Activation of GIRK Channels by Muscarinic Receptors and Group II Metabotropic Glutamate Receptors Suppresses Golgi Cell Activity in the Cochlear Nucleus of Mice

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Submitted 14 April 2006; accepted in final form 14 July 2006

Irie, Tomohiko, Iwao Fukui, and Harunori Ohmori. Activation of GIRK channels by muscarinic receptors and group II metabotropic glutamate receptors suppresses Golgi cell activity in the cochlear nucleus of mice. J Neurophysiol 96: 2633–2644, 2006. First published July 19, 2006; doi:10.1152/jn.00396.2006. Granule cells and parallel fiber circuits in the dorsal cochlear nucleus (DCN) play a role in integration of multimodal sensory with auditory inputs. The activity of granule cells is regulated through inhibitory connections made by Golgi cells. Golgi cells in turn probably receive parallel fiber inputs and regulate activity of the DCN. We have investigated the electrophysiological properties of Golgi cells using the whole cell patch-clamp method in slices made from transgenic mice that express green fluorescent protein driven by the promoter of metabotropic glutamate receptor subtype 2. Stimulation of auditory nerve fibers (ANFs) and of parallel fibers evoked glutamatergic excitatory postsynaptic currents (EPSC) through AMPA receptors. The strengths and latencies of these inputs differed, however. ANF stimulation evoked EPSCs after 4.7 ± 0.4 ms, whereas parallel fiber stimulation evoked EPSCs after 1.4 ± 0.2 ms that were on average 2.5 times as large. The multiple peaks and prolonged activity suggest the presence of polysynaptic connections between ANFs and Golgi cells. Agonists for group II metabotropic glutamate receptors (mGluRs) and for muscarinic receptors induced membrane hyperpolarization and suppressed the firing of Golgi cells by activating G-protein-coupled inward rectifier K⁺ (GIRK) channels. These results strongly suggest that Golgi cells were regulated through the combined activities of glutamatergic and cholinergic synapses, which presumably regulated the temporal firing patterns of granule cells and through them the activity of principal cells of the DCN.

INTRODUCTION

The cochlear nuclei (CN) are made of two distinct parts in mammals: the dorsal cochlear nucleus (DCN) and the ventral cochlear nucleus (VCN) (Oertel 1991). The VCN is unlayered, whereas the DCN is layered and many of its cell types are similar to those of the cerebellum (Oertel and Young 2004). The role of the DCN is not completely understood, but it has been suggested that the DCN plays a role in localizing sound sources using spectral cues (Kanold and Young 2001; May 2000; Oertel and Young 2004; Sutherland et al. 1998).

The principal cells of the DCN, cells the axons of which project out of the nucleus, are the fusiform cells and giant cells. These cells receive excitatory inputs from auditory nerve fibers (ANFs) and parallel fibers. Parallel fibers, the axons of granule cells, provide input to the molecular layer of the DCN, and convey multimodal sensory information (Mugnaini et al. 1980b; Oertel and Young 2004). Granule cells are excitatory neurons located in granule cell domains. Some granule cells lie in the fusiform cell layer of the DCN and others on the surface of the VCN. Granule cells are associated with inhibitory interneurons, Golgi cells. Golgi cells have a dense axonal plexus around granule cells and are presumed to have modulatory effects on the activity of granule cells (Ferragamo et al. 1998; Mugnaini et al. 1980a).

Identification of Golgi cells is difficult because they are small and interspersed among granule cells in small numbers. Only a few recordings have been made from Golgi cells in vitro (Ferragamo et al. 1998), where Golgi cells were identified morphologically by biocytin-staining combined with electrical-recording. However, to understand the function in the CN, detailed electrical properties and physiological roles of Golgi cells remain to be elucidated. The experiments reported in this paper were done in transgenic mice that express green fluorescent protein (GFP) under the control of a promoter for metabotropic glutamate receptor subtype 2 (mGluR2) (Watanabe and Nakanishi 2003; Watanabe et al. 1998). Because Golgi cells are known to express mGluR2, the expression of GFP facilitated the identification of the cells (Ohishi et al. 1998).

The present whole cell patch recording experiments in slices revealed the intrinsic electrical properties of Golgi cells, and the glutamatergic modulation of the membrane potential through group II mGluRs and cholinergic modulation through muscarinic receptors. Hyperpolarization of the membrane potential was mediated by the activation of GIRK channels. We conclude that these metabotropic receptors have modulatory effects on the activity of a local network formed with granule cells and in this way affect the firing patterns of parallel fibers.

METHODS

Transgenic mice

The IGI7 line of homozygous transgenic mice expressing the fusion protein of GFP and human interleukin-2 receptor α subunit were used (Watanabe and Nakanishi 2003; Watanabe et al. 1998). Animals were kept and used according to the regulations of the Animal Research Committee, Graduate School of Medicine, Kyoto University.

