Calcium Channels Involved in Synaptic Transmission From Reticulospinal Axons in Lamprey

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INTRODUCTION

Presynaptic receptors act to modulate the amount of transmitter released at different synapses in various regions in the CNS (Rudomin et al. 1998). Different mechanisms have been shown to underlie this presynaptic modulation of synaptic transmission, including those affecting calcium channels (Dittman and Regehr 1996; Dunlap and Fischbach 1981; Wu and Sagau 1994), potassium channels (Mouginot et al. 1998), or unidentified targets of the “release machinery” (Fatt and Katz 1952). Calcium is required for transmitter release, and the types of calcium channels involved have been characterized in different regions of the mammalian brain. In most of the so-far-studied synapses in the brain, transmitter release depends on calcium influx through P/Q- and N-type channels, with P/Q-type channels playing a major role (Dunlap et al. 1995; Regehr and Mintz 1994; Takahashi and Momiyama 1993; Wheeler et al. 1994; Wu and Sagau 1994). In the spinal cord, however, the different calcium channel subtypes involved in excitatory transmission and their modulation by presynaptic receptors are relatively less well characterized.

In vertebrates, including lampreys, descending reticulospinal neurons can initiate activity in the spinal locomotor networks (Brodin et al. 1988; Jordan et al. 1992; Shik and Orlovsky 1976). In contrast to sensory and network interneurons (Alford et al. 1991; El Manira et al. 1997), phasic presynaptic modulation of reticulospinal axons was not detected during locomotion in the lamprey (Alford et al. 1991). Recently, however, these axons have been shown to receive excitatory synaptic inputs, which might increase their excitability (Cochilla and Alford 1997). In the lamprey synaptic transmission from reticulospinal axons is presynaptically inhibited by 5-hydroxytryptamine (Buchanan and Grillner 1991; Shupliakov et al. 1995), which appears to act through mechanisms separate from calcium influx through voltage-activated channels (Shupliakov et al. 1995). Activation of presynaptic metabotropic glutamate receptors (mGluRs) can also inhibit synaptic transmission from reticulospinal axons (Krieger et al. 1996). Although the reticulospinal synapse has been extensively used for analyzing the mechanisms of exocytosis and presynaptic inhibition, the types of calcium channels responsible for transmitter release and their modulation have not been characterized.

We examined the types of calcium channels participating in synaptic transmission from reticulospinal axons and whether they are subject to modulation by mGluRs that mediate presynaptic inhibition. The types of calcium channels present in the somata of spinal neurons were characterized and present a pharmacological profile similar to that found in mammalian CNS neurons (El Manira and Bussières 1997). They were thus subdivided into N-, L-, P/Q-, and R-type channels, with N-type channels representing the larger part of the calcium current. On the postsynaptic side N- and P/Q-type calcium channels are involved in activating calcium-dependent potassium channels, which generate the late afterhyperpolarization after a spike. (Wikström and El Manira 1998). We here show by using specific agonists and antagonists for the different calcium channels that presynaptic N-, P/Q-, and R-type calcium channels participate in reticulospinal synaptic transmission, with N-type channels playing the major role. We also provide evidence that the presynaptic inhibition evoked by the mGluR...
agonist L(+)-2-amino-4-phosphonobutyric acid (L-AP4) does not involve modulation of voltage-activated calcium channels.

