Involvement of Calcium in Rhythmic Activity Induced by Disinhibition in Cultured Spinal Cord Networks

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Darbon, Pascal, Christophe Pignier, Ernst Niggli, and Jürg Streit. Involvement of calcium in rhythmic activity induced by disinhibition in cultured spinal cord networks. J Neurophysiol 88: 1461–1468, 2002; 10.1152/jn.00213.2002. Disinhibition of rat spinal networks induces a spontaneous rhythmic bursting activity. The major mechanisms involved in the generation of such a bursting are intrinsic neuronal firing of a subpopulation of interneurons, recruitment of the network by recurrent excitation, and autoregulation of neuronal excitability. We have combined whole cell recording with calcium imaging and flash photolysis of caged-calcium to investigate the contribution of \([Ca^{2+}]_i\) to rhythmogenesis. We found that calcium mainly enters the neurons through voltage-activated calcium channels and \(N\)-methyl-b-aspartate (NMDA) channels as a consequence of the depolarization during the bursts. However, \([Ca^{2+}]_i\) could neither predict the start nor the termination of bursts and is therefore not critically involved in rhythmogenesis. Also calcium-induced calcium release is not involved as a primary mechanism in bursting activity. From these findings, we conclude that in the rhythmic activity induced by disinhibition of spinal cord networks, the loading of the cells with calcium is a consequence of bursting and does not functionally contribute to rhythm generation.

INTRODUCTION

Two types of spontaneous rhythmic activity are observed in spinal cord preparations in vitro: fictive locomotion (Cazalets et al. 1990; Grillner et al. 1991) and synchronous bursting induced by disinhibition of the network through the blockade of \(GABA_A\) and glycine receptors. The latter rhythms are well documented in the isolated spinal cord (Bracci et al. 1996), in organotypic spinal slice cultures (Ballerini et al. 1999; Streit 1993; Tscharter et al. 2001), and in cultures of dissociated spinal neurons (Gross et al. 1982; Streit et al. 2001). Although they are clearly distinct from the rhythms involved in the control of locomotion, they are probably produced by similar networks (Streit et al. 2001; Tscharter et al. 2001). The study of these rhythms may therefore reveal some of the mechanisms involved in rhythm generation of spinal networks. Furthermore, similar rhythms are found in other neuronal structures: in slices of cortex (Sanchez-Vives and McCormick 2000), hippocampus (Traub and Jefferys 1994), olfactory bulb (Puopolo and Belluzzi 2001), thalamus (Jonkheer et al. 1993), hypothalamus (Poulain 2001), and brain stem (Koshiya and Smith 1999), as well as in culture of dissociated cortical (Maeda et al. 1995) and hypothalamic neurons (Müller and Swandulla 1995). The underlying mechanisms may therefore represent basic features of neuronal networks.

We have previously proposed that the major mechanisms involved in the generation of disinhibition-induced bursting in spinal cultures are intrinsic neuronal firing, recruitment of the network by recurrent excitation and autoregulation of neuronal excitability (Darbon et al. 2002). Neuronal excitability is proposed to be regulated, among other factors, by internal calcium concentration (Turrigiano et al. 1994). Changes in intracellular calcium during rhythmic network activity have been described in cortical (Robinson et al. 1993) and brain stem cultures (Baker et al. 1995). Such changes may be caused by an entry of calcium through voltage-dependent channels and/or through receptor-operated channels like NMDA channels (Barish 1991; Usachev and Thayer 1997). In addition, changes in intracellular calcium may be amplified by the contribution of calcium from intracellular stores.

The role of such changes is not clear. It could be that they are simply a side effect of bursting, or, alternatively they may be involved in rhythm generation (Baker et al. 1995). To approach this question, we have investigated the contribution of \([Ca^{2+}]_i\) to rhythmogenesis by combining intracellular calcium imaging with patch-clamp recording. We found that calcium mainly enters the neurons through voltage-activated calcium and NMDA channels as a consequence of the depolarization during the bursts. Calcium levels could predict neither the start nor the termination of bursts and are therefore not critically involved in rhythmogenesis.

