Experimental and Modeling Studies of Novel Bursts Induced by Blocking Na\textsuperscript{+} Pump and Synaptic Inhibition in the Rat Spinal Cord

ALDO ROZZO,1,2 LAURA BALLERINI,1,2 GILDA ABBATE,3 AND ANDREA NISTRI1,2

1Biophysics Sector and 2Istituto Nazionale Fisica della Materia Unit, International School for Advanced Studies (SISSA), 34014 Trieste; and 3Department of Mathematics, Avogadro University of Western Piedmont, 13100 Vercelli, Italy

Received 14 January 2002; accepted in final form 25 April 2002

Rozzo, Aldo, Laura Ballerini, Gilda Abbate, and Andrea Nistri. Experimental and modeling studies of novel bursts induced by blocking Na\textsuperscript{+} pump and synaptic inhibition in the rat spinal cord. J Neurophysiol 88: 676–691, 2002; 10.1152/jn.00027.2002. This study addressed some electrophysiological mechanisms enabling neonatal rat spinal networks in vitro to generate spontaneous rhythmicity. Networks, made up by excitatory connections only, after block of GABAergic and glycnergic transmission, develop regular bursting (disinhibited bursts) suppressed by the Na\textsuperscript{+} pump blocker strophanthidin. Thus the Na\textsuperscript{+} pump is considered important to control bursts. This study, however, shows that, after about 1 h in strophanthin solution, networks of the isolated spinal cord surprisingly resumed spontaneous bursting (“strophanthin bursting”), which consisted of slow depolarizations with repeated oscillations. This pattern, recorded from lumbar ventral roots, was synchronous on both sides, of irregular periodicity, and lasted for \( \geq 12 \) h. Assays of \( ^{86}\text{Rb}^\text{+} \) uptake by spinal tissue confirmed Na\textsuperscript{+} pump block by strophanthidin. The strophanthin rhythm was abolished by glutamate receptor antagonists or tetrodotoxin, indicating its network origin. \( N\)-methyl-\( \alpha \)-aspartate (NMDA), serotonin, or high K\textsuperscript{+} could not accelerate it. The size of each burst was linearly related to the length of the preceding pause. Bursts could also be generated by dorsal root electrical stimulation and possessed similar dependence on the preceding pause. Conversely, disinhibited bursts could be evoked at short intervals from the preceding one unless repeated pulses were applied in close sequence. These data suggest that rhythmicity expressed by excitatory spinal networks could be controlled by Na\textsuperscript{+} pump activity or slow synaptic depression. A model based on the differential time course of pump operation and synaptic depression could simulate disinhibited and strophanthidin bursting, indicating two fundamental, activity-dependent processes for regulating network discharge.

Intronction

Spinal networks can generate a range of rhythmic electrical discharges comprising oscillations and bursting via intrinsic central pattern generators (CPGs; Marder and Calabrese 1996), which are thought to play an important role in the development of network connectivity (Feller 1999). A characteristic rhythm pattern, readily studied in model preparations like the neonatal rat spinal cord in vitro, is fictive locomotion, which consists in rapidly alternating ventral root (VR) oscillations evoked by bath-applied excitatory substances (reviewed by Kiehn and Kjaerulff 1998; Kiehn et al. 2000) or by dorsal root (DR) stimuli (Marchetti et al. 2001a). During fictive locomotion, the CPG expresses a rhythmic excitatory drive, mainly of glutamatergic nature, with alternating discharges due to patterned inhibition mediated by glycine and GABA\textsubscript{A} receptors.

Conversely, a distinct, rhythmic spinal activity develops spontaneously when glycine and GABA\textsubscript{A} receptors are blocked (Bracci et al. 1996a). This pattern, termed disinhibited bursting, is made up by synchronous, regular, slow bursts with intraburst oscillations. Multi-site recordings from organotypic slice cultures have shown that disinhibited bursting is due to a wave of excitation caused by firing of spontaneously active neurons in the ventral horn and spreading widely through the spinal network via recurrent excitation (Tschetter et al. 2001), while the dorsal horn is not implicated in this phenomenon (Ballerini et al. 1999). Although the question of whether fictive locomotion and disinhibited bursting are generated by the same CPG is not fully resolved, current evidence favors a common rhythmonic network (Beato and Nistri 1999) and accords with the notion that each segment of the spinal cord contains a unit burst generator coupled to other segments to express rhythmicity (Grillner et al. 1991). The strength of intersegmental excitatory (Beato and Nistri 1999) or inhibitory (Marchetti and Nistri 2001) connections is variable and can be changed by neuromodulators (Marchetti and Nistri 2001). While the disinhibited rhythm is clearly unable to support locomotion (even though it generates rhythmic muscle contractions; Tschetter et al. 2001), it remains an interesting paradigm to study the mechanisms enabling a mammalian spinal CPG (functionally made up by excitatory connections only and with preserved cytoarchitecture) to generate spontaneous bursts and to control their duration. A similar approach has also been used to study network bursting in the rat hippocampus (Staley et al. 2001; Troub and Miles 1991).

Former studies of bursting activity in spinal (Fedruch et al. 1999; Keefer et al. 2001; Streit 1993; Tabak et al. 2001) or hippocampal (Staley et al. 1998, 2001) networks in vitro have indicated synaptic depression to be important to control onset and termination of burst episodes. Conversely, in the rat isolated spinal cord, disinhibited bursting crucially depends on the electrogenic Na\textsuperscript{+}/K\textsuperscript{+} pump activity, because pharmacological block of this transporter completely disrupts regular rhythmicity (Ballerini et al. 1997). Recent preliminary experiments, however, indicated that, after a long period (about 1 h) of burst...
suppression by pump inhibitors, a novel type of spontaneous bursting emerged (Rozzo et al. 2000). This unexpected finding prompted us to investigate the properties of this phenomenon, because it might help to understand the most basic mechanisms responsible for rhythmicity. Hence, the aims of this study were as follows: 1) to compare the electrophysiological properties of this late bursting with those of the standard disinhibited rhythm and the respective weight of Na⁺ pump activity versus synaptic depression; 2) to confirm, with biochemical methods, the effective inhibition of the Na⁺ pump by a blocker like strophanthidin; and 3) to develop a model, based on experimental data, to describe the spontaneous rhythmicity of excitatory networks in the rat spinal cord.

METHODS

Experimental preparation, electrophysiology, and data analysis

Experiments were performed on thoracosacral spinal cord preparations isolated from neonatal Wistar rats (5–7 days old) under urethane anesthesia (0.2 ml ip of a 10% wt/vol solution) as previously described (Bracci et al. 1998). This procedure is in accordance with the regulations of the Italian Animal Welfare Act and is approved by the local authority veterinary service.

Each preparation was placed in a small recording chamber and continuously superfused (7.5 ml · min⁻¹) with saline solution of the following composition (in mM): 113 NaCl, 4.5 KCl, 1 MgCl₂ 7H₂O, 2 CaCl₂, 1 NaH₂PO₄, 25 NaHCO₃, and 11 glucose, gassed with 95% O₂-5% CO₂ (pH 7.4 at room temperature). Drugs were bath-applied via the superfusing solution.

DC-coupled VR recordings (usually L₄, L₅, or L₆ VRs) were obtained with glass suction microelectrodes containing an Ag-AgCl pellet and filled with saline solution. Responses were amplified, digitized at 1 or 10 kHz using Axoscope software (Axon Instruments Inc., versions 7 and 8), displayed on a linear chart recorder, and stored on hard-disk and on video tape for further analysis. As detailed elsewhere (Beato and Nistri 1999; Bracci et al. 1996a,b), the following parameters were measured when recording bursting activity: time to plateau, plateau amplitude, burst decay time, burst duration, interburst interval (period), and pause between bursts. Burst duration was measured as the time during which the VR polarization level remained above a preset noise threshold, usually set at 25 times the SD of baseline noise. We also measured the frequency of oscillations within a single burst. Data were quantified as means ± SD with n = number of preparations (unless otherwise indicated). For each preparation, data analysis was based on the average of ≥10 burst episodes. Statistical significance was assessed with the Student’s t-test or analysis of variance (ANOVA), depending on whether data were normally or not normally distributed. The accepted level of significance was P = 0.05.