**METHODS**

**Electrophysiology experiments**

Experiments were performed on adult lampreys (*Lampetra fluviatilis*), which were anesthetized with tricaine methane sulphonate (MS-222). The spinal cord was dissected free from the notochord and mounted ventral side up in a cooled (8–12°C) Sylgard-lined chamber, which was perfused continuously with physiological solution with the following composition (in mM): 138 NaCl, 2.1 KCl, 1.8 CaCl$_2$, 1.2 MgCl$_2$, 4 glucose, 2 HEPES, 0.5 l-glutamine, and 0.5 mg/ml BSA (Sigma, St. Louis, MO). Albumin was added to avoid binding of ω-agatoxin IVA (ω-Aga) and ω-conotoxin GVIA (ω-CgTx) to the perfusion tubes. The physiological solution was bubbled with O$_2$, and the pH was adjusted to 7.4 with NaOH. Intracellular recordings were made from gray matter neurons with thin-walled microelectrodes filled with K acetate (4 M). Compound excitatory postsynaptic potentials (EPSPs) were elicited by stimulation of the medial fiber tract on the ventral surface of the spinal cord with a suction electrode. The EPSPs occurred at a constant latency and followed high-frequency (10 Hz) stimulation, suggesting that they were monosynaptic. The stimulated descending fibers could include reticulospinal axons and propriospinal axons. However, excitatory propriospinal axons run more laterally than the reticulospinal axons, have a slower conduction velocity, and evoke EPSPs with a smaller amplitude and without electrical component (Buchanan and Grillner 1987; Dale 1986). The large electrical component of the compound EPSP evoked by stimulation of the medial fiber tract where reticulospinal axons run is characteristic of reticulospinal transmission (Rovainen 1974), suggesting that the compound EPSP was evoked primarily by activation of reticulospinal axons. We will thus refer to the compound EPSP as reticulospinal-evoked EPSP. The axons were stimulated at a frequency of 0.2 Hz to obtain EPSPs with stable amplitudes (Brodin et al. 1994; Krieger et al. 1996; Pieribone et al. 1995). The peak amplitude of EPSPs was measured in control and in the presence of the different agonists and antagonists. Strychnine (5 μM) and D(-)-amino-5-phosphonopentanoic acid (AP5, 100 μM) were added to the perfusion solution to eliminate glycine- and N-methyl-D-aspartate (NMDA)-mediated synaptic transmission, respectively. Intraxonal recordings were made from reticulospinal axons identified by their location and conduction velocity (>2 m/s) to test the effect of mGluR activation on the calcium influx during action potentials. In these experiments TEA (5 mM) and 4-AP (200 μM) were added to the perfusing solution to block K$^+$ channels. Intracellular recordings were made in bridge mode or discontinuous current-clamp mode with an Axoclamp 2A (Axon Instruments, Foster City, CA). Axon Instrument software (pClamp) was used for data acquisition and analysis on a PC computer equipped with an A/D interface (Digidata 1200). Paired t-test was used for statistical comparisons with P < 0.05 taken as expressing significant difference between means.

**Cell dissociation**

Larval lampreys (*Petromyzon marinus*) were used for culture of reticulospinal neurons. The animals were anesthetized, eviscerated, and dissected in cooled, oxygenated physiological solution (described previously). To label descending reticulospinal neurons with fluorescein-coupled dextran amines (FDA), a brain stem/spinal cord preparation was used. FDA was applied to a freshly cut spinal cord, and the preparation was subsequently washed with Ringer to remove excess FDA. After 48 h of transport time at 8°C to allow labeling of reticulospinal neurons, the brain stem/spinal cord was incubated at room temperature in collagenase (1 mg/ml, 30 min; Sigma, St. Louis, MO) and then in protease (2 mg/ml, 45 min; Sigma) diluted in Leibovitz’s L-15 culture medium (Sigma). Thereafter the brain stem/spinal cord was washed and triturated through a sterilized pipette. The supernatant containing the dissociated cells was distributed in 35-mm petri dishes and incubated at 15°C for 1–15 days.

