PEPTIDERIC EXCITATORY AND INHIBITORY SYNAPSES IN
MAMMALIAN SYMPATHETIC GANGLIA: ROLES OF
SUBSTANCE P AND ENKEPHALIN

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ABSTRACT

Electrophysiological and neurochemical experiments were made to examine possible
transmitter roles of substance P (SP) and enkephalin in guinea pig mesenteric ganglia. Repetitive
stimulation of the dorsal root produced noncholinergic slow excitatory
postsynaptic potential (e.p.s.p.) in postganglionic cells. Capsaicin caused a marked
increase in the release of SP from the ganglia in a Ca-dependent manner but did not
change the release of vasoactive intestinal polypeptide. The noncholinergic slow
e.p.s.p. evoked by preganglionic stimulation was markedly depressed after capsaicin
treatment, whereas cholinergic fast e.p.s.p. was not affected by the drug. These
results suggest that capsaicin is a selective SP-releaser and abolishes noncholinergic
slow e.p.s.p. by depleting SP that is released as transmitter from axon collateral
terminals of certain primary afferent neurons.

Conditioning repetitive stimulation of preganglionic nerves produced a prolonged
and naloxone-reversible inhibition of cholinergic fast e.p.s.p. During this inhibition
the quantum content of fast e.p.s.p.s was reduced to 45–63% of the control, whereas
the quantal unit of e.p.s.p.s and the amplitude of acetylcholine-induced depolarization
of postganglionic cells were slightly increased. [D-Ala²]-Met-enkephalinamide
exerted a naloxone-reversible inhibition of SP release from the ganglia. The present
findings suggest that there exists enkephalinergic presynaptic inhibition of cholinergic
and SP-mediated transmissions in mammalian sympathetic ganglia.

KEY WORDS  substance P / enkephalin / neurotransmitter / peptidergic synapse / sympathetic
ganglion

The discovery of a variety of peptides in both
central and peripheral nervous systems has
stimulated widespread interest in physiological
functions of the neuropeptides (for review see
reference 6). The idea of peptide neurotransmitters was first proposed for the undecapeptide,
substance P (SP), in the spinal cord where SP is a
leading candidate of transmitter released from
certain primary afferent terminals (14, 18). To substantiate further the transmitter roles of
the neuropeptides, it is essential to demonstrate
electrophysiologically the peptidergic synaptic
potentials or synaptic processes. Although this
attempt was confronted with considerable
difficulties at central synapses mainly because of
the structural complexity, rather good evidence
is now accumulating for peptidergic excitatory
postsynaptic potentials (e.p.s.p.s) at peripheral
synapses (7, 8, 13).

Several peptides including enkephalin, SP,
and vasoactive intestinal polypeptide (VIP) were
shown by immunohistochemical techniques to
occur in nerve terminals in mammalian sympathetic ganglia (5, 6, 15, 19). Our previous study on mammalian prevertebral sympathetic ganglia suggested that SP is a transmitter of noncholinergic slow e.p.s.p. generated in ganglion cells by activation of certain primary afferent fibers passing through the ganglia (13, see also reference 17). Furthermore, it was suggested that enkephalin serves as presynaptic inhibitory transmitter that reduces the release of acetylcholine (ACh) and SP in the ganglia (12, 13). In the present study we have further studied the possible transmitter roles of SP and enkephalin in guinea pig mesenteric ganglia. Our results suggest that the classical cholinergic transmission in the mesenteric ganglia is modulated by peptidergic transmitters, SP and enkephalin.

