Available online at www.sciencedirect.com
ScienceDirect
Neuropharmacology 51 (2006) 1038–1046
NEURO PHARMACOLOGY
www.elsevier.com/locate/neuropharm

Effects of flufenamic acid on fictive locomotion, plateau potentials, calcium channels and NMDA receptors in the lamprey spinal cord

Di Wang, Sten Grillner, Peter Wallén*

Department of Neuroscience, Nobel Institute for Neurophysiology, Karolinska Institute, SE-171 77 Stockholm, Sweden

Received 2 April 2006; received in revised form 22 June 2006; accepted 23 June 2006

Abstract

A Ca^{2+}-activated, non-selective cation current (I_{CAN}) has been suggested to contribute to plateau potentials in lamprey reticulospinal neurons, providing the drive for locomotor initiation. Flufenamic acid (FFA) is commonly used as a blocker of I_{CAN}. To explore the effects of FFA on spinal locomotor pattern generation, we induced fictive locomotion in the isolated lamprey spinal cord. Bath-applied FFA (100–200 μM) caused a marked reduction of amplitude and regularity of the locomotor burst activity. We next analyzed the NMDA-induced membrane potential oscillations in single spinal neurons. The duration of depolarizing plateaus was markedly reduced when applying FFA, suggesting an involvement of I_{CAN}. However, in experiments with intracellular injection of the Ca^{2+} chelator BAPTA, and in the presence of the K_{Ca}-channel blocker apamin, no support was found for an involvement of I_{CAN}. We therefore explored alternative explanations of the effects of FFA. FFA reduced the size of the slow, Ca^{2+}-dependent afterhyperpolarization, suggesting an influence on calcium channels. FFA also reduced the NMDA component of reticulospinal EPSPs as well as NMDA-induced depolarizing responses, demonstrating an influence on NMDA receptors. These non-selective effects of FFA can account for its influence on fictive locomotion and on membrane potential oscillations and thus, a specific involvement of the I_{CAN} current in the lamprey spinal cord is not supported.

Keywords: I_{CAN}; Network; Central pattern generator; Membrane potential oscillations; NMDA

1. Introduction

Calcium-activated, non-selective (CAN) cation currents (I_{CAN}) have been shown to generate long-lasting depolarizing plateaus in several classes of neurons (Partridge et al., 1994; Zhang et al., 1995; Fraser and MacVicar, 1996; Klink and Alonso, 1997; Morisset and Nagy, 1999; cf. Wilson et al., 1996; Congar et al., 1997). The non-steroid anti-inflammatory drug flufenamic acid (FFA) has been commonly used as a blocker of I_{CAN} in different systems (Morisset and Nagy, 1999; Pena et al., 2004; cf. Lee et al., 1996).

In the lamprey brainstem, CAN channels have been suggested to contribute to plateau potentials in reticulospinal neurons, providing the drive for locomotor initiation (Viana Di Prisco et al., 2000). It is not known, however, if CAN channels also contribute to the NMDA-induced membrane potential oscillations in neurons of the lamprey spinal cord, nor is it known if these channels contribute during locomotor pattern generation in the spinal network.

The lamprey central nervous system is a well-studied model for the cellular and network bases of locomotion (Grillner et al., 2001; Grillner, 2003). Locomotor-like activity can be induced in the isolated lamprey spinal cord by application of excitatory amino acids such as *N*-methyl-D-aspartate (NMDA) (Grillner et al., 1981). The operation of the spinal neuronal network underlying locomotion has been studied in considerable detail (Grillner et al., 2001). Spinal neurons may exhibit NMDA-induced membrane potential oscillations that persist after the blockade of Na^+ currents with

* Corresponding author. Tel.: +46 8 524 86913; fax: +46 8 349544.
E-mail address: peter.wallen@ki.se (P. Wallén).

0028-3908/$ - see front matter © 2006 Elsevier Ltd. All rights reserved.
doi:10.1016/j.neuropharm.2006.06.012

tetrodotoxin (TTX). These pacemaker-like oscillations are present in neurons of the locomotor network and are believed to be important for the maintenance of a slow and stable locomotor activity (Brodin and Grillner, 1986; Wallén and Grillner, 1987). The TTX-resistant oscillations induced by NMDA normally have an amplitude of 15–40 mV and exhibit several distinct phases: a rapid depolarization when NMDA receptor channels open and Na^+ and Ca^{2+} ions flow into the cell, a maintained depolarized phase, and a rapid repolarization mediated by the activation of Ca^{2+}-dependent K^+ channels (K_{Ca}) and closure of NMDA channels (Wallén and Grillner, 1987). When intracellular Ca^{2+} levels have decreased and fewer K_{Ca} channels are being activated, the membrane potential will again start to depolarize and when the level is reached at which NMDA channels open, the next rapid depolarization phase will start and a new oscillatory cycle will follow.

In this study, we investigate the effects of FFA on the spinal locomotor pattern generation and plateau potentials, calcium channels and NMDA receptors. On the network level, FFA reduced the amplitude and regularity of the locomotor burst activity, and on the single cell level FFA shortened NMDA-induced plateau depolarizations, suggesting an involvement of I_{CAN}. However, in a separate set of experiments, not utilizing FFA, intracellular injection of the Ca^{2+} chelator BAPTA in the presence of the K_{Ca}-channel blocker apamin did not result in any shortening of the depolarization plateau, giving no support for an involvement of I_{CAN} in lamprey spinal cord neurons. Instead, FFA has here been shown to have non-specific effects on glutamatergic synaptic transmission, particularly on the NMDA receptor-mediated component and on the electrotonic, gap junction-mediated component, and on Ca^{2+} channels. These non-selective effects of FFA can account for its influence on fictive locomotion and on membrane potential oscillations. Some of these results have been presented in preliminary form (Wang et al., 2001).