Whole cell recordings were performed from FDA-labeled reticulospinal neurons with an Axopatch 200A patch-clamp amplifier. Neurons were clamped at a holding potential of −90 mV, and the calcium current elicited by 80-ms depolarizing voltage steps to −10 or 0 mV, applied at 10-s intervals. In some experiments the calcium current was studied with a voltage-ramp protocol from −100 to 100 mV in 400 ms, repeated every 15 s. Linear leak and residual capacitive currents were subtracted on-line with a P4 subtraction protocol (4 steps, one-fourth of the test pulse, averaged and scaled for each test pulse). Data acquisition and analysis were performed with pClamp software. The cells were perfused through a gravity-driven system with a solution containing (in mM) 114 NaCl, 10 TEA, 1 KCl, 1.2 MgCl$_2$, 5 CaCl$_2$, 10 glucose, 10 HEPES, and 0.001 TTX, with pH adjusted to 7.6. For whole cell recordings, the pipettes were filled with a solution containing (in mM) 110 CsCH$_3$SO$_3$−, 10 EGTA, 10 glucose, 10 HEPES, 5 MgCl$_2$, 1 CaCl$_2$, 2 ATP, 0.4 GTP, and 8 phosphocreatinine, pH 7.6 adjusted with CsOH. The osmolarity was adjusted to 270 mOsm.

**Drugs**

Stock solutions of (±)Bay K 8644 (Bay K, RBI, Natick, MA) and nimodipine (RBI) were prepared as 10-mM stock solutions in ethanol and stored at −20°C. ω-CgTx (Peptides Institute, Barnet, UK) and ω-Aga (Peptides Institute) were prepared as 0.1-mM stocks in distilled water and stored at −20°C. L-AP4 (Tocris Cookson, Bristol, UK) was prepared as 100-mM stock in 100 mM NaOH and stored at −20°C. Cadmium (Cd$^{2+}$; Sigma, St. Louis, MO) was freshly prepared during each experiment. The different drugs were added to the perfusing solution. In experiments in which the intact spinal cord was used, the chamber volume was ~1.5 ml, and the flow rate of the perfusion was 1 ml/min and was kept constant throughout the experiment.

**RESULTS**

The subtypes of high-voltage activated (HVA) calcium channels involved in synaptic transmission from reticulospinal to spinal cord neurons were analyzed as well as the possibility of a presynaptic modulation of calcium channels accounting for the group III mGluR-mediated depression of reticulospinal synaptic transmission.

**Presynaptic calcium channel subtypes involved in transmitter release from reticulospinal axons**

The effect of the specific N-type antagonist ω-CgTx was tested on the amplitude of compound EPSPs (n = 6). In the spinal cord neuron shown in Fig. 1, application of 1 μM ω-CgTx markedly reduced the amplitude of the chemical component of the EPSP (66 ± 2%; results are expressed as means ± SD) without further decrease at a higher concentration of the toxin (3 μM; 66.5 ± 3%, Fig. 1, A and B). The remainder of the chemical component of the EPSP was completely blocked by cadmium (200 μM), and the electrical component remained unchanged (Fig. 1, A and B). The resting membrane potential and the input resistance of the postsynaptic neuron (as judged by the constancy of the electrotonic component of the EPSP) were not altered by ω-CgTx or any of the other calcium channel agonists and antagonists tested in this study. These results indicate that N-type channels are present.
in the synaptic sites of reticulospinal axons and that they play a role in mediating transmitter release.

Blockade of P/Q-type channels with \( \omega \)-Aga (200 nM) also reduced the amplitude of the EPSP (Fig. 2, A and B), the chemical component of which was completely blocked by cadmium (200 \( \mu \)M). L-type channels thus do not contribute to synaptic transmission at reticulospinal synapses.

