Role of the calcium-binding protein parvalbumin in short-term synaptic plasticity

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GABAergic (GABA = \(\gamma\)-aminobutyric acid) neurons from different brain regions contain high levels of parvalbumin, both in their soma and in their neurites. Parvalbumin is a slow Ca\(^{2+}\) buffer that may affect the amplitude and time course of intracellular Ca\(^{2+}\) transients in terminals after an action potential, and hence may regulate short-term synaptic plasticity. To test this possibility, we have applied paired-pulse stimulations (with 30- to 300-ms intervals) at GABAergic synapses between interneurons and Purkinje cells, both in wild-type (PV\(^{-}\)/+ ) mice and in parvalbumin knockout (PV\(^{-}\)/−) mice. We observed paired-pulse depression in PV\(^{+}\)/+ mice, but paired-pulse facilitation in PV\(^{-}\)/− mice. In paired recordings of connected interneuron-Purkinje cell pairs, dialysis of the presynaptic interneuron with the slow Ca\(^{2+}\) buffer EGTA (1 mM) rescues paired-pulse depression in PV\(^{-}\)/− mice. These data show that parvalbumin potently modulates short-term synaptic plasticity.

GABA | cerebellum | basket cells | stellate cells | Purkinje cells

The immediate consequences of past neuronal activity on synaptic strength are often examined by measuring the ratio (called paired-pulse ratio, or PPR) between the mean synaptic current in response to a test stimulation over that obtained with a conditioning stimulus. If the PPR is larger than 1, the synapse is considered as facilitating, whereas values smaller than 1 are characteristic of depressing synapses. However, facilitation and depression presumably coexist in all experimental conditions, and the PPR that is measured may be viewed as a balance between these two competing processes (1, 2).

Current hypotheses link facilitation to the fact that some of the Ca\(^{2+}\) ions entering the presynaptic terminal during the first stimulus are still present when the second stimulus is delivered (3, 4). Several modes of action have been envisaged for the residual calcium. It could act by binding to high affinity sites of the normal exocytosis machinery (5), by binding to special sites responsible for facilitation (2, 6), or by modulating the degree of saturation of high affinity Ca\(^{2+}\) buffers (7, 8), but direct evidence in favor of any of these possibilities is still lacking.

Depression is a complex phenomenon including both pre- and postsynaptic components. It may involve depletion of a readily releasable pool of vesicles, saturation or desensitization of postsynaptic receptors, or still other processes (7, 9).

Calcium-binding proteins such as parvalbumin (PV), calretinin, and calbindin D\(_{28k}\) are important modulators of intracellular calcium dynamics in neurons (10) and could therefore influence both facilitation and depression. Effects of these calcium buffers are determined by their affinities for Ca\(^{2+}\) ions and by the kinetics (on and off rates) of binding and release of Ca\(^{2+}\). PV is in this regard interesting because it has a slow dissociation rate (about 1 s\(^{-1}\)) and a slow apparent association rate (about 10\(^7\) M\(^{-1}\)s\(^{-1}\)), due to the fact that Mg\(^{2+}\) ions compete with Ca\(^{2+}\) ions for binding. As a result of this situation, PV tends to accelerate the initial phase of decay of Ca\(^{2+}\) transients rather than slowing it down, as fast buffers would (11, 12). Furthermore PV reduces the peak calcium transients much less efficiently than fast buffers do (11, 12). Specific subclasses of GABAergic (GABA = \(\gamma\)-aminobutyric acid) neurons from different brain regions contain high levels of PV both in their soma and in their processes, but the physiological role of PV in brain neurons remains unclear (10, 13, 14).

In the present work, we took advantage of the recent development of a strain of PV-deficient (PV\(^{-}\)/−) mice (15) to investigate the effects of PV on short-term synaptic plasticity, using a PPR protocol. PV\(^{-}\)/− mice have a locomotor behavior that can be distinguished from wild-type mice and show an increased susceptibility toward epileptic seizures\(^5\). Because PV is highly expressed in axons and terminals of cerebellar interneurons (basket and stellate cells (14)), we centered our investigation on the synapse formed between these interneurons and Purkinje cells.