DR electrical stimuli, delivered via miniature bipolar suction electrodes, were employed to elicit evoked VR responses (recorded from the ipsilateral VR of the same segment). In all instances, stimulus intensity (1–to 20-V range; 0.1–1 ms duration) was calculated in terms of threshold (Th), defined as the minimum intensity to elicit a detectable response from the homolateral VR (on average Th = 1.9 ± 0.6 V; n = 7). DR compound action potentials were recorded from one severed end of isolated DRs while the other end was stimulated (1 Hz; 0.1 ms; 7–10 × Th) and averaged from ≥30 responses. VR responses to train of DR stimuli were measured as recently reported (Barbieri and Nistri 2001).

Na⁺/K⁺ pump activity

This activity was biochemically assayed according to the method by Longo et al. (1991), using, as an index, the transport of radioactive Rb⁺ (Rb⁸⁶; Amersham Pharmacia Biotech), because the Na⁺/K⁺ pump does not discriminate between K⁺ and Rb⁺ and can thus transport Rb⁸⁶ into cells.

For this purpose, spinal slices (350–400 μm thick) were cut in ice-cold solution with a Vibratome and allowed to recover for ≥1 h before use. The slices were preincubated (at room temperature) in control solution (normal saline solution) or in the presence of Na⁺ pump blockers (4 μM strophanthidin or 50–200 μM ouabain) for different times (30–60 min), after which 0.1 mM Rb⁸⁶ was added. Rb⁸⁶ initial specific activity was 37 MBq/ml and was calculated just prior to use on the basis of Rb⁸⁶ half-life (18.7 days). After 1.5–11.5 min of Rb⁸⁶ incubation, slices were collected and washed three times in isotonic saline solution (NaCl 150 mM, ice cold), to remove the isolate from the extracellular space. The slices were transferred into 1.5-ml tubes and dissolved with NaOH (0.2–0.5 N, 500 μl). Radioactivity was measured with a β counter and referred to the amount of protein content measured with a spectrophotometric assay (BioRad protein assay, based on the Bradford dye-binding procedure; Bradford 1976). Heat-inactivated (55°C for 60 min) spinal cord samples were used to assess nonspecific Rb⁸⁶ binding.

In control conditions, Na⁺ pump activity was expressed as total Rb⁸⁶ uptake (without addition of blockers) after subtracting nonspecific binding. Any residual Rb⁸⁶ signal left in the samples in the presence of saturating concentrations (50–200 μM) of the irreversible Na⁺ pump inhibitor ouabain was used to measure Rb⁸⁶ accumulation into cells via pump-independent mechanisms (mainly passive distribution). To correlate electrophysiological data with Na⁺ pump inhibition, Rb⁸⁶ uptake was measured after 30–60 min incubation with 4 μM strophanthidin (the concentration used for all electrophysiological experiments). Since ouabain induces a gradual increase in neuronal conductance (Willis et al. 1974) and loss of electrical responses (Ballerini et al. 1997), electrophysiological experiments on long-term inhibition of the Na⁺ pump were carried out in the presence of strophanthidin.

Modeling of spinal network activity

In accordance with the study by Tabak et al. (2000), model equations were implemented within XPPAUT (freely available software by G. B. Ermentrout, http://www.pitt.edu/~phase/), a general purpose interactive package for numerically solving and analyzing differential equations. XPPAUT includes a tool for calculation of bifurcation diagrams (AUTO). Simulations were performed using the Runge-Kutta integration method with a time step of 0.1 (dimensionless units). We confirmed that the results were unchanged when the time step was 0.01. Simulations were run on Linux PC.

RESULTS

Na⁺ pump inhibition

Our previous experiments have demonstrated that 4 μM strophanthidin fully disrupts disinhibited bursting (Ballerini et al. 1997). This treatment was adopted also for the present study, but it required prior validation that Na⁺ pump activity was effectively inhibited under the present experimental conditions. The spinal cord Na⁺ pump activity was measured in terms of its ability to accumulate Rb⁸⁶ (Bowen 1992; Longo et al. 1991) in control solution and in the presence of saturating concentrations of ouabain (50–200 μM; 30–60 min) or strophanthidin (4 μM; 30–60 min). Because data at 30- and 60-min incubations with each blocker were the same, they were pooled together. Likewise, as there was no difference between the effect of ouabain at 50 or 200 μM concentrations, results were also pooled together.

The time profile plot of Fig. 1 shows that, in control condi-
tions, spinal cord slices took up Rb\(^{86}\) linearly during the first 12 min. In the presence of 50–200 \(\mu M\) ouabain, Rb\(^{86}\) uptake was strongly inhibited (\(P < 0.0001\)), leaving only a small residual activity most likely reflecting passive redistribution of this isotope. In the presence of strophanthinid (4 \(\mu M\)), Rb\(^{86}\) accumulation was also significantly (\(P < 0.0001\)) smaller than in control and was as low as the one observed with ouabain, confirming that pharmacological treatment with strophanthinid fully inhibited Na\(^{+}\) pump activity. To infer the contribution by Na\(^{+}\) pump block to excitability of spinal pathways, we next studied the effect of strophanthinid on synaptic transmission induced by DR stimuli in control saline.

**Strophanthinid-induced effects on electrically evoked synaptic transmission**

We first examined the action of 4 \(\mu M\) strophanthinid on VR responses induced by single pulses (one every 30 s) applied to one DR at the same segmental level. While the average size of the compound action potential recorded from isolated DRs was unaffected (97 ± 16%; \(n = 7\)), neither the peak of the VR reflex nor the magnitude of the late reflex component was changed (112 ± 29% and 97 ± 33%, respectively; stimulus = 5 \(\times\) Th; \(n = 10\)). Associated changes in VR polarization level were 0.42 ± 0.31 mV (\(n = 10\)).

Substantial changes were, however, observed when standard protocols for DR stimulus trains (1 Hz, 20 pulses, 1 ms, 10 \(\times\) Th, applied after about 30 min in strophanthinid solution; Barbieri and Nistri 2001; Sivilotti et al. 1993), as exemplified in Fig. 2A in which averaged VR responses in control or strophanthinid solution, are superimposed. The amplitude of cumulative depolarization was largely reduced by strophanthinid while the half decay time was significantly prolonged as quantified in Fig. 2, B and C. In control saline, after the first two responses to the train, cumulative depolarization developed biphasically with an initially higher rate of rise, which then slowed down (Barbieri and Nistri 2001; Sivilotti et al. 1993). In strophanthinid solution, the rate of rise was as slow as the late phase in control solution (Fig. 2D). These changes might have been partly or wholly due to the neuronal depolarization developed in the presence of strophanthinid. To check for this possibility, we investigated the effect of NMDA (4 \(\mu M\); \(n = 5\)), which induced comparable baseline depolarization. In the presence of NMDA, the cumulative depolarization amplitude was 0.35 ± 0.11 mV, a value similar to the one in the presence of strophanthinid (see Fig. 2B). Nevertheless, in NMDA solution, the rate of rise of cumulative depolarization remained biphasic and its half decay time was 16.8 ± 4.8 s, a value analogous to control (see Fig. 2C).

