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IPSPs Strongly Inhibit Climbing Fiber–Activated $[Ca^{2+}]_i$ Increases in the Dendrites of Cerebellar Purkinje Neurons

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The interaction between the excitatory climbing fiber (CF) response and stellate cell inhibition was studied in guinea pig Purkinje cells in sagittal slices from the cerebellar vermis. Sharp microelectrode recordings from the soma or dendrites were combined with high-speed fluorescence imaging of intracellularly injected fura-2. In this way both the electrical responses and the associated $[Ca^{2+}]_i$ changes could be monitored at the same time. Usually simultaneously activated inhibition caused almost no change to the somatically recorded CF response. However, the inhibition caused a strong reduction in the CF-associated $[Ca^{2+}]_i$ increase which normally was widespread in the dendrites. This effect was graded; stronger inhibition caused a larger and more widespread reduction in the $[Ca^{2+}]_i$ change that was greatest in the more distal dendrites. Sometimes the reduction was over 90% in the distal dendrites and occasionally it was localized to only a single dendritic branch. Both the inhibitory postsynaptic potential (IPSP) and the associated reduction in the CF-induced $[Ca^{2+}]_i$ change were blocked by bicuculline, a $GABA_A$ receptor antagonist.

Dendritic recordings showed that each CF response evoked a 2–3 msec wide action potential. The amplitude of this action potential was reduced in a graded manner by the IPSP in parallel with the reduction in the $[Ca^{2+}]_i$ change. Varying the time between the activation of the IPSP and the CF response showed that both the reduction in the $[Ca^{2+}]_i$ change and the action potential amplitude occurred in a narrow time window of about 8–10 msec, about the rise time of the IPSP. Together these results indicate that the CF response activates a fast dendritic Ca^{2+} spike that causes most of the $[Ca^{2+}]_i$ increase, both of which can be blocked by an inhibitory shunting conductance. This interaction provides a means whereby Ca^{2+}-dependent dendritic mechanisms can be modulated without affecting the immediate output of the Purkinje cell.

[Key words: Purkinje cell, climbing fiber, dendrite, calcium, inhibition, stellate cell, fura-2, imaging]

The climbing fiber (CF) synapse on Purkinje cells is unusual. Activation of this single distributed synapse evokes a large, all-or-none excitatory postsynaptic potential (EPSP) that causes a burst of several action potentials in the Purkinje cell axon. Several functions have been suggested for the CF synapse. One role, based on the possibility that the Purkinje cell is an important locus of plasticity in cerebellar function, suggests that CF activation plays an instructional role in modifying future Purkinje cell excitability (Marr, 1969; Albus, 1971). A more detailed incarnation of this idea proposes that the CF input generates a rise in intracellular calcium concentration ($[Ca^{2+}]_i$) that can combine with parallel fiber (PF) activation to produce long-term depression (LTD) of subsequent parallel fiber EPSPs (Ito, 1989). A second proposed function (e.g., Llinás, 1984) stresses the role of the CF as a cerebellar afferent, whose most important function is to relay and amplify a CF spike through the Purkinje cell with a burst of action potentials to the cerebellar nuclei. A third view of the CF response, less concerned with the broader issues of cerebellar function, has also been proposed (Llinás, 1984; Hounsgaard and Midtgaard, 1989). This view suggests that CF activity prevents the firing of Na^+ spikes in the time interval immediately following the CF response, possibly by activating one or more Ca^{2+}-dependent K^+ conductances. These views are not mutually exclusive.

The large CF synaptic current evokes a regenerative response in the Purkinje cell. The exact nature of this response has not been completely determined, but it clearly includes a Ca^{2+} component in the dendrites (Ross and Werman, 1987). Because the CF voltage response is so large and regenerative, it is relatively insensitive to IPSPs or PF-generated EPSPs. Indeed, our own recordings (as illustrated in this paper) show almost no modification to the somatically recorded CF response by simultaneously active stellate cell inhibitory inputs. However, it is possible that significant interactions between these GABA-mediated IPSPs and the CF response take place on the dendrites of the Purkinje cell without being reflected in the somatic recordings or the immediate output of the cell. These interactions might involve modulation of the $[Ca^{2+}]_i$ changes caused by the CF synapse as well as changes in the electrical response. This kind of interaction was suggested by Ekerot and Kano (1985) in their analysis of LTD in the rabbit cerebellum. They suggested that LTD could be prevented by simultaneously activating CF inputs and off-beam PF fiber pathways that activate mainly inhibitory inputs to the Purkinje cell. This proposal is consistent with earlier experiments by Hounsgaard and Yamamoto (1979), who showed that GABA could prevent the generation of CF-induced Ca^{2+} spikes in Purkinje cell dendrites. More directly, Midtgaard (1992) recently demonstrated that stellate cell activity in the turtle cerebellum can block the generation of Ca^{2+} action potentials.

These ideas can be explored by directly examining $[Ca^{2+}]_i$

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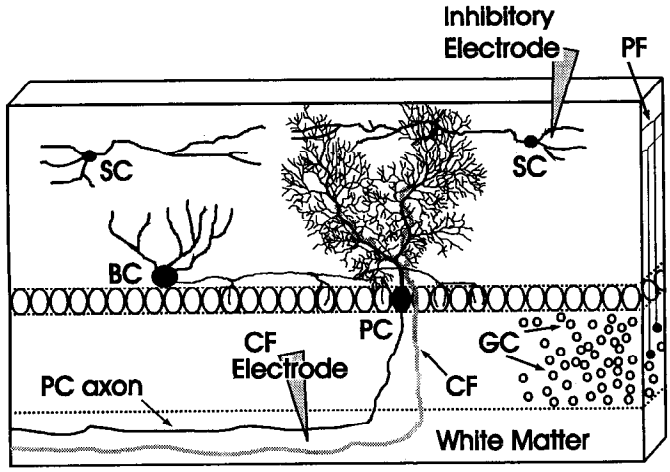

Figure 1. Schematic diagram of the arrangement of electrodes for most of these experiments. The electrode over the white matter was used to stimulate the CF input. The electrode to the side of the Purkinje cell near the edge of the molecular layer near the pial surface was used to stimulate the IPSP. It was positioned after the shape of the cell was known from observing the fura-2 fluorescence. In most experiments intracellular recording and dye injection were from a microelectrode in the soma (not shown). For some experiments the dendrites were impaled. *PC,* Purkinje cell; *SC,* stellate cell; *BC,* basket cell; *GC,* granule cell; *PF,* parallel fiber.

changes in Purkinje cells caused by interacting synaptic events. Previous imaging experiments in acute slices (Ross and Werman, 1987; Konnerth et al., 1992; Miyakawa et al., 1992; Midtgaard et al., 1993) demonstrated that there is a large, widely distributed, transient increase in $[Ca^{2+}]_i$ in the dendrites associated with each CF response. The spatial distribution of these $[Ca^{2+}]_i$ changes is not constant. One factor affecting this distribution is an A-like potassium conductance intrinsic to the dendritic membrane (Chan et al., 1989; Miyakawa et al., 1992; Midtgaard et al., 1993). These observations suggest that other dendritic conductances, activated synaptically, also could influence the CF-activated $[Ca^{2+}]_i$ changes.

To test this possibility we characterized the interactions between the CF response and IPSPs in sagittal slices from the guinea pig cerebellum. $[Ca^{2+}]_i$ changes were measured with high-speed fluorescence imaging (Lasser-Ross et al., 1991) of individual Purkinje cells filled with fura-2 (Grynkiewicz et al., 1985) while simultaneous intracellular recordings were made from either the soma or the dendrites. The results show that these IPSPs can dramatically attenuate the amplitude of $[Ca^{2+}]_i$ changes associated with the CF response without affecting the somatically recorded electrical response. In addition, dendritic recordings reveal that the reduction in Ca^{2+} entry is due to the prevention of a CF-activated Ca^{2+} spike by the inhibitory conductance. The results are consistent with both an instructional role and relay role for the CF synapse since the $[Ca^{2+}]_i$ changes can be regulated without modulating the output of the cell.