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Histochemical examination

Fourteen transgenic mice (P20–P30) were used for histochemical examination. They were killed with ether, and the brain was removed after transcardial perfusion with 5 mM phosphate-buffered saline (PBS; pH 7.4), followed by 4% (wt/vol) formaldehyde in PBS. When anti-GABA rabbit antibody (Sigma, St. Louis, MO) was used, PBS perfusion was followed by 2% (wt/vol) formaldehyde and 0.01% glutaraldehyde (vol/vol) in PBS. The brain was postfixed 1 h at room temperature. The brain was then cryoprotected with 30% (wt/wt) sucrose in PBS and sliced into 40-μm-thick coronal sections using a cryostat (CM3050S; Leica, Nussloch, Germany). Detailed procedures of immunoperoxidase staining and double-immunofluorescence histochemistry were given in a previous report (Furuta et al. 2004). Anti-GFP guinea pig antibody (0.2 μg/ml) (Tamamaki et al. 2000) was used for immunoperoxidase staining with biotinylated anti-guinea pig IgG (10 μg/ml, Chemicon, Temecula, CA; Fig. 1, C and D) and in several double-immunofluorescence histochemistry experiments (Fig. 1, A, B, E, and F) with Alexa488-conjugated anti-guinea pig IgG goat antibody (4 μg/ml, Molecular Probes, Eugene, OR). The following secondary antibodies and chemicals were used in double-immunofluorescence histochemistry: anti-metabotropic glutamate receptor 2 and 3 (mGluR2/3) rabbit antibody (1 μg/ml, Chemicon; Fig. 1, A and B), anti-GABA rabbit antibody (1:1,000-diluted; Fig. 1E), biotinylated anti-rabbit IgG donkey antibody (10 μg/ml, Molecular Probes; Fig. 1, A, B, and E), and Alexa594-conjugated streptavidin (1 μg/ml, Molecular Probes; Fig. 1, A, B, and E). Signal strength of immunoreactivity against vesicular acetylcholine transporter (VACt) was amplified by a tyramide amplification system (TSA Fluorescence System; PerkinElmer, Boston, MA), where anti-VACt goat antibody (1:4,000-diluted, Chemicon), peroxidase-conjugated anti-goat IgG donkey antibody (1:100-diluted, Chemicon), and Cy3-conjugated tyramide (PerkinElmer) were used (Fig. 1F). Immunofluorescence
was observed under a confocal microscope (LSM5 Pascal, Zeiss, Oberkochen, Germany) with the following appropriate filter sets: Alexa488 (excitation, 450–490 nm; emission, 514–565 nm), Alexa594 (excitation, 530–585 nm; emission, 615 nm and long path), and Cy3 (excitation, 530–585 nm; emission, 615 nm and long path). When double immunofluorescence of GFP and VACHT was observed, the confocal depth was <1 μm.

Measurement of cell body areas and mapping of Golgi cells

Cell body areas were measured with National Institutes of Health Image (NIH, Bethesda, MD) after immunoperoxidase staining using anti-GFP antibody (Fig. 1, C, D, and G). Location of Golgi cells in the CN was mapped on the drawing made from the middle section of 5 coronal sections obtained from a single animal (Fig. 1H). Cytoarchitectonic regions were named according to Jaarsma et al. (1998).

Slice preparations for electrophysiological experiments

Mice (P16–P20) were killed with ether and decapitated. The brain stem was dissected from the skull and placed in dissection saline containing (in mM) 122 NaCl, 2.5 KCl, 0.1 CaCl2, 5 MgCl2, 1.25 NaH2PO4, 10 HEPES, 17 d-glucose, 0.4 ascorbic acid, 3 myo-inositol, and 2 sodium pyruvate bubbled with 100% O2, pH 7.4 adjusted with NaOH. When ANFs were electrically stimulated, a single parasagittal slice of 300- to 500-μm thickness containing the CN and ANFs was prepared from a single animal using a tissue slicer (PR7; Dosaka, Kyoto, Japan, Figs. 4, A–G, and 11). In the other experiments, coronal brain stem slices (300 μm thick) containing the CN were used (Figs. 2, 3, 4H, and 5–10). These slices were then incubated in oxygenated artificial cerebrospinal fluid (ACSF) at 36°C for 1 h before use. ACSF contained (in mM) 120 NaCl, 2.5 KCl, 2 CaCl2, 1 MgCl2, 26 NaHCO3, 1.25 NaH2PO4, 17 d-glucose, 0.4 ascorbic acid, 3 myo-inositol, and 2 sodium pyruvate bubbled with 5% CO2-95% O2. BAPTA was loaded into the cells (Fig. 4), slices were incubated in 50 μM BAPTA/AM (Dojindo, Kumamoto, Japan) in ACSF at 36°C for 1 h. Slices were mounted on a recording chamber on the stage of an upright microscope (BX50W1; Olympus, Tokyo, Japan), perfused continuously with ACSF at the rate of 5 ml/min by a peristaltic pump (P-3; Amersham Biosciences, Arlington Heights, IL), and maintained at 33°C (DTC300, Diamedical, Tokyo, Japan). The volume of ACSF in the chamber was 1 ml. Cells were visualized with a ×60 objective lens with Nomarski optics using an IR-CCD camera (C5999; Hamamatsu Photonics, Hamamatsu, Japan). GFP-positive cells were visualized and selected using epifluorescence optics (Olympus).

Electrophysiological recordings

Whole cell recordings were made with a patch-clamp amplifier (Axopatch 200B, Axon Instruments, Union City, CA). The resting membrane potential was measured immediately after the whole cell recording was achieved. Cells were further investigated if the potential was more negative than −50 mV. Patch pipettes were made from thin-walled borosilicate glass capillaries (GC150TF-100; Harvard, Holliston, MA) and had a resistance of 3–5 MΩ when filled with a K-gluconate-based internal solution containing (in mM) 125 K-glucuronate, 10 KCl, 3 MgCl2, 0.1 EGTA, 5 Na2-ATP, 5 Na2-phosphocreatine, 0.3 Na2-GTP, and 10 HEPES-KOH, pH 7.3. The K-glucuronate-based internal solution was used unless otherwise stated. In some experiments (Figs. 5D and 8B), Na2-GTP was substituted with GDPβS trillium salt (GDPβS, 1 mM Sigma). Pipettes were coated with a silicone resin (Sylgard; Dow Corning Asia, Tokyo, Japan) and fire polished before use. The electrode capacitance and series resistance (7–15 MΩ) were estimated and compensated electronically by 80–95%. For recording excitatory postsynaptic currents (EPSCs), miniature EPSCs (mEPSCs) and miniature inhibitory postsynaptic currents (mIPSCs), a CsCl-based internal solution of the following composition was used (in mM): 140 CsCl, 3 MgCl2, 0.1 EGTA, 5 Na2-ATP, 5 Na2-phosphocreatine, 0.3 Na2-GTP, 2 QX-314 (Alomone Labs, Jerusalem, Israel), and 10 HEPES-CsOH, pH 7.3. The liquid-junction potential (10 mV, K-glucuronate-based internal solution; 5 mV, CsCl-based internal solution) was measured between the patch electrode and the bath and was corrected.

