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ORIGINAL ARTICLE

Dexmedetomidine and clonidine inhibit the function of $Na_V1.7$ independent of α_2-adrenoceptor in adrenal chromaffin cells

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Abstract
Purpose Besides being administered systemically for sedation and analgesia, α_2-agonists such as dexmedetomidine and clonidine have been administered with intrathecal, epidural, or perineural injections, leading to an antinociceptive effect at the spinal cord or peripheral nerve level. However, the mechanism for this remains unclear. In the present study, we examined whether dexmedetomidine and clonidine could inhibit the function of tetrodotoxin-sensitive Na^+ channels, which play important roles in the generation of pain.
Methods Cultured bovine adrenal chromaffin cells expressing the tetrodotoxin-sensitive $Na_v1.7$ Na^+ channel isoform were incubated in KRP buffer containing 2 μCi $^{22}NaCl$ for 5 min without or with dexmedetomidine or clonidine in the absence or presence of veratridine, α-scorpion venom, β-scorpion venom, *Ptychodiscus brevis* toxin-3 or ouabain. Cells were then washed and counted radioactively.
Results Dexmedetomidine and clonidine reduced veratridine-induced $^{22}Na^+$ influx via $Na_v1.7$ in a concentration-dependent manner (EC_{50} = 50 μM and 530 μM), even in the presence of ouabain, an inhibitor of Na^+, K^+-ATPase. Dexmedetomidine and clonidine shifted the concentration–response curve of veratridine for $^{22}Na^+$ influx downward without altering the EC_{50} of veratridine. Atipamezole and yohimbine, α_2-antagonists, did not prevent the inhibition of veratridine-induced $^{22}Na^+$ influx by dexmedetomidine. Dexmedetomidine and clonidine combined with lidocaine induced more inhibition of veratridine-induced $^{22}Na^+$ influx than each drug did individually. Atipamezole and yohimbine did not prevent the lidocaine-enhancing effect of dexmedetomidine and clonidine.
Conclusion Dexmedetomidine and clonidine inhibit the function of $Na_v1.7$ independent of α_2-adrenoceptor. These results may lead to a deeper understanding of the peripheral antinociceptive effects of α_2-agonists.

Keywords Adrenal chromaffin cell · Dexmedetomidine · Clonidine · Tetrodotoxin-sensitive Na^+ channels · $Na_V1.7$

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Introduction

Dexmedetomidine and clonidine are α_2-adrenoceptor agonists that have been used clinically for sedation and analgesia. These α_2-agonists have been administered through various methods involving systemic, intrathecal, epidural, or peripheral perineural injections such as axillary block [1–3]. α_2-Adrenoceptors are expressed on brainstem nuclei,

neurons in the superficial laminae of the spinal cord, and primary afferent terminals [3, 4]. These findings support the idea that α_2-agonists exert an antinociceptive effect via α_2-adrenoceptors not only at the brainstem but also at the spinal and peripheral nerve levels. When administered systemically, α_2-agonists activate α_2-adrenoceptor in the locus coeruleus (located bilaterally in the upper brainstem), providing sedation and an analgesic sparing effect; in particular, dexmedetomidine is widely used for critically ill patients in intensive care units to avoid depressing respiratory drive [1]. However, the antinociceptive mechanism that occurs at the spinal cord or peripheral nerve level is still rather poorly understood, because it has been reported that α_2-agonists alone or in combination with local anesthetics exert a local anesthetic-like nerve-block effect or enhance the nerve-block effects of local anesthetics [3]. These interactions between α_2-agonists and local anesthetics were considered to be due to (1) the inhibition of conduction in nerve fibers through interaction with Na^+ channels [5] and/or (2) the local vasoconstriction mediated by α_2-adrenoceptors [6].

In dorsal root ganglion (DRG) neurons, voltage-dependent Na^+ channels play important roles in the generation of pain [7, 8]. The DRG neurons express both tetrodotoxin-sensitive and tetrodotoxin-resistant Na^+ channels. The dysregulation of the expression or activity of these Na^+ channels contributes to neuropathic, inflammatory, or diabetic neuropathy pain [8]. A recent study reported that α_2-agonists directly inhibited tetrodotoxin-resistant Na^+ channels in rat DRG neurons [9]. However, it remains unclear whether α_2-adrenoceptor agonists can inhibit the function of tetrodotoxin-sensitive Na^+ channels.

