Role of the Electrogenic Na/K Pump in Disinhibition-Induced Bursting in Cultured Spinal Networks

P. Darbon, A. Tscheter, C. Yvon, and J. Streit
Institute of Physiology, University of Bern, 3012 Bern, Switzerland

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INTRODUCTION

Rhythm generation is an important feature of neural networks in the brain and in the spinal cord. In the latter, rhythm-generating networks, the central pattern generators, are involved in the control of repetitive movements like those used during locomotion (Grillner et al. 1998). Although in early embryonic spinal networks rhythms may originate from motor neurons (Hanson and Landmesser 2003; Wenner and O’Donovan 2001), they are clearly produced by networks of spinal interneurons in the postnatal vertebral spinal cord (Cazalets et al. 1996; Grillner et al. 1998; Kiehn and Kjaerulff 1998). Such rhythms include fictive locomotion and synchronous bursting. The former is commonly induced by excitatory transmitters like NMDA and 5-HT and involves alternation of neuronal bursting. The latter is induced by removing synaptic inhibition pharmacologically (disinhibition) and is characterized by a synchronous slow bursting (Bracci et al. 1996; Cowley and Schmidt 1995). Although fictive locomotion requires at least a few intact spinal segments (Ballion et al. 2001; but see also Demir et al. 2002), disinhibition-induced bursting is also seen in slice cultures and dissociated cultures of the spinal cord (Ballerini and Galante 1998; Streit 1993; Tscheter et al. 2001). Furthermore, similar rhythms have been described in many other preparations (Muller and Swandulla 1995; Sanchez-Vives and McCormick 2000).

Rhythm generation in disinhibited networks is based on intrinsic spiking in some neurons, network recruitment by recurrent excitation through glutamate synaptic transmission, and a network refractory period after the bursts, which is caused by a suppression of network excitability. However, the mechanisms involved in the suppression of network excitability are still unknown. Both synaptic and neuronal excitability mechanisms were discussed in previous works (O’Donovan and Rinzel 1997; Tabak et al. 2001). We previously suggested that autoregulation of the neuronal excitability may be the major mechanism leading to suppression of network excitability (Darbon et al. 2002b). Such autoregulation could involve calcium-dependent K⁺ conductances (El Manira et al. 1994), the electrogenic Na/K pump, or slow inactivation of Na conductances (Pfeiderer et al. 1996). Using calcium imaging and patch clamp, we previously showed that calcium levels in neurons could predict neither the start nor the termination of bursts, and are therefore not critically involved in rhythm generation. In line with these findings, apamin and charybdotoxin, which block calcium-dependent potassium channels, had no effect on bursting parameters (Darbon et al. 2002a).

The blockade of the electrogenic Na/K pump, however, disrupted the rhythm induced by disinhibition in whole spinal cord preparation (Ballerini et al. 1997). Furthermore, pump currents are involved in rhythm generation in invertebrates (Tsiay and Chen 1995), brain stem motoneurons (Del Negro et al. 1999), and dopaminergic neurons (Shen and Johnson 1998).

In the isolated spinal cord, the role of the pump in spinal interneurons could only be assumed because there was no direct experimental access to these neurons. We therefore investigated the contribution of the electrogenic pump to the membrane potential of spinal interneurons and to rhythm generation in networks of spinal interneurons in dissociated and asynchronous background activity. Later, the burst rate decreased to initial values and the bursts became shorter and smaller. In single neurons, we observed an immediate depolarization of the membrane during the interburst intervals concomitant with the rise in burst rate. This depolarization was more pronounced during disinhibition than during control, suggesting that the pump was more active. Later a decrease in burst rate was observed and, in some neurons, a complete cessation of firing. Most of the effects of strophantidin could be reproduced by high K⁺-induced depolarization. During prolonged current injections, spinal interneurons exhibited spike frequency adaptation, which remained unaffected by strophantidin. These results suggest that the electrogenic Na/K pump is responsible for the hyperpolarization and thus for the changes in excitability during the interburst intervals, although not for the spike frequency adaptation during the bursts.

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organotypic cultures. Indeed Ballerini et al. (1999) showed that the fast and regular rhythmic activity of interneurons in the spinal organotypic culture was a useful model for investigating spinal rhythmic activity. We tested the hypothesis that the role of the pump in rhythm generation can be fully attributed to its hyperpolarizing effect, by comparing the effects of the pump blocker strophanthidin on rhythm generation to those of a general depolarization induced by high [K+]o. Finally we investigated whether the pump is involved in spike frequency adaptation in spinal interneurons. Indeed spike frequency adaptation has been proposed to be one of the mechanisms underlying locomotor rhythm generation in the lamprey brain stem-spinal cord (Grillner et al. 1998) and is used as a key parameter in the modeling of rhythmic bursting in a large network of neurons (Latham et al. 2000).

