DUAL ACTIONS OF SUBSTANCE P ON THE MOTONEURONS OF THE NEWBORN RAT SPINAL CORD *IN VITRO*

KAYOKO ONODERA and AKIRA TAKEUCHI

Department of Physiology, School of Medicine, Juntendo University, Hongo, Bunkyo-ku, Tokyo 113, Japan

ABSTRACT

The actions of substance P (SP) on the motoneurons were investigated using the isolated newborn rat spinal cord. Potential changes were recorded intracellularly from the motoneurons. The bath application of SP produced a depolarization of the motoneuron membrane, accompanied by an increase in the membrane potential fluctuation. When the Ca concentration in the perfusing fluid was decreased to 1/10, the synaptic transmission was blocked, and the depolarization produced by SP was suppressed, together with a decrease in the membrane potential fluctuation. In a solution containing tetrodotoxin (TTX), the synaptic potentials were abolished and the depolarization induced by SP was decreased. Membrane fluctuations during rest and the action of SP disappeared in the TTX solution. In a solution containing DL-2-amino-5-phosphonovaleric acid (APV), the SP-induced depolarization was suppressed, but a potential fluctuation remained. The results suggest that the bath application of SP induced the release of the endogenous transmitter, possibly glutamate, and produced a depolarization of the motoneuron membrane, partly through the activation of *N*-methyl-D-aspartate (NMDA) receptor. At higher concentrations, in addition to the presynaptic action, SP acted on the postsynaptic membrane and depolarized the motoneuron.

Substance P (SP) produces a depolarization of spinal motoneurons, and experimental evidence suggests that this neuropeptide may function as a neurotransmitter in synapses in the spinal cord (for reviews, see 18, 23). However, considerable controversy exists concerning the mechanism underlying this depolarization. The bath application of SP to the rat or frog spinal cord causes a depolarization associated with an increase in the membrane conductance of the motoneuron (16, 17, 22). The iontophoretic application of SP to cat motoneurons produces either no change (33) or a decrease (13) in the membrane conductance. The time course of SP action is generally slow compared to that of the excitatory synaptic potential or to the glutamate action (4, 7, 14, 25, 33; however, see 24).

In the present study, attempts were made to

characterize the action of SP on the spinal motoneurons under various conditions, *i.e.* in media containing low Ca, tetrodotoxin (TTX) and antagonists for amino acid receptors. The results suggest that the depolarization produced by the bath application of SP at lower concentrations (less than about 200 nM) is largely attributable to the evoked release of the transmitter which depolarizes the motoneuron through the activation of amino acid receptors. At higher concentrations, the postsynaptic action of SP was also involved.

MATERIALS AND METHODS

Wistar rats aged from 6 to 9 days were killed under ether anesthesia, and the spinal cord with attached dorsal and ventral roots (L4-L6) were dissected in an oxygenated artificial solu-

Fig. 1 Effects of SP on the motoneuron membrane. A: The SP-induced depolarizations recorded intracellularly from a spinal motoneuron of a 7-day-old rat. SP was applied into the perfusion fluid during the period indicated by the bar. The numbers under the bar indicate the concentrations of SP in nM. The resting potential was 58 mV. B: Dose-depolarization relationship. The potential fluctuations are indicated in the inset. Upper trace, before the SP application. Lower trace, at the peak of depolarization produced by 200 nM SP.

tion at 20°C (21). After removing the pia mater as much as possible, the spinal cord was sagittally hemisected and fixed in a small bath (200 μ l in volume) with the hemisected side downwards. A small amount of α -cyanoacrylate (aron alpha, Toa Gosei Kagaku) was used to secure the fixation of the preparation. The preparation was superfused with the oxygenated solution at the rate of 3 ml/min at 29°C. The dorsal and ventral roots were placed on silver electrodes for stimulation.