MATERIALS AND METHODS

Electrophysiological Experiments

Potentials were recorded intracellularly from neurons in the inferior mesenteric ganglia isolated from male guinea pigs (200—400 g). The animals were stunned and bled. The ganglia with associated nerves were isolated and incubated in a dish filled with physiological solution containing collagenase (0.01—0.02%), which facilitated later microelectrode impalement. The preparation was pinned out in a recording chamber perfused with modified Tyrode solution of the following composition (mM): NaCl 138.6, KCl 3.4, CaCl₂ 2.5, MgCl₂ 1.2, NaHCO₃ 21, NaH₂PO₄ 0.6, glucose 10; the solution was bubbled continuously with 95% O₂, 5% CO₂ gas mixture and kept at a temperature of 35—37°C. In some experiments, to block synaptic transmission in the ganglia, the preparations were perfused with a solution containing low-Ca and high-Mg. The preganglionic nerves, i.e. lumbar splanchnic and intermesenteric nerves were taken up into one or two suction electrodes for stimulation. When the effects of conditioning stimulation on cholinergic fast e.p.s.p. were examined, a small bundle of lumbar splanchnic nerve was taken up into a stimulating suction electrode to evoke test fast e.p.s.p.s, and conditioning stimuli were given to the remaining lumbar splanchnic and/or intermesenteric nerves via separate one or two suction electrodes. The intensity and duration of stimuli were 0.5—10V and 0.2—0.5 msec, respectively. Intracellular recordings from ganglion cells were made with microelectrodes filled with 2M potassium acetate and having resistances of 40—80 MΩ. Signals were led to a preamplifier and displayed on an oscilloscope and a pen recorder. A conventional bridge circuit incorporated in the preamplifier was used to pass current through the recording microelectrode. In some experiments, a signal averager was used for measuring mean sizes of responses. Peptides and drugs were dissolved in Tyrode solutions and applied by perfusion. For iontophoretic application of ACh, microelectrodes were filled with 1M ACh solution. The resistance of the ACh-containing electrodes was in the range of 80—150 MΩ. Results were obtained from neurons giving action potentials of more than 70 mV in response to depolarizing current pulses injected through the recording electrode.

RESULTS


There is neurochemical as well as morphological evidence that SP-containing terminals in the inferior mesenteric ganglion are derived from
Fig. 1 Synaptic potentials recorded from a neuron in the inferior mesenteric ganglia which was isolated together with preganglionic nerves connected to ventral and dorsal roots (for details see text). A: Cholinergic fast e.p.s.p.s and noncholinergic slow e.p.s.p. following repetitive stimulation (20 Hz for 10 sec) given either to the ventral root (VR) or dorsal root (DR) of L3 spinal segment. B and C: Fast sweep traces recorded during the stimulation of the ventral and dorsal roots, respectively. Note that the cholinergic fast e.p.s.p.s were produced only in response to ventral root stimulation (B).

certain primary afferent neurons with their cell bodies in the dorsal root ganglia (3, 13). If these SP-containing neurons are responsible for generating the noncholinergic slow e.p.s.p., one would expect that the slow e.p.s.p. is evoked in postganglionic cells following activation of the dorsal root. This was tested in the experiment illustrated in Fig. 1. We dissected the inferior mesenteric ganglia together with the preganglionic nerve trunks connected with the ventral and dorsal roots of spinal nerves. When recorded intracellularly from neurons in the inferior me- senteric ganglia, repetitive stimulation of the dorsal root (L3) produced the slow e.p.s.p. (Fig. 1A) that were not affected by cholinergic antagonists (hexamethonium and atropine). Dorsal root stimulation, however, did not evoke the cholinergic fast e.p.s.p.s (Fig. 1C). In contrast, stimulation of the ventral root generated only cholinergic fast e.p.s.p.s (Fig. 1, A and B). The results are consistent with previous neuro-chemical and morphological evidence (3, 13) and suggest that SP-containing primary afferent neurons with the cell bodies in the spinal ganglia send their axon collaterals to form synapses on the principal cells in the inferior mesenteric ganglion and to generate the noncholinergic slow e.p.s.p.