Parts of this work have been presented in abstract form (Callaway et al., 1992).

Materials and Methods

Sagittal slices, 150 μm thick, from the cerebellar vermis of young (3–5 week, 250–350 gm) Hartley guinea pigs (Charles River Laboratories, Wilmington, MA) were prepared as previously described (Llinás and Sugimori, 1980a; Lev-Ram et al., 1992). They were placed in a chamber with a glass coverslip bottom, and held in place with a single piece of platinum placed across the tissue away from the region of interest. Near, but not covering, the target region, small pieces of coverslip kept the slice flat. Submerged slices were maintained at 30–33°C and continuously superfused with standard Krebs saline of the following composition (in mM): 124 NaCl, 5 KCl, 1.2 NaH_2PO_4, 2.4 $CaCl_2$, 1.3 $MgSO_4$, 26 $NaHCO_3$, 10 glucose, pH 7.4, equilibrated with 95% O_2–5% CO_2 (Llinás and Sugimori, 1980a). For some experiments 10 μM bicuculline methiodide (RBI, Natick, MA) or 1 μM tetrodotoxin (TTX, Sigma, St. Louis, MO) was added to this solution.

The chamber was mounted on the stage of an inverted microscope (IMT-2F, Olympus, Cherry Hill, NJ). A dissecting microscope was attached to and aligned with the inverted microscope to view the same field as the substage objective. The dissecting microscope was used to view the slice from above and position the intracellular and stimulating electrodes.

Glass microelectrodes were pulled from 1.5 mm o.d., thick-wall, Ω-dot capillary tubing (#1511-M, Glass Company of America, Bargaintown, NJ). The tips were filled by capillary action with 2–4 mM fura-2 free acid (#F-1200, Molecular Probes, Eugene, OR) dissolved in 200 mM KAc, and the shanks were backfilled with 4 M KAc. Typical starting electrode resistances were 80–120 MΩ. The electrometer was a Dagan 8100 operated in the bridge mode. Bipolar stimulating electrodes were made from 100 μm diameter tungsten wire that was insulated except for the etched tip. The end of one wire was extended slightly in front of the second to cause a more focused stimulation. All electrodes approached the slice almost vertically and were connected to hydraulic micromanipulators (Narashige, Japan) that were mounted on the stage of the inverted microscope. In this way impalements and electrode positions could be preserved while changing focus or the field of view.

Microelectrodes were tracked through the slice at either the cell body layer or in the molecular layer up to two-thirds the distance to the pial surface from the cell body layer. In general, we ignored cells in the upper half of the slice and impaled cells in the lower half. This choice improved the visibility of the fluorescence from the fura-2-filled cells by reducing light scattering from the intervening cells in the light path.

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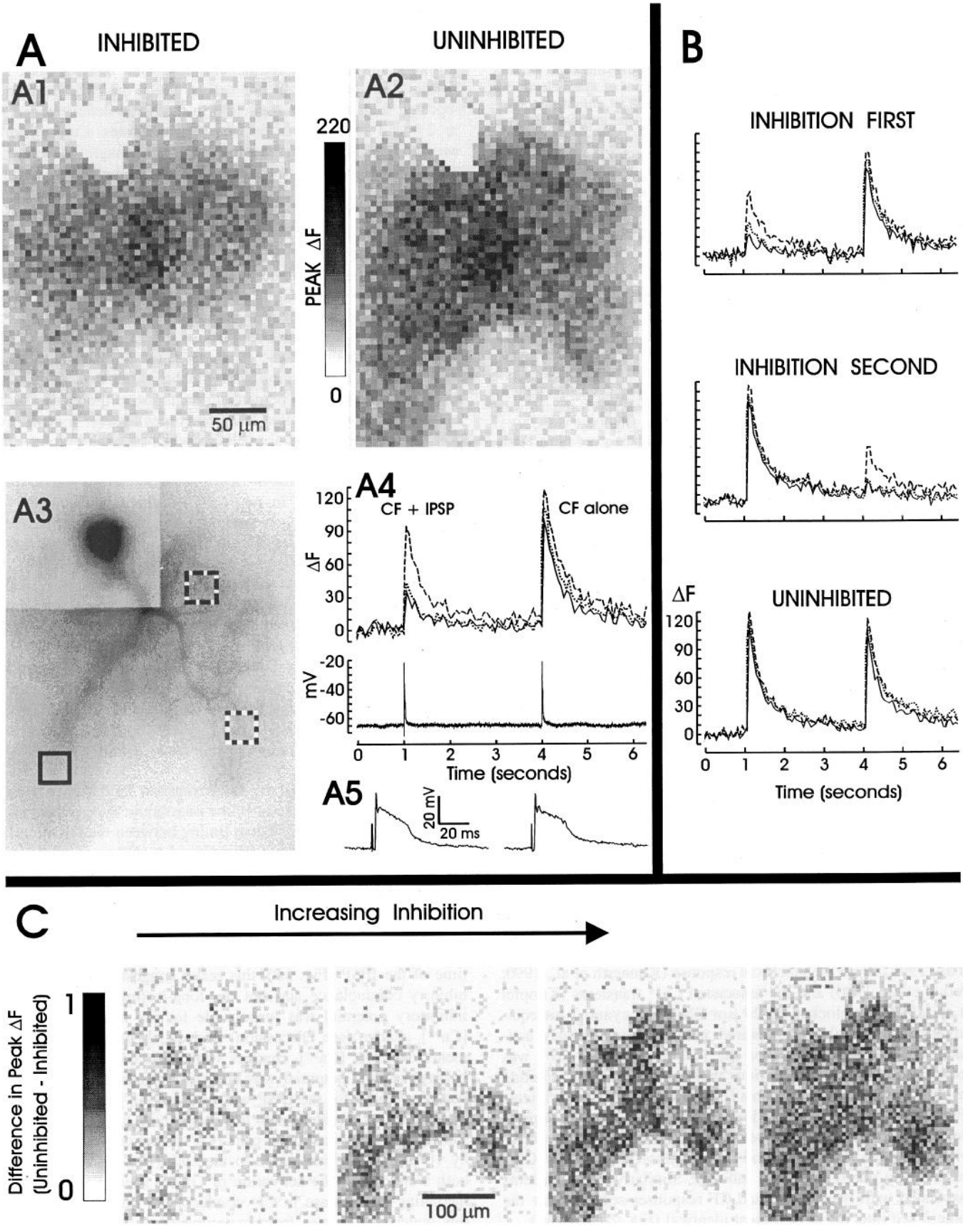
Figure 2. IPSPs reduce the magnitude of CF-induced fura-2 fluorescence changes in the dendrites. *A3,* An unbinned fluorescence image of the fura-2-filled Purkinje neuron (380 nm excitation). Two images are combined together to show both the somatic and dendritic regions in focus. *A4,* Time-dependent fluorescence changes from the one proximal and two distal regions selected by boxes on the image of the cell. The *solid, dashed,* and *dotted traces* correspond to the *solid, dashed,* and *dotted boxes.* Below the fluorescence traces is the time course of the somatically recorded electrical signal. The two CF responses at 1 and 4 sec are shown expanded below these traces in *A5.* At the time of the first CF response an inhibitory stimulus was also applied (*CF + IPSP*). During the second response no inhibitory stimulus was given (*CF alone*). At all three locations the fluorescence change was smaller during the first stimulus, while the electrical response was about the same in both cases. *A1,* Gray-scale image of the change in spatial distribution of fura-2 fluorescence between a time just before the first stimulus and the time of the peak of this response. *A2,* The spatial distribution of ΔF for the second response. The inhibited response has a smaller and more spatially restricted fluorescence change. The lack of response in the cell body is because the camera was saturated at that location. *B,* The effect of inhibition is independent of the order of stimulation. The traces show the time course of the fluorescence changes from the same three regions as in *A3.* In the *top panel,* inhibition was paired with the first CF stimulus. In the *middle panel,* inhibition was paired with the second CF stimulus. In the *bottom panel,* no inhibitory stimulus was given. *C,* The effect of inhibition is graded and strongest in the most distal dendrites. Each gray-scale image shows the difference between the CF responses with and without inhibition; that is, like the difference between images *A2* and *A1.* Thus for the *first panel,* with a very weak inhibitory stimulus, there was almost no fluorescence reduction, the difference was small, and the image is almost white. With increasing intensity of the inhibitory stimulus the reduction in fluorescence occurs first in the distal dendrites and later in the proximal dendrites. Image scales are normalized (0–1) to the maximum difference for this set of trials.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After impaling a Purkinje cell the indicator was iontophoresed into the neuron with a steady hyperpolarizing current (usually less than 1 nA) for 15–30 min. At the end of this period the current could be reduced without spontaneous firing of Ca^{2+} or Na^{+} spikes.

