5-HT and dopamine modulates CaV1.3 calcium channels involved in postinhibitory rebound in the spinal network for locomotion in lamprey

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Wang D, Grillner S, Wallén P. 5-HT and dopamine modulates CaV1.3 calcium channels involved in postinhibitory rebound in the spinal network for locomotion in lamprey. J Neurophysiol 105: 1212–1224, 2011. First published January 12, 2011; doi:10.1152/jn.00324.2009.—Postinhibitory rebound (PIR) can play a significant role for producing stable rhythmic motor patterns, like locomotion, by contributing to burst initiation following the phase of inhibition, and PIR may also be a target for modulatory systems acting on the network. The current aim was to explore the PIR in one type of interneuron in the lamprey locomotor network and its dependence on low voltage-activated (LVA) calcium channels, as well as its modulation by 5-HT and dopamine. PIR responses in commissural interneurons, mediating reciprocal inhibition and left-right alternation in the network, were significantly larger than in motoneurons. The L-type calcium channel antagonist nimodipine reduced PIR amplitude by ~50%, whereas the L-channel agonist BAY K 8644 enhanced PIR amplitude, suggesting that LVA calcium channels of the L-subtype (CaV1.3) participate in the PIR response. The remainder of the response was blocked by nickel, indicating that T-type (CaV3) LVA calcium channels also contribute. No evidence was obtained for the involvement of a hyperpolarization-activated current. Furthermore, 5-HT, acting via 5-HT1A receptors, reduced PIR, as did dopamine, acting via D2 receptors. Coapplication of nimodipine caused no further PIR reduction, indicating that these modulators target CaV1.3 channels specifically. These results suggest that PIR may play a prominent role in the generation of alternating network activity and that the CaV1.3 and CaV3 subtypes of LVA calcium channels together underlie the PIR response. 5-HT and dopamine both target PIR via CaV1.3 channels, which may contribute significantly to their modulatory influence on locomotor network activity.

central pattern generator; commissural interneuron; L-type calcium channels; low voltage-activated calcium channels; motoneuron

THE LAMPREY NERVOUS SYSTEM has provided important insights into the detailed operation of the spinal network generating locomotion at the cellular, synaptic, and network levels (see Grillner 2003, 2006). The intrinsic membrane properties at potentials near firing threshold of the rhythmically active network neurons are of particular significance. One such property is the postinhibitory rebound (PIR) depolarization that occurs following a period of membrane potential hyperpolarization. PIR has been described in motoneurons in the lamprey spinal cord (Matsushima et al. 1993), and it is a common property of rhythm-generating networks in both vertebrates and invertebrates (Angstadt and Friesen 1993a,b; Angstadt et al. 2005; Satterlie 1985; Serrano et al. 2007; Roberts and Tunstall 1990; Roberts et al. 1995, 2008; Bertrand and Cazalets 1998; Fan et al. 2000). In several cases, the PIR property is targeted by modulatory systems influencing network operation (e.g., Angstadt and Friesen, 1993a; Angstadt et al. 2005; Peck et al. 2001; Merrywest et al. 2003; Matsushima et al. 1993). The PIR can be due to, for instance, a hyperpolarization-activated depolarizing current (Ih; Angstadt et al. 2005; Bertrand and Cazalets 1998; Harris-Warrick et al. 1995), or a low-voltage activated (LVA) calcium current (Fan et al. 2000; Matsushima et al. 1993). In lamprey spinal neurons, LVA calcium channels have been shown to contribute to the PIR (Matsushima et al. 1993; Tegnér et al. 1997; Wang et al. 2008).

Although the presence of PIR in lamprey spinal neurons is long since established (Matsushima et al. 1993; Tegnér et al. 1997), and its significance for the generation of alternating network activity is suggested from modeling studies (Tegnér et al. 1997), a more detailed characterization of the PIR property has been lacking. The present investigation was therefore undertaken to explore PIR in neurons of the spinal locomotor network in lamprey, the iconic bases of the rebound response as well as the modulatory actions exerted on the PIR property. We first compared the PIR response in motoneurons and one type of commissural inhibitory interneuron with a long, descending contralateral axon (Buchanan 1982), providing reciprocal inhibition in the locomotor network, to subsequently analyze the ionic mechanisms (subtypes of ion channels) underlying the PIR. Since the spinal 5-HT and dopamine systems are known to be turned on during locomotion in the lamprey (Grillner 2003; Schotland et al. 1995; Zhang and Grillner 2000), we also explored whether these systems affect the PIR. Dopamine has a complementary action to that of 5-HT and is coreleased from the same plexus (Grillner 2003; Schotland et al. 1995). Parts of these results have been reported in abstract form (Wang et al. 2006, 2008).

MATERIALS AND METHODS

In Vitro Lamprey Spinal Cord Preparation

All experimental procedures were approved by Stockholms Norra Försöksdjurssetiska Nämnd, according to the Swedish regulations for the care and use of laboratory animals.

Adult male and female lampreys (Lampetra fluviatilis or, in a few initial experiments on commissural interneurons, Ichthyomyzon unicuspis) were kept in freshwater aquaria at 4–6°C. When the average PIR responses in commissural interneurons recorded in Ichthyomyzon were compared with those recorded in Lampetra, response amplitudes were in the same range, and thus the experiments on the two species yielded indistinguishable results. In previous studies, no differences in cellular properties of spinal neurons in the two species have been observed. Animals were anesthetized by immersion in a solution of...
tricaine methanesulfonate (MS-222; 100 mg/l; Sigma, St. Louis, MO) and then decapitated caudal to the gills. The in vitro preparation consisted of the spinal cord (8–14 segments long; a total of 113 experiments were performed) either attached to the notochord or in isolation (Fig. 1A; Wallén et al. 1985). The spinal cord was isolated from the notochord and fixed in a Sylgard-lined (Dow Corning, Midland, MI) open perfusion chamber with the ventral side up and the meninges removed. The chamber was maintained at 6–10°C and continuously perfused with HEPES-buffered, oxygenated physiological solution containing (in mM) 138 NaCl, 2.1 KCl, 1.8 CaCl₂, 1.2 MgCl₂, 4 glucose, and 20 NaHCO₃, adjusted to pH 7.4. In the case of *Ichthyomyzon*, the solution had a slightly different composition (in mM): 91 NaCl, 2.1 KCl, 2.6 CaCl₂, 1.8 MgCl₂, 4 glucose, and 20 NaHCO₃, adjusted to pH 7.6 by 95% O₂-5% CO₂.