2. Methods

Adult male and female lampreys (*Lampetra fluviatilis* or *Ichthyomyzon unicuspis*) were kept in fresh-water aquaria at 4–6 °C. Results obtained did not differ between the two species. All experimental procedures were approved according to the Swedish regulations for the care and use of laboratory animals. Animals were anaesthetized by immersion in a solution of tricaine methane sulfonate (MS-222, 100 mg/l, Sigma) and then decapitated caudal to the gills. The in vitro preparation consisted of the spinal cord (8–14 segments long) either attached to the notochord or in isolation (Fig. 1; Wallén et al., 1985). For intracellular recordings, the spinal cord was isolated from the notochord and fixed in a Sylgard-lined (Dow-Corning Corp., USA) open perfusion chamber with the ventral side up, and the meninges removed. The chamber was maintained at 6–10 °C and was continuously perfused with physiological solution containing (in mM): 138 NaCl, 2.1 KCl, 1.8 $CaCl_2$, 1.2 $MgCl_2$, 4 glucose, 2 HEPES, and 0.5 L-glutamine, bubbled with O_2 and adjusted to pH 7.4 with NaOH. In the case of *Ichthyomyzon,* the solution had a slightly different composition (in mM): 91 NaCl, 2.1 KCl, 2.6 $CaCl_2$, 1.8 $MgCl_2$, 4 glucose, and 20 $NaHCO_3$ and was buffered to pH 7.6 by bubbling with 95%O2–5%CO_2. Motoneurons and interneurons were recorded in discontinuous current clamp (DCC) mode or bridge mode using an Axoclamp-2A amplifier (Axon Instruments Inc., USA), and data was stored and analyzed on a PC-computer after A/D conversion under software control

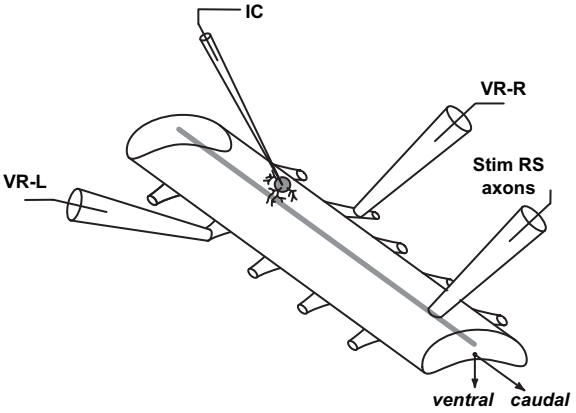

Fig. 1. Schematic drawing (not to scale) of the lamprey spinal cord in the recording configuration used. Suction electrodes were placed on two ventral roots (VR) on the left (L) or right (R) side of the spinal cord segment, and used to record the burst activity; one suction electrode was located on the surface of the spinal cord for extracellular stimulation of reticulospinal axons (RS), while postsynaptic responses were recorded in individual neurons. A microelectrode was introduced in the neuron for intracellular (IC) recording in current clamp mode.

(PClamp 8.0, Axon Instruments Inc., USA). The membrane potential of the cell was kept constant by current injection in DCC mode. Somata of neurons in the lateral gray matter were impaled with microelectrodes filled with 3 M KAc and 0.1 M KCl (resistance 40–70 MΩ). Neurons were stimulated to produce action potentials by intracellular current injection, using a variety of stimulus paradigms like single and trains of brief (2 ms) depolarizing pulses. A programmable pulse stimulator unit (Master-8, A.M.P.I., Israel) was used to set and deliver stimulus protocols. Fictive locomotor burst activity was recorded extracellularly from the cut ventral roots, which were sucked into glass tip suction electrodes (Fig. 1).

For pharmacological analysis, the normal physiological solution was replaced with solutions containing different drugs and/or different concentrations of ions.

To induce fictive locomotor activity as well as TTX-resistant membrane potential oscillations, *N*-methyl-D-aspartate (NMDA, Tocris) was added to a final concentration of 100–150 μM (Grillner et al., 1981; Wallén and Grillner, 1987). To block action potentials, 1.5 μM TTX (Sigma) was added to the perfusion medium.

Reticulospinal EPSPs were evoked by extracellular stimulation of reticulospinal axons using a glass tip suction electrode placed on the spinal cord surface. These EPSPs have chemical and electrical components (Brodin et al., 1988). The electrotonic synaptic component was isolated by perfusing with 200 μM cadmium chloride (Sigma) a broad range Ca^{2+} channel blocker. Apamin (Sigma) was dissolved in phosphate-buffered saline (PBS) and frozen in stock vials. Just before use, it was thawed and mixed with physiological solution to the desired final concentration (2.5 μM; Cangiano et al., 2002). The fast calcium chelator 1,2-bis-(2-aminophenoxy)-ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid (BAPTA; tetrapotassium salt, Sigma) was dissolved in 3 M KCl to a final concentration of 200 mM, and intracellularly injected from the recording microelectrode. Tetraethyl ammonium (TEA; 5 mM, Sigma) was added in the perfusion solution to achieve a prominent calcium component of the action potential. 2,3-dihydro-6-nitro-7-sulfamoyl-benzo(f)quinoxaline (NBQX 1 μM, Sigma) was added to the perfusion solution to isolate the NMDA component of the EPSP. NMDA-receptor mediated depolarizing responses were evoked by direct activation using local application of NMDA from a pressure pipette (1 mM) in the presence of TTX (1.5 μM). The effects of flufenamic acid (FFA; 100–200 μM, Sigma) were investigated by bath application during extra- and/or intracellular recording.

For quantitative analysis of drug effects on the cycle structure of NMDA-induced, TTX-resistant oscillations (Figs. 3–5), values of the normalized half width (see Results) were measured over 20–30 cycles and plotted as means ± S.D. Student's *t*-test was used for statistical comparisons.

3. Results

3.1. Effects of flufenamic acid on the spinal locomotor network

In the spinal cord, fictive locomotion can be elicited by application of excitatory amino acids such as NMDA, and the motor output can be recorded from the ventral roots (Grillner et al., 1981). To explore whether FFA, as an I_{CAN} antagonist, would influence the lamprey spinal locomotor network, we first tested its effect on fictive locomotion (Fig. 2). FFA was added to the solution during ongoing fictive locomotion induced by bath application of NMDA (100 μM). Under control conditions, regular alternating burst activity appeared in the ventral roots with a frequency of approximately 3 Hz (Fig. 2A). The rectified and filtered traces of the neurograms (below each raw trace in Fig. 2) illustrate further the degree of regularity in burst amplitude and burst rate. When the spinal cord was perfused with 100 μM FFA in addition to NMDA, the amplitude of the locomotor burst activity was reduced, and the bursting became more irregular, which is particularly evident from the filtered traces (Fig. 2B, $n = 7$). There was no consistent change of burst rate; in three cases it was increased, while in four others it was instead decreased. The effect of FFA was persistent and recovery upon wash-out was only partially obtained in some cases.