Stimulating electrodes were positioned just above the slice without making contact with the tissue, allowing movement of these electrodes after impalement. The electrode for CF activation or antidromic stimulation was positioned over the white matter near the granule cell layer and opposite the Purkinje cell soma. After filling the cell with fura-2, and when the dendritic arbors were visible, the electrode evoking IPSPs was positioned over the molecular layer near the pial surface and "off beam" from the impaled neuron (see schematic drawing, Fig. 1). Usually a location could be found where the IPSPs showed little apparent contamination from simultaneously evoked PF EPSPs in somatic recordings. Because of the distal location of this electrode we assume that the majority of activated interneurons were stellate cells, although we have no independent evidence for this assumption. Stimulating pulses were 100–200 μsec in duration. The intensity for CF activation was adjusted to be just suprathreshold for the all-or-none CF response. The intensity of the inhibitory stimulation depended on the experiment.

$[Ca^{2+}]_i$ measurements. Fura-2 fluorescence was viewed through a 20×, 0.75 NA dry fluorescence objective (Nikon, Garden City, NY). With this lens the entire dendritic arbor was within the camera field and we could focus through to the top of the slice. The excitation light at 380 nm (10 nm FWHM) was from a 75 W Xenon lamp regulated with a standard power supply (Model 1600, Opti-quip, Highland Mills, NY). Emission light was selected with a 475 nm long-pass glass filter. Static pictures and high-speed image sequences were detected with a cooled CCD camera system (Photometrics, Tucson, AZ) using techniques modified from those previously described (Lasser-Ross et al., 1991). In this system, digitized image sequences were directly stored in the memory of a Gateway 486-25 computer through an AT200 camera controller (Photometrics). Synchronized electrical measurements were digitized with a stand-alone (Iotech, Cleveland, OH) ADC488 analog-to-digital convertor and later transferred to the computer. The camera head contained a half-masked Thompson 7883 CCD chip operated in the frame-transfer mode. With the 20× objective each of the exposed 288 × 384 pixels detected light from exactly 1 μm^2 in the slice. In a typical experiment groups of 5 × 5 pixels were binned together (Lasser-Ross et al., 1991) and 200 pictures were recorded with a frame interval of 30–35 msec.

For these experiments we were mostly interested in the change in the CF-evoked $[Ca^{2+}]_i$ transient caused by the inhibitory input to the cell. The important information in this kind of experiment could be derived from a comparison of the changes in fura-2 fluorescence (ΔF) or fractional change ($\Delta F/F$) under different experimental conditions, without actually converting these values to $[Ca^{2+}]_i$. Leaving the records in these forms avoided problems with calibration and corrections due to slice autofluorescence (Lev-Ram et al., 1992). To correspond with physiological expectations a reduction in fluorescence intensity is plotted upwards in all figures.

Results

Climbing fiber activation causes a large, widespread increase in $[Ca^{2+}]_i$ in Purkinje cell dendrites that rapidly recovers to baseline levels (Fig. 2*A2*; see also Ross and Werman, 1987; Miyakawa et al., 1992). Both the electrical response (Konnerth et al., 1990; Perkel et al., 1990) and the associated Ca^{2+} transients (Knopfel et al., 1990) are blocked by 20 μm CNQX (6-cyano-7-nitroquinoxaline-2,3-dione). When paired with a simultaneously activated IPSP the magnitude of the $[Ca^{2+}]_i$ change ($-\Delta F$) was reduced at all dendritic locations without significantly affecting the time course of the transients (Fig. 2*A1*). Control experiments (Fig. 2*B*) showed that the reduction was not due to the depression of the response in the second trial since pairing the IPSP with either the first or second CF response reduced the Ca^{2+} transient by about the same amount. Similarly, the transients associated with two uninhibited CF responses separated by the same 3 sec interval were almost identical (Fig. 2*B*, bottom).

Previously (Miyakawa et al., 1992), we noted that the spatial distribution of the $[Ca^{2+}]_i$ changes following repetitive stimulation was variable. However, in cells with resting potentials below −60 mV and no spontaneous activity the magnitude of the reduction in peak $[Ca^{2+}]_i$ amplitude was quite reproducible if the cell was stimulated at frequencies less than 0.5 Hz (Fig. 2*B*, bottom). For example, in one cell in which the experiment was repeated four times the reduction in the distal dendrites was 93 ± 2% and 76 ± 2% in the proximal dendrites. Consequently, we could design experiments to compare the magnitude and spatial distribution of the transient reduction under different stimulation paradigms. When an IPSP was evoked with low stimulus intensity concurrently with the CF activation, the reduction in peak $[Ca^{2+}]_i$ was mostly in the distal dendrites (Fig. 2*C*, left panel). As the inhibitory stimulus intensity was increased the reduction in the distal dendrites increased. In addition, there was a reduction in peak $[Ca^{2+}]_i$ at more proximal locations. This trend continued with even higher stimulus intensities. In some cells we could eliminate over 90% of the CF-associated $[Ca^{2+}]_i$ increase at distal dendritic locations (Fig. 3). Transients measured in the somatic region were much smaller than those from the dendrites. Also, the percentage reduction at the soma caused by inhibition was smaller and sometimes absent. Twenty-five cells were analyzed. In six cells in which the inhibition in the distal dendrites was greater than 50% and less than 90% the reduction in the soma was 8–18% and the reduction 30 μm from the soma was 6–22%. In two cells in which inhibition in the distal dendrites was greater than 90%, the reduction in the soma was 27 and 30%, and at 30 μm from the soma the reduction was 30 and 40%.

This pattern of weak inhibition causing a reduction in the $[Ca^{2+}]_i$ change in the distal dendrites, and stronger inhibition causing a more widespread reduction, was commonly observed. In a few experiments (e.g., Fig. 4) inhibition caused a reduction in only a single branch. Success in achieving this selective inhibition seemed to depend critically on the positioning of the inhibitory electrode along the pial surface and the intensity of stimulation. It also helped to have widely separated arbors. The inhibited branch was always ipsilateral to the off-beam stimulating electrode.