**Drugs**

Nimodipine [1,4-dihydro-2,6-dimethyl-4-(3-nitrophenyl)-3,5-pyridine dicarboxylic acid 2-methylethyl 1-methylethyl ester; Tocris, Bristol, UK] and BAY K 8644 [1,4-dihydro-2,6-dimethyl-5-nitro-4-(2-trifluoromethyl)phenyl]pyridine-3-carboxylic acid methyl ester; Sigma] were prepared just before application in 10 and 1 µM stock solutions with methanol and then diluted to 10 and 1 µM, respectively, in physiological solution. ZD 7288 (4-ethylphenylamino-1,2-dimethyl-6-methylaminopyrimidinium chloride; Tocris) was prepared as a 5 mM stock solution in water and then diluted to 50 µM immediately before application. Nickel chloride was prepared at a 400 µM concentration in physiological solution. 5-HT and 8-OH-DPAT [(R)-(+)-8-hydroxy-2-(di-n-propylamino)tetralin hydrobromide; Sigma], were mixed with the physiological solution at the reported concentrations just before application. Dopamine and TNPA [(R=)-2,10,11-trihydroxy-N-propynoraporphine hydrobromide hydrate; Sigma] were prepared just before application in 100 mM stock solutions with ethanol and then diluted to 100 µM in physiological solution. None of the drugs used had any consistent influence on the resting membrane potential.

**Neuron Recording and Identification**

Lamprey spinal gray matter neurons (n = 135) were recorded in discontinuous current-clamp (DCC) mode (sampling rate 3–4 kHz) using an Axoclamp 2A amplifier (Axon Instruments, Foster City, CA), and data were stored and analyzed on a personal computer after analog-to-digital conversion under software control (pClamp 8.0; Axon Instruments). The membrane potential of the cell was controlled by current injection in DCC mode. Somata of neurons in the lateral gray matter were impaled with microelectrodes filled with 3 M K-acetate and 0.1 M KCl (resistance 30–50 MΩ). A programmable pulse stimulator unit (Master-8; A.M.P.I., Jerusalem, Israel) was used to set and deliver stimulus protocols.

Motoneurons were identified by injecting single depolarizing current pulses to evoke action potentials, which produced unit spikes...
occurring one for one at constant latency in a nearby ipsilateral ventral root that was sucked into a glass-tip suction electrode (Fig. 1A). Commisural, caudally projecting interneurons [corresponding to CC interneurons as described by Buchanan (1982)] were identified either physiologically, by stimulating cell bodies intracellularly and testing for spikes in the contralateral and caudal end of the spinal cord (distance 10–20 segments from the recorded cell), or anatomically by retrograde labeling. For physiological identification, the caudal end of the spinal cord was cut 2–3 mm along the midline and the halves were drawn into the tips (diameter 350–500 μm) of separate glass suction electrodes for unit recording (Fig. 1A). The mean axonal conduction velocity of recorded commissural interneurons was 1.49 ± 0.44 m/s (mean ± SD; n = 5), corresponding to previously published data for inhibitory commissural interneurons in the lamprey spinal cord (Buchanan 1982). For anatomical identification, commissural interneurons were retrogradely prelabeled using dextran tetramethyl-rhodamine (3,000 MW; Molecular Probes, Eugene, OR). Pieces of spinal cord (n = 26) with the underlying notochord (8–10 segments long) were isolated, and dorsal meninges were removed. A transverse cut was made with the tip of a fine scalpel from the lateral edge to near the midline, and a small amount of the amonic powder of the dye was placed inside the cut with a fine needle tip. The spinal cord was then kept in cold physiological solution during 24 or 48 h to allow retrograde transport of the dye. Experiments were performed in a fluorescence microscope setup (Carl Zeiss, Göttingen, Germany), allowing visualization of prelabeled cells at high resolution. Cells selected for analysis (n = 31) were located at least four segments rostral and contralateral to the cut. After recording, cells were filled with a different fluorescent dye (fluor-4 or calcium green 1; Molecular Probes) from the microelectrode, to allow confirmation of the cell identity.

Stimulus Protocol and Response Analysis

PIR responses were induced by applying rectangular hyperpolarizing current pulses (1.0 nA × 100 ~ 900 ms) at different holding potential levels in DCC mode. During pharmacological experiments, the holding potential was always kept constant at the same level in control recordings and after drug applications. The threshold for action potential generation was determined by depolarizing the membrane potential by current injection until spiking started to occur. Only those neurons whose resting membrane potentials were in the range of −60 to −72 mV and with spike thresholds of −50 mV or lower were selected for analysis. For a quantitative analysis of the PIR response, its amplitude was measured at the maximum peak depolarization, with the membrane potential level before the stimulus pulse taken as the baseline. Thirty sweeps of recorded responses were averaged for each experimental condition and for each cell. PIR amplitudes were plotted as means ± SE, as either absolute or relative values, normalized to the maximal response in the cell. Two-group comparisons were made, using Student’s t-test, between the control group and each test group (e.g., altered test pulse duration or after drug application), as indicated in bar graphs.

RESULTS

The PIR response occurs at membrane potentials near spike threshold and at the termination of a hyperpolarizing event, like the phase of inhibition during rhythmic locomotor activity (Angstadt et al. 2005; Bertrand and Cazalets 1998; Friesen 1994; Matsushima et al. 1993). The PIR is normally characterized by the facts that it will increase in amplitude at more depolarized holding potentials and that it will also increase with the amplitude and duration of a hyperpolarizing test pulse (Bertrand and Cazalets 1998; Matsushima et al. 1993; Tegnér et al. 1997; Angstadt et al. 2005; cf. Surges et al. 2006). Thus the PIR can be expected to increase in size as the membrane potential approaches the threshold for spiking and also to become larger if the preceding hyperpolarizing event will bring the membrane potential to a more negative level and for a longer period of time.

