Cerebellar granule cells in culture: Monosynaptic connections with Purkinje cells and ionic currents

(excitatory postsynaptic potential/patch-clamp)

TOMOO HIRANO, YOSHIHIRO KUBO, AND MICHAEL M. WU

Department of Neurobiology, Institute of Brain Research, School of Medicine, University of Tokyo, Tokyo, Japan

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ABSTRACT Electrophysiological properties of cerebellar granule cells and synapses between granule and Purkinje cells were studied in dissociated cultures. Electrophysiological properties of neurons and synapses in the mammalian central nervous system are best studied in dissociated cell cultures because of good target cell visibility, control over the contents of the extracellular solution, and the feasibility of whole-cell patch electrode recording, which has been a powerful technique in analyzing biophysical properties of ionic channels in small cells. We have applied this whole-cell recording technique to cultured cerebellar granule cells whose electrophysiological properties have been almost unknown because of their small cell size. In this study, simultaneous intracellular recordings from presynaptic granule and postsynaptic Purkinje cells demonstrated that granule cells made functional monosynaptic connections with Purkinje cells in dissociated cell cultures. Further, the existence of Na, Ca, and K channels in granule cells is demonstrated by external ion substitutions.

The cerebellar cortex has drawn much attention from neuroscientists because of its highly regular neuronal organization and circuit (1, 2). Purkinje and granule cells constitute major elements in the cortex. Purkinje cells are the only neurons that send outputs from the cerebellar cortex, whereas granule cells are the most numerous and the only known excitatory neurons in the cerebellar cortex. Granule cells relay the mossy fiber input, one of the two major inputs to Purkinje cells. Although there are many electrophysiological works on Purkinje cells *in vivo* and *in vitro* slice preparations (1, 2), very little work has been done on granule cells. Electrophysiological works have been conducted on cells in dissociated cerebellar cultures (3-5), but in such studies only Purkinje cells were identified (5). Recently, granule cell-rich dissociated cell cultures have been established (6, 7). Using these cultures we identified granule cells as small neurons that establish monosynaptic excitatory connections with Purkinje cells and applied to them the whole-cell voltage- or current-clamp technique. We then analyzed their ionic currents and synaptic effects on Purkinje cells.

METHODS

Culture methods of granule and Purkinje cells in rats were similar to those described (5, 8). For Purkinje cell cultures rat fetuses were sacrificed on approximately embryonic day 20, and for granule cell cultures 1-week-old rats were sacrificed. Cerebella were dissected out, freed of meninges, and incubated for 4 min (fetuses) or 13 min (newborn) at room temperature in Ca- and Mg-free Hanks' balanced salt solution containing 1% trypsin and 0.05% DNase. After three washings with Ca- and Mg-free Hanks' balanced salt solution the

tissue was dissociated by triturating with a fire-polished Pasteur pipette in Ca-free Hanks' balanced salt solution containing 0.05% DNase and 12 mM MgSO₄. The cells were centrifuged at 150 × *g* at 4°C and the pelleted cells were resuspended at a concentration of about 10⁶ cells per ml in a defined medium (9). One milliliter of the cell suspension from newborn rats was plated first in a Petri dish (3.5 cm in diameter) containing several pieces of heat-sterilized, poly(L-lysine)-coated coverslips, and then 1 ml of fetal cell suspension was added. One-half of the culture medium was exchanged with fresh medium once a week. Electrophysiological experiments were conducted after placing a coverslip into a trough mounted on an inverted microscope with Nomarski optics. All experiments were performed at 20-23°C unless otherwise stated. A granule cell was whole-cell voltage-clamped or current-clamped with a S-3666 patch-clamp amplifier (Nihon Kohden) with a feedback resistor of 100 MΩ. Fire-polished patch electrodes filled with intracellular KCl or CsCl intracellular solution (≈10-MΩ resistance) were used. All current traces were filtered with an 8-pole Bessel low-pass filter at 1 kHz. Recording of Purkinje cell membrane potential was performed with a conventional dc amplifier after penetration with a microelectrode filled with 3 M potassium acetate or with a fire-polished patch pipette, similar to ones used for granule cells, filled with intracellular potassium acetate, citrate, or fluoride solution. The composition of KCl intracellular saline was 145 mM KCl/5 mM EGTA/10 mM Hepes titrated with KOH, pH 7.4, and the composition of normal external saline was 140 mM NaCl/5 mM KOH/10 mM Hepes/2 mM CaCl₂/1 mM MgCl₂/17 mM glucose, pH 7.4. The composition of CsCl intracellular solution was the same as that of KCl intracellular solution except KCl and KOH were substituted by CsCl and CsOH, respectively. Intracellular potassium acetate, citrate, and fluoride solutions were made by replacing chloride with acetate, citrate, and fluoride, respectively. Na-free saline was the same as normal external saline except that NaCl was replaced with tetramethylammonium chloride. Ca-free saline was the same as normal external saline except that CaCl₂ was replaced by MgCl₂.

