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Synaptic transmission between rat cerebellar granule and Purkinje cells in dissociated cell culture: Effects of excitatory-amino acid transmitter antagonists

(synaptic current/glutamate/aspartate/patch clamp)

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ABSTRACT **Monosynaptic excitatory connections between cerebellar granule and Purkinje cells were studied in dissociated cell cultures, and identification of the transmitter and the postsynaptic receptor at this synapse was pharmacologically investigated. The presynaptic granule cell and the postsynaptic Purkinje cell were voltage- or current-clamped simultaneously, and the excitatory postsynaptic current induced by the granule cell was examined. The neurons and monosynaptic excitatory connections were identified as in our earlier study. Several pairs of granule and Purkinje cells were stained with Lucifer yellow and propidium iodide, respectively, and their morphology was examined after electrophysiological recording. The monosynaptic excitatory postsynaptic current was suppressed by 1 mM kynurenate, an antagonist for excitatory-amino acid receptors, but was little affected by 0.2 mM DL-2-amino-5-phosphonovalerate, a selective antagonist of *N*-methyl-D-aspartate receptors. Glutamate and aspartate induced inward current in the Purkinje cells. These currents were suppressed by kynurenate at 1 mM. DL-2-Amino-5-phosphonovalerate at 0.2 mM suppressed the inward current induced by 100 μM aspartate but did not affect the inward current induced by 10 μM glutamate. These results are consistent with the idea that glutamate, or a glutamate-like substance, but not aspartate is the transmitter released at the synapse between granule and Purkinje cells and that non-*N*-methyl-D-aspartate receptor channels are functioning in the postsynaptic membrane.**

Glutamate and aspartate have been considered as candidates for transmitters in many excitatory synapses in the central nervous system, and receptors for these amino acids have been classified into *N*-methyl-D-aspartate (*N*-Me-D-Asp) and non-(*N*-Me-D-Asp) receptors (1–3). It has been suggested that glutamate is the transmitter at the synapse between cerebellar granule and Purkinje cells (4), and the results of an *in vivo* pharmacological experiment suggest that the postsynaptic receptors at the synapse are of the non-*N*-Me-D-Asp type (5). However direct confirmation that glutamate is the transmitter and direct identification of the postsynaptic receptor at the synapse between granule and Purkinje cells are still lacking. In experiments with intact cerebella or in slice preparations, the structural complexity makes it difficult to isolate direct effects of agonists or antagonists on a particular synapse or cell.

The previous study (6) has established the presence of monosynaptic connections between rat cerebellar Purkinje and granule cells in dissociated cell cultures. In these cultures, the good visibility and accessibility of the neurons makes it possible to identify and simultaneously record from granule and Purkinje cells. In addition, the external ionic environment can be manipulated. We will provide further physiological and morphological information about these neurons and the synapse, based on experiments where granule and Purkinje cells were simultaneously clamped in the whole-cell configuration. Furthermore, we offer more direct pharmacological information that helps to identify the transmitter and the postsynaptic receptor at the synapse between the granule and Purkinje cells.

METHODS

Methods for culturing rat granule and Purkinje cells were the same as described previously (6, 7).

All electrophysiological data were obtained at room temperature (20–22°C). Neurons growing on coverslips were placed into a trough mounted on a Zeiss inverted microscope and observed with Nomarski optics. We used neurons cultured for 27–38 days. The density of neurons at the time of experiment was 5–50 cells per mm^2 for Purkinje cells and 500–3000 cells per mm^2 for small neurons. The Purkinje cell soma was voltage-clamped with an EPC-7 patch-clamp amplifier (List). Another EPC-7 amplifier was used to voltage-clamp or current-clamp the granule cell. Firepolished and Sylgard-coated patch pipettes of 3–6 MΩ were used for Purkinje cells, and pipettes of 5–10 MΩ were used for granule cells. All current traces were filtered at 3 KHz with a 3-pole Bessel low-pass filter.

