Ziconotide Inhibits KCL-Induced Increase in Intracellular Calcium in Rat Sensory Neurons: A Peripheral Mechanism of Analgesic Action

Zikonotid Sıçan Duyusal Nöronlarında KCL ile İndüklenen Hücre İçi Kalsiyum Artışını İnhibe Eder: Analjezik Etkinliğin Bir Periferal Mekanizması

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ABSTRACT Objective: Ziconotide, a novel non-opioid intrathecal analgesic agent with higher analgesic activity than morphine, has been recently approved for the treatment of severe and cronic pain. However, the exact cellular mechanism of the analgesic effects of ziconotide is not well documented. The aim of this study was to investigate the effects of ziconotide on KCl-induced intracellular calcium ($[Ca^{2+}]_i$) signaling in cultured rat dorsal root ganglion (DRG) neurons. **Material and Methods:** DRG neurons were grown in primary culture, following enzymatic and mechanical dissociation of ganglia from 1 or 2-day-old Wistar rats. DRG neurons were loaded with calcium-sensitive dye Fura-2 AM (1 μmol). Initialy calcium-sensitive dye loaded cells were stimulated with application of KCl (30 mM) alone (control). Then, ziconotide (1 nM,10 nM and 1 μM)+KCl were applied. Finally, these cells were stimulated with application of KCl again and intracellular calcium ($[Ca^{2+}]_i$) responses were quantified by the changes in the ratio of 340/380 nm using fluorescence calcium imaging system. **Results:** Ziconotide [1 nM (n=17), 10 nM (n=23) and 1 μM (n=37)] dose-dependently reduced the increase of $[Ca^{2+}]_i$ which was elicited by 30 mM KCl (high K^+) in a rather irreversible manner. Moreover, pretreatment with 1 μM ziconotide effectively prevented $[Ca^{2+}]_i$ response to the high K^+. **Conclusion:** It was concluded that ziconotide reduced the membrane depolarisation-induced increase in $[Ca^{2+}]_i$, in concentration dependent and a partially irreversible manner in rat DRG neurons, providing evidence for peripheral antinociceptive action of this drug. The effects on $[Ca^{2+}]_i$ may indicate that ziconotide reduces release of pro-nociceptive neurochemicals in the peripheral pain pathway.

Key Words: Pain; ganglia, spinal; calcium; ziconotide

ÖZET Amaç: Zikonotid; şiddetli ve kronik ağrının tedavisi amacıyla son günlerde onaylanan, opioid ilaç olmayan, intratekal olarak uygulanan ve morfinden daha etkili yeni bir analjezik ajandır. Ancak zikonotidin analjezik etkilerinin kesin hücresel mekanizması tüm açıklığıyla ortaya konulmamıştır. Bu çalışmanın amacı zikonotidin kültüre edilmiş sıçan dorsal kök gangliyonu (DKG) nöronlarında KCl'ün indüklediği hücre içi kalsiyum sinyalleşmesi üzerine etkisinin incelenmesiydi. **Gereç ve Yöntemler:** DKG nöronlarının primer kültürü, 1 veya 2 günlük Wistar cinsi sıçanlardan enzimatik ve mekanik ayrıştırma yoluyla elde edildi. DKG nöronları kalsiyum duyarlı boya olan Fura-2 AM (1 μmol) ile yüklendi. İlk olarak kalsiyum duyarlı boya ile yüklenen hücreler, KCl'nin (30 mM) yalnız başına uygulanmasıyla uyarıldı (kontrol). Daha sonra zikonotid (1 nM,10 nM ve 1 μM)+KCl uygulandı. Son olarak bu hücreler KCl uygulaması ile tekrar uyarıldı ve hücre içi kalsiyum ($[Ca^{+2}]_i$) cevapları, flüoresan kalsiyum görüntüleme sistemi vasıtasıyla 340/380 nm oransal değişimi kullanılarak belirlendi. **Bulgular:** Zikonotid [1 nM (n=17), 10 nM (n=23) ve 1 μM (n=37)], 30 mM KCl (yüksek K^+) ile indüklenen $[Ca^{+2}]_i$ artışını doz bağımlı olarak geri dönüşümsüz bir şekilde azalttı. Aynı zamanda 1 μM zikonotidin ön muamele şeklinde uygulanması yüksek K^+'nın meydana getirdiği $[Ca^{+2}]_i$ artış cevabını engelledi. **Tartışma:** Zikonotidin sıçan DKG nöronlarında KCl'nin neden olduğu $[Ca^{+2}]_i$ artışını konsantrasyon bağımlı azaltması ve bu etkiyi kısmen geri dönüşümsüz olarak meydana getirme bulgusu, bu ilacın periferal antinosiseptif etki mekanizmasına yönelik kanıt olarak öne sürülebilir. $[Ca^{+2}]_i$ üzerinde meydana gelen bu etkiyle, zikonotid periferal ağrı yolaklarındaki pro-nosiseptif nörokimyasalların salınımı azaltabilir.