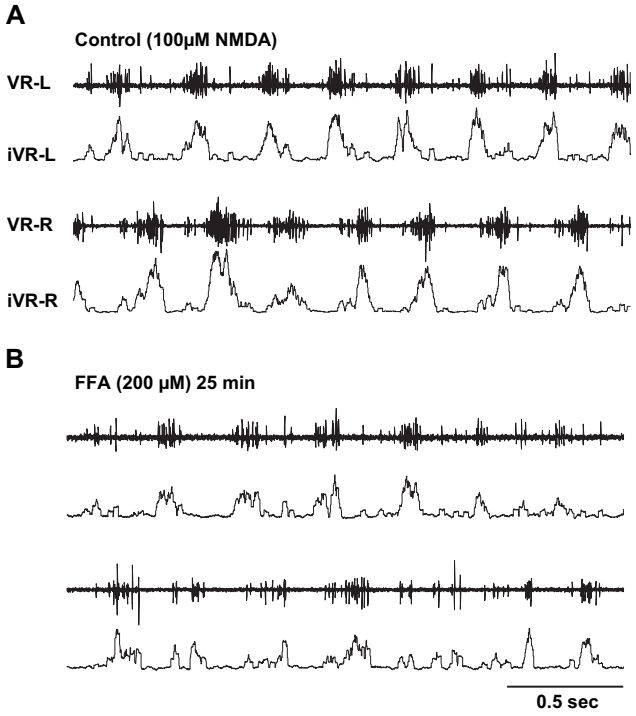

Fig. 2. Effects of flufenamic acid (FFA) on activity of the spinal locomotor network. Fictive locomotor activity was induced by bath application of *N*-methyl-D-aspartate (100 μM NMDA), and was recorded from the ventral roots. The rectified and filtered (integrated) version of the ventral root recording (iVR) is shown below each raw trace. (A) Perfusion with only NMDA (100 μM) induced regular rhythmic activity with a burst frequency of approximately 3 Hz. (B) Addition of FFA (200 μM, 25 min) reduced the amplitude of the activity and the burst pattern became less regular.

3.2. Effects of FFA on NMDA-induced plateau potentials

During fictive locomotion induced by NMDA, spinal neurons not only fire bursts of action potentials, but they may also display membrane potential oscillations due to intrinsic properties. These pacemaker-like oscillations are NMDA-receptor dependent and can be observed after addition of TTX, which blocks voltage-dependent Na^+ channels and synaptic transmission mediated by Na^+ action potentials. The NMDA-induced TTX-resistant oscillations (Fig. 3A) consist of a rapid depolarization mediated by the Na^+ and Ca^{2+} influx via an opening of the NMDA channels and to some degree by the opening of voltage-dependent Ca^{2+} channels. It is followed by a prolonged plateau depolarization and a subsequent rapid repolarization, which terminates the plateau, dependent on K_{Ca} channels activated by the Ca^{2+} entry, and on the closure of the NMDA channels (Wallén and Grillner, 1987).

Application of FFA (150 μM) produced a marked, time-dependent reduction of the amplitude and duration of the depolarizing plateaus (Fig. 3B–E), and eventually the oscillations ceased at a hyperpolarized level (Fig. 3F). Similar results were obtained in all neurons tested ($n = 6$). These effects have been further analyzed and illustrated in Fig. 3G. The duration of the depolarized plateau, expressed as the width at half maximal amplitude (“half width”; see Fig. 3H), was normalized to the cycle duration and plotted for all six neurons tested. Although the half width showed considerable variability between cells, FFA consistently and significantly reduced the duration of the depolarized plateau in all cells.

Calcium-dependent potassium (K_{Ca}) channels play a critical role in the termination of the depolarized plateaus (El Manira et al., 1994). To avoid an interaction between K_{Ca} channels that will terminate the plateaus, and a putative Ca^{2+}-dependent I_{CAN} that would prolong the plateau, K_{Ca} channels were blocked with apamin. Application of apamin (2.5 μM) produced a marked prolongation of the plateau depolarization during NMDA oscillations (Fig. 4 A,B; cf. El Manira et al., 1994). In the presence of apamin, FFA (200 μM) still reduced the depolarization plateau markedly (Fig. 4C–E). This effect was seen in all of three neurons tested (Fig. 4F). Despite the variability in half width between cells, FFA significantly reduced the duration of the depolarized plateau in all cells, thus also in the presence of apamin. This would be expected if putative CAN channels were to contribute to the maintenance of the depolarized plateau.

3.3. Effects of the Ca^{2+} chelator BAPTA on NMDA-induced plateau potentials

Since I_{CAN} is activated by intracellular Ca^{2+}, the Ca^{2+} chelator BAPTA would be expected to prevent its activation. This allows for a separate test of an involvement of I_{CAN}, not utilizing FFA. Intracellular administration of BAPTA through the microelectrode caused a prolongation of the NMDA plateaus (Fig. 5 A, B, $n = 3$). This can be ascribed to a reduced activation of K_{Ca} channels. If I_{CAN} is present in these neurons,