The rebound response was investigated in neurons recorded in the quiescent lamprey spinal cord by injection of hyperpolarizing current pulses. The PIR was either subthreshold or gave rise to an action potential. With a more depolarized holding potential, sometimes not only a single spike but a burst of several spikes could be generated. In the first part of this study, the presence of a PIR response was established and described in one subtype of commissural interneuron, projecting caudally in the contralateral spinal cord and corresponding to the large CC interneurons described by Buchanan (1982). We use the term “commissural” for this interneuron type in our report because of its functional homology to commissural interneurons described in other spinal cord model systems [e.g., frog tadpole (Roberts and Tunstall 1990; Roberts et al. 2008) or mouse (Quinlan and Kiehn 2007)]. The PIR response in commissural interneurons was compared with that in motoneurons by systematically delivering test pulses of varying amplitude and duration and at different holding potentials (cf. Matsushima et al. 1993; Tegnér et al. 1997). In the second part of the study, we investigated the subtypes of ion channels underlying the PIR response. Finally, in the last part of the study, we examined the modulatory actions of 5-HT and dopamine on the PIR.

Postinhibitory Rebound in Commissural Interneurons and Motoneurons

The PIR in commissural interneurons. Figure 1B shows a rebound response in a commissural interneuron, evoked by a hyperpolarizing current pulse (~1.0 nA × 100 ms) from a holding potential of ~61 mV. The potential dependence of the PIR in commissural interneurons is apparent in Fig. 1C and was investigated by applying hyperpolarizing current pulses (~1.0 nA × 100 ms) at different holding potentials in DCC mode (see MATERIALS AND METHODS), set to different levels within 15 mV from the threshold level for spike initiation. In all commissural interneurons tested (n = 5 in this part of the study), the PIR amplitude increased with a more depolarized holding potential (Fig. 1, C and D). The maximal PIR amplitude observed in commissural interneurons was ~4 mV at holding potentials that did not elicit an action potential. At membrane potentials closer to threshold, commissural interneurons would instead spike on the PIR. Figure 1D illustrates the increasing PIR with membrane depolarization and shows that upon a further small depolarizing shift of holding potential (from ~59 to ~58 mV), faithful spiking occurred on the PIR.

The dependence of the PIR on the membrane potential level reached during a hyperpolarizing current pulse was investigated by delivering a series of hyperpolarizing pulses (100 ms) of increasing amplitude (from ~0.2 to ~1.0 nA) at a constant holding potential level (~58 mV in Fig. 1E). The progressive increase in PIR amplitude with more negative membrane potentials during the prepulse is evident.

The dependence of the PIR in commissural interneurons on the duration of the hyperpolarizing current pulse was studied by delivering a series of pulses (~1.0 nA) of increasing...
duration from a set holding potential level (−58 mV in Fig. 1F). The amplitude of the PIR increased markedly with pulse duration (Fig. 1, F and G). Figure 1G shows one example where the amplitude of the PIR increased with a pulse duration from 20 to 80 ms. At 60 ms, occasional action potentials were elicited, and at 80 ms they occurred with each PIR.

The PIR in motoneurons. Figure 2A illustrates a PIR depolarization evoked in a motoneuron by a hyperpolarizing current pulse (−1.0 nA × 100 ms, holding potential −61 mV). We analyzed the motoneurons in the same way as the commissural interneurons in Fig. 1. In 24 of 30 motoneurons, the amplitude of the PIR was <1 mV; in the remaining 6 motoneurons, the PIR amplitude was between 1 and 1.5 mV. The potential dependence of the PIR is apparent (Fig. 2, B and C) and was investigated by applying hyperpolarizing current pulses (−1.0 nA × 100 ms) at different holding potential levels within 15 mV from the threshold level for spike initiation. The PIR amplitude increased gradually with more depolarized holding potentials in all motoneurons (n = 30; Fig. 2B), and spiking could occur at more depolarized levels (Fig. 2C). Faithful spiking with each pulse was followed by multiple spikes on further depolarization. In this case, the 2nd and 3rd spikes were triggered by the PIR following the preceding postspike after-hyperpolarization (see Matsushima et al. 1993; Tegnér et al. 1997). Multiple spiking on the rebound was also observed in commissural interneurons.

Also in motoneurons, the PIR increased, from a set holding potential, with a more negative membrane potential level reached during the hyperpolarizing current pulse (Fig. 2D). Similarly, the PIR increased with the duration of the hyperpolarizing current pulse at a set holding potential level (Fig. 2E). The summary plot in Fig. 2F shows that the mean PIR amplitude increased significantly (P < 0.01–0.001; n = 24) with increasing stimulus pulse duration.

Comparison of the PIR in commissural interneurons and motoneurons. To compare the postinhibitory rebound response in commissural interneurons and motoneurons, we analyzed responses in relation to the membrane potential reached during the hyperpolarizing prepulse and also in relation to its duration (Fig. 3). The dependence of the PIR amplitude on the level of hyperpolarization during the prepulse is illustrated in Fig. 3A1 for one commissural interneuron and one motoneuron. The PIR amplitude increased, from the same holding potential, with more negative potentials in both cell types, but with a more pronounced increase in the commissural interneuron in which the PIR amplitude reached 2 mV. The difference in PIR amplitude, and in the slope of its increase, is within the same range of membrane potential (−60 to −65 mV in Fig. 3A1). In Fig. 3A2, the corresponding plots for all commissural interneurons studied (n = 5) are shown. Figure 3A3 illustrates the PIR amplitude increase with more negative prepulse potentials in six motoneurons with a holding potential and spike threshold similar to those of the commissural interneurons (holding potential range −55 to −60 mV; spike threshold range −54 to −59 mV). It is clear that in commissural interneurons, the amplitude of the PIR following a hyperpolarizing prepulse will increase more markedly with more negative membrane potential levels during the prepulse, and reach higher values, than the...
Ionic Bases for the Postinhibitory Rebound Response