METHODS

 Cultures

Dissociated cultures were made from the whole spinal cord of rats at embryonic age 14 (E 14). The cultures were prepared as previously described (Streit et al. 2001). Briefly, the fetuses were delivered by caesarian section from rats anesthetized with 0.4 ml Vetanarcol (pentobarbiturate i.m.) killed by decapitation. The treatment of the animals was in accordance with guidelines approved by Swiss local authorities. Their backs were isolated from their limbs and viscera and cut into 225-μm-thick transverse slices with a tissue chatter. Slices of all regions of the spinal cord without dorsal root ganglia were exposed to a 0.3% trypsin solution for 3 min at 37°C. After that they were mechanically dissociated by forcing them through fine-tipped pipettes several times. The cells were plated into microwells at a density of

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75,000/150 µl and were maintained in microwells containing 150 µl nutrient medium and incubated in a 5% CO₂-containing atmosphere at 36.5°C. MEM Eagle (Sigma, Fluka Chemie AG, Buchs, Switzerland), supplemented with fetal bovine serum 10%, glucose 0.2%, B 27 and Glutamax (Gibco BRL, Life Technologies AG, Basel), was used. Half of the medium was changed weekly.

**Patch-clamp recordings**

The whole cell patch-clamp recording technique (Hamill et al. 1981) was employed by using either an Axoclamp-2B (Axon Instruments, Foster City, CA) amplifier or an Axopatch 200 (Axon Instruments). Currents were filtered at 3–5 kHz and sampled at 5–10 kHz using an A/D converter and either pClamp 8 (Axon Instruments) or LabView acquisition software (National Instruments, Ennetbaden, Switzerland). Recording electrodes were pulled from filamented borosilicate glass capillaries (GC150F, Harvard Apparatus GmbH, March-Hugstetten, Germany) on a horizontal puller (DMZ, Zetz Instrumente GmbH, München, Germany). Typical pipette resistance was around 5 MΩ. Cells were observed in whole cell current clamp. Recordings were made in a special chamber mounted on a microscope from cultures of 3–6 weeks of age. The culture medium was replaced by an extracellular solution containing (in mM) 145 NaCl, 4 KCl, 1 MgCl₂, 2CaCl₂, 5 HEPES, 2 Na-pyruvate, and 5 glucose at pH 7.4. The bath solution was continuously superfused at a minimum rate of 1 ml/min. All recordings were made at room temperature (22–26°C).

**Confocal recordings**

Cells were imaged with a ×40 oil-immersion objective (Fluor, NA = 1.3; Nikon, Japan). Fluo3 was excited with 488-nm line of an argon-ion laser (Model 5000, Ion Laser Technology, Salt Lake City, UT) at 5 µW. The fluorescence was detected at 540 ± 15 nm with the photomultiplier tube of a laser-scanning confocal system (MRC 1000, Bio-Rad, Glattbrugg, Switzerland) operated in Time Course Software Module (TCSM) mode or in line-scan mode. The sampling rate in TCSM mode was 2 Hz (unless otherwise stated). In line-scan mode, the speed was set to 6 ms per line. The 512 lines recorded in one frame corresponded to 3.072 s. To load the dye into the cells, fluo3 (50 µM) was added to the pipette solution or the cultures were incubated with membrane permeable fluo3AM (5 µM) at RT for 30 min (following by a 30-min pause for de-esterification). The calibration of the calcium signal is in terms of percentage of fluorescence change (ΔF) relative to the resting fluorescence level (F0), allowing comparison among cells of magnitude of the changes. Fluorescence images were processed using a customized version of the public domain National Institutes of Health Image program. To avoid the bleaching of fluo3, the laser power was set to 3% and cells were illuminated every 0.5 s. Moreover, the stability of the baseline was used as a criterion of absence of bleaching. When the drift of the baseline was >20% after 1 h, the recording was discarded.

