CHOLINERGIC TRANSMISSION IN THE RECURRENT FACILITATORY PATHWAY OF THE SPINAL MOTONEURON OF THE TOAD

Shiushi Matsuura*

Department of Physiology, Faculty of Medicine, Kyoto University, Kyoto, Japan

Summary 1. The VR-EPSP of the spinal motoneuron of the toad was investigated by intracellular recording. Concerning the size, time course, and effect of membrane potential displacements, no essential difference was found between the VR-EPSP in the toad and that in the frog. A remarkable facilitatory effect of the DR-EPSP on the VR-EPSP was observed.
2. The effects of various pharmacological agents on the VR-EPSP were tested by topical application using a double- or triple-barrel microelectrode, or by application to the perfusion fluid through the vascular system.
3. The VR-EPSP was increased in size and the decaying phase was prolonged by topical application of physostigmine. Acetylcholine was effective in increasing the size of the VR-EPSP when applied topically or through the vascular system.
4. A transient depolarization, which may be called acetylcholine potential, was recorded intracellularly when acetylcholine was applied extracellularly by a brief outward current from the microelectrode filled with acetylcholine.
5. Curare was found to have no effect on the VR-EPSP, while nicotine and atropine were effective in depressing the VR-EPSP.
6. Cholinergic transmission at the synapse between motor axon collaterals and motoneurons in the toad was demonstrated. It was, furthermore, pointed out that the present investigation did not exclude the possibility of electrical interaction between motoneurons.

Since Eccles et al. (1954) showed that impulses in collaterals from mammalian spinal motor axons exert inhibitory action on the motoneuron via the Renshaw
cell, a number of reports have appeared concerning the functional significance of the collaterals of mammalian spinal motor axons (Granit et al., 1957; Kuno, 1959; Wilson, 1959; Brooks and Wilson, 1959; Granit et al., 1960; Wilson et al., 1960; Eccles et al., 1961; Wilson and Burgess, 1962a, b).

In the frog spinal cord, on the other hand, Kubota and Brookhart (1963) and Katz and Miledi (1963) have found graded postsynaptic depolarization (VR-EPSP) in the motoneuron after ventral root stimulation. After systematic investigation of this VR-EPSP, Kubota and Brookhart (1963) have concluded that the VR-EPSP is produced by recurrent collaterals which presumably terminate monosynaptically on the nonsomatic portion of the motoneuron, i.e., on the distal portion of motoneuron dendrites. Furthermore, through their preliminary trials with pharmacological agents, they have suggested a cholinergic nature of the termination of recurrent collaterals on motoneurons.

However, Grinnell (1966) has recently shown that the so-called VR-EPSP may originate from interaction between motoneurons, possibly in such a way that overlapping dendrites of adjacent motoneurons interact with each other electrically. In his pharmacological investigation, the possibility of chemical transmission in the recurrent facilitatory pathway has been excluded.

The results of the present investigation seem to provide evidence to support the idea that the VR-EPSP originates from the release of acetylcholine from the recurrent collateral of the motor axon. It will be shown that the size of the VR-EPSP of the spinal motoneuron of the toad is remarkably altered by drugs that affect cholinergic transmission. A preliminary report of the present investigation has already appeared (Araki and Matsuura, 1965).

METHODS

Toads were used in all experiments. They were anesthetized with Nembutal (25 mg/kg). The spinal cord was exposed and the animal was firmly suspended in a rigid metal frame. In some cases, the spinal cord and the brain were perfused through the vascular system with Ringer's solution, which was oxygenated by continuous bubbling of 95% O₂ and 5% CO₂ and was of the following composition (mm): NaCl, 111; KCl, 2.0; CaCl₂, 1.8; NaHCO₃, 2.0; and glucose, 1 g/liter. In some later experiments, excised spinal cords were used. When necessary, the excised spinal cord was perfused with Ringer's solution through the anterior spinal artery.

The ninth and tenth ventral and dorsal roots were cut and mounted on a respective pair of silver-silver chloride electrodes for stimulation. Glass micro-electrodes filled with 3M KCl or 2 M potassium citrate were used for intracellular potential recording from motoneurons. The DC resistance of the micro-electrodes usually ranged from 10 to 15 MΩ. A bridge circuit was used for displacement of the membrane potential. The cathode-follower output was connected to the
two channels of a 502 Tektronix oscilloscope.

