Effects of Baclofen on Spinal Reflexes and Persistent Inward Currents in Motoneurons of Chronic Spinal Rats With Spasticity

Y. Li, X. Li, P. J. Harvey, and D. J. Bennett
Centre for Neuroscience, University of Alberta, Edmonton, Alberta T6G 2S2, Canada

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Li, Y., X. Li, P. J. Harvey, and D. J. Bennett. Effects of baclofen on spinal reflexes and persistent inward currents in motoneurons of chronic spinal rats with spasticity. J Neurophysiol 92: 2694–2703, 2004; 10.1152/jn.00164.2004. In the months after spinal cord injury, motoneurons develop large voltage-dependent persistent inward currents (PICs) that cause sustained reflexes and associated muscle spasms. These muscle spasms are triggered by any excitatory postsynaptic potential (EPSP) that is long enough to activate the PICs, which take >100 ms to activate. The PICs are composed of a persistent sodium current (Na PIC) and a persistent calcium current (Ca PIC). Considering that Ca PICs have been shown in other neurons to be inhibited by baclofen, we tested whether part of the antispastic action of baclofen was to reduce the motoneuron PICs as opposed to EPSPs. The whole sacral spinal cord from acute spinal rats and spinal chronic spinal rats (with sacral spinal transection 2 mo previously) was studied in vitro. Ventral root reflexes were recorded in response to dorsal root stimulation. Intracellular recordings were made from motoneurons, and slow voltage ramps were used to measure PICs. Chronic spinal rats exhibited large monosynaptic and long-lasting polysynaptic ventral root reflexes, and motoneurons had associated large EPSPs and PICs. Baclofen inhibited these reflexes at very low doses with a 50% inhibition (EC50) of the mono- and polysynaptic reflexes at 0.26 ± 0.07 and 0.25 ± 0.09 (SD) μM, respectively. Baclofen inhibited the monosynaptic reflex in acute spinal rats at even lower doses (EC50 = 0.18 ± 0.02 μM). In chronic (and acute) spinal rats, all reflexes and EPSPs were eliminated with 1 μM baclofen with little change in motoneuron properties (PICs, input resistance, etc.), suggesting that baclofen’s antispastic action is presynaptic to the motoneuron. Unexpectedly, in chronic spinal rats higher doses of baclofen (20–30 μM) significantly increased the total motoneuron PIC by 31.6 ± 12.4%. However, the Ca PIC component (measured in TTX to block the Na PIC) was significantly reduced by baclofen. Thus baclofen increased the Na PIC and decreased the Ca PIC with a net increase in total PIC. By contrast, when a PIC was induced by 5-HT (10–30 μM) in motoneurons of acute spinal rats, baclofen (20–30 μM) significantly decreased the PIC by 38.8 ± 25.8%, primarily due to a reduction in the Ca PIC (measured in TTX), which dominated the total PIC in these acute spinal neurons. In summary, baclofen does not exert its antispastic action postsynaptically at clinically achievable doses (<1 μM), and at higher doses (10–30 μM), baclofen unexpectedly increases motoneuron excitability (Na PIC) in chronic spinal rats.

INTRODUCTION

Baclofen has been used widely as an anti-spasticity drug since the 1970s (Hudgson and Weightman 1971; Hudgson et al. 1972). Because it reduces muscle tone and spasms with similar efficacy in patients with spasticity caused by complete or incomplete spinal cord injury, or from cerebral origin (Albright et al. 1991; Davidoff 1985; Metz 1998; Meythaler et al. 2001), it is assumed to act mainly at the spinal cord level. This assumption has been confirmed by the recent finding that the effect of baclofen is more potent when applied intrathecally than orally (Azouvi et al. 1996; Kamensek 1999; Ochs et al. 1989). GABAB receptors are distributed extensively in the spinal cord, especially presynaptically on the primary sensory afferent terminals (Price et al. 1984; Yang et al. 2001). Baclofen, as a potent GABAB receptor agonist, has been shown in many studies to decrease synaptic transmission by binding to the presynaptic GABAB receptors at the afferent terminal through a second-messenger pathway, ultimately decreasing the calcium influx and neurotransmitter release (Batueva et al. 1999; Bussieres and El Manira 1999; Curtis et al. 1997; Miller 1998). In addition, baclofen’s binding to the presynaptic GABAB receptors can also decrease the neurotransmitter release by activating potassium channels (Gage 1992), thus contributing to its presynaptic inhibitory effect.

Recently, major postsynaptic effects of baclofen have also been reported in moto- and interneurons (Russo et al. 1998; Svirskis and Hounsgaard 1998; Voisin and Nagy 2001); this could also contribute to baclofen’s antispastic action. In motoneurons of normal animals (or humans) with intact spinal cord and brain stem, there are voltage-dependent persistent inward currents (PICs) that, once activated, can remain active for many seconds after stimulation, producing sustained depolarizations (plateau potentials) and firing (self-sustained firing), thus greatly increasing their excitability (Bennett et al. 1998; Gorassini et al. 2002; Hounsgaard et al. 1984; Lee and Heckman 1998a,b; Schwindt and Crill 1984). The PICs are composed of a low-threshold persistent calcium current, carried by Cav1.3 L-type calcium channels, and a TTX-sensitive persistent sodium current (Chandler et al. 1994; Hounsgaard and Kiehn 1989; Lee and Heckman 2001; Li and Bennett 2003; Schwindt and Crill 1995). Large PICs are not present in motoneurons immediately after spinal cord injury because of the massive loss of brain-stem-derived monoamines that normally facilitate PICs (Hounsgaard et al. 1988). Exogenous application of metabotropic receptor agonists (such as 5-HT, norepinephrine, and muscarine receptors) can enhance PICs and thus recover plateaus and self-sustained firing after acute spinal transection or in vitro slice injury (Hounsgaard and Kiehn 1989; Lee and Heckman 1998a,b; Svirskis and Hounsgaard 1998). Baclofen has been shown recently to decrease the amplitude of these enhanced PICs in motoneurons of turtle spinal cord slices (Svirskis and Hounsgaard 1998) and to decrease spontaneously occurring PICs in deep dorsal horn.
neurons of turtles and rats (Russo et al. 1998; Voisin and Nagy 2001), raising the interesting possibility that baclofen’s clinical action may be partly postsynaptic by decreasing the PICs that play an important role in the production of spasticity (Bennett et al. 2001a,b; Li et al. 2004a).