EPSCs were recorded in the presence of 20 μM bicuculline (Sigma) and 1 μM strychnine (Sigma) to block GABA-A and glycine receptors, respectively. EPSCs and excitatory postsynaptic potentials (EPSPs) were elicited by electrical stimulation with a bipolar tungsten electrode placed on the root of the auditory nerve in the slice (Figs. 4, A–D and 11) or placed on parallel fibers (~200 μm dorsal to the cell body (Figs. 4H and 6). When parallel fibers were stimulated, atropine was included in ACSF. The stimuli were biphasic square-wave voltage pulses of 0.3- to 24-V amplitude and 0.1-ms duration and were applied every 5 s in most cases. A train of 10 stimuli was delivered at 30-s intervals (Fig. 6A). When mEPSCs were recorded, 1 μM tetrodotoxin (TTX; Sankyo, Tokyo, Japan) was further included in the ACSF, and the membrane was held at −70 mV. Frequency of occurrence of mEPSCs, their amplitude, 10–90% rise time, and decay time constant were measured off-line. The occurrence of mEPSC and

FIG. 2. Membrane excitability of Golgi cells. A: voltage response to a small hyperpolarizing current (~30 pA, 200-ms duration) fitted by a double-exponential function (thin line). Two time constants are indicated. B: voltage responses to depolarizing and hyperpolarizing current pulses (~300–400 pA, 100-pA increment, 300-ms duration). Golgi cells fired in burst with firing frequency adaptation and showed a depolarizing voltage sag when hyperpolarized. C: firing frequencies were plotted against the injected current intensity. Different symbols indicate different cells (n = 5). D: instantaneous frequency was calculated from the interspike interval from traces in B and was plotted at the time of the 2nd spike peak of the pair. E: voltage responses to hyperpolarizing current pulses (~300–0 pA, 50-pA increment, 300-ms duration) for the control (Ea) and in ZD7288 (50 μM, Eb). A, B, and Eb, was recorded in the same cell. F: current-voltage relationship calculated by measuring voltage amplitudes at the pulse end (ε in Ea and ε in Eb). Application of ZD7288 increased voltage amplitudes significantly (*P < 0.05 by paired t-test). Here and in the following figures, error bars indicate SE.
EPSC was detected using a threshold of 3 SD of current noise above the baseline. Ensemble averaging of mEPSC (Fig. 3B) was made by aligning traces to the point corresponding to 50% rise time. When mEPSCs were recorded (Fig. 3, F and G), TTX and blockers of ionotropic glutamate receptors were included in ACSF as follows, and mIPSCs were recorded (Fig. 3, F). mEPSCs was recorded at –70 mV and blocked by strychnine (1 μM). G: ensemble averaged mIPSC from 30 events recorded from a single cell and was curved fitted as in B by a thin line.

FIG. 3. Properties of miniature excitatory and inhibitory postsynaptic currents (mEPSCs and mIPSCs). A: mEPSCs recorded at –70 mV. Ten consecutive traces were superimposed. mEPSCs (Aa) were blocked by application of DNQX (20 μM, Ab). B: ensemble averaged mEPSC from 30 events recorded from a single cell and was curved fitted by a single exponential function (thin line). C–E: amplitude, 10–90% rise time, and decay time constant of mEPSCs with binning of 10 pA (C), 0.1 ms (D), and 0.2 ms (E). Mean ± SE is indicated in each histogram. F: mIPSCs was recorded at –70 mV and blocked by strychnine (1 μM). G: ensemble averaged mIPSC from 30 events recorded from a single cell and was curved fitted as in B by a thin line.

Measurement of cell capacitance and resistance

Input capacitance and resistance of cells were measured by injecting a small hyperpolarizing current (–30 to –50 pA, 200- to 300-ms duration) in current clamp (e.g., Fig. 2A) or by applying a small voltage step (–5 mV, 20 ms duration) from –60 mV holding potential in voltage clamp. Both voltage responses and current transients took double exponential time course. The faster component in current clamp reflected the dendrites, therefore the slower component was chosen as the membrane time constant of the cell body (Rall 1969). In voltage clamp, the fast current transient reflects the charging current of somatic capacitance, and was estimated by subtraction of the slow current transient (Llano et al. 1991). The somatic capacitance \( C_m \) was then estimated from the electronic charge \( Q \) carried by the...
RESULTS

Immunohistochemical characterization of GFP-positive cells and possible innervation of Golgi cells by VACHT-immunopositive terminals

Immunopositivity for group II mGluRs was tested in GFP-positive cells by double labeling with anti-mGluR2/3 antibody and anti-GFP antibody (Fig. 1, A and B). mGluR2/3 immunoreactivity was observed in 96.3% (208/217) of GFP-positive cells, whereas GFP immunoreactivity was observed in all mGluR2/3-positive cells (208 cells). Therefore GFP-positive cells overlapped completely the mGluR2/3-positive cells.

Golgi cells are immunopositive for GABA (Kolston et al. 1992) and glutamate decarboxylase (Mugnaini 1985). Among GFP-positive cells, large cells were GABA-positive (96.8%, 122/126 cells; Fig. 1E, arrows), whereas small cells were GABA-negative (0%, 0/284 cells). Among GABA-positive cells, there were significant populations of GFP-negative cells (69%, 82/119 cells; Fig. 1E, double arrowheads). These cells might be stellate cells and/or cartwheel cells (Kolston et al. 1992; Mugnaini 1985).

Small GFP-positive cells (Fig. 1, Ba and D) are likely to be unipolar brush cells (UBCs) because of the presence of a single stubby dendrite (Jaarsma et al. 1998), and the large cells (Fig. 1, Aa and C) Golgi cells because they have multipolar dendrites (Ferragamo et al. 1998; Mugnaini et al. 1980b). Figure 1G shows the measure of somatic area of GFP-positive cells and demonstrates two distinct populations (open columns):

Data acquisition and analysis

Data were filtered at 5 kHz with a four-pole low-pass filter with Bessel characteristics and sampled at 10–200 kHz by a 12-bit A/D converter (ADM-8298BPC, Micro Science, Tokyo, Japan) with a data-acquisition program written in-house. Some data were recorded with DAT (RD-125T; TEAC), and these data were played back and printed using an analog thermal array recorder (RTR-1100; Nihon Kohden, Tokyo). Off-line analysis of data was made using Axograph 4.8. (Axon Instruments, Union City, CA). Data are given as means ± SE (n = numbers of cells). Statistical significance was tested with one-way ANOVA and a post hoc (Bonferroni/Dunn) test, unless otherwise stated.

