Intracellular study of rat entopeduncular nucleus neurons in an in vitro slice preparation: response to subthalamic stimulation

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Responses of rat entopeduncular nucleus (EP) neurons after stimulation of the subthalamic nucleus (STh) and the morphology of the EP neurons were studied using brain slice preparations. EP neurons were classified into two types based on their electrophysiological properties as reported previously. Of 87 EP neurons, 72 were Type I and the rest were Type II. Synaptic responses to STh stimulation were different in these two cell types. STh stimulation evoked excitatory postsynaptic potentials (EPSPs) followed by strong inhibitory postsynaptic potentials (IPSPs) in Type I neurons and EPSPs without strong IPSPs in Type II neurons. The EPSPs were considered to be monosynaptic because no large change in the latency (1.7 ± 0.5 ms) resulted by alteration of stimulus intensity. The EPSPs were reversibly suppressed by kynurenate in a dose-dependent manner. Bath application of (+)-tubocurarine (10–50 μM) had no effect on EPSPs or IPSPs. Bath application of bicuculline methiodide (50–100 μM) markedly suppressed IPSPs evoked by STh stimulation and at the same time increased the amplitude and duration of EPSPs without affecting the latency. In the presence of bicuculline methiodide, EPSPs could induce plateau potentials and slow action potentials. Some Type I and Type II neurons were intracellularly labeled by biocytin. Type I neurons were located throughout the EP but Type II neurons were located mainly in the dorsal portion of the EP. Medium sized somata of both Type I and Type II neurons were spine-free and fusiform or round in shape. They had 3–4 thick primary dendrites with diameters of 2–5 μm that branched into thin secondary dendrites. The secondary and tertiary dendrites of Type I neurons were sparsely covered with spines. Dendritic terminals of some Type I neurons had complex arborizations with abundant spines and appendages. The dendrites of Type II neurons were generally smooth and had no complex arborizations at their terminals.

INTRODUCTION

The entopeduncular nucleus (EP) of the rat, a structure homologous to the internal segment of the globus pallidus in the primate, is located among the fibers of the internal capsule immediately caudal to the globus pallidus (GP) and rostral to the subthalamus (STh). The EP receives major afferents from the neostriatum, the GP, and the STh. In addition, EP neurons receive projections from the cerebral cortex, the substantia nigra (SN), and the pedunculopontine tegmental nucleus. It is well demonstrated in the rat that STh projection to the EP arises from neurons whose bifurcating axons also innervate the GP and the SN. Previous electrophysiological and neurotracing studies have indicated that the EP contains two groups of projection neurons. One group projects to the centromedian-parafascicular thalamic nuclei, the ventroanterior-ventrolateral thalamic nuclei, and the PPN. The other projects to the lateral habenular nucleus. Because of these anatomical connections, the EP is considered to be a major output nucleus of the basal ganglia.

STh projection to the EP is considered to be one of the important circuits in movement control. Pathological or experimental lesion of the SThs in the primate can lead to a violent form of hyperkinesia called hemiballism. The synaptic nature of STh inputs to the EP, however, is a matter of controversy. Electrophysiological studies, utilizing extracellular recording technique, have suggested that STh inputs to the SN were excitatory. However, STh inputs to the EP were reported to be inhibitory and GABAergic. Our recent intracellular study of rat brain slice preparations demonstrated that stimulation of the SThs induced monosynaptic excitatory postsynaptic potentials (EPSPs) in SN neurons. The same group of STh neurons innervate to the EP and the SN, therefore, the inputs to the two nuclei are expected to be the same.

Our recent study, using intracellular recording techniques and in vitro brain slice preparations, indicated that the EP consists of at least two types of neurons having different electrophysiological properties. Type I neu-
ions, which comprise about 80% of the EP neurons recorded, are characterized by a strong, time-dependent, anomalous rectification, a weak spike adaptation, and a strong rebound excitation, at the offset of a hyperpolarizing pulse. Type II neurons, of which fewer were recorded in the EP, are characterized by no apparent rectification, a strong spike adaptation, and a ramp-shaped repolarization, similar to the A-current. The aims of the present electrophysiological study, using combined electrophysiological and anatomical techniques, were to examine the postsynaptic potentials evoked in the two types of EP neurons after stimulation of the STh and to examine the morphology of these recorded neurons.