To determine whether the Ca^{2+} transient inhibition was due to a conductance increase or to the membrane hyperpolarization caused by the IPSP, the relative timing between the CF stimulus and the IPSP stimulus was varied (Fig. 5*A*). When the IPSP was activated more than a few milliseconds after the CF there was almost no reduction in the peak $[Ca^{2+}]_i$ change. Similarly, when the IPSP was activated more than 10 msec before the CF there was little change in the Ca^{2+} transient. Since the time interval of effective inhibition corresponded approximately to the rise time of the IPSP (Fig. 5*B*), this result suggested that a fast inhibitory conductance and not the long-lasting hyperpolarizing inhibitory potential was responsible for the reduction in peak $[Ca^{2+}]_i$ change (see Discussion). Indeed, in many such experiments dendritic $[Ca^{2+}]_i$ changes were reduced even though no hyperpolarizing potential was observed, suggesting that the reversal potential for the IPSP was near resting potential in those experiments. Consistent with this hypothesis, both the IPSP (not shown) and the reduction in peak $[Ca^{2+}]_i$ change were eliminated by 10 μM bicuculline added to the bath (Fig. 6), showing that both were due to the activation of $GABA_A$ receptors, probably mediating an increase in Cl^- conductance. Further support for this conclusion comes from experiments in which we tried to mimic the IPSP hyperpolarization by injecting current into the soma during the CF response (Fig. 7). In the illustrated experi-

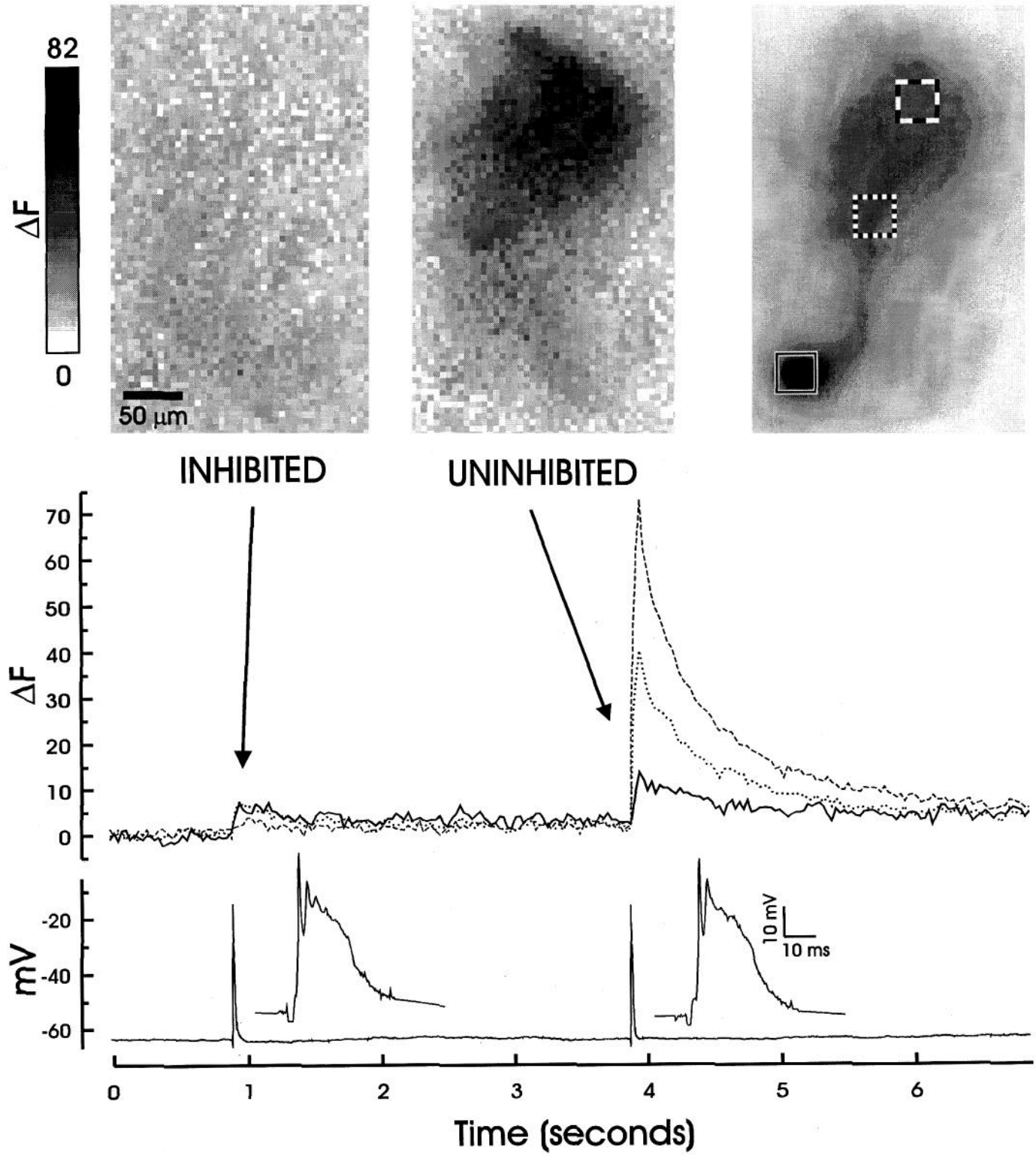

Figure 3. The reduction in CF-induced $[Ca^{2+}]_i$ can be almost complete. Panels and traces as in Figure 2*A*, except that the unbinned reference image is in the *upper right panel.* The traces and the gray-scale images show that the reduction in the dendrites was over 90%. The reduction in the soma was smaller but still significant. The enlargements of the electrical traces show that the somatically recorded response was hardly changed. In this figure and others, *INHIBITED* refers to the case when the CF and inhibitory stimuli were given together, and *UNINHIBITED,* to the case with only the CF stimulus.