In order to test the effects of various pharmacological agents, the following methods were employed. (A) A double-barrel microelectrode was used. One pipette was for intracellular recording. The other one, filled with acetylcholine chloride or physostigmine salicylate, was located just outside the motoneuron and acetylcholine or physostigmine was applied to the motoneuron electrophoretically. The tip distance between the two pipettes was usually 10–20 μ. In some instances, a three-barrel microelectrode was used. The third pipette was filled with 0.7% NaCl solution and Na+ ions were applied as the control. (B) A double-barrel microelectrode was used as in A, but drugs were applied by pressure. (C) A single microelectrode was used and drugs were applied to the perfusion fluid. Drugs used in the present experiment were acetylcholine chloride, physostigmine salicylate, d-tubocurarine chloride, atropine sulfate, and nicotine tartrate.

The room temperature was in the range from 14 to 19°C.

RESULTS

1. VR-EPSP in the toad

Graded depolarization, which is characteristic of excitatory postsynaptic potential, resulting from ventral stimulation (VR-EPSP) of the motoneuron of the toad was observed in about 25% of the 172 motoneurons tested and the maximum amplitude was found to be 2–4 mV. The configuration of the VR-EPSP of the toad was not essentially different from that of the frog reported by Kubota and Brookhart (1963), Katz and Millesi (1963), and Grinnell (1966).

Examples of the VR-EPSP in the toad motoneuron are illustrated in Fig. 1. A small negative deflection, which was attributable to the field potential produced by antidromic invasion in adjacent motoneurons, was followed by a delayed depolarization, a VR-EPSP. When the VR-EPSP was not produced, only negativity was observed and the potential decayed to the original baseline quickly. The stimulus strength was progressively increased from A to D. As the antidromic volley was increased, there was an increase of the VR-EPSP, and a spike potential was evoked by the VR-EPSP in D. The VR-EPSP in another motoneuron shown in E was preceded by a relatively large negative deflection. When the stimulus strength was increased, a spike potential was produced by the VR-EPSP (F), while further increase in stimulus strength evoked an antidromic spike (G), which has a shorter latency than the former. It was found that the size of the spike potential evoked by the VR-EPSP was usually 1.0–11.6% larger (mean: 5.2% in 12 cells) than that evoked antidromically in the same motoneuron.

The VR-EPSP was not changed by electrically induced alteration of the resting potential and the spike potential of antidromic invasion was not reduced in amplitude during the VR-EPSP.
Fig. 1. Records A–D show VR-EPSP's recorded from a motoneuron with an intracellular microelectrode. The antidromic stimulus strength was progressively increased from A to D. Records E–G show responses of another motoneuron to antidromic stimulation, the strength of which was increased from E to G. Note that a spike potential was evoked by the VR-EPSP in D and F, while an antidromic spike was produced in G.

A spike potential of the motoneuron was relatively easily evoked by summation of VR-EPSP's produced by stimulation of different ventral roots. When antidromic stimulation was applied during the course of the EPSP resulting from dorsal root stimulation (DR-EPSP), a remarkable summation of the EPSP's, resulting in an initiation of a spike potential, was also observed.

It was noticed that there was a deep depression of the VR-EPSP when the antidromic stimulation was applied during the phase of the after-hyperpolarization that follows a spike potential. This may be attributable to an increase in potassium conductance of the motoneuron membrane during after-hyperpolarization (COOMBS et al., 1955). Remarkable depression of the VR-EPSP was seen, particularly at the early stage of after-hyperpolarization. Consequently, spike potentials were difficult to produce by VR-EPSP following repetitive antidromic stimulation at relatively high frequencies. On the other hand, antidromic spikes were elicited with higher frequency stimulation in the same motoneuron.
2. Pharmacological investigation of VR-EPSP

In the present investigation, pharmacological tests on the VR-EPSP of the toad's motoneuron were carried out with three classes of drugs: an anticholinesterase drug, depressants of cholinergic transmission, and the suspected transmitter, acetylcholine. Various methods of drug application were employed as described in "METHODS," above.

Effects of anticholinesterase drug

If the production of the VR-EPSP is attributable to the release of acetylcholine from motor axon collaterals, prolongation of the VR-EPSP may be expected when an anticholinesterase drug is applied. In the present investigation, the VR-EPSP's tested were increased in size and the decaying phase was prolonged by topical application of physostigmine in 5 out of 6 cases tested.