**Flash lamp photolysis**

Ultraviolet flashes (Strobex 238/278, Chawick, El Monte, CA, 230 W) were used to photolyse intracellular DM-nitrophen in an epi-illuminated arrangement and were generated with a frequency-tripled neodymium: yttrium-aluminum garnet laser [Surelite-II, Continuum, Santa Clara, CA; wavelength: 355 nm, maximum energy: 20.7 mJ (as described in DelPrincipe et al. 1999)].

**Analysis**

Event detection and further analysis were done off-line using the computer program IGOR (WaveMetrics, Lake Oswego, OR). In patch-clamp experiments, the bursts were defined as periodic membrane depolarizations either completely crowned with spikes at high-frequency (repetitive firing burst) or crowned with only a few spike at the beginning (plateau burst) (see also Darbon et al. 2002). The burst rate was calculated from the burst period (measured from the start of a bursting event to the beginning of the next one). In calcium imaging experiments, the variation of fluorescence level were displayed as fast and transient peaks detected with a built-in peak detection function. The peak frequency was calculated from the peak period measured as the peak-to-peak time. The peak duration was measured as the difference between the time at 50% of the rising phase and at 50% of the decreasing phase of the peak. All the results are given in means ± SE (unless otherwise stated).

**Chemicals**

Bicuculline, strychnine, dl-2-amino-5-phosphonovaleric acid (APV), 6-cyano-7-nitroquinoxalene-2,3-dione (CNQX), caffeine were purchased from Sigma; TTX, ryanodine from Alomone Labs (Jerusalem, Israel); Fluo3 (pentapotassium salt) and Fluor3 acetoxyethyl (AM) ester from TecLabs (Austin, TX).

**RESULTS**

Rhythmic activity in calcium imaging and whole cell patch-clamp recording

To assess the involvement of calcium in the bursting activity, we have measured by confocal microscopy the intracellular calcium level in 133 cells from 31 dissociated 3- to 6-wk-old cultures of embryonic rat spinal cord. Before the disinhibition induced by co-application of bicuculline and strychnine (B + S), the cells displayed irregular fast transients of the fluorescence level (Fig. 1), which were usually not synchronized between individual cells. When B + S was added to the bath, the cells showed large periodic calcium transients (42.47 ± 2.7% ΔFI/F, n = 133, ranging from 4.96 to 251.55% ΔFI/F), which were synchronized in all cells of the recorded optical field. The frequency of the periodic calcium transients (calculated from the peak-to-peak time) was 4.17 ± 0.1 peaks/min. In addition the baseline fluorescence level increased by 14.51 ± 0.5% ΔFI/F. The periodicity of the calcium signals under disinhibition looked similar to the periodic bursting activity observed in whole cell patch-clamp recording under the same conditions (Darbo et al. 2002). Whole cell recordings were established in 87 cells with an average resting potential of ~48.85 ± 0.85 mV. When disinhibited by strychnine and bicuculline, they showed a regular pattern of rhythmic activity (Fig. 2) consisting of bursts of action potentials alternating with more or less silent hyperpolarized periods, the interburst intervals. The average burst rate was 4.95 ± 0.28 bursts/min, the average burst duration was 8.04 ± 0.64 s and the average membrane potential in the interburst intervals was −58.06 ± 1.16 mV.

These data suggested that calcium transients accompanied the bursting. To find out whether calcium transients simply follow the bursts or whether they may be involved in the induction of bursts, we investigated the temporal relationship between the bursts and the calcium transients. In experiments where calcium imaging was combined with whole cell recordings, the rapid depolarization at the beginning of the bursts
always preceded the calcium rise (Fig. 3, n = 8). This finding suggests that the calcium rise is a consequence of bursting and not its prerequisite. This was confirmed in experiments in which intracellular calcium was rapidly increased by flash photolysis of DM-nitrophen without inducing bursts (n = 5, see also Fig. 7). Altogether these findings suggest that calcium transients represent a loading of the neurons with calcium that was induced by the bursts.

Pathways of calcium entry

The loading of the cells with calcium during the bursts can occur in three ways: calcium influx through calcium-permeable glutamate receptors or voltage-dependent calcium channels (VDCCs) or calcium release from internal calcium stores. The best known calcium-permeable glutamate receptors are the NMDA receptors (Mayer et al. 1987), although some AMPA receptors can also be calcium permeable depending on their subunit composition.
Calcium peaks to 69.0 during the bursts. APV decreased the mean amplitude of the NMDA antagonist APV on calcium loading of the neurons (Ballerini et al. 1999; Streit 1993).