Materials and Methods

Immunohistochemistry. Sagittal cerebellar slices (100 \(\mu\)m thick) were prepared as described previously (18) and then fixed for 1 h with paraformaldehyde in PBS. Sections were incubated overnight with the rabbit PV antiserum PV-28 (Swant, Bellinzona, Switzerland), diluted 1:50 in PBS. After rinsing with PBS containing 1% albumin, sections were incubated with the secondary antibody (fluorescein anti-rabbit; Vector Laboratories) diluted 1:500 at 4°C in a dark room. Immunoreactive neurons were then visualized with a confocal microscope (Axiovert 135TV; Zeiss), using an illumination wavelength of 488 nm.

Western Blot Detection of PV in the Cerebellum. Brains were removed from killed mice and separated into forebrain and cerebellum. An approximately 5-fold larger volume of PBS (pH 7.4) containing 2 mM EDTA and a protease inhibitor mixture (Roche, Mannheim, Germany) was added, and the tissue was disrupted by using a Polytron Homogenizer (Kinematica, Lucerne, Switzerland). The suspension was centrifuged at 15,000 \(\times\) g for 30 min at 4°C, and protein concentration of the supernatant was determined by using the Bradford assay (Bio-Rad). Proteins were separated by SDS/PAGE (15% polyacrylamide), trans...
ferred to Zeta probe-membranes (Bio-Rad), and immunostained with the antiseraum PV4064 (1:1000) as described before (19). PV-immunoreactive bands were visualized by using 4-chloraniphol/hydrogen peroxide as a chromogen. A parallel gel was stained with Coomassie blue to check for even loading.

**ELISA.** The sandwich ELISA for PV is very similar to the one published in detail for calretinin (20, 21). ELISA plates were pretreated with the monoclonal mouse anti-PV antibody PV235 (Swant; 0.2 mg/ml in bidistilled water, diluted 1:50 in 100 mM NaHCO3, pH 8.0) for 16–24 h at room temperature. After blocking additional protein binding sites, purified recombinant protein in PV was considered as satisfactory and the PPR measure-
instable response past the threshold, the location of the stimulating electrodes was determined by using sagittal cerebellar slices (180 µm thick), which were prepared as described previously (18). Animal age ranged from 7 to 12 days.

Slices were perfused (1.5 ml/min) with an extracellular saline containing (in mM): 125 NaCl, 2.5 KCl, 1.25 NaHPO4, 26 NaHCO3, 2 CaCl2, 1 MgCl2, and 10 glucose. NBQX (10 µM) and D-AP5 (50 µM) were added to block α-amino-3-hydroxy-5-methyl-4-isoxazolpropionic acid (AMPA)-kainate and N-methyl-D-aspartate (NMDA) receptors respectively.

Tight-seal whole cell recordings were used to record inhibitory postsynaptic currents (IPSCs). The intracellular saline for Purkinje cells contained (in mM): 150 CsCl, 4.6 MgCl2, 10 Hepes, 4 Na-ATP, 0.4 Na-GTP, 1 EGTA, and 0.1 CaCl2. Series resistances ranged from 4 to 9 MΩ and were partially compensated (60–90%). Cell potential was held at −60 mV. All recordings were performed at room temperature.

**Extracellular Stimulations.** The stimulating electrode was a glass pipette of similar size to those used for patch-clamp of Purkinje cells, filled with a Hepes-buffered extracellular saline solution. The reference electrode for the stimulation circuit was made with a platinum wire wrapped around the stimulating electrode. A voltage pulse (10–60 V) or a current pulse (100–800 µA) was applied through the electrode during 50–200 µs. Special precautions were taken to maximize the chances that data represented reliable stimulations of a single presynaptic cell. The stimulation electrode was placed at various locations in the molecular layer, and a series of increasing stimulations were applied until single eIPSCs were evoked. If a clear intensity of protein in PV was measured in PV mice and a complete lack of signal revealed a reduced signal in PV+/- mice and a complete lack of signal in PV+/- mice (Fig. 1C). The results were further supported by a quantitative ELISA assay. PV levels were 8.7 ± 1.6 µg/mg of protein in PV+/- mice and 3.4 ± 0.6 in PV+/- mice (40% compared with PV+/-), and no detectable levels were measured in PV+/- mice. The results from Western blot analysis and ELISA are in agreement with previous studies on fast-twitch muscles where no detectable levels of PV could be found in PV-deficient mice (15).