Voltage-dependent Na^+ channels consist of the principal α subunit without or with the β_1–β_4 subunit [10, 11]. The α subunit consists of four homologous domains (I–IV), each containing six transmembrane segments (S1–S6), and forms the ion-pore and the toxin-binding sites (e.g., site 1 for tetrodotoxin/saxitoxin, site 2 for veratridine, and site 5 for *Ptychodiscus brevis* toxin-3 [PbTx-3]) [11–14]. The nine α subunits ($Na_v1.1$–$Na_v1.9$) arise from nine different genes (SCN1A–SCN5A and SCN8A–SCN11A) [11]. The β subunits are type 1 transmembrane proteins, regulating the gating kinetics and cell surface targeting of the Na^+ channel [10].

In adrenal chromaffin cells (embryologically derived from neural crest), the Na^+ channel α subunit is $Na_v1.7$ [the tetrodotoxin/saxitoxin-sensitive human neuroendocrine type Na^+ channel α-subunit (hNE-Na)] encoded by SCN9A, as in sympathetic and sensory ganglia [8, 10, 11, 15–18]. Surprisingly, gain-of-function/loss-of-function mutations of $Na_v1.7$ in human patients have shown that $Na_v1.7$ is the first Na^+ channel isoform to be causally involved in intolerable pain syndromes/insensitivity of pain syndrome [19, 20], so $Na_v1.7$ has became the molecular target of pain treatment. We previously showed that $^{22}Na^+$ influx assay with Na^+ channel openers (e.g., veratridine) is a useful method for examining the pharmacological function of $Na_v1.7$ in adrenal chromaffin cells [21]. In the present study, we show that (1) dexmedetomidine and clonidine inhibit veratridine-induced $^{22}Na^+$ influx via tetrodotoxin-sensitive $Na_V1.7$ independent of α_2-adrenoceptor, and (2) dexmedetomidine and clonidine in combination with lidocaine enhances the inhibition of veratridine-induced $^{22}Na^+$ influx by each drug individually in adrenal chromaffin cells.

Materials and methods

Materials

Eagle's minimum essential medium was obtained from Nissui Seiyaku (Tokyo, Japan). Dexmedetomidine and atipamezole were from Orion Corporation Orion Pharma (Turku, Finland). Cytosine arabinoside, clonidine, veratridine, ouabain, α-scorpion venom (*Leiurus quinquestriatus*), β-scorpion venom (*Centruroides sculpturatus*), yohimbine and lidocaine were from Sigma–Aldrich (St. Louis, MO, USA). *Ptychodiscus brevis* toxin-3 (PbTx-3) was from Latoxan (Westbury, NY, USA). $^{22}NaCl$ (6–17 Ci/mmol) was from GE Healthcare (Piscataway, NJ, USA).

Primary culture of adrenal chromaffin cells

Isolated bovine adrenal chromaffin cells were cultured (4×10^6 per dish, 35 mm diameter; BD Falcon, Franklin Lakes, NJ, USA) in Eagle's minimum essential medium containing 10% newborn calf serum and 3 μM cytosine arabinoside to suppress the proliferation of nonchromaffin cells under 5% CO_2/95% air in a CO_2 incubator for 3 days (60–62 h) [21, 22].

$^{22}Na^+$ influx assay

Cells were washed with ice-cold Krebs–Ringer phosphate (KRP) buffer (values in mM: 154 NaCl, 5.6 KCl, 1.1 $MgSO_4$, 2.2 $CaC1_2$, 0.85 NaH_2PO_4, 2.15 Na_2HPO_4, 5 glucose, and 0.5% bovine serum albumin, pH 7.4), and incubated at 37°C with 2 μCi $^{22}NaCl$ in 1 mL of KRP buffer for 5 min without or with veratridine, dexmedetomidine, clonidine, ouabain, atipamezole, yohimbine, PbTx-3, α-scorpion venom, β-scorpion venom and lidocaine. The cells were then washed with ice-cold KRP buffer, solubilized in 10% Triton X-100, and counted for radioactivity [21, 22].

Statistical methods

The $^{22}Na^+$ influx assay was performed in triplicate, and all experiments were repeated at least three times (mean ± SEM). Significance ($P < 0.05$) was determined by one-way ANOVA with post hoc means comparison by the Newman–Keuls multiple range test. Student's t test was used when the means of two groups were compared.

