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Localization of L-type calcium channel $Ca_V1.3$ in cat lumbar spinal cord – with emphasis on motoneurons

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Abstract

Voltage-dependent persistent inward currents (PICs) which underlie the plateau potentials are an important intrinsic property of spinal motoneurons. Electrophysiological experiments have indicated that a subtype of the low threshold L-type calcium channel, $Ca_V1.3$, mediates this current. In mouse and turtle lumbar spinal cord it has been shown that these channel proteins are mainly found on motoneuron dendrites. In the present study we have used immunohistochemistry to locate these channels in lumbar spinal neurons, especially motoneurons, of the cat. The results indicate that $Ca_V1.3$ immunoreactivity was unevenly distributed among the laminae of the spinal grey matter. The small neurons in superficial dorsal horn (laminae I–III) were sparsely and weakly labelled, while large neurons in ventral horn were frequently and densely labelled. Groups of motoneurons in lamina IX that were immunoreactive to choline acetyltransferase also co-expressed $Ca_V1.3$. The immmunoreactivity was mainly associated with neuronal somata and proximal dendrites. Double staining with antibodies against $Ca_V1.3$ and MAP2 (a dendritic marker) showed that some fine fibres, which may include distal dendrites, were also labelled. These results in the cat spinal cord show some differences from studies in mouse and turtle motoneurons where the immunoreactivity against this channel was mainly localized to the dendrites.

Keywords: Lumbar spinal cord; Motoneuron; Persistent inward currents; Plateau potential; L-type calcium channel; Immunohistochemistry

Voltage-dependent persistent inward currents (PICs) make important contributions to the intrinsic properties of spinal motoneurons [7,11,22,23]. These PICs manifest themselves as "plateau potentials" and may cause self-sustained firing following brief activation of the motoneurons. In a physiological context these PICs are assumed to provide amplification of the normal synaptic input to the motoneurons. Several investigations have demonstrated that nifidepine-sensitive L-type calcium channels are responsible for a major part of the PICs in turtle [9], mouse [3], and rat motoneurons [15]. Electrophysiological evidence suggests that these PICs are located mainly at dendritic regions, a strategic position to amplify synaptic inputs [2,3,5,6,10,12,25].

L-type calcium channels are encoded by *Ca_V1*-genes and include four different subtypes referred to as *$Ca_V1.1$–$Ca_V1.4$* [16]. Only *$Ca_V1.2$* and *$Ca_V1.3$* are expressed in the central nervous system, including the spinal cord [3,4,8,13,24,27]. Electrophysiological properties of the motoneuron PICs suggest that they depend on $Ca_V1.3$ channels [7]. Initial immunohistochemical studies in rats using antibodies against the $Ca_V1.3$ channel protein (produced in Catterall's laboratory) demonstrated immunoreactivity mainly in the region of the somata and proximal dendrites of spinal motoneurons [27]. However, in recent studies in mice [3,13] and turtles [24], the distal dendritic regions of spinal motoneurons have shown strong immunoreactivity for $Ca_V1.3$ channels. Since many of the studies on the electrophysiological aspects of PICs in spinal motoneurons are performed in the cat, and since there may be species differences in the neuronal $Ca_V1.3$ channel distribution, the aim of the present study was to extend these immunohistochemical studies to cat motoneurons.

All experiments were conducted in accord with the guidelines of *EU Directive 86/609/EEC* and were approved by the Danish Council for Animal Experiments. Four adult cats (2.5–4.5 kg body weight) were used in this study. Three animals

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were anaesthetized with isoflurane (2.0–2.5%) and decerebrated by either ligating the basilar and both common carotid arteries (anaemic decerebration) or by a coronal knife lesion between the cranial and caudal colliculi (intercollicular decerebration with removal of the rostral part). Electrophysiological recording was obtained from cervical or lumbar motoneurons for 10–20 h in each of these animals (for detailed experiment protocol, see [12]). Subsequently, the animals were perfusion fixed for immunohistochemical analysis (see below). One cat was used only for immunohistochemical analysis. Anaesthesia was induced in this animal by isoflurane followed by Nembutal (50 mg/kg body weight, i.p.). Perfusion fixation was performed transcardially with 0.9% saline containing 7500 IU/L heparin followed by 2 L 0.1 M cold phosphate buffered-saline (PBS) containing 4% paraformaldehyde, 0.36% L-lysine and 0.05% sodium *m*-periodate. The whole spinal cord was then removed immediately and postfixed in the same fixative solution overnight at 4 °C. The next day, the spinal cord was dissected into smaller segments and cryoprotected in PBS with 30% sucrose up to 48 h at 4 °C. Selected segments from the lumbar spinal cord (L4–L7) were cut transversely into 40 μm-thick sections using a sliding microtome.

Every fifth transverse section was processed for $Ca_V1.3$ immunohistochemistry. Rabbit anti-$Ca_V1.3$a polyclonal antibody (AM9742) was raised against a segment of C-terminus of a long-spliced variant of rat neuronal L-type calcium channel subunit $Ca_V1.3$a, LDC5, and affinity purified on a Sepharose-conjugated LDC6 peptide (for details see [28]). The specificity of this antibody was verified by Western Blot using cat spinal tissues (data not shown).