The dependence of the PIR response amplitude on the membrane potential reached during the hyperpolarizing prepulse (A) and the stimulus pulse duration (B) was compared in MNs and CINs. A1: the relationship between PIR amplitude and the membrane potential reached during the hyperpolarizing prepulse (prepulse MP) is plotted for 1 CIN and 1 MN. Note the steeper increase and higher values of the PIR amplitude in the CIN with more negative potential levels. A2: data compiled from 5 CINs. The PIR amplitude increased markedly in all cells with more negative membrane potentials reached during the prepulse. A3: corresponding plot with data compiled from 6 MNs. In all cells the PIR amplitude increased with more negative membrane potentials reached during the prepulse, but less steeply and to lower values than in CINs. B: the PIR amplitude also increased with longer stimulus pulse durations in both CINs and MNs. In CINs the PIR amplitudes reached significantly higher values than in MNs at all tested pulse durations. Data were compiled from 5 CINs and 14 MNs. Statistical comparisons in B were made using Student’s t-test, with data plotted as means ± SE. *P < 0.05; **P < 0.001, significant difference between CINs and MNs. +P < 0.05, significant difference for CINs between the 20- and 100-ms groups.

PIR of motoneurons. This difference is thus not due to differences in the level of hyperpolarization reached during the prepulse in the two types of neurons, since it is manifested within the same potential range.

The effect of altering the duration of the prepulse is summarized in Fig. 3B. The PIR amplitude increased with the duration of the prepulse in both motoneurons and commissural interneurons, again with the PIR reaching significantly larger values in commissural interneurons (P < 0.05 at 20 ms, P < 0.001 at 40–100 ms). Thus, with a longer period of hyperpolarization, and with more negative potential levels reached, the rebound response is augmented much more markedly in commissural interneurons compared with motoneurons.

These results suggest that the PIR property in commissural interneurons of the lamprey locomotor network may play a significant role in the operation of the network, as suggested previously in modeling studies in which postinhibitory rebound in this class of network neurons was found to be important, in particular for the regulation of rhythm frequency (Tegnér et al. 1997).

Ionic Bases for the Postinhibitory Rebound Response

Involvement of LVA calcium channels of the L-type (CaV1.3). LVA calcium channels have been shown to contribute to the generation of the PIR response in lamprey spinal neurons (Matsushima et al. 1993; Tegnér et al. 1997), but which subtype is responsible (e.g., L or T) has not been determined. L-type (CaV1.3) LVA channels in rodents are activated at relatively hyperpolarized membrane potentials (~55 mV in Xu and Lipscombe 2001 and in Lipscombe et al. 2004; Koschak et al. 2001), near the threshold for spiking. This channel subtype may thus possibly contribute to the PIR response. We investigated the possible involvement of L-type LVA channels, which, like high voltage-activated (HVA) calcium channels of the L-type, are sensitive to dihydropyridines, albeit at micromolar concentrations (Koschak et al. 2001; Xu and Lipscombe 2001; Perrier et al. 2002).

Figure 4A shows that the PIR amplitude was decreased by the L-type calcium channel antagonist nimodipine (10 μM). This decremental effect was seen in all 11 cells tested (4 commissural interneurons, 3 motoneurons, and 4 unidentified neurons) and is illustrated in Fig. 4B for 4 of the cells, in which the relative change of the PIR amplitude has been plotted as a function of time after the start of nimodipine application. The degree of recovery varied between cells, with two of them reaching complete recovery. Figure 4C shows that spiking on the rebound response was also compromised after application of nimodipine, with recovery on washout.

The decremental effect of nimodipine on the PIR was also demonstrated in identified commissural interneurons (n = 4; Fig. 4, D and E). As in other neurons, the PIR amplitude in commissural interneurons was decreased by ~50% by nimodipine (cf. Fig. 4F). The time course of the decrease appeared somewhat faster in commissural interneurons (compare B and E in Fig. 4; note the difference in time scales), most likely due to the smaller volume of the preparation chamber used for experiments on commissural interneurons. In one of the interneurons, recovery was obtained on washout of the blocker. Figure 4F illustrates the dose dependence of the nimodipine effect in three spinal neurons (unidentified). Four doses between 5 and 15 μM were applied to each cell, and a steady-state PIR reduction was allowed to be reached between each application. The PIR amplitude at each dose was compared with control values (100%). All four doses of nimodipine significantly decreased the amplitude of postinhibitory rebound depolarizations in all neurons tested and with recovery on washout. No further decrease was observed between 10 and 15 μM.

The action of the L-type calcium channel agonist BAY K 8644 was also investigated. The amplitude of the rebound depolarization was increased by BAY K application (1 μM) in all four cells tested (1 motoneuron and 3 unidentified cells; Fig. 4G), up to more than twice the control PIR amplitude. In Fig. 4H, data from all four cells have been combined and plotted as means ± SE for each minute before and during BAY K application. The mean PIR amplitude was significantly increased (P < 0.05) during the first 3 min of BAY K application, with a tendency to remain augmented during the remainder of the experiment.

These combined results with nimodipine and BAY K 8644 application (Fig. 4) thus suggest that LVA calcium channels of the L-type (CaV1.3) contribute by at least 50% to the postinhibitory rebound response in both motoneurons and commissural interneurons.

Effects of the Ih current blocker ZD 7288 on PIR amplitude. A possible contribution of an Ih current to the rebound response was also investigated. Involvement of an Ih current is as a rule associated with a voltage “sag” of a slowly depolarizing membrane potential level during long hyperpolarizing pulses.
To test for a possible involvement of an $I_h$ current, we applied the commonly used $I_h$ current blocker ZD 7288 (Gasparini and DiFrancesco 1993; Harris and Constanti 1995). The amplitude of the rebound depolarization was nevertheless decreased by ZD 7288 application, as illustrated for a motoneuron in Fig. 5A (500-ms pulse; only the rebound response is shown). This effect was seen in all 11 cells tested (3 commissural interneurons, 3 motoneurons, and 5 unidentified neurons), 4 of which showed recovery on washout (Fig. 5B).