Results

Effects of dexmedetomidine and clonidine on veratridine-induced $^{22}Na^+$ influx in cultured bovine adrenal chromaffin cells

In cultured bovine adrenal chromaffin cells, veratridine causes a persistent influx of $^{22}Na^+$ for at least 5 min, which passes through $Na_V1.7$ [12]. As shown in Fig. 1, veratridine (100 μM) increased $^{22}Na^+$ influx by 210.3 ± 12.5 nmol/4×10^6 cells/5 min, and dexmedetomidine and clonidine inhibited veratridine-induced $^{22}Na^+$ influx in a concentration-dependent manner (IC_{50} = 50 and 530 μM, respectively). The basal measure of $^{22}Na^+$ influx was not changed by dexmedetomidine and clonidine at any concentration used, and significant reductions due to dexmedetomidine and clonidine were observed at 10 and 300 μM.

When cells were stimulated with various concentrations of veratridine, which binds to site 2, segment 6 of domain I (IS6) [14], veratridine raised $^{22}Na^+$ influx in a concentration-dependent manner. The inhibitory effects of dexmedetomidine (10 and 100 μM) and clonidine (300 and 1000 μM) were not overcome even when the concentration of veratridine was increased to 500 μM (Fig. 2a, b).

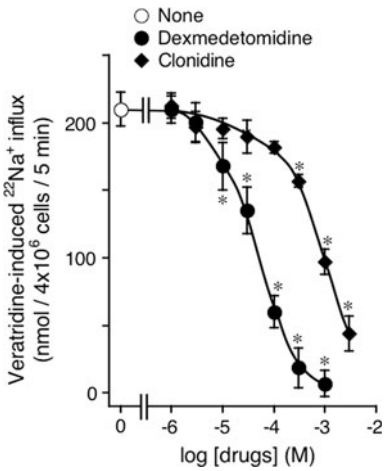

Fig. 1 Concentration-dependent inhibitions of veratridine-induced $^{22}Na^+$ influx via $Na_V1.7$ by dexmedetomidine and clonidine. Cells were incubated at 37°C for 5 min in 1 ml KRP buffer containing 2 μCi $^{22}NaCl$ without or with 100 μM veratridine in the absence or the presence of various concentrations of dexmedetomidine and clonidine. Cells were then washed, solubilized, and counted for radioactivity. Basal values at 37°C in the absence of veratridine were not changed by dexmedetomidine and clonidine, and were subtracted from the data. Mean ± SEM (n = 3). *$P < 0.05$, compared with cells that were not treated with dexmedetomidine and clonidine

Effects of dexmedetomidine and clonidine on $^{22}Na^+$ influx caused by veratridine in combination with ouabain

We previously reported that spontaneous or veratridine-induced Na^+ influx increased the activity of Na^+,K^+-ATPase, whereby Na^+, once it entered chromaffin cells, was continuously pumped out [21]. As shown in Fig. 2a, veratridine (100 μM)-induced $^{22}Na^+$ influx was still decreased by dexmedetomidine and clonidine even in the presence of ouabain at 100 μM, a concentration that completely blocks the Na^+,K^+-ATPase [12]. Thus, the reductions in the veratridine-induced $^{22}Na^+$ influx caused by dexmedetomidine and clonidine were not due to any modification of the Na^+,K^+-ATPase activity.

Atipamezole and yohimbine: no antagonism of the inhibitory effect of dexmedetomidine on veratridine-induced ^{22}Na influx

Dexmedetomidine and clonidine are well-known α_2-adrenoceptor agonists. Therefore, we examined whether atipamezole and yohimbine, which were α_2-antagonists, could antagonize the inhibitory effects of dexmedetomidine and clonidine on veratridine-induced $^{22}Na^+$ influx. As shown in Fig. 3a, b, atipamezole and yohimbine themselves inhibited veratridine-induced $^{22}Na^+$ influx. Consequently, we chose a concentration of 10 μM for atipamezole and yohimbine, because this concentration had few or no inhibitory effects on veratridine-induced $^{22}Na^+$ influx and could antagonize up to 100 μM dexmedetomidine [23, 24]. We added 10 μM atipamezole or 10 μM yohimbine to 10–100 μM dexmedetomidine (Fig. 3c); however, atipamezole and yohimbine did not prevent the decrease in veratridine-induced $^{22}Na^+$ influx caused by dexmedetomidine.