Weeks after spinal cord transection in rats (chronic spinal rats) the plateaus and PICs in motoneurons below the injury recover spontaneously without exogenous application of metabotropic receptor agonists (Bennett et al. 2001a,b; Li and Bennett 2003). With these recovered PICs (and thus recovered motoneuron excitability) combined with a loss of descending inhibition over spinal reflexes after spinal cord injury, a brief dorsal root stimulation can produce long-lasting exaggerated reflexes or spasms, which reflect an essential characteristic of spasticity (Bennett et al. 2001a,b; Li et al. 2004a). The recovered PICs in chronic spinal rats are mediated by a low-threshold L-type calcium current (Ca PIC, as in the turtle motoneurons and rat deep dorsal horn neurons) and a persistent sodium current (Na PIC) (Li and Bennett 2003). Considering that baclofen can inhibit the PIC mediated by L-type calcium currents in normal motoneurons, we examined in the present paper whether baclofen acts similarly in chronic spinal rats. Surprisingly, we found that baclofen did not decrease the PICs, even at high doses, and instead somewhat increased the PICs.

Spasticity is usually absent in acutely injured animal preparations, and it only gradually develops with chronic injury (>1 mo) (Bennett et al. 1999). However, much of our understanding of baclofen’s mechanisms of action comes from normal intact or acutely injured animal preparations that do not exhibit the hyperexcitability of the chronic spinal spastic state. Thus in the present paper we studied how baclofen reduced spasticity in adult female Sprague-Dawley rats (>60 days old, n = 17) and spastic rats with chronic spinal cord injury (>90 days old, n = 16) included in the present study. For the spastic rat, a complete spinal cord transection was made at the S4 sacral level when the rat was 50 days old (Bennett et al. 1999). Usually, within 30 days, dramatic spasticity developed in the tail muscles, which were innervated by sacrocaudal motoneurons below the level of the injury. Only rats >50 days (50–120 days) post injury with clear spasticity were included in the present study. The details of the chronic transection and spasticity assessment are described in Bennett et al. (1999, 2004). All experimental procedures were approved by the University of Alberta animal welfare committee.

METHODS

Both normal adult female Sprague-Dawley rats (>60 days old, n = 17) and spastic rats with chronic spinal cord injury (>90 days old, n = 16) were included in the present study. For the spastic rat, a complete spinal cord transection was made at the S4 sacral level when the rat was 50 days old (Bennett et al. 1999). Usually, within 30 days, dramatic spasticity developed in the tail muscles, which were innervated by sacrocaudal motoneurons below the level of the injury. Only rats >50 days (50–120 days) post injury with clear spasticity were included in the present study. The details of the chronic transection and spasticity assessment are described in Bennett et al. (1999, 2004). All experimental procedures were approved by the University of Alberta animal welfare committee.

In vitro preparation

Two in vitro preparations were employed in the present study: one for intracellular recording and one for root reflex recording. For both preparations, normal and chronic spinal rats were deeply anesthetized with urethane (0.18 g/100 g; with a maximum of 0.45 g for rats >250 g), and the whole sacrocaudal spinal cord was removed and placed in a dissection dish filled with modified artificial cerebrospinal fluid (mACSF) at 20–21°C. For the root reflex preparation, all the dorsal and ventral roots below the level of the injury were kept and gently separated (see details in Li et al. 2004b). After an hour’s rest in the dissection chamber, the cord was transferred to the recording chamber, where it was immersed in continuously flowing (5 ml/min) normal ACSF (nACSF), maintained at 24–25°C (see Li et al. 2004b for procedure details). In the recording chamber, the cord was supported on a small piece of paper mesh and secured by passing pins through the end of the cord and through the lateral vasculature and connective tissue and into the silicone elastomer (Sylgard) base below the nappy paper. For the intracellular recording preparation, everything was the same as the root reflex preparation except that all the dorsal roots attached to the cord were cut off except the main caudal dorsal root (i.e., Cauda, which was attached to the caudal equina), and the cord was glued (super glue; RP 1500, Adhesive Systems) on its dorsal surface to a small piece of nappy paper (with the ventral side facing up) to increase stability (see Li and Bennett 2003 for details).