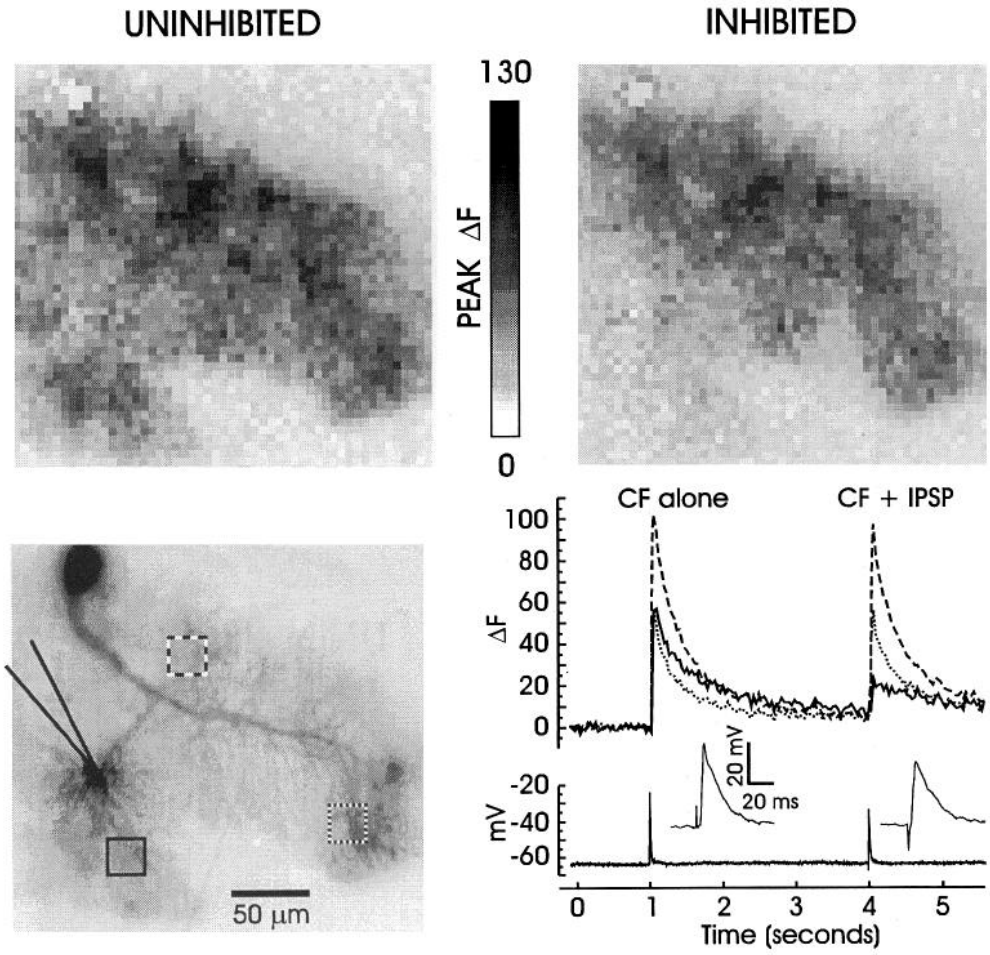

Figure 4. Inhibition sometimes reduces the $[Ca^{2+}]_i$ transient in a single branch. Neuron impaled in a dendrite (indicated with a *V*) and filled from that location. Gray-scale images and time-dependent traces as in Figure 2. The inhibited image and time-dependent traces show that the fluorescence change was reduced most strongly in the impaled branch. The stimulating electrode for inhibition was positioned to the left of that branch. Note that the dendritic electrical recording shows that inhibition caused a reduction in the CF response at that location.

ment, current injection hyperpolarized the soma by more than 15 mV, at least twice the amplitude of the somatically recorded IPSP which was effective in reducing the peak $[Ca^{2+}]_i$ change. Yet in this case there was no change (or possibly a slight increase) in the Ca^{2+} transient. Similar results were found with small current injections following dendritic impalements (not shown). However, much larger somatic current pulses, that hyperpolarized the membrane by more than 50 mV, did reduce the peak $[Ca^{2+}]_i$ change (not shown).

Dendritic recordings

A remarkable feature of these experiments was that there was little change in the somatically recorded CF electrical response when the IPSP reduced the dendritic $[Ca^{2+}]_i$ change (Figs. 2*A*, 3). Even when inhibition was strong, reducing the $[Ca^{2+}]_i$ transient by 90%, there was little change in the peak somatic CF potential change. The only effect was a somewhat variable small reduction in the shoulder that followed the peak CF response.

Dendritic recordings consistently showed a single fast action potential, about 2–3 msec wide, on top of the CF response. Figure 8*A* shows that when the CF response was paired with an IPSP the spike was reduced in amplitude and became broader. These effects were accentuated with increasing stimulation strength until the spike was eliminated altogether. This reduction in spike amplitude paralleled the reduction in peak $[Ca^{2+}]_i$ changes measured simultaneously with the CCD camera. Experiments shifting the time between the CF and inhibitory activation, similar to those shown above for the Ca^{2+} transients, showed that the time window for spike reduction was also about 10 msec (Fig. 8*B*). These results strongly suggest that the fast, CF-induced dendritic action potentials are a Ca^{2+} spike, and that this spike is largely responsible for the CF-associated $[Ca^{2+}]_i$ increase.

Additional evidence for the conclusion that these 2–3 msec wide spikes are Ca^{2+} spikes comes from experiments where we directly evoked spikes of similar width with strong dendritic depolarization. These spikes were unaffected by TTX while the small, simultaneously recorded Na^+ spikes were blocked (Fig. 9; see also Llinás and Sugimori, 1980a,b). TTX also had no effect on the associated dendritic $[Ca^{2+}]_i$ changes (not shown). In no experiment were we able to evoke a large dendritic action potential that was sensitive to TTX.

Large plateau potentials

Ekerot and Oscarsson (1981), in an *in vivo* study of the cat cerebellum, noted that the CF response was often followed by a plateau potential which they suggested was the main source of the dendritic $[Ca^{2+}]_i$ increase. They proposed that inhibition truncates this plateau, reduces the $[Ca^{2+}]_i$ increase, and prevents the induction of LTD (Campbell et al., 1983; Ekerot and Kano, 1985). Indeed, they found that off-beam activation of parallel fibers (which synaptically activate inhibitory interneurons) lessens the chance of inducing LTD. In our slice experiments we did not observe a long-lasting plateau in neurons with 30–50 MΩ input resistance and resting potentials lower than −60 mV. However, in more depolarized cells (possibly due to impalement

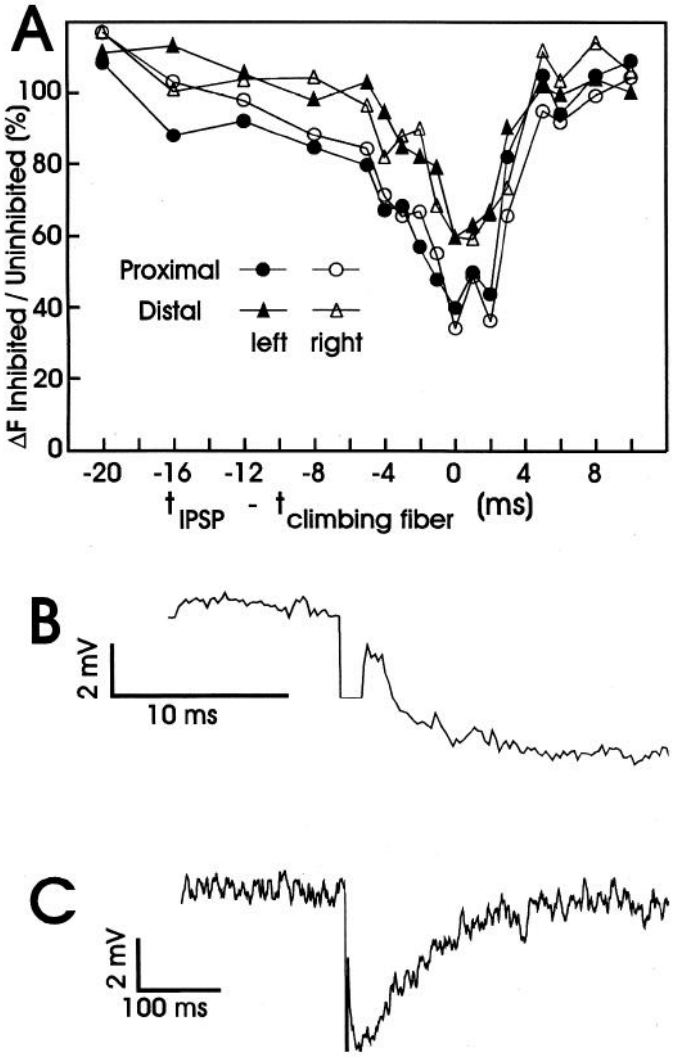

Figure 5. The inhibitory effect occurs in a narrow time window around the moment of CF activation. *A,* The traces show the ratio of the magnitude of the fluorescence changes (ΔF) following inhibited and uninhibited CF stimuli. Data from four locations in the dendrites, two proximal and two distal. At all locations the maximum effect occurs between 6 msec before the CF stimulus and 4 msec after. However, there is still some inhibition when the IPSP is activated at earlier times. *B* and *C,* Somatic electrical recordings of the IPSP, activated alone, for the cell used in *A*. *B,* The early part of the IPSP is shown on the same time scale as the plot in *A*. Note that the time window for inhibition corresponds approximately to the rise time of the IPSP. *C,* The full time course of the IPSP. The recovery time (which matched the recovery time of a hyperpolarizing pulse) is much longer than the inhibition time window.