The motoneurons were impaled with a conventional intracellular microelectrode filled with 1 M K-acetate (80–150 M Ω). Antidromic stimulation was applied to the ventral roots to identify the motoneuron. When the motoneuron was successfully impaled, the membrane potential was stable for several hours, and the resting potential was measured at the end of the experiments. Potential changes were

recorded on a pen recorder and stored in an FM tape recorder for later analysis. The composition of the solution used was (mM); NaCl, 130; KCl, 4.5; CaCl₂, 2.0; glucose, 11.0; and NaHCO₃, 10.0. The pH was 7.0 after saturation with 95% O₂ : 5% CO₂. Drugs used were substance P (SP, Sigma), DL-2-amino-5-phosphonovaleric acid (APV, Sigma and Tocris), D-2-amino-4-phosphonobutyric acid (APB, Tocris) and tetrodotoxin (TTX, Sankyo). For the application of SP, various concentrations of SP were injected into the circulating fluid for 40 sec.

RESULTS

Effects of Substance P

The bath application of SP exerted a powerful depolarizing action on the motoneurons. Fig. 1 shows an example recorded intracellularly

Fig. 2 Effects of the low Ca solution on the action of SP. A: The SP-induced depolarization recorded intracellularly from a motoneuron. Depolarization by 50 nM SP (a), and by 2 μ M SP (b). The resting potential was 66 mV. In the low Ca solution, the Ca concentration was reduced to 0.2 mM. B: Dose-depolarization relationship, in the control solution ($\bullet$), and in the low Ca (0.2 mM Ca-1.8 mM Mg) solution ($\circ$). Mean of 23 motoneurons. Bars indicate SE. C: Membrane potential fluctuation before (a), and during the application of 2 μ M SP (b). In the low Ca solution, both the resting and SP-induced potential fluctuations were suppressed.

from a motoneuron. A depolarization of about 4 mV was produced by the bath application of 10 nM SP, and this increased up to about 18.5 mV with 2 μ M SP. The degree of depolarization varied from preparation to preparation, and larger depolarization was usually recorded from motoneurons that showed intense spontaneous synaptic activities. The SP-induced depolarization was always accompanied by an increase in the potential fluctuations, which may have been caused by synaptic potentials impinging on the motoneuron (17). An example is shown in the inset in Fig. 1B. The upper trace shows the membrane potential fluctuation just before the application of SP, and the lower trace was recorded at the peak of depolarization produced by

200 nM SP (Fig. 1A, b). The potential fluctuation increased from the resting value of 0.45 mV rms to 0.97 mV rms during the SP depolarization. Since motoneurons showed burst-like spontaneous synaptic activities, the potential fluctuation was recorded at intervals between the spontaneous synaptic activities. The increase in the potential fluctuation suggests that SP induced the release of the transmitter(s) from presynaptic elements.

The rise and fall of depolarization produced by lower concentrations of SP was relatively fast. As the concentration of SP was increased, usually over the micromolar range, the declining phase was remarkably prolonged, although the rising phase was not changed or even became steeper. In Fig. 1A, the duration of

depolarization was about 1.5 min with the application of 50 nM SP, and it increased to more than 4 min by 2 μ M SP. During the action of SP at higher concentrations, the spontaneous synaptic activities were sometimes suppressed. A possible reason for this depression may be the release of the inhibitory transmitter (15).

Effects of the Low Ca Solution on SP Actions

The increase in the potential fluctuation produced by SP may be due to transmitter release from the presynaptic elements. When the concentration of Ca in the bath solution was reduced to 0.2 mM, substituted with Mg or Mn, the spontaneous synaptic activities were suppressed (Fig. 2C), and the synaptic potentials evoked by dorsal root stimulation were blocked in about 10 min. Substitution with Mn was more effective in the suppression of potential changes. As shown in Fig. 2A, the bath application of 50 nM SP produced a depolarization of about 4 mV in the control solution, and it was decreased to about 1 mV in the low Ca (0.2 mM-Ca, 1.8 mM-Mg) solution. In the low Ca solution, depolarization produced by SP lower than 200 nM was suppressed to about 30% of the control, while depolarization produced by 2 μ M SP in the low Ca solution was almost the same or only slightly less than in the control solution (Fig. 2B). In the low Ca solution the SP-induced potential fluctuation was depressed, but was still observed (Fig. 2C). Therefore, transmitter release may not have been completely blocked in the low Ca solution used in the present experiment. The rising phase of the SP-induced depolarization was steeper in the control than in the low Ca solution, suggesting a contribution of transmitter release (see also 24).