Root reflex recording

Each dorsal and ventral root (usually S3, S4, and Caudal ventral and dorsal) was mounted on a silver-chloride wire above the ASCF of the recording chamber and covered with a 1:1 mixture of petroleum jelly (Vaseline) and mineral oil for monopolar stimulation and recording (Li et al. 2004b). To record the monosynaptic reflex in the ventral root, the dorsal roots were stimulated at 0.05 Hz, 0.1 ms, 0.1 mA (10 times threshold, 10×T; T = 0.01 mA), i.e., a 20-s delay existed between each stimulation to avoid reflex depression (Bennett et al. 2004). To record the polysynaptic reflex, the dorsal roots were stimulated similarly, except sometimes with a higher repetition rate (0.5 Hz, 0.1 ms, 0.1 mA) to facilitate the long-lasting polysynaptic reflexes (Li et al. 2004b). This stimulation intensity was chosen to be supramaximal to evoke mono- and polysynaptic reflexes reliably. The ventral root reflexes were amplified by 5,000 times with a custom-built preamplifier, low-pass filtered at 3 kHz, high-pass filtered at 100 Hz and recorded with a data-acquisition system sampling at 6 kHz (Axonoscope 8; Axon Instruments). The monosynaptic reflex was recognized by its large amplitude and short delay (~3 ms, including peripheral delay), and the peak amplitude was measured with Axonoscope 8. The polysynaptic reflex was quantified by rectifying the data and averaging over a 1-s window starting 50 ms after the stimulus using Matlab (Mathworks). Because the input resistance of the extracellular ventral roots recordings was similar from rat to rat, we averaged the reflex responses across rats without correcting for this resistance.

Intracellular recording

The same intracellular recording procedures described previously were employed (Li and Bennett 2003) and are only briefly summarized here. The long ventral roots (usually sacral S4 and caudal Cauda) and caudal equina (which had attached caudal dorsal roots, Cauda) were mounted on silver-chloride wires above the nACSF and covered with high vacuum grease (Dow Corning). Sharp intracellular recording electrodes were made from thick-wall glass capillaries (WPI GC 150F-10, 1.5 mm OD) with a micropipette puller (Sutter P-87 puller), filled with a 1:1 mixture of 2 M K-Acetate and 2 M KCl and beveled down to 20–30 MΩ on a rotary grinder (Sutter, BV-10, fine 006 beveling stone). Electrodes were advanced perpendicularly into the
ventral surface of the cord with a stepper-motor micromanipulator (660, Kopf) to penetrate motoneurons. Motoneurons were identified by antidromic ventral root stimulation. Only motoneurons with a stable penetration, resting potential less than −60 mV, spike amplitude >60 mV, and reliable repetitive firing were included in the study. An Axoclamp2b intracellular amplifier (Axon Instruments) running in either discontinuous current-clamp modes (DCC, switching rate 7–10 kHz, output bandwidth 3.0 kHz) or discontinuous voltage-clamp modes (gain: 1–2.5 nA/mV) was used to collect the data. The basic properties of the motoneurons, such as cell resistance, firing threshold and firing level (voltage threshold of first spike) were measured during current ramps in DCC mode as described in Li and Bennett (2003).

Slow triangular current ramps (0.4 nA/s) and voltage ramps (standard speed: 3.5 mV/s, varied from 2 to 5 mV/s) were applied to the motoneurons to evoke firing and quantify the PICs. During the current ramps (in current-clamp), the PICs that contributed to sustained firing (self-sustained firing) were estimated from the difference between the injected current required to terminate firing (Istart) and the current required to start firing (ΔI = Iend − Istart) (see Fig. 4A) (see also Bennett et al. 2001b). During the voltage ramps (in voltage-clamp), the PICs caused a negative-slope region in the current-voltage (I-V) relation, and this negative-slope region caused the plateau behavior and self-sustained firing seen in current clamp (Li and Bennett 2003). To obtain an estimation of the passive leak current that summed with the PICs to give the total recorded current, a linear relation was fit to the subthreshold current response in the linear region 10 mV below the negative-slope region onset, and extrapolated to more positive voltages (leak current, thin triangular line overlaying current; Fig. 4E). The amplitude of the PIC was then estimated by subtracting this leak current from the recorded current; this difference is indicated by the arrow in Fig. 4E (see details in Fig. 1 of Li and Bennett 2003).

Reflexes and excitatory post synaptic potentials (EPSPs) were also recorded during intracellular recordings, in response to brief stimulation of the Ca2+ dorsal root.

Drugs and solutions

Two kinds of ACSF were used in the experiments: nACSF in the recording chamber and mACSF in the dissection chamber. The composition of nACSF was (in mM) 118 NaCl, 24 NaHCO3, 2.5 CaCl2, 3 KCl, 1 MgSO4, 12 D-glucose, and 1 kynurenic acid; the latter is a non-specific blocker of glutamate transmission (Kekesi et al. 2002). Both kinds of ACSF were saturated with 95% O2–5% CO2 and maintained at pH 7.4. Drugs added to the nACSF in (Kekesi et al. 2002). Both kinds of ACSF were saturated with 95% O2–5% CO2 and maintained at pH 7.4. Drugs added to the nACSF in the experiments included: 0.01–30 μM (±) baclofen (Sigma), 1–2 μM TTX (RBI), 10–20 μM nimodipine (Sigma), 400 μM Cd2+ (Sigma), and 10 μM 5-HT (Sigma). Baclofen was mixed in 1 mM as stock; TTX, 5-HT and Cd2+ were dissolved at high concentrations (×100 of final concentration) as stock. Nimodipine was dissolved in DMSO before each experiment (100–200 mM). These drugs were then diluted to the desired concentration in nACSF. The DMSO concentration was <0.02% in the final nACSF solution and had no effect on PICs/plateaus (Li and Bennett 2003).

Data analysis

Data were analyzed in Clampfit 8.0 (Axon Instruments) and Matlab (Mathworks). Figures were made in SigmaPlot (Jandel Scientific). The effective concentration of baclofen to reduce the reflex by 50% (EC50) in each rat was obtained by fitting a sigmoid curve to the relation between the percentage inhibition of reflex amplitude and the drug concentration (log). Data were shown as averages ± SD. A Student’s t-test was used to test for statistical differences, with a significance level of P < 0.05.