MATERIALS AND METHODS

A total of 35 male Sprague–Dawley rats (150–250 g) were used. In 5 animals, coronal transection of the brain at the level immediately caudal to the STh and rostral to the GP were made by using a Halazs knife under Nembutal (45 mg/kg) anesthesia 6–10 days prior to the recording session. The transections were used to eliminate fibers originating from the brainstem and passing through the STh. Detailed procedures for slice preparation and intracellular recordings are described elsewhere. Shortly, the animals were decapitated, the brains were rapidly removed and blocks containing the region of the EP were obtained. Parasagittal slices (400 μm thick) were cut from the block on a Vibratome. Electrical stimulation (up to 300 μA, 200 μs in duration) was applied through a bipolar electrode placed on the surface of the slice. Microelectrodes filled with 2 M potassium methylsulfate or 1.5 M KCl were used for intracellular recordings from EP neurons. The Krebs solution was composed of (mM): NaCl 124, KCl 5.0, KH2PO4 1.24, NaHCO3 26, CaCl2 2.4, MgSO4 1.3, and glucose 10. Bicuculline methiodide (Sigma, 50–100 μM), tubocurarine (10–50 μM), and kynurenic acid (Sigma, 0.3–1.0 mM) were dissolved in the superfusion medium.

In some experiments, intracellularly recorded neurons were labeled with biocytin in order to identify their location and morphology. In these experiments, animals were briefly perfused through the aorta with cold (10 °C) oxygenated Krebs solution under ketamine (80 mg/kg) and xylazine (13 mg/kg) anesthesia before decapitation. Intracellular labeling and histochemical procedures for biocytin were performed according to the method described by Horikawa and Armstrong. Microelectrodes used for intracellular labeling were filled with 2% biocytin (Sigma) in 0.5 M potassium acetate (pH 7.2–7.4). Biocytin was injected through the electrode by passing 1–2 nA alternative rectangular current pulses of 200 ms duration at 1 Hz for 5–10 min. After biocytin injections, slices were immersed in a fixative (a mixture of 4% paraformaldehyde and 0.02% picric acid) and kept overnight at 4 °C. The fixative was then replaced with KPBS (0.02 M potassium phosphate buffered (pH 7.4) saline) and kept (overnight to 2 weeks) until processing for biotin histochemistry. The slices were frozen and cut into 50 μm sections parallel to the surface, using a microtome. The sections were collected in KPBS, rinsed several times, and incubated in the avidin-biotin HRP complex for 1–2 h. The sections were rinsed and reacted with diaminobenzidine (0.06%) and H2O2 (0.003%) in KPBS for 10–20 min.

RESULTS

Responses to STh stimulation

A total of 87 EP neurons were recorded and analyzed in this study. They were classified into two types based on their electrophysiological properties as reported elsewhere. Of those 87 neurons, 72 were Type I and the rest were Type II. It was found that synaptic responses to STh stimulation were different in these two cell types. Frequently, STh stimulation evoked small amplitude (less than 2 mV) triphasic (i.e., negative-positive-negative) field potentials with a latency of about 1 ms (Fig. 2). The triphasic field potentials were not affected by application of Co ions but were suppressed by tetrodotoxin (TTX), indicating that the field potentials reflect the spike activity of axons at the recording foci (Fig. 2). Of 72 Type I neurons examined, 48 evoked postsynaptic potentials after stimulation of the STh. The postsynaptic potentials consisted of small depolarizing potentials and hyperpolarizing potentials (Figs. 1A and 2A). The amplitude of depolarizing potentials evoked by STh stimulation was decreased by injection of depolarizing current and increased by injection of hyperpolarizing current. The depolarizing potential could occasionally trigger an action potential. The amplitude of hyperpolar-
The polarity of hyperpolarizing potentials could be reversed by injection of a strong current or by injection of Cl ions through KCl-filled recording electrodes (not shown). These results indicate that the depolarizing and the hyperpolarizing potentials evoked by STh stimulation are EPSPs and inhibitory postsynaptic potentials (IPSPs), respectively. The EPSPs were considered to be monosynaptic because the change in stimulus intensity resulted in gradual changes of the amplitude of the EPSPs with very small (i.e. less than 0.5 ms) changes in the latency (Fig. 1B). The latency of EPSPs evoked by STh stimulation was 1.7 ms ± 0.5 (mean ± S.D., n = 48). Precise latency of IPSPs could not be determined as their initial parts were masked by the preceding EPSPs. However, the latency was estimated from IPSPs evoked during depolarization of neurons to a level close to the reversal potential of the preceding EPSPs. The estimated latency was between 2 and 6 ms.

Of 15 Type II neurons examined, 4 evoked depolarizing potentials after stimulation of the STh. Unlike Type I neurons, the depolarization was not followed by strong IPSPs. The depolarizing potentials were considered EPSPs because they were able to trigger spikes and also...
hyperpolarizing current increased and depolarizing current decreased their amplitude (Fig. 1C). The latency of EPSPs decreased gradually but only slightly (i.e. less than 0.5 ms) upon increase in the stimulus intensity, indicating that the nature of the response was monosynaptic (Fig. 1D). The latency of EPSPs induced by STh stimulation in
Type II neurons was 1.7 ± 0.6 ms and was not different from that seen in Type I neurons (mean ± S.D., n = 4).

