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Calcium is an intracellular mediator of the climbing fiber in induction of cerebellar long-term depression

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ABSTRACT **In cerebellar Purkinje cells, conjunctive stimulation of parallel fibers and the climbing fiber causes long-term depression of parallel fiber–Purkinje cell transmission. It has been postulated that calcium is an intracellular mediator of the climbing fiber to induce this synaptic modification. To directly test the hypothesis, a calcium-chelating agent, EGTA, was intracellularly injected into Purkinje cells. In these injected cells, conjunctive stimulation failed to induce depression. Instead, it caused potentiation similar to that observed after repetitive stimulation of parallel fibers alone.**

A unique and characteristic feature of the cerebellar cortical network structure is that the final output neuron, the Purkinje cell (PC), receives two distinct types of excitatory afferents: numerous parallel fibers (PFs), which are axons of cerebellar granule cells; and a single but powerful climbing fiber (CF), which derives from the inferior olive (1, 2). Synapses supplied by PFs to PCs exhibit prominent activity-dependent modifiability. A long-term depression (LTD) is induced by conjunctive stimulation of PFs with a CF (3–5), whereas a potentiation is produced by repetitive stimulation of PFs alone (5). This modifiability of PF–PC synapses would explain the role of the cerebellum in motor learning (1, 6).

Involvement of calcium in induction of LTD has been postulated (1, 3–5) from the findings that the CF response is associated with a large amount of calcium influx to PCs (7–9) and that strong postsynaptic inhibition of PC dendrites, which presumably depresses the calcium influx, prevented the induction of LTD (4). However, there has been no direct evidence for this hypothesis. Here, I report that when EGTA was injected into PCs, conjunctive CF–PF activation no longer produced LTD but, rather, elicited a potentiation. This observation suggests that calcium chelation by EGTA cancels the action of CF impulses in inducing LTD, consequently leaving the same potentiating effect as with PF stimulation alone (5). The present results provide direct evidence for a role of calcium in induction of LTD.

MATERIALS AND METHODS

Parasagittal slices (each 300–330 μm thick) were prepared from guinea pig cerebellar vermis and maintained *in vitro* as described (5). Slices were prepared using a Microslicer (Dosaka EM, Kyoto) and preincubated for >1 hr in a standard medium containing 124 mM NaCl, 5 mM KCl, 1.24 mM KH_2PO_4, 1.3 mM $MgSO_4$, 1.24 mM $CaCl_2$, 26 mM $NaHCO_3$, and 10 mM glucose and equilibrated with a 95% O_2/5% CO_2 gas mixture. The temperature was kept constant at 36°C.

Intracellular recordings were made from PC somata or proximal dendrites. PCs were identified by their characteristic CF responses and/or antidromic spike responses (2). A stimulating electrode was placed at the superficial molecular layer for stimulating PFs with positive–negative current pulses. Another stimulating electrode was inserted into white matter for stimulating CFs. Since stimulation of the white matter often elicited inhibitory postsynaptic potentials (IPSPs) in PCs via a pathway through mossy fibers, granule cells, and inhibitory interneurons (5), picrotoxin (40 μM) was added to the medium to eliminate possible influences of the inhibition on LTD (4).

EGTA was injected (10) into a PC iontophoretically through a recording microelectrode containing 2.0 M potassium acetate and 0.5 M K_2EGTA. Hyperpolarizing currents were applied in the form of steps of 0.5–0.8 nA in amplitude and 0.6 s in duration, intermittently once every 1 s for 10–15 min. The same amount of current was injected through an electrode filled with 2.5 M potassium acetate alone as a control. Since it took >15 min to obtain baseline responses after EGTA injection, the onset time of the EGTA injection and that of LTD testing were separated by >30 min, which allowed sufficient diffusion of EGTA throughout the dendrites.

RESULTS

Only cells with resting membrane potentials more negative than −55 mV and CF responses larger than 50 mV in amplitude were used. When the resting membrane potential was less negative than −60 to −65 mV, small hyperpolarizing currents (<1.2 nA, constant through an impalement) were passed through a recording electrode to avoid spontaneous membrane activities and prevent spike discharges from being triggered by PF-mediated excitatory postsynaptic potentials (EPSPs). Pulses applied to PFs were usually adjusted to 10–40 μA in amplitude and 200–300 μs in duration to evoke EPSPs of 5–8 mV in amplitude in PCs. Slices in which current pulses of >60 μA were required to evoke EPSPs of this amplitude range were not used. Seven EGTA-injected cells and six control cells satisfied the above criteria through a sufficient span of time for the present study.

EGTA injection did not produce a significant effect on PF-evoked responses. The amplitudes of PF-mediated EPSPs were in the same range of 5–8 mV in both groups. With regard to the time course of PF-mediated EPSPs, the time to peak was 7.2 ± 1.3 ms in EGTA-injected cells and 7.7 ± 1.4 ms in control cells ($P > 0.5$, Student's t test; mean ± SD); the half-width (duration of the EPSP at half-maximal amplitude) was 35.1 ± 7.2 ms and 37.6 ± 9.0 ms, respectively ($P > 0.7$).