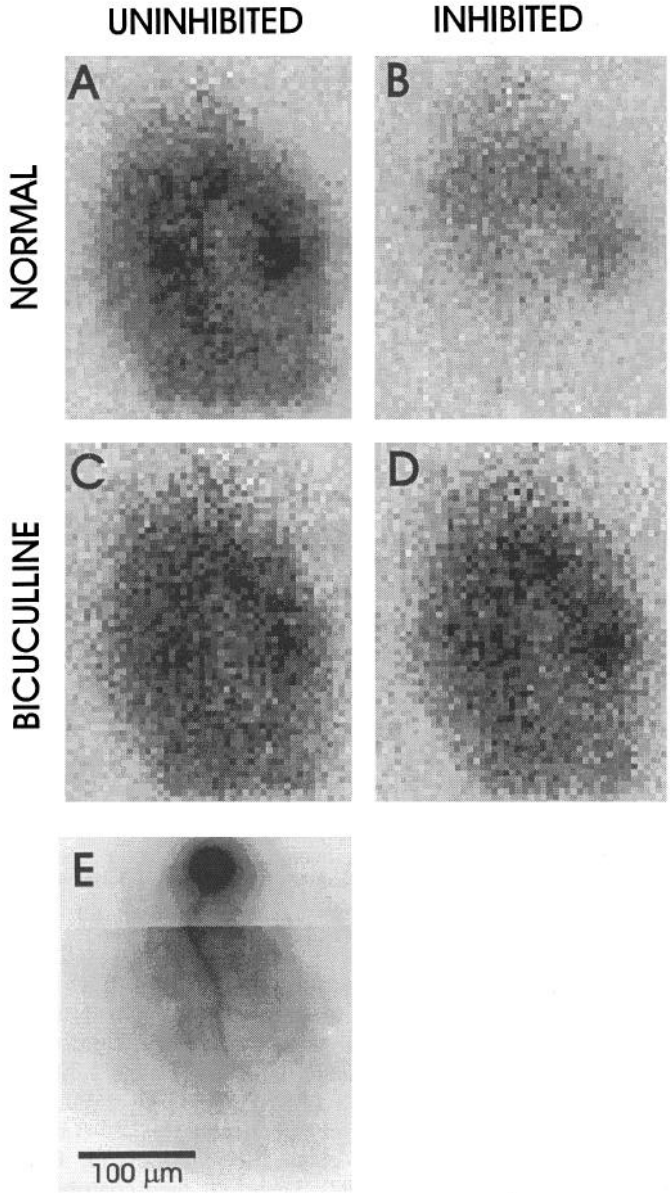

Figure 6. Bicuculline (10 μM) blocks the reduction in the CF-induced $[Ca^{2+}]_i$ change caused by the inhibitory stimulus. *A,* Gray-scale image of spatial distribution of the peak CF-induced fluorescence change. *B,* Reduced fluorescence change when CF is paired with inhibition. *C* and *D,* Same protocols as in *A* and *B* except with bicuculline in the bath. Stimulating the inhibitory pathway (*D*) does not affect the fluorescence change. *E,* Unbinned cell image (most of the dendrites are blurred due to light scattering in the tissue). Same gray scale for *A–D. Black* is largest fluorescence change, *white* is no change.

injury) the immediate CF response was sometimes followed by a long-lasting plateau which caused a significant increase in dendritic $[Ca^{2+}]_i$ (Fig. 10). This plateau (which was preceded by the typical Ca^{2+} spike and wide EPSP in dendritic recordings) was easily truncated by an inhibitory input (see also Midtgaard, 1992). In no experiment was this plateau-related $[Ca^{2+}]_i$ increase confined to a fraction of the dendrites, and inhibition always reduced the transients in all locations.

We cannot be sure that the conditions in our slice experiments precisely matched those in whole animal experiments. Therefore, it is possible that these plateau potentials and the $[Ca^{2+}]_i$ changes they evoke occur more often in *in vivo* conditions than in the slice environment. However, Sakurai (1987, 1990) showed that the Ca^{2+}-dependent induction of LTD occurred in the absence of large plateau potentials using experimental conditions similar to our own. Therefore, experiments at depolarized resting potentials were not pursued further.

Discussion

Source of CF-induced $[Ca^{2+}]_i$ increase

Previous experiments (Ross and Werman, 1987; Konnerth et al., 1992; Miyakawa et al., 1992; Midtgaard et al., 1993) have clearly shown that there is a large transient increase in $[Ca^{2+}]_i$ over most of the dendrites when the CF is activated. However, the

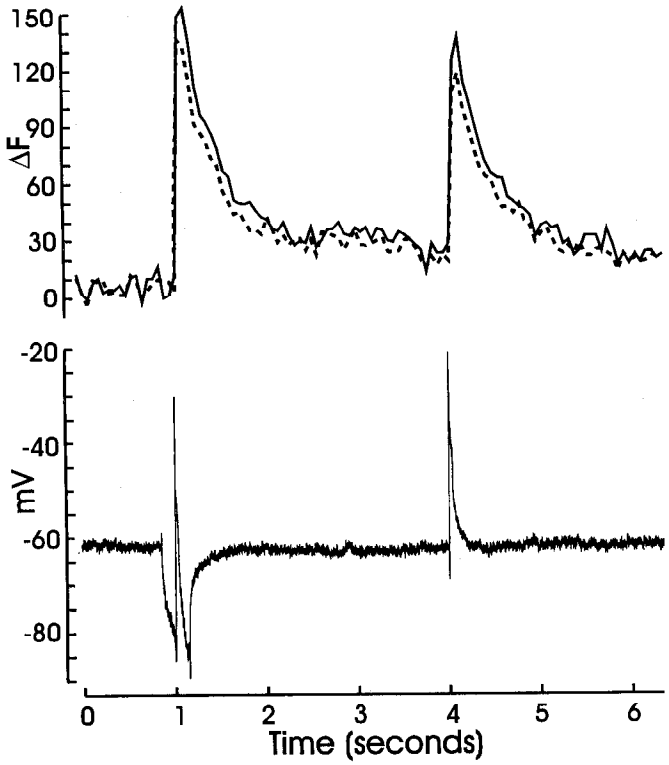

Figure 7. Moderate somatic hyperpolarization does not reduce the CF-associated $[Ca^{2+}]_i$ increase. During the time of the first CF response current was injected through the microelectrode to hyperpolarize the soma at least 15 mV. However, the fluorescence traces from representative proximal (*solid*) and distal (*dashed*) dendritic locations show that the $[Ca^{2+}]_i$ changes were not reduced.