The composition of the external saline was 140 mM NaCl/5 mM KOH/10 mM Hepes/2 mM $CaCl_2$/1 mM $MgCl_2$/17 mM glucose, pH 7.4. No $MgCl_2$ was added to the Mg-free saline. Composition of the intracellular solution was 140 mM glucuronate/160 mM KOH/7 mM HCl/5 mM EGTA/10 mM Hepes/2 mM MgATP/1 mM $MgCl_2$, pH 7.3. The Mg-free intracellular solution contained neither MgATP nor $MgCl_2$. Junction potential between this intracellular solution and the external solution was 14 mV (intracellular solution being negative), which was measured with a 3 M KCl electrode as previously described (8). All potential values were corrected for the junction potential.

In several experiments the Purkinje cell was stained intracellularly with propidium iodide, and the granule cell was stained with Lucifer yellow. Neurons were stained with patch pipettes containing the intracellular solution and 0.1–0.2% Lucifer yellow CH (Sigma) or 0.2–0.4% propidium iodide (Sigma). Photographs were taken shortly after the removal of the pipettes. The excitation wavelength was 485 nm.

Kynurenate, DL-2-amino-5-phosphonovalerate (APV), γ-D-glutamylglycine (all from Sigma), and tetrodotoxin (TTX)

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Abbreviations: EPSC, excitatory postsynaptic current; IPSC, inhibitory postsynaptic current; APV, DL-2-amino-5-phosphonovalerate; *N*-Me-D-Asp, *N*-methyl-D-aspartate; TTX, tetrodotoxin.