(Asher et al. 1988). In the spinal cord, the recurrent excitation underlying bursting depends on the opening of AMPA and NMDA receptors (Ballerini et al. 1999; Streit 1993).

To determine the contribution of the NMDA receptors to the calcium entry into the neurons, we investigated the effect of the NMDA antagonist APV on calcium loading of the neurons during the bursts. APV decreased the mean amplitude of the calcium peaks to 69.0 ± 3% (n = 44) of the control value under disinhibition (see Table 1). The total amount of calcium inflow, which is best represented by the area of the peak, was also reduced to 71.3 ± 3.2% (n = 44). APV decreased the average burst duration to 75.8 ± 6.6% of the control, while leaving the duration of the intervals between the bursts unchanged (Table 1 and Fig. 4A). It thus increased the burst rate as well as the rate of the calcium peaks to 127 ± 9.8 (n = 13) and 118.8 ± 4.8% (n = 44), respectively. The shortening of the bursts may partly explain the decrease in the area of the calcium peaks, however, not the decrease in the peak amplitude. When the NMDA-Rs are blocked, only the AMPA-Rs are responsible for the depolarization of the membrane. The depolarization and the evoked spikes activate VDCCs, which open another pathway for the calcium inflow. Therefore any changes in the amount of depolarization and in the spike rate during the burst have to be considered to estimate the contribution of NMDA-Rs to the total amount of calcium inflow during the burst. As expected, APV partly repolarized the membrane potential during the bursts from −28.0 ± 3.0 to −30.9 ± 2.3 mV (n = 10). However, in parallel it increased the spike rate during the bursts from 23.4 ± 5.8 to 33.3 ± 5.9 Hz. Because the spike rate was increased, calcium inflow during the spikes through high-voltage-activated calcium channels was probably also increased and certainly not reduced. The reduction in the amplitude of the calcium peak by APV is therefore most likely due to the contribution of the NMDA channels to the calcium inflow.

The contribution of AMPA receptors to the internal calcium signal cannot be tested in a similar way because the blockade of AMPA receptors leads to a complete cessation of bursting in most of the cultures. When the AMPA receptors were partly inhibited by low doses of CNQX (1 µM), similar effects on the depolarization and the spike rate during the bursts were seen as with APV: the membrane potential repolarized from −36.3 ± 2.2 to −39.6 ± 1.9 mV (n = 18) while the spike rate increased from 26.8 ± 4.8 to 36.4 ± 6.0 Hz. In parallel, the burst rate was reduced while the burst duration was unchanged in most cases.

### Table 1. Effect of various antagonists on the features of the rhythmic activity observed with patch-clamp or calcium imaging

<table>
<thead>
<tr>
<th></th>
<th>Burst</th>
<th>Interburst</th>
<th>Calcium Peak</th>
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<tr>
<td></td>
<td>Frequency</td>
<td>Duration</td>
<td>Frequency</td>
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<tr>
<td>B + S (20 µM + 1 µM)</td>
<td>85</td>
<td>100**</td>
<td>100</td>
</tr>
<tr>
<td>+ APV (50 µM)</td>
<td>13</td>
<td>127.5 ± 9.8**</td>
<td>75.8 ± 6.6*</td>
</tr>
<tr>
<td>+ CNQX (1 µM)</td>
<td>20</td>
<td>73.1 ± 10.1**</td>
<td>95.6 ± 13.1NS</td>
</tr>
<tr>
<td>+ Ryanodine (100 µM)</td>
<td>7</td>
<td>94 ± 7.4NS</td>
<td>92.2 ± 6.8NS</td>
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B + S, bicuculline and strychnine; APV, 2-amino-5-phosphonovaleric acid; CNQX, cyan-7-nitroquinoloxalene-2,3-dione. * Significant (P < 0.05); ** highly significant (P < 0.01); NS nonsignificant.