Anahtar Kelimeler: Ağrı; gangliyonlar, spinal; kalsiyum; zikonotid

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"Pain is an unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage" (definition promoted by the International Association for the Study of Pain).[1] Pain sense plays a critical role in protective responses of body to injury or harmful stimuli.

Following the excitation of peripheral pain receptors (nociceptors), pain information is transmitted from peripheral nociceptors to higher levels of the central nervous system through pain pathways involving dorsal root ganglion (DRG) neurons, spinal cord and brainstem.[2,3] Complex cellular mechanisms including release of proalgesic agents through variety of mechanisms including the intracellular calcium signaling are involved in the process of spinal nociceptive transmission.[4,5]

Primary sensory neuronal cell bodies (DRG and trigeminal neurons) are present in the pathway for afferent nociceptive signals. It has been shown that following tissue injury, the expression and function of ligand and voltage-gated ion channels in DRG neurons are altered as a process of protective mechanisms including alarming state through pain signal. Thus, making ion channels and signaling in these peripheral sensory neurons as one of the best molecular target for identifying induction and transmission of pain signals, and also for development of progressive antagonists as analgesics.[5-7]

Currently, the most commonly used drugs for pain relief include opioid analgesics and nonsteroidal anti-inflammatory drugs (NSAIDs).[8] However, long-term use of these drugs often cause serious side effects, ineffectiveness and addiction. The search for better analgesics has never halted and numoreous classes of agents including antiepileptic and antidepressants have been determined to be effective clinically as adjunctive drugs for the relief of chronic pain, and in particular, neuropathic pain.[9,10] In progressed stage of intense painful conditions when opioids failed to provide sufficient analgesy, the patients are exposed to invasive therapies, such as spinal cord stimulation or intrathecal drug administration.

Ziconotide is the synthetic equivalent of ω-MVIIA toxin of the fish-hunting marine snail, Conus magus.[11] Intrathecal ziconotide has been established to block N-type calcium channels as the first new, proven mechanism for the curing of chronic pain.[11] Ziconotide, a state-independent $Ca_v2.2$ (N-type) blocker, was approved in the United States in 2004, approval in the European Union was than followed, for the management of severe chronic pain in patients who were refractory to systemic analgesics, adjunctive therapies, or intolerant.[12]

Despite the clarity of its effect on calcium channels, the exact mechanism of the analgesic effects, and possible peripheral involvement of the ziconotide effect is not well documented. Hence the aim of this study was to investigate the actions of the novel nonopioid intrathecal agent ziconotide on KCl-induced calcium signaling in isolated rat sensory neurons using calcium imaging.

MATERIAL AND METHODS

PREPARATION AND PRIMARY CULTURE OF RAT DRG NEURONS

Protocols of this study was approved by the Animal Ethical Committee of the University of Firat and the study was carried out according to "Guide for the Care and Use of Laboratory Animals (www.nap.edu/ca ta log/5140.html). Primary cultures of rat sensory neurons from dorsal root ganglia were used in this study, as described previously.[13] Briefly, DRG cells were obtained from Wistar rats (aged 1-2 days, either sex, n=9) following decapitation. Their spinal cords were removed, and DRGs at the cervical, thoracic, lumbar and sacral levels were removed (~45-50/pup) and temporarily collected in culture medium containing neurobasal-A medium with B27 (Invitrogen, Paisley, UK), 5 mM glutamine (Invitrogen, Paisley, UK), and penicillin (5000 IU/mL)-streptomycin (5000 mg/mL), $NaHCO_3$ (14 mM).