To test whether nimodipine and ZD 7288 blocks distinct components of the PIR response, we applied the two drugs in combination (Fig. 5, C–I). The amplitude of the postinhibitory rebound depolarization was, as described above, reduced by nimodipine, and with the addition of ZD 7288, no further reduction was observed in any of 8 cells tested (4 commissural interneurons and 4 motoneurons; Fig. 5C). Figure 5D shows the time course of the initial nimodipine effect in a motoneuron, followed by ZD 7288, which did not cause a further reduction of the PIR amplitude, followed by a partial recovery on washout. The lack of a further reduction of PIR amplitude with the addition of ZD 7288 is further illustrated in Fig. 5F for four cells, two of which were motoneurons.

The effects of combined applications of nimodipine and ZD 7288 on the PIR of identified commissural interneurons are illustrated in Fig. 5, G–I. After application of nimodipine, the amplitude of the rebound depolarization was, as described above, reduced by nimodipine, and with the addition of ZD 7288, no further reduction was observed in any of 8 cells tested (4 commissural interneurons and 4 motoneurons; Fig. 5C). Figure 5D shows the time course of the initial nimodipine effect in a motoneuron, followed by ZD 7288, which did not cause a further reduction of the PIR amplitude, followed by a partial recovery on washout. The lack of a further reduction of PIR amplitude with the addition of ZD 7288 is further illustrated in Fig. 5F for four cells, two of which were motoneurons.

The effects of combined applications of nimodipine and ZD 7288 on the PIR of identified commissural interneurons are illustrated in Fig. 5, G–I. After application of nimodipine,
Fig. 5. Tests for other components of the PIR response in addition to the L-type (CaV1.3) calcium channel component. A: the hyperpolarization-activated depolarizing current (Ih) blocker ZD 7288 (50 μM) reduced the PIR response in 1 MN (induced by a −0.5-nA × 700-ms hyperpolarizing pulse; only the response following the pulse is shown). Holding potential was kept constant at −55 mV. B: time course of PIR amplitude reduction after application of ZD 7288. The PIR response was reduced in all 4 neurons (2 MNs), with recovery on washout. Open circles indicate onset of washout in each experiment. C: bath application of nimodipine (10 μM) reduced PIR amplitude in 1 MN (cf. Fig. 4A), and after ZD 7288 was added, no further reduction was seen (30 sweeps were averaged and filtered). Holding potential was kept constant at −65 mV. D: time course of nimodipine and ZD 7288 effects on PIR amplitude in a MN. Nimodipine application resulted in a gradual reduction of the PIR amplitude from −0.7 mV to 0.2 mV. Addition of ZD 7288 in the presence of nimodipine caused no further reduction. Partial recovery was seen on washout. Each point is the mean of 30 stimuli. E: effect of nickel application in combination with nimodipine and ZD 7288. Plot shows the time course of the PIR amplitude change after reduction to −50% following nimodipine application. PIR amplitude was maintained at the same level after addition of ZD 7288 (cf. D) but decreased to almost 0% after the addition of nickel (100 μM). F: summary plot of the combined effects of nimodipine, ZD 7288, and nickel. Nimodipine reduced PIR amplitude to −50% in all 4 neurons (2 MNs), with no further reduction after the addition of ZD 7288, whereas the addition of nickel further decreased PIR amplitude. Complete or partial recovery was seen on washout. Each bar value is the mean of the last 5 time-point recordings during each drug combination (30 stimuli at each time point were averaged). Statistical comparisons were made using Student’s t-test, with data plotted as means ± SD. *P < 0.05; **P < 0.01; ***P < 0.001 vs. nearest data group to left. G: time course of nimodipine and ZD 7288 effects on PIR amplitude in a CIN. Nimodipine application resulted in a gradual reduction of the PIR amplitude from −2 mV to 0.8 mV. Addition of ZD 7288 in the presence of nimodipine caused no further reduction. Partial recovery was seen on washout. Each point is the mean of 30 stimuli. H: reversed order of drug application. An initial application of ZD 7288 (50 μM) reduced PIR amplitude in a CIN, and after nimodipine was added, no further reduction was obtained. Holding potential was kept constant at −61 mV. I: time course of nimodipine and ZD 7288 effects on PIR amplitude in two CINs with reversed order of drug applications. An initial application of ZD 7288 (CIN1) or nimodipine (CIN2) reduced PIR amplitude by −50% in both interneurons, and with a second application of nimodipine and ZD 7288, respectively, no further reduction of PIR amplitude was seen. Addition of nickel (100 μM) practically abolished the PIR in both interneurons. Partial recovery was obtained in 1 of them (CIN2).

causing the previously described reduction in PIR amplitude, an addition of ZD 7288 did not cause any further reduction in the commissural interneuron illustrated in Fig. 5G. To further corroborate this finding, the order of application of the two drugs was reversed in three additional interneurons. ZD 7288 first caused a reduction of the PIR amplitude, and when followed by nimodipine, no further decrease was observed (Fig. 5H). Figure 5I illustrates the time course of the effects in two commissural interneurons, where ZD 7288 application was followed by nimodipine in one of them (CIN1) and the order of application was reversed for the other (CIN2). In neither case did the addition of the second drug cause any further reduction of PIR amplitude. These results show that nimodipine and ZD 7288 block the same component of the PIR response and that ZD 7288 may have an antagonistic effect on L-type (CaV1.3) LVA calcium channels in both commissural interneurons and motoneurons. There is thus no evidence for a contribution of an Ih current to the PIR in lamprey spinal neurons.