FIG. 5. DCG-IV induced a membrane hyperpolarization through G-protein-coupled inward rectifier K⁺ (GIRK) channels. A: bath application of DCG-IV (1 μM) induced a hyperpolarizing response. Input resistance was monitored by applying hyperpolarizing current pulses (~200 pA, 200-ms duration) every 5 s. A bar labeled ± s.d. indicates the compensation of membrane potential by current injection (+80 pA, in this case). B: averaged and expanded traces from A. a’ was the ensemble average of 3 records near a, and b’ was the average of 2 records near b in A. C: current responses in voltage-clamp experiments (from −30 to −120 mV, 10-mV step, 300-ms duration; holding potential, −50 mV) in 10 mM K⁺-containing ACSF; Ca the control; Cb, in DCG-IV; Cc, DCG-IV-induced currents (Cb minus Ca). D: current responses after loading the cell with GDPβS (1 mM) through the patch electrode. The protocol of voltage clamp and DCG-IV application were the same as C. E: current-voltage relationship of DCG-IV-induced currents normalized by cell capacitance (○), showing inward rectification. The reversal potential was −66.2 ± 1.4 mV (n = 5). ○, from cells recorded with GDPβS. The reduction of DCG-IV-induced currents was significant (*P < 0.05 and #P < 0.01 by unpaired t-test).

FIG. 6. Group II mGluRs-dependent afterhyperpolarization. A: voltage responses evoked by high-frequency stimulation (100 Hz, a train of 10 stimuli) of the parallel fiber in the presence of strychnine, bicuculline, and atropine. Action potentials were truncated. Long-lasting afterhyperpolarization was abolished by application of antagonist of group II mGluRs (LY341495, 1 μM). B: amplitude of afterhyperpolarization was significantly reduced by LY341495 (*P < 0.05 by paired t-test). C and D: occlusion of afterhyperpolarization by DCG-IV. Voltage responses were evoked similarly as in A. C: train of 15 stimuli was applied at 100 Hz. The resting membrane potential was set at −55 mV by current injection in the control (+50 pA) and in DCG-IV (+140 pA). Action potentials were truncated. D: application of DCG-IV significantly reduced the afterhyperpolarization (#P < 0.01 by paired t-test).
cells <70 μm² and cells >90 μm² (n = 306 cells). Based on the dendritic shapes (Fig. 1, C and D), these two populations probably correspond to UBCs (gray columns, n = 32 cells) and Golgi cells (filled columns, n = 37 cells).

The location of Golgi cells was mapped in Fig. 1H (see Methods). Golgi cells were localized in the superficial granular layer (SGL) of the VCN, granule cell lamina (La), and fusiform cell layer (FCL) and some in the deep region (DR) of the DCN. The finding that their location overlapped with the cholinesterase-positive area (Osen and Roth 1969; Osen et al. 1984; Yao and Godfrey 1995) indicates that Golgi cells can receive cholinergic innervation. VAChT is a marker for cholinergic esterase-positive area (Osen and Roth 1969; Osen et al. 1984; Yao and Godfrey 1995). Double immunolabeling with antibodies for GFP and VAChT showed VAChT-immunopositive puncta closely apposed to the GFP-positive somata (Fig. 1F, a and b; arrowheads) and to the proximal dendrites (Fig. 1Fb; arrow).

Membrane excitability of Golgi cells

Electrical recordings were made from 140 Golgi cells. The resting membrane potential was on average –60.0 ± 0.89 mV (n = 137). Action potentials were generated spontaneously in three cells and were prevented by hyperpolarizing current.

Input capacitance and resistance of cells were measured at the resting potential either from small hyperpolarizing voltage responses in current clamp or from capacitive current transients in voltage clamp. The membrane hyperpolarization in current clamp was well fitted by a double-exponential function (Fig. 2A); the time constant was 5.5 ± 0.4 ms for 62.7 ± 7.0% of the response and 28.5 ± 1.8 ms for the remaining slower component (n = 53). The slower component was chosen as the membrane time constant of the cell body (see Methods). Input capacitance and resistance of cells, thus measured, were 103 ± 6.6 pF and 296 ± 17 MΩ, respectively (n = 53). In voltage clamp, the capacitance of cell body was calculated from the fast current transient. Input capacitance and resistance of cells were 31 ± 1.5 pF and 250 ± 10 MΩ, respectively (n = 87). Current injection evoked a burst of action potentials and a hyperpolarization with a depolarization sag (Fig. 2B). The firing frequency increased with the injected current (Fig. 2C). Figure 2C shows that the discharge rate increased monotonically to a maximum firing frequency of ~100 Hz with depolarizing currents of 400–700 pA (99.1 ± 6.3 Hz, n = 14). When large (300–400 pA) currents were injected, the firing rate adapted (Fig. 2B; top and 2nd traces). The adaptation was stronger when a larger current was injected (Fig. 2D).

In responses to hyperpolarizing current, the voltage sag was eliminated after application of I_h blocker, ZD7288 (50 μM, Fig. 2E). The voltage at the end of pulses of injected current (open circle in Fig. 2Ea and filled circle in Fig. 2Eb) was measured and then the current-voltage relationship was plotted in Fig. 2F, showing that the voltage response was increased significantly by the application of ZD7288 (n = 5; P < 0.05 by paired t-test).

Properties of mEPSCs and mIPSCs

Golgi cells are interspersed among granule cells. It has been suggested that they receive excitatory synapses from parallel fibers and some inhibitory synapses (Mugnaini et al. 1980a).