The ganglia were treated enzymatically with collagenase (0.125% in culture medium for 13 min, Sigma-Aldrich, Seelze, Germany) and trypsin (0.25% in PBS for 6 min, Sigma-Aldrich, Seelze,

Germany). Then the cells were mechanically dissociated by trituration.

Preplates of the cells were prepared and after 3-4 h. The neurons were washed off the preplate to give a concentrated preparation of sensory neurons which were then finally plated on laminin-poly-D-lysine coated coverslips and bathed in culture medium. The cultures were maintained at 37°C in humidified air with 5% CO_2 and used 6 hours and 2 days after plating.

RATIOMETRIC INTRACELLULAR CALCIUM IMAGING

$[Ca^{2+}]_i$ was measured as described previously, after a slight modification.[14] In $[Ca^{2+}]_i$ measurement, the DRG neurons were incubated with calcium-sensitive dye 1 μM fura-2/AM ester (Molecular probes Inc, 1mM stock in dimethylsulphoxide, DMSO) for 60 min in imaging bath solution at 37°C in incubator, then washed 3-4 times for 20 min with imaging bath solution. The imaging bath solution contained (in mM): 130.0 NaCl, 3.0 KCl, 0.6 MgCl2, 2.0 CaCl2, 1.0 NaHCO3, 5.0 glucose, and 10.0 HEPES. The pH was adjusted with NaOH to 7.4 and the osmolarity was adjusted to 310-320 mOsm by sucrose. All imaging experiments were performed in the dark, at the room temperature.

DRG neurons viewed under an inverted Nikon TE 2000 S microscope (S-fluor, 40X oil, 1.3 NA) attached with a CCD camera (ORCA 285, Hamamatsu Photonics, Hamamatsu, Japan). Fura-2 fluorescence was recorded at 510 nm during alternating excitation at 340 and 380 nm using computer controlled filter wheel (Sutter Instruments, USA). Calculations and analysis of $[Ca^{2+}]_i$ were performed off-line on a computer with an image processor and data-analysis software (sPCI, Hamamatsu Photonics, Herrsching, Germany). Fluorescence intensity of individual cells was determined over time by selecting a region of interest (ROI) using the imaging software.

The imaging bath solution containing high K^+ (30mM) was used to activation of voltage-gated Ca^{2+} channels and large transient increases in $[Ca^{2+}]_i$ by membrane depolarization. For each experiment, cells were stimulated three times with consistent duration of Hi K^+ application.

Ziconotide was tested on response to the second stimulus by 5 min pre-treatment before second application of HiK^+. The actions of ziconotide on the peak amplitude of the fluorescence ratio (340/380 nm) response was measured.

Fura-2AM was obtained from Invitrogen (Basel, Switzerland), dissolved in DMSO. The final concentration of DMSO in the bathing solution did not exceed 0.2% (v/v), which did not elicit any change in $[Ca^{2+}]_i$ by itself in control experiment. Ziconotide was obtained from Bachem AG (Bubendorf, Switzerland). Stocks of ziconotide were prepared in imaging bath solution and stored at -20°C.

STATISTICAL ANALYSIS

Data are given as mean±standard deviation (SD). Differences between the first application of HiK^+ alone, second application of HiK^+ + ziconotide and third application of HiK^+ alone on $[Ca^{2+}]_i$ were calculated by means of one-way analysis of variance followed by a post-hoc Tukey HSD test. $p<0.05$ was accepted as evidence of significance.

RESULTS

Figure 1 shows that ziconotide dose-dependently reduced the increase in $[Ca^{2+}]_i$ elicited by 30 mM KCl. First, we tested the effects of ziconotide on basal $[Ca^{2+}]_i$. Treatment with ziconotide alone did not evoke any $[Ca^{2+}]_i$ change in DRG neurons. Figure 1A-B shows that pretreatment with ziconotide (1 μM) prevented $[Ca^{2+}]_i$ increase in response subsequent application of HiK^+.