**Effects of Capsaicin**

Capsaicin, the pungent factor in red pepper, was previously shown to act on certain primary afferent neurons (9, 16) and to cause a release and depletion of SP in the spinal cord (4, 11, 20). Since the mesenteric ganglia of the guinea pig contain a large amount of SP (13), one may expect that capsaicin causes a release of SP from the mesenteric ganglia. This was indeed the case as shown in Table 1. When the ganglia were incubated with the solution containing capsaicin, the release of immunoreactive SP from the ganglia was markedly increased. The amount of SP released in capsaicin solution (0.9—1.5 μM) was about 5 times larger than the spontaneous release. The effect of capsaicin was observed in normal Tyrode solution containing 3 mM Ca but not in the medium containing low-Ca and high-Mg. According to our preliminary radioimmunoassays, the mesenteric ganglia of the guinea pig contain a large amount of VIP, the content in the inferior mesenteric ganglion being 1.7 pmol/mg protein which is rather higher than that of SP (1.0 pmol/mg protein). The release of VIP from the mesenteric ganglia, however, was not changed by capsaicin (Table 1). The results suggest that capsaicin acts selectively on SP-containing preganglionic fibers and evokes SP release therefrom in a Ca-

<table>
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<tr>
<th>Control</th>
<th>Capsaicin (0.9—1.5 μM)</th>
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<tr>
<td>A B</td>
<td>A B</td>
</tr>
<tr>
<td>SP (fmol)</td>
<td>32±12(5) 16±1(4)</td>
</tr>
<tr>
<td>VIP (fmol)</td>
<td>45±13(4)</td>
</tr>
</tbody>
</table>

Each value represents mean±SEM and number of determinations in parentheses.
Since SP was shown to exert a potent depolarizing action on ganglion cells (2, 12, 13), capsaicin is expected to produce a depolarization of postganglionic cells via SP released from nerve terminals. As shown in Fig. 2, application of capsaicin (0.3–3 μM) evoked a depolarization of neurons in the inferior mesenteric ganglia. The amplitude of capsaicin-induced depolarization was 14.2 ± 1.7 mV (mean ± SEM of the measurements in 11 cells). The action of capsaicin persisted after the ganglia were treated with cholinergic antagonists, i.e. hexamethonium (200 μM) and atropine (2 μM) (Fig. 2B). As expected from the results of our release experiments, capsaicin exerted a depolarizing action in normal Tyrode solution but not in the low-Ca (0.1 mM) and high-Mg (20 mM) medium where synaptic transmission in the ganglia was completely blocked.

If SP is a transmitter released from primary afferent terminals to mediate the noncholinergic slow e.p.s.p. in the mesenteric ganglia (13), it is reasonably expected that the slow e.p.s.p. would be abolished after a prolonged treatment of the ganglia with capsaicin which probably depletes SP from nerve terminals. This possibility was tested in the experiment illustrated in Fig. 2. Following application of capsaicin (0.2–2 μM), ganglion cells were initially depolarized, and in some cells repetitive firing of action potentials was induced by the depolarization (Fig. 2B). This depolarization subsided gradually during the period of 15–30 min in capsaicin-containing solution. The noncholinergic slow e.p.s.p. evoked by stimulation of the lumbar splanchnic and intermesenteric nerves were markedly depressed during the treatment with capsaicin (Fig. 2B), and this depression persisted after washing out the drug (Figs. 2C and 3A). The amplitude of the slow e.p.s.p. recorded during the period of 20–60 min after the end of capsaicin treatment for 10–30 min was 18.5 ± 5.1% of the control size (mean ± SEM of the measurements in 8 cells).
Fig. 3 Synaptic potentials and SP-induced depolarizing responses recorded from a neuron in the inferior mesenteric ganglion before and after a prolonged treatment with capsaicin. A: Cholinergic fast e.p.s.p.s and noncholinergic slow e.p.s.p.s evoked by repetitive stimulation (20 Hz for 3 sec) of the lumbar splanchnic nerves. B: Cholinergic fast e.p.s.p.s evoked by single stimuli (1 Hz) to the same nerves. Each record consists of 8–9 superimposed traces. C: Depolarizations produced by SP (0.1 μM). SP-containing solutions were perfused during the periods indicated by horizontal bars. 1, responses in control solution. 2, responses obtained 20–50 min after the end of capsaicin treatment (0.6 μM for 25 min).

