that the depression of the slow IPSP following application of met-enkephalin is due to the hyperpolarization of the ganglionic neurones. To examine this possibility the relation between slow IPSP amplitude and external K^+ concentration was studied. When the external K^+ concentration is altered, E_K and V_m change to a different extent and the driving force E_K-V_m changes accordingly. The results of one experiment are depicted in Figure 4. Similar results were obtained in three other experiments of this nature. The values for V_m and E_K were calculated by the Goldman and the Nernst equation respectively, assuming standard values for the intracellular ion concentrations (Na^+: 53 mM; K^+: 144 mM) and for the permeability ratio P_{Na}/P_K (0.04; Plonsey and Fleming, 1969). The upper abscissa in Figure 4 depicts the calculated values for E_K-V_m corresponding to the external K^+ concentrations used (lower abscissa). It is clear from this Figure that the amplitude of the slow IPSP is linearly related to E_K-V_m. This is in keeping with the hypothesis that E_K-V_m is the driving force for the slow IPSP. By dividing the measured value of the potential across the sucrose gap by the calculated value of V_m one can obtain a reasonable estimate of the short-circuit factor of the sucrose gap (0.17–0.3). With this factor and by extrapolation from Figure 4 the hyperpolarization necessary to cause a depression of the slow IPSP by 40% can be estimated (assuming E_K remains constant). The value obtained in this way was much larger (5–10 times) than the hyperpolarization produced by met-enkephalin.

The sympathetic ganglion contains two types of neurones, classified as B and C neurones respectively, and the slow IPSP occurs in C neurones only (Nishi, 1976). So it is possible that only these neurones are hyperpolarized by met-enkephalin. Even if it is assumed that C neurones comprise 25% of the total neuronal population, the depression of the slow IPSP cannot be attributed entirely to the hyperpolarization. Thus it is concluded that an additional mechanism is responsible for the depression of slow synaptic inhibition by met-enkephalin.

Dopamine-induced hyperpolarizations

The slow IPSP is brought about by the release of a catecholamine, probably dopamine (Libet, 1970), from an interneurone which in turn is activated by a muscarinic cholinergic synapse (Nishi, 1976). Thus, the depression of the slow IPSP may be caused by a reduction in the amount of transmitter released from the nerve terminals, or a decreased sensitivity of the postsynaptic membrane to the transmitter. To study the influence of met-enkephalin on the sensitivity of the ganglion to exogenous dopamine, small amounts of the transmitter were applied just over the ganglion with the aid of the micro-ejection device. A single

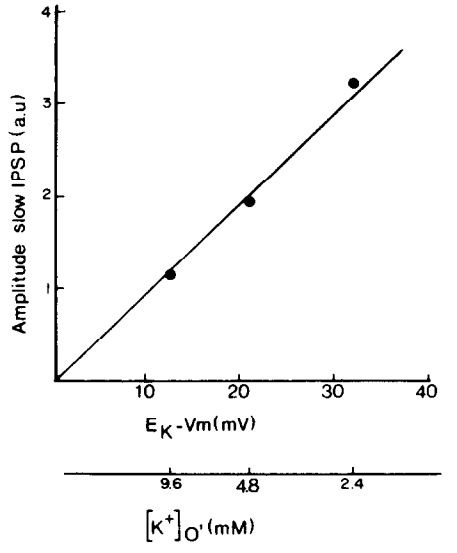

Fig. 4. Relation between amplitude of slow IPSP expressed in arbitrary units (a.u.) and E_K-V_m (upper abscissa), calculated by the Nernst and Goldman equation, respectively. The lower abscissa ($[K^+]_o$) depicts the external K^+ concentrations used.

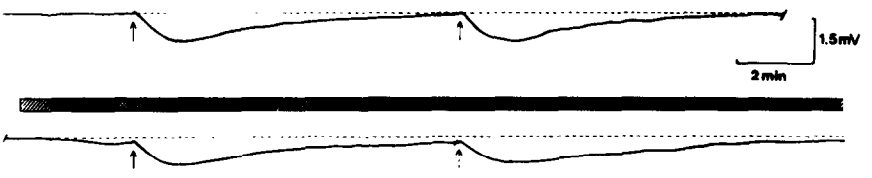

Fig. 5. Effect of met-enkephalin on transient hyperpolarizations produced by microapplication of dopamine (1 μl; 100 mM) just over the ganglion (arrows). Horizontal bar indicates application of 1 μM met-enkephalin. Dashed line denotes base line voltage across the sucrose gap. Note the hyperpolarization induced by met-enkephalin.

ejection of dopamine produced a transient hyperpolarization with a duration of several minutes and with an amplitude of 0.5–3.0 mV in different experiments (Fig. 5). If the ejections were repeated at intervals of 10 min, the amplitude of the hyperpolarizations remained virtually constant for a period of several hours. After application of 1 μM met-enkephalin, only a slight decrease in amplitude of the dopamine-induced hyperpolarizations was observed, as illustrated in Figure 5. In 5 experiments the mean amplitude was reduced by 13.8 $\pm$ 6.1%. This amount of reduction can be accounted for by the met-enkephalin-induced hyperpolarization of the ganglionic neurones, as discussed in the previous section. After washing the preparation with met-enkephalin-free Ringer the amplitude of the dopamine induced hyperpolarization returned to its original level. In these experiments a small, persistent, hyperpolarization of the ganglion was observed shortly after the application of met-enkephalin (see Fig. 5). These results suggest that the postsynaptic sensitivity of the ganglionic neurones to dopamine is hardly affected by met-enkephalin.