In summary, Na\(^{+}\) pump inhibition had no effect on impulse conduction and only a modest one on polysynaptic responses at low stimulus rate. With stimulus trains at 1 Hz, Na\(^{+}\) pump inhibition largely reduced cumulative depolarization; it linearized its development and lengthened its decay time. The latter two effects were not due to network depolarization induced by strophanthinid. Our observations indicate that in control conditions, operation of the Na\(^{+}\) pump enabled early, rapid response summation because it presumably played a role in maintaining ionic gradients despite intense excitation.
Strophanthidin-induced late bursting

Application of strychnine (1 μM) plus bicuculline (20 μM) generates regular disinhibited bursting (Bracci et al. 1996a) as exemplified by records in Fig. 3A. In accordance with previous results (Ballerini et al. 1997), strophanthidin (4 μM) suppressed a disinhibited bursting within 10 min from the start of application (data not shown). Slow bursts were replaced by irregular, short-lasting discharges of varying amplitude while VRs depolarized to a stable level. However, in this study, when the application of strophanthidin was prolonged long after bursting suppression (approximately 1 h), bursting appeared as shown in Fig. 3B (same preparation as in Fig. 3A). This novel pattern was termed strophanthidin bursting, and because experimental conditions did not differ from those of our previous study (Ballerini et al. 1997), the only condition to observe its emergence was sustained exposure (1 h or more) to strophanthidin solution. Appearance of strophanthidin bursting eliminated the fragmented discharges left from the disruption of the disinhibited rhythm, indicating that these two activities could not co-exist. Strophanthidin bursting was observed in 130 preparations with a latency of 60 ± 10 min from the application of this drug.

Figure 3, C and D, (time base faster than in A and B) contrasts, for the same preparation, the properties of a typical disinhibited burst (C) with those of a strophanthidin burst (D), which had longer duration (see slower time calibration), reduced plateau amplitude (see different voltage scale), different frequency of intraburst oscillations, and slower decay. These properties are summarized in Table 1 for a random sample of seven spinal cords. It is noteworthy that periodicity of stro-
Pharmacology of strophanthidin bursting

Such an activity was completely dependent on intact glutamatergic transmission because application of a mixture of the AMPA/kainate receptor antagonist 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) (10 μM) and the NMDA receptor antagonist d-APV (50 μM) rapidly and completely abolished bursting (n = 8). CNQX (10 μM) largely depressed bursting as shown by the continuous slow trace in Fig. 5A, VR discharges could still be evoked by strong (5 × Th) DR stimuli (see asterisks in Fig. 5A). This block was, however, reversible after a few minutes of washout of CNQX (see last event in Fig. 5A). Similar data were obtained from eight preparations.

Figure 5B shows an example of the reversible blocking action of d-APV (50 μM) on bursting activity, although DR stimulation (5 × Th; asterisks) could still evoke VR discharges. During washout of d-APV, DR stimuli generated bursts again while recovery of spontaneous bursting was obtained a few minutes later (data not shown). Similar data were obtained from nine preparations. Strophanthidin bursting was

Table 1. Comparison of burst characteristics during disinhibited rhythm and strophanthidin bursting

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Disinhibited Rhythm</th>
<th>Strophanthidin Bursts</th>
</tr>
</thead>
<tbody>
<tr>
<td>Duration (s)</td>
<td>5.8 ± 2.1</td>
<td>154 ± 72</td>
</tr>
<tr>
<td>Plateau amplitude (mV)</td>
<td>1.48 ± 0.53</td>
<td>0.89 ± 0.43</td>
</tr>
<tr>
<td>Interburst interval (s)</td>
<td>26.3 ± 9.2</td>
<td>412 ± 373</td>
</tr>
<tr>
<td>Interburst interval CV (%)</td>
<td>18.2 ± 9.6</td>
<td>58.6 ± 11.8</td>
</tr>
<tr>
<td>Intraburst oscillation frequency (Hz)</td>
<td>3.2 ± 0.8</td>
<td>1.0 ± 0.79</td>
</tr>
<tr>
<td>Intraburst oscillation frequency CV (%)</td>
<td>25.2 ± 6.1</td>
<td>60.2 ± 11.2</td>
</tr>
<tr>
<td>Decay time (s)</td>
<td>5.5 ± 1.1</td>
<td>138 ± 64</td>
</tr>
</tbody>
</table>

Data are from seven randomly chosen preparations before and after applying strophanthidin.

Strophanthidin bursting was characterized by a very large CV value, indicating its irregular occurrence, unlike the low CV value for disinhibited bursting. Strophanthidin bursts contained rhythmic oscillations present throughout most of each burst episode and displaying periodicity similar to the one of disinhibited bursting. Strophanthidin bursts contained rhythmic oscillations present throughout most of each burst episode and displaying periodicity similar to the one of disinhibited bursting, although with higher CV values that reflect the considerable slowing down of the oscillation frequency toward the end of each burst.

The characteristic features of strophanthidin bursting led to further analysis of the underlying mechanisms, because they could cast light into the processes enabling regular network rhythmicity.

Figure 4 (top row) shows histograms relative to 107 consecutive events of strophanthidin bursting recorded from one preparation with stable activity for ≥12 h. Note that burst periodicity, duration, and amplitude were widely distributed. We then attempted to study if there was any correlation between various parameters of strophanthidin bursts. Figure 4 (bottom row) shows plots of burst amplitude, preceding pause, or subsequent pause versus burst duration for the same data shown in Fig. 4 (top). Burst duration was strongly correlated to burst amplitude and to the length of the preceding pause, but not to its subsequent pause. Thus burst duration, which should reflect the length of synchronous network discharges, depended on the extent of neuronal recruitment (i.e., burst amplitude), was shaped by network activity during the preceding pause, and had little influence on subsequent interburst behavior.

Strophanthidin bursts retained some aspects of disinhibited bursts like abrupt onset (see Fig. 3, C and D) and synchronous occurrence at segmental and inter-segmental level when recorded bilaterally from L2–L4 VRs (data not shown). Furthermore, strophanthidin bursting was not due to gradual metabolic disturbance of the spinal cord, reflecting the deleterious effects of sustained Na⁺ pump inhibition. In fact, this form of bursting was continuously observed for ≥12 h without significant change in burst characteristics. In particular, bursts after 1–2 h in strophanthidin solution were as large as those after about 12 h (0.8 ± 0.4 and 0.9 ± 0.4 mV, respectively; n = 10), had similar periodicity (383 ± 209 and 508 ± 267 s, respectively) and average duration (130 ± 70 and 177 ± 75 s, respectively).
promptly suppressed by tetrodotoxin (TTX; 0.25–1 μM; n = 8) without leaving any residual, spontaneous, or evoked activity recorded from VRs. Likewise, in the presence of CNQX (10 μM) and D-APV (50 μM), there was neither spontaneous nor electrically evoked activity. The gap junction blocker carbene-xolone (200 μM) did not inhibit bursting (n = 4). These observations indicate that strophanthidin bursting was a network-mediated phenomenon, required intact glutamatergic transmission, and was conveyed to motoneurons via spike-dependent activity.

We also investigated if strophanthidin bursting could be up-regulated by excitatory agents like NMDA (4–6 μM; n = 9), serotonin (4–12 μM; n = 8), or high K+ (5.5–8.5 mM; n = 5), all of which evoke fictive locomotor rhythms (Kiehn et al. 2000) and accelerate disinhibited bursting (Bracci et al. 1996b, 1998). Each one of these treatments produced a stereotypic response consisting of a burst that reached plateau, lost its oscillations, and remained depolarized as long as the excitatory agent was applied. Recovery to baseline and return of spontaneous bursting was, however, always obtained after washout. Despite fine titration of the concentration range of these excitatory agents, it was never possible to maintain bursting or to avoid the subsequent depolarization plateau. Hence, concurrent block of Cl−-mediated inhibition and of the Na+ pump caused spinal networks to respond to bath applied agents with a large, sustained depolarization. Because excitatory agents did not modulate bursting, we wondered whether a brief and potent activation of the spinal network by synchronically released transmitters could. To explore this issue, we examined the effect of DR stimulation on strophanthidin bursting.