In Fig. 2, the effect of physostigmine on the VR-EPSP is shown. A double-barrel microelectrode was used in this experiment. Record A shows the control VR-EPSP recorded from inside a motoneuron by one of the pipettes of the double-barrel microelectrode. The other pipette filled with 10 mM physostigmine salicylate

![Fig. 2. The effect of physostigmine on the VR-EPSP. Record A shows the control VR-EPSP; B and C were recorded during and a few minutes after application of physostigmine, applied from one of the pipettes of a double-barrel microelectrode by an outward current of $9 \times 10^{-8}$A. Note the spike initiation and the enhanced and prolonged VR-EPSP in B and C, respectively. Salicylate ions were applied by an inward current of the same intensity in D.](image)
S. MATSUURA

(dissolved in Ringer’s solution) was located just outside the motoneuron. As seen in Fig. 2B, the VR-EPSP was increased in size and gave rise to a spike potential when physostigmine was applied to the motoneuron electrophoretically by a cationic current of $9 \times 10^{-8}$A (duration, 30 sec). A few minutes after the end of drug application, the spike potential disappeared and an enhanced and somewhat prolonged VR-EPSP remained (Fig. 2C). These effects were not observed when salicylate ions were applied by an anionic current of the same intensity as shown in Fig. 2D.

Effects of acetylcholine

It was observed in the present investigation that the VR-EPSP increased in size when acetylcholine was applied to the motoneuron by various methods, i.e., by the electrophoretic method, by pressure, and by adding it to the perfusion fluid. Figure 3 shows the effect of acetylcholine on the VR-EPSP. In this case, about $1 \times 10^{-6}$ ml of 1 m acetylcholine chloride was applied by pressure from one of the pipettes of the double-barrel microelectrode that was connected to a microsyringe via a polyethylene tube filled with paraffin oil. Care was taken to prevent diffusion of acetylcholine from the microelectrode by applying an appropriate amount of a bucking current between the microelectrode and the indifferent electrode. Figures 3 A and B show intracellularly recorded VR-EPSP’s evoked by single and triple antidromic stimulations in different cells. Immediately after application of acetylcholine, the size of the VR-EPSP increased in C, and a spike potential appeared from the VR-EPSP in D.

![Fig. 3. The effect of acetylcholine on the VR-EPSP. A and B show control VR-EPSP's evoked by single and triple antidromic stimulations, respectively, in different cells. C and D were recorded after application of acetylcholine by pressure. Note an increase of the size of the VR-EPSP in C and the production of a spike potential in D.](image-url)
Figure 4 provides another example that indicates the effect of acetylcholine on the VR-EPSP. In record A, the control VR-EPSP evoked by a stimulus applied to the ventral root is shown with high and low amplification. The VR-EPSP increased in size and initiation of a spike potential was observed 10 sec after $1.5 \times 10^{-5}$ g (0.2 ml, $5 \times 10^{-4}$ M) of acetylcholine was administered to the perfusion fluid, as shown in Fig. 4B. However, when a much greater amount of acetylcholine (0.5 ml, $1 \times 10^{-3}$ M) was administered to the perfusion fluid, responses to ventral root stimulation were depressed and the VR-EPSP decreased in size (this is not shown in the figure).

It may be anticipated that, if the synapses between motor axon collaterals and motoneurons are cholinergic, an acetylcholine potential, which has been shown in the endplate by Del Castillo and Katz (1955) may be observed when acetylcholine is iontophoretically applied to the motoneuron. Although a typical acetylcholine potential as shown in the endplate was rather difficult to demonstrate, a transient depolarization which may be attributable to the transient acetylcholine emission from the microelectrode induced by a brief outward current was observed in a few cases. Figure 5 shows an example of such an acetylcholine potential. In this experiment a three-barrel microelectrode was used, as illustrated in Fig. 5E. One pipette was inside the motoneuron, while two other pipettes, filled with 1 M acetylcholine chloride solution and 1 M NaCl solution respectively, were located just outside the motoneuron. Figure 5A shows a VR-EPSP evoked by the antidromic stimulation, while records B–D show intracellular
potential changes recorded with high and low amplification following application of acetylcholine or Na⁺ ions. Unfortunately, artifacts due to the current application were considerably large. However, in Figs. 5 B and D, a transient depolarization is seen like a hump on the declining phase of the beams following application of acetylcholine by an outward current of \(2 \times 10^{-7} \text{A}\). In Fig. 5C, Na⁺ ions were applied by an outward current of \(2.2 \times 10^{-7} \text{A}\). In this case, no such depolarization was seen.

