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Research report

Clinically relevant concentration of pregabalin has no acute inhibitory effect on excitation of dorsal horn neurons under normal or neuropathic pain conditions: An intracellular calcium-imaging study in spinal cord slices from adult rats

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ABSTRACT

Pregabalin is thought to exert its therapeutic effect in neuropathic pain *via* binding to α2δ−1 subunits of voltage-gated calcium (Ca^{2+}) channels. However, the exact analgesic mechanism after its binding to α2δ−1 subunits remains largely unknown. Whether a clinical concentration of pregabalin (≈10 μM) can cause acute inhibition of dorsal horn neurons in the spinal cord is controversial. To address this issue, we undertook intracellular Ca^{2+}-imaging studies using spinal cord slices with an intact attached L5 dorsal root, and examined if pregabalin acutely inhibits the primary afferent stimulation-evoked excitation of dorsal horn neurons in normal rats and in rats with streptozotocin-induced painful diabetic neuropathy. Under normal conditions, stimulation of a dorsal root evoked Ca^{2+} signals predominantly in the superficial dorsal horn. Clinically relevant (10 μM) and a very high concentration of pregabalin (100 μM) did not affect the intensity or spread of dorsal root stimulation-evoked Ca^{2+} signals, whereas an extremely high dose of pregabalin (300 μM) slightly but significantly attenuated Ca^{2+} signals in normal rats and in diabetic neuropathic (DN) rats. There was no difference between normal rats and DN rats with regard to the extent of signal attenuation at all concentrations tested. These results suggest that the activity of dorsal horn neurons in the spinal cord is not inhibited acutely by clinical doses of pregabalin under normal or DN conditions. It is very unlikely that an acute inhibitory action in the dorsal horn is the main analgesic mechanism of pregabalin in neuropathic pain states.

1. Introduction

Pregabalin and gabapentin are analgesic agents prescribed routinely for neuropathic pain due to conditions such as diabetic neuropathy, and post-herpetic neuralgia (Dworkin et al., 2003; Rice and Maton, 2001; Rosenstock et al., 2004). The α2δ−1 subunit of voltage-gated calcium (Ca^{2+}) channels (VGCCs) in the central nervous system (CNS) is a high affinity-binding site for these gabapentinoids (Bian et al., 2006; Field et al., 2006). This type of binding is considered to be a prerequisite for their therapeutic action (Field et al., 2006). However, the exact analgesic mechanisms after binding of gabapentinoids to α2δ−1 subunits are largely unknown. Classically, it was thought that these compounds act acutely on VGCCs expressed at primary afferent terminals in the dorsal horn of the spinal cord and reduce release of excitatory neurotransmitters, thereby acutely inhibiting the excitability of dorsal horn neurons. Nevertheless, the cellular mechanisms underlying their mode of action are incompletely understood because high-dose gabapentin (1 mM) does not significantly affect the kinetics or function of Ca^{2+} channels in dorsal root ganglionic neurons of adult rats (Dooley et al., 2007; Hendrich et al., 2008). Several electrophysiological studies have reported differing results with regard to the effects of pregabalin and gabapentin on synaptic transmission in the dorsal horn. For example, Moore et al. (2002) reported that gabapentin has no acute presynaptic inhibitory effect on identified primary afferent Aδ- and C-fiber terminals in adult rats. Other scholars have reported acute presynaptic inhibitory effects in normal immature rats or young mice (Matsuzawa et al., 2014; Shimoyama et al., 2000). Surprisingly, a presynaptic inhibitory effect of gabapentin was reported to occur under hyperalgesic conditions due to diabetic neuropathy, but not under normal conditions (Patel et al., 2000). In addition to these suggested acute effects, some convincing chronic actions of gabapentinoids have been reported. To this end, gabapentin and pregabalin can enter cells *via* the L-amino acid transporter system, and act

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intracellularly on components of the vesicular exocytotic process, leading to inhibition of VGCC trafficking to primary afferent terminals in the dorsal horn (Bauer et al., 2009; Hendrich et al., 2008). Furthermore, gabapentinoids may inhibit abnormal excitatory synaptogenesis after injury to peripheral nerves by binding to the $\alpha 2\delta-1$ subunit, which is also a binding site of neuronal thrombospondin secreted from astrocytes (Christopherson et al., 2005; Eroglu et al., 2009). Thus, gabapentinoids seem to have various target sites for their chronic action. More support for the chronic (rather than acute) action of these compounds has been provided because typically it takes at least several days for pregabalin to reveal its analgesic effect in patients suffering neuropathic pain (Freeman et al., 2008; Stacey et al., 2008), even though plasma and cerebrospinal fluid (CSF) concentrations of pregabalin peak within a few hours after its oral administration (Buvanendran et al., 2010). Collectively, these clinical observations (together with the results of some basic research studies) argue against the acute inhibitory effects of pregabalin in the dorsal horn of the spinal cord being its main analgesic mechanism.

Intracellular Ca^{2+}-imaging is widely used to monitor the action potential activity in neuronal tissues (Grienberger and Konnerth, 2012; Mao et al., 2001). We have developed a Ca^{2+}-imaging method to study gross neuronal activity in the spinal cord of adult rats. Using this method, we examined whether pregabalin applied at clinically relevant concentrations can acutely inhibit the extent and spatiotemporal spread of excitation in the dorsal horn neurons of normal rats, and of rats with painful diabetic neuropathy.