METHODS

Cultures

All cultures were made from the spinal cord of rats at embryonic age 14. The cultures were prepared in the same way as described previously (Braschler et al. 1989; Darbon et al. 2002b; Tschetter et al. 2001). Embryos were delivered by cesarean section from deeply anesthetized rats (0.4 ml pentobarbital intramuscularly) and killed by decapitation. After the delivery of the embryos, the mother rat was killed by intracardial injection of pentobarbital. Animal care was in accordance with guidelines approved by Swiss local authorities. The backs of the embryos were isolated from their limbs and viscera and cut into 225-μm-thick transverse slices with a tissue chopper. For the dissociated cultures, slices from all regions of the spinal cord, without dorsal root ganglia, were exposed to a 0.3% trypsin solution for 3 min at 37°C. They were subsequently mechanically dissociated by forcing them through fine-tipped pipettes several times. The cells were plated on multielectrode arrays (MEAs) or on glass coverslips at a density of 150,000 or 75,000/150 mm2, respectively. MEAs were produced as described previously (Tschetter et al. 2001) and coated for 1 h with diluted (1:50) Matrigel (Falcon/Biocoat, Becton Dickinson AG, Basel, Switzerland). The glass coverslips were coated with polylysine (1 mg/ml overnight at 37°C or Matrigel (1:50). Cells were restricted to an area around the electrodes (about 50 mm2) using cloning glass or Matrigel (1:50). Cells were maintained in culture dishes containing 150,000 or 75,000 ml of nutrient medium and incubated in a 5% CO2–95% O2 atmosphere at 36.5°C for ≤12 wk. Defined, serum-free Neurobasal medium (Gibco BRL, Life Technologies AG, Basel, Switzerland), supplemented with B27 and Glutamax (both Gibco BRL), was used for the MEA cultures and some of the cultures on glass coverslips. The other cultures were kept in a MEM Eagle’s medium supplemented with 10% fetal bovine serum, 0.2% glucose, B27, and Glutamax (Life Technologies AG). Half of the medium was changed weekly.

For the organotypic cultures, the spinal cord slices with their attached dorsal root ganglia were fixed on MEAs using reconstituted chicken plasma (Cocalico Biologicals, Reamstown, PA) coagulated by thrombin (Sigma, Fluka Chemie AG, Buchs, Switzerland). Cultures were maintained in sterile plastic tubes containing 3.5 ml of nutrient medium and incubated in roller drums rotating at 120 rph in a 5% CO2-containing atmosphere at 36.5°C. The medium had the following composition: 79% Dulbecco’s MEM with Glutamax, 10% fetal bovine serum (Life Technologies), 10% H2O, and 5 ml/l 2.5 S nerve growth factor (Alomone Labs, Jerusalem, PA). During the first week of incubation a medium with 10 ml/l glucose was used. Half of the medium was removed and replaced with fresh medium weekly.

Recordings

Recordings were made in a chamber mounted on an inverted microscope (Nikon, Tokyo, Japan) from cultures of 1–3 wk of in vitro age (organotypic cultures) and 4–12 wk (dissociated cultures). Within these periods no systematic changes of network activity with age were recorded, suggesting that the cultures had reached a steady state. More time was needed in dissociated cultures to reach the steady state compared with slice cultures, probably reflecting the fact that the networks had to regrow entirely after dissociation. The medium was replaced by an extracellular solution containing (in mM): NaCl, 145; KCl, 4; MgCl2, 1–2; CaCl2, 2; HEPES, 5; Na-pyruvate, 2; glucose, 5, at pH 7.4. Recordings were performed in the absence of a continuous flow of solution with solution changes every 10–15 min or in the presence of continuous superfusion at 1 ml/min. No differences in the patterns of activity were seen between these 2 protocols. All recordings were made at room temperature.

MEA recording and analysis

MEAs contained 68 electrodes, laid out either in the form of a rectangle (dissociated cultures) or in the form of a hexagon or 4 pentagons (organotypic cultures). In the latter, the electrodes were spaced less densely than normal (distance between electrodes was 400 instead of 200 μm) and both the leads and the electrodes were made of platinum (see Fig. 1A). Channels showing activity (usually 10–30 for the dissociated cultures and 20–50 for the slice cultures) were selected by eye and their recordings digitized at 6 kHz per channel, visualized, and stored on hard disk using custom-made virtual instruments within Labview (National Instruments, Ennetbaden, Switzerland) as described previously (Tschetter et al. 2001). Detection of the extracellularly recorded action potentials and further analysis were performed off-line using the software package IGOR (WaveMetrics, Lake Oswego, OR) as described previously (Tschetter et al. 2001). In organotypic cultures, the electrodes recorded fast, medium, and slow voltage transients (Fig. 1A), whereas in dissociated cultures, usually only the fast and sometimes also the slow transients were seen. The medium and slow transients probably correspond to local field potentials and changes in the extracellular milieu (see Tschetter et al. 2001 for further discussion). Only the fast transients were considered for further analysis. They correspond to action potential in neuronal somata or axons (single-unit activity). These fast voltage transients often appeared in clusters (multiunit activity) originating from closely spaced other neurons or axons (multiunit activity). These fast voltage transients could not be clearly detected. For the analysis of the spinal network activity, a detector was designed to find fast voltage transients, and its output was defined as an “event.” This activity detector reliably translated single-unit activity into single events ≥250 Hz. Above this level, multiunit activity was translated into trains of events, which were arbitrarily set to a frequency of 333 Hz. The electrical noise of individual channels was very stable. The selectivity of event detection was controlled using recordings obtained in the presence of tetrodotoxin (TTX, 1.5 μM) as a zero reference. No attempt was made to sort spikes seen by one electrode. The processed data were visualized in the form of raster plots and network activity plots (Fig. 1A).