**DR stimulation during strophanthidin bursting**

We first tested the effect of a single DR stimulus on VR discharges. Figure 6 compares the excitability of the same preparation stimulated with just one DR pulse (1 × Th) under various experimental protocols. In control solution this stimulus generated a threshold response (Fig. 6B), which is better seen at high gain (Fig. 6A). During a pause between disinhibited bursts recorded later from the same preparation, an identical pulse now generated a large burst (amplitude = 1.45 mV; duration = 9.4 s) with superimposed oscillations (Fig. 6C), while smaller stimuli were ineffective. During a pause between strophanthidin bursts, the same pulse also generated a large burst (amplitude = 1.32 mV) with repeated oscillations and with long duration (157.8 s; Fig. 6D). Thus, in the continuous presence of strophanthidin (plus strychnine and bicuculline), spinal networks displayed the same excitability threshold to afferent stimuli as they had in the presence of strychnine and bicuculline alone.

Figure 7, A and B, shows that DR-evoked bursts depended on the length of the preceding interburst interval. This phenomenon is quantified in Fig. 7C for the same preparation illustrated in Fig. 7, A and B: the correlation between burst amplitude and preceding silence was very similar for spontaneous and evoked bursts. However, evoked bursts (the amplitude of which was measured at the event plateau vs. baseline) were on average significantly larger (and longer) than spontaneous ones (Fig. 7D) once analogous interburst intervals were observed. Similar data were obtained with three other preparations.

Figure 8 shows that, on the same preparation during strophanthidin bursting, changing stimulus intensity from 3 × Th to 5 or 8 × Th strongly accelerated the average interburst oscillation frequency (from 0.3 to 0.5 or 0.6 Hz, respectively). In conjunction with such an oscillation frequency increase, there was an associated rise in background discharge activity developing in the troughs between oscillatory peaks. In fact, whereas at 3 × Th intensity oscillation peaks were followed by relatively quiet phases, with stronger stimuli the asynchronous discharge activity rose largely. Pooling data from a sample of preparations tested with a close range of stimulus intensities indicated that for 3–4 × Th stimuli, the intraburst oscillation...
frequency was 0.45 ± 0.1 Hz, while for 6–8 × Th stimuli, the oscillation frequency was 0.67 ± 0.16 (n = 5; P < 0.007). There was, however, no significant change in burst duration or amplitude. These average oscillation frequencies are somewhat lower than those found during spontaneous bursts (Table 1). Single DR pulses therefore generated standard bursts with frequency modulation of their oscillations.

Previous experiments have indicated that disinhibited bursts can be entrained, on a 1:1 basis, by a train of DR pulses applied at various frequencies (Bracci et al. 1997). We next examined if strophanthidin bursting could show similar properties. Figure 9, A and B, shows examples of responses induced by electrical stimuli applied every 45 or 15 s. At the lower frequency (Fig. 9A; asterisks mark electrically evoked responses), the first pulse induced a burst while subsequent ones evoked shorter VR discharges (comprising one or more events) superimposed on a declining baseline. At the higher frequency (Fig. 9B), pulses (after the first one) elicited a single discharge made up by a fast transient followed by a slow component, riding over a persistent baseline depolarization. Figure 9C shows the relative amplitude of slow responses (measured from the immediately preceding level of VR polarization) for a series of DR pulses at varying frequency. Despite changing frequency, each pulse generated a slow response that remained at relatively stable amplitude throughout the stimulus train. When the pulse interval was set at 45 s, spontaneous bursts could occasionally appear (as shown by the filled circle in Fig. 9C), but never in coincidence with the earliest part of the evoked event. At the faster rates of stimulation, spontaneous bursts did not appear. Collectively, these results suggest that, during strophanthidin

![Figure 7](image7.png)

**FIG. 7.** Effects of DR stimuli during strophanthidin bursting. A and B show, from the same preparation, records depicting evoked events (asterisks; 2 × Th, 0.1 ms) induced at varying interval from preceding spontaneous events. C: plot of burst amplitude (open circles, spontaneous; stars, evoked) vs. preceding silence. Relation is linear in both cases and with similar slope. Mean amplitude of events comprised between 2 vertical dotted lines (100-s epoch) in C is shown in D. Evoked bursts have significantly higher amplitude (P < 0.001).

![Figure 8](image8.png)

**FIG. 8.** Increasing stimulus strength enhances oscillation frequency. Records shows strophanthidin bursts (displayed on relatively fast time base) induced by DR electrical pulses of 3 intensities (3, 5, and 8 × Th, indicated along each trace). With stronger pulses, oscillations develop at higher frequency on a background of strong discharge activity, which becomes clearly visible in the troughs between oscillation peaks. All data are from the same preparation.

![Figure 9](image9.png)

**FIG. 9.** Effects of stimulus trains on strophanthidin bursting. A and B: examples of VR responses induced by electrical stimuli (asterisks in A) applied every 45 (A) or 15 (B) s, respectively. Note that, at the higher frequency (B), each pulse after the first one elicits a single discharge, riding over a persistent baseline depolarization. C: relative amplitude of slow responses (measured from the immediately preceding level of polarization) for a series of DR pulses at varying frequency for the same preparation. Filled circle indicates spontaneous burst.
bursting, spinal networks could be entrained to generate synaptic discharges in response to repeated stimuli.

Studies carried out on organotypic spinal cultures (Streit 1993) and the chick embryo spinal cord (Tabak et al. 2000) have indicated that one important mechanism to ensure burst termination is synaptic depression, which is responsible for reducing excitability of spinal networks between burst episodes. It appeared therefore necessary to explore whether an analogous process might have played a major role in spontaneous rhythmic activity of the neonatal rat spinal cord. Figure 10A shows that, for disinhibited bursting, a single DR pulse (5 × Th), applied immediately after the end of the intraburst oscillation as soon as the burst started decaying back to baseline, promptly induced another burst with initial fast peak, sustained firing, and oscillations. On average, we found that the duration of bursts electrically evoked immediately after spontaneous disinhibited bursts was 92.2 ± 19.9% (n = 10). However, as previously reported by Bracci et al. (1997), when electrical pulses were repeatedly applied, subsequent bursts became shorter (from 4.2–2.3 s) although their amplitude was similar (1.90, 1.79, and 1.76 mV, respectively, vs. 1.94 mV control; Fig. 10B). Hence, a phenomenon similar to synaptic depression could appear in the disinhibited neonatal spinal cord, but it had a time course slower than the duration of a standard disinhibited burst. It is worth noting that, under our experimental conditions, other processes like metabolic pump activities or persistent changes in voltage-dependent ionic currents might have mimicked a condition looking like slow synaptic depression.

Conversely, a single pulse of similar intensity applied near the end of a strophanthidin burst generated a burst of similar peak amplitude (0.610 vs. 0.605 mV, respectively), but of much shorter duration (59.6 vs. 90.0 s, respectively) and lacking oscillations (Fig. 10C; same preparation as in A and B). On average, the duration of strophanthidin bursts electrically induced immediately after spontaneous ones was 43.4 ± 22.2% (n = 5; P < 0.05).

Modeling

We assumed that the same interneuron network (made up by excitatory connections only because of pharmacological block of fast synaptic inhibition) was responsible for either disinhibited or strophanthidin bursting. In fact, it seemed advantageous to seek a unitary theory to account for both bursting modes intended as state variations taking place within the same network.

Minimum requirements for the present model to simulate disinhibited as well as strophanthidin bursting were as follows: 1) ability to produce oscillatory bursts with distinctive properties depending on the different pharmacological treatment, and 2) generation of characteristic bursts when the network excitability was suddenly raised to mimic afferent fiber stimulation. The difference in intraburst frequency between strophanthidin and disinhibited bursts (Table 1) was mathematically accounted for by a change in the temporal characteristics of the network system, although identification of underlying cellular processes will need future experimental work. Additionally, we considered that duration of simulated bursts had to be related to the preceding silent interval observed experimentally (Fig. 4; Tschelter et al. 2001). Furthermore, we supposed that slow neuronal depression (perhaps due to gradual build-up of synaptic fatigue; Tabak et al. 2000) was contributing to ending a single strophanthidin burst rather than a single disinhibited burst (see experiments with electrical DR stimuli; Fig. 10). Indeed, after each spontaneous disinhibited burst, one state characteristic of the network was its rapid recovery of excitability, which we proposed to be due to Na⁺ pump operation.