To examine the properties of excitatory and inhibitory receptors, mEPSCs and mIPSCs were recorded from Golgi cells using a CsCl-based internal solution (see Methods). Ten consecutively recorded traces were superimposed to show the occurrence of mEPSCs (Fig. 3Aa). These mEPSCs were blocked by DNQX (20 μM, Fig. 3Ab), indicating mEPSCs were mediated by AMPA receptors. Figure 3B shows ensemble averaged mEPSC from 30 events recorded from a single cell. The decay time course was fitted by a single-exponential function with a time constant of 1.10 ms. Amplitude, 10–90% rise times, and decay time constant of mEPSCs were plotted on histograms (Fig. 3, C–E).

When excitatory synaptic currents were blocked, we could record mIPSCs (Fig. 3F) (see Methods). mIPSCs were blocked by strychnine (1 μM), indicating that they were mediated through glycine receptors. Figure 3G shows an ensemble average of mIPSC from 30 events recorded from a single cell. The decay time course was fitted by a single exponential function and the time constant was 107 ms. Amplitude, 10–90% rise time, and decay time constant of mIPSCs were 31.9 ± 0.7 pA, 4.9 ± 0.7 ms, and 100 ± 3.5 ms, respectively (from 180 events, −70 mV holding potential, n = 4 cells).

EPSC evoked by the stimulation of ANFs and parallel fibers

We recorded EPSCs that were evoked by the stimulation of the auditory nerve root in the presence of strychnine and bicuculline. Figure 4A shows that evoked EPSCs have multiple peaks at a fixed stimulus intensity (24 V) recorded at holding potentials of −50 and +50 mV. EPSCs were blocked by DNQX (20 μM), indicating that they were mediated through AMPA receptors (n = 5). IPSCs were not evoked in ACSF containing DNQX, indicating that IPSCs are mediated through a polysynaptic pathway.

When the stimulus intensity was changed, several distinct current peaks were observed (Fig. 4B, 3.1 V). At larger stimulus intensity, the timing of the first two peaks became fixed (↑ and ▲, 7–13 V). Other peaks appeared at random intervals (Fig. 4B, some of them indicated by ○). The presence of distinct current peaks might indicate that Golgi cells are excited through multiple pathways. The responses with the shortest latencies might arise directly from ANFs, and the later responses might be mediated through excitatory interneurons. We measured the latencies of peaks of EPSCs from the stimulation artifacts while applying repetitive stimuli at constant intensity (Fig. 4, C and D). Ten consecutive traces were superimposed in Fig. 4Ca. An event histogram (Fig. 4Cb) was made by counting the peak time with 1-ms bin width from 70 consecutive records from one cell (Fig. 4Ca, ↑; 4Cb, ▲). The height of peaks in the histogram decreased progressively, indicating that the probability of later EPSCs was reduced progressively. The latency for the first EPSC ranged from 2.5 to 8.3 ms with a mean 4.7 ± 0.2 ms (n = 22). Ferragamo et al. (1998) reported a shorter latency for the first EPSP (~1.3 ms) to ANF stimulation than our observation (see Discussion).

To reduce the release probability of transmitters, the slice was incubated with BAPTA/AM, a membrane-permeable Ca^{2+} chelator. Under these conditions, the incidence of multiple peaks was reduced (Fig. 4D). After the primary EPSC, only few synaptic responses were observed (Fig. 4Da, ↑; 4Db, ▲). These observations, too, were consistent with late EPSCs...
being mediated through a polysynaptic pathway. Figure 4, E and F, shows histograms made from populations of cells in normal slices (control) and in slices after incubation with BAPTA/AM, respectively. The onset time of the first EPSC was defined as 0 ms. The event histogram of the control shows a prolonged occurrence of synaptic activities but not in the BAPTA/AM (Fig. 4F). Although each individual histogram had multiple peaks in the control, the accumulated histogram does not because the interval of peaks differed from cell to cell. The cumulative frequency plot of the event histogram shows that 80% of synaptic events occurred within 4 ms after BAPTA/AM; however, it took 17 ms in the control (Fig. 4G).

Figure 4H shows an EPSC evoked by the stimulation of parallel fibers (12 V, −70-mV holding potential). The latency was 1.4 ± 0.2 ms (n = 5) and was shorter than that of EPSC evoked by ANF stimulation (4.7 ± 0.4 ms, n = 22); this strongly suggests that Golgi cells are innervated by parallel fibers. The amplitude of EPSCs evoked by parallel fiber stimulation was 1.040 ± 85 pA (n = 5) and was 2.5-fold larger than that of EPSCs evoked by ANF stimulation (400 ± 78 pA, n = 7). Parallel fiber inputs were more synchronized and were robust than ANF inputs. This may indicate more importance in parallel fiber inputs; however, it might also be a consequence of a shorter distance of parallel fiber inputs and the disruption of long ANFs inputs.

Membrane hyperpolarization induced by an agonist for group II mGluRs

We tested the effect of DCG-IV under current clamp (Fig. 5, A and B). Bath application of DCG-IV (1 μM) induced a membrane hyperpolarization (10 mV; Fig. 5A) with the average hyperpolarization being −8.1 ± 0.4 mV (n = 4). Figure 5B shows a voltage response of control (a′) and during application of DCG-IV, after adjusting the membrane potential by current injection (b′). Input resistance measured at the peak voltage deflection was decreased to 85% of the control. This hyperpolarization may be due to an increase of K+ conductance because $E_K$ was −105 mV, whereas $E_{Cl}$ was −66 mV as calculated from the Nernst equation at 33°C. We tested the current response to DCG-IV in ACSF that contained 10 mM K+ and the $E_K$ of which was −69 mV by applying a series of voltage pulses from the holding potential of −50 mV. Application of DCG-IV induced a slight positive shift of the holding current (30 pA) and the current response to the step voltage change was increased (Fig. 5Cb). Figure 5Cc shows subtracted currents; i.e., currents in DCG-IV minus control at corresponding membrane voltages. Current-voltage relationship was plotted in Fig. 5E (●) after normalization by the cell capacitance (pA/pF). The amplitude of the DCG-IV-induced current was measured as the time average between 50 and 300 ms of the subtracted current. The current-voltage relationship demonstrated an inward rectification. The reversal potential was −66.2 ± 1.4 mV (n = 5) and was close to $E_K$ (−69 mV) in 10 mM K+−containing ACSF. Because group II mGluRs are known to be coupled with GIRK channels in cerebellar UBCs (Knollfach and Kemp 1998), we tested the effect of GDPβS. GDPβS is a nonhydrolysable analogue of GDP and is expected to suppress G-protein-mediated signaling. DCG-IV-induced currents were markedly reduced by loading GDPβS into the cells through the patch electrodes (Fig. 5De). The slope of the current-voltage relationship after normalization by the cell capacitance (○, n = 5) was significantly reduced by GDPβS (P < 0.05 at −40 and −80 mV, P < 0.01 at −50, −90 to −120 mV by unpaired t-test). Ba2+ is a blocker of inward rectifier K+ current (Hagiwara et al. 1976; Ohmori 1980), and DCG-IV did not induce any current in the presence of 200 μM BaCl2 (traces not shown, n = 3). These results indicate that group II mGluRs were coupled to GIRK channels.