Whole cell patch-clamp recording and analysis

Intracellular voltage and current measurements were obtained from individual neurons using the whole cell patch technique (Hamill et al. 1981) with an Axoclamp 2B amplifier (Axon Instruments, Union City, CA). The patch pipettes were filled with a solution containing (in mM): K-glutamate, 100; KCl, 20; HEPES, 10; Mg-ATP, 4; Na2-GTP,
0.3; Na₂-phosphocreatine, 10; pH 7.3 (with KOH). The electrodes had a resistance of 4–5 MΩ. No series resistance compensation was applied. Native resting membrane potentials were in the range of −40 to −60 mV (mean −47.36 ± 0.9 mV, n = 37). Cells with a potential less negative than −40 mV were discarded. Current recordings were digitized, visualized, and stored on computer using pClamp (Axon Instruments, Foster City, CA) software. They were analyzed off-line using custom made programs in IGOR (WaveMetrics). Bursts were defined based on the intervals between two subsequent spikes. An interspike interval shorter or longer than a defined threshold indicated the start or the end of a burst.

Statistics

Averages are expressed as means ± SE. Unless stated otherwise, the level of significance is set at P < 0.05.

Chemicals

All agents were applied by exchanging the bath solution for a solution containing the drugs. The following agents were used: bicuculline methochloride (Tocris, Anawa Trading SA, Wangen, Switzerland), strychnine, APV, CNQX, TTX (all Sigma, Buchs, Switzerland), strophanthidin (Alomone, Jerusalem, Israel).

RESULTS

The blockade of the Na/K pump disrupts disinhibition-induced bursting

In organotypic slice cultures of fetal rat spinal cord, a slow type of network bursting appears after the fast synaptic inhibition has been blocked pharmacologically by a combination of strychnine and bicuculline (Ballerini et al. 1997; Streit 1993; Tschelter et al. 2001). This bursting consists of episodes of simultaneous oscillations of activity in the whole slices (Fig. 1, A and B). The oscillations are composed of activity waves, which usually start on one side of the central fissure and spread to the rest of the slice (Tschelter et al., 2001). The episodes last for 10–40 s, during which the oscillation frequency decreases from around 5–8 to 1–3 Hz. In the intervals between such bursts the network is silent with occasional asynchronous activity. The same patterns of disinhibition-induced bursting were found in the isolated spinal cord of the neonatal rat (Bracci et al. 1996). There, they are disrupted when the Na/K pump is blocked by strophanthidin, ouabain, or 0 K⁺ (Ballerini et al. 1997), suggesting a crucial involvement of the pump in rhythm generation. To reveal the contribution of the pump to the mechanisms of rhythm generation, we studied the effects of strophanthidin on disinhibition-induced bursting in organotypic and dissociated cultures of the spinal cord.

In organotypic cultures (n = 10), the blockade of the pump by strophanthidin (10 µM) rapidly decreased the interburst interval duration and increased the rate of asynchronous “background” activity during the intervals. Within 1–2 min these effects led to a confluence of the bursts and thus to continuous oscillations. After 2 min these oscillations had a mean period of 1.8 ± 0.3 s (n = 5). They became slower and less regular with time. After 10 min, the mean period had significantly increased to 10.5 ± 2.2 s (n = 5, P < 0.005) with a parallel increase in the coefficient of variation from 0.37 ± 0.04 (after 2 min) to 0.57 ± 0.06 (P < 0.05). Over the same period, however, the amplitude and the duration of the activity waves did not change significantly (amplitude: 1823 ± 405 events/s after 10 min vs. 1958 ± 444 events/s after 2 min; duration: 0.31 ± 0.05 s after 10 min vs. 0.26 ± 0.02 s after 2 min). The asynchronous background activity between the waves was highest during the first 2 min of strophanthidin application (51 ± 14 events/s, n = 4) and decreased thereafter to 6.5 ± 1.2 events/s after 10 min (P < 0.05). This background activity was much higher than the background activity seen between the bursts before strophanthidin application (0.62 ± 0.16 events/s, P < 0.0005). During the initial application of strophanthidin, however, it was comparable to the background activity seen within the bursts between the activity waves (66 ± 9 events/s).

We previously showed that disinhibition also induces bursting in dissociated cultures (Streit et al. 2001). Compared with organotypic cultures, burst rates in dissociated cultures are higher and bursts are shorter (Streit et al. 2001; Tschelter et al. 2001; compare also Figs. 1 and 2). Bursts in dissociated cultures usually consist of decreasing sustained activity and not of
oscillations (Fig. 2A). Strophanthidin had a biphasic effect on disinhibition-induced network bursting in dissociated cultures (Fig. 2, n = 11) similar to that observed in organotypic cultures. First, it led to an increase in the burst rate coincident with the bursts becoming smaller and the asynchronous background activity between the bursts becoming more intense. After 1–2 min of strophanthidin application, the burst rate had increased by 242 ± 35% (n = 6, P < 0.001), the burst amplitude had decreased by 45 ± 10% (P < 0.01), the burst duration was more or less unchanged (−22 ± 14%, P = 0.17), and the background activity in the bursts was augmented by more than a factor of 10 (+1,169%), P < 0.05). Thereafter, the rate, amplitude, and duration of the bursts, as well as the background activity decreased. After 5–10 min of strophanthidin application the burst rate and the background activity had roughly returned to control values (4.6 ± 1.3 vs. 5.7 ± 0.8 and 8.8 ± 2.6 vs. 11.5 ± 5.1, P > 0.05), whereas the burst amplitude and duration were strongly decreased by 75 ± 5 and 73 ± 9% (P < 0.001) compared with control.