**Extracellular Stimulations.** To record interneuron-evoked IPSCs (eIPSCs), whole-cell recordings were obtained in Purkinje cells. Double pulses were applied through an extracellular pipette located in the lower part of the molecular layer to stimulate the axons of stellate or basket cells. Interstimulus intervals (ISI) ranged from 30 to 300 ms. For ISIs shorter than 30 ms, eIPSCs overlap heavily. We did not study PPRs at such short ISIs to avoid complications due to possible postsynaptic effects (9). In PV+/- mice, the amplitude of the second eIPSC was smaller than that of the first for ISIs ranging from 30 to 300 ms (Fig. 2A). The PPR was 0.84 ± 0.05 at 30 ms and 0.86 ± 0.04 at 100 ms (n =
12 cells; Fig. 2C). Application of the GABA$_B$ antagonist CGP55845A (1 μM) did not modify the PPR ($n = 7$, $P = 0.84$), indicating that the PPR was independent of the activation of presynaptic GABA$_B$ receptors. These results show that interneuron-Purkinje cell synapses are depressing in control juvenile mice, as they are in juvenile rats (26). In PV$−/−$ mice, however, the mean amplitude of the second eIPSC was larger than that of the first one for ISIs ranging from 30 to 100 ms. PPR values were $1.17 \pm 0.08$ at 30 ms and $1.04 \pm 0.05$ at 100 ms ($n = 19$ cells; Fig. 2B and C). By comparison to the PV$+/+$ mice, the PPR was significantly different at 30-, 70-, and 100-ms ISI. These results indicate that, for ISIs of 100 ms or less, elimination of PV converts depressing synapses into facilitating ones.

**Paired Recordings.** To gain further insight into the mechanisms responsible for the PPR changes, we compared the properties of GABAergic synapses in PV$−/−$ and PV$+/+$ mice by using connected pairs (stellate cell-Purkinje cell or basket cell-Purkinje cell). This method had two advantages over extracellular stimulation. First, possible changes in release probability could be evaluated by analyzing failure rates. Secondly, manipulations of the presynaptic Ca$^{2+}$ buffer could be attempted. We were in particular interested to see whether adding EGTA to the presynaptic cell in PV$−/−$ mice could rescue the PV$+/+$ phenotype. EGTA was selected for these rescue experiments because it is, like PV, a slow Ca$^{2+}$ buffer (11, 12, 27).

After the switch to whole-cell configuration in the interneuron, pairs of depolarizing steps were applied under somatic voltage clamp. This protocol led to the generation of action potentials that propagated along the axon (22) and gave rise to an eIPSC when the recorded Purkinje cell was synaptically connected.

In the PV$−/−$ pairs, when the interneuron was recorded with a low concentration of EGTA (50 μM; standard presynaptic solution), the PPRs measured at 30 ms and 70 ms ISI were $1.22 \pm 0.05$ and $1.14 \pm 0.11$, respectively ($n = 13$, Fig. 3A and C). The PPRs measured in these conditions were similar to the ones measured when interneurons were extracellularly stimulated. In contrast, when the interneuron was recorded with 1 mM EGTA, the PPRs measured at 30 ms and 70 ms ISI were $0.94 \pm 0.04$ and $1.01 \pm 0.1$, respectively (Fig. 3B and C). At 30 ms ISI the PPR was significantly lower than the one measured with extracellular stimulation or with a low EGTA concentration. In the PV$−/−$
pairs, the percentage of failure was not significantly modified by EGTA [8 ± 3% with 50 μM EGTA and 6 ± 5% with 1 mM EGTA (P = 0.74)].