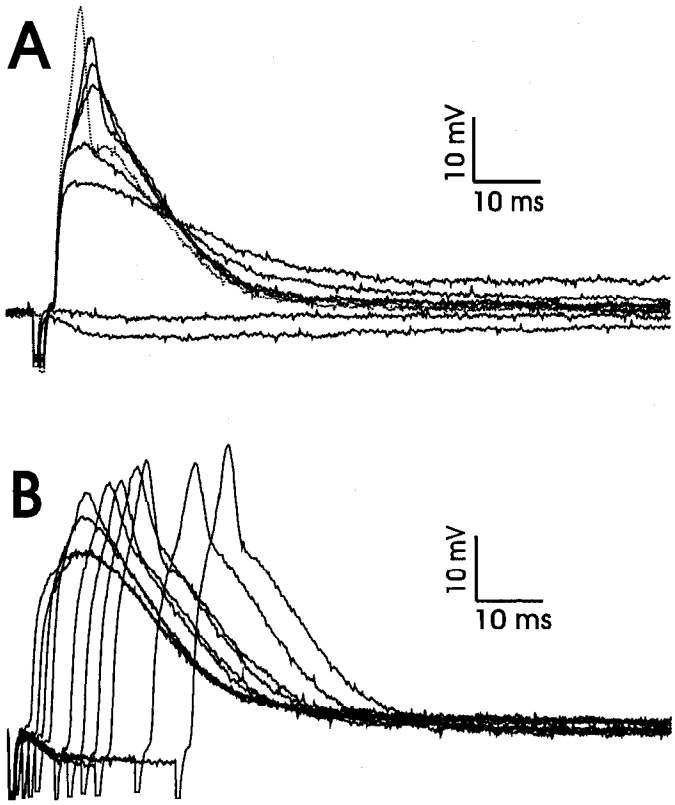

Figure 8. Varying the intensity of the inhibitory stimulus or the time relative to the CF stimulus blocks the fast CF-induced dendritic action potential in a graded manner. Microelectrode in a dendrite about one-third of the way toward the pial surface from the soma. *A*, The traces show the CF response recorded at this location with varying intensities of the inhibitory stimulus. The highest amplitude response, without inhibition (*dotted line*), shows a 2–3 msec wide action potential activated by the CF synaptic potential. Increasing inhibition gradually reduces the amplitude of this spike until it is completely eliminated, leaving only the synaptic potential. The IPSP alone, for two stimulation intensities, is also shown. *B*, Traces show dendritically recorded CF responses as the CF stimulation is delayed with respect to the time of an inhibitory stimulus of constant intensity. When both stimuli are given at the same time the CF response is rounded with no spike. With increasing delay the spike appears, reaching full amplitude for delays greater than about 13 msec.

specific underlying event that is the source of this $[Ca^{2+}]_i$ increase has been the subject of some debate. One possibility is that Ca^{2+} enters through ligand-gated channels. Glutamate receptors on adult Purkinje cells are of the AMPA category, blockable by CNQX (Konnerth et al., 1990; Perkel et al., 1990; Miyakawa et al., 1992), and not very permeable to Ca^{2+} (Linden et al., 1993; Schneggenburger et al., 1993; Sorimachi, 1993; however, see Brorson et al., 1992). Therefore, their contribution to these signals, relative to voltage-gated entry, is not expected to be substantial. Further arguments against the significance of this pathway are (1) Ca^{2+} transients are evoked synchronously over all parts of the cell, including the fine dendrites where there are no CF synaptic contacts (Miyakawa et al., 1992); and (2) the CF electrical response remains even after most of the $[Ca^{2+}]_i$ increase has been blocked by synchronously activated IPSPs (this paper).

Another possibility is that a component of the $[Ca^{2+}]_i$ increase could come from release of internal stores. Such a mechanism would have to include a voltage-mediated step, since all significant $[Ca^{2+}]_i$ increases are associated with regenerative electrical events. Previously (Miyakawa et al., 1992), we suggested that the very fast onset and time to peak of the CF signals was incompatible with a release mechanism where diffusion of a second messenger (including Ca^{2+} itself) over distances greater than 1–2 μm played a significant role. These new experiments add no additional evidence concerning this possibility. Release from internal stores following bath application of glutamate (Llano et al., 1991) or long-lasting depolarizing pulses (Llano et al., 1994) has been demonstrated. However, a role for this pathway has yet to be established for synaptically activated events; all Ca^{2+} signals reported in this paper are consistent with Ca^{2+} entry through voltage-sensitive channels.

These experiments do help distinguish which of the voltage-sensitive mechanisms contribute to CF-induced $[Ca]_i$ increases in different parts of the cell. Possible components include the synaptic potential itself, the 15–20 msec wide "climbing fiber response" recorded in the soma, a Ca^{2+} spike in the dendrites, or a dendritic Ca^{2+}-dependent plateau potential. The Ca^{2+} spike must be the most significant Ca^{2+} source, since the IPSP blocked the dendritically recorded Ca^{2+} spike simultaneously with most of the dendritic $[Ca^{2+}]_i$ increase. It also follows that the large EPSP which generated the Ca^{2+} spike cannot be a major source of dendritic $[Ca^{2+}]_i$ since this potential was still present after the inhibitory input blocked the $[Ca^{2+}]_i$ increase (Figs. 3, 8). The plateau potential which follows the large CF response is also an insignificant component, since the $[Ca^{2+}]_i$ peaks at all locations within 7–8 msec from the onset of the CF response (Miyakawa et al., 1992) while the plateau potential persists for 15–20 msec.

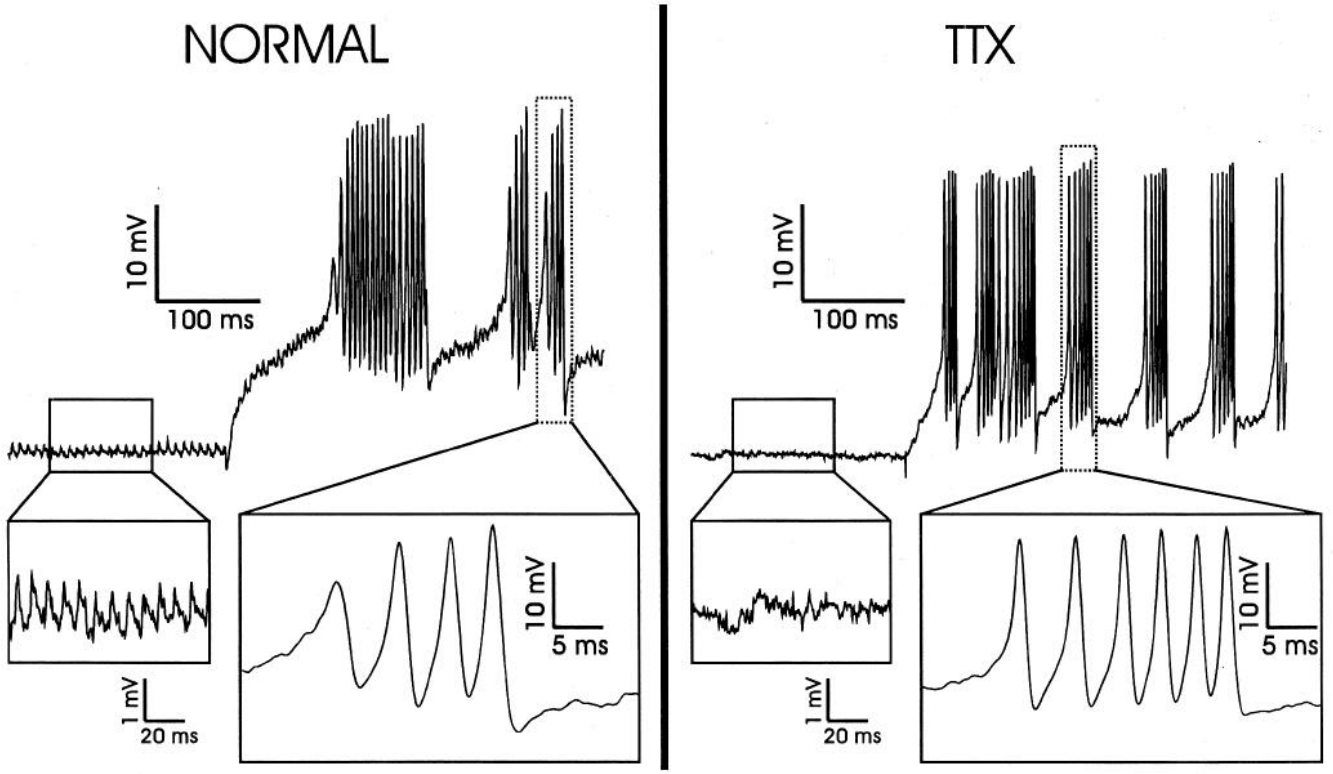

Figure 9. Large amplitude fast dendritic spikes are not sensitive to TTX, while small amplitude spikes are blocked by this toxin. *A,* Dendritic recording in normal saline. A steady depolarizing current evoked repetitive small amplitude action potentials. About halfway through the trace a further depolarization evoked two bursts of large amplitude spikes. The *blowups* show parts of the trace in detail. Note that the large amplitude spikes are only 2–3 msec wide. *B,* Same protocol after adding TTX to the bath. The small spikes are eliminated but the fast, large amplitude spikes remain.