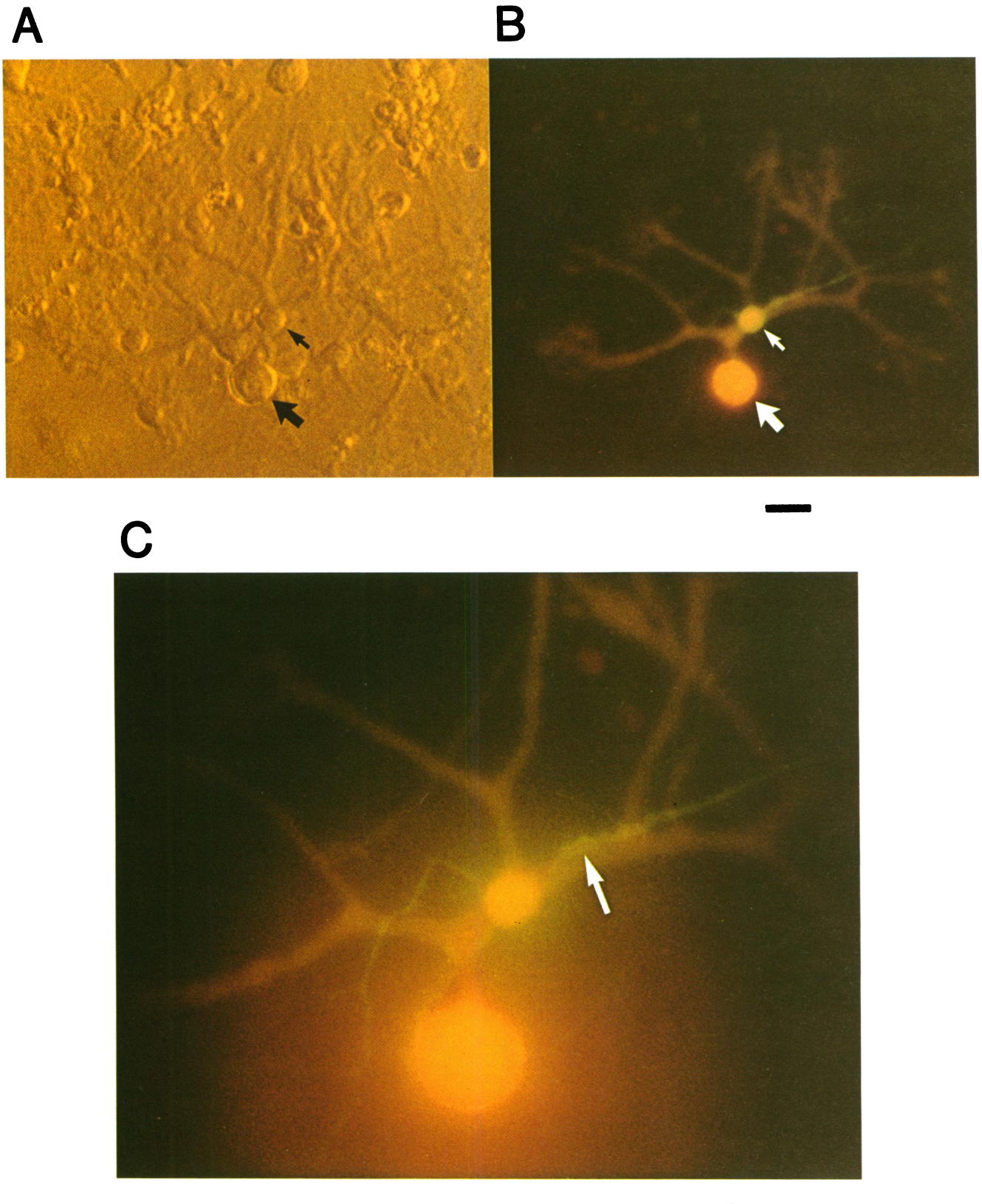

FIG. 1. Cultured granule and Purkinje cells that had a monosynaptic excitatory connection. (*A*) Photograph taken with Nomarski optics before the experiment. Large and small arrows indicate Purkinje and granule cell somas, respectively. (*B* and *C*) Fluorescent photographs of the same neurons taken after the experiment. Large and small arrows in *B* indicate the Purkinje and granule cell somas, and an arrow in *C* indicates a possible synaptic site, where a neurite of the granule cell swells and appears to contact a Purkinje cell dendrite. Bar = 20 μm.

(Sankyo, Tokyo) were applied to the bath solution. Glutamate and aspartate were applied locally to the somatic region through a glass capillary with a tip diameter of 2–4 μm. Positive pressure of about 70 kPa was applied to the capillary for 1 sec.

RESULTS

We identified Purkinje and granule cells on morphological and electrophysiological criteria as done in a previous study

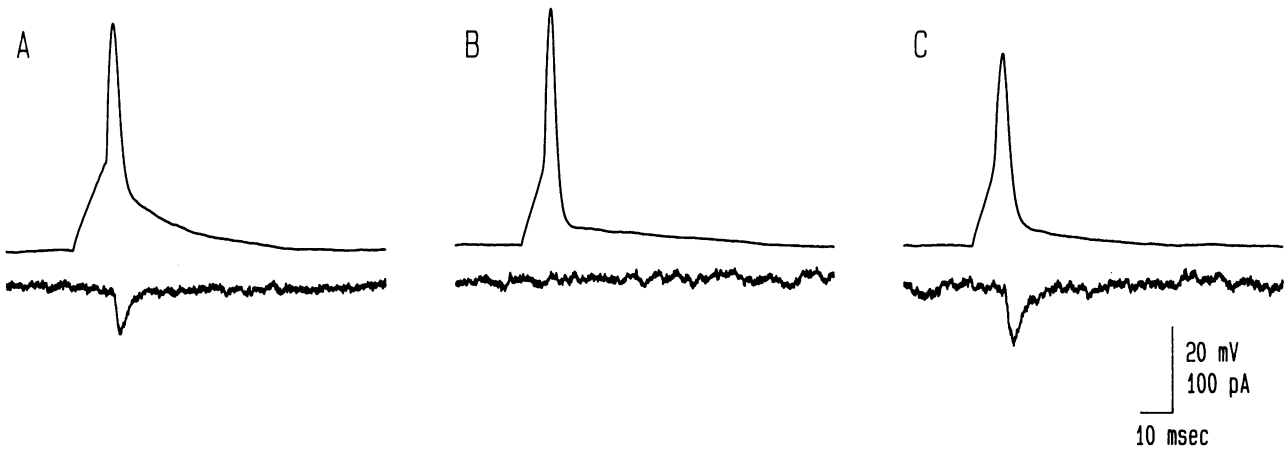

FIG. 2. Effect of 1 mM kynurenate on the monosynaptic EPSC in a Purkinje cell. Simultaneous whole-cell recordings were made from the granule and Purkinje cell somas. Upper traces show the membrane potential of a current-clamped granule cell, and the lower traces show the current of a Purkinje cell voltage-clamped at −60 mV. After the action potential of the granule cell, the inward EPSC was recorded in the Purkinje cell (*A*). (*A*) Records in normal external solution. (*B*) Records in normal solution containing 1 mM kynurenate. (*C*) Records after washing away kynurenate with normal solution. The resting potential of the granule cell was −64 mV (*A*), −75 mV (*B*), and −75 mV (*C*).