Whole cell patch-clamp recordings from spinal interneurons showed a regular pattern of rhythmic activity when disinhibited by strychnine and bicuculline (Darbon et al. 2002b). This pattern consisted of bursts of strong depolarization with spikes followed by more or less silent hyperpolarized intervals. When the Na/K pump was blocked under disinhibition, the membrane potential observed during the intervals rapidly depolarized from −56.2 ± 2.8 to −46.1 ± 2.3 mV (n = 11; see Fig. 2B) within the first 2 min of strophanthidin application. This phase the burst rate increased by 67.3 ± 18.3% and 7 out of 11 cells started to spike between the bursts, making the bursts more difficult to define. These findings corresponded well with the observed increase in burst rate and background activity at the network level. After the first 2 min, the depolarization became much slower and the membrane potential finally stabilized at −37.8 ± 2.6 mV. During this period, the burst rate decreased and died out in some of the cells. After 5 min of strophanthidin application, 6 out of 11 cells were tonically firing with sporadic bursts (Fig. 2B). In the other 5 cells, the intrinsic spike rate decreased and later stopped. There was no significant difference in the strophanthidin-induced depolarization between these 2 groups of cells (14.3 ± 4.0 mV in silent cells vs. 18.3 ± 4.5 mV in spiking cells, P = 0.54). After washout of strophanthidin, the membrane potential during the interburst intervals was not significantly different from that before the blockade (−55.5 ± 2.4 mV, n = 7, P = 0.51). Five out of 7 cells fully restored their previous rhythm. The 2 remaining cells recovered only to 66.4 ± 2.5% of the burst rate they had before the blockade of the pump.

Together these findings reveal the time course of strophanthidin-induced disruption of disinhibition-induced bursting. Strophanthidin causes an initial rapid depolarization of the neurons leading to an increase in burst rate and in background activity. This initial phase is followed by a second period during which the membrane stabilizes to a depolarized level and both the burst rate and the background spiking decrease. Furthermore, these results suggest a strong contribution of the Na/K pump to the resting membrane potential during burst intervals. Therefore we next investigated this contribution in more detail.

Contribution of the Na/K pump to the membrane potential

The rapid initial depolarization induced by strophanthidin is most probably caused by the blockade of the current produced by the electrogenic activity of the Na/K pump. This current is directly proportional to the level of activity of the pump. Pump activity is known to be regulated by the spike rate in the network through the Na⁺-loading of the cells (Shen and Johnson 1998). Therefore we wanted to know whether the depolarization induced by strophanthidin is dependent on the overall network activity. To investigate this, we compared, in 6 cells, the effects of strophanthidin under control conditions (with native spontaneous activity as illustrated in Fig. 3A) to that under disinhibition (as illustrated in Fig. 3B). However, as we previously showed (Streit et al. 2001; Tscherver et al. 2001) in control conditions, spontaneous activity was less intense and less regular than with disinhibition. In control conditions, strophanthidin (10 μM) significantly depolarized the cell from −49.4 ± 2.7 to −37.9 ± 4.6 mV (P < 0.02, Fig. 3D, columns ctrl and ctrl+stro). Then strophanthidin was washed out and the cells fully recovered their previous membrane potential. There was no significant difference between the control value of membrane potential (Fig. 3D, column ctrl) and the washout one (Fig. 3D, column ctrl2: −48.6 ± 3.7 mV, P = 0.77). After
the washout, we induced disinhibition, which caused a significant hyperpolarization of the membrane during the interburst intervals from $-48.6 \pm 3.7$ to $-54.7 \pm 3.8$ mV ($P < 0.04$, Fig. 3D, columns ctrl2 and bs). After disinhibition, the addition of strophanthidin produced a highly significant depolarization to $-35.1 \pm 4$ mV ($P < 0.008$, Fig. 3D, columns bs and bs+stro). The depolarization of 11.5 $\pm 3.6$ mV induced by strophanthidin in control condition (black column in Fig. 3E) was significantly less than the depolarization of 19.6 $\pm 3.4$ mV ($P < 0.04$) induced during disinhibition (gray column in Fig. 3E). The difference between the effects of strophanthidin with or without inhibition approximately corresponded to the hyperpolarization induced by disinhibition (6.1 $\pm 2.2$ mV; white column in Fig. 3E). Indeed there was no significant difference ($P = 0.62$) between the depolarization induced by the blockade of the pump during disinhibition (19.6 $\pm 4.7$ mV) and the sum of the depolarization induced by the blockade of the pump in control (11.5 $\pm 3.6$ mV) and of the hyperpolarization induced by the disinhibition (6.1 $\pm 2.2$ mV). This indicates that strophanthidin fully removed the hyperpolarization induced by disinhibition.