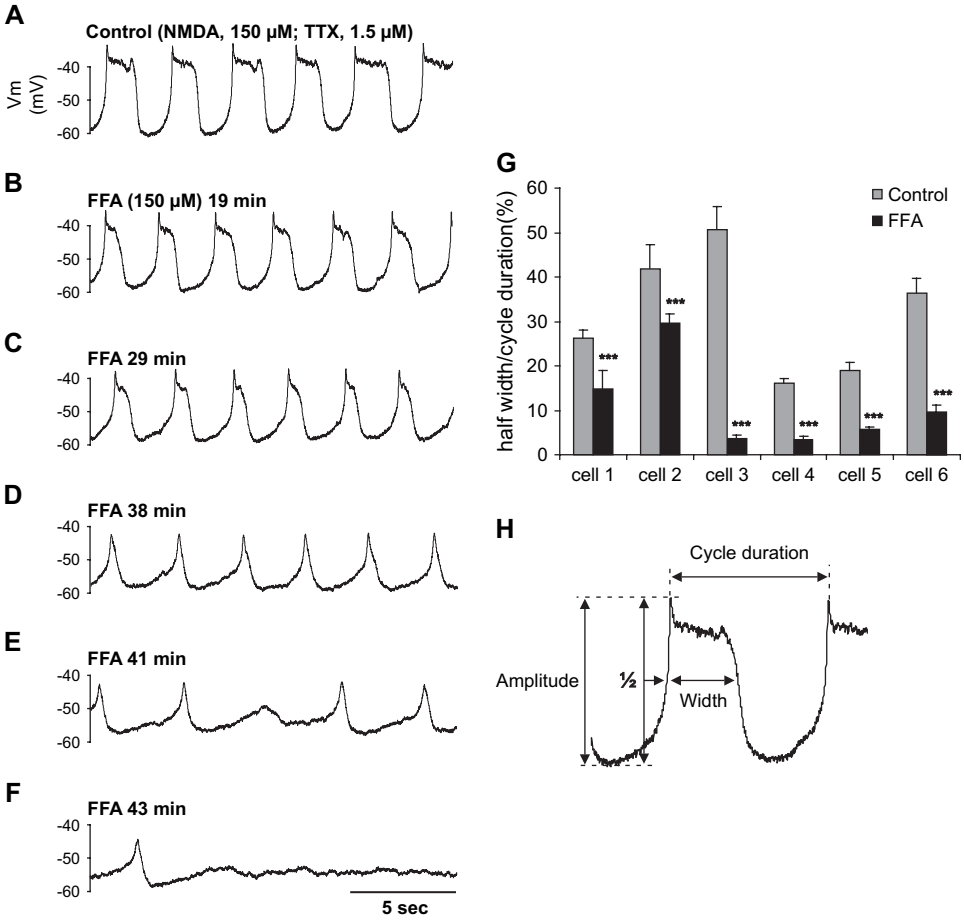

Fig. 3. Effects of FFA on NMDA-induced TTX resistant membrane potential oscillations. Intracellular recording from a spinal neuron using discontinuous current clamp. (A) Bath application of 150 μM NMDA and 1.5 μM TTX induced membrane potential oscillations. (B–F) FFA (150 μM) progressively reduced the amplitude and duration of the plateau depolarizations, which eventually ceased. The average trough membrane potential (−58 mV in control) was kept constant by DC-current injection during the experiment. (G) Cycle structure effects of FFA on NMDA-induced membrane potential oscillations. The width of the depolarization phase, measured at its half maximal amplitude (half width) was plotted as the proportion of the cycle duration for the 6 cells tested. The half width/cycle duration (%) was significantly reduced after FFA bath perfusion in all 6 cells ($P < 0.01$). (H) Graphical representation of parameters measured: Oscillation amplitude, as trough-to-peak; cycle duration, between peaks; width of plateau depolarization, at half maximal amplitude.

these results suggest that the influence of K_{Ca} channel activation on the plateau duration would nevertheless be dominating.

For this reason, we next attempted to reduce the contribution of K_{Ca} channels by administrating apamin (2.5 μM; Fig. 5C–E). Also under these conditions, BAPTA injection caused an increase in plateau duration, rather than a shortening (Fig. 5 D, E, $n = 3$). This effect was seen in all of three neurons tested (Fig. 5F). Despite the variability in half width between cells, BAPTA significantly increased the duration of the depolarized plateau in all cells, hence also in the presence of apamin. This prolongation is likely to be due to blockade of the activation of remaining K_{Ca} channels that had not been blocked by the apamin administration (cf. Cangiano et al., 2002). This is in contrast to findings in reticulospinal neurons, where BAPTA injection has been reported to indeed shorten the depolarized plateau (Viana Di Prisco et al., 2000). The present findings thus do not provide support for the presence of an I_{CAN} current in lamprey spinal neurons.

3.4. Effects of FFA on calcium channels and the slow AHP

On the basis of the results described above, where no support for an involvement of I_{CAN} could be demonstrated, we explored alternative explanations of the effects of FFA. One possibility would be that FFA influences Ca^{2+} channels, and thereby the slow afterhyperpolarization (sAHP) which follows the action potential, and which is dependent on K_{Ca} channel activation. Clearly, an effect on the slow AHP would influence the firing properties of single neurons as well as the activity of the burst-generating spinal network. After administering FFA, the sAHP was markedly reduced in amplitude (Fig. 6A; summed sAHP following a train of three spikes). A similar reduction in sAHP amplitude was seen in all of four cells tested (Fig. 6B). In the presence of TEA, a potassium channel blocker, the action potential is prolonged, largely due to an increased Ca^{2+} influx (Hill et al., 1985). Correspondingly, the K_{Ca} channel-dependent sAHP is increased in amplitude in the presence of TEA. After

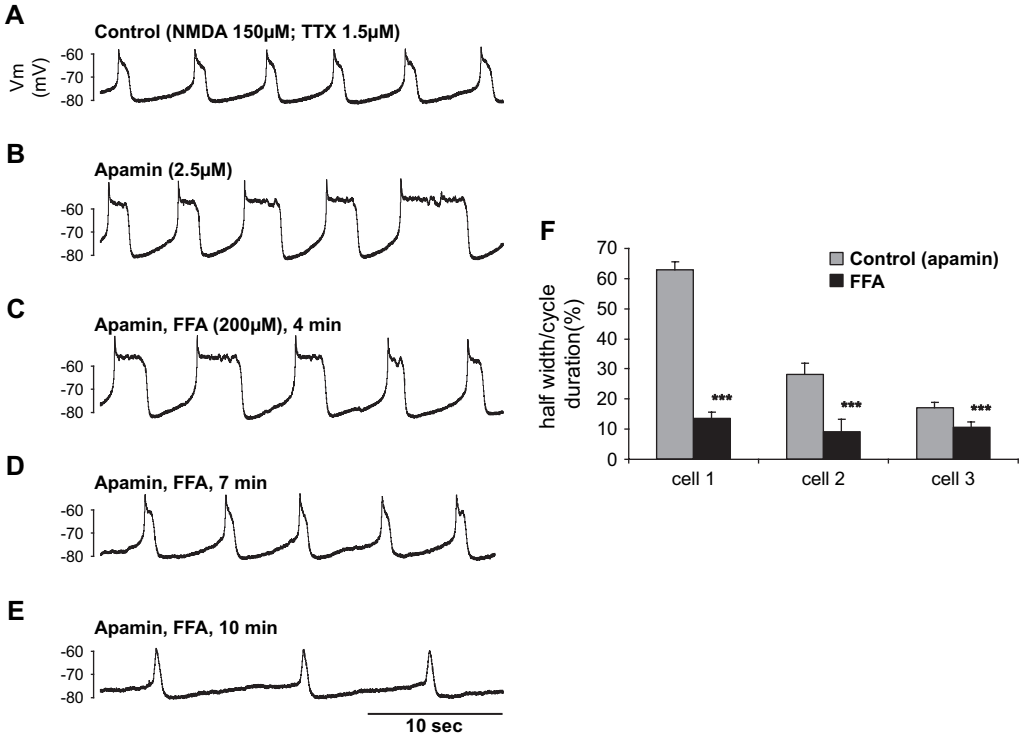

Fig. 4. Effects of FFA on prolonged oscillation plateaus, induced in the presence of the K_{Ca} channel blocker apamin. (A) Control recording from a spinal neuron (resting membrane potential: −78 mV) showing membrane potential oscillations, induced by perfusion of NMDA (150 μM) together with tetrodotoxin (TTX 1.5 μM). (B) Bath application of apamin (2.5 μM) produced a marked prolongation of the depolarizing plateau. (C–E) In the presence of apamin, perfusion of FFA (200 μM) gradually reduced both the amplitude and duration of the plateau. (F) In the presence of apamin, the half width of the depolarization phase was significantly reduced by FFA in all of three cells tested ($P < 0.01$).

administering FFA in the presence of TEA, the amplitude of the sAHP was again reduced in all of four cells tested (Fig. 6 C,D). At the same time, the duration of the prolonged action potential was significantly reduced (Fig. 6E, $n = 3$). These findings thus suggest that FFA indeed influences Ca^{2+} channels.