(6). A large neuron with a round soma of ≈20 μm diameter and with flat dendrites a few μm in width (for example, Fig. 1*A*, large arrow) was identified as a Purkinje cell; a small neuron with a soma of 5–10 μm diameter with fine neurites (≈1 μm) (Fig. 1*A*, small arrow), and which induced a monosynaptic excitatory postsynaptic current (EPSC) in a Purkinje cell, was identified as a granule cell. We recorded from about 120 pairs (a Purkinje cell and a small neuron located within 100 μm from the Purkinje cell) and found that 37 pairs showed direct connections. Among those pairs, 33 had monosynaptic excitatory connections, and the small neurons in these pairs were identified as granule cells. The direct effect of granule cells on Purkinje cells *in vivo* is known to be only excitatory (4). In the remaining 4 pairs, we recorded either monosynaptic inhibitory postsynaptic currents (IPSCs, 3 pairs) or electrotonic coupling (one pair). In these 4 pairs we assumed that the small neurons were not granule cells but were instead small stellate, basket, or some other cells.

Fig. 2 shows simultaneous recordings from a Purkinje cell and a small cell with characteristic granule cell morphology. The small cell was current-clamped, and the Purkinje cell soma was voltage-clamped at −60 mV. The small cell was excited by an outward-current pulse. After the action potential of the small cell, transient inward current was recorded in the Purkinje cell. This inward current was an EPSC because the amplitude decreased with depolarization and increased with hyperpolarization, and it was inhibited by kynurenate at 1 mM (Fig. 2*B*), an antagonist for excitatory amino acids. This EPSC was monosynaptic because of the constant and short latency of 3.5 ± 0.1 msec (mean ± SD, $N = 11$). Thus, the small cell was identified as a granule cell based on its morphology and its monosynaptic excitatory connection to a Purkinje cell. The latency was measured as the time interval between the onset of the action potential in the presynaptic neuron and the onset of the EPSC in the postsynaptic neuron. Our criterion for monosynaptic connections was that the latency be 6.5 msec or less. The latency varied from one pair of cells to another and was between 2.5 and 6.5 msec; however, it was constant for each pair. We occasionally recorded an EPSC with a latency greater than 10 msec; in these cases, the latency was variable even within each pair. We assumed that these were polysynaptic connections.

In several paired recordings, the granule cell was stained with Lucifer yellow, and the Purkinje cell was stained with propidium iodide. Their morphology was observed after electrophysiological measurements. Fig. 1 shows a stained pair in which the granule cell induced a monosynaptic EPSC in the Purkinje cell. Both the granule and Purkinje cells showed typical morphology for these cultured neurons. The wide flat stem dendrites of the Purkinje cell were clearly visible after staining, and fine dendritic arborizations were seen at the ends of the main branches. The granule cell was