**Effect of combined application of \( \omega \)-Aga and \( \omega \)-CgTx on reticulospinal synaptic transmission**

To determine if synaptic transmission from reticulospinal axons is only supported by calcium influx through N- and P/Q-type channels, the effect of simultaneous blockade of these channels was tested. Combined application of \( \omega \)-Aga and \( \omega \)-CgTx caused a more pronounced depression of the EPSP compared with that of either blocker applied alone. In the experiment shown in Fig. 4, application of \( \omega \)-Aga (200 nM) reduced the amplitude of the EPSP. Subsequent application of \( \omega \)-Aga and \( \omega \)-CgTx (1 \( \mu \)M) further depressed the amplitude of pyridine agonist Bay K (2 \( \mu \)M; \( n = 5 \)) changed the amplitude of the EPSP (Fig. 3, A and B), the chemical component of which was completely blocked by cadmium (200 \( \mu \)M). L-type channels thus do not contribute to synaptic transmission at reticulospinal synapses.
The possibility of an inhibition of the calcium current by L-AP4 was further analyzed on somata of identified reticulospinal neurons in culture. Whole cell patch-clamp recordings were performed from FDA-prelabeled reticulospinal neurons, and the effect of L-AP4 was tested on the calcium current (n = 6). L-AP4 (100 μM) had no effect on the amplitude of the HVA calcium current elicited by test voltage steps to 0 mV from a holding potential of −90 mV (Fig. 6, A and B). The I-V plot (Fig. 6C) shows that L-AP4 did not affect the low-voltage calcium current, activated at −50 mV, or the HVA calcium current activated by voltage steps to different potentials. To determine if activation of group III mGluRs affects calcium influx during action potentials in the reticulospinal axons, intra-axonal recordings were performed, and the effect of L-AP4 was tested on the calcium component of action potentials elicited in the presence of the potassium channel blockers TEA and 4-AP. The calcium component of action potentials recorded in the reticulospinal axons was not affected by L-AP4 (Fig. 6D; n = 3). These results provide evidence that L-AP4 does not affect calcium influx through voltage-activated channels at the soma or the axon of reticulospinal neurons.

DISCUSSION

Reticulospinal transmission is mediated primarily by N-type calcium channels and to a lesser degree by P/Q- and R-type calcium channels

Calcium influx through N-type channels represents the majority (68%) of the total calcium current in somata of spinal neurons (El Manira and Bussières 1997) and reticulospinal neurons (El Manira and Bussières, unpublished). P/Q-type calcium channels represent −6% of the total calcium current in the soma of spinal neurons (El Manira and Bussières 1997). The chemical component of the EPSP was strongly reduced by 1 μM ω-CgTx (67%) and was also decreased by 200 nM ω-Aga (27%). The combined application of these two blockers was never able to completely block the chemical component of the EPSPs (84.5% reduction), demonstrating that synaptic transmission from reticulospinal axons also involves calcium entering through other channel types, presumably corresponding to R-type channels described in other systems. Our results thus show that calcium influx through N-type channels contributes predominantly to transmitter release at the reticulospinal synapse. P/Q- and R-type channels also contribute to trans-
input resistance of the postsynaptic neurons was not changed by the calcium channel antagonists as shown by the constancy of the electrotonic component of the EPSP (Figs. 1, 2, 4, and 5). Third, \( \omega \)-CgTx could possibly depress the EPSP amplitude by changing the conductance of the postsynaptic receptor channels mediating the EPSP. This seems unlikely, however, because it has previously been shown that \( \omega \)-CgTx does not change the amplitude of EPSP mediated by activation of non-NMDA receptors (Pfrieger et al. 1992) or the amplitude of glutamate-induced depolarization (Gruner and Silva 1994), which argues against an effect of \( \omega \)-CgTx on postsynaptic \( \alpha \)-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors. Furthermore, \( \omega \)-Aga seems not to affect kainate-induced inward current in Purkinje cells (Regehr and Mintz 1994). This strongly suggests that the decrease of the EPSP amplitude by \( \omega \)-CgTx and \( \omega \)-Aga is mediated by an effect on presynaptic calcium current. We thus conclude that reticulospinal synaptic transmission is mediated primarily by calcium influx through presynaptic N-type channels and to a lesser extent through P/Q- and R-type channels.

In many regions of the mammalian brain, extensive studies analyzed the role of the different calcium channel subtypes in supporting synaptic transmission (Dunlap et al. 1995; Reuter 1996). This study is the only one available regarding the function and modulation of presynaptic calcium channel terminals of descending reticulospinal neurons in the spinal cord. In the \textit{Xenopus} embryo, propriospinal inhibitory transmission is largely mediated by N-type calcium channels (Wall and Dale 1994). In the rat spinal cord propriospinal inhibitory transmission is supported by P/Q- and to lesser extent by N-type calcium channels (Takahashi and Momiyama 1993). Synaptic transmission between ventral horn neurons in culture was shown not to be affected by \( \omega \)-CgTx, whereas that of DRG neurons was reduced (\( \sim 50\% \)) (Yu et al. 1992).