Contribution of T-type calcium channels to the PIR. Nimodipine and ZD 7288 reduced the PIR amplitude by ∼50%, separately or in combination (Fig. 5, E, F, I). To investigate the possibility that the remainder of the rebound response could be due to the activation of LVA calcium channels of the T-type (CaV3), we applied the commonly used T-channel blocker...
nickel (Lee et al. 1999; Molineux et al. 2006; Obejero-Paz et al. 2008) after the spinal cord had been superfused with nimodipine and ZD 7288. After addition of nickel (400 μM, a concentration sufficient to cause substantial block of different subtypes of T channels; cf. Lee et al. 1999), the PIR decreased further, to be markedly reduced or even abolished (Fig. 5, E, F, I). This effect was seen in all six neurons tested, including two commissural interneurons and two motoneurons, with partial or full recovery on washout (Fig. 5, E, F, I). These results thus indicate that in addition to the involvement of L-type (CaV1.3) LVA calcium channels, T-type (CaV3) LVA calcium channels may also contribute to the PIR in lamprey spinal commissural interneurons and motoneurons.

5-HT and Dopamine Modulation of Postinhibitory Rebound

Since both the 5-HT and dopamine systems are turned on when the locomotor network is active and are known to modulate HVA calcium channels of the N- and L-types, it is pertinent to also ask whether the postinhibitory rebound is modulated, and if so, by targeting L-type (CaV1.3) LVA calcium channels. 5-HT modulation of the PIR. The amplitude of the rebound depolarization was decreased by 5-HT application (2.5 μM; Fig. 6A), with complete or partial recovery on washout. This effect was seen in all nine cells tested (4 commissural interneurons, 3 motoneurons and 2 unidentified cells). Figure 6B illustrates the relative change of the PIR amplitude as a function of time after the start of 5-HT application. Figure 6, C and D, illustrates the corresponding decremental effect of 5-HT on the PIR amplitude in identified commissural interneurons. Partial recovery on washout could be obtained in one of the four interneurons (Fig. 6D, CIN 2).

Rebound spiking was also reduced by 5-HT (n = 10; 6 motoneurons and 4 unidentified neurons; kept at similar holding potentials). In Fig. 6E, the PIR spike in a motoneuron was effectively eliminated by 5-HT, with spiking reappearing on washout.

5-HT inhibits N-type calcium channels via 5-HT1A receptors in lamprey spinal neurons (Hill et al. 2003). To investigate whether the effects of 5-HT on the PIR are also mediated via 5-HT1A receptors, we applied the specific 5-HT1A agonist 8-OH-DPAT (1 μM). As with 5-HT, the amplitude of the rebound depolarization was decreased by 8-OH-DPAT application in both commissural interneurons and motoneurons, and recovered on washout (Fig. 7, A and C). This effect was seen in all nine neurons tested (4 commissural interneurons, 1 motoneuron, and 4 unidentified cells), as illustrated in Fig. 7, B and D. 8-OH-DPAT also reduced rebound spiking, as shown in two additional neurons. After a hyperpolarizing current pulse in a motoneuron, PIR spikes were evoked with each stimulus (Fig. 7E; 30 sweeps superimposed). After 8-OH-DPAT application, rebound spiking was gradually compromised and recovered on washout (Fig. 7E). These findings thus show that 5-HT exerts a modulatory influence on the postinhibitory rebound, presumably through 5-HT1A receptors, in lamprey spinal neurons, including identified commissural interneurons and motoneurons.

Dopamine modulation of the PIR. As with 5-HT, application of dopamine (50 μM) reduced the amplitude of the PIR in both commissural interneurons and motoneurons (Fig. 8, A and B). This effect was seen in all nine cells tested (3 commissural interneurons, 1 motoneuron, and 5 unidentified cells) as illustrated in Fig. 8, A–D. The relative amplitude of the PIR was reduced in all neurons and also recovered, partially or com-

Fig. 6. 5-HT modulation of the PIR response. A: the PIR amplitude was markedly reduced after bath application of 5-HT (2.5 μM) in a MN and partially recovered on washout (30 sweeps were averaged and filtered). Holding potential was kept constant at −58 mV. B: 5-HT application resulted in a reduction of PIR amplitude in all 5 neurons tested (3 MNs). The relative PIR amplitude in each cell was plotted vs. time after 5-HT application. Each point is the mean of 30 stimuli. After washout, partial or nearly complete recovery was seen in all 5 cells. Open circles indicate onset of washout in B and D. C: the PIR amplitude was markedly reduced after bath application of 5-HT (2.5 μM) in a CIN and partially recovered on washout. Holding potential was kept constant at −58 mV. D: 5-HT application resulted in a reduction of PIR amplitude in all 4 CINs investigated. The relative PIR amplitude in each cell was plotted vs. time after 5-HT application. Each point is the mean of 30 stimuli. Partial recovery could be obtained in 1 of the interneurons (CIN2). E: in this MN (spike threshold −51 mV), 5-HT application (5 μM, 15 min) eliminated rebound spiking, which recovered on washout at the same holding potential (−53 mV). Bottoms of traces were truncated.
Fig. 7. 5-HT<sub>1A</sub> receptors are involved in the 5-HT depression of the rebound response. A: after application of the 5-HT<sub>1A</sub> receptor agonist 8-OH-DPAT (1 µM), the PIR amplitude was markedly reduced in a MN. Recovery was seen on washout (30 sweeps were averaged and filtered). Holding potential was kept constant at −65 mV. B: 8-OH-DPAT application resulted in reduction of the PIR amplitude in all 5 neurons tested (1 MN). The relative PIR amplitude in each cell was plotted vs. time after 8-OH-DPAT application (control PIR was set to 100%). Each point is the mean of 30 stimuli. After washout, partial or complete recovery was seen in all 5 cells. In B and D, open circles indicate onset of washout. C: application of 8-OH-DPAT (1 µM) also reduced the PIR amplitude in a CIN. Recovery was seen on washout (30 sweeps were averaged and filtered). Holding potential was kept constant at −66 mV. D: 8-OH-DPAT application resulted in reduction of the PIR amplitude in all 4 CINs tested. The relative PIR amplitude in each interneuron was plotted vs. time after 8-OH-DPAT application (control PIR was set to 100%). Each point is the mean of 30 stimuli. After washout, partial or complete recovery was seen in all 4 interneurons. E: modulation of rebound spiking by 8-OH-DPAT. In this MN (spike threshold −60 mV, holding potential −62 mV), hyperpolarizing current pulses (−1.0 nA × 100 ms) produced rebound spiking at a short delay with each stimulus (30 sweeps superimposed). After 8-OH-DPAT application, rebound spiking was gradually compromised, to recover on washout.