At this stage, the depolarizing response of the postganglionic cells to SP was as large as that recorded before capsaicin treatment (Fig. 3C). The cholinergic fast e.p.s.p.s evoked by single stimuli to the same nerves, in contrast, were not affected by capsaicin treatment (Fig. 3B). The most likely explanation for the depression of the noncholinergic slow e.p.s.p. following capsaicin treatment is that capsaicin acts selectively on SP-containing primary afferent fibers and evokes a release of SP and a consequent depletion of readily releasable SP from the axon terminals which generate the noncholinergic slow e.p.s.p.

Neurally Evoked Presynaptic Inhibition of Cholinergic Fast e.p.s.p.

Previous studies on the prevertebral sympathetic ganglia demonstrated that immunoreactive enkephalin exists in the nerve terminals of preganglionic fibers (15, 19) and that the opioid peptides inhibit cholinergic fast e.p.s.p. by presynaptic mechanism (12). These results suggest the existence of enkephalinergic presynaptic inhibition of cholinergic transmission in the ganglia. This possibility was tested in the following experiments. First, we explored the effects of conditioning preganglionic stimulation on the cholinergic fast e.p.s.p. in the inferior mesenteric ganglion. As shown in Fig. 4, the conditioning stimulation at 50 Hz for 8 sec produced a prolonged inhibition of the fast e.p.s.p. (see also Fig. 5A). The maximal degree and half-decay time of the inhibition were 70.5% and 56.4 sec, respectively (mean of the results from 12 cells). This inhibition can not be attributed to a homosynaptic depression of the fast e.p.s.p.s because the test and conditioning stimuli were given to different preganglionic nerves (see Methods). As shown in Fig. 4, the inhibitory effect of conditioning preganglionic stimulation on the e.p.s.p.s was abolished by naloxone (3 μM), suggesting that enkephalin is involved in this neurally evoked inhibition.
Fig. 5 Effects of conditioning stimulation on cholinergic fast e.p.s.p. and ACh-induced depolarization recorded from a neuron in the inferior mesenteric ganglion. A: Test e.p.s.p.s evoked by single stimulation (1 Hz) of a bundle of the lumbar splanchnic nerves. Each record consists of 8-9 superimposed traces. B: Averaged ACh-induced depolarizations. ACh was applied iontophoretically with current pulses (1.5 nA, 10 msec) at 0.5 Hz, and 8 responses were averaged every 20 sec. 1, responses recorded during the control period. 2, responses recorded during the period of 10-30 sec after the end of conditioning stimulation (50 Hz for 8 sec) given to the lumbar splanchnic and intermesenteric nerves.

Table 2 Effects of Conditioning Stimulation (50 Hz for 8 sec) of Preganglionic Nerves on the Quantal Size and Quantal Content of Cholinergic Fast e.p.s.p.s in Control Medium (A) and Naloxone (3 μM)-Containing Medium (B)

<table>
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<th>Cell</th>
<th>Quantal size (mV)</th>
<th>Quantal content</th>
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<tr>
<td></td>
<td>Control</td>
<td>Conditioned</td>
</tr>
<tr>
<td>1A</td>
<td>0.61</td>
<td>0.69</td>
</tr>
<tr>
<td>B</td>
<td>0.64</td>
<td>0.61</td>
</tr>
<tr>
<td>2A</td>
<td>0.46</td>
<td>0.59</td>
</tr>
<tr>
<td>B</td>
<td>0.54</td>
<td>0.54</td>
</tr>
<tr>
<td>3A</td>
<td>0.82</td>
<td>1.04</td>
</tr>
<tr>
<td>B</td>
<td>0.74</td>
<td>0.73</td>
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Mean ± SEM

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<tr>
<th></th>
<th>Control</th>
<th>Conditioned</th>
<th>Ratio</th>
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<td>1.23±0.05</td>
<td>0.54±0.05</td>
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Test e.p.s.p.s were evoked at 1 Hz, and each value was determined from about 100 test responses during the control period and the conditioned period 10-120 sec after the end of conditioning stimulation (for further details see text and Methods).