These data show that the switch from paired-pulse depression to paired-pulse facilitation in the PV−/− can be reversed by dialyzing EGTA into the presynaptic interneuron. Furthermore, the failure data suggest that addition of a slow buffer such as EGTA does not modify the release probability.

In PV+/+ pairs, when the interneurons were recorded with the standard solution (with 50 μM EGTA), the PPRs measured at 30 ms and 70 ms were 0.94 ± 0.05 (n = 11) and 0.94 ± 0.06 (n = 6, Fig. 4). These values are much lower than the corresponding values in PV−/− mice, but they are slightly higher than those obtained with extracellular stimulation in PV+/+ mice. When the interneurons were recorded with 1 mM EGTA in PV+/+ experiments, the PPRs measured at 30 ms and 70 ms dropped to 0.82 ± 0.03 (n = 9) and 0.81 ± 0.08 (n = 7), respectively, that is, to values very close to the ones measured with extracellular stimulation. The percentage of failures with 50 μM EGTA was not significantly different from that in 1 mM EGTA (14 ± 4% instead of 12 ± 5%; P = 0.83).

These results indicate that, in PV+/+ mice, as in PV−/− mice, addition of EGTA to presynaptic neurons shifts the PPR to lower values without affecting the failure rate of the first IPSC. In PV+/+ paired experiments, a difficulty arises from the fact that the high affinity Ca2+-binding sites of PV also have a moderately high affinity for Mg2+ such that PV under basal intracellular Ca2+ is largely in the Mg2+-bound form (28). If the Mg2+ concentration chosen for the pipette does not match that of the cytosol, establishment of the new Mg2+ concentration on presynaptic whole-cell recording will change the buffering power of PV. Physiological values of the resting free Mg2+ concentration [Mg2+]i in neurons are around 300 μM (29). Because
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Slow buffers such as EGTA affect transmitter release after an unconditioned stimulus only if release sites are not in direct vicinity to the site of Ca2+ influx, the voltage-dependent Ca2+ channels (8). In some preparations [squid giant synapse (27) and pyramidal-pyramidal synapses (31)], where influx and release are apparently tightly coupled together, EGTA does not inhibit markedly the probability of release, whereas in other preparations [crayfish neuromuscular junction (32) or hippocampal mossy fiber synapse (33, 34)], where the coupling is looser, a significant reduction is observed. Our finding that the probability of failures is not different in PV+/+, PV−/−, and in PV−/− strains, and that it is not modified by addition of EGTA in PV−/− mice (Table 1), suggests that the interneuron-Purkinje cell synapse falls in the first category, where release sites are closely associated to voltage-dependent Ca2+ channels.