EGTA injection did not affect the peak amplitude of CF responses, which was 67.2 ± 8.1 mV in EGTA-injected cells and 70.0 ± 5.1 mV ($P > 0.5$; mean ± SD) in control cells. However, the half-width of CF responses tended to be longer in EGTA-injected cells (4.73 ± 0.81 ms) than in control cells (3.80 ± 0.81 ms), though the difference between these did not

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Abbreviations: PC, Purkinje cell; PF, parallel fiber; CF, climbing fiber; LTD, long-term depression; IPSP, inhibitory postsynaptic potential; EPSP, excitatory postsynaptic potential.

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reach a significant level ($0.1 > P > 0.05$). This slight prolongation of CF response could be explained by suppression of calcium-dependent processes that may contribute to repolarization of CF response, such as activation of calcium-sensitive potassium (11) and chloride (12) channels and inactivation of calcium channels (13). Another obvious effect of EGTA injection on CF responses was disappearance or diminution (Fig. 1*E*) of the hyperpolarization that slowly developed in PCs after conjunctive stimulation (Fig. 1*B*) (5). This observation suggests that hyperpolarization is calcium dependent and ensures that EGTA was effectively injected in the way presently employed.

The same conjunction paradigm as in the previous study (5) was employed: CF and PFs were stimulated conjointly at 4 Hz for 25 s, with CF stimulation preceding PF stimulation by 10 ms. In control cells, conjunctive stimulation induced LTD (Fig. 1 *A* and *C*) in five of six cells examined (Fig. 2), as previously demonstrated (3–5). By contrast, in EGTA-injected cells, conjunctive stimulation of a CF and PFs failed to produce LTD of PF-mediated EPSPs. Instead, it potentiated PF-mediated EPSPs (Fig. 1 *D* and *F*). The potentiation was observed in six of seven cells (Fig. 2; $P < 0.001$, in a two-way analysis of variance, program package SAS GLM). Similar to the potentiation induced by stimulation of PFs alone (5), the potentiation was not associated with changes in the input resistance (Fig. 1 *A* and *C*; 19.6 ± 1.6 MΩ, before conditioning and 19.5 ± 3.0 MΩ, 6–15 min after conditioning; $P > 0.8$) or the membrane potentials (68.7 ± 2.3 mV and 68.5 ± 2.1 mV, respectively; $P > 0.8$).

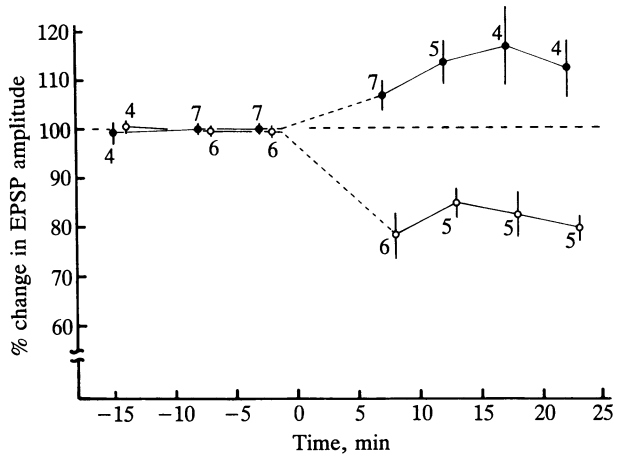

FIG. 2. Plots of amplitude of PF-mediated EPSPs before and after conjunctive stimulation. ●, EGTA-injected cells; ○, control cells. Time 0 indicates the end of conjunctive stimulation. Vertical bars represent ±SEM. The values at each plotted point represent the number of cells involved in the measurements. Student's *t* test shows a significant difference between test and control groups: $P < 0.001$ ($t = 10$ min), $P < 0.005$ ($t = 15$, 20, and 25 min).

DISCUSSION

Previous studies indicate that there are abundant voltage-gated calcium channels over PC dendrites (8) and that a considerable amount of calcium entry is associated with CF activity (7–9). On these bases, it has been hypothesized that a large depolarization evoked by an excitatory action of a CF activates voltage-gated calcium channels, through which calcium flowed into PC dendrites; in turn, this would trigger intracellular processes, in concert with simultaneously active PF synapses, presumably with activated quisqualate-type receptors (14), leading to LTD (1, 3). It has been shown that strong inhibition of PC dendrites through activation of stellate cells (15) during conjunctive stimulation blocks the induction of LTD (4). This suggests that induction of LTD requires