RESULTS

A total of 17 acute spinal rats and 13 chronic spinal rats were included in the present study. For the 17 acute spinal rats, 6 were used to record the root reflexes and 11 were used for intracellular recording. For the 16 chronic spinal rats, 7 were used for the root reflex recording and 9 were used for intracellular recording.

The motoneurons of chronic spinal rats had an average input resistance of 7.70 ± 4.14 MΩ, resting membrane potential of −75.9 ± 7.1 mV, firing level of −44.6 ± 4.6 mV (during the slow current ramp), and spike height of 71.7 ± 8.85 mV (with antidromic ventral root activation). Motoneurons of acute spinal rats had an input resistance of 4.67 ± 1.58 MΩ, resting membrane potential of −74.9 ± 8.7 mV, firing level of −47.2 ± 9.8 mV, and spike height of 66.1 ± 2.59 mV. No significant differences were found between acute and chronic spinal rats in these membrane properties.

Effect of baclofen on root reflexes of chronic spinal rats

In chronic spinal rats, when a single stimulation pulse was applied to the dorsal roots (10×T), the ventral roots usually responded with a large monosynaptic reflex (3- to 5-s latency, depending on root lengths, Fig. 1C) followed by a long-lasting polysynaptic reflex (usually ~2 s long, Fig. 1A), which is the counterpart of the long-lasting spastic reflex and spasms seen in these spinal rats when they were awake (Bennett et al. 1999, 2004). The monosynaptic reflex in chronic spinal rats was on average 3.85 ± 2.37 mV in amplitude (peak amplitude), and the polysynaptic reflex was 0.48 ± 0.31 mV.

FIG. 1. A low concentration of baclofen (1 μM) was sufficient to block mono- and polysynaptic long-lasting spastic reflexes in chronic spinal rats. A: long-lasting spastic reflex recorded from the ventral root of a chronic spinal rat, triggered by a single dorsal root stimulation pulse (0.1 mA, 0.1 ms). Note the length of the reflex (~2 s). B: 1 μM baclofen completely eliminated the long-lasting reflex. C and D: expanded version of A and B to show the exaggerated monosynaptic reflex in chronic spinal rats, which is also completely eliminated by 1 μM baclofen. E: only a small monosynaptic reflex was recorded from the ventral root of acute spinal rats by the same stimulation in (A). F: 1 μM of baclofen completely eliminated the monosynaptic reflex.
(mean rectified response in a 1-s window; see METHODS; n = 7). When increasing concentrations (0.01, 0.03, 0.1, 0.3, 1 μM) of baclofen were applied to the bath, the monosynaptic and the long-lasting polysynaptic reflexes were reduced almost simultaneously. For example, at a concentration of 0.3 μM, the monosynaptic reflex was inhibited by 67.81 ± 21.86%, and the long-lasting polysynaptic reflexes were inhibited by 69.99 ± 19.59% (Fig. 2, B and C). Both the monosynaptic reflex and the long-lasting reflexes of these chronic spinal rats were completely eliminated at 1 μM (Fig. 1, B and D). The EC50 of baclofen for the monosynaptic reflex was 0.26 ± 0.07 μM, and the EC50 of baclofen for the polysynaptic reflexes was 0.25 ± 0.09 μM (n = 7, Fig. 2, B and C). There was no significant statistic difference between these numbers, suggesting the blockades of both reflexes were mediated by the same underlying mechanisms, most probably through decreased presynaptic neurotransmitter release.

**Effect of baclofen on EPSPs and membrane properties of motoneurons of chronic spinal rats**

To further determine whether baclofen blocked the reflexes by decreasing neurotransmitter release presynaptically or by changing motoneuron membrane properties postsynaptically, we recorded intracellularly from the motoneurons while stimulating the dorsal roots (n = 6). A single stimulation pulse to the dorsal roots triggered a long-lasting reflex discharge similar to that recorded from the ventral roots (Fig. 3A). Hyperpolarization of the motoneuron membrane, to eliminate the action of voltage-dependent PICs and spiking, reduced the duration of the response substantially, indicating that the motoneuron PICs produced the many-second-long response, as described previously (Bennett et al. 2001b; Li et al. 2004a). However, under these hyperpolarized conditions, a 0.5-s-long response (hyperpolarized EPSPs, Fig. 3B) remained that represented the synaptic input unaffected by postsynaptic PICs. Without hyperpolarization, this synaptic input normally triggered the PICs that ultimately caused the long-lasting reflex discharge (Fig. 3A) (for details, see Bennett et al. 2001; Li et al. 2004a). The hyperpolarized EPSPs had monosynaptic components as indicated in Fig. 3B. Application of 1 μM baclofen eliminated these monosynaptic EPSPs (Fig. 3D, cell hyperpolarized) and accordingly eliminated the long-lasting reflexes (Fig. 3C; cell not hyperpolarized), consistent with the finding that this dose of baclofen eliminated the ventral root reflexes (Fig. 1, described in the preceding text). Thus baclofen’s major antispastic action is to block the synaptic input to the motoneurons. Higher doses of baclofen (20–30 μM) also completely eliminated the EPSPs.

Postsynaptically, baclofen had only weak effects that could not contribute to its antispastic action. That is, in chronic spinal rats baclofen (1–30 μM, n = 6) did not significantly change the motoneuron resting membrane potential (change of 0.06 ± 1.92 mV), input resistance (change of −0.81 ± 1.78 MΩ), or the current threshold to evoke firing (change of 0.07 ± 1.19 nA). Baclofen did significantly decrease the spike threshold (by 4.05 ± 3.09 mV) and increase the PIC amplitude (see details in the following text), but these effects increased the motoneuron excitability. Thus baclofen’s antispastic action is not to decrease the motoneuron excitability, but to block the synaptic inputs to the motoneurons.