Finally, we compared, in 2 pools of cells (Fig. 3F), the effect of the blockade of the pump on the membrane potential in control conditions ($n = 11$) and in the absence of fast synaptic transmission ($n = 12$). In the presence of disinhibition, bursting was suppressed by blockade of the fast glutamatergic transmission by the AMPA/kainate antagonist CNQX (10 $\mu$M) and the NMDA antagonist APV (50 $\mu$M), and the membrane potential returned to control values, although some slow synaptic events could persist (see Fig. 3C under strophanthidin). Under these conditions, when no spiking was present, strophanthidin (10 $\mu$M) induced a depolarization of 12.8 $\pm 2.6$ mV (white column in Fig. 3F, $n = 12$), which was not significantly different from the depolarization induced by strophanthidin in control condition (13.1 $\pm 2.6$ mV, black column in Fig. 3F, $n = 11$, $P = 0.93$).
Taken together, these findings suggest that under control conditions and even in the absence of spiking a basic level of pump activity is present, which contributes by approximately 13 mV to the membrane potential. During disinhibition, pump activity is increased and thus causes a hyperpolarization of the membrane, which plays a major role in silencing the network during the interburst intervals.

The effects of strophanthidin can be reproduced by the depolarization of the neurons in the network

We further investigated whether the effects of strophanthidin on the disinhibition-induced bursting could be reproduced by a simple depolarization of the neurons in the network. Because the rhythm was network driven, any changes at the single-cell level would not modify the rhythm. Therefore we depolarized the network by increasing the extracellular concentration of K⁺. Between normal [K⁺]ₑ and 10.5 mM of [K⁺]ₑ, the burst rate increased by 79 ± 52% and the duration of the bursts decreased by 42 ± 18%. Between 10.5 and 12.6 mM of [K⁺]ₑ, the burst disappeared and only tonic spiking persisted. Above 12.6 mM, most of this firing also stopped.

The range of K⁺ concentrations where bursting disappeared corresponded to a range of membrane potential depolarizations between 12.5 ± 2.8 and 17.9 ± 2.9 mV, which was also the range of strophanthidin-induced depolarizations. Increasing the external K⁺ depolarized the cells according to the Nernst equation. Figure 4B compares the experimental data with the theoretical line calculated from the Nernst equation for the different extracellular concentrations of potassium.

The effects of high [K⁺]ₑ on network activity was also tested in 17 dissociated cultures using MEAs (Fig. 5A). Between normal [K⁺]ₑ and 10 mM, the burst rate increased by a maximum of 166 ± 49% (P < 0.01, n = 10), with individual maxima for each culture between 8 and 10 mM [K⁺]ₑ. At higher [K⁺]ₑ in the range between 9 and 11 mM bursting first became less frequent (51 ± 42% ns compared with control, P = 0.28) and finally ceased. The cessation of bursting could occur immediately after the solution change or 1–10 min after the solution change, depending on [K⁺]ₑ. Concomitantly, with increasing [K⁺]ₑ, the bursts became shorter and smaller (burst

![FIG. 4. Depolarizing neurons with high [K⁺]ₑ mimics effect of the blockade of the pump. A: example of whole cell current-clamp recordings at 5 different [K⁺]ₑ. Various [K⁺] solutions were randomly applied. Dashed line indicates membrane potential level in control condition (4 mM of [K⁺]ₑ). Note stop of bursting between 10.45 and 12.6 mM [K⁺]ₑ. V_m: −55.1 mV. B: plot of membrane potential as a function of log [K⁺]ₑ. Line represents theoretical value calculated from Nernst equation. n = 6 cells; SE bars are too small to be visible. Data form dissociated cultures.](image)

![FIG. 5. High [K⁺]ₑ fully mimics effect of strophanthidin in dissociated cultures, although not in slice cultures. A: in dissociated cultures, increasing [K⁺]ₑ first leads to an increase in burst rate and a shortening of bursts. At higher concentrations, bursts are decreasing in amplitude and finally disappear. Concomitantly asynchronous background activity is increasing. Solution changes are indicated by large artifacts. B: in slice cultures, high [K⁺]ₑ leads to an increase in burst rate and at higher concentrations, to a decrease in burst amplitude and duration. It does not reproduce early effects of strophanthidin, which constitute a confluence of bursts. All data under B are from same experiment. Solution changes are indicated by large artifacts (arrows).](image)
duration: $-79 \pm 8\%$; $n = 5$, $P < 0.001$, amplitude: $-30 \pm 7\%$, $P < 0.05$). Above 10–11 mM $[K^+]_o$, bursting ceased and only the background activity remained. Background activity increased with increasing $[K^+]_o$, reaching a maximum at around 9–12 mM ($+393 \pm 157\%$, $n = 17$, $P < 0.05$). At higher $[K^+]_o$, it rapidly dropped to values around zero as a function of $[K^+]_o$ and of time. In 12 mM $[K^+]_o$, background activity was 75% ± 60% ($n = 11$) above control in the first 2 min after change of solution. After 7 min, it was roughly at control level (14 ± 46%). In 16 mM $[K^+]_o$, the corresponding values were $-90 \pm 5$ and $-96 \pm 2\%$ of control.

The qualitative similarity between the biphasic effects of strophanthidin and of high $[K^+]_o$ were confirmed by the quanti-tative comparison of the early effects of strophanthidin with the effects of high $[K^+]_o$ in the range of 8–11 mM. From all the described parameters, only the changes in burst duration were significantly different between these two groups (no change in early strophanthidin, decrease in high $[K^+]_o$, $P < 0.05$). These findings thus suggest that the depolarization of the neurons attributed to the blockade of the electrogenic pump is the major mechanism involved in the early changes in network activity induced by strophanthidin.