In the somatic region the recorded CF response was not significantly affected by distal inhibitory inputs. Since the magnitude of the $[Ca]_i$ changes in the somatic region was small compared to the changes in the dendrites, the somatic potentials probably do not involve regenerative Ca^{2+} components (Lev-Ram et al., 1992; Midtgaard et al., 1993). One interpretation of the somatic CF response is that, in addition to the synaptic potential, this event includes a regenerative Na^+ component supported by noninactivating Na^+ channels (Llinás and Sugimori, 1980a; Chan et al., 1989).

Significance of inhibitable $[Ca^{2+}]_i$ changes

Since simultaneously activated inhibition caused little or no change in the somatically recorded CF response, there was no effect on the pattern of action potentials in the Purkinje cell axon generated by the CF. Therefore, the interaction of these two in-

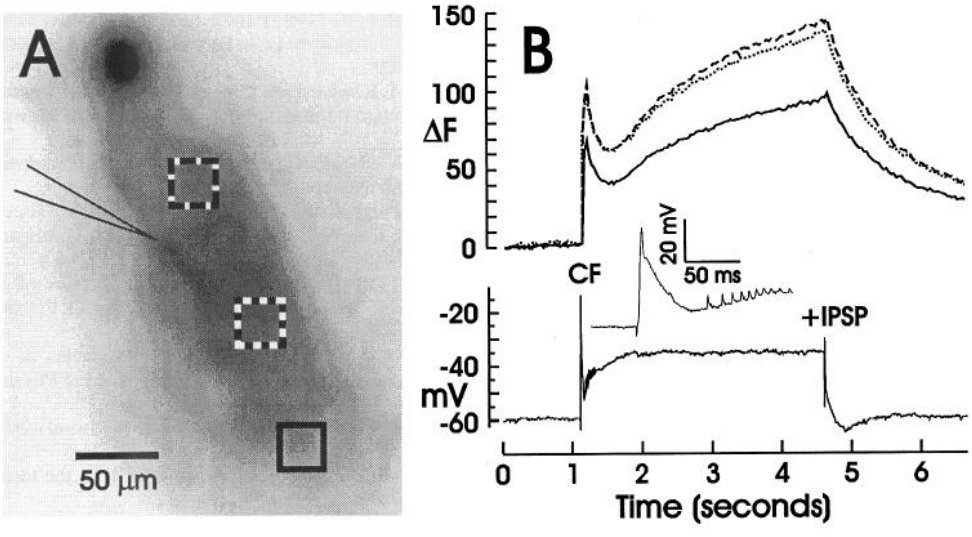

Figure 10. Climbing fiber stimulation sometimes evokes a calcium-dependent plateau which can be truncated by inhibition. *A,* Image of fura-2-filled cell showing electrode in dendrite. *B,* Fluorescence changes recorded at three dendritic locations (shown in *A*) when the cell was first stimulated via the CF and then with the CF and the inhibitory input together (*+IPSP*). The CF stimulus alone caused the cell to generate a plateau potential which continuously increased $[Ca^{2+}]_i$ at all locations. The inhibitory stimulation ended the plateau and allowed the $[Ca^{2+}]_i$ to return to resting levels. The *inset* shows the response to the CF stimulus. Note the Ca^{2+} spike on the immediate response and the burst of Na^+ spikes on the rising edge of the plateau potential. Cell was held at resting potential with a small hyperpolarizing current to prevent spontaneous plateau potentials.

puts in no way resembles the usual forms of synaptic integration in dendrites. The fact that a Ca^{2+} spike was prevented in the dendrites had no consequence for the immediate output of the cell. The importance of this interaction probably lies in the reduction in the $[Ca^{2+}]_i$ change which was caused by the spike.

There is little hard evidence concerning the role of elevated $[Ca^{2+}]_i$ levels in dendrites. One possible function is to activate K^+ conductances. An increase in K^+ conductance would shunt EPSPs in the period following a CF response, making the cells less excitable. Such a reduction in Purkinje cell firing rate has been observed in many experiments *in vivo* (e.g., Sato et al., 1993). A second possibility is that the elevated $[Ca^{2+}]_i$ causes dendritic release of some neurotransmitter, peptide, or other substance. While such a mechanism has been suggested (e.g., Getchell and Shepherd, 1975), there is little data so far to confirm it.

A third possibility is that the elevated $[Ca^{2+}]_i$ interacts with PF inputs to cause LTD of subsequent PF EPSPs (Sakurai, 1987; Ito, 1989). Data concerning this kind of interaction have been presented (e.g., Sakurai, 1990; Konnerth et al., 1992). Interestingly, these experiments used $GABA_A$ blockers to facilitate the induction of LTD. Without these blockers this form of depression was difficult to induce (Schruers and Alkon, 1993).

For each of these possibilities a strong reduction in the $[Ca^{2+}]_i$ increase would reverse the effects. That is, there would be less activation of K^+ conductances and a reduced hiatus in simple spike firing; depression of PF EPSPs following conjunctive activation would be prevented; and there would be less secretion from the dendrites.

Localization of the inhibitory effect

The CF-induced $[Ca^{2+}]_i$ increase was strongly reduced only when the inhibitory input was activated in a time window of about 10 msec around the time of the CF input (Fig. 5). This interval is about the duration of the rise time of the IPSP (Fig. 5) and of inhibitory postsynaptic currents (IPSCs) recorded in voltage-clamped Purkinje cells using whole-cell recordings (7–13 msec, Vincent et al., 1992), and much less than the duration of the IPSP. These experiments strongly suggest that the inhibitory input prevented the generation of a Ca^{2+} spike in the dendrites by causing a conductance shunt and not by hyperpolarizing the dendrites. Further evidence for this conclusion came from experiments showing that hyperpolarizing the soma during the CF response had no significant effect on the magnitude of the CF-induced $[Ca^{2+}]_i$ changes (Fig. 7).

A conductance shunt localized on a particular dendritic branch could prevent a Ca^{2+} spike from firing on that branch without affecting the generation of a spike on other branches in the dendritic arbor. Localized inhibition was occasionally observed in some experiments (Fig. 4). In contrast, any effect directly due to the hyperpolarization caused by the inhibitory input would be effective all over the dendrites, since Purkinje cells are compact below the threshold for most active conductances (Shelton, 1985; Rapp et al., 1994).

In our experiments we activated many stellate and possibly some basket interneurons which, together, make contacts over many branches of a Purkinje cell. Therefore, the $[Ca^{2+}]_i$ increase usually was inhibited over a large part of the dendrites, and inhibition of single branches was infrequently observed. Under physiological conditions, however, when individual stellate cells are active, it is possible that the effects of inhibition could be more focused. Thus local inhibition would provide a mechanism to restrict the effects of transient changes in $[Ca^{2+}]_i$ (and the consequences of these changes) to selective locations in the dendrites.

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