It has been reported that the SP-induced depolarization was larger in the low Ca solution than that in the control solution (24). The reason for the different results obtained in the present experiments is not clear, but it may be attributed to the differences in the experimental methods. In the above-mentioned study, the depolarization of motoneurons was recorded extracellularly from the ventral root, and the ionic composition of the solution used was slightly different from that in our present experiment. Lowering the Ca concentration reduced the spontaneous synaptic activity, and the membrane resistance of the motoneu-

rons may have been increased, resulting in an increase in the electrotonic spread of depolarization to the ventral root.

As will be discussed in later sections, the SP-induced depolarization may be partly due to the activation of the *N*-methyl-D-aspartate (NMDA) receptor by the released endogenous transmitter. When the Ca concentration was reduced, Mg or Mn was used to replace Ca. Since these divalent cations inhibit NMDA receptor (1, 19), the suppression of SP action in the low Ca solution might be partly postsynaptic.

Effects of TTX on SP Action

When the perfusion fluid was changed to that containing TTX (1×10^{-7} g/ml), background synaptic noise disappeared, and, in many cases, membrane potential was slightly hyperpolarized (7.3 ± 0.54 mV, $n=4$). As shown in Fig. 3A, application of 200 nM SP produced a depolarization of about 8.5 mV in the control solution, and it was abolished in the TTX-solution. Application of 2 μ M SP produced a small depolarization, the peak amplitude being about 2 mV compared to 12 mV in the control solution, and the onset of depolarization was remarkably delayed. The slow onset of SP depolarization was a consistent observation in the TTX-solution. No potential fluctuation was noted during the depolarization. As summarized in Fig. 3B, the action of SP was about ten times stronger in the control solution than in the TTX-solution (see also 24).

Effects of APV on SP Action

The above results suggest that the bath application of SP caused the release of the endogenous transmitter. If the released transmitter is glutamate or related amino acids, it is expected that the depolarization produced by SP may be suppressed by the antagonist against amino acid receptors. Recent pharmacological experiments suggest that there are three types of amino acid receptors in the spinal cord (31), and APV is the most potent and specific antagonist against the NMDA receptor (2).

When the spinal cord was perfused with APV, the background synaptic activities were reduced and the synaptic transmission was blocked in about 10 min, leaving the fast component of dorsal root-evoked synaptic poten-

Fig. 3 Effects of TTX on the SP action. A: The SP-induced depolarization recorded intracellularly from a motoneuron. SP was applied during the period indicated by the bar; the concentrations are indicated in nM. In 10^{-7} g/ml TTX solution, the SP-induced depolarization was depressed. The resting potential was 64 mV. B: The dose-depolarization relationship in control solution (●) and in 10^{-7} g/ml TTX solution (○). Mean of seven motoneurons. Bars indicate SE.

tials relatively unaffected (Fig. 4A, c) (6). The concentration of APV necessary to block the synaptic transmission varied from preparation to preparation, but it was within the range from 20 to 200 μ M. An example is shown in Fig. 4A. A depolarization of 12 mV was produced by bath application of 50 nM SP in the control solution, and it was almost completely suppressed in the APV-solution (200 μ M), leaving a small increase in the potential fluctuation. Bath application of 500 nM SP produced a depolarization of 11.5 mV in the same motoneuron and it was decreased to 3.5 mV in the APV-solution. Application of 500 nM SP in the APV-solution produced a large potential fluctuation (Fig. 4A, b). Since the fast component of dorsal root-evoked synaptic potential, which is probably monosynaptic, is

resistant to APV (6), the large potential fluctuation brought about by 500 nM SP might be due to transmitter release from the monosynaptic input. After washing the spinal cord with the control solution, the synaptic transmission and the SP-induced depolarization were almost completely restored in about 10 min (Fig. 4A). Similar but slightly less marked effects were obtained with 1 mM D-APB. This drug is an unspecific antagonist against amino acid receptors, and the monosynaptic and polysynaptic transmission were more or less equally inhibited, as shown in Fig. 4A, c (3). Perfusion with a solution containing 1 mM D-APB decreased the spontaneous synaptic activities, and depolarization produced by SP was reversibly depressed by the D-APB-solution (Fig. 4A, a and b).