In organotypic cultures, however, the early effects of strophanthidin could not be reproduced by high $[K^+]_o$. As illustrated in the experiment in Fig. 5B, elevating the $[K^+]_o$ from 4 to 8 mM decreased the burst period by 54% ± 8% and the burst duration by 33% ± 10% ($n = 5$, compare Ballerini et al. 1999; Fig. 7). However, none of the $[K^+]_o$ concentrations tested (6, 8, 9, 10, 11 mM) led to a confluence of the bursts. In contrast, we observed a confluence of the bursts after a short transient increase in burst rate (as illustrated in the bottom trace of Fig. 5B and in Fig. 1) in all of 10 slice cultures as an early effect of strophanthidin application. Between 8 and 11 mM $[K^+]_o$, bursts became shorter, rare, and irregular (see Fig. 5B, middle trace). Their intraburst structure changed from oscillations to mostly single activity waves. These changes developed within minutes after the change in $[K^+]_o$. After 5–10 min in $[K^+]_o$, of 8–11 mM (varying between the cultures) the mean period of the activity waves was 19.6 ± 4.1 s, with a CV of 0.63 ± 0.12 ($n = 9$). These values were not significantly different from those seen after prolonged application of strophanthidin ($P > 0.05$; compare Fig. 5B, middle trace to Fig. 1). At even higher concentrations of $[K^+]_o$, the bursts disappeared completely and only background activity in some channels persisted, which, as in dissociated cultures, decreased with increasing $[K^+]_o$.

**Strophanthidin has no effect on spike frequency adaptation during bursts**

We previously showed that under disinhibition the spike rate is decreasing during the burst in the whole network (network activity) as well as in individual cells (Darbon et al. 2002b). This could be the result of a decrease in the number of active cells in the network (and thus in the average input to each neuron) or of spike frequency adaptation in individual neurons. When short depolarizing current pulses were injected in the absence of fast synaptic input, most interneurons showed prac-tically no spike frequency adaptation (Ballerini et al. 1999; Darbon et al. 2002b). However, when long pulses mimicking the duration and the period of the bursts were injected (in the presence of bicuculline, strychnine, CNQX, and APV to block fast synaptic transmission), considerable spike frequency adaption occurred, which was paralleled by a diminution and a slowing of the spikes (see Fig. 6). The amount of adaptation was dependent on the amplitude of the injected current: at higher intensity (140–250 pA), the adaptation was more pronounced and firing could cease before the end of the pulse (see Fig. 6A). We wanted to know whether the Na/K pump was involved in this type of spike frequency adaptation. Therefore we investigated the effect of strophanthidin on the firing induced by long current pulses (in the absence of fast synaptic transmission; $n = 14$). When a current pulse with a constant amplitude was injected, strophanthidin caused an increase in spike frequency adaptation, meaning that spiking died out more readily (see Fig. 6B). However, this effect was clearly attributed to the well-known depolarizing effect of strophanthidin (see Fig. 6B), which shifted the response curve of the neurons (spike frequency vs. amplitude of injected current) to the left (data not shown). When this depolarization was compensated for by the injection of a steady bias current, spike frequency adaptation as well as the response curve were not affected by strophanthidin (see Fig. 6C). The repetitive injection of long depolarizing current pulses caused a hyperpolariza-tion of the membrane in the intervals between the pulses (in 13 of 14 experiments; see Fig. 6, A and B). The amount of hyperpolarization seemed to depend on the number of spikes evoked by the current pulses. The higher the spike rate (as illustrated in Fig. 6A, pulses 10–12), the larger was the obs-erved hyperpolarization ($-5.6 \pm 0.9 \text{ mV}$, $n = 4$). When the firing ceased during the pulse because of strong depolarization (as illustrated in Fig. 6A, pulses 16–18), the hyperpolarization between the pulses was reduced by 53 ± 2%. When the stimulation was too small to trigger spikes no hyperpolariza-tion was observed and when the stimulation triggered spikes at low frequency the amplitude of the hyperpolarization was decreased by 69 ± 14% (as illustrated in Fig. 6A, pulses 2–4). No hyperpolarization was seen when the current pulses were injected in the presence of strophanthidin (in the presence of a bias current compensating the strophanthidin-induced depolarization; data not shown). These findings, although not excluding other possible mechanisms, fit well to the hypothesis that the activation of the Na/K pump by the high spike rate during the bursts hyperpolarizes the cells.

Together, these findings show that the effects of strophan-thidin on the response curve of neurons and on spike frequency adaptation are entirely attributed to the depolarization of the cells and that spike frequency adaptation is not affected by the blockade of the Na/K pump.

**DISCUSSION**

The main result of this study is that the involvement of the Na/K pump in rhythm generation in cultured spinal networks is mostly attributable to its contribution to the membrane poten-tial of spinal interneurons. This contribution is especially im-portant during rhythmic activity when the activity of the pump is increased; however, the pump is not involved in the mech-anisms of spike frequency adaptation during bursts.