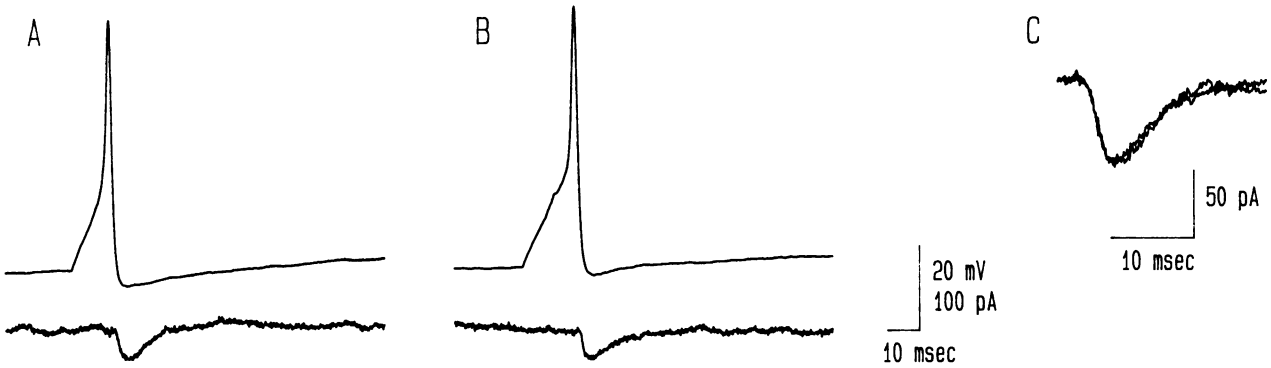

FIG. 3. Effects of APV and Mg-free solution on the monosynaptic EPSC. (*A*) Records in normal external solution. (*B*) Records in normal solution containing 0.2 mM APV. Upper traces show the potential of the current-clamped granule cell. Lower traces show the currents of the Purkinje cell voltage clamped at −60 mV. The resting potential of the granule cell was −62 mV (*A*) and −58 mV (*B*). (*C*) The monosynaptic EPSC was recorded in a Purkinje cell voltage-clamped at −60 mV with the Mg-free intracellular solution. Four traces in normal saline and four traces in Mg-free saline were separately averaged and then superimposed.

a bipolar cell with a small soma and with thin and long neurites. There was a slight swelling of a neurite of the granule cell along a dendrite of the Purkinje cell (designated by an arrow in the higher-magnified photograph, Fig. 1*C*). Similar swellings were demonstrated to be presynaptic terminals at the electron microscopic level in the cultured spinal neurons (9). Therefore, the swollen area, which appears to contact the Purkinje cell dendrite, may correspond to a presynaptic site. Such apparent contacts of granule and Purkinje cells were seen for all stained cell pairs that had excitatory synaptic connections of a short latency (seven/seven). In contrast, no such contact was observed for pairs without monosynaptic connections (three/three).

Time course of the EPSC varied considerably from one pair of cells to another, probably due to variation in location of the synaptic site. The rise time (time interval between the onset and the peak) of EPSCs varied from 3 to 5 msec, the average ± SD being 3.8 ± 0.8 msec (N = 29), and the half-maximum-amplitude width ranged from 5.5 to 21 msec, the average being 9.4 ± 3.9 msec (N = 28). Amplitudes of the EPSCs also varied from one pair to another, ranging from 20 pA to >800 pA; large EPSCs elicited an action potential in the unclamped region of the cell. The average amplitude was 160 ± 160 pA (N = 29). The amplitude of the EPSC varied little for some pairs (for example, 70 ± 2 pA, N = 6) but varied in other cases (for example, 130 ± 45 pA, N = 5).

We observed that the time course of the monosynaptic IPSCs was slower than that of the EPSCs. The rise times of the IPSCs were 7, 6.5, and 3.5 msec, and the half-amplitude widths were 50, 60, and 30 msec. Therefore, an IPSC can be distinguished from EPSC, not only by the direction of the current but also by its time course.

The addition of kynurenate at 1 mM to the bath solution abolished the monosynaptic EPSC without suppressing the action potential of the presynaptic granule cell (five/five) (shown in Fig. 2); this effect was reversible. Kynurenate also suppressed all spontaneous EPSCs in Purkinje cells. In contrast, kynurenate had little effect on the spontaneous IPSCs present in Purkinje cells. γ-D-glutamylglycine at 1 mM also suppressed monosynaptic and spontaneous EPSCs in Purkinje cells (data not shown). Fig. 3 *A* and *B* shows the effect of 0.2 mM APV. APV at 0.2 mM had little effect on the EPSC. The average amplitude of the EPSC in the solution containing 0.2 mM APV was 96 ± 4% (five pairs) of that in normal solution. The effect of Mg-free solution on the monosynaptic EPSC was also examined. A Purkinje cell was voltage-clamped with a patch pipette containing Mg-free intracellular solution, and the external saline was changed from normal saline to Mg-free saline while recording the monosynaptic EPSC. No clear effect of the Mg-free condition on the monosynaptic EPSC was seen as shown in Fig. 3*C* (five/five).