Group II mGluRs-mediated afterhyperpolarization evoked by high-frequency synaptic input

Dendrites and somata of cochlear nuclear Golgi cells are immunoreactive for mGluR2/3 (Fig. 1Ac) (Jaarsma et al. 1998). In the cerebellum, parallel fiber stimulation at high frequency induced a long-lasting hyperpolarization of Golgi cells because of the activation of mGluR2 (Watanabe and Nakaniashi 2003). Therefore we measured the responses to parallel fiber stimulation at high-frequency (100 Hz, 10–30 train); strychnine, bicuculline, and atropine were included in ACSF. High-frequency stimulation (Fig. 6A; 100 Hz, a train of 10 stimuli) evoked action potentials followed by a long-lasting afterhyperpolarization; the hyperpolarization was significantly reduced by application of LY341495, a selective antagonist of group II mGluRs (1 μM, Fig. 6, A and B, P < 0.05 by paired t-test). Furthermore, we tested a possible occlusion of the long-lasting afterhyperpolarization by the activation of group II mGluRs by DCG-IV (Fig. 6, C and D). In DCG-IV, the hyperpolarized membrane potential was compensated by depolarizing current injection. The afterhyperpolarization was significantly reduced by application of DCG-IV (1 μM, Fig. 6D, P < 0.01 by paired t-test). The application of neither DCG-IV nor LY341495 affected the amplitude of EPSCs evoked by the parallel fiber stimulation (DCG-IV, 92 ± 17%, n = 5; LY341495, 102 ± 8%, n = 5). These results indicate that the afterhyperpolarization was induced by the activation of postsynaptic group II mGluRs.

Membrane hyperpolarization induced by cholinergic agonists

Golgi cells seem to be innervated by cholinergic fibers (Fig. 1F), leading us to test whether they respond to cholinergic agonists. Bath application of carbachol (100 μM) induced a membrane hyperpolarization (−7 mV), accompanied by a decrease in the input resistance (Fig. 7A). All cells tested were hyperpolarized (Table 1). Application of ACh (100 μM-1 mM) induced a similar response (traces not shown, Table 1).

Both nicotinic receptors (Happe and Morley 1998; Yao and Godfrey 1999a) and muscarinic receptors (Yao and Godfrey 1995; Yao et al. 1996) are expressed in the CN. However, bath application of nicotine (10 μM) did not affect the membrane potential or the input resistance of Golgi cells (Fig. 7B; n = 5). On the other hand, muscarine (30 μM) induced a membrane hyperpolarization (−6 mV), together with a decrease in the input resistance, mimicking the effects of carbachol (Fig. 7, C and D, Table 1). Furthermore, atropine (5 μM), an antagonist of muscarinic receptors, attenuated the muscarine-induced hyperpolarization (Fig. 7C; 5 of 5 cells tested). These results indicate that cholinergic responses were mediated through muscarinic receptors.
Current–voltage relationship of muscarine-induced current

The muscarine-induced current was measured under voltage-clamp. From a holding potential of –50 mV in ACSF that contained 10 mM K\(^+\), a series of voltage pulses was applied in the absence and presence of muscarine. Application of muscarine induced a positive shift of the holding current (20 pA) and the currents evoked by pulses were increased (Fig. 8Ab). Figure 8Ac shows subtracted currents at corresponding membrane voltages. Current-voltage relationship of the difference currents is plotted in Fig. 8C after normalization by the cell capacitance. The current-voltage relationship demonstrated an inward rectification. The reversal potential was \(-66.2 \pm 1.4\) mV (\(n = 6\)). The reversal potentials were plotted as a function of the external K\(^+\) concentration on a semi-logarithmic coordinate (Fig. 8D, ●). These plots were well fitted by the Nernst equation indicated by a straight line (Fig. 8D). Muscarine-induced currents were reduced markedly by intracellular application of GDP\(\beta\S\) (Fig. 8Bc). The current-voltage relationship showed a significant reduction by GDP\(\beta\S\) (Fig. 8C, ○, \(P < 0.05\) at \(-50, -60, -100,\) and \(-110\) mV, \(P < 0.01\) at \(-120\) mV by unpaired \(t\)-test). Muscarine did not induce current in the presence of 200 \(\mu\)M BaCl\(_2\) (traces not shown, \(n = 3\)). These results indicate that muscarinic receptors were coupled with GIRK channels in Golgi cells.

Slow membrane hyperpolarization blocked by atropine

The localization of VACHT-immunopositive puncta with Golgi cells (Fig. 1F) indicates that muscarinic receptors could be activated synaptically. Figure 9Aa shows superimposed responses in 10 mM K\(^+\)-containing ACSF to voltage pulses as indicated at the top. Aa, control; Ab, in muscarine; Ac, muscarine-induced currents (Ab minus Aa). B: current responses after loading the cell with GDP\(\beta\S\) (1 mM). The other experimental protocol was the same as in A. C and D: activation of GIRK channels. C: current-voltage relationship of muscarine-induced currents normalized by cell capacitance (●), showing inward rectification. ○, from cells loaded with GDP\(\beta\S\). Reduction of muscarine-induced current was significant (*\(P < 0.05\) and #\(P < 0.01\) by unpaired \(t\)-test). D: reversal potentials were plotted against \([\text{K}^+]_o\) on a semi-logarithmic coordinate. Straight line indicates the Nernst relationship.