DISCUSSION

The present experiments show that met-enkephalin induced a hyperpolarization of ganglionic neurones and, in addition, caused a depression of slow synaptic inhibition in frog sympathetic ganglion. D-ala-met-enkephalinamide and morphine produced similar effects. The hyperpolarization as well as the depression of slow synaptic inhibition were antagonized by naloxone, indicating that both effects are mediated by specific opiate receptors.

Whether all ganglionic neurones are hyperpolarized by met-enkephalin or only a certain type of neurones, e.g. C-neurones which generate the slow IPSP, is a question that needs further study. If it is assumed that all neurones are equally affected, the met-enkephalin-induced hyperpolarization at the cellular level is estimated to be 1–2.2 mV after correction for the short circuit factor of the sucrose gap. A similar hyperpolarization has been recorded intracellularly in myenteric neurones and in cultured spinal neurones after application of opiates and opioids and is probably responsible for the depression of neuronal firing by these agents (North, 1976; North and Williams, 1976, 1977; Barker *et al.*, 1978). Thus, it appears that membrane hyperpolarization is a more general effect of enkephalins, resulting in a decreased neuronal excitability.

The mechanism by which enkephalins cause a hyperpolarization of the neuronal membrane is not known. Since in the present experiments the hyperpolarization persisted during the time met-enkephalin was applied it is not likely that this effect is brought about by the enkephalin-induced release of some substance. It has been suggested that enkephalins and opiates act directly on the neuronal membrane and cause an increase in ionic permeability (North, 1976; North and Williams, 1977; Barker *et al.*, 1978).

In addition to the hyperpolarization met-enkephalin caused a decrease in amplitude of the slow IPSP by as much as 40%. By varying the external K^+ concentration it was shown that this effect was not due to the hyperpolarization itself. The sensitivity of the ganglion to exogenous dopamine, the putative transmitter of the slow IPSP, was hardly affected by met-enkephalin. The slight decrease in amplitude of the dopamine-induced hyperpolarizations can be accounted for by the hyperpolarization of ganglionic neurones by met-enkephalin. These results strongly suggest that the depression of the slow IPSP is presynaptic in nature and may be due to a decrease induced by met-enkephalin in the amount of transmitter released from the nerve terminals upon stimulation. Enkephalins have in fact been shown to inhibit the release of a number of (putative) transmitter substances from various nervous tissue preparations, presumably by a direct action on presynaptic nerve terminals (for references, see Introduction). Recently opiate receptors located presynaptically on nerve terminals have been demonstrated (Lamotte *et al.*, 1976; Pollard *et al.*, 1977; 1977).

At present there is no information available on the mechanism of presynaptic inhibition of transmitter release by enkephalins (Starke, 1977). It is generally accepted that presynaptic inhibition in the spinal cord is brought about by depolarization of primary afferent nerve terminals via axo-axonic synapses (Schmidt, 1971). This depolarization is considered to result in a decrease of the amplitude of the action potential propagating towards the nerve terminal by virtue of inactivation of the sodium carrying system. Because of the steep relationship between action potential amplitude and transmitter release, the amount of transmitter released will by considerably reduced. This hypothesis

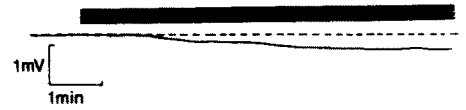

Fig. 6. Hyperpolarization of the terminal part of the preganglionic nerve after application of 5 μM morphine, as indicated by horizontal bar. The sucrose gap was applied across the preganglionic nerve and morphine was applied to the ganglionic side of the preparation. Dashed line indicates baseline voltage across sucrose gap.

tacitly assumes that the nerve action potential is actively conducted into the nerve terminal, an implication which has not been verified experimentally. It is also possible, however, that the action potential spreads passively to the nerve terminal. If this is the case a reduction of its amplitude may be brought about by a hyperpolarization of the terminal membrane.

The sympathetic ganglion offers a unique opportunity for studying the effects of drugs on the membrane potential of presynaptic nerve terminals. When the sucrose gap is applied across the preganglionic nerve, changes in membrane potential of that part of the preganglionic nerve located within the ganglion can be recorded (Koketsu and Nishi, 1968). This was done in three experiments in which morphine (5 μM) was applied to the ganglion under study. In all three cases a small hyperpolarization of the terminal part of the preganglionic nerve was observed, as illustrated in Figure 6. The hyperpolarization reached its maximum within 5–10 min and remained constant during the time morphine was applied. After washing the preparation with morphine-free Ringer the potential returned to its original level. These results indicate that opiates, and possibly opioids, are capable of hyperpolarizing the presynaptic nerve membrane, which may result in a decrease of the action potential and consequently of the amount of transmitter released. Thus, it may be that the hyperpolarization of the postsynaptic membrane and the decrease in the amount of transmitter released from the presynaptic nerve terminal by met-enkephalin are both due to the same mechanism. It should be noted, however, that the depression of the slow IPSP originates in interneuronal nerve terminals, the membrane potential changes of which cannot be recorded by the sucrose gap technique.

The results reported here are in accordance with the hypothesis that enkephalins act as inhibitory transmitters. They produce a hyperpolarization of the postsynaptic membrane, resulting in a decreased neuronal excitability, and cause a reduction in the amount of transmitter released from the nerve terminal, possibly via a hyperpolarization of the presynaptic nerve membrane. The depression of the slow IPSP observed here, however, may result in an increased transmission across the sympathetic ganglion due to disinhibition. Such a disinhibition may well account for some of the excitatory effects of enkephalins in the central nervous system (Zieglgänsberger *et al.*, 1978).

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