**Effect of baclofen on ventral root reflexes of acute spinal rats**

In acute spinal rats, after a dorsal root stimulation pulse, only a monosynaptic reflex could be recorded on the ventral roots (Fig. 1E), consistent with the lack of spastic reflexes seen in the acute spinal state (Bennett et al. 1999; Li et al. 2004b). This monosynaptic reflex had a latency of 3–5 ms and an average amplitude of 0.72 ± 0.48 mV (n = 6), significantly smaller than of chronic spinal rats. During baclofen application, the amplitude of this monosynaptic reflex started to decrease at the low 0.1 μM concentration and was completely eliminated at 1 μM (Fig. 1F). At a concentration of 0.3 μM, 92.92 ± 11.31%
of the monosynaptic reflex was eliminated, a significantly larger decrease than that of chronic spinal rats at this dose (which was 68%, Fig. 2, A and B). In addition, the EC_{50} of baclofen for the monosynaptic reflex was 0.18 ± 0.02 μM (n = 6, Fig. 2A), significantly lower than that of the chronic spinal rats (0.26 μM). These results suggest that chronic spinal rats have a moderately lower (30%) sensitivity to baclofen than do acute spinal rats, which might be due to a down regulation/desensitization of GABA_B receptors or decreased background GABA levels after chronic spinal cord injury (see discussion).

Effect of baclofen on EPSPs and membrane properties of motoneurons of acute spinal rats

When recorded intracellularly, mono- and long-polysynaptic EPSPs in acute spinal rats were, as in chronic spinal rats, triggered by a single stimulation pulse. Therefore these long EPSPs emerged acutely with injury (data not shown, see Fig. 9C of Li et al. 2004a for detail). Unlike in chronic spinal rats, these long EPSPs never triggered any long-lasting reflexes, nor were they amplified by depolarization, consistent with the small PICs seen in acute spinal rats. Low doses of baclofen (1 μM) eliminated the EPSPs as did higher doses (20–30 μM), indicating that baclofen, as in the chronic spinal rats, eliminated the monosynaptic reflex mainly by decreasing presynaptic neurotransmitter release in the acute spinal rats.

Baclofen did not have an obvious effect on the basic membrane properties of motoneurons of acute spinal rats. That is, baclofen (1–30 μM, n = 6) did not significantly change the motoneuron resting membrane potential (ΔV = −0.35 ± 4.02 mV), the input resistance (ΔR = −0.12 ± 0.46 MΩ), the spike threshold (ΔV = −0.93 ± 0.64 mV), or the current threshold (ΔI = 0.12 ± 0.45 nA) in acute spinal rats.

There was an inhibitory effect of high dose baclofen on the PICs, described in the following text. However, because these PICs are too slow (time constant of activation >100 ms) to play a major role in monosynaptic reflexes (Li et al. 2004a), the reduction in monosynaptic reflexes in acute spinal rats is not mediated by postsynaptic actions.

Effect of baclofen on the PICs in chronic spinal rats

As reported previously for motoneurons of chronic spinal rats, a subthreshold plateau potential followed by self-sustained firing could be activated with slow triangular current ramps without any exogenous neuromodulator application (Fig. 4A) (see Bennett et al. 2001a for detail). The PIC that caused this plateau and self-sustained firing was quantified with slow voltage ramps, under voltage-clamp conditions (Fig. 4E; peak of the leak subtracted PICs indicated by arrow; thin line indicates leak current). The PICs were always large enough to generate a negative-slope region in the current-voltage relation (at downward deflection during upward ramp in Fig. 4E). Unexpectedly, application of doses of baclofen sufficient to block the spastic reflexes (1–30 μM) never decreased the total PIC, even though these doses had previously been shown to be effective in reducing PIC in turtle motoneurons and dorsal horn neurons (Russo et al. 1998; Svirskis and Hounsgaard 1998).

Furthermore, 20–30 μM baclofen increased, rather than decreased, the total PIC as follows [n = 6; opposite of Svirskis and Hounsgaard (1998)]; 1 μM had no effect on the PICs. First of all, the amplitude of the initial peak of the PIC (peak PIC on upward ramp, after leak subtraction; arrow in Fig. 4E) increased significantly with baclofen from an average of 2.52 ± 1.52 to 3.20 ± 1.70 nA. The sustained peak of the PIC (peak PIC on downward current ramp) (Li and Bennett 2003) also increased significantly from 2.06 ± 1.34 to 2.65 ± 1.48 nA. Thus there was a 31.6 ± 12.4% increase in the initial peak and 40.5 ± 24.6% increase in the sustained peak (summarized in Fig. 6A). Also, the width of the classic N-shaped dip in current formed by the negative-slope region (width of valley; see double arrow in Fig. 4G) (see also details in Li and Bennett 2003) significantly increased from 12.51 ± 4.22 to 18.73 ± 5.57 mV.

Even though the PIC was increased, this did not lead to increased self-sustained firing, as quantified by the difference between the current to start and stop firing ΔI (see METHODS) on the triangular current injections. That is, ΔI did not significantly increase with baclofen, although it did increase somewhat, from 0.90 ± 0.41 to 1.02 ± 0.31 nA. This discrepancy may be because the total PIC is made up of both an L-type calcium current (Ca PIC) and a TTX-sensitive persistent sodium current (Na PIC). The Ca PIC is primarily responsible for the long-lasting self-sustained firing, whereas the Na PIC produces less self-sustained firing (Li and Bennett 2003; Harvey and Bennett, unpublished data). Thus it is possible that baclofen may increase the total PIC by increasing only the Na PIC and thus not markedly increasing the self-sustained firing. This idea is also supported by the fact that the voltage spike threshold of motoneurons decreased significantly, by 4.05 mV ± 3.09 mV, after high doses of baclofen, and we have previously shown the spike threshold to be linked with the Na PIC (see discussion for detail).