As a starting point, we applied the model derived from bursting of the chick embryo spinal cord (Tabak et al. 2000) to our data. However, unless an extra variable was added, that model was unable to simulate disinhibited rhythms. Our own model relied on two ordinary differential equations to display various dynamic network behaviors.

The system was represented by the following equations

\[ \tau_a \dot{a} = \alpha (a - 1 - s) d - a = \frac{1}{1 + e^{-(1 + s + 2 a \phi_d)}} - a \]  

(1)

and

\[ \tau_d \dot{d} = \delta (a - d) = \frac{1}{1 + e^{-2 a \phi_d}} - d \]  

(2)

Using Eqs. 1 and 2, it was possible to obtain three state diagrams (Fig. 11A) representing average network activity (see similar representation by Tabak et al. 2000) and constructed with the following parameters on the assumption that there was a sigmoidal function relating network activity to network input (Tabak et al. 2000; Wilson and Cowan 1972): \(a\), an index of network activity such as the fraction of neurons firing at a given time; \(d\), a fast variable representing the fraction of network neurons not affected by depression; \(\tau_a\) and \(\tau_d\), their time constants; \(n\), the fraction of synapses unaffected by slow depression; \(s\), the fraction of neurons inhibited as a consequence of Na⁺ pump activity; \(\upsilon\), the neuronal firing threshold.

\(J\) Neurophysiol • VOL. 88 • AUGUST 2002 • \textcopyright \textit{www.jn.org}
FIG. 11. Three state diagrams representing average network activity. A: in each graph, $a$ is an index of network activity while $d$ is a fast variable representing the fraction of network neurons unaffected by depression. The 3 panels represent the curve $t \rightarrow [a(t), d(t)]$ in the $d$-$a$ plane for decreasing values of $n(1 - s)$, namely the overall number of active elements in the network (available synapses and neurons). In the left panel, there is only a stable, periodic orbit that corresponds to the bursting state with oscillations. As the arrows show, regardless of the initial state of the system, after a small interval of time, the system reaches the periodic orbit. Middle panel represents a state that can shift between resting and bursting activity. Thus, depending on the initial state of the system, the behavior is completely different; above the 2 critical lines depicted in the middle panel, whenever the initial activity is sufficiently high, the system will oscillate. Below the critical lines the system reaches a rest state with activity close to 0. In the right panel, the only stable orbit is the rest equilibrium with activity close to 0. Note that, even if the initial state of the system is with high activity (e.g., network stimulation), the system reaches a rest condition after a single burst response indicated by the upper orbit. In other words, this state corresponds to the silent period between bursts. Right panel shows a state diagram on slower time scale. B–D: comparison between disinhibited (left) and strophanthidin (right) bursts generated by network modeling. $a$, index of network activity; $n$, fraction of synapses unaffected by slow depression; $s$, fraction of neurons inhibited as a consequence of Na$^+$ pump activity. Time is in arbitrary units.
(presumed to be constant); and \( k_s \), the sigmoid function slope. When applied to \( d \), threshold and slope become \( v_d \) and \( K_d \).

The average neuronal activity expressed as \( a \) (at time \( t \)) generated a series of signals returning to the same cells as \( n \cdot d \cdot a \), that is, their neuronal input was inclusive of some activity-dependent loss of synaptic activity. The effective input fed back to the network then became \((1 - s) \cdot n \cdot d \cdot a\). The value \( n(1 - s) \) represented the overall number of synapses and neurons available for network activity. The variables \( d, n, \) and \( s \) could be described by sigmoidal functions (Tabak et al. 2000).

If the system relied on \( a \) and \( d \) only, the solution depended on the value of the following parameters: \( \tau_p, \tau_d, n(1 - s), v, v_d, k_s, k_{ds} \) and \( k_d \). By plotting \( a \) versus \( d \), we could show three basic network states when \( n(1 - s) \) decreased from left to right (Fig. 11A).

In particular, in Fig. 11A (left), \( n(1 - s) \) was sufficiently large so that the system displayed a stable cycle, as indeed should occur during a burst when the system follows the frequency of intraburst oscillations. The middle shows that, when \( n(1 - s) \) was getting smaller, there was a region of bistability in which the system could either continue oscillating or switch off. In the latter case, it was possible to evoke a burst when the system was turned off as the stimulus made the network to return to the excited state. The right panel of Fig. 11A shows that when \( n(1 - s) \) was very small, there was only one stable state corresponding to the pause observed between bursts. The system therefore shifted among these three states during both disinhibited and strophanthin bursting, although the speed of transition in the case of strophanthin bursting was considerably slower. The transition between the three states could be described with differential equations for \( n \) and \( s \), although for strophanthin bursting the value of \( s \) became minimal.

It is assumed that \( \text{Na}^+ \) pump operation could not immediately inhibit network discharges because it required a certain time to re-establish ionic gradients to generate neuronal hyperpolarization. If there was modest neuronal activity, the pump work required to obtain the correct ionic gradients was small and created a comparatively brief delay before the pump could repolarize neurons and regenerate their excitability. If there was intense neuronal firing, longer pump operation was necessary to reset the system to its ground state. When the \( \text{Na}^+ \) pump was pharmacologically inhibited, the time required to inhibit burst discharge became much longer, implied by an even slower process. In this framework, we may therefore predict two distinct cases: disinhibited bursting with burst termination due to \( s \), and strophanthin bursting with burst termination due to \( n \). Our approach does not exclude that other solutions (for example changing the parameter \( J \) as in Tabak et al. 2000) may produce analogous modeling data. The simulated neuronal system relies on a large number of constants, not always validated experimentally, which means that bursting patterns deduced from modeling are to be viewed as plausible states of the network operation.

The equation describing \( n \) is as follows

\[
\frac{dn}{dt} = \frac{1}{(1 + e^{-v_n/k_n})} - n
\]

While the equation for \( s \) is given by

\[
\tau_s \frac{ds}{dt} = \frac{m_s}{(1 + e^{-(v_s - v_d)/k_d})} - s
\]

Note that \( m_s \) expresses the interneuron fraction reactivated (during the time unit) by membrane hyperpolarization due to maximal electrogenic ion transport (primarily \( \text{Na}^+ \) pump activity). The value of \( m_s \) was fixed at 1 for disinhibited bursting, and at 0.1 for strophanthin bursting (we avoided setting it to 0 because additional transport mechanisms might have partly compensated for lack of \( \text{Na}^+ \) pump activity).

The value \( \tau_s(a) \) is given by

\[
\tau_s(a) = \frac{\tau_s}{(1 + e^{-(v_s - v_d)/k_d})}
\]

The parameter \( \tau_s(a) \), representing the time constant for the process to obtain \( s \), is therefore crucial to determine the duration of disinhibited bursts.

Figure 11, B–D, compares the temporal changes in \( a, n, \) and \( s \) values simulated during disinhibited (left) or strophanthin (right) bursting, while Table 2 shows the values assigned to the variables used for burst simulations. The values pertaining to the parameters \( a \) and \( d \) were increased in the case of strophanthin bursting to reflect the objectively longer duration of such bursts (indicative of persistent network discharge, \( a \)), and their stronger dependence on synaptic depression (shown by data in Fig. 10C).