**Contribution of the pump to the resting membrane potential**

The Na/K pumps maintain $[\text{Na}^+]$ and $[\text{K}^+]$ gradients across the cell membrane. In addition, they are electrogenic, meaning
that they create a hyperpolarizing current (pump current) arising from the exchange, at each translocation cycle, of 3 Na\(^+\) for 2 K\(^+\). The inhibition of the pump thus has 2 effects: it immediately blocks the pump current and it causes a slow redistribution of Na\(^+\) and K\(^+\). We revealed the contribution of the electrogenic pump current to the membrane potential of cultured spinal interneurons by the early and rapid depolarization induced by strophanthidin. In control conditions, this depolarization was about 13 mV, comparable to the contribution of the pump current to the membrane potential calculated by Lauger (1984) and measured by Senatorov et al. (1997) in rat auditory thalamus (8.8 mV), by Munakata et al. (1998) in rat neostriatal neurons (12.1 ± 1.3 mV), or by Dobretsov et al. (1999) in rat dorsal root ganglia neurons (about 20 mV). In the absence of fast synaptic transmission, the component of the pump current induced a level of hyperpolarization (about 13 mV) similar to that during spontaneous activity. This level is comparable to the 17.5 ± 1.5 mV recorded by Ross and Soltész (2000) in the presence of APV, CNQX, TTX, and bicuculline in interneurons of rat dentate gyrus. Taken together, these findings therefore suggest that the pump operates at a basic steady-state level even when spiking is heavily decreased or blocked.

**Upregulation of the pump during rhythmic activity**

In hippocampal CA1 neurons, the Na/K pump activity is increased after the focal application of glutamate (Thompson and Prince 1986). Del Negro et al. (1999) recently proposed for bursting trigeminal motoneurons that the role of the pump is multifaceted and that the pump current is composed of both steady-state and dynamic components. The steady-state component affects the voltage trajectory most effectively when the membrane conductance is low, which corresponds to the interburst interval. The dynamic component is specifically activated during the burst because of spike-mediated enhanced Na\(^+\) influx. This dynamic component constrains intraburst spike frequency and generates a transient postburst AHP. The authors proposed that the dynamic component of the pump current was responsible for burst termination in trigeminal motoneurons under experimental conditions in which Ca\(^{2+}\)-dependent K\(^+\) currents were blocked (with Ba\(^{2+}\) and apamin). In our experiments the disinhibition of the network induced rhythmic activity made of bursts, which were interspaced with silent intervals. The membrane potential during these intervals was hyperpolarized compared to the control conditions in the absence of disinhibition (Darbon et al. 2002b). The blockade of the pump during disinhibition provoked a depolarization of about 20 mV, which was stronger than in control conditions (see Fig. 3). The difference in membrane potential between the basic level and the “dynamic” level of pump activity was similar to the amount of hyperpolarization induced by disinhibition. This suggests that during disinhibition the supplementary Na\(^+\) influx during the bursts accelerates the pump turnover (Shen and Johnson 1998) and this higher level of activity of the pump during the burst is responsible for the hyperpolarization of the membrane potential after the bursts (postburst AHP). In line with this hypothesis, the upregulation of the pump could be mimicked in single cells in the absence of network activity by mimicking bursts by current injection (see Fig. 6A). However, a transient post-
burst AHP was absent in many interneurons in spinal cultures during disinhibition-induced bursting. In these neurons, however, the membrane was hyperpolarized during the interburst intervals compared with control values. When the burst rate was decreased by a low dose of CNQX (1 µM), a slow transient postburst AHP was revealed in 50% of these cells (Darbon et al. 2002b). This suggests that, at the burst rates observed in our preparations, disinhibition produces a persistent upregulation of the pump.

Involvement of the pump in rhythm generation

We previously showed that rhythm generation in disinhibited spinal cultures depends on intrinsic spiking in some interneurons, recruitment of the network by recurrent excitation through glutamatergic synapses, and autoregulation of neuronal excitability (Darbon et al. 2002b). The latter leads to a silencing of intrinsically firing interneurons after the bursts probably caused by the hyperpolarization during the intervals. The role of the electrogenic pump in the maintenance of the neuronal excitability is well established. In a study in rat neostriatal neurons, Munakata et al. (1998) proposed that the Na/K pump directly affected the neuronal excitability. The acceleration of the pump after long-lasting discharge, which increased the Na⁺ influx a produced a hyperpolarization of the membrane and thus functioned as a negative feedback mechanism against overexcitement. On the other hand, the decrease in the pump activity at hyperpolarized potential may play a significant role in stabilizing the membrane potential. Shen and Johnson (1998) assumed that the ability to generate pump currents at high density may be a necessary condition for enabling burst firing during NMDA-induced bursting in rat midbrain dopamine neurons.

In line with a crucial role of the pump in rhythm generation, we found that strophanthidin immediately led to an increase in burst rate in conjunction with an early depolarization and an increase in the intrinsic spike rate of the cells (asynchronous background activity). As pointed out above, the pump current was the major source of the hyperpolarization of the neurons during the intervals. Thus, when the pump current was blocked, the neurons depolarized, with the consequence that they started to intrinsically fire action potentials (at least a fraction of them) and thereby increased the burst rate.

We confirmed the role of depolarization of the neurons for accelerating the rhythms by depolarizing all neurons in the culture with increasing [K⁺]ᵣ. We have shown (Fig. 4) that an increase of [K⁺]ᵣ between 8 and 10.5 mM depolarized the interburst potential in the same range as initially induced by the blockade of the Na/K pump and caused the same increase in burst rate and background activity as seen as an early effect of strophanthidin. These findings suggest that the early effects of strophanthidin, at least in dissociated cultures, can be entirely explained by the depolarization induced by the blockade of the pump current. Similar conclusions were reached by Del Negro et al. (1999). Higher [K⁺]ᵣ transiently increased the intrinsic background activity of the neurons but decreased the rate, regularity, and amplitude of the bursts and finally stopped bursting. The same effects were seen with prolonged application of strophanthidin, which further depolarized the neurons, although a full blockade of bursting was usually not reached. The blockade of the pump impaired the extrusion of Na⁺ ions and the import of K⁺ ions, which progressively modified these ion gradients and further depolarized the cells. With time, the slow increase of the depolarization brought more and more cells in a nonfiring state during the bursts attributed to the inactivation of sodium channels, thus reducing the rate, amplitude, and duration of the bursts recorded with MEAs (Fig. 2A). When the number of cells available for activation was no longer sufficient to recruit the network, bursting ceased and only intrinsic spiking in some neurons remained.