Considering that the total PIC is composed of large sodium (Na PIC) and calcium (Ca PIC) components and considering that the Ca PIC has previously been shown to be decreased by baclofen (Svirskis and Hounsgaard 1998), it stands to reason that baclofen may increase the PIC by selectively increasing the Na PIC component as suggested indirectly by the data described in the previous paragraph. Indeed, we show in the next few sections that baclofen has an interesting mixed effect where it increases the Na

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Hysteresis was significantly reduced by the application of baclofen (30 \mu M) and offset of (0.61 ± 0.27 nA) or (6.93 ± 2.30 mV, respectively), without significant change of the onset voltage of PICs, again suggesting that baclofen actually decreased the Ca PIC portion of the total PIC as shown in the other neurons (Russo et al. 1998; Svirskis and Hounsgaard 1998; Voisin and Nagy 2001).

We also tested the application of TTX (2 \mu M) after baclofen application (Fig. 4). In TTX and baclofen, a large calcium plateau potential could be revealed during a current ramp because the spikes were also blocked (Fig. 4C) (see details in Li and Bennett 2003). However, the underlying PIC was smaller than before TTX application because of the block of the TTX-sensitive Na PIC (Fig. 4, F and G) (see Li and Bennett 2003). The remaining PIC and plateau were eliminated by 20 \mu M nimodipine (Fig. 4, D and H) and thus were mediated by L-type calcium channels. These results indicate that both Na and Ca PICs persist with baclofen. Thus while baclofen likely increases the Na PIC and decreases the Ca PIC (see preceding text), it does not completely block the Ca PIC.

**Effect of baclofen on PICs induced by 5-HT in acute spinal rats**

In acute spinal rats, there was usually no subthreshold plateau or self-sustained firing (Fig. 5A); accordingly, there was no PIC large enough to produce a negative-slope region in the voltage-current relation (Fig. 5E) (as in Li and Bennett 2003). That is, in current-clamp recording, when a triangular current ramp was applied to the motoneuron, the motoneuron
usually started and stopped firing at the same current level; in voltage-clamp recording, when a triangular voltage ramp was applied, the recorded current response was nearly linear (Fig. 5, A and E). To facilitate PICs in these motoneurons, 10–30 μM 5-HT was added to the bath (n = 6). This induced self-sustained firing, a plateau (see ∆f) during a current ramp (Fig. 5B) and a moderate PIC (leak subtracted PIC, arrow of Fig. 5F) measured under voltage-clamp.

When baclofen (20–30 μM) was added to these 5-HT-treated cells (n = 6), the PICs were decreased: the opposite action to that seen in chronic spinal rats but consistent with a dominant decrease in the Ca PIC (as in Svirskis and Hounsgaard 1998). That is, the initial peak amplitude of the PICs decreased significantly with baclofen from an average of 2.83 ± 0.97 to 1.93 ± 1.32 nA, and the sustained peak amplitude decreased significantly from 2.91 ± 1.08 to 2.19 ± 1.32 nA, respectively, which was about a 38.8 ± 25.8% decrease in the initial peak amplitude and a 29.3 ± 24.3% decrease in the sustained peak amplitude (Fig. 5, C and G, summarized in Fig. 6B). The PIC onset voltage, \( V_{onset} \), did not change significantly with baclofen (changed by 1.41 ± 2.65%). Lower doses of baclofen (1 μM, n = 4) had no effect on the PICs. For the cell shown in Fig. 5, the remaining small PIC after baclofen was almost completely eliminated by TTX (Fig. 5, D and H), suggesting that it was a Na PIC that remained in baclofen and that any Ca PIC was inhibited by baclofen prior to the TTX. However, in other cells where TTX was added first to block the Na PIC, to leave only a pure Ca PIC (see following text), baclofen only often partly blocked this Ca PIC, suggesting that baclofen only incompletely blocked the calcium mediated portion of the PICs (see following text, n = 4). Together, these results suggest that in acute spinal rats baclofen acts to reduce the total PIC by reducing the Ca PIC.

The three cells with the largest PICs induced by 5-HT (of 6 cells total) exhibited very slow firing at de-recruitment (Fig. 5B, C), which we have previously shown to result from subthreshold oscillations of a large Na PIC (Li et al. 2004a). Interestingly, these cells with the presumably largest Na PICs showed the least percent reduction in total PICs with baclofen, with one cell showing no effect of baclofen. We suspected, therefore that baclofen might selectively inhibit the Ca PIC, and facilitate the Na PIC (as mentioned above for chronic spinal rats; also see discussion). To test this idea, PICs were induced by 5-HT (10 μM) in 4 additional cells and then TTX (2 μM) was added to block the Na PIC prior to baclofen application (and also to block any spike mediated transmission), thus leaving the Ca PIC in isolation (Li and Bennett 2003). In all these cells, there was a clear calcium-mediated plateau and Ca PIC with characteristic hysteresis (not shown) (Li and Bennett 2003). The application of baclofen (20–30 μM) decreased these Ca PICs markedly. That is, the initial peak amplitude of the PICs decreased significantly with baclofen from an average of 1.74 ± 0.59 nA to 0.97 ± 0.32 nA, and the sustained peak amplitude decreased significantly from 1.69 ± 0.65 nA to 1.01 ± 0.40 nA respectively, which was
about a 57.2 ± 16.4% decrease in the initial peak amplitude and 60.5 ± 13.8% decrease in the sustained peak amplitude. The remaining PIC in TTX/5-HT/baclofen was eliminated by a calcium blockade with Cd^{2+}, showing that it was indeed a Ca PIC. Importantly, the absolute reduction of the PIC by baclofen after TTX (0.77 nA initial PIC) was not significantly different from the reduction of the total PIC in cells not treated with TTX (0.90 nA, see preceding text) and was, on average, smaller, suggesting that baclofen’s inhibitory action is primarily to reduce the Ca PIC and that baclofen moderately increased the Na PIC. Furthermore, the percent reduction in PIC by baclofen was significantly larger after TTX application (almost doubled), also consistent with the suggestion that baclofen primarily inhibits the Ca PIC, and thus removing the Na PIC with TTX reveals a much larger direct effect of baclofen on the Ca PIC.