The target for the dopamine modulation of the PIR was examined in an analogous fashion. Application of nimodipine caused a marked reduction in PIR amplitude, and after addition of DA, no further reduction was observed (Fig. 9, D–F). The same effect was seen in all three neurons tested, one of which was a motoneuron (Fig. 9F); a clear recovery on washout was obtained in two of the cells. The time course of the change in PIR amplitude is illustrated for the motoneuron in Fig. 9E. Thus, as for 5-HT, Ca<sub>V1.3</sub> LVA calcium channels may be the target for the dopamine modulation of the PIR response in lamprey spinal neurons.

**DISCUSSION**

In the present study we explored the existence of postinhibitory rebound in one type of commissural interneuron, with a long, descending contralateral axon, providing reciprocal inhibition in the spinal locomotor network in lamprey, and made a comparison to PIR responses in motoneurons. We also investigated the nature of the LVA calcium channels involved in the PIR in lamprey spinal neurons, as well as the putative modulatory action on the PIR exerted by two major modulatory systems in the lamprey spinal cord, the 5-HT and dopamine systems.

**PIR in Commisural Interneurons of the Lamprey Locomotor Network**

In a cyclic network that involves alternating excitation and inhibition, it is likely that a PIR would contribute to burst initiation following the phase of inhibition, and thereby to a well-coordinated burst pattern (Angstadt and Friesen 1993a,b; Angstadt et al. 2005; Satterlie 1985; Serrano et al. 2007; Roberts and Tunstall 1990; Roberts et al. 1995, 2008; Matsu...
The ionic mechanisms underlying the PIR have previously been shown to involve an activation of LVA calcium channels (Matsushima et al. 1993; Tegnér et al. 1997). LVA channels inactivate at depolarized membrane potential levels (Carbone and Lux 1984), and during rhythmic network activity, this inactivation will be reduced, or even removed, during a period of hyperpolarization. The subsequent activation of the LVA current at the transition to the depolarized phase will then induce a rebound depolarization, shifting the membrane potential toward the threshold for the action potential.

The subtype(s) of LVA calcium channels responsible for the PIR depolarization in lamprey neurons had previously not been determined. We therefore investigated the possible contribution of L-type calcium channels, of which the form containing the \( \alpha_{L} \)-subunit (\( \alpha_{L} \)) has been shown to activate at relatively hyperpolarized membrane potentials, whereas the \( \alpha_{T} \)-subunit, activates at potentials about 25 mV less negative (Xu and Lipscombe 2001; cf. Koschak et al. 2001). The \( \alpha_{L} \) subunit has been reported to activate at about −55 mV with physiological concentrations of calcium (2 mM; Lipscombe et al. 2004). With higher concentrations of calcium or barium, more depolarized activation levels have been reported (e.g., −46 mV with 15 mM barium, Koschak et al. 2001; cf. Lipscombe et al. 2004). In lamprey spinal gray matter neurons during voltage clamp, an LVA calcium current was observed to activate at −55 mV (Matsushima et al. 1993; Tegnér et al. 1997), and a corresponding current was observed and reported to activate at −60 mV in spinal sensory dorsal cells (Christenson et al. 1993; see also Wang et al. 2009). L-type LVA channels (\( \alpha_{L} \)) have also been implicated in the generation of plateau potentials in turtle spinal motoneurons (Perrier et al. 2002; Perrier and Hounsaard 2003). Together, these findings suggest that L-type calcium channels of the LVA type (\( \alpha_{L} \)) are not only present in lamprey spinal neurons but also that this channel

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**Ionic Bases for the Postinhibitory Rebound Response**

The ionic mechanisms underlying the PIR have previously been shown to involve an activation of LVA calcium channels (Matsushima et al. 1993; Tegnér et al. 1997). LVA channels inactivate at depolarized membrane potential levels (Carbone and Lux 1984), and during rhythmic network activity, this inactivation will be reduced, or even removed, during a period of hyperpolarization. The subsequent activation of the LVA current at the transition to the depolarized phase will then induce a rebound depolarization, shifting the membrane potential toward the threshold for the action potential.
subtype has an activation voltage range that will make it contribute to the PIR response.

L-type calcium channels are blocked by dihydropyridines, with the CaV1.3 form requiring higher concentrations, in the micromolar range, than the CaV1.2 form (Xu and Lipscombe 2001; Koschak et al. 2001). In the present study, nimodipine (at 10 μM) markedly reduced the PIR depolarization, and in addition, the L-type channel agonist BAY K 8644 enhanced the PIR. These results thus suggest that LVA calcium channels of the L-type (CaV1.3) contribute to the postinhibitory rebound response in lamprey spinal neurons. Note that although nimodipine has been reported to also block potassium channels (Fagni et al. 1994; Sodickson and Bean 1996; Zhang and Gold 2009), such a spurious effect of nimodipine is unlikely to occur in the present experiments and affect the PIR. In the studies of the blocking effect of dihydropyridines on potassium channels (Fagni et al. 1994; Zhang and Gold 2009), BAY K 8644 had a blocking action similar to that of nimodipine, whereas this compound had an incremental effect on the PIR in the present experiments (Fig. 4, G and H), as expected for an LVA calcium channel agonist. Furthermore, if a potassium channel block by nimodipine were to occur, this would be expected to counteract the blocking effect on LVA calcium channels in the case of the PIR response (cf. Zhang and Gold 2009), and thus the combined application of nimodipine and nickel would not be expected to be able to abolish the PIR, which was indeed observed (Fig. 5, E, F, I).