Both glutamate and aspartate induced inward currents in Purkinje cells (Fig. 4 *A* and *D*), as reported previously in slice preparations (10, 11). We used a glass capillary with a tip diameter of 2–4 μm to apply 10 μM glutamate or 100 μM aspartate to the somatic region of a Purkinje cell. TTX at 0.3 μM was used in some experiments to suppress synaptic activity, which was presumably due to spike generation in presynaptic neurons. Positive pressure was applied for 1 sec to the glass capillary. The average amplitudes of the inward current induced by 10 μM glutamate and 100 μM aspartate were 500 ± 240 pA (N = 10) and 240 ± 150 pA (N = 17), respectively. Fig. 4 also shows that 1 mM kynurenate reduced both glutamate- and aspartate-induced currents and that this effect was reversible. The amplitudes of glutamate- and aspartate-induced current in the solution containing 1 mM kynurenate were 27 ± 17% (N = 6) and 20 ± 12% (N = 5), respectively, of those in normal solution. Fig. 5 shows

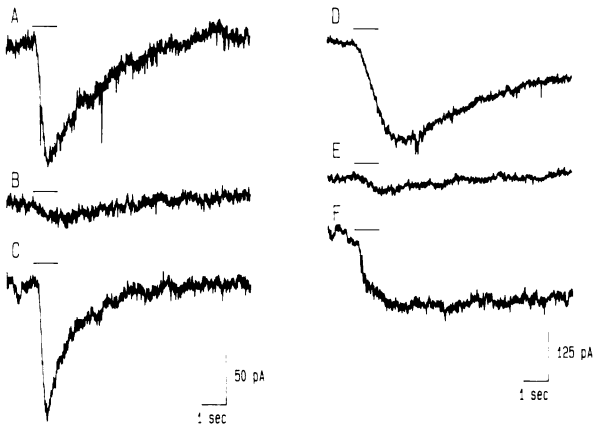

FIG. 4. Effects of 1 mM kynurenate on the glutamate (*A*, *B*, and *C*)- and aspartate (*D*, *E*, and *F*)- induced inward currents. The Purkinje cell soma was voltage clamped at −60 mV, and 10 μM glutamate or 100 μM aspartate was applied for 1 sec (at times indicated by the bars above the current traces) through a glass capillary. (*A* and *D*) Records in normal solution containing 0.3 μM TTX. (*B* and *E*) Records in normal solution containing 1 mM kynurenate and 0.3 μM TTX. (*C* and *F*) Records in normal solution containing 0.3 μM TTX after washing out kynurenate.

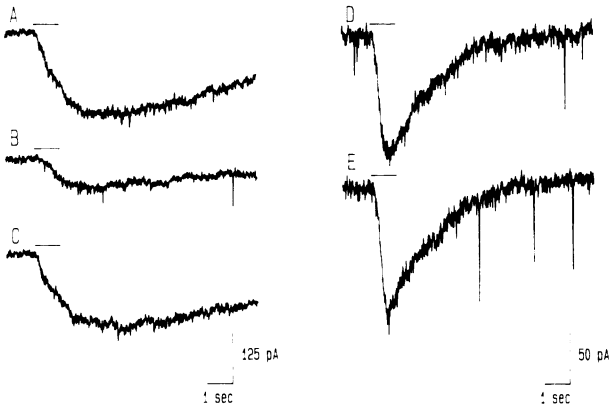

FIG. 5. Effects of 0.2 mM APV on aspartate (*A*, *B*, and *C*)- and glutamate (*D* and *E*)- induced inward currents. The Purkinje cell soma was voltage-clamped at −60 mV, and 100 μM aspartate or 10 μM glutamate was applied for 1 sec (at times indicated by the bars above the current traces) through a glass capillary. (*A* and *D*) Records in normal solution containing 0.3 μM TTX. (*B* and *E*) Records in normal solution containing 0.2 mM APV and 0.3 μM TTX. (*C*) Record in normal solution containing 0.3 μM TTX after washing out APV.

the effects of 0.2 mM APV on currents induced by glutamate or aspartate. APV at 0.2 mM had little effect on the inward current induced by 10 μM glutamate but substantially reduced the current induced by 100 μM aspartate. The amplitudes of glutamate- and aspartate-induced currents in the solution containing 0.2 mM APV were 96 ± 8% (N = 7) and 21 ± 12% (N = 6), respectively, of those in normal solution. The APV inhibition of the aspartate-induced current was also reversible.

