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β-POMPILIDOTOXIN MODULATES SPONTANEOUS ACTIVITY AND PERSISTENT SODIUM CURRENTS IN SPINAL NETWORKS

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Abstract—The origin of rhythm generation in mammalian spinal cord networks is still poorly understood. In a previous study, we showed that spontaneous activity in spinal networks takes its origin in the properties of certain intrinsically spiking interneurons based on the persistent sodium current (I_{NaP}). We also showed that depolarization block caused by a fast inactivation of the transient sodium current (I_{NaT}) contributes to the generation of oscillatory activity in spinal cord cultures. Recently, a toxin called beta-pompilidotoxin (β-PMTX) that slows the inactivation process of tetrodotoxin (TTX) -sensitive sodium channels has been extracted from the solitary wasp venom. In the present study, we therefore investigated the effect of β-PMTX on rhythm generation and on sodium currents in spinal networks. Using intracellular recordings and multielectrode array (MEA) recordings in dissociated spinal cord cultures from embryonic (E14) rats, we found that β-PMTX reduces the number of population bursts and increases the background asynchronous activity. We then uncoupled the network by blocking all synaptic transmission (APV, CNQX, bicuculline and strychnine) and observed that β-PMTX increases both the intrinsic activity at individual channels and the number of intrinsically activated channels. At the cellular level, we found that β-PMTX has two effects: it switches 58% of the silent interneurons into spontaneously active interneurons and increases the firing rate of intrinsically spiking cells. Finally, we investigated the effect of β-PMTX on sodium currents. We found that this toxin not only affects the inactivation of I_{NaT} but also increases the peak amplitude of the persistent sodium current (I_{NaP}). Altogether, theses findings suggest that β-PMTX acting on I_{NaP} and I_{NaT} enhances intrinsic activity leading to a profound modulation of spontaneous rhythmic activity in spinal networks.

Key words: persistent sodium currents, β-pompilidotoxin, spinal cord networks, spontaneous activity.

Rhythmic activity is a common feature of neuronal networks in the CNS. This collective neuronal electrical behavior sustains key neurophysiological functions as sleep (Steriade, 2001), breathing (Rekling and Feldman, 1998), development (O'Donovan, 1999) as well as locomotion (Grillner, 2003). In spinal cord, neuronal networks are able to self-generate rhythmic activity which drives the repetitive limb movement underlying locomotion (Grillner, 2006). This intrinsic spinal circuit is called central pattern generator (CPG) and has been extensively studied using different models, preparations and approaches (for review see Alford et al., 2003; Barbeau et al., 1999; Grillner, 2006; Kiehn, 2006). However, whether rhythm generation is supported by cellular pacemaker properties (Wallen and Grillner, 1987; Tresch and Kiehn, 2000; Tazerart et al., 2008; Ziskind-Conhaim et al., 2008), by emergent properties of the network as proposed for the respiratory center in the brainstem (Feldman and Del Negro, 2006) or by both (Brocard et al., 2010) remains an open question.

Organotypic and dissociated fetal rat spinal cord cultures exhibit spontaneous rhythmic activity which propagates to the whole preparation (Streit et al., 2001; Tscherter et al., 2001). Such *in vitro* preparations conserve basic components of rhythm generation and are able to produce different rhythmic activities in given conditions (Streit et al., 2006). This activity consists of repetitive population bursts which appear spontaneously in organotypic cultures and which can be induced by disinhibition in dissociated cultures. This pattern of activity consists of episodes of high activity which propagates in a wave-like manner through the whole network. In previous studies, we have shown that population bursts are initiated by a group of excitatory neurons which are driven by a subthreshold depolarizing conductance called persistent sodium currents (I_{NaP}) (Darbon et al., 2004). This current allows neurons to be spontaneously active. Some of these intrinsically active neurons may recruit other neurons by recurrent excitation via the (+/–)-α-amino-3-hydroxy-5-methylisoxazole-4-proprionic acid hydrate (AMPA)/Kainate and (*N*-methyl-D-aspartate (NMDA) receptors and thus cause population bursts (Legrand et al., 2004; Streit et al., 2001; Tscherter et al., 2001). In addition, the termination of the burst requires different accommodation processes as the spike frequency adaptation and the up regulation of the Na/K pump (Darbon et al., 2002, 2003). The fast intra-burst oscillations present in organotypic cultures were also observed in dissociated cultures after application of low doses of riluzole (2 μM), which increase fast sodium current inactivation and partially decrease I_{NaP} (Yvon et al., 2007). Riluzole indeed caused early depolarization block, increased the frequency adaptation of firing in spinal neurons and shifted the inactivation curve of the transient sodium current (I_{NaT}) to the left. We therefore proposed that the fast suppression of the