Inspection of Fig. 11, B–D, (left) indicates that, in the case of disinhibited bursting, when \( a \) was elevated (during the oscillatory phase of a disinhibited burst), the value of \( \tau_s(a) \) was large so that the peak value of \( s \) (i.e., inhibition of a substantial fraction of neurons) was rather slowly reached. When \( s \) reached a value critical to suppress bursting (corresponding to the large value of \( m_s \), data not shown), \( a \) became quite small and the burst terminated. Figure 12A reveals that the present model could generate spontaneous regular discharges similar to recorded disinhibited bursts, although simulated bursts lacked the short plateau phase with elevated firing just before the oscillatory phase. Figure 12B shows that, at the end of a spontaneous disinhibited burst, a single electrical stimulus (indicated by an asterisk and mimicking the experimental protocol shown in Fig. 10A) elicited a burst shorter but otherwise similar to the previous one (as observed experimentally in Fig.

| Table 2. Values of constants used for burst simulations |
|-----------------|-----------------|
| Disinhibited Bursting | Strophanthin Bursting |
| \( \tau_p \) | 0.25 | 2 |
| \( \theta_d \) | 0.18 | 0.18 |
| \( K_a \) | 0.15 | 0.05 |
| \( \tau_d \) | 0.5 | 0.5 |
| \( \delta_d \) | 0.5 | 0.5 |
| \( K_d \) | 0.2 | 0.2 |
| \( \delta_s \) | 0.3 | 0.3 |
| \( K_s \) | 0.01 | 0.01 |
| \( m_s \) | 1 | 0.1 |
| \( \tau_s \) | 4000 | 4000 |
| \( \delta_m \) | 0.14 | 0.14 |
| \( K_m \) | 0.02 | 0.02 |
| \( \tau_s \) | 500 | 500 |
| \( \delta_s \) | 0.25 | 0.25 |
| \( k_s \) | 0.05 | 0.05 |

All values are dimensionless.

Note that \( m_s \) expresses the interneuron fraction reactivated (during the time unit) by membrane hyperpolarization due to maximal electrogenic ion transport (primarily \( \text{Na}^+ \) pump activity). The value of \( m_s \) was fixed at 1 for disinhibited bursting, and at 0.1 for strophanthin bursting (we avoided setting it to 0 because additional transport mechanisms might have partly compensated for lack of \( \text{Na}^+ \) pump activity).

The value \( \tau_s(a) \) is given by

\[
\tau_s(a) = \frac{\tau_s}{(1 + e^{-(v_s - v_d)/k_d})}
\]
10A), because at this time point the value of $\tau_{a}(a)$ had become sufficiently small (see Fig. 11D, left). However, a further stimulus (see Fig. 12B) induced an even shorter burst (compare it with experimental data in Fig. 10B). The reason for the ability of the network to produce bursts following repeated stimuli lies in the relatively small and slow variation in $n$ value during a single disinhibited burst (this value is shown superimposed on bursting activity; Fig. 12). With closely repeated bursts induced by electrical pulses, $n$ (despite its slow change) could reach a value sufficient to reduce network activity (see Fig. 10B for experimental data and Fig. 12B for simulated ones).

In the case of strophanthidin bursting (Fig. 11, B–D, right; note time scale slower than in left), $m_s$ was small because of Na$^+$ pump inhibition. Thus the variable responsible for burst termination became $n$ (Fig. 11C, right) as the number of active synapses fell below a certain value. The slow recovery of $n$ influenced the time necessary to reset the network for subsequent bursting and presumably played a role in the observed correlation between pause and burst size (see Fig. 4). It is noteworthy that our modeling approach did not include the long transition phase of irregular asynchronous activity before strophanthidin bursting developed. Thus our simulation brought about a sudden conversion of bursting mode from disinhibited patterns to strophanthidin-type patterns. We can only surmise that the $m_s$ value became gradually smaller as the Na$^+$ pump activity was blocked and another transport mechanism slowly emerged. At steady-state conditions $m_s$ was assigned a small, yet finite value (0.1).

Simulated data for strophanthidin bursting (Fig. 12C) show that an electrical pulse (marked by a asterisk) shortly after the burst end could evoke a small burst only. In practice, the end of one strophanthidin burst was apparently determined by the number of excitatory synapses remaining active. Note that the present model could not simulate the irregularity of strophanthidin bursting because it was based on a system of ordinary

---

**FIG. 12.** Characteristics of simulated bursts. A: rhythmic bursts are observed when the fraction of synapses unaffected by slow depression is comparatively high and the fraction of neurons inhibited by Na$^+$ pump activity is high (see Fig. 11, B–D, left). This condition resembles disinhibited bursting. B: bursts induced by DR stimuli (asterisks) applied during disinhibited rhythm (see A) generated by network modeling. Note that the left vertical scale refers to $a$ (network activity), while the right scale refers to $n$ (fraction of synapses unaffected by slow depression). Superimposed plot of the variable $n$ demonstrates how this value progressively decreases with each burst. C: burst induced by DR pulse (asterisk) applied after a strophanthidin-like burst generated by network modeling. In this case, the superimposed plot of $n$ shows a large fraction of synapses affected by synaptic depression when Na$^+$ pump activity is blocked. $a$, index of network activity; time units (abscissa) are arbitrary. DR stimulation is simulated by suddenly shifting $a$ to 0.6.
differential equations which, by definition, is deterministic. It would be possible to add a stochastic variable to this model to describe the high variability of strophanthidin bursting. Nevertheless, as this approach would simply bring a further variable into the model and would not improve its ability to describe burst evolution and termination, it was not further pursued.

Finally, it should be considered that the network output was measured via motoneuron discharges (via VRs), and thus our readout was an indirect index of the interneuronal activity responsible for bursting (Bracci et al. 1996a; present data with glutamate receptor blockers or TTX). This condition inevitably distorts the real behavior of interneurons, but it has the experimental advantage of showing the integrative properties of the network at its final output stage and of demonstrating the signal sent to peripheral targets. Simulated behavior depicted in Figs. 11B and 12 reflects interneuronal activity (not their average membrane potential changes that remain unknown even experimentally), which might follow a time course different from the changes in VR polarization level integrating the average membrane potential of motoneurons and their axons. This condition may explain, for example, the slower decay of recorded bursts versus simulated ones. It seems likely that motoneuron intrinsic properties conferred this characteristic component to the bursting event.

DISCUSSION

The principal finding of this study is the report of a new type of spontaneous activity (termed strophanthidin bursting) displayed by the neonatal rat spinal cord following block of Cl–-mediated inhibition and Na+ pump operation. This novel bursting was a long-lasting, network-mediated phenomenon apparently dependent on slowly developing synaptic depression within an excitatory network, indicated by experimental and modeling data. Strophanthidin bursting therefore seemed to be a very elementary form of synchronized network discharge, which is useful to understand the basic processes responsible for rhythmogenic activity in this area of the mammalian CNS.

Strophanthidin, an effective inhibitor of the Na+ pump, shaped VR responses to repeated DR stimuli

The similar effects of ouabain (strong, irreversible inhibitor of Na+/K+ ATPase) and strophanthidin on 86Rb+ uptake (Longo et al. 1991) indicated that Na+ pump activity was fully blocked with short or long incubation by either glycoside. This result was relevant to the present electrophysiological experiments because the electrogenic pump was as much inhibited after 30 min of 4 μM strophanthidin incubation, when the disinhibited rhythm was completely disorganized, as it was after 60 min when the new spontaneous pattern appeared.

In accordance with previous data (Ballerini et al. 1997), strophanthidin application per se did not elicit spontaneous rhythmicity and induced only a modest VR depolarization, likely due to loss of the background hyperpolarizing pump current (Li and Stys 2001; Shen and Johnson 1998). The insignificant effects of pump-activity block on DR impulse conduction, reflex threshold, and amplitude when responses were evoked at low stimulus rate suggest that, at room temperature, the role of the pump on synaptic transmission in the absence of intense spiking activity was small. However, with stimulus trains inducing strong depolarization and repetitive firing, blocking the Na+ pump operation with strophanthidin strongly decreased cumulative depolarization, which grew monotonically at a slow rate and decayed very slowly. Note that repeated stimuli at high rates were not delivered when preparations were bathed in strophanthidin, strychnine, and bicuculline solution.

It may be concluded that Na+/K+ ATPase activity normally limited network signal summation velocity and sped up recovery from persistent excitation. Thus after electrogenic pump block, the network was partly depolarized and generated slowly, incrementing excitation during high-frequency firing.