α-Scorpion venom, β-scorpion venom, and PbTX-3: no interaction with dexmedetomidine and clonidine at the binding sites of their toxins

We examined whether dexmedetomidine and clonidine could directly inhibit Na^+ channels by using α-scorpion venom, β-scorpion venom, and PbTX-3, because these toxins that bind to distinct sites at the Na^+ channels are useful probes for characterizing the structure and function of Na^+ channels [12]. α-Scorpion venom, which binds to site 3 (IS5-S6), β-scorpion venom, which interacts with site

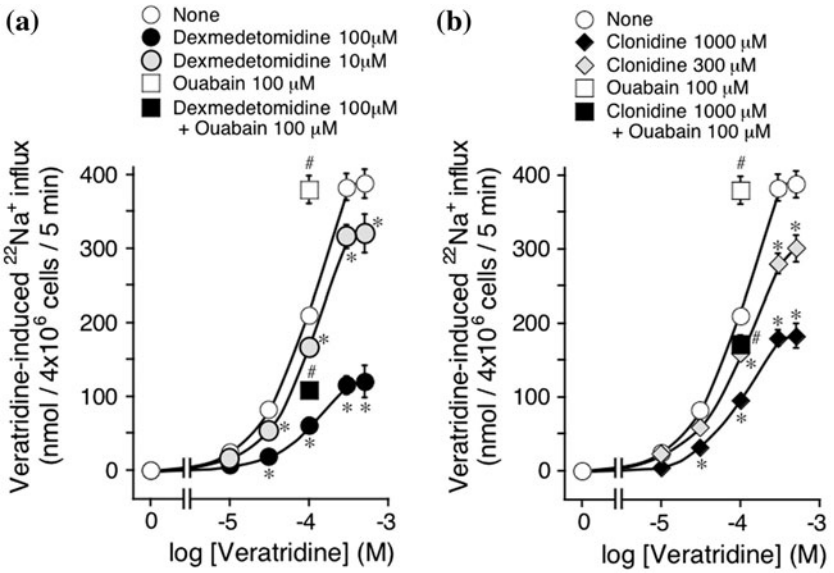

Fig. 2 Effects of dexmedetomidine and clonidine on $^{22}Na^+$ influx measured in the absence and presence of various concentrations of veratridine and ouabain. Cells were incubated at 37°C for 5 min in 1 ml KRP buffer containing 2 μCi $^{22}NaCl$ without or with **a** 10 μM and 100 μM dexmedetomidine, or **b** 300 and 1000 μM clonidine in the absence or presence of increasing concentrations of veratridine. 100 μM veratridine-induced $^{22}Na^+$ influx was also measured without or with **a** 100 μM dexmedetomidine or **b** 1000 μM clonidine in the presence of 100 μM ouabain. Basal values at 37°C were subtracted from the data. In **a** and **b**, *some bars* that indicate the SEM are included within the *symbols* because the SEM is smaller than the symbol. Mean ± SEM ($n = 3$). $^*P < 0.05$, compared with cells that were not treated with dexmedetomidine and clonidine; $^{\#}P < 0.05$, compared with 100 μM veratridine alone within each untreated and dexmedetomidine- or clonidine-treated cell group

4 (IIS1-S2, IIS3-S4), and PbTx-3, which binds to site 5 (IS6) [14], caused only a slight increase of $^{22}Na^+$ influx over the basal value (Fig. 4). Dexmedetomidine and clonidine did not reduce $^{22}Na^+$ influx caused by either α-scorpion venom, β-scorpion venom, or PbTx-3. α-Scorpion venom and β-scorpion venom, as well as PbTx-3, potentiated the $^{22}Na^+$ influx caused by veratridine 3.5-, 1.9-, and 3.7-fold, respectively, as reported previously [12]. Veratridine-induced $^{22}Na^+$ influx was potentiated by either venom/toxin even in the presence of dexmedetomidine or clonidine, although absolute values of $^{22}Na^+$ influx remained decreased in dexmedetomidine- or clonidine-treated cells as compared to untreated cells. In addition, PbTx-3 in combination with either α-scorpion venom or β-scorpion venom augmented veratridine-induced $^{22}Na^+$ influx to a greater extent than either venom/toxin did individually, and this enhancement occurred even in the presence of dexmedetomidine or clonidine, as in the untreated cells.

Lidocaine: enhancement of the inhibitory effects of dexmedetomidine and clonidine on veratridine-induced ^{22}Na influx

We examined how dexmedetomidine and clonidine interacted with lidocaine. Each agent was used at its IC_{50} concentration (50 μM dexmedetomidine, 530 μM clonidine, and 50 μM lidocaine). We previously reported that lidocaine inhibited veratridine-induced $^{22}Na^+$ influx in a concentration-dependent manner ($IC_{50} = 47.8$ μM) [25]. As shown in Fig. 5, dexmedetomidine with lidocaine and clonidine with lidocaine inhibited veratridine-induced $^{22}Na^+$ influx by 34.7 and 39.0%, respectively. 10 μM Atipamezole and 10 μM yohimbine could not prevent the lidocaine-enhancing effects of dexmedetomidine and clonidine (Fig. 5).