In the presence of nimodipine, nickel further reduced, or even abolished, the remainder of the PIR response. Although nickel may also affect HVA calcium channels (Zamponi et al. 1996; cf. Lee et al. 1999), it seems highly likely that under the present conditions the effect of nickel on the subthreshold PIR is due to a block of T-type (CaV3) calcium channels (Molineux et al. 2006; Obejero-Paz et al. 2008). Thus the L-type (CaV1.3) and T-type (CaV3) of LVA calcium channels could together account for the PIR response in commissural interneurons and motoneurons in the lamprey spinal cord.

The possibility that an I\textsubscript{h} current could also be involved was tested by applying the commonly used I\textsubscript{h} blocker ZD 7288 (Gasparini and DiFrancesco 1997; Harris and Constanti 1995), which, however, did not influence the PIR response in the presence of nimodipine. When applied alone, ZD 7288 did reduce the PIR response, thus suggesting the possibility that this compound may act on L-type calcium channels in lamprey spinal neurons. Also, in other systems, ZD 7288 has been shown to affect subtypes of calcium channels (Felix et al. 2003; Sanchez-Alonso et al. 2008). These findings, together with the fact that no evidence for a voltage sag of the I\textsubscript{h} type was found even with longer hyperpolarizing pulses (Bertrand and Cazalets 1998; Serrano et al. 2007), therefore suggest that there is no significant contribution from an I\textsubscript{h} current to the PIR response in lamprey spinal commissural interneurons and motoneurons. The depolarizing slope that was detected in a few cases in the present study (Fig. 4) was also seen in previous studies (Matsushima et al. 1993; Tegnér et al. 1997); however, this slope only occurs at very depolarized holding potentials close to threshold and could be accounted for by the development of an LVA calcium current during the prepulse. The existence of such a depolarizing slope thus does not indicate the involvement of an I\textsubscript{h} current in the generation of the rebound response in lamprey spinal neurons (cf. Tegnér et al.
the modeling studies discussed above. Deinactivation of LVA
findings of a modulatory depression of the PIR by 5-HT and
conclusion is corroborated by our distinct alternation between hemisegments in the lamprey lo-
interneurons, a prominent postinhibitory rebound may occur in both commissural interneu-
5-HT and dopamine both give rise to a slowing of the locomotor rhythm (Harris-Warrick and Cohen 1985; Schotland et al. 1995). These effects have been ascribed to the previously established modulation of N-type calcium channels (CaV2.2; Hill et al. 2003; Wikström et al. 1999), leading to a reduced postinhibitory rebound and its modulation by serotonin in excitatory swim motor neurons of the medicinal leech. J Comp Physiol A Neuroethol Cell 204.

postinhibitory rebound response, presumably in combination with T-type (CaV3) channels and without an involvement of an I_h current. The 5-HT- and dopamine-induced modulation of the postinhibitory rebound is due to a depressing action possibly exerted mainly on CaV1.3 calcium channels.

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An I_h current has, on the other hand, been demonstrated in neurons of the lamprey striatum (Ericsson et al. 2010).

It thus appears that ZD 7288 blocks the L-type calcium channel-dependent portion of the PIR. It therefore seems per-
tinent to caution for the usage of ZD 7288 when testing for a contribution by an I_h current to postinhibitory rebound. It has been common practice to assign a role for I_h in PIR on the basis of blocking experiments using ZD 7288 (Gasperini and Di-Francesco 1997; Sekirnjak and du Lac 2002; cf. Surges et al. 2006).

5-HT and Dopamine Modulation of Postinhibitory Rebound

Serotonergic and dopaminergic modulatory effects in the lamprey spinal cord are due to release from a ventromedial plexus of small neurons and fibers along the spinal cord, and it has previously been established that 5-HT and dopamine are coreleased from this plexus (Schotland et al. 1995). Network interneurons and motoneurons extend their medial dendritic processes into the plexus, and both 5-HT and dopamine influence the rhythmic activity of the locomotor network (Schotland et al. 1995; Wallén et al. 1989). Our present findings that these two modulatory systems also affect the PIR (as is the case for the GABAergic system; Matsushima et al. 1993; Tegnér et al. 1993) thus add to the diversity of membrane properties that are targeted by the different modulatory systems. The present results further suggest that both 5-HT and dopamine may exert their action on L-type CaV1.3 calcium channels. Furthermore, the LVA calcium current was depressed by 5-HT_1A and dopamine D_2 agonists, respectively, in both commissural interneurons and motoneurons.

5-HT and dopamine both give rise to a slowing of the locomotor rhythm (Harris-Warrick and Cohen 1985; Schotland et al. 1995). These effects have been ascribed to the previously established modulation of N-type calcium channels (CaV2.2; Hill et al. 2003; Wikström et al. 1999), leading to a reduced slow afterhyperpolarization (Schotland et al. 1995; Wallén et al. 1989). Presynaptic effects have also been suggested to contribute to the action on the locomotor rhythm (Schwartz et al. 2005; Svensson et al. 2003). The influence demonstrated in the present study on the PIR in commissural interneurons would also act toward a slowing of the rhythm, since a reduced PIR would give a slower and less efficient switch of alternation between inhibition and excitation (Tegnér et al. 1997). 5-HT and dopamine thus modulate several different molecular targets, all of which act in a synergistic fashion on the network level.

In conclusion, the present results show that in commissural interneurons, a prominent postinhibitory rebound may occur after a period of inhibition. Although not readily testable by a specific blockade during fictive locomotion, the PIR in commissural interneurons may be of particular importance for a distinct alternation between hemisegments in the lamprey locomotor network. This conclusion is corroborated by our findings of a modulatory depression of the PIR by 5-HT and dopamine, both of which reduce the burst frequency, and by the modeling studies discussed above. Deinactivation of LVA calcium channels of the L-type (CaV1.3) contributes to the postinhibitory rebound response, presumably in combination with T-type (CaV3) channels and without an involvement of an I_h current. The 5-HT- and dopamine-induced modulation of the postinhibitory rebound is due to a depressing action possibly exerted mainly on CaV1.3 calcium channels.

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