3.5. *Effects of FFA on NMDA receptors and gap junctions*

Since both the rhythmic network activity and the NMDA-induced plateau potentials depend on the activation of NMDA receptors, we also tested whether FFA would directly influence these receptors. We first investigated if FFA would affect synaptic transmission between ventromedial reticulospinal axons and neurons in the spinal gray matter. Intracellular recordings were made in postsynaptic cells during extracellular stimulation of reticulospinal axons (see Fig. 1). The reticulospinal EPSP in spinal neurons is composed of a mixed chemical EPSP with an NMDA-component and a non-NMDA-component, and an early electrical component mediated by gap junctions (Ohta and Grillner, 1989). To test whether FFA would influence the NMDA component of the reticulospinal EPSP, the non-NMDA-component was blocked. After bath application of NBQX, an AMPA/kainate receptor antagonist, the remaining EPSP thus includes an electrical and an NMDA component. After administration of FFA, the NMDA-EPSP amplitude was markedly reduced (Fig. 7A). This effect was seen in all of three cells tested (Fig. 7B). To further determine whether the reduction of the NMDA-EPSP amplitude is caused by a direct influence on NMDA receptors, or indirectly by an effect on glutamate release, we also tested FFA on NMDA depolarizations induced by direct, local application of NMDA in the presence of TTX (Fig. 7C). FFA caused a marked reduction in size of the NMDA-induced depolarization ($n = 3$). These results thus suggest that FFA also influences NMDA receptors.

After administration of the Ca^{2+} channel blocker Cd^{2+}, all chemical synaptic transmission will be absent, leaving only the electrical component of the EPSP. FFA depressed also the remaining electrical EPSP (Fig. 7D, $n = 3$). The possibility that FFA might influence the presynaptic action potential in the reticulospinal axon, which indirectly would affect the size of the electrical EPSP, was tested with intra-axonal recordings. No significant change of amplitude or shape of the action potential in reticulospinal axons was found upon FFA administration ($n = 5$; not illustrated). These results therefore indicate that FFA also affects gap junctions.

4. Discussion

A calcium-activated non-selective (CAN) cation current (I_{CAN}) plays a crucial role in important functions in many neurons because of its involvement in generating a maintained

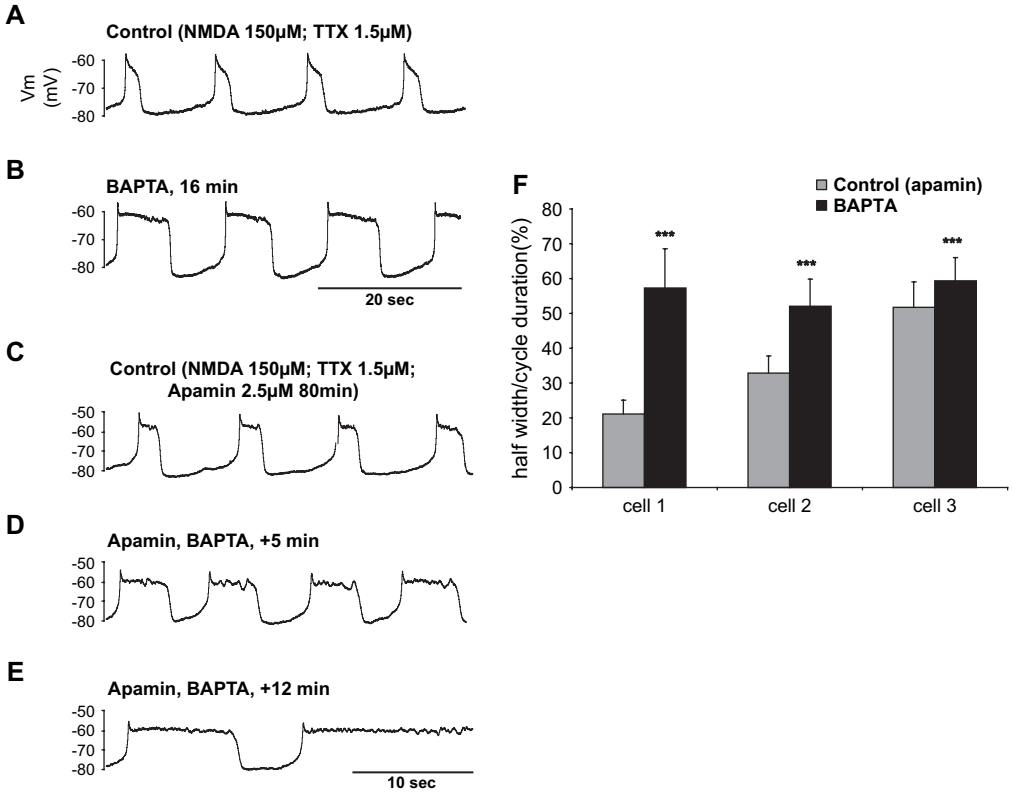

Fig. 5. Effects of BAPTA in combination with apamin on NMDA-induced plateau potentials. (A) Control recording from a spinal neuron (resting membrane potential: −78 mV) showing membrane potential oscillations, induced by perfusion of NMDA (150 μM) together with tetrodotoxin (TTX 1.5 μM). (B) After 16 min of intracellular BAPTA injection (200 mM in microelectrode), plateau depolarizations were prolonged. (C–E) In the presence of apamin, BAPTA still prolonged plateau depolarizations. (C) Control recording after preincubation of the spinal cord with apamin (2.5 μM) for 80 min. (D,E) After 5 and 12 min of intracellular BAPTA injection, the depolarized plateaus were progressively prolonged. (F) In the presence of apamin, the half width of the depolarization phase was significantly prolonged by BAPTA in all of three cells tested ($P < 0.01$).

depolarization of the cell, such as during bursting activity of pacemaker neurons (Partridge et al., 1994; Zhang et al., 1995; Fraser and MacVicar, 1996; Klink and Alonso, 1997; Morisset and Nagy, 1999). Flufenamic acid (FFA), a class of non-steroid anti-inflammatory drugs (NSAIDs) that are derivatives of diphenylamine-2-carboxylate (DPC), has been utilized as a blocker of I_{CAN} in a number of cell types. FFA blocks I_{CAN} in molluscan bursting neurons (Lee et al., 1996) and the CAN channel-dependent plateau in deep dorsal horn neurons (Morisset and Nagy, 1999), and a block of CAN channels may contribute to the protective effect of FFA from ischemic injury in retinal neurons (Chen et al., 1998).