Comparing strophanthidin bursting with disinhibited bursting

The disinhibited rhythm (Ballerini et al. 1997; Bracci et al. 1996a,b) is, of course, unphysiological for the neonatal rat spinal cord, because it appears after blocking GABA and glycine-mediated inhibition, which in rodents, develops during late embryonic life (at E18.5 and E20.5, respectively) to allow signal alternation between motoneuron pools (Nishimaru and Kudo 2000). However, blocking fast, chloride-mediated inhibition largely simplifies the rhythmogenic network and enables preliminary studies of the processes that allow a mammalian excitatory network (with normal architecture) to develop and organize rhythmicity made up of bursts with regular oscillations (Bracci et al. 1996a,b). Likewise, other immature spinal preparations without inhibition generate rhythmic activity (Sernagor et al. 1995). It should also be noted that, in embryonic life, developing networks communicate via excitatory connections only (Habets et al. 1987; Jackson et al. 1982) and generate synchronous discharges believed to be essential for pruning and sculpting neuronal circuits (Tosney and Landmesser 1985). Hence, disinhibited rhythmicity resembles the early type of collective network burst. As the output of all neurons in this network is similar and synchronous, it can be regarded as the same through various lumbar segments and representative of two network states, namely strong firing during bursts and minimal firing during pauses (in analogy with the rat hippocampus; Cohen and Miles 2000). These characteristics are also useful to develop models based on neuronal field operation (Staley et al. 2001; Tabak et al. 2000; Tsodyks et al. 2000). In this study, one unexpected evolution of disinhibited activity, after about 1 h of strophanthidin application, was the emergence of very slow, spontaneous bursting, termed “strophanthidin bursting.” When strophanthidin bursts were compared with disinhibited rhythm, they showed duration and inter-burst interval values one order of magnitude larger, while both activities were synchronous in all lumbar VRs. Although strophanthidin bursts were characterized by a smaller peak amplitude and were occurring very irregularly, burst episodes contained oscillations lasting longer than those observed during disinhibited bursts. As strophanthidin bursts did not gradually decline over several hours of continuous recording, their stability suggests lack of metabolic poisoning of spinal neurons during sustained Na+/K+ pump inhibition.

The transition from disinhibited to strophanthidin bursting went through an intermediate stage of rapid discharges, replac-
ionizing the slow rhythm observed in strychnine and bicuculline. However, once strophanthidin bursting was fully generated, these fast discharges completely disappeared. The most parsimonious hypothesis for this observation is that the same neuronal network, which generated disinhibited bursting, was converted into strophanthidin bursting mode. This view accords with recent work indicating a surprising degree of flexibility of spinal interneuronal networks with multifunctional character (Jankowska 2001).

Mechanisms for delayed onset and maintenance of strophanthidin bursting delayed onset

Ion transport mechanisms other than the Na⁺ pump might have been facilitated to allow emergence of network bursting. Future studies should aim at clarifying their identity because this approach may shed light on the processes enabling a network to recover from disruption of rhythmicity. During this phase prodromic to strophanthidin bursting, presence of rapid electrical discharges suggested that the network was not fully inactivated by sustained depolarization.

Once bursting started, spinal neurons must have pumped out their excessive Na⁺ load to preserve excitability. A possible candidate for the mechanism of Na⁺ extrusion would be the electrogenic Na⁺/Ca²⁺ exchanger (Fujioka et al. 1998). This process should lead to intracellular Ca²⁺ rise and facilitation of neurotransmitter release, a phenomenon elegantly demonstrated in model cells like chromaffin cells (Tang et al. 2000). If a similar process occurred in the rat spinal cord, it should have stimulated glutamate release from network interneurons and contributed to burst generation. Our experiments have, in fact, demonstrated (with the use of glutamate receptor antagonists) that glutamatergic transmission was essential for strophanthidin bursting. Within this framework, an additional source of glutamate release might have been the reverse operation of the glutamate membrane carrier that can take place when the sodium pump is inhibited (Li and Stys 2001). It is tempting to speculate that the combined activity of these mechanisms designed to re-establish ionic gradients could also enhance excitatory transmitter release and prompt the onset of strophanthidin bursting.

Pharmacology of strophanthidin bursting

Since application of TTX completely inhibited spontaneous and DR-evoked bursting, it appears that this activity was generated by the interneuronal network and recorded via motoneuron pools. Although CPG signals are usually transmitted to motoneurons via network spike firing, it has been recently suggested that a stable motoneuron rhythm could originate from synchronized oscillations of motoneurons coupled via gap junctions (Tresch and Kiehn 2000). As the gap-junction blocker carbeneoxonol did not modify strophanthidin bursting, this communication process was not apparently needed under the present conditions.

Whereas non-NMDA glutamate receptors alone are sufficient to drive the disinhibited rhythm (Bracci et al. 1996a), activation of AMPA/kainate and NMDA receptors was necessary for strophanthidin bursting as demonstrated by the current experiments with the receptor antagonists CNQX or D-APV. After suppression of bursting by either antagonist, strong DR pulses could still induce short VR discharges, indicating that at least one class of excitatory synaptic receptors remained functional at network level.

In the neonatal rat spinal cord, high K⁺, NMDA, or serotonin are well-known excitatory agents to induce (alone or in combination) fictive locomotion (Bracci et al. 1998; Cazalets et al. 1992; Cowley and Schmidt 1997; Kiehn and Kjaerulf 1996; Kudo and Yamada 1987). These excitatory agents also decrease interburst interval and burst duration during disinhibited rhythm (Bracci et al. 1996a,b, 1998).

During strophanthidin bursting, the same excitatory agents (NMDA, serotonin, or high K⁺), at a concentration sufficient to induce detectable VR depolarization, evoked a single large burst followed by a silent, stable plateau phase. Drug washout induced slow VR repolarization and subsequent recovery of spontaneous activity. Multi-site recording of the NMDA receptor-mediated disinhibited rhythm generated by dissociated spinal neurons in chronic culture also shows that application of NMDA completely disrupts periodic oscillations (Keefer et al. 2001). Thus blocking the Na⁺ pump made the spinal network functionally analogous to dissociated cultures in which rhythmicity-controlling mechanisms were less efficient than those normally found in the intact tissue.

Burst onset and termination

Burst onset and termination during the activity of the spinal locomotor network of the lamprey has been attributed to the interplay between synaptic mechanisms and intrinsic conductances (Grillner et al. 1991). Somewhat analogous, though not identical, considerations have been applied to the tadpole (Dale 1995), rat spinal cord (Kiehn et al. 2000), and rat hippocampus (Menendez de la Prida and Sanchez-Andres 2000). Conversely, spontaneous rhythmic bursts of the chick embryo spinal cord are thought to originate from network-driven motoneurons that activate Renshaw cells, which in turn, depolarize a large number of spinal neurons owing to the excitatory nature of GABA and glycine-mediated transmission at this early stage of development (Wenner and O’Donovan 2001).

Multi-site recordings from rat organotypic slice preparations during disinhibited bursting (Tscherter et al. 2001) have suggested that bursting starts at a certain site when there is a critical number of intrinsically active neurons (usually in the ventral area) with efficient synaptic connections, and that bursting does not require pacemaker cells as it simply depends on network excitability. A wave of excitation then spreads rapidly, via recurrent excitation, to engulf virtually all cells in the ventral and central areas (dorsal horn neurons are much less involved in this phenomenon). During the pause between bursts, network activity becomes low and has minimal spatial propagation.