Tissue sections were rinsed in 0.1M PBS for 10 min and then incubated in 0.3% H_2O_2 in PBS for 30 min. To minimize non-specific staining, the sections were preincubated in PBS containing 0.3% Triton X-100 (PBS-T), 2% bovine serum albumin (BSA) and 5% normal goat serum (NGS) for 1 h. The sections were then incubated in the same solution containing primary rabbit anti-$Ca_V1.3$a antibody (1:500) for 2–3 days at 4 °C. The sections were sequentially incubated with biotinylated goat anti-rabbit IgG (1:500; Dako, Denmark) in PBS-T with 1% BSA and 2% NGS and avidin–biotin complex (ABC, 1:100; Vector Labs, Burlingame, CA) in PBS-T for 1 h each. Finally, the sections were incubated for 5–10 min in 0.05 M Tris buffer (pH 7.5) containing 0.04% diaminobenzidine tetrahydrochloride and 0.01% H_2O_2. Following this, they were dried, cleared and coverslipped.

To identify motoneurons, neuronal dendrites and $Ca_V1.3$ channels in the spinal cord, double-immunostaining was carried out using goat anti-choline acetyltransferase (ChAT) polyclonal antibody (1:100) or mouse anti-MAP2 (microtubule-associated proteins) monoclonal antibody (1:500; both antibodies were from Chemicon, Temecula, CA) and rabbit $Ca_V1.3$a antibody. $Ca_V1.3$-immunolabelling was performed first, as described above, up to the ABC incubation. After washing with PBS-T, the sections were then incubated in biotinylated tyramide (1:500, TSA indirect; NEN Life Science Products) in PBS containing 0.005% H_2O_2 for 5 min followed by washing with PBS and incubation in streptavidin-Alexa Fluor 488 (1:100) in PBS-T for 1 h. The sections were then washed and incubated in goat anti-ChAT or mouse anti-MAP2 antibodies in PBS-T with 2% BSA and 5% normal donkey serum (NDS) or NGS overnight at 4 °C. Subsequently the sections were incubated in donkey anti-goat Alexa Fluor 568 or goat anti-mouse Alexa Fluor 568 (1:100–200) in PBS-T with 1% BSA and 2% NDS or NGS for 1 h. All fluorescent-labelled secondary antibodies were from Molecular Probes (Eugene, OR). The sections were mounted with Fluorescent Mounting Medium (Dako, Denmark).

Transverse sections adjacent to those immunostained for $Ca_V1.3$ were stained with thionine to reveal the general cytoarchitecture of the spinal cord. Control immunohistochemical staining was done using the same procedures as described above except that either the primary antibodies were omitted or the primary antibodies were absorbed with the antigen sequence against which the antibody was raised. No specific staining was present in these control sections.

The sections were observed with a conventional light, epifluorescence microscope (Axioplan2, Zeiss, Germany) or a laser scanning confocal microscope (Leica TCS SP2 system) equipped with argon and helium-neon lasers. Images were captured digitally (AxioCam camera, Zeiss, Germany) and processed with Adobe Photoshop CS2 (version 9.0).

The $Ca_V1.3$a staining pattern was the same for all four cats; no differences were seen between the three cats that were perfused after electrophysiological studies and the cat that was perfused directly. $Ca_V1.3$a immunoreactive-like ($Ca_V1.3$a-IR) neurons were seen throughout the lumbar spinal grey matter (Fig. 1A). No axon-like profiles were labelled in the grey matter. No dendrites, axons or glial cells were labelled in the white matter except for those associated with an occasionally labelled neuron.

The frequency and intensity of labelled neurons varied depending on the Rexed lamina of the grey matter. The frequency increased gradually from the superficial dorsal horn to the ventral horn (Fig. 1). When compared with adjacent Nissl stained sections, only a small fraction of neurons in laminae I–III were labelled (Fig. 1A–C). Whereas many neurons were labelled in laminae IV–VI and X, nearly all neurons were labelled in laminae VII–IX (Fig. 1A and B). The $Ca_V1.3$a labelled neurons may include projection neurons, interneurons and motoneurons. Some neurons in lamina I and certain large neurons in laminae IV–VI might be projection neurons (Fig. 1C and D). Most ChAT-negative neurons in laminae I–VII are likely to be interneurons, while ChAT-positive neurons in laminae VIII and IX are probably motoneurons (Fig. 2A1–A3). The intensity of $Ca_V1.3$a-IR also varied among neurons located in different laminae. The large neurons in lamina IX, probably motoneurons, were the most intensely labelled neurons. Some relatively large neurons in the deep dorsal horn (laminae IV–VI) and in the region surrounding the central canal were also strongly labelled.

In the large (moto)neurons the immunoreactivity of the dendrites could be traced up to 100–200 μm along the proximal dendrites. As the labelling pattern was punctate it was difficult to follow the dendrites distally. However, there were indeed quite a few small $Ca_V1.3$a-IR dots scattered within motoneuronal area in lamina IX (Fig. 1E, 2B1, 3A1). Previous