To determine the nature of the inhibition, we examined the effect of conditioning preganglionic stimulation on the ACh-sensitivity of postganglionic cells. The responses to ACh were evoked by iontophoretic application of the drug. ACh-induced depolarizations were not reduced but rather slightly increased during the period of 10-60 sec after the end of the conditioning stimulation (Fig. 5B), whereas the mean sizes of the fast e.p.s.p.s recorded from the same cell were reduced to 15-60% of the control during the same period (Fig. 5A). The results suggest that the mechanism of this neurally evoked inhibition is presynaptic. This was further supported by
quantal analysis of cholinergic fast e.p.s.p. The results are summarized in Table 2. We analysed amplitude histograms of test e.p.s.p.s before and after conditioning preganglionic stimulation. The quantal content of the e.p.s.p.s was estimated from the proportion of failures to total test stimuli (70-200 trials). During the period of 1–2 min after the termination of conditioning stimulation, the number of failures increased to 3–6 times of that observed during the control periods (see Fig. 5A), and quantum content of e.p.s.p.s decreased to 54 ± 5% of the control value (Table 2). In contrast, the quantal size of e.p.s.p.s was slightly increased after the conditioning stimulation. The increases of quantal size (Table 2) and the amplitudes of the ACh-induced response (Fig. 5) can be explained by the increase of membrane resistance due to the noncholinergic slow e.p.s.p. produced by preganglionic stimulation (cf. reference 13). The input resistance of the postganglionic cells were in fact increased slightly after conditioning stimulation (not illustrated). The effect of conditioning stimulation to reduce the quantum content was abolished by naloxone (Table 2). The results are consistent with the hypothesis that enkephalin liberated from nerve terminals by preganglionic repetitive stimulation produces presynaptic inhibition of cholinergic transmission in the mesenteric ganglia.

**Effect of Opioid Peptide on the Release of SP from the Ganglia**

Our previous results suggested that enkephalin depresses the noncholinergic slow e.p.s.p. by presynaptic mechanism (13). To test this hypothesis we examined the effect of [D-Ala²]-Met-enkephalinamide (DAEA), a potent analog of Met-enkephalin, on the release of SP from the mesenteric ganglia. In each series of experiments, we measured the release of SP evoked by 50 mM K, first in the medium containing DAEA (3 μM), and then in the medium containing DAEA (3 μM) plus naloxone (2–5 μM). As shown in Table 3, high K-evoked release of SP was significantly increased by adding naloxone to DAEA-containing medium. Naloxone alone was without effect on the spontaneous release of SP. These results suggest that enkephalin acts on the opiate receptors of preganglionic axon terminals containing SP and inhibits the noncholinergic slow e.p.s.p. by reducing the release of SP therefrom.

**DISCUSSION**

The results of the present study strongly suggest the existence of three distinct peptidergic synapses in guinea pig mesenteric ganglia: 1) SP synapses on the principal adrenergic neurons; 2) enkephalinergic synapses on the cholinergic preganglionic nerve terminals; 3) enkephalinergic synapses on the SP-containing nerve terminals. Fig. 6 is a schematic representation of these synaptic connections in relation to other neuronal pathways. Our findings, in agreement with

<table>
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<th>SP released (fmol)</th>
<th>DAEA (3 μM)</th>
<th>DAEA (3 μM) and naloxone (2–5 μM)</th>
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<tr>
<td>3 mM K</td>
<td>15 ± 7</td>
<td>18 ± 9</td>
</tr>
<tr>
<td>50 mM K</td>
<td>34 ± 6</td>
<td>73 ± 17</td>
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Each value represents mean ± SEM of four separate experiments.
recent studies by Jan, Jan and Kuffler (7, 8), support the notion that the cholinergic transmission conveying main signals from preganglionic to postganglionic neurons in sympathetic ganglia is under the influence of peptidergic excitatory and inhibitory transmitters.