The possibility that the STh stimulation-evoked responses in EP neurons are due to undesirable stimulation of passing fibers was tested by experiments using slices obtained from 5 rats whose brains were chronically cut in two places immediately caudal to the STh and rostral to the GP. Intracellular recordings were very difficult to obtain in these slices. All of the 5 successfully recorded neurons were Type I and evoked EPSP-IPSP sequences after stimulation of the STh. The responses were very similar to those evoked in slices obtained from intact animals (Fig. 1E).

In order to examine the involvement of excitatory amino acids in the EPSPs evoked by STh stimulation, the effects of kynurenic acid (a broad-spectrum excitatory amino acid antagonist) were tested in Type I neurons. The EPSPs were reversibly suppressed by kynurenic acid in a dose-dependent manner (0.3–1 mM) without affecting the field potentials which precede the EPSP (n = 6, Fig. 2B, C). Kynurenic acid also slightly (up to 50%) reduced the amplitude of IPSPs. There were no detectable changes in the membrane potential or the membrane input resistance by kynurenic acid. Bath application of (+)-tubocurarine (10–50 μM) had no effect on EPSPs or IPSPs (not shown).

Bath application of bicuculline methiodide (50–100 μM) markedly suppressed IPSPs evoked by STh stimulation and at the same time increased the amplitude and duration of EPSPs without affecting the latency (n = 8) (Figs. 2D and 3). In the presence of bicuculline methiodide, EPSPs could induce plateau potentials (Fig. 3A) and slow action potentials (Fig. 3D). An injection of short duration hyperpolarizing current pulse could terminate the plateau potential (Fig. 3A). EPSPs induced by STh stimulation in the presence of bicuculline methiodide were markedly suppressed by application of kynurenic acid (1 mM) and failed to induce the slow action potential (Fig. 3E). The plateau potential, however, could be induced by strong STh stimulation, even when EPSPs were largely suppressed by kynurenic acid (Fig. 3B).

**Intracellular labeling of recorded EP neurons**

Eight Type I and three Type II neurons were intracellularly labeled by biocytin. They were reconstructed from serial sections with the aid of a drawing tube in order to analyze their morphological characteristics. Type I neurons were located throughout the EP but Type II neurons were located mainly in the dorsal portion of the EP. Overall appearance of these neurons was similar. Somata of both Type I and Type II neurons were spine-free and fusiform or round in shape with the shortest diameter being 10–15 μm and the longest diameter being 18–25 μm (Fig. 4). They had 3–4 thick primary dendrites with diameter of 2–5 μm that branched into thin secondary dendrites. The dendritic field of these neurons extended for 600–800 μm in the sagittal plane, and 100–200 μm in the coronal plane. Their axon was thin, smooth, of constant diameter, and originated from a soma or proximal dendrite. No axon collaterals were found in these neurons. The secondary and tertiary dendrites of Type I neurons were sparsely covered with spines. The dendritic terminals of 4 Type I neurons formed a complex of arborizations with abundant spines and appendages (Fig. 4A–C). The dendrites of Type II neurons were smooth and had no complex arborizations at their terminus (Fig. 4D, E).

**DISCUSSION**

Stimulation of the STh evoked EPSPs followed by IPSPs in Type I, and EPSPs without strong IPSPs in Type II EP neurons. The EPSPs observed in both types are considered to be due to an activation of STh afferent because (i) the latency showed only a small (i.e. less than 0.5 ms) change upon increase in stimulus intensity, (ii) the existence of STh projections to the EP is well demonstrated by both anatomical and electrophysiological studies, (iii) the latency of EPSPs (mean = 1.7 ms) coincides well with the conduction time of STh axons to the EP plus a standard synaptic delay, and (iv) EPSPs evoked by STh stimulation were not affected by chronic transections placed caudal to the STh and rostral to the GP. Transections were made to eliminate possible involvement of brainstem and cortical afferent. Some Type-I and Type-II neurons failed to evoke postsynaptic responses after STh stimulation. It is not certain if STh fails to innervate all EP neurons or if some STh fibers are cut during preparation of the brain slice. The present study suggests that the transmitter of STh efferent includes excitatory amino acid (i.e. glutamate or its related compound) because the short-latency EPSPs evoked by STh stimulation was greatly decreased by application of kynurenic acid (up to 1 mM), a broad spectrum excitatory amino acid antagonist. Results of previous immunohistochemical studies are consistent with this concept. Neurons in the STh are immunoreactive for glutamate. Because the EP contains a small number of cholinergic and the EP might receive brainstem cholinergic afferent, effects of (+)-tubocurarine were also examined. The results suggested that nicotinic responses are not involved in EPSPs evoked in EP neurons after stimulation of the STh.