**Table 1. Response amplitude of Golgi cells to cholinergic agonists**

<table>
<thead>
<tr>
<th>Agonist</th>
<th>Response Amplitude, mV</th>
<th>20–80% Fall Time of Hyperpolarization (s)</th>
<th>No. of Cells Tested</th>
<th>No. of Hyperpolarized Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACh (100 (\mu)M-1 mM)</td>
<td>(-5.9 \pm 0.5)</td>
<td>16 ± 1.0</td>
<td>7</td>
<td>7</td>
</tr>
<tr>
<td>Carbachol (100 (\mu)M)</td>
<td>(-6.4 \pm 0.7)</td>
<td>19 ± 2.2</td>
<td>8</td>
<td>8</td>
</tr>
<tr>
<td>Muscarine (30 (\mu)M)</td>
<td>(-6.2 \pm 0.4)</td>
<td>19 ± 1.4</td>
<td>28</td>
<td>26</td>
</tr>
</tbody>
</table>

Values are means ± SE. ACh, acetylcholine.
Muscarinic reduction of neuronal excitation

Figure 10A shows a series of records from a single cell. Figure 10Aa shows control responses to current pulses of varying strength and polarity (450, 50, and –50 pA; 300-ms duration). Small depolarizing current pulse (50 pA) caused the cell fired repetitively at 13.3 Hz (Fig. 10Aa; thicker trace). Application of muscarine (30 μM) induced a membrane hyperpolarization (Fig. 10Ab; 7 mV). A similar current pulse (50 pA) evoked only a single action potential (Fig. 10Ab; thicker trace). When the membrane hyperpolarization was compensated by current injection, the same depolarizing current pulse (50 pA) induced repetitive firing at ~10 Hz (Fig. 10Ac; thicker trace). The effects of muscarine on firing frequency are summarized in Fig. 10B. Figure 10Bb shows expanded plots. Application of muscarine significantly reduced the firing frequency at the current range of 20–100 pA (Fig. 10Bb; n = 6, *P < 0.05 in the range of 20–100 pA, between control and muscarine). At a large current injection, the firing frequency was not significantly different (P > 0.05 in the range of 150–450 pA). Figure 10C shows the input resistance measured by a small hyperpolarizing current injection (−50 pA); input resistance in the control (199 ± 14 ΩM; n = 6) was significantly (P < 0.05) larger than in the presence of muscarine (154 ± 14 ΩM; n = 6). After compensation for hyperpolarization of the resting potential by current injection, the input resistance was still smaller than under control conditions. The difference, however, was not significant (P = 0.12). Figure 10, D and E, shows the threshold potential and threshold current, measured from single action potentials evoked by a short current injection (3-ms duration, 10-pA increments). The threshold potential was not significantly different; however, the threshold current was significantly larger in the presence of muscarine (186 ± 31 pA; n = 8) than in control (78 ± 24 pA; n = 8; *P < 0.01). After compensation for the hyperpolarization of the resting potential by current injection, the threshold current became the same as the control. These results indicate that muscarine reduced the excitability of the Golgi cells by increasing the threshold current.

Muscarinic suppression of action potential generated by synaptic input

Activation of muscarinic receptors hyperpolarized the membrane and reduced the excitability of Golgi cells. Therefore the generation of action potentials by ANF stimulation might be suppressed by muscarine. When the membrane potential was maintained at −60 mV, action potentials were evoked. The
stimulus intensity was reduced to the minimum level required to evoke action potentials without failure (firing probability 1). Application of muscarine induced membrane hyperpolarization (−5 mV) and suppressed action potential generation (Fig. 11A); inset shows EPSPs after magnification. The firing probability calculated from 10 consecutive stimuli was significantly reduced to 0.1 ± 0.05 (Fig. 11B, filled bar; n = 6, P < 0.01 by paired t-test). In contrast, glutamatergic excitatory synaptic transmission itself was not affected by AChe application (100 μM); neither the peak amplitude of EPSCs evoked by ANF stimulation (106 ± 22%, n = 5) nor the frequency of spontaneous mEPSCs (110 ± 15%, n = 4) was affected.

**DISCUSSION**

Estimation of cell capacitance

We estimated Golgi cell capacitance in both current and voltage clamp and the results were 103 ± 6.6 and 31 ± 1.5 pF, respectively. Both of these estimates were larger than the calculation from the Golgi cell diameter (5.4 pF); assuming a spherical shape of the cell (13.0 ± 0.3 μm, n = 20) and the specific capacitance 1 μF/cm². Ferragamo et al. (1998) reported the membrane time constant of 5.1 ms in one Golgi cell with an input resistance of 131 MΩ. The capacitance could be 38 pF. This is close to our electrophysiological estimation and is larger than the estimate from cell diameter. Therefore all these electrophysiological experiments might indicate that a large fraction (84–95%) of the measured cell capacitance reflects the capacitance of dendrites and/or axons.

Similarity between cochlear nuclear and cerebellar Golgi cells

Golgi cells are inhibitory interneurons that would control firing in granule cells of the cerebellum and the CN. The firing in cochlear nuclear Golgi cells seems to be regulated much like in cerebellar Golgi cells (Dieudonné 1998; Watanabe and Nakanishi 2003). They fire repetitively and show spike frequency adaptation. Their excitability is regulated by the hyperpolarization-activated mixed cation conductance that underlies Iₚₙ and by GIRK channels. GIRK channels are in turn regulated through group II mGluRs.

Cerebellar Golgi cells are thought to perform a gain control function; the synaptic interaction between Golgi cells and granule cells is proposed to adjust the threshold for granule cell firing so that the local granule cell activity remains within some operational range (Albus 1971; Marr 1969). Cochlear nuclear Golgi cell axon terminals would make inhibitory synapses onto the granule cell dendrites, and these structures are the same as those observed in the cerebellum (Mugnaini et al. 1980a). Therefore cochlear Golgi cells may also have a function to control the output gain of granule cells. We need further evidence to clarify this point.