**Effects of depressants of cholinergic transmission**

The effect of depressants of cholinergic transmission, such as \(d\)-tubocurarine chloride, atropine sulfate, and nicotine tartrate on the VR-EPSP was tested by adding them to the perfusion fluid. It was found that \(d\)-tubocurarine chloride at a concentration of \(10^{-4} \text{M}\) was ineffective in depressing the VR-EPSP, in agreement with the work of GRINNELL (1966) on the VR-VRP. The ineffectiveness of curare on the cholinergic synapses on Renshaw cells in the cat was reported by ECCLES *et al.* (1954, 1956).
Contrary to the findings of Grinnell (1966) based on the ventral root potential resulting from ventral root stimulation (VR-VRP), nicotine tartrate at a concentration of $1 \times 10^{-3}$ M was considerably effective in reducing the size of the VR-EPSP, as is seen in Fig. 6. Record A shows a VR-EPSP and a DR-EPSP evoked by stimulation of the ventral and dorsal roots respectively. After application of $1 \times 10^{-3}$ M of nicotine tartrate to the perfusion fluid, the VR-EPSP was depressed in a few minutes, as shown in Fig. 6 B, while the DR-EPSP remained unchanged. The recovery of the diminished VR-EPSP was rather slow. Figure 6C was taken about 2 hr after replacing the perfusion fluid with normal Ringer’s solution. In this record, the DR-EPSP was diminished in size, probably due to slight damage of the motoneuron on account of the prolonged stay of the microelectrode, but the VR-EPSP recovered fairly well. Atropine at a concentration of $1 \times 10^{-4}$ M was found to have a depressant action on the VR-EPSP. The effect was, however, somewhat less potent than that of nicotine.

Fig. 6. The effect of nicotine on the VR-EPSP. In record A, control VR-EPSP and DR-EPSP are shown. Record B was obtained after application of $1 \times 10^{-3}$ M of nicotine tartrate to the perfusion fluid. Note the reduction in the size of the VR-EPSP. Record C was obtained about 2 hr after replacing the perfusion fluid with normal Ringer’s solution.

DISCUSSION

The amplitude of the spike potential evoked by the VR-EPSP has been found to be usually larger than that of the antidromically initiated spike potential at the same motoneuron. This may be attributable to the partial summation of the spike potential and the VR-EPSP, in addition to the fact that an antidromically evoked spike may be generated more asynchronously than that evoked synaptically (Coombs et al., 1955). The fact that the VR-EPSP is not changed by electrically induced alterations of the resting potential and that the spike potential of antidromic invasion is not reduced in amplitude during the VR-EPSP were shown
by Kubota and Brookhart (1963), and these facts have also been confirmed in the present study. On the basis of these results it could be inferred that the VR-EPSP may be initiated in the dendritic membrane, as has been suggested. The increased potassium conductance of not only the somatic but possibly also the dendritic portion of the motoneuron membrane may contribute to the suppression of the VR-EPSP during the after-hyperpolarization. On the basis of the results mentioned above, a dendritic origin for the VR-EPSP seems to be most probable.

It has been postulated that the same chemical transmitting substance is employed at all junctions operated by a particular cell (Dale, 1934, 1952). This postulate has led Eccles et al. (1954) to the expectation that the synaptic transmitter by which motor axon collaterals activate Renshaw cells is identical with that by which the motor nerve fibers activate muscle fibers. Their expectation that acetylcholine may be the central transmitter at the synapse between motor axon collaterals and Renshaw cells was tested pharmacologically, and they obtained results that confirm Dale's principle.

In analogy with cholinergic transmission at the terminal of motor axon collaterals in the cat, Kubota and Brookhart (1963) suggested cholinergic transmission at the synapse between motor axon collaterals and motoneurons in the frog. However, their evidence was rather scanty. They mentioned only that the VR-EPSP was altered by either succinylcholine chloride, decamethonium bromide, or curare in preliminary trials.

As far as the present investigation is concerned, the cholinergic nature of the synapse between motor axon collaterals and motoneurons seems to have been demonstrated. The pharmacological tests with the anticholinesterase drug, with depressants of cholinergic transmission, and with the suspected transmitter, acetylcholine, support the idea that the VR-EPSP is produced by the release of acetylcholine from the terminal of the recurrent collaterals.

Several points of disagreement have been found between the present investigation and the results described by Kubota and Brookhart (1963) and by Grinnell (1966) on the effect of drugs on the VR-EPSP or on the VR-VRP. The former authors reported that the VR-EPSP was altered by curare, but this was not the case in the present investigation. The effects of nicotine and atropine, both of which depressed the VR-EPSP in the present study, were contrary to the results described by the latter author, who found neither drug to have any appreciable effect on the VR-VRP. It is not clear why these discrepancies exist.