![ FIG. 3. Combined recording of intracellular calcium variation and electrical activity. A: The pattern of the electrical activity of 1 cell is identical to the pattern of the periodic calcium transients observed in 5 other cells of the same network. B: in another network, the simultaneous combined recording of the intracellular calcium signal at fast sampling rate (5 Hz) and of the membrane potential in the same cell shows that the burst starts before the calcium rise.](http://jn.physiology.org/)

![ FIG. 4. Effects of antagonists of the N-methyl-D-aspartate and AMPA receptors (NMDA-Rs and AMPA-Rs) on the shape of calcium transients. A: average of 11 peaks recorded in the same experiment under disinhibition by strychnine and bicuculline (- - -). Average of 10 peaks (---) after blockade of the NMDA-Rs by bath application of APV (50 µM). B: average of 5 peaks recorded in the same experiment under disinhibition by strychnine and bicuculline (---). Average of 4 peaks (---) after the partial blockade of the AMPA-Rs by bath application of 6-cyano-7-nitroquinoloxalene-2,3-dione (CNQX, 1 µM).](http://jn.physiology.org/)
experiments with low doses of CNQX (see Table 1). However, CNQX did not change the amplitude of the calcium peaks, although it reduced the area of the peaks to 71.8 ± 4.2% (Fig. 4B, n = 19).

A direct determination of the contribution of the VDCCs to the calcium inflow is difficult because a block of these channels will suppress synaptic transmission, which is a prerequisite for the recurrent excitation underlying bursting. Nevertheless, in few experiments bursts were long enough to compare the spike rate during the bursts with the shape of the calcium transients. In these experiments (Fig. 5), the internal calcium seemed to be correlated to the spike rate during the bursts: it increased at high rates and decreased at low rates. This finding is in agreement with the hypothesis of a major contribution of Ca\(^{2+}\) entry through VDCCs to the internal Ca\(^{2+}\) signal. From these findings, we conclude that the major pathways of the calcium inflow are the VDCCs and the NMDA-Rs. However, we cannot completely exclude a minor contribution of the AMPA-Rs.

**Internal calcium stores**

In many systems the release of calcium from internal stores contributes to transients in internal calcium. A prominent pathway is the calcium-induced calcium release (CICR) through the ryanodine receptors located on the internal membrane of the calcium stores (Verkhratsky and Shmigol 1996). It is still controversial whether this mechanism significantly contributes to neuronal functions like transmitter release or neuronal excitability (Carter et al. 2002; Emptage et al. 2001; Llano et al. 2000). However, it has been shown that CICR is induced by Ca\(^{2+}\) influx via NMDA receptors (Emptage et al. 1999) and by short bursts of action potentials (Usachev and Thayer 1997). To investigate a possible contribution of CICR to the bursting mechanisms, we have blocked the ryanodine receptors with high doses of ryanodine (100 μM) to stop the release of calcium from the internal stores. Under these conditions, there were no significant changes either on burst parameters like duration and rate or on the amplitude and area of the calcium signal (Table 1). We conclude that CICR does not significantly contribute to the calcium transients during bursting and that it is not involved as a primary mechanism in bursting.