It has been reported previously that FFA will abolish the sensory stimulus-evoked, maintained depolarizing plateau of reticulospinal (RS) neurons in the lamprey brainstem, indicating an involvement of I_{CAN} in these neurons (Viana Di Prisco et al., 2000). The present study was initiated to clarify if I_{CAN} may also be present in spinal neurons of the lamprey and if this current would contribute to rhythm generation in the spinal locomotor network. It was shown that FFA influences both the amplitude of rhythmic burst activity and the regularity of the pattern during fictive locomotion in the isolated lamprey spinal cord. In addition, FFA markedly decreased the amplitude and duration of the depolarizing plateau phase during NMDA-induced membrane potential oscillations.

4.1. FFA effects on the locomotor pattern and on plateau potentials

If I_{CAN} were involved in plateau potentials and during locomotor pattern generation in the lamprey, one would expect that a specific I_{CAN} blockade would influence the amplitude of bursting and the regularity of the locomotor pattern, which indeed occurred after administration of FFA. Intracellular Ca^{2+}, through its role in the activation of putative CAN channels and of calcium-dependent potassium channels, is of key importance during membrane potential oscillations. In bursting neurons, Ca^{2+} influx through NMDA channels and voltage-gated calcium channels may activate nearby CAN channels, which might then contribute to the maintenance of depolarization during the burst.

Since FFA is a non-specific drug that has been shown to influence other currents than the CAN current (e.g. Ottolia and Toro, 1994; Harks et al., 2001; cf. Schiller, 2004; Tryba et al., 2006, and below), we also performed independent tests for an involvement of CAN channels in lamprey spinal neurons without using FFA, by manipulating the effects of intracellular Ca^{2+}. After intracellular injection of the calcium chelator BAPTA, the depolarized plateaus of NMDA-induced membrane potential oscillations were prolonged (Fig. 5 A, B), suggesting that the dominating role of

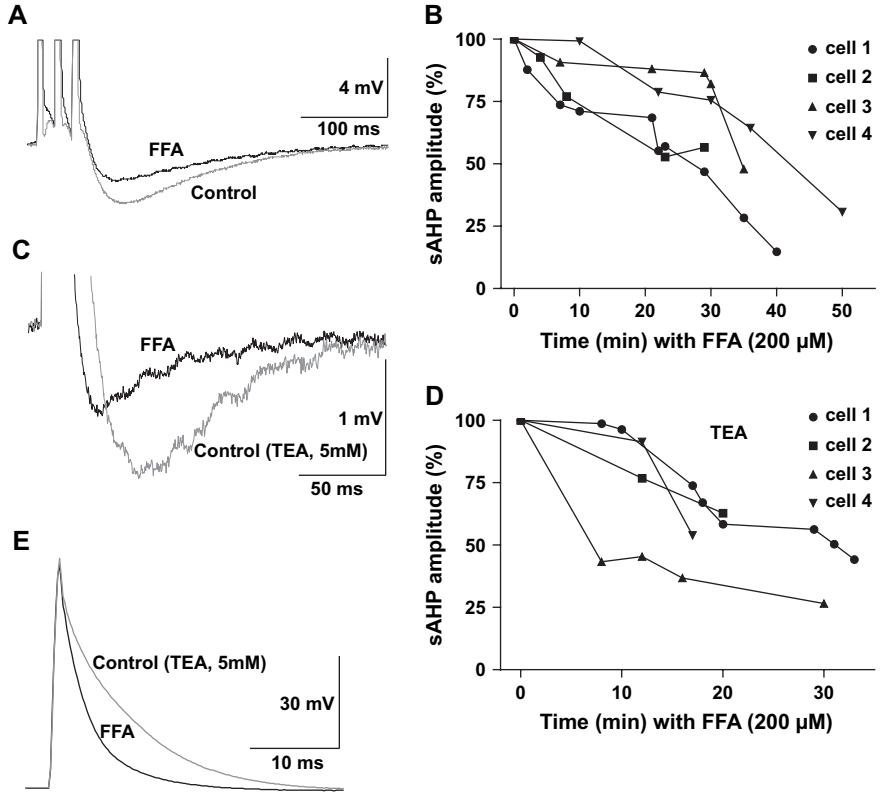

Fig. 6. Effects of FFA on calcium channels and the slow afterhyperpolarization (sAHP). (A) Effect of FFA on the sAHP. The sAHP, which is dependent on K_{Ca} channel activation, was here summed following a train of three action potentials (20 ms interval) (Control, gray trace), and was reduced after administering FFA (200 μM). (B) In all of four cells tested, the amplitude of the sAHP was reduced following FFA application (relative change of sAHP amplitude plotted versus time of FFA application). (C) Effect of FFA on the TEA-sAHP. The sAHP was increased in the presence of TEA, a potassium channel blocker (5 mM; Control, gray trace; single action potential), and was markedly reduced after administering FFA (200 μM). (D) In all of four cells tested in the presence of TEA, the amplitude of the sAHP was reduced following FFA application (relative change of sAHP amplitude plotted versus time of FFA application). (E) Effect of FFA on the prolonged action potential of the same spinal neuron as in C (Control, gray trace) in the presence of TEA (5 mM). Upon administration of FFA (200 μM), the duration of the broadened action potential was significantly reduced. Traces are averages of 30–40 individual trials.

intracellular Ca^{2+} is to activate K_{Ca} channels which take part in the repolarization and termination of the plateau. Accordingly, after blockade of K_{Ca} channels with apamin, plateau depolarizations also become prolonged (Fig. 4 A, B; cf. El Manira et al., 1994). Thus, with the effect of intracellular Ca^{2+} on K_{Ca} channels being compromised by apamin blockade, one would expect that, if CAN channels would contribute to the depolarized plateau, BAPTA injection would now cause a shortening of the plateau. This, however, did not occur (Fig. 5C–F); instead BAPTA still resulted in a prolongation, presumably due to an incomplete block by apamin (cf. Cangiano et al., 2002). This test, without utilizing FFA, thus failed to demonstrate any contribution of I_{CAN} to the depolarized plateau and hence, CAN channels may not be present in spinal neurons of lamprey, at least not to a degree that would render them a significant role during NMDA-induced membrane potential oscillations. An alternative interpretation may be considered, which is that BAPTA injection using sharp electrodes would have failed to penetrate the cellular compartments to a degree sufficient to influence the activation of putative CAN channels. This possibility seems unlikely, however, since BAPTA injection readily reduces the activation of K_{Ca} channels, leading to a delayed repolarization and prolongation of the depolarized plateau (Fig. 5). Besides, in the work on lamprey reticulospinal neurons (Viana Di Prisco et al., 2000; cf. below), which are larger in size than the spinal neurons studied here, injection of BAPTA from sharp microelectrodes was reported to be effective in influencing CAN channels.