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Abbreviations: CNQX, 6-cyano-7-nitroquinoxaline-2,3-dione; D-APV, D-2-amino-5-phosphopentanoic acid; HEPES, 4-(2-hydroxyethyl)-piperazin-1-ethansulphonic acid; I_{NaP}, persistent sodium currents; I_{NaT}, transient sodium current; MEA, multielectrode array; TTX, tetrodotoxin; β -PMTX, β-pompilidotoxin.

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network activity during oscillations is mainly mediated by depolarization block caused by a fast inactivation of the I_{NaT} (Czarnecki et al., 2008; Yvon et al., 2007).

Recently, toxins called α- and β-pompilidotoxin (α- and β-PMTX) have been extracted from the solitary wasp venom (Konno et al., 1997, 1998). It was reported that both toxins slow the sodium channel inactivation process inducing a long burst of action potentials in axons of the walking leg of the lobster (Kinoshita et al., 2001; Konno et al., 2000; Sahara et al., 2000). Their effects on sodium channel inactivation can be measured as an increase of the time constant of the decay of I_{NaT} and a steady state portion of inactivation. The reduction of fast inactivation increases persistent currents (Grieco and Raman, 2004; Kinoshita et al., 2001; Sahara et al., 2000; Schiavon et al., 2010), promotes action potential generation and increases firing rate (Khaliq and Raman, 2006; Miyawaki et al., 2002). α and β toxins are distinct by only one amino-acid conferring to β-PMTX an efficiency that is five fold higher than in the α-form (Konno et al., 2000; Sahara et al., 2000).

In the present study, we therefore chose β-PMTX to further study the role of sodium current inactivation in the generation of spontaneous activity in spinal cord cultures. We characterized the effect of β-PMTX on spontaneous and intrinsic activity in dissociated spinal cultures on multielectrode arrays (MEAs) and investigated its effect on sodium currents at the cellular level in spinal neurons. We found that β-PMTX slows inactivation of I_{NaT} in all neurons and increases I_{NaP} in a subtype of non spiking neurons. The result of this effect is an increase of the firing rate of spontaneously active neurons and the switch of some silent neurons into spontaneously active cells. β-PMTX thus promotes intrinsic activity at the network level leading to a profound modulation of spontaneous rhythmic activity.