DISCUSSION

The identification of neurons has been a problem in the study of synapses between cultured neurons from the mammalian central nervous system. Rat cerebellar culture is one of the few culture systems where both presynaptic and postsynaptic neurons can be identified. Cultured cerebellar Purkinje cells (12, 13) and granule cells (14, 15) were identified for electrophysiological studies primarily on morphological

grounds. Cell-specific monoclonal antibodies have supported the morphological identification of cultured cerebellar Purkinje cells (7, 16, 17). A previous study (6) reported the formation of excitatory synaptic connections between cultured granule and Purkinje cells and thus added a physiological criterion for the identification of a granule cell (excitatory connection to a Purkinje cell). In the present study several pairs of granule and Purkinje cells, which had been studied electrophysiologically, were also stained intracellularly with fluorescent dyes. When the morphology of these cells was examined in more detail, they resembled the stained Purkinje and granule cells in other studies on cerebellar cultures (7, 12, 14, 17). Common features are a large round soma, wide flat stem dendrites and fine dendritic arborizations for the Purkinje cell, and a small soma with a few thin, long neurites for the granule cell.

It has been suggested that glutamate is the transmitter of the synapse between granule and Purkinje cells (4). Our experimental results give further pharmacological support that glutamate or a glutamate-like substance is the transmitter at the synapse. We found that kynurenate, a specific excitatory-amino acid antagonist (18, 19), suppressed both the EPSC and the glutamate-induced current. We also reported that APV, which reduced the aspartate-induced current, but had little effect on the glutamate-induced current, had little effect on the EPSC.

Excitatory-amino acid receptors have been classified into *N*-Me-D-Asp and non-*N*-Me-D-Asp receptors (1, 2). The involvement of both non-*N*-Me-D-Asp and *N*-Me-D-Asp receptors has been demonstrated for synaptic transmissions in the spinal cord (20). The results that both Mg-free solution and APV had little effect on the EPSC suggest that the major postsynaptic receptor channel at the synapse between granule and Purkinje cells in our rat cerebellar cultures is of a non-*N*-Me-D-Asp type. APV is known as a specific *N*-Me-D-Asp antagonist (1, 2, 21), and Mg is known to block *N*-Me-D-Asp-receptor channels (22, 23).

Non-*N*-Me-D-Asp receptors have been further subdivided into quisqualate and kainate receptors (1, 2). However, a selective antagonist is lacking, and the results of an experiment in cultured spinal motoneurons suggest that quisqualate and kainate interact with the same receptor (3). Kano and Kato recently proposed (5) that the postsynaptic receptor at the granule and Purkinje cell synapse is of the quisqualate type based on an *in vivo* experiment that demonstrated the interaction between quisqualate and parallel fiber stimulation. We did several preliminary experiments to determine whether quisqualate or kainate receptors were functioning as the postsynaptic receptors. However, results were not conclusive. Both quisqualate and kainate induced currents in the Purkinje cell, and we could not find a selective antagonist for either quisqualate or kainate receptors. We observed that the monosynaptic EPSC became smaller in the presence of quisqualate, becoming undetectable when saturating concentrations of quisqualate (1 μM) were present in the bath, a result which may indicate that quisqualate binds to the postsynaptic receptors. However, we could not do the same experiment with kainate. Kainate induced such large inward currents in both granule and Purkinje cells that we were unable to isolate the effect on the EPSC. We also found that quisqualate and kainate responses in a Purkinje cell were not additive. When quisqualate was applied to a Purkinje cell in a saturating concentration of kainate (1 mM), no further increase of the inward current occurred, suggesting that quisqualate and kainate responses in Purkinje cells are not independent and, consequently, may be analogous to the response of spinal motoneurons (3).

Thus, our results are consistent with the idea that glutamate or a glutamate-like substance is the transmitter at the synapse between granule and Purkinje cells and that the major postsynaptic receptor channel is of a non-*N*-Me-D-Asp type.

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