A similar disruption of bursting resulting in rare, irregular short bursts was reported by Ballerini et al. (1997) in the isolated spinal cord of the neonatal rat. In this study, only late effects of strophanthidin or ouabain were reported, probably reflecting the slow diffusion of these lipophilic substances in the spinal cord preparation. We have observed a discrepancy between the early effects of strophanthidin in dissociated cultures and in organotypic cultures. Although in dissociated cultures, the early effects of strophanthidin were almost fully reproduced by depolarizing the network with high [K⁺]ᵣ (only the burst duration changes were different in both protocols), this was not the case in organotypic cultures. In the latter, strophanthidin caused a rapid confluence of the bursts (see Fig. 1), which was not seen with high [K⁺]ᵣ. One possible explanation for this discrepancy may be that strophanthidin, because of its lipophilic nature, may be unevenly distributed in the slice during early exposure. Such an uneven distribution would lead to the coexistence of depolarized and not yet depolarized regions of the network, favoring the activation of the network and thus preventing the shortening of the bursts seen in high [K⁺]ᵣ.

Such a hypothesis would also explain the lack of burst shortening in spite of the increase in burst rate in dissociated cultures during early strophanthidin exposure. However, other explanations related to the differences in the ion distribution in the extracellular space between the two regimes must also be considered. Interestingly, in the isolated spinal cord, slow irregular rhythms reappeared after prolonged exposure to strophanthidin for several hours (Rozzo et al. 2002), suggesting that the preparation did not die as a result of the blockade of the pump.

In summary, the contribution of the pump to rhythm generation in spinal cultures may be dual: first, to bring a significant fraction of neurons below firing level to silence the network after the bursts; and second, to remove inactivation from sodium conductances inactivated by depolarization to prepare the recruitment of the network for the subsequent burst episode.

Spike frequency adaptation

There might be a dual role of the electrogenic pump during bursting activity. We have shown that the pump is used to maintain the hyperpolarization in the interburst intervals and therefore to pace the bursting. On the other hand, the increase in internal sodium produced by the repetitive spiking during the bursts may result in a slow development of a hyperpolarizing current and a decrease in discharge rate (spike frequency adaptation). Del Negro et al. (1999) showed that, in trigeminal motoneurons, the Na/K
pump current influenced the intraburst spike frequency. Blockade of the pump during simulated bursts shifted the f-t relationship in an upward direction, suggesting that the pump current was activated during simulated bursts and contributed to spike frequency adaptation. On the other hand, reduction of the pump activity did not produce a significant change in any of the phases of spike frequency adaptation in a study of rat hypoglossal motoneurons (Powers et al. 1999).

In our experiments, simulated bursts displayed spike frequency adaptation during prolonged pulses (Fig. 6). The blockade of the pump by strophanthidin, however, left the spike frequency adaptation unchanged when the strophanthidin-induced depolarization was compensated for (Fig. 6C).

The theoretical effect of the pump current on spike frequency is a slowly accumulating hyperpolarizing current decreasing the discharge rate and slowly hyperpolarizing the plateau level. In our experiments, however, the plateau slightly depolarized instead. Also, the response curves of the neurons were identical before and after pump blockade (after injection of a bias current to compensate the strophanthidin-induced depolarization). Therefore the spike frequency adaptation observed in our experiment was not dependent on the Na/K pump activity. The slow depolarization of the pulse plateau and the progressive decrease in spike amplitude going parallel to the spike frequency adaptation suggest that it was a consequence of a slow inactivation of sodium channels (Fleidervish et al. 1996; Toib et al. 1998).

In summary, our results show that in spinal cultures the addition of the Na/K pump to rhythm generation can be of a slow inactivation of sodium channels (Fleidervish et al. 1999). The theoretical effect of the pump current on spike frequency is a slowly accumulating hyperpolarizing current decreasing the discharge rate and slowly hyperpolarizing the plateau level. In our experiments, however, the plateau slightly depolarized instead. Also, the response curves of the neurons were identical before and after pump blockade (after injection of a bias current to compensate the strophanthidin-induced depolarization). Therefore the spike frequency adaptation observed in our experiment was not dependent on the Na/K pump activity. The slow depolarization of the pulse plateau and the progressive decrease in spike amplitude going parallel to the spike frequency adaptation suggest that it was a consequence of a slow inactivation of sodium channels (Fleidervish et al. 1996; Toib et al. 1998).

In summary, our results show that in spinal cultures the contribution of the Na/K pump to rhythm generation can be attributed to its hyperpolarizing effects, but not to a role of the pump in spike frequency adaptation. The latter must be considered as an additional mechanism influencing burst termination.

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Present address of A. Tschetter: Department of Neuroscience, University of Roma “Tor Vergata,” 00133 Rome, Italy.

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