**Is calcium involved in the regulation of excitability?**

We have previously suggested that the major mechanisms involved in bursting are spontaneous spiking in a subpopulation of neurons, recurrent excitation and an autoregulation of excitability (Darbon et al. 2002). Excitability may be regulated by the internal calcium concentration through calcium-dependent potassium channels. If this is true for spinal neurons, an increase in the internal calcium concentration should cause a hyperpolarization and a decrease in excitability. We have tested this hypothesis by using caffeine (20 mM) to transiently increase the internal calcium in all neurons of the network. Indeed caffeine induced a huge calcium peak with a mean area of 209.89 ± 21.8% (n = 38, range: 106.6–1,035.6%) of the values under B + S (Fig. 6). During this calcium peak, caffeine provoked a fast transient hyperpolarization of the membrane potential (−2.53 ± 0.57 mV lasting 10–30 s, n = 7), during which the burst duration largely decreased to 27.1% of the B + S value. This hyperpolarization induced by caffeine was independent of the rhythmic activity because it could still be provoked when the activity was blocked by CNQX (10 μM) and APV (50 μM). These observations show that a high concentration of intracellular calcium decreases excitability. However, the calcium peaks during the bursts were much smaller than the peaks induced by caffeine (Fig. 6). Therefore we next investigated whether a decrease in excitability induced by the intracellular calcium peaks during the burst may be sufficient to determine the end of the bursts. If this would be true, intracellular calcium should steadily increase during the bursts until reaching a threshold value, which terminates the burst. This was clearly not found. In all cells in which calcium peaks were measured in combination with whole cell recordings, the calcium peaks were reached before the end of the bursts (see Fig. 3). Furthermore, when in a cell the calcium was increased during the burst by flash photolysis of caged calcium, the burst was neither stopped nor was the cell hyperpolarized (Fig. 7, n = 5, for levels of the discharged energy ranging from 70 to 160 J).

The average time to peak to the calcium transients was significantly shorter than the average burst duration (4.9 ± 0.4
Mechanisms involved in bursting contribute to shape the rhythmic patterns. Calcium transients occur during bursting, but the main source is calcium entry mainly through NMDA channels, VDCCs, and calcium-activated potassium channels. The estimation of the calcium entry is mainly NMDA channels, VDCCs, and calcium-activated potassium channels.

**DISCUSSION**

In this paper, we have investigated the hypothesis that changes in [Ca$^{2+}$]i in interneurons of cultured spinal cord networks are involved in the regulation of rhythmic bursting, which is induced by disinhibition. Our findings suggest that although calcium transients occur during bursting, they are mainly the consequence of calcium inflow through VDCCs and NMDA channels during the bursts and do not critically contribute to shape the rhythmic patterns.

**Mechanisms involved in bursting**

The blockade of GABAA and glycine receptors induces the disinhibition of the whole network leading to a rhythmic activity throughout the entire culture. In previous studies (Darbon et al. 2002; Streit et al. 2001), we have reported on extracellular and intracellular activity in disinhibited dissociated cultures of rat spinal cord. We have shown that intrinsic spiking in some neurons, recurrent excitation through glutamatergic synaptic transmission, and autoregulation of neuronal excitability mainly controls the pattern of rhythmic activity induced by disinhibition. A similar mechanism has been proposed to underlie the slow oscillations in cortical slices (Sanchez-Vives andMcCormick 2000). In the context of this hypothesis, the autoregulation of neuronal excitability plays an important role in the shaping of the rhythms. In the cortical slices, spike frequency adaptation based on calcium- and sodium-dependent potassium conductances has been proposed to cause the decrease in excitability during the bursts and the slow afterhyperpolarization following the bursts (Sanchez-Vives andMcCormick 2000). Calcium-activated potassium conductances together with calcium inflow through NMDA channels are also reported to be the major rhythmicogenic mechanism in lamprey spinal cord (Grillner et al. 1995). In spinal interneurons in culture, however, spike frequency adaptation is not prominent during the injection of sustained current pulses (Ballerini et al. 1999; Darbon et al. 2002). Although there is a slow afterhyperpolarization following the bursts in about half of the interneurons, this can therefore not easily be attributed to an accumulation of calcium or sodium-dependent potassium conductances (Darbon et al. 2002). In addition, apamine, a blocker of one of the calcium-dependent potassium channels, has no effect on burst duration. Other mechanisms must therefore be considered.