RESULTS

Fig. 1 shows a photograph of the culture observed with Nomarski optics. The large round cell with a diameter of about 20 μm indicated with a large arrow is a Purkinje cell, and the small cell with a diameter of 9 μm pointed to by a small arrow is a granule cell. Identification of Purkinje cells was done previously with monoclonal antibodies (5, 8). The most numerous neurons in culture, such as the small cell indicated in Fig. 1, have been regarded as granule cells (5-7, 9), because granule cells are most numerous in the cerebellum and such cultured neurons have been stained with a

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Abbreviations: EPSP, excitatory postsynaptic potential; IPSP, inhibitory postsynaptic potential.

FIG. 1. A Purkinje cell (indicated by large arrow) and a granule cell (indicated by small arrow) in dissociated cell culture, observed under Nomarski differential interference optics. (Bar = 20 μm .) The granule cell was identified as a cell having a monosynaptic excitatory connection with a Purkinje cell. The culture was used 28 days after dissociation.

granule cell-specific monoclonal antibody (7). Further, these small neurons have been stained by the Karnovsky method (10), indicating the presence of acetylcholinesterase, which is known to be possessed by granule cells (11). Thus, most of these small cultured neurons must be granule cells; however, it is likely that some are cerebellar inhibitory interneurons, such as basket and stellate cells. For this reason, granule cells cannot be identified solely by cell size. We, therefore, identified granule cells by showing electrophysiologically these cells to be excitatory.

To identify a candidate cell as a granule cell having excitatory synaptic connections with Purkinje cells, simultaneous intracellular recordings from a Purkinje cell and the candidate cell were performed. A candidate cell was whole-cell voltage-clamped or current-clamped with a patch-recording amplifier using intracellular K or Cs solution, and the intracellular recording of the Purkinje cell was done with a conventional intracellular amplifier using a microelectrode filled with 3 M potassium acetate or a patch pipette filled with potassium fluoride or citrate intracellular solution. Acetate, citrate, and fluoride are known not to pass through the inhibitory Cl channel in spinal motoneurons (12). Fig. 2*a* shows a series of simultaneous recordings. A granule cell was current-clamped. Seal resistances were as high as 10 G Ω and input resistances after breaking the patch were 0.5–1 G Ω . The resting membrane potential ranged from –60 to –85 mV. Following an action potential of the granule cell, a depolarizing potential of a uniform size (4.1 ± 0.3 mV, mean $\pm$ SD, $n = 7$) was recorded in a Purkinje cell with a constant latency [measured as the time from the onset of the action potential or early inward current (vertical broken lines and leftmost arrows in Fig. 2*a*) to the onset of postsynaptic potential (rightmost arrows in Fig. 2*a*)] of 4.1 ± 0.1 msec ($n = 7$). Such a depolarizing potential was identified as an excitatory postsynaptic potential (EPSP) because its amplitude decreased