These results are at variance with findings from lamprey reticulospinal neurons, where not only FFA application, but also BAPTA injection blocked the sensory stimulus-evoked, maintained depolarizing plateau (Viana Di Prisco et al., 2000). In these neurons the plateau is, however, of a different character, being non-regenerative and long-lasting (tens of seconds). In lamprey spinal neurons, the NMDA plateau potentials are regenerative at a rate corresponding to low to medium locomotor frequencies (up to about 2 Hz). Activation of K_{Ca} channels significantly contributes to the repolarization phase during these plateau oscillations (Fig. 4; cf. El Manira et al., 1994), while these channels may contribute less to the plateau properties of reticulospinal neurons (cf. Viana Di Prisco et al., 2000). Moreover, the contribution of voltage-activated calcium channels to the plateau properties seems minor, or even absent, in reticulospinal neurons (Viana Di Prisco et al., 2000; cf. Wallén and Grillner, 1987).

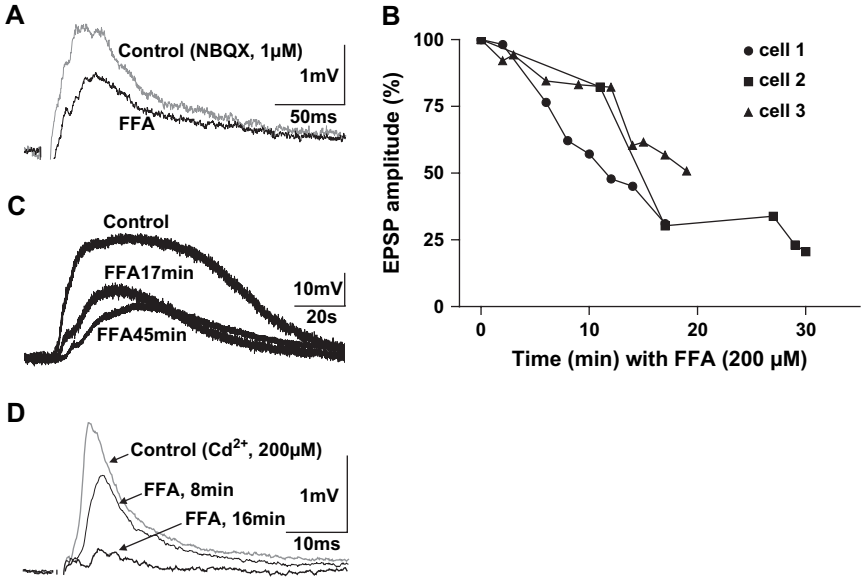

Fig. 7. Effects of FFA on NMDA receptors and gap junctions. (A) Compound NMDA-receptor mediated EPSP in a spinal cord neuron, evoked by extracellular stimulation of reticulospinal axons, in the presence of NBQX (1 μM) a non-NMDA receptor blocker (Control, gray trace). In the presence of FFA (200 μM), the amplitude of the EPSP was markedly depressed. (B) In all of three cells tested, the amplitude of the NMDA-EPSP was reduced following FFA application (relative change of EPSP amplitude plotted versus time of FFA application). (C) Membrane potential depolarization induced by direct, local application of NMDA, in the presence of TTX to avoid synaptic activation. In the presence of FFA (200 μM), the amplitude of the NMDA-receptor mediated depolarization was markedly reduced. (D) In the presence of cadmium chloride (200 μM), a Ca^{2+} channel blocker, only the electrical, gap junction mediated EPSP remains (Control, gray trace). Upon application of FFA (200 μM), the amplitude of the electrical EPSP was progressively reduced, as evident from the traces taken at 8 and 16 min after onset of FFA application. Traces are averages of 30–40 individual trials.

4.2. The mechanisms of FFA effects on the locomotor pattern and on plateau potentials

What then are the alternative mechanisms underlying the effects of FFA on the locomotor pattern and the plateau potentials in spinal neurons? The data presented demonstrate that FFA has several non-selective effects on lamprey spinal cord neurons. The amplitude of the slow AHP, as well as the duration of the action potential in TEA, was markedly reduced upon administration of FFA, suggesting an influence on calcium channels. Furthermore, the NMDA component of the EPSP from reticulospinal axons was reduced in size by FFA, as were NMDA-induced depolarizations, demonstrating an influence on NMDA receptors (cf. Chen et al., 1998; Lerma and Martin del Rio, 1992). Both of these influences will clearly lead to effects also on the locomotor pattern produced by the spinal network and on the NMDA-dependent plateau potentials. For example, an influence on calcium channels will affect both the slow AHP of bursting neurons and the oscillatory properties leading to alterations of the rhythm (cf. Wallén and Grillner, 1987; El Manira et al., 1994; Grillner et al., 2001). A blockade of NMDA receptor activation will obviously affect synaptic transmission within the network, and will also compromise the ability to generate membrane potential oscillations.

FFA also influenced gap-junction mediated synaptic transmission, and even if the detailed role of gap junctions in the locomotor network remains to be explored, such an effect of FFA may clearly also contribute to its influence on the locomotor burst pattern. It is worth noting that FFA and other fenamates have been shown to block gap junctions in other cell types, including neuroblastoma cells (Harks et al., 2001; Srinivas and Spray, 2003; cf. Bruzzone et al., 2005).

In conclusion, our findings demonstrate several non-selective effects of FFA. These effects may account for the observed influence of FFA on fictive locomotion and on membrane potential oscillations in the spinal cord and thus, a specific involvement of the I_{CAN} current in lamprey spinal neurons is not supported. Furthermore, a word of caution may be raised towards the usage of FFA as a blocker of this current also in other systems.

Acknowledgments

We are grateful to Dr. R.H. Hill for his valuable comments on the manuscript, and to Monica Bredmyr for her skilful technical assistance. This study was supported by the Swedish Research Council (Medicine, project no. 3026, and Natural and Engineering Sciences, project no. 1496), the European Union (project no. QLG3-CT-2001-01241), the Marianne and Marcus Wallenberg Foundation, and the Karolinska Institute Foundations.