**Sources of calcium**

Bursting is paralleled by a loading of the neurons with calcium. Similar findings have been made in cortical (Robinson et al. 1993) and hippocampal cultures (Bacciet et al. 1999). Combined patch and calcium imaging recordings show that the increase in calcium always follows the depolarization in single neurons. Therefore, the calcium peaks are most likely caused by calcium inflow during depolarization. Possible pathways of calcium entry are mainlly NMDA channels, VDCCs, and AMPA channels. In addition, the loading of the cells with calcium may be augmented by CICR. The estimation of the relative contributions of the sources of calcium is compromised by the fact that the various sources mutually depend on each other. The sequence of events at the beginning of a burst is first, the opening of the AMPA channels leading to a rapid membrane depolarization, thus removing the magnesium block of the NMDA channels (Ascher et al. 1988) and permitting the calcium inflow. The depolarization leads to the opening of the VDCCs, which increases the calcium inflow. When the AMPA receptors are blocked, this will therefore influence all three pathways. When the NMDA receptors are blocked, the NMDA and the VDCC pathways will be affected. The NMDA antagonist APV slightly repolarized the membrane potential during the bursts as expected; however, it surprisingly increased the spike rate, probably due to a relaxation of part of the sodium channels from inactivation. Because most of the calcium inflow goes through high-threshold-activated calcium channels, which open at approximately $-20$ mV (Carbone and Lux 1987), this mainly occurs during the spikes. Indeed, the calcium concentration followed the spike rate during long bursts with large variations in spike rate (Fig. 5). The reduction of the
calcium peak by APV may therefore rather represent an underestimation of the contribution of the NMDA channels to the calcium inflow due to the partial compensation of the effect by the VDCCs. We have made no attempt to determine experimentally the contribution of the AMPA channels to the calcium inflow. AMPA receptors can also be calcium permeable, depending on their subunit composition; but this is probably only a small percentage of all receptors (Pellegrini-Giampietro et al. 1997). Also CICR from internal stores is excluded as a significant calcium source during the bursts because ryanodine at concentrations where it blocks CICR had no effect on the calcium peaks. In summary, we conclude from our findings that the calcium is entering the cells mainly through VDCCs (−2/3) and NMDA channels (−1/3).

Involvement of calcium in rhythm generation?

Although we found no evidence for an involvement of calcium-dependent potassium currents in rhythm generation, calcium may regulate bursting through other mechanisms. Turrigiano et al. (1994) proposed that neuronal excitability is regulated by intracellular calcium. Calcium inactivates for example VDCCs thus leading to a decrease in calcium currents. Such a mechanism has been shown to decrease excitability and cause conduction failures in dorsal root ganglion cells of spinal slice cultures (Lüscher et al. 1994, 1996). Furthermore this mechanism may also lead to a decrease in the probability of synaptic release (Jia and Nelson 1986) and thus to a depression of synaptic efficacy during bursting. In addition, calcium directly inactivates NMDA channels (Legendre et al. 1993), again leading to synaptic depression. However, as argued in previous papers (Darbon et al. 2002; Streit 1993), synaptic depression is probably more involved in fast oscillations than in slow bursting.

Disinhibition-induced bursting is characterized by a strong correlation between the burst duration and the duration of the preceding interval (Streit 1993; Streit et al. 2001; Tabak et al. 2001; Tscherter et al. 2001). Such a finding suggests, that the termination of the bursts is a deterministic, whereas the initiation is a more variable process (Tabak et al. 2001). The bursts may be terminated by the accumulation of calcium up to a certain threshold. The following two findings argue against this hypothesis: first, the peak of the calcium concentration was always reached before the termination of the bursts. In some experiments, the calcium concentration followed the spike rate and thus showed large variations during the bursts. Therefore bursts ended at variable calcium concentrations, which is not compatible with a deterministic process based on a calcium threshold. Second, a rapid increase in calcium during the burst did not terminate the burst.

We have also tested the hypothesis that the variability in burst initiation may be caused by the variability in the internal calcium concentration, assuming that bursts may be initiated at a defined low calcium concentration. During the intervals between the bursts, the calcium concentration slowly decreased, the new bursts, however, started at variable levels of calcium. Again, this variability argues against a role of calcium in burst initiation. In line with our conclusions that internal calcium plays a minor role in rhythm generation is the finding that the blockade of CICR has no effect on bursting.

In summary we have found that during disinhibition-induced bursting spinal interneurons are loaded with calcium. This calcium enters the cells through NMDA and voltage-dependent calcium channels, which are activated during the bursts. The loading of the cells is a consequence of bursting and does not functionally contribute to rhythm generation.

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