**Excitatory SP Synapses on Adrenergic Principal Cells**

It was previously suggested that SP-containing fibers generating the noncholinergic slow e.p.s.p. in the mesenteric ganglia are the peripheral branches of primary afferent neurons with their cell bodies in the spinal ganglia (13). This was further supported by the present finding that repetitive stimulation of the dorsal root produces noncholinergic slow e.p.s.p. in postganglionic cells (Fig. 1). There is also morphological evidence that certain afferent fibers traverse the inferior mesenteric ganglion on their way from visceral receptors to their cell bodies in the dorsal root ganglia (3). It is therefore probable that axon collaterals of these fibers make SP synapses with peripheral neurons in the mesenteric ganglia as well as with central neurons in the spinal cord (Fig. 6). Thus, the excitability of adrenergic principal cells innervating visceral organs seems to be modulated by a peripheral reflex via visceral afferent fibers containing SP. Similar axon reflex pathways formed by somatic afferent neurons have long been known to produce vasodilatation which is in all likelihood mediated by SP (1, 6; cf. Fig. 6).

The present study indicates that the action of capsaicin is specific to SP-containing primary afferent neurons because the drug evoked a release of SP but not of VIP from the mesenteric ganglia and depressed the noncholinergic slow e.p.s.p. but not cholinergic fast e.p.s.p. These results suggest that capsaicin abolishes the noncholinergic slow e.p.s.p. by a depletion of SP from the primary afferent terminals in the ganglia. Thus it is likely that capsaicin serves as a useful tool to elucidate SP-mediated synapses formed by certain primary afferent neurons in central and peripheral nervous systems. Recently it has been shown that capsaicin affects the nociceptive transmission and produces analgesia without alteration in motor functions (21). It is therefore probable that SP is released in the spinal cord as a transmitter from primary afferent neurons conveying pain sensation, although it remains to be revealed whether SP-mediated synaptic potentials in spinal neurons are fast, slow or both.

**Inhibitory Enkephalinergic Synapses on ACh- and SP-Containing Nerve Terminals**

It has been proposed by many authors that enkephalin serves as inhibitory transmitter at central and peripheral synapses (10, 12). To substantiate this hypothesis, it is of crucial importance to reveal inhibitory synaptic processes that are induced by activation of enkephalin-containing nerve fibers and are reversed by opiate antagonists. In this study we showed that preganglionic stimulation produces a naloxone-reversible inhibition of cholinergic fast e.p.s.p.s in the mesenteric ganglia and that the mechanism of this neurally evoked inhibition is presynaptic. Previously enkephalin was shown in sympathetic ganglia to inhibit cholinergic e.p.s.p. by presynaptic mechanism (12). Furthermore, the enkephalin-containing terminals in the ganglia are thought to be derived from preganglionic neurons (15, 19). Therefore the present findings suggest that enkephalin released from preganglionic nerve terminals plays a role of transmitter mediating presynaptic inhibition of cholinergic fast e.p.s.p. in the ganglia.

Since an opioid peptide, DA EA, inhibits both the noncholinergic slow e.p.s.p. (13) and the release of SP from the ganglia (Table 3), it is also suggested that there exists an enkephalinergic pathway that exerts presynaptic inhibition of SP-mediated slow e.p.s.p. in the mesenteric ganglia. This is consistent with the results of a previous study by Jessell and Iversen showing that enkephalin reduces the release of SP evoked by high K from spinal trigeminal nucleus (10). It is therefore likely that enkephalinergic fibers form synapses on both peripheral and central branches of primary afferent neurons and inhibit synaptic transmission by inhibiting the release of SP from their terminals (Fig. 6).

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REFERENCES

mesenteric ganglion of the guinea pig. Identification of the cells of origin in dorsal root ganglia. *Brain Res.* 126, 149–153

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