during depolarization and because spontaneous inhibitory postsynaptic potentials (IPSPs) appeared as hyperpolarizing potentials in the same record. The EPSPs were also elicited by spontaneous action potentials of the granule cell with the same latency (4.1 ± 0.1 msec, $n = 14$). The relatively short and constant latency suggests that this EPSP was induced monosynaptically. In two experiments we increased the temperature of the experimental bath while recording the EPSP. The latency changed from 3.0 ± 0.1 msec ($n = 14$) at 23°C to 1.0 ± 0.1 msec ($n = 14$) at 31°C and from 4.1 ± 0.1 msec ($n = 13$) at 23°C to 2.1 ± 0.1 msec ($n = 13$) at 28.5°C (Fig. 2*b*). The calculated Q_{10} values were 3.9 and 3.4, respectively.

Obviously, the neurites originating from the granule cell body, where the patch-clamp electrode was located, were not voltage-clamped, as indicated by a long latency of inward current after the onset of depolarizing pulses (Fig. 2*b*). Thus, in these experiments the action potential initiated somewhere away from the soma may have propagated toward the presynaptic terminal and generated the synaptic potential in the Purkinje cell. With a decreased space constant at increased temperature, the spike initiation site may have moved closer to the cell body. If this complication occurred, the conduction time, and therefore the measured latency, would have increased at higher temperature. Consequently, the apparent Q_{10} values may be underestimated.

Assuming a Q_{10} of 3.5, latencies of 8.4 msec at 20°C and 5.8 msec at 23°C will be reduced to 1 msec at 37°C. Taking into account the conduction time of presynaptic axons, this suggests that latencies of <6 msec at 20–23°C are within the physiological range of monosynaptic connections, since the synaptic delay between granule and Purkinje cells in the rat was reported as 0.7 msec (13) and that of synapses on spinal motoneurons in the cat was reported as 0.3 msec (14). We succeeded in recording postsynaptic potentials in Purkinje cells that followed preceding presynaptic spikes in candidate

FIG. 2. Simultaneous intracellular recording from presynaptic neurons and postsynaptic Purkinje cells. (a) Simultaneous membrane potential recordings from a granule cell (upper trace) and a Purkinje cell (lower trace) in normal saline. The membrane potential of the granule cell was recorded with intracellular KCl solution in a constant-current mode of the patch-clamp amplifier. The numbers on the left of each trace indicate membrane potentials (mV). The action potential was evoked by injecting 60 pA of depolarizing current for 30 msec. Vertical broken lines and leftmost arrows indicate the onset of action potentials. The vertical scale bar for the granule cell membrane potential = 25 mV. Lower traces show membrane potential changes of a Purkinje cell (ac recording; time constant = 100 msec). Rightmost arrows indicate the onset of EPSPs. The vertical scale bar = 5 mV. These potential records from the Purkinje cell were obtained with a conventional intracellular amplifier for potential recording. The Purkinje cell patch pipette was filled with intracellular KF solution, in which Cl of intracellular KCl was substituted with F. The recorded Purkinje and granule cells are indicated with arrows in Fig. 1. (b) Simultaneous recording from a presynaptic granule cell and a postsynaptic Purkinje cell at different temperatures: 23°C (upper traces) and 28.5°C (lower traces). The granule cell was voltage-clamped at -40 mV from the holding potential of -60 mV, and the current record is shown in the upper trace of each pair. Vertical broken lines and leftmost arrows indicate the onset of inward currents from which the synaptic latency was measured. The lower trace of each pair shows the membrane potential recorded from a Purkinje cell. Rightmost arrows indicate the onsets of EPSPs. The synaptic latency changed from 4.1 ± 0.1 msec ($n = 13$) to 2.1 ± 0.1 msec ($n = 13$) by increasing temperature from 23°C to 28.5°C. (c) Simultaneous recording from a presynaptic inhibitory neuron and a Purkinje cell. The presynaptic neuron was whole-cell voltage-clamped and recorded currents are shown in the upper trace of each pair. The clamp potentials were -40 and -35 mV. The lower trace of each pair shows the membrane potential changes of the postsynaptic Purkinje cell recorded with a conventional intracellular microelectrode filled with 3 M potassium acetate. The IPSP in the Purkinje cell appeared after the initial inward current of a presynaptic cell with a short and constant latency. Rightmost arrows indicate the onset of IPSPs.