Fig. 4 Effects of APV and D-APB on the SP-induced depolarization. A: Depolarizations produced by bath application of 50 nM SP (a) and 500 nM SP (b). (c), dorsal root-evoked synaptic potentials. All the records were obtained intracellularly from the same motoneuron. The resting potential was 64 mV. The concentration of APV was 200 μM and that of D-APB was 1 mM. B: The dose-depolarization relationship in the control (●) and 200 μM APV solution (○). Mean of six motoneurons. Bars indicate SE.

DISCUSSION

The bath application of SP exerted a powerful depolarizing action on motoneurons, and the depolarization was accompanied by an increase in the membrane potential fluctuation, which may have been caused by a barrage of synaptic potentials impinging on the motoneuron membrane. These observations suggest that the depolarization of motoneuron induced by SP at relatively lower concentrations (less than about 200 nM) may be largely attributable to the evoked release of endogenous transmitter. This possibility may be supported by the observation that the depolarization and the membrane potential fluctuation produced by SP at lower concentra-

tions were suppressed or abolished in the low Ca solution and in the TTX-solution.

The present results indicate that the SP-induced depolarization was suppressed by APV and D-APB. This suggests that the transmitter released by SP may be glutamate and/or aspartate. Since APV is a specific antagonist against the NMDA receptor and it depresses mainly polysynaptic pathways (6; see also Fig. 4A, c), SP may act on the interneurons and cause the release of transmitter that activates the NMDA receptor. It has also been observed that in the APV-solution application of SP produced a large potential fluctuation that may be due to activation of the fast component of the synaptic potentials (Fig. 4A, b). Thus, SP may also activate the mono-

synaptic pathways, and causes the release of a transmitter that acts on the non-NMDA receptor.

It will be shown in the following paper that the bath application of SP causes a significant increase in the release of glutamate and that the evoked release of glutamate is almost completely inhibited in the TTX-solution, whereas the aspartate release is small and not influenced by TTX (12). These observations together with the present results suggest that SP acts presynaptically and causes the release of glutamate that depolarizes the motoneuron. Although relatively little is known about the presynaptic mechanism (15), the presynaptic action of SP on motoneurons was about ten times more potent than its postsynaptic action; this type of action may have physiological importance. Presynaptic action of SP has been suggested in several synapses, e.g., autonomic ganglia (5, 8, 29, 32), the spinal cord (11, 16) and the neuromuscular junction (27).

In TTX and low Ca solutions, application of SP at higher concentrations produced a depolarization which was slow in its time course. If transmitter release induced by SP was suppressed or abolished in the low Ca or TTX-solution, a slow depolarization might be a characteristic action of SP on the motoneuron membrane. A slow action of neuropeptide has been observed in several neurons including sympathetic ganglia (9, 10, 30), cultured neurons (20, 26) and rat motoneuron (28). These actions are accompanied by a decrease in the membrane conductance.

It has been shown that bath application of SP to the spinal cord of newborn rats (22) and frogs (16, 17) causes an increase in the membrane conductance of the motoneurons. On the other hand, SP applied iontophoretically to cat motoneurons produces either no change (33) or a decrease in the membrane conductance (13). These conflicting results may be due to the difference in animal species and in the experimental techniques, but at the same time may be partly attributable to the dual actions of SP. In the bath application, the presynaptic action of SP may be pronounced, whereas the iontophoretically applied SP may act mainly on the motoneuron membrane *per se*.

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