2. Results

2.1. Effect of streptozotocin injection on blood glucose and nociceptive thresholds

In normal saline-injected rats (hereinafter referred to as "normal rats") (n =50), blood glucose remained unchanged throughout the entire experimental period (Fig. 1A). Repeated-measures two-way ANOVA for blood glucose concentrations revealed a significant main effect of streptozotocin ($F_{1,98}=1791$, $P<0.0001$) as well as a significant main effect of time after injection ($F_{2,196}=825.4$, $P<0.0001$). Moreover, there was a significant interaction effect between treatment and time after injection ($F_{2,196}=865.1$, $P<0.0001$). As shown in Fig. 1A, *post hoc* analysis indicated that rats treated with a single injection of streptozotocin (60 mg/kg i.p.) (n=50) developed hyperglycemia that was evident on the third day after injection and continued to the final experiment of spinal cord extraction and slice preparation [($P<0.0001$ versus normal rats, (degrees of freedom, df=294) and respective baseline (df=196,), Sidak's multiple comparisons test following repeated-measures two-way ANOVA]. In accordance with a previous study (Malcangio and Tomlinson, 1998), streptozotocin-injected diabetic rats showed a significant increase in mechanical sensitivity, and thus hereinafter are referred to as "diabetic neuropathic" (DN) rats. Repeated-measures two-way ANOVA for withdrawal thresholds revealed a significant main effect of streptozotocin ($F_{1,98}=590.8$, $P<0.0001$) as well as a significant main effect of time after injection ($F_{4,392}=123$, $P<0.0001$). Moreover, there was a significant interaction effect between treatment and time after injection ($F_{4,392}=198.3$, $P<0.0001$). As revealed by *post hoc* analysis, differences in thresholds for paw withdrawal between DN (n=50) and normal (n=50) rats became significant 2 weeks after injection ($P<0.0001$, versus normal rats, Sidak's multiple comparisons test following repeated-measures two-way ANOVA, df=490, Fig. 1B), and this mechanical hypersensitivity continued throughout the experimental period ($P<0.0001$, versus respective baseline, Sidak's multiple comparisons test following repeated-measures two-way ANOVA, df=392, Fig. 1B). Thus, compared with normal controls, DN rats used for in vitro experiments as a model of painful neuropathy displayed hyperglycemia and clear signs of hypersensitivity.

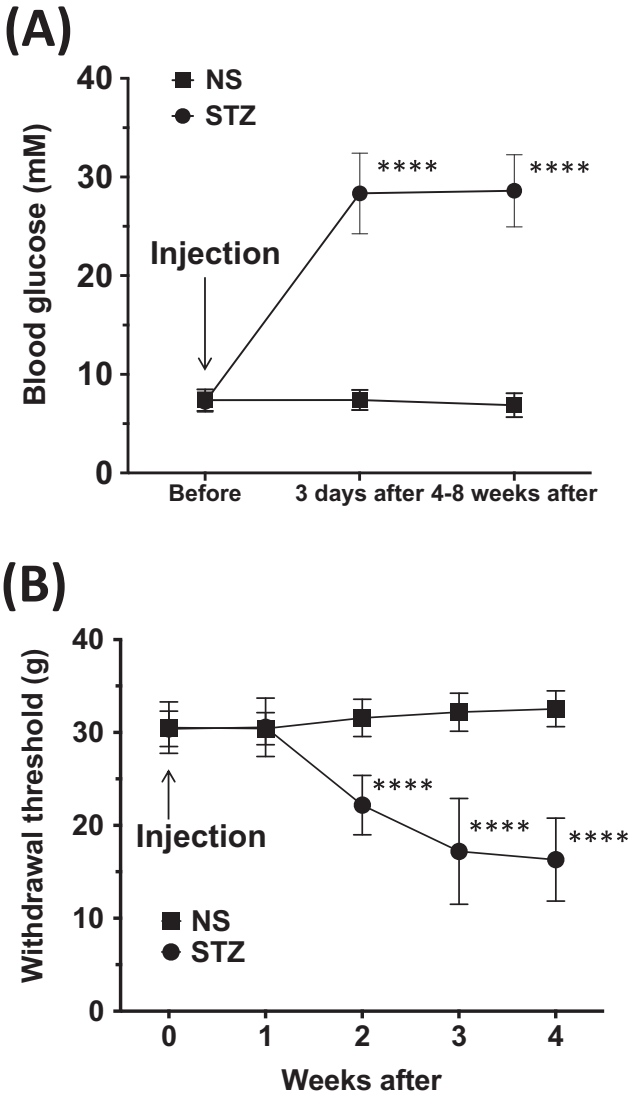

Fig. 1. Effect of normal saline (NS) or streptozotocin (STZ, 60 mg/kg, i.p.) injection on (A) blood glucose concentrations and (B) hind-paw withdrawal thresholds in Wistar rats (n=50 for both groups). (A) Diabetes mellitus was confirmed 3 days after the injection of streptozotocin. (B) Hind-paw withdrawal thresholds were reduced significantly in diabetic rats starting from the second week after injection. ****$P<0.0001$, compared with normal rats and the respective baseline. Data are the mean ± SD; Sidak's multiple comparisons test following repeated-measures two-way ANOVA.

2.2. Dorsal root stimulation-evoked A- and C-fiber-mediated Ca^{2+} signals in the dorsal horn (intracellular Ca^{2+}-imaging)

Intensity and spread of neural excitation in the dorsal horn were visualized using intracellular Ca^{2+} fluorescence signals. Single electrical stimulation of the dorsal root at C-fiber intensity evoked a biphasic change in optical signals mainly in the medial-half of lamina II (SG) and lamina III. The initial phase was due to the input *via* primary afferent A ($A\delta$)-fibers, and the next phase (after a delay of 20–30 ms) was due to the input *via* A- and C-fibers. Excitation started at the medial-third of SG and extended laterally (to the middle-third of SG) and ventrally (to lamina III) (Fig. 2, Video data). Most intense signals were observed in the medial-third of SG, so this location was used for evaluation of the effect of pregabalin on the intensity of neural excitation. Middle-