Table 1. Summary of simultaneous recordings in 13 experiments

Exp.	Distance, μ m	Evoked potential	Clamp mode	Latency, msec	Amplitude, mV	Rise-time, msec	Half-height width, msec
1	—*	EPSP	VC	3.0 ± 0.1 (14)	—†	—	—
2	20	EPSP	VC	3.2 ± 0.2 (9)	4.1 ± 1.9 (10)	4.3 ± 1.9 (10)	—†‡§
3	30	EPSP	CC	3.7 ± 0.1 (9)	3.1 ± 0.3 (9)	3.6 ± 0.3 (9)	10.4 ± 1.5 (6)
4	30	EPSP	CC	4.1 ± 0.1 (7)	4.1 ± 0.3 (7)	5.5 ± 0.3 (7)	11.2 ± 1.5 (7)
5	40	EPSP	VC	4.1 ± 0.4 (13)	—†	—	—
6	—*	EPSP	VC	4.5, 4.0	3.6, 3.4	—‡	—
7	65	EPSP	VC	5.0 ± 0.6 (13)	2.6 ± 1.2 (12)	4.6 ± 0.9 (9)	9.3 ± 1.7 (6)
8	25	EPSP	CC	5.7 ± 0.2 (6)	7.5 ± 2.1 (6)	5.7 ± 0.6 (6)	12.0 ± 2.0 (6)
9	—*	IPSP	VC	3.3 ± 0.2 (13)	2.8 ± 0.3 (15)	4.4 ± 0.8 (14)	23.0 ± 1.9 (15)
10	25	EPSP	CC	6.9 ± 0.8 (5)	3.4 ± 0.4 (5)	7.3 ± 0.9 (5)	13.8 ± 4.8 (5)
11	75	EPSP	VC	7.9 ± 0.9 (5)	6.3 ± 0.8 (6)	5.6 ± 0.5 (6)	11.2 ± 1.8 (6)
12	65	EPSP	VC	19.5 ± 3.6 (13)	5.3 ± 0.9 (8)	—§	—
13	25	IPSP	VC	11.1 ± 1.3 (12)	7.6 ± 0.7 (12)	3.6 ± 0.3 (12)	27.9 ± 2.4 (11)

Data from experiments 1–9 are considered indicative of monosynaptic connections between presynaptic cells and Purkinje cells because of the short latencies of Purkinje cell postsynaptic potentials. Connections in experiments 12 and 13 are considered polysynaptic because of their long and variable latencies. Connections in experiments 10 and 11 are neither clearly monosynaptic nor clearly polysynaptic. Note that the half-height width of the two IPSPs (experiments 9 and 13) was about twice that of an EPSP. All experiments were carried out at 20–23°C. All values (mean $\pm$ SD) were measured from clear traces only. Numbers in parentheses indicate the sample number. Distance is measured from the center of the granule cell to the center of the Purkinje cell. Clamp mode of granule cell: VC, voltage-clamp; CC, current-clamp.

*No photograph taken.

†Action potential generated by EPSP.

‡EPSP followed by an IPSP.

§EPSP followed by a polysynaptic EPSP.

¶Local dendritic spike elicited by the EPSP.