Discussion

Inhibition of tetrodotoxin-sensitive $Na_V1.7$ activity by dexmedetomidine and clonidine

In the present study, we demonstrated that dexmedetomidine (1 μM–1 mM) and clonidine (1 μM–3 mM) reduced veratridine-induced $^{22}Na^+$ influx ($IC_{50} = 50$ and 530 μM, respectively) in a concentration-dependent manner in cultured bovine adrenal chromaffin cells (Fig. 1). Our study also provides evidence that the combination of dexmedetomidine with lidocaine and that of clonidine with lidocaine enhanced the inhibitory effect of each drug on $Na_V1.7$ (Fig. 5).

Na^+ channels contribute to the hyperexcitability of DRG neurons associated with pain. DRG neurons express

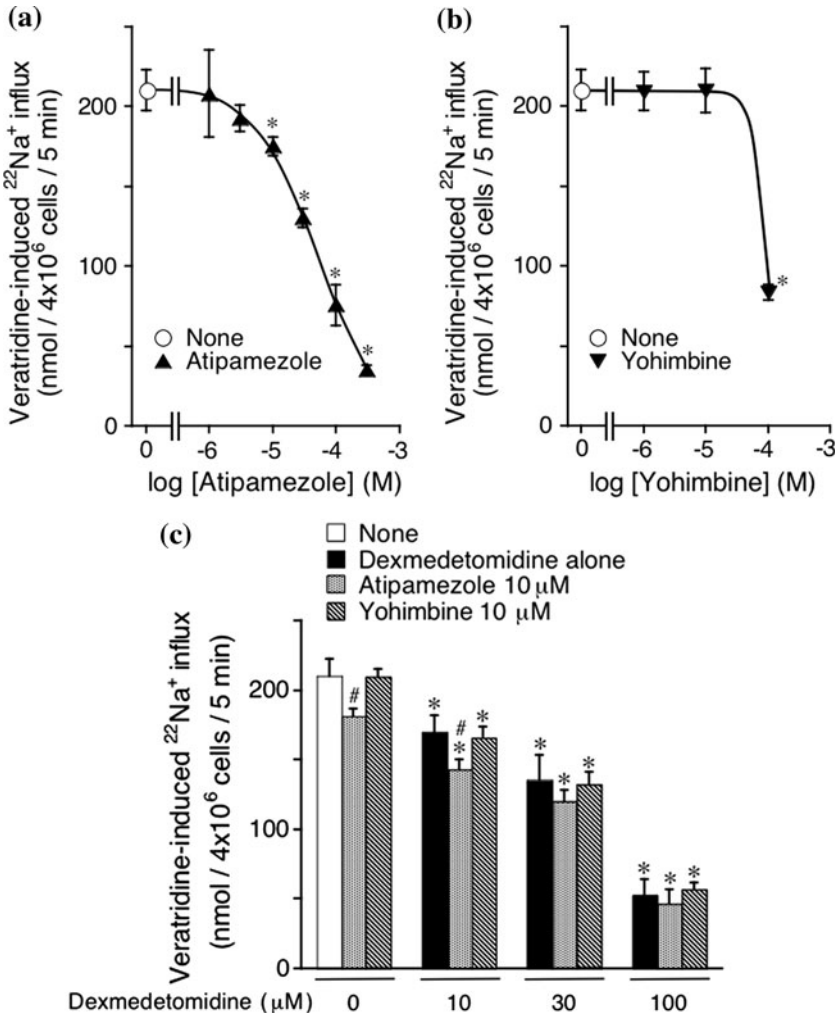

Fig. 3 No antagonism by atipamezole and yohimbine of the inhibitory effect of dexmedetomidine on veratridine-induced $^{22}Na^+$ influx. Cells were incubated at 37°C for 5 min in 1 ml KRP buffer containing 2 μCi $^{22}NaCl$ without or with various concentrations of **a** atipamezole and **b** yohimbine in the absence or presence of 100 μM veratridine. Basal values at 37°C were subtracted from the data. Mean ± SEM ($n = 3$). $^*P < 0.05$, compared with atipamezole- and yohimbine-nontreated cells. **c** Cells were incubated at 37°C for 5 min in 1 ml KRP buffer containing 2 μCi $^{22}NaCl$ and 100 μM veratridine without or with 10 μM atipamezole or 10 μM yohimbine in the absence (*open columns*) or presence (*solid columns*) of 10–100 μM dexmedetomidine. Basal values at 37°C were subtracted from the data. Mean ± SEM ($n = 3$). $^*P < 0.05$, compared with cells that were not treated with dexmedetomidine; $^{\#}P < 0.05$, compared with cells that were not treated with atipamezole and yohimbine within each dexmedetomidine-nontreated and dexmedetomidine-treated cell group