The effects of EGTA and PV on the PPR may be understood by considering their effects on the Ca2+ transient on a time scale of milliseconds, i.e., after the collapse of channel-associated Ca2+ domains. EGTA or PV do not reduce markedly the peak Ca2+ transient resulting from voltage-dependent Ca2+ influx, but they accelerate the initial rate of decay of this transient (11, 12, 35). The acceleration of the Ca2+ decay is likely to reduce the residual Ca2+ concentration and hence to inhibit facilitation (35). The effects on the PPR due to the elimination of PV and of its replacement with EGTA are in agreement with this expectation. Examination of the time course of the PPR in the various experimental conditions suggests that it is facilitation, not depression, that is modified. Facilitation decays as a function of one or several seconds (1, 37). We find that the effects of PV deletion or of EGTA addition on the PPR decay quickly with the ISI of one or several seconds (1, 37). We find that the effects of PV deletion or of EGTA addition on the PPR decay quickly with the ISI of one or several seconds (1, 37). We find that the effects of PV deletion or of EGTA addition on the PPR decay quickly with the ISI of one or several seconds (1, 37). We find that the effects of PV deletion or of EGTA addition on the PPR decay quickly with the ISI of one or several seconds (1, 37). We find that the effects of PV deletion or of EGTA addition on the PPR decay quickly with the ISI of one or several seconds (1, 37). We find that the effects of PV deletion or of EGTA addition on the PPR decay quickly with the ISI of one or several seconds (1, 37). We find that the effects of PV deletion or of EGTA addition on the PPR decay quickly with the ISI of one or several seconds (1, 37). We find that the effects of PV deletion or of EGTA addition on the PPR decay quickly with the ISI of one or several seconds (1, 37). We find that the effects of PV deletion or of EGTA addition on the PPR decay quickly with the ISI of one or several seconds (1, 37). We find that the effects of PV deletion or of EGTA addition on the PPR decay quickly with the ISI of one or several seconds (1, 37). We find that the effects of PV deletion or of EGTA addition on the PPR decay quickly with the ISI of one or several seconds (1, 37). We find that the effects of PV deletion or of EGTA addition on the PPR decay quickly with the ISI of one or several seconds (1, 37). We find that the effects of PV deletion or of EGTA addition on the PPR decay quickly with the ISI of one or several seconds (1, 37). We find that the effects of PV deletion or of EGTA addition on the PPR decay quickly with the ISI of one or several seconds (1, 37). We find that the effects of PV deletion or of EGTA addition on the PPR decay quickly with the ISI of one or several seconds (1, 37). We find that the effects of PV deletion or of EGTA addition on the PPR decay quickly with the ISI of one or several seconds (1, 37). We find that the effects of PV deletion or of EGTA addition on the PPR decay quickly with the ISI of one or several seconds (1, 37). We find that the effects of PV deletion or of EGTA addition on the PPR decay quickly with the ISI of one or several seconds (1, 37). We find that the effects of PV deletion or of EGTA addition on the PPR decay quickly with the ISI of one or several seconds (1, 37). We find that the effects of PV deletion or of EGTA addition on the PPR decay quickly with the ISI of one or several seconds (1, 37). We find that the effects of PV deletion or of EGTA addition on the PPR decay quickly with the ISI of one or several seconds (1, 37). We find that the effects of PV deletion or of EGTA addition on the PPR decay quickly with the ISI of one or several seconds (1, 37). We find that the effects of PV deletion or of EGTA addition on the PPR decay quickly with the ISI of one or several seconds (1, 37). We find that the effects of PV deletion or of EGTA addition on the PPR decay quickly with the ISI of one or several seconds (1, 37). We find that the effects of PV deletion or of EGTA addition on the PPR decay quickly with the ISI of one or several seconds (1, 37). We find that the effects of PV deletion or of EGTA addition on the PPR decay quickly with the ISI of one or several seconds (1, 37). We find that the effects of PV deletion or of EGTA addition on the PPR decay quickly with the ISI of one or several seconds (1, 37).

Table 1. Properties of the basket/stellate cells-Purkinje cells connections

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<thead>
<tr>
<th>Property</th>
<th>+/+</th>
<th>−/−</th>
</tr>
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<tbody>
<tr>
<td>Amplitude, nA</td>
<td>0.48 ± 0.13 (13)</td>
<td>0.54 ± 0.19 (10)</td>
</tr>
<tr>
<td>Failure, %</td>
<td>13 ± 5 (13)</td>
<td>13 ± 5 (10)</td>
</tr>
<tr>
<td>Var./mean, pA</td>
<td>110 ± 46 (13)</td>
<td>87 ± 19 (10)</td>
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Numbers in parentheses indicate the number of paired recordings in each condition. The last line indicates the variance-to-mean ratio of peak IPSCs in each condition.

The close agreement between the former results and those obtained with paired recordings of PV−/− mice with 1 mM EGTA and low Mg2+ (ii) intermediate values near 0.95 (paired recordings of PV+/+ mice with 50 mM EGTA and high Mg2+, or paired recordings of PV−/− mice with 1 mM EGTA); (iii) high values near 1.20 (PV−/− mice, either with extracellular stimulations or with paired recordings by using 50 mM EGTA).

Discussion

The principal results of this study are that elimination of PV changes a depressing synapse into a facilitating one, and that adding back EGTA in PV−/− mice restores the wild-type phenotype.