Figures 1B and 2 show the percentages of fluorescence ratio values: 96.7±8.2 % and 97.6±6.4 % after application of second HiK^+ with low dose of ziconotide (1 nM) and third treatment with HiK^+, respectively (n=17).

Figure 3 shows the percentages of fluorescence ratio values: 90.7±4.1 % and 98.3±5.8 % after application of the second HiK^+ with 10 nM ziconotide ($p<0.001$) and third application of HiK^+, respectively (n=23).

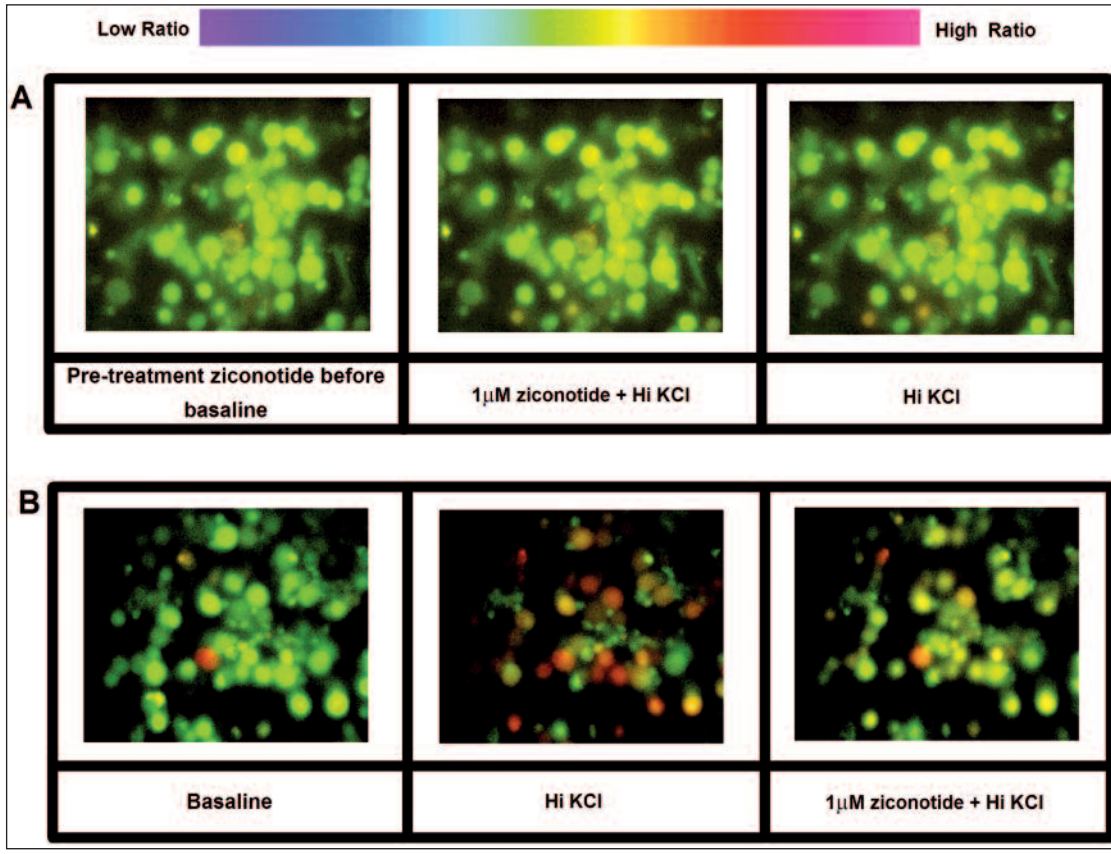

FIGURE 1: Effects of 1 μM ziconotide on total fluorescence ratio **(A)** Pre-treatment of ziconotide (1 μM) blocked of $[Ca^{2+}]_i$ increase elicited by HiK^+, insert showing an original response of fluorescence ratio from DRG neurons, **(B)** then under control conditions transient increase in $[Ca^{2+}]_i$ were observed in response to depolarisation of the membrane by application of bath imaging solution containing HiK^+, 1 μM ziconotide were applied during the second HiK^+-evoked depolarisation.
(See color figure at http://tipbilimleri.turkiyeklinikleri.com/)

Figure 4 shows the percentages of fluorescence ratio values: 62.1±8.5% and 65.8±8.7% after treatment of the second HiK^+ with high doses of ziconotide (1 μM) ($p<0.001$) and third application of HiK^+, respectively (n =37).