References

Brodin, L., Grillner, S., 1986. Effects of magnesium on fictive locomotion induced by activation of *N*-methyl-D-aspartate (NMDA) receptors in the lamprey spinal cord in vitro. Brain Res. 380, 244–252.

Brodin, L., Grillner, S., Dubuc, R., Ohta, Y., Kasicki, S., Hökfelt, T., 1988. Reticulospinal neurons in lamprey: transmitters, synaptic interactions and their role during locomotion. Arch. Ital. Biol. 126, 317–345.

Bruzzone, R., Barbe, M.T., Jakob, N.J., Monyer, H., 2005. Pharmacological properties of homomeric and heteromeric pannexin hemichannels expressed in Xenopus oocytes. J. Neurochem. 92, 1033–1043.

Cangiano, L., Wallén, P., Grillner, S., 2002. Role of apamin-sensitive K_{Ca} channels for reticulospinal synaptic transmission to motoneuron and for the afterhyperpolarization. J. Neurophysiol. 88, 289–299.

Chen, Q., Olney, J.W., Lukasiewicz, P.D., Almli, T., Romano, C., 1998. Fenamates protect neurons against ischemic and excitotoxic injury in chick embryo retina. Neurosci. Lett. 242, 163–166.

Congar, P., Leinekugel, X., Ben-Ari, Y., Crepel, V., 1997. A long-lasting calcium-activated nonselective cationic current is generated by synaptic stimulation or exogenous activation of group I metabotropic glutamate receptors in CA1 pyramidal neurons. J. Neurosci. 17, 5366–5379.

El Manira, A., Tegnér, J., Grillner, S., 1994. Calcium-dependent potassium channels play a critical role for burst termination in the locomotor network in lamprey. J. Neurophysiol. 72, 1852–1861.

Fraser, D.D., MacVicar, B.A., 1996. Cholinergic-dependent plateau potential in hippocampal CA1 pyramidal neurons. J. Neurosci. 16, 4113–4128.

Grillner, S., McClellan, A., Sigvardt, K., Wallén, P., Wilén, M., 1981. Activation of NMDA-receptors elicits ''fictive locomotion'' in lamprey spinal cord in vitro. Acta Physiol. Scand. 113, 549–551.

Grillner, S., Wallén, P., Hill, R., Cangiano, L., El Manira, A., 2001. Ion channels of importance for the locomotor pattern generation in the lamprey brainstem-spinal cord. J. Physiol. 533, 23–30.

Grillner, S., 2003. The motor infrastructure: from ion channels to neuronal networks. Nature Rev. Neurosci. 4, 573–586.

Harks, E.G., de Roos, A.D., Peters, P.H., de Haan, L.H., Brouwer, A., Ypey, D.L., van Zoelen, E.J., Theuvenet, A.P., 2001. Fenamates: a novel class of reversible gap junction blockers. J. Pharmacol. Exp. Ther. 298, 1033–1041.

Hill, R.H., Århem, P., Grillner, S., 1985. Ionic mechanisms of 3 types of functionally different neurons in the lamprey spinal cord. Brain Res. 358, 40–52.

Klink, R., Alonso, A., 1997. Ionic mechanisms of muscarinic depolarization in entorhinal cortex layer II neurons. J. Neurophysiol. 77, 1829–1843.

Lee, R.J., Shaw, T., Sandquist, M., Partridge, L.D., 1996. Mechanism of action of the non-steroidal anti-inflammatory drug flufenamate on $[Ca^{2+}]_i$ and Ca^{2+}-activated currents in neurons. Cell Calcium 19, 431–438.

Lerma, J., Martin del Rio, R., 1992. Chloride transport blockers prevent *N*-methyl-D-aspartate receptor-channel complex activation. Mol. Pharmacol. 41, 217–222.

Morisset, V., Nagy, F., 1999. Ionic basis for plateau potentials in deep dorsal horn neurons of the rat spinal cord. J. Neurosci. 19, 7309–7316.

Ohta, Y., Grillner, S., 1989. Monosynaptic excitatory amino acid transmission from the posterior rhombencephalic reticular nucleus to spinal neurons involved in the control of locomotion in lamprey. J. Neurophysiol. 62, 1079–1089.

Ottolia, M., Toro, L., 1994. Potentiation of large conductance KCa channels by niflumic, flufenamic, and mefenamic acids. Biophys. J. 67, 2272–2279.

Partridge, L.D., Muller, T.H., Swandulla, D., 1994. Calcium-activated nonselective channels in the nervous system. Brain Res. Rev. 19, 319–325.

Pena, F., Parkis, M.A., Tryba, A.K., Ramirez, J.M., 2004. Differential contribution of pacemaker properties to the generation of respiratory rhythms during normoxia and hypoxia. Neuron 43, 105–117.

Schiller, Y., 2004. Activation of a calcium-activated cation current during epileptiform discharges and its possible role in sustaining seizure-like events in neocortical slices. J. Neurophysiol. 92, 862–872.

Srinivas, M., Spray, D.C., 2003. Closure of gap junction channels by arylaminobenzoates. Mol. Pharmacol. 63, 1389–1397.

Tryba, A.K., Pena, F., Ramirez, J.M., 2006. Gasping activity in vitro: a rhythm dependent on 5-HT2A receptors. J. Neurosci. 26, 2623–2634.

Viana Di Prisco, G., Pearlstein, E., Le Ray, D., Robitaille, R., Dubuc, R., 2000. A cellular mechanism for the transformation of a sensory input into a motor command. J. Neurosci. 20, 8169–8176.

Wallén, P., Grillner, S., 1987. *N*-methyl-D-aspartate receptor-induced, inherent oscillatory activity in neurons active during fictive locomotion in the lamprey. J. Neurosci. 7, 2745–2755.

Wallén, P., Grillner, S., Feldman, J.L., Bergelt, S., 1985. Dorsal and ventral myotome motoneurons and their input during fictive locomotion in lamprey. J. Neurosci. 5, 654–661.

Wang, D., Grillner, S., Wallén, P., 2001. A Ca^{2+} activated cation current (I_{CAN}) may contribute to spinal locomotor pattern generation in lamprey. Soc. Neurosci. Abstr., 730.2.

Wilson, G.F., Richardson, F.C., Fisher, T.E., Olivera, B.M., Kaczmarek, L.K., 1996. Identification and characterization of a Ca^{2+}-sensitive non-specific cation channel underlying prolonged repetitive firing in Aplysia neurons. J. Neurosci. 16, 3661–3671.

Zhang, B., Wootton, J.F., Harris-Warrick, R.M., 1995. Calcium-dependent plateau potentials in a crab stomatogastric ganglion motor neuron. II. Calcium-activated slow inward current. J. Neurophysiol. 74, 1938–1946.