\textbf{L-type channels do not mediate reticulospinal transmission}

Neither the dihydropyridine agonist Bay K nor the antagonist nimodipine affected the amplitude of the EPSPs evoked by stimulation of reticulospinal axons. L-type channels are present in somata of spinal neurons and represent \( \sim 12\% \) of the total HVA current (El Manira and Bussières 1997). They are strongly (\( > 200\% \)) potentiated by Bay K at low-voltage steps. The lack of effect of dihydropyridines on the monosynaptic EPSPs indicates that L-type channels do not contribute to release of transmitter evoked by single action potentials. However, the possibility that presynaptic L-type channels may be involved in high-frequency–induced transmission cannot be ruled out. Such an analysis was precluded because of the strong depression of synaptic transmission and the depolarization of the membrane potential occurring during high-frequency activation of the reticulospinal synapse (see Pieribone et al. 1995; Shupliakov et al. 1997). Synaptic transmission in hippocampus (Takahashi and Momiyama 1993; Wheeler et al. 1994; Wu and Sagau 1994) and cerebellum (Regehr and Mintz 1994; Takahashi and Momiyama 1993) is not affected by dihydropyridine agonists and antagonists. L-type calcium channels have however been shown to support synaptic transmission from bipolar cells of the goldfish retina. In these neurons dihydropyridine antagonists block the presynaptic calcium current, which results in a depression of synaptic transmission (Tachibana et al. 1993).
Conversely, Bay K increases both presynaptic calcium levels and the amplitude of the postsynaptic current, whereas blockade of N-type calcium channels has no effect.

**mGluR-mediated presynaptic inhibition does not involve modulation of presynaptic calcium channels**

The depression of reticulospinal transmission by L-AP4 persisted after blockade of N-type calcium channels. L-AP4 could, however, mediate presynaptic inhibition through an action on P/Q- and/or R-type calcium channels. This seems unlikely because L-AP4 can depress the EPSP amplitude to a larger degree than that obtained by a block of P/Q- and R-type calcium channels (see Krieger et al. 1996). This suggests that the previously reported mGluR-mediated presynaptic inhibition of reticulospinal axons (Krieger et al. 1996) is not mediated through inhibition of presynaptic calcium channels. L-AP4 also had no effect on the calcium current recorded in the somata of reticulospinal neurons or on the calcium component of action potentials in reticulospinal axons (Fig. 6). In a recent publication, Cochilla and Alford (1998) also reported that mGluR agonists did not affect the presynaptic calcium current. Thus mGluR-mediated presynaptic inhibition of reticulospinal transmission does not appear to involve inhibition of calcium influx during action potentials.

Activation of mGluRs has been shown to inhibit the calcium current in neurons from different regions of the brain. In hippocampus neurons, mGluRs inhibit N-, L-, and P/Q-type calcium channels (Sahara and Westbrook 1993; Wheeler et al. 1994). In cortical neurons, group I and II (Choi and Lovinger 1996) and group III mGluRs (Stefani et al. 1998) have been shown to inhibit both N- and P/Q-type channels. Furthermore, in the calyx of Held, L-AP4 decreases transmitter release by inhibiting presynaptic calcium influx via P/Q-type channels (Takahashi et al. 1996). This is in contrast to the findings in the lamprey spinal cord where mGluRs may be acting directly on the release process and/or by affecting other channels (Cochilla and Alford 1998; this study). An effect of mGluRs on the release machinery has been shown both in hippocampus and cerebellum, where activation of mGluRs reduced the frequency...
but not the amplitude of miniature synaptic events (Llano and Marty 1995; Poncer et al. 1995).

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REFERENCES