DISCUSSION

Data from this study showed that ziconotide inhibited the Ca^{2+} transients, which are key for the modulation of cell membrane excitability and neurotransmitter release, evoked with 30 mM K^+ in concentration dependent manner in cultured rat sensory neurons. Inhibition of calcium entry into DRG neurons may mediate the antinociceptive action of ziconotide. Thus, inhibitory effects of several agents including antiepileptics and local

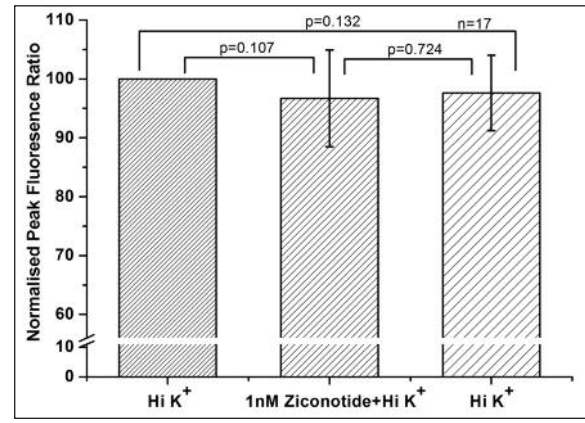

FIGURE 2: Effects of 1 nM ziconotide on total fluorescence ratio. The first peak represents response to first application of 30 mM KCl (Hi K^+), second response represent response to application of Hi K^+ before 5 min pre-treatment with 1 nM ziconotide, and the last peak represents response to third application of Hi K^+ alone (one-way analysis of variance followed by a post-hoc Tukey HSD test). Similar results were obtained from different experiments and the mean results (expressed as mean ± SD) are derived from three different dishes.

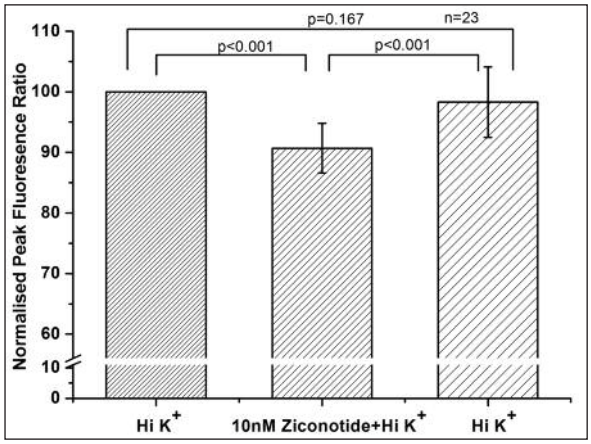

FIGURE 3: Effects of 10 nM ziconotide on total fluorescence ratio. The first peak represents response to first application of 30 mM KCl (Hi K^+), second response represents response to application of Hi K+ before 5 min pre-treatment with 10nM ziconotide and the last peak represents response to third application of Hi K^+ alone, n.s: not significant and p<0.01(one-way analysis of variance followed by a post-hoc Tukey HSD test). Similar results were obtained from different experiments and the mean results (expressed as mean ± SD) are derived from three different dishes.