EXPERIMENTAL PROCEDURES

Cultures

Cultures were made from the spinal cord of Wistar rats (Janvier, Le Genest St. Isle, France) at embryonic age 14. The cultures were prepared as described previously (Streit et al., 2001). The embryos were delivered by caesarean section from deeply anaesthetized animals (0.4 ml pentobarbital i.m., Streuli Pharma AG, Uznach, Switzerland) and killed by decapitation. Following the delivery of the embryos, the mother was killed by intracardiac injection of pentobarbital. This procedure guarantees a minimal suffering of animal (grade 0). The number of animal used to prepare the culture was kept minimal. In addition, animal handling procedure was in accordance with guidelines approved by Swiss local authorities (Amt für Landwirtschaft und Natur des Kantons Bern, Veterinärdienst, Sekretariat Tierversuche, approvals No. 61/05 and 45/08). The backs of the embryos were isolated from their limbs and viscera and cut into 225 μm thick transverse slices with a tissue chopper. Slices of all regions of the spinal cord, without dorsal root ganglia, were exposed to a 0.3% trypsin solution for 3 min at 37 °C. Then they were mechanically dissociated by forcing them through fine-tipped pipettes several times. The cells were plated on MEAs or on glass coverslips at a density of 150,000 or 75,000/150 μl, respectively. MEAs were produced as described previously (Tscherter et al., 2001) and coated for 1 h with diluted (1:50) Matrigel® (Falcon/Biocoat, Becton Dickinson AG, Switzerland). The cells were restricted to an area around the electrodes (ca. 50 mm^2) using cloning glass cylinders attached to the MEAs or coverslips with silicone sealant. They were maintained in culture dishes containing 150 μl of nutrient medium and incubated in a 5% CO_2/95% air atmosphere at 36.5 °C for up to 12 weeks. Serum-free Neurobasal™ medium (Gibco BRL, Life Technologies AG, Switzerland) supplemented with B 27 and Glutamax (both Gibco BRL) was used for the MEA and glass coverslip cultures. Half of the medium was changed weekly.

Recordings

Recordings were made in a chamber mounted on an inverted (patch-clamp) or upright (MEA) microscope (Nikon, Japan) from cultures of 4–6 weeks of *in vitro* age. The medium was replaced by an extracellular solution containing (in mM): NaCl, 145; KCl, 4; $MgCl_2$, 2; $CaCl_2$, 2; HEPES, 5; Na-pyruvate, 2; glucose, 5 at pH 7.4. Recordings were made with solution changes every 10–15 min. All recordings were made at room temperature (22–26 °C).

MEA recording and analysis

MEAs contained 68 electrodes, laid out in the form of a rectangle. Channels showing activity (usually 10–40) were selected by eye and their recordings digitized at 6 kHz, visualized and stored on hard disc using custom virtual instruments within Labview® (National Instruments) as described previously (Streit et al., 2001). Detection of the extracellularly recorded action potentials and further analysis were done offline in the software package IGOR (WaveMetrics Inc., Lake Oswego, OR, USA) as described previously (Tscherter et al., 2001). Signal detection was based on standard deviation with the threshold set at three times the noise level of each channel. The electrical noise of individual channels was usually stable throughout the experiments. If this was not the case, the results from the electrode were discarded. The detected signals were fast voltage transients ($<$4 ms), which correspond to single action potentials in neurons or axons (single-unit activity). These transients often appeared in clusters (multi-unit activity) originating from closely timed action potentials of several neurons or axons seen by one electrode. When they appeared at more than 250 Hz ($=$ upper limit of temporal resolution of the detector) they could not be clearly separated from each other and therefore such activity was set by definition to a level of 333 Hz (see Tscherter et al., 2001). No attempt was made to sort spikes seen by one electrode. The selectivity of event detection was controlled using recordings obtained in the presence of tetrodotoxin (TTX, 1.5 μM) as a zero reference. The processed data were displayed in the form of network activity plots or distribution plot. Network activity plots show the total activity of all selected channels within a sliding time window of 10 ms, shifted by 1 ms intervals. Synchronized activity seen in the network activity plot was called a population burst. The activity outside the population bursts was called asynchronous activity. The asynchronous activity represents the activity of the intrinsic spiking cells. The intrinsic spiking cells are cells in which intracellular recordings still exhibit spiking activity in the absence of synaptic transmission. Distribution plots show filled circles whose diameters are proportional to the total activity of the electrodes projected on a picture of the slice culture. Total activity is measured as the mean of the activity per second detected by each electrode during the whole recording period (usually 10 min).

Whole cell patch clamp recording and analysis

Intracellular current and voltage measurements were obtained from individual neurons in cultures on glass coverslips using the whole cell patch technique with an Axoclamp 2 B amplifier (Molecular Devices Inc., Union City, CA, USA). For the measurement of sodium currents in voltage-clamp, the patch pipettes were filled with solution containing (in mM): K-gluconate, 120; KCl, 10;