**Discussion**

Our results demonstrate that very low concentrations of baclofen (≤ 1 μM) are sufficient to block both monosynaptic and long-lasting spastic reflexes in chronic spinal rats and the underlying EPSPs recorded intracellularly. These same low concentrations of baclofen have no major inhibitory effect on postsynaptic motoneuron properties and in particular do not reduce the PICs that are known to play a major role in producing long-lasting spastic reflexes. Thus at doses where the reflexes are inhibited (similar to the clinical dose range, see following text), baclofen’s antispastic action is purely presynaptic. Considering that long-lasting spastic reflexes are mediated by postsynaptic PICs in the motoneurons that are triggered by polysynaptic EPSPs (Fig. 2) (Li et al. 2004a), baclofen must act to block these spastic reflexes by simply blocking the polysynaptic EPSPs so that the PICs are not triggered rather than blocking the PICs directly. The polysynaptic reflex decreased at similar doses to the monosynaptic reflex (similar dose-response curve slope and EC_{50}), suggesting similar mechanisms underlying the two blockades, most probably by reducing neurotransmitter release. Interestingly, the monosynaptic reflex of acute spinal rats was inhibited at a lower dose than that of the chronic spinal rats, suggesting there might be decreased sensitivity/number of GABA_{A} receptors or less GABAergic inhibition after chronic injury. Baclofen did have postsynaptic effects at higher doses (significant at 20–30 μM), but these were unexpectedly excitatory in chronic spinal rats, increasing the PICs and decreasing the spike threshold voltage. In normal motoneurons of acute spinal rats, high-dose baclofen decreased the PICs induced by 5-HT, consistent with baclofen’s inhibitory action in normal turtle motoneurons and deep dorsal horn neurons (Russo et al. 1998; Svirsks and Hounsgra 1998; Voisin and Nagy 2001).

The different effects of baclofen on acute and chronic spinal rats in both root reflexes and PICs were unexpected. The reasons for such effects are discussed in the following text in addition to their possible implications in treating spasticity with baclofen in spinal-cord-injured humans.

**Decreased GABAergic inhibition after chronic spinal cord injury**

It has been shown that there are abundant GABA_{A} receptors in the primary afferent terminals in many vertebrates (Price et al. 1984; Yang et al. 2001). Baclofen can strongly activate these receptors, and through the second-messenger system, coupled to the calcium and potassium channels (activating inward rectifying potassium channels and inhibiting most types of calcium channels) (Misgeld et al. 1995), substantially decrease the neurotransmitter release; in some preparations, as in the present study, this blockade can be complete (Jimenez et al. 1991; Kangrga et al. 1991). Because the blockade of the monosynaptic EPSPs occurs at a low baclofen concentration (≤ 1μM) where there are no major changes in postsynaptic motoneuron properties (Edwards et al. 1989; Lev-Tov et al. 1988), this blockade is likely mediated primarily by decreased presynaptic neurotransmitter release from the primary afferent terminals (Dolphin et al. 1990; Miller 1998; Takahashi et al. 1998). The fact that the mono- and polysynaptic long-lasting reflexes are inhibited by identical doses of baclofen (EC_{50} = 250–260 nM) suggests that baclofen acts with equal efficacy in the mono- and polysynaptic pathways. Thus polysynaptic pathways are likely also inhibited by presynaptic inhibition of the afferent terminals, like the monosynaptic reflex. However, we cannot rule out baclofen’s action on interneurons in the polysynaptic reflex pathway.

Interestingly, the doses of baclofen needed to inhibit the monosynaptic reflex in chronic spinal rats were moderately higher than in acute spinal rats (i.e., the dose-response curve was shifted to the right in chronic spinal rats, EC_{50} was 40% higher). The reason for this decreased sensitivity of chronic spinal rats to baclofen is still uncertain, although there may be less background GABA available and thus less GABAergic inhibition in chronic spinal rats, so that more baclofen would be needed to reach a similar level of inhibition. This idea is consistent with the decreased presynaptic inhibition of the monosynaptic reflex in the lower limbs of patients with upper motoneuron disease (Ashby and McCrea 1987; Iles and Roberts 1986; Nielsen et al. 1995). The decreased GABAergic inhibition may also explain why the monosynaptic reflex increases in amplitude in chronic spinal rats (see Results) and could also contribute to triggering the exaggerated long-lasting reflexes. Indeed, our unpublished data suggest that there are GABAergic interneurons in both the ipsilateral and contralateral sacral spinal cord that provide a strong inhibition of the monosynaptic reflex in normal intact rats because the size of the ipsilateral monosynaptic reflex increases dramatically after removal of the contralateral cord with a sagittal hemisection (publication in preparation). Similar decreases in GABA levels have also been observed in the superficial dorsal horn after chronic peripheral nerve injury (Moore et al. 2002). In addition, fewer GABA receptors after chronic spinal cord injury due to receptor degeneration (Castro-Lopes et al. 1995; Price et al. 1987) or changes in GABA_{A}-coupled second-messenger systems after chronic injury may also explain the decreased efficacy of baclofen after chronic injury.