Synaptic inputs to Golgi cells

Stimulation of ANFs evoked multiple synaptic responses in Golgi cells. The earliest arrived after latencies of −5 ms. The later EPSCs occurred over periods that lasted >100 ms (Fig. 4). These findings are largely consistent with those of Ferragamo et al. (1998). These authors observed both sharply timed early synaptic responses and long-lasting depolarizations that lasted 30–120 ms in response to stimulation of ANFs. These authors also concluded that ANFs form both monosynaptic and/or polysynaptic connections with Golgi cells. However, the shortest latencies we observed differed, ranging 2.5–8.3 ms with a mean 4.7 ms (n = 22), whereas Ferragamo et al. reported −1.3 ms as the latency of the first EPSPs to stimulation of ANFs. This difference may be due to several factors, including the age of mice, the distance of stimulus to Golgi cells, and some disruption of monosynaptic connection by slicing.

Stimulus to parallel fibers, the axons of granule cells, also evoked EPSCs in Golgi cells (Fig. 4H). It is possible that these responses reflect direct excitation of Golgi cells by granule cells. Anatomical evidence indicates that Golgi cells receive inputs from parallel fibers and also from mossy fibers (Mugnaini et al. 1980a). Granule cells receive inputs from widespread areas of the brain associated with multiple sensory modalities, such as the cuneate nucleus, vestibular afferents, pontine nuclei, unmyelinated auditory nerve fibers, the octopus cell area of the VCN, the inferior colliculus, and the auditory cortex (Oertel and Young 2004). Some of these inputs terminate on granule cells as mossy fiber endings. One of the origins of mossy fibers is the lateral part of the cuneate nucleus, which mediates discriminative touch and proprioception reflecting the position of head and pinna (Ryugo et al. 2003). Multimodal information is likely to be transmitted to Golgi cells via parallel fibers and possibly via mossy fibers.

The decay time constant of spontaneous mEPSCs recorded in Golgi cells was 1.1 ± 0.06 ms (Fig. 3E). Gardner et al. (1999) studied mEPSCs systematically in the CN and found a correlation between the decay time constant and the type of synaptic inputs. They reported that mEPSC recorded in bushy, octopus, T stellate, and tuberculoventral cells have significantly faster decay time constants (0.35–0.40 ms) than those recorded in fusiform and cartwheel cells (1.32–1.99 ms). The former four cell types receive their excitatory input mostly from ANFs, and the fast decay time constants might contribute to encoding the timing information (Trussell 1999). The latter two cell types are targets of parallel fibers. The mEPSCs recorded in Golgi cells were slower than those in neurons of...
the VCN and similar to those measured in the targets of parallel fibers.

Golgi cells are contacted by inhibitory synaptic boutons (Mugnaini et al. 1980a, and Ferragamo et al. 1998) recorded glycinergic IPSP from Golgi cells. They suggested that the origin of the IPSP was D stellate cells, which receive ANF innervation and project to granule cell lamina (Oertel et al. 1990). We speculate that absence of evoked IPSC in ACSF containing DNQX was due to blocked synaptic transmission between ANFs and D stellate cells. Although we have not studied inhibitory inputs systematically in Golgi cells, the observation of glycinergic mIPSC (Fig. 3F) was consistent with previous reports (Oertel et al. 1990).

**Origin of the cholinergic projection**

Cholineric inputs to the CN originate from the superior olivary complex and projections are made through the olivocochlear bundle and trapezoid body (Godfrey et al. 1987a,b). These pathways originate from the medial olivocochlear cells and small cells in the ventral nucleus of the trapezoid body and form a part of the auditory efferent projection (Sherriff and Henderson 1994; Smith and Spirou 2001). Therefore the cholineric inputs we observed are likely to arise from these cells (Figs. 1F and 9).

**Possible physiological roles of group II mGluRs and muscarinic receptors**

We demonstrated that group II mGluRs were coupled with GIRK channels in cochlear Golgi cells. Similar coupling was reported in Xenopus oocyte expression system (Saugstad et al. 1996; Sharon et al. 1997), cerebellar UBCs (Knoflach and Kemp 1998), and cerebellar Golgi cells (Watanabe and Nakaniishi 2003). Cerebellar UBCs were immunoreactive for anti-mGluR2/3 antibody (Jaarsma et al. 1998), and an agonist for group II mGluRs activated GIRK channels (Knoflach and Kemp 1998). In hippocampal pyramidal cells and lateral parabrachial neurons, GIRK channels are activated by G-protein-coupled receptors (Andrade et al. 1986; Christie and North 1998). We also demonstrated that muscarinic receptors and group II mGluRs activated GIRK channels in cochlear nuclear Golgi cells; this suggests that these two metabotropic receptors and their signaling pathways converge in the activation of GIRK channels.

In cerebellar Golgi cells, facial stimulation is known to evoke action potentials and was followed by a long-lasting decrease of firing in vivo (Vos et al. 1999); this silencing of firing was induced by the activation of postsynaptic mGluR2 (Watanabe and Nakaniishi 2003). We demonstrated an afterhyperpolarization that followed high-frequency stimulation of parallel fibers (Fig. 6). This afterhyperpolarization would be expected to result in the decrease of firing in cochlear nuclear Golgi cells. The other projection of olivocochlear efferent neurons (Sherriff and Henderson 1994; Smith and Spirou 2001) may lead to cholinergic suppression of Golgi cells. Therefore Golgi cells are likely to play some crucial roles in modulating the granule cell activities by integrating variety of inputs; leading to the disinhibition of granule cells and then through the activity of parallel fibers, the modulation of principal cells in the DCN.

**ACKNOWLEDGMENTS**

We thank Dr. Donata Oertel for carefully reading the manuscript and for valuable comments and suggestions, Drs. S. Nakanishi and D. Watanabe for the transgenic mice, Dr. H. Kubo for reading the manuscript, Drs. T. Kaneko and K. Nakamura for the antibody and for technical assistance in immunohistochemistry, and M. Fukao for machining the equipment.

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**GRANTS**

This study was supported by the Grants-in-Aid (17023027–0133) from the Ministry of Education to H. Ohmori.

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