tetrodotoxin-sensitive Na^+ channel α-subunits ($Na_V1.7$, $Na_V1.1$, $Na_V1.6$, $Na_V1.2$, and $Na_V1.3$) and tetrodotoxin-resistant Na^+ channel α-subunits ($Na_V1.8$ and $Na_V1.9$). Of these Na^+ channels, $Na_V1.7$, $Na_V1.8$, and $Na_V1.9$ are preferentially expressed in DRG neurons. In peripherally axotomized spinal sensory neurons, the expressions of $Na_V1.8$ and $Na_V1.9$ were downregulated, whereas the $Na_V1.3$ level was upregulated [26, 27]. It has been suggested that $Na_V1.7$ also plays a role in eliciting intolerable pain in inflammation [28, 29] and diabetic neuropathy [30, 31]. Therefore, the $Na_V1.8$, $Na_V1.7$, $Na_V1.9$ and $Na_V1.3$ channels have emerged as key molecules involved in the generation of intolerant pain associated with neuronal injury and inflammation [19]. Since 2004, gain-of-function/loss-of-function mutations of $Na_V1.7$ in human patients had been discovered [14, 19, 32–34]. One gain-of-function mutation of $Na_V1.7$ was responsible for the inherited form of erythermalgia, an intermittent burning pain syndrome in the distal extremities [32]. Another gain-of-function mutation of $Na_V1.7$ accounted for paroxysmal extreme pain syndrome characterized by paroxysmal visceral pain [33]. "Channelopathy-associated insensitivity to pain" was caused by a loss-of-function mutation of $Na_V1.7$; the patients had never felt pain, but had the correct perceptions of other sensations (e.g., warm/cold) [34]. These findings are very exciting and important, because they are the first human diseases to provide clear evidence that the mutation of this ion channel is involved in intolerable pain syndromes/insensitivity to pain syndrome, and that $Na_V1.7$ can play a crucial role in nociception.

Several studies using an electrophysiological technique have demonstrated that the tetrodotoxin-resistant Na^+ current or an action potential combining the tetrodotoxin-sensitive and tetrodotoxin-resistant Na^+ currents is inhibited by α_2-agonists [9, 35–39]. However, the effect of α_2-agonists on the tetrodotoxin-sensitive Na^+ channel has been unclear. In our present study, we utilized adrenal chromaffin cells expressing the tetrodotoxin-sensitive $Na_V1.7$ channel to check whether dexmedetomidine and clonidine inhibit the tetrodotoxin-sensitive Na^+ channel. The degree of inhibition of the Na^+ current by the α_2-agonists differed depending on the type of Na^+ channel or nerve [9, 35–39]. The IC_{50} of

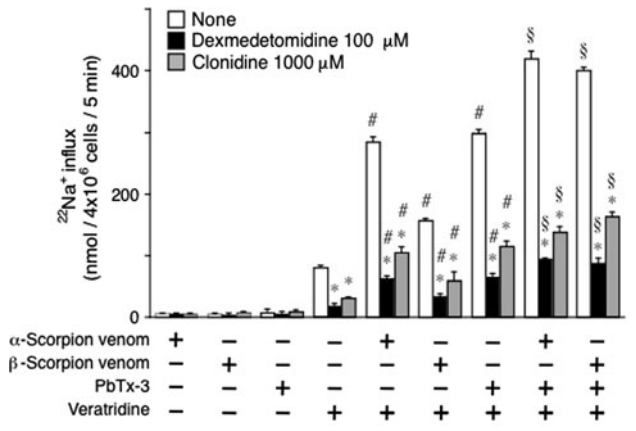

Fig. 4 Effects of dexmedetomidine and clonidine on $^{22}Na^+$ influx caused by veratridine, α-scorpion venom, β-scorpion venom, and PbTX-3, measured in the absence and presence of various concentrations of veratridine and ouabain. Cells were incubated at 37°C for 5 min in 1 ml KRP buffer containing 2 μCi $^{22}NaCl$ without (*open columns*) or with (*solid columns