Slow buffers such as EGTA affect transmitter release after an unconditioned stimulus only if release sites are not in direct vicinity to the site of Ca2+ influx, the voltage-dependent Ca2+ channels (8). In some preparations [squid giant synapse (27) and pyramidal-pyramidal synapses (31)], where influx and release are apparently tightly coupled together, EGTA does not inhibit markedly the probability of release, whereas in other preparations [crayfish neuromuscular junction (32) or hippocampal mossy fiber synapse (33, 34)], where the coupling is looser, a significant reduction is observed. Our finding that the probability of failures is not different in PV+/+, PV−/−, and in PV−/− strains, and that it is not modified by addition of EGTA in PV−/− mice (Table 1), suggests that the interneuron-Purkinje cell synapse falls in the first category, where release sites are closely associated to voltage-dependent Ca2+ channels.

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Throughout the 30- to 300-ms range in the wild type (Fig. 2C) suggests that, in this condition, facilitation is negligible.

Thus, our results are most simply explained by assuming that PV and EGTA specifically and completely inhibit facilitation. Both buffers act presumably by reducing the residual Ca\(^{2+}\) of depression expected from vesicle deprivation, thus preventing the potentially deleterious effects of excessive Ca\(^{2+}\) accumulation in the synaptosome cell.

With 730 \(\mu M\) Mg\(^{2+}\), adding 1 mM EGTA in PV--/+ mice restored the same PPR as that obtained in PV+/+ mice with 50 \(\mu M\) EGTA (Fig. 5). This result indicates that the speed of Ca\(^{2+}\) removal is the same for 950 \(\mu M\) EGTA and for the (unknown) concentration of PV prevailing in PV--/+ mice. Because the dissociation rate constants of Ca\(^{2+}\)-EGTA and Ca\(^{2+}\)-PV, 0.3 s\(^{-1}\) (41) and 1 s\(^{-1}\) (12), respectively, are small compared with the inverse of the ISI (33 s\(^{-1}\)), the rate of Ca\(^{2+}\) decay is determined by the rate of binding of Ca\(^{2+}\) to the buffers. Assuming apparent binding rates of 2.7 \(\times 10^{4}\) M\(^{-1}\)s\(^{-1}\) for EGTA (42) and of 4.3 \(\times 10^{4}\) M\(^{-1}\)s\(^{-1}\) for PV [calculated assuming a value of 730 \(\mu M\) for [Mg\(^{2+}\)]], according to (12), it may be calculated that the concentration of PV in PV+/--/+ mice is 600 \(\mu M\). This is a large value, which is however in line with the earlier estimate of 1 mM obtained with immunogold staining in axons and presynaptic terminals of cerebellar stellate and basket cells (14). With a concentration of this order of magnitude, it is hardly surprising that PV exerts potent effects on short-term synaptic plasticity in this preparation.

A striking correlation was found in various brain regions (hippocampus, neostriatum, and frontal cortex) between the presence of PV and the firing pattern of neurons: unlike other GABAergic neurons, PV-containing neurons are always able to fire repetitively at a high rate (10, 43, 44). In line with this correlation, we note that cerebellar stellate and basket cells fire in bursts at high rate in vivo (16). The present results, together with previous ones (11, 12), suggest two ways by which the presence of PV in the presynaptic neuron could contribute to maintain synaptic transmission during bursts. First, PV helps bringing down the presynaptic Ca\(^{2+}\) quickly, thus preventing the potentially deleterious effects of excessive Ca\(^{2+}\) accumulation in the synaptosome cell. Second, PV maintains the strength of the postsynaptic neuron near its resting level by preventing cumulative facilitation, thus permitting nearly linear information processing in the postsynaptic neuron as a function of action potential number. It should be pointed out, however, that the removal of facilitation would work only as long as the incoming Ca\(^{2+}\) does not saturate PV. Thus, for prolonged bursts at high rates of firing, presynaptic Ca\(^{2+}\) accumulation and postsynaptic facilitation are expected to reappear (12), unless depression takes the lead (17, 37).

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