FIG. 3. Whole-cell clamp recordings of ionic currents from granule cell somata with internal KCl (*a*) or CsCl (*b–d*) and normal (*a* and *b*), Na-free (*c*), or Ca-free (*d*) external solutions. Each recorded cell was identified as a granule cell by its excitatory connection with a nearby Purkinje cell. The granule cells were voltage-clamped for 50 msec at depolarized potentials, as shown on the left of each trace, from a holding potential of -60 mV. (*a*) Voltage-clamp recording with internal KCl in normal external saline. From the onset of the voltage-clamp pulse, outward (upward on the trace) capacitive transient, early inward current (downward deflection indicated with an open arrow) and outward current and following late inward current (indicated with a filled arrow) were recorded. (*b*) With internal Cs solution and in normal saline, outward current decreased, and not only early inward current (indicated with an open arrow) but also late inward current (indicated with a filled arrow) became clearer. (*c*) Records in Na-free external saline and internal Cs solution. Late inward current (indicated with a filled arrow) remained, but the early inward component almost completely disappeared. Records in *b* and *c* are from the same cell. The delayed onset of inward current at -15 mV is probably due to poor space clamping of dendrites. (*d*) Records in Ca-free external saline and internal Cs. Early inward current remained (indicated with an open arrow) and late inward current disappeared.

cells in 13 experiments (Table 1). In all, among these 13 we observed postsynaptic potentials with a latency of <6 msec ($20\text{--}23^\circ\text{C}$) in 9 experiments. These 9 were regarded as monosynaptic connections. Among these 9 we only once observed an inhibitory connection (Fig. 2*c*), indicating that the recorded presynaptic cell was other than a granule cell. This result suggests that most of the small neurons ($10\text{ }\mu\text{m}$ or less) in our cultures were granule cells. However, careful identification of granule cells was necessary because a small number of inhibitory neurons certainly contaminated the culture, as reported previously (15). All granule cell data in this report come from cells identified electrophysiologically, as described above.

When a granule cell soma was whole-cell voltage-clamped at a more positive potential than -40 mV from the holding potential of -60 mV with a patch pipette filled with KCl intracellular solution, first outward capacitive transient, early inward current (downward deflection indicated with an open arrow) and then outward current and following late inward current (indicated with a filled arrow) were recorded (Fig. 3*a*). The early inward current decreased while the outward current increased in amplitude as the voltage-clamp pulse became more positive. The late inward current became less clear with the development of outward current at potential levels more positive than -15 mV. To identify charge carriers of the early inward current, outward current, and late inward current, the ionic composition of internal and external solutions was changed. When the granule cell soma was voltage-clamped with internal Cs solution, outward current disappeared and late inward current was clearly observed (Fig. 3*b*). As Cs is known to block voltage-dependent K channels, the outward current recorded with internal K solution is assumed to be K current. When Na ions in the external solution were replaced by tetramethylammonium ions, early inward current disappeared, leaving late inward current relatively unchanged (Fig. 3*c*). And when Ca ions in the external solution were replaced by Mg ions, late inward current disappeared, leaving early inward current relatively unchanged (Fig. 3*d*). These ion substitution exper-

iments verify that the early inward current is carried by Na and late inward current is carried by Ca.

DISCUSSION

The present study demonstrated functional monosynaptic connections in dissociated cell culture between two identified neurons in the mammalian central nervous system, the cerebellar granule and Purkinje cells. The dissociated cell culture is an excellent experimental system with which to study functional properties of synapses of central nervous systems because of the good target cell visibility and accessibility and also because of the feasibility of applying refined electrophysiological techniques such as the patch (whole-cell) clamp. However, so far no synapse between two clearly identified neurons from the mammalian central nervous system has been studied in dissociated cell cultures, because of the difficulty in identifying neurons. The identification of cultured neurons is crucial because synaptic properties depend on the various combinations of presynaptic and postsynaptic neurons, and these combination-specific properties ultimately determine the functional role of the neural circuitry. In invertebrates, synapses were formed between identified neurons in culture (16, 17). In mammals, synaptic formation between sensory neurons of dorsal root ganglia and spinal neurons have been reported (18, 19); however, spinal neurons were not further identified. The present study shows a way to examine the specific synaptic mechanism between two identified mammalian central neurons and also opens the possibility of modifying its function in isolation from other neurons. Further, this study also enabled us to study previously uncharacterized ion channels responsible for spike generation in the small, but physiologically important, granule cell.

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