**Paradoxical effect of baclofen on PICs in acute and chronic spinal motoneurons**

One unexpected finding of the present study is that baclofen increased the amplitude of the PICs in chronic spinal rats (at 20–30 μM). By contrast, in normal motoneurons of acute spinal rats, baclofen decreased the PICs induced by 5-HT, consistent with baclofen’s inhibitory action in normal turtle...
motoneurons and in turtle and rat deep dorsal horn neurons (Russo et al. 1998; Svirskis and Hounsgaard 1998; Voisin and Nagy 2001). In turtle neurons, baclofen hyperpolarizes the membrane and decreases the membrane conductance; however, it has been proposed that baclofen exerts its effect mainly by intracellularly inhibiting the L-type calcium channels, which mediate the activation of PICs and the associated plateau potentials in these neurons (Russo et al. 1998; Svirskis and Hounsgaard 1998).

L-type calcium currents also mediate PICs and plateau potentials in motoneurons of chronic spinal rats. However, this Ca PIC makes up only about half of the initial peak of the total PIC; the other half is mediated by a persistent sodium current, Na PIC (Li and Bennett 2003). By contrast, in turtle motoneurons and deep dorsal horn interneurons, a Na PIC has not been reported to play a major role (Hounsgaard and Kiehn 1989; Russo and Hounsgaard 1996). We have found that baclofen increases PICs in chronic spinal rats by facilitating the Na PIC more than it decreases the Ca PIC. This idea is supported by several facts. First, baclofen decreases the Ca PIC (measured in TTX or inferred from hysteresis), even though it increases the total net PIC (measured without TTX present), which we know is composed of just two currents: Na PIC and Ca PIC.

Second, although baclofen increases the PIC amplitude, the self-sustained firing (indicated by Δf) measured in current-clamp recording is not significantly increased. L-type calcium currents play a major role in producing the self-sustained firing while persistent sodium currents play a smaller role (Li and Bennett 2003; Li et al. 2004a); therefore increased PICs without changes in Δf are more consistent with an increased Na PIC. Third, the spike threshold decreases by ~4.05 mV with high concentration of baclofen (see Results). The persistent sodium current appears to be tightly linked to the spike threshold, with a threshold that is always a few millivolts lower than the spike threshold, and it is important in initiating firing (Lee and Heckman 2001; Li and Bennett 2003). Thus the lowering of the spike threshold may result from a facilitation of the persistent sodium current by baclofen.

In summary, the net effect of baclofen depends on the balance of two opposite effects: a facilitation of the Na PIC and an inhibition of the Ca PIC. A large portion of the PIC is mediated by Na PICs in motoneurons of chronic spinal rats and thus the facilitatory effect of baclofen on the Na PIC dominates. In contrast, in motoneurons of acute spinal rats the PIC induced by 5-HT often has a larger Ca PIC than the Na PIC, and therefore the inhibitory effect of baclofen on the Ca PIC dominates, and the net PIC is reduced by baclofen.

Anti-spasticity effect of baclofen on rats and humans

The dose-response relation for baclofen is very steep. That is, the dose of baclofen to inhibit 50% of the mono- and polysynaptic reflexes (EC50 0.26 μM and 0.25 μM, respectively) is very close to the concentration of baclofen that blocks all the reflexes (mono- and polysynaptic reflexes, 1 μM, ~4 times that of the EC50). This very steep, dose-response curve is consistent with our in vivo studies in chronic spinal rats, where we found it difficult to increase the dose to an effective antispastic dose without producing a total loss of all reflexes and side effects such as loss of bladder control (unpublished results). A similar phenomenon has been observed in humans. In spastic patients, the therapeutic plasma baclofen concentration is ~100–650 ng/ml (i.e., 0.47–3.04 μM) (Aisen et al. 1992; Knuttson et al. 1974), and the maximal safe dose should not exceed 1 μg/ml (4.68 μM). At a concentration of 2 μg/ml, which is also about four times the therapeutic concentration of baclofen, serious toxicity including muscle flaccidity occurs (Flanagan 1998). Finally, with a plasma-to-cerebrospinal fluid concentration ratio of ~8:1 (Knutsson et al. 1974), the therapeutic concentration of orally administered baclofen in the CSF is in the low nanomolar range (0.06–0.38 μM), very close to the effective baclofen concentration in our ACSF.

Various researchers have demonstrated that baclofen relieves spasticity at the spinal cord level and mainly decreases the neurotransmitter release by presynaptic inhibition (Abbruzzese 2002; Davidoff 1985). In our experiments, the low concentrations of baclofen, equivalent to the clinical doses (<1 μM), do not have obvious effects on the postsynaptic motoneuron properties, even though baclofen blocks monosynaptic and long-lasting reflexes at these low doses. Thus baclofen likely blocks spasticity in the chronic spinal rats by the same presynaptic mechanisms proposed in humans. The similarity between the effect of baclofen in chronic spinal rats and spastic patients with chronic spinal cord injury suggests a similarity in the mechanisms of spasticity in the two systems and provides another piece of evidence that chronic sacral spinal rats may be a good model to develop new anti-spasticity drugs (Bennett et al. 1999; Li et al. 2004a). For example, drugs that effectively inhibit the motoneuron PICs involved in spasticity (Li and Bennett 2003), without blocking the presynaptic neurotransmitter release (unlike baclofen), might be more effective anti-spastic agents.

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