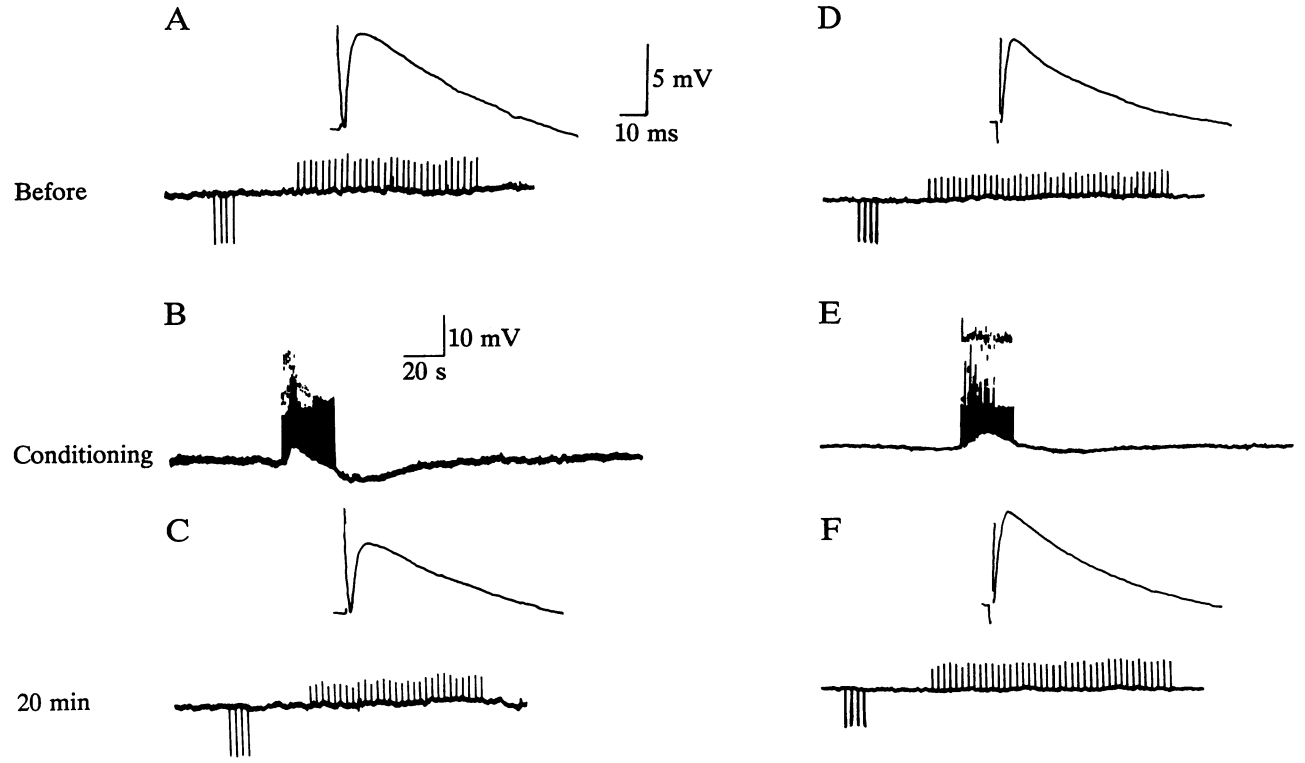

FIG. 1. Effects of conjunctive CF–PF stimulation (4 Hz for 25 s) in control (*A–C*) and EGTA-injected (*D–F*) cells: Strip chart records of the membrane potential of PCs, several minutes before, during, and 20 min after conditioning, respectively. In *A*, *C*, *D*, and *F*, upper deflections indicate PF-mediated EPSPs, and downward deflections indicate hyperpolarization induced by intracellular injection of a dc current step (0.5 nA, 200 ms). The respective averaged EPSPs are shown in the *Insets*. The calibration bar in *B* is for chart records in *A–F*; that in *A* is for averaged EPSPs in *Insets* in *A*, *C*, *D*, and *F*.

sufficient depolarization of postsynaptic PCs; this effect has been assumed to be due to depression of calcium-dependent dendritic potentials associated with CFs. The present study provides direct evidence for this view. Thus, the CF would act as a potent calcium injector to PC dendrites and, in turn, calcium would play the role of intracellular messenger of a CF in induction of LTD. Therefore, when calcium is chelated by EGTA, the action of CF input would be canceled at the level of the second messenger. Under this condition, conjunctive stimulation would have the same effect as repetitive stimulation of PFs alone. This accounts for the finding that conjunctive stimulation elicited potentiation in EGTA-injected cells.

The hippocampus is also believed to play an important role in learning and memory (cf. ref. 16), where marked synaptic plasticity, long-term potentiation (LTP), has been demonstrated (17, 18). It is suggested that calcium plays a key role in induction of LTP (10). In the hippocampal CA1 area and dentate gyrus, it is suggested that calcium influx leading to LTP is mediated by *N*-methyl-D-aspartate (NMDA) receptor-coupled channels (19). However, the contribution of NMDA receptor-coupled channels would be very small, if present at all, in the cerebellar cortex, because adult PCs are almost devoid of NMDA sensitivity (20, 21). Thus calcium is commonly involved in the mechanism of memory formation in the mammalian brain in the cerebrum and cerebellum, although the signals mediated by calcium and the outcome produced by it are quite different in the two structures.

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