It is well demonstrated by electrophysiological and anatomical studies that the majority of STh neurons in the rat give rise to collateral projections to both the EP...
and the SN7,14,20,21,37. Our previous electrophysiological study involving intracellular recordings from rat brain slice preparations showed that STh stimulation evoked short-latency monosynaptic EPSPs in SN neurons28. Electron microscopic analysis of STh axon terminals labeled with both intracellular injection of HRP and the anterograde neurotracer Phaseolus vulgaris-leucoagglutinin revealed that they made asymmetric synaptic contacts with dendritic shafts of GP and SN neurons6,20. These results are consistent with the present finding that STh inputs to the EP are excitatory.

It has been recently reported that, in the primate, a kynurenic acid blockade of glutamatergic neurotransmission in the medial segment of the GP leads to a dyskinesia which is indistinguishable from that induced by STh lesion14. STh glutamatergic efferent may exert tonic regulation of posture and movement control.

Previous unit recording studies reported an inhibition of firing in EP neurons after stimulation of the STh13,26. It is likely that the direct monosynaptic STh-EP input was not able to direct, in these studies, because of predominant overlapping IPSPs which were induced by polysynaptic pathways or stimulation of undesirable pathway fibers.

In Type I neurons, STh stimulation evoked IPSPs that succeeded EPSPs. We consider that IPSPs must include GABAergic inputs, which open Cl-channels, since application of bicuculline methiodide suppressed the IPSPs, and intracellular injection of Cl ions reversed their polarity in a depolarizing direction. There are three possible origins of the IPSPs: first, is an antidromic activation of striato-SN and GP-SN fibers having collaterals in the EP. These projections are known to be GABAergic10,27. Because chronic transection placed rostral to the GP preserved the IPSPs, the most likely candidates responsible for the IPSPs are the axons of GP neurons, which are known to project both to the EP and the STh5,38. The second possible origin of the IPSPs is an activation of local inhibitory circuits disynaptically involving axon collaterals of EP neurons. The third, is an activation of inhibitory STh-EP projection. The second possibility is unlikely, since IPSPs were preserved when preceding EPSPs were strongly suppressed by kynurenic acid. The third possibility is also unlikely since only a few STh neurons are immunoreactive for glutamate decarboxylase or GABA2,32-35.

We have obtained two types of EP neurons which have different electrical membrane properties and different postsynaptic responses to STh stimulation. Observation of intracellularly labeled neurons suggested that their overall appearance of these two types of neurons is similar while some differences exist in their distal dendrites. Anatomical and electrophysiological studies have demonstrated that the EP contains a heterogeneous population of neurons. Immunocytochemical studies showed that the EP has three chemically distinct types of neurons: GABA, somatostatin and substance P1,16,33,34 (although GABA and somatostatin or substance P frequently coexist in the same neurons). In morphological studies, utilizing the Golgi method, Iwahori and Mizuno distinguished two types of EP neurons based on the size of the soma17. Conversely, Dvergsten et al. considered the EP to have only one cell type8. Anatomical axon tracing and electrophysiological studies suggested that EP was composed of two populations of neurons in terms of their output organization9,20,30. Van der Kooy and Carter demonstrated, in their study utilizing a fluorescent double-labeling technique, that the neurons in the rostral portion of EP projected to the lateral habenular nucleus, whereas the neurons in the caudal portion of EP projected to the other thalamic nuclei and the pedunculopontine tegmental nucleus36. They suggested that the EP might have two functionally separate subdivisions: the rostral limbic portion and the caudal motor portion. Fillion and Harnois have reported, in their electrophysiological study, that the rostro-medial part of EP neurons mainly projected to the lateral habenular nucleus9. Larsen and Sutin demonstrated that EP neurons with high frequency discharge which were inhibited by STh stimulation projected to the lateral habenular26. They also reported that EP neurons with low frequency discharge were not affected by STh stimulation and many of them were antidromically activated by the stimulation of the ventroanterior nucleus of the thalamus. Type I neurons are found throughout the EP, while Type II neurons are found mainly in the caudal part. Furthermore, our preliminary study utilizing a combination of intracellular labeling with biocytin and fluorescent retrograde labeling with rodamine latex microspheres, showed that some Type I neurons were labeled retrogradely from either the lateral habenular nucleus or the ventroanterior of the thalamus, whereas Type II neurons were labeled only from the lateral habenular nucleus.

The neostriatum and the STh receive excitatory inputs from the sensorimotor cortex22,24,40. Striato-EP projections are known to be inhibitory and GABAergic10,27. The present study indicates that the STh-EP projections are excitatory and glutamatergic. Therefore, EP neurons may converge cortex-derived neostriatal inhibitory and cortex-derived STh excitatory inputs.

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