Our recording conditions did not allow us to identify the relative contribution of certain membrane or cellular mechanisms to burst initiation for strophanthidin or disinhibited bursting. However, we can exclude some and consider others probable. By blocking all main components of synaptic inhibition pharmacologically (Bracci et al. 1996b), a role for synaptic inhibition to produce the burst pause was made very unlikely. Furthermore, in the neonatal rat spinal cord, Renshaw cell activity is inhibitory, not excitatory (Marchetti et al. 2001b). In analogy with the data from organotypic slice prep-
arations (Ballerini et al. 1999; Tschetter et al. 2001), it is suggested that, under the present experimental conditions, disinhibited burst onset was caused by synchronous firing of a certain number of spontaneously active cells recruiting most other neurons via recurrent excitation. This phenomenon might have occurred periodically and regularly during disinhibited bursting because the Na$^+$ pump operation routinely restored neuronal excitability after each burst. We suggested that, under these conditions, regular rhythmicity was based on the hyperpolarizing action by the Na$^+$ pump (Ballerini et al. 1997), which had a dual action: 1) to bring a significant fraction of neurons below firing level to switch off bursting, and 2) to reactivate neuronal conductances inactivated by depolarization so as to recruit cells for the subsequent burst episode.

During strophanthidin bursting, however, bursting episodes were irregular and typically very long. Data collected during 12-h continuous strophanthidin bursting (without further experimental interference by stimuli or other substances) were used to investigate the distribution and correlation of burst parameters. As reported for other preparations (Staley et al. 1998; Tabak et al. 2001), high correlation was observed between amplitude and duration (or between amplitude and recovery time) and between preceding pause and duration (or preceding pause and amplitude). Our interpretation of these results is that the level of network activity (expressed as burst amplitude or duration) depended on the preceding silent period. On the other hand, there was no significant correlation between burst duration and pause after each event (or between amplitude and pause). It seems likely that, once generated, bursts terminated in view of some intrinsic mechanisms that turned off network activity (Tabak et al. 2000; Tschetter et al. 2001).

High correlation between preceding pause and burst size (amplitude and duration) has also been observed in cultured spinal cord slices (Tschetter et al. 2001) and dissociated spinal neurons (Keefe et al. 2001; Streit 2001), and attributed to synaptic depression (probably due to presynaptic transmitter depletion; Lev-Tov and Pinco 1991; Streit 1993), which is a form of short-term plasticity at certain brain synapses (Stevens and Wesseling 1999). In chick embryo spinal cord, similar results suggested that a burst episode was controlled by activity-dependent network depression (O’Donovan 1999), a notion incorporated into a recent model (Tabak et al. 2000). In particular, two types of synaptic depression are reputed to operate, one (with rapid kinetics) responsible for intraburst oscillations, and the other one (with slow kinetics) responsible for burst periodicity (Tabak et al. 2000). On the basis of these considerations, it seemed plausible that synaptic depression played a role in strophanthidin bursting. Tests were therefore carried out to establish the ability of the network to generate discharges in response to electrical stimuli during strophanthidin or disinhibited bursting.

Effects of DR stimulation on strophanthidin or disinhibited bursting

The value of stimulus threshold for observing VR responses was apparently unmodified by strychnine and bicuculline, demonstrated by the fact that the same pulse intensity was necessary for a minimal response in control solution or for a stereotypic burst when applied during the silent period of the disinhibited rhythm (see also Bracci et al. 1997) or strophanthidin bursting.

In strophanthidin solution, burst amplitude after a DR stimulus depended on the preceding pause. Furthermore, there was a significant increase in intraburst oscillation frequency induced by raising stimulus intensity. Stronger stimuli presumably activated a higher number of afferent DR fibers (Marchetti et al. 2001a) to release a proportionally larger amount of excitatory transmitter: this phenomenon should have recruited more network elements that could oscillate at higher rate. While the identity of the mechanisms generating intraburst oscillations in the neonatal rat spinal cord remains unknown, the present data suggest that oscillations were facilitated by more intense synaptic activation. In particular, increasing stimulus intensity brought about a significant rise in the oscillation frequency and disclosed intense background discharges between the oscillation peaks. Although extracellular recording was unsuited to quantify such a background activity, it seems clear that, during the oscillatory phase, the spinal network was more (rather than less) excitable.

More widespread and efficient activation of the network by electrical stimuli possibly accounted for the larger amplitude of evoked bursts. Note also that, while changing the intensity of a single pulse modulated oscillation frequency, changing the frequency of pulses abolished them all together. It seems likely that the process underlying intraburst oscillations could be dissociated from the mechanisms responsible for burst size.

When trains of stimuli were applied at various frequencies, entrainment of strophanthidin bursts was difficult and limited to the low frequency range. In this sense, there were two opposing factors: at low frequencies (for example 1/45 s) spontaneous bursts occasionally re-emerged, while with faster frequencies there was a sustained baseline depolarization influencing individual burst duration. These observations suggest that slowly incrementing synaptic depression was an important factor to terminate such bursts. This suggestion has been put forward before to explain strychnine-induced bursts in organotypic cultures (Streit 1993) or the spontaneous activity of the chick embryo spinal cord (Tabak et al. 2000). In contrast, disinhibited rhythm can be entrained to frequencies near 0.25 Hz (Bracci et al. 1997), suggesting that, in this case, synaptic fatigue was a much less prominent phenomenon or that network recovery from it was so fast that it was not influential for bursting behavior.

Further insight into network excitability and fatigue onset was obtained by applying pulses at the end of a burst episode. When a single pulse was delivered at the end of a strophanthidin burst, the evoked burst was significantly shorter and lacked oscillations (although the early burst peak remained the same). When the same protocol was applied during disinhibited rhythm, the evoked burst had similar duration as the preceding spontaneous response and still manifested oscillations. However, when successive electrical pulses were applied, evoked bursts became shorter and lost oscillations. This was not just the result of network rapid entrainment because the last burst was briefer than the immediately preceding one. The present data suggest that, in general, spinal network excitability was not completely depressed at the end of a burst because fast discharges could always be induced by electrical stimulation during strophanthidin (Fig. 9) or disinhibited (Bracci et al. 1997) bursting.
Because reproducibility of a burst episode immediately after one burst or during a pulse train was very limited in strophanthidin solution, it seems likely that these phenomena were the manifestation of slowly developing synaptic depression that curtailed network activity and was probably responsible for the irregular nature of this rhythm. In contrast, disinhibited rhythms were less susceptible to synaptic fatigue unless stimuli were repeatedly applied at close interval. It is noteworthy that, during disinhibited bursting of the rat spinal cord, such events remain very regular in amplitude and duration, yet they show rapid adaptation in their duration when evoked by electrical stimuli at various frequencies (Bracci et al. 1997). Although this might be interpreted as a prima facie case of synaptic depression regulating burst activity, tests with electrical pulses applied at very short interval after a burst failed to demonstrate network inability to generate a new burst promptly (see Fig. 10). We suggest that, in addition to correlation between burst parameters, direct electrophysiological tests are useful to differentiate synaptic depression from other mechanisms.

A recent study carried out on the chick spinal cord (Tabak et al. 2001) has shown that reduction in network excitatory drive can account for burst onset and periodicity, and that, whenever the excitatory network is partly impaired, the excitability of the remaining network is increased via compensatory rise in synaptic efficacy. This phenomenon would thus render this activity robust to network disruptions. In the present experiments it seems likely that strophanthinid application led to reduction in the glutamatergic excitatory drive because bursting did require AMPA and NMDA receptor activation. However, it should also be noted that strophanthidin bursting was not very robust because it was readily suppressed by even modest concentrations of excitatory agents.

These observations were used for the present model in which two distinct forms of bursting were simulated on the basis of their control by Na$^+$ pump activity or slow synaptic depression. We assumed that Na$^+$ pump kinetics were faster than those of synaptic depression as suggested by the current experimental observations. Simulations were run by keeping the number of variables to a minimum and produced bursting patterns with characteristics similar to those experimentally observed in the neonatal rat spinal cord. For this reason, simulations were run by keeping the number of variables to a minimum and produced bursting patterns with characteristics similar to those experimentally observed in the neonatal rat spinal cord. This allowed us to reproduce the bursting patterns observed experimentally in the neonatal rat spinal cord.

REFERENCES


J Neurophysiol • VOL 88 • AUGUST 2002 • www.jn.org