When the effects of drugs on the VR-EPSP in the present investigation are compared with those on Renshaw cell activation by motor axon collaterals in the cat, the cholinergic transmission of which has been clearly demonstrated by Eccles et al. (1954, 1956) and Curtis and Ryall (1966a, b), it has been made clear that acetylcholine has an excitatory effect and curare has no effect on both synapses. But nicotine induced a depression of the VR-EPSP in the toad, while
it enhanced Renshaw cell activation in the cat. This depression of the VR-EPSP may be associated with desensitization of the motoneuron, as a high concentration of nicotine was applied to the perfusion fluid, compared with the small amount of nicotine applied electrophoretically to the Renshaw cell. It was reported that, although substances with nicotinic properties were the most powerful excitants, cholinomimetics was probably associated with desensitization of Renshaw cells (CURTIS and RYALL, 1966a, b), and Renshaw cells showed depressed activity when a large amount of acetylcholine or cholinomimetics was ejected. Therefore, no essential difference seems to exist between the synapse which motor axon collaterals make on motoneurons in the toad and that which motor axon collaterals make on Renshaw cells in the cat.

It was observed in the present investigation that the VR-EPSP increased in size when acetylcholine was applied to the motoneuron by the electrical method and by pressure. Similar enhancement of the VR-EPSP was also observed when 0.2 ml of $5 \times 10^{-6}$ M acetylcholine was added to the perfusion fluid. These observations seem to indicate no occurrence of desensitization as might be expected from the fact that comparatively smaller doses of acetylcholine were applied and the period of application was not so long in these cases. On the other hand, the depression of the VR-EPSP with addition of the larger amount of acetylcholine (0.5 ml, $1 \times 10^{-3}$ M) to the perfusion fluid may depend on desensitization of the motoneuron.

Although the acetylcholine potentials recorded in the present experiments were not typical ones like those observed in the endplate, the existence of a cholinergic synapse on the motoneuron seems to have been established. The recording of a typical acetylcholine potential may have been difficult due to the location of the synapse, which is presumably not on the soma but on dendrites. At present, there is no conclusive evidence as to whether the recorded acetylcholine potential was produced only by the activation of the synapse originating from the motor axon collaterals, since there is, thus far, little information available on the other cholinergic fibers of the frog spinal cord. Recently, histochemical identification of cholinesterase in the amphibian spinal cord was reported by CHACKO and Cerf (1960). They have shown that specific-type acetylcholinesterase is distributed principally in the perikarya of the motoneuron, extending varying distances into the cell processes.

Challenging the chemical theory of the origin of the VR-EPSP, the interaction theory postulated by WASHIZU (1960) and GRINNELL (1966) has appeared. The former author reported the possibility of an interaction between motoneurons through dendritic bridges, though he reported no EPSP-like prepotential induced by antidromic stimulation. The latter author has tended to exclude the role of a chemical synapse in the production of the VR-EPSP on the basis of studies of the VR-EPSP and in particular of the VR-VRP. His results indicate that VR-VRP showed behavior different from the ventral root potential resulting from
the stimulation of dorsal root (DR-VRP), dorsal root potential resulting from the stimulation of dorsal root (DR-DRP), or dorsal root potential resulting from the stimulation of ventral root (VR-DRP), the production of which is thought to be due to the activation of chemical synapses, with regard to the effects of temperature, pharmacological agents, Ca++, and Mg++ ions, and GRINNELL thus proposed electrical transmission. In that case, however, either a slow conduction velocity of motor axon collaterals to the presumed termination, the dendrite, or a disynaptic connection between motor axon collaterals and motoneurons should be assumed in order to give an account of the latency of the VR-EPSP following the stimulation of ventral root. No evidence rejecting the electrical transmission theory has been obtained in the present investigation.

However, the chemical theory and the interaction theory may not be mutually exclusive. The synapse between motor axon collaterals and motoneurons in the amphibian spinal cord may be of a dual character, i.e., both chemical and electrical transmission may be operating. Further study will be required to clarify the functional relation between these electrical and chemical synapses.

The author wishes to express thanks to Professor T. Araki for his kind advice and criticism throughout this investigation and for his help in preparing the manuscript, and also to Dr. K. Endo for his help throughout the investigation.

REFERENCES


CHOLINERGIC TRANSMISSION IN MOTONEURONS