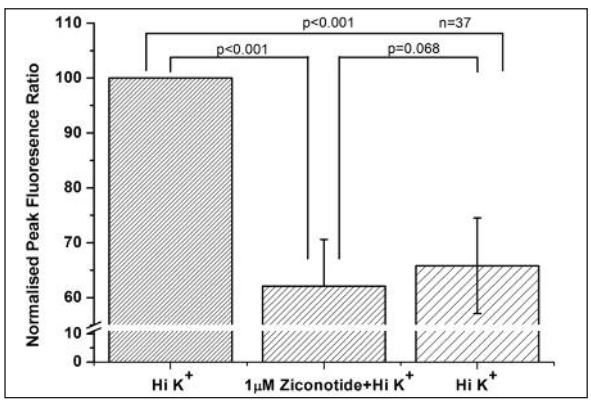

FIGURE 4: Effects of 1 μM ziconotide on total fluorescence ratio. The first peak represents response to first application of 30 mM KCl (Hi K+), second response represents response to application of Hi K+ before 5 min pre-treatment with 1 μM ziconotide and the last peak represents response to third application of Hi K+ alone, and p<0.001(one-way analysis of variance followed by a post-hoc Tukey HSD test). Similar results were obtained from different experiments and the mean results (expressed as mean ± SD) are derived from three different dishes.

anesthetics on calcium entry into DRG neurons may mediate the antinociceptive action.[15,16] Nociceptor-specific N-type calcium channel blockers have better selectivity, which results from a greater binding affinity for N-type calcium channels in DRG neurons.[17] It is clear that these channels are critically involved in the development of hyperalgesia and allodynia associated with neuropathic pain, as well as inflammatory pain.[18,19]

There are previous clinical and experimental studies providing evidence that ziconotide has analgesic activity.[20,21] Ziconotide is effective in reducing tactile allodynia in rats.[22] This study by Scott et al. also showed that intrathecal morphine was effective in decreasing tactile allodynia, but it was less selective and less potent than ziconotide.[22] Additionally, ziconotide has a highly effective analgesic activity, about 800 times stronger than that of morphine.[21] It is suggested that intrathecal injection of this non-opioid drug produces potent analgesia by efficient suppression of N-type calcium channels in humans.[23] Furthermore, ziconotide produced a significant anticonvulsant effect on amygdaloid kindled seizures in rats, through mechanism of blockade of N-type calcium channels mediated neurotransmitter release in the brain.[24] Therefore, since increase in free intracellular calcium is central to neurotransmitter release, inhibition of $[Ca^{2+}]_i$ increase in response to High K^+ stimulation may mean suppression of pro-algesic neurotransmitter(s). In the central and peripheral nervous systems, ziconotide inhibits voltage sensitive calcium channels, especially the N-type ($Ca_v2.2$).[25,26] Ziconotide is thought to modify nerve transmission and act by blocking these calcium channels. It has already been suggested that, in the dorsal horn of spinal cord, voltage sensitive calcium channels may be inhibited by ziconotide, supplying a base for analgesic pharmacotherapy.[27,28] Although the effects of ziconotide on membrane calcium channels have been shown, our results are novel since we showed that ziconotide inhibited the membrane depolarization-induced increase in intracellular calcium in cultured rat sensory neurons.[29] Our finding is novel for indication of effectiveness of ziconotide on pain signaling at the periphery.

Clinical results indicate that higher doses of ziconotide, a state-independent $Ca_v2.2$ blocker, may have some side effects such as nausea, vomiting, confusion, postural hypotension, nystagmus/amblyopia, abnormal gait, urinary retention, reduced

level of consciousness, dizziness or lightheadedness, weakness, visual problems (eg. double vision).[30] With suitable utilization, many studies have shown that ziconotide is a safe and effective intrathecal analgesic when used alone or in combination with other intrathecal analgesics.[31-34]

N triazole oxindole, TROX-1, another novel blocker of N-type calcium channels, is a potent and state-dependent agent. TROX-1 has been shown to be effective in a number of animal models for pain.[35,36] In a recent study it was shown that TROX-1 blocks calcium influx in DRG neurons via N-type calcium channels.[36] However, unlike ziconotide, TROX-1 is not so selective as it also blocks the $Ca_v2.1$, $Ca_v2.2$ and $Ca_v2.3$ calcium channel subtypes.

In conclusion, we found that ziconotide reduced the increase in intracellular calcium, induced by KCl, in a partially irreversible and concentration-dependent manner in rat DRG neurons, and we suggest that this mechanism may contribute to the antinociceptive efficacy of ziconotide at the peripheral level. The detailed consequence of this inhibitory action of ziconotide on calcium signaling in this nociceptive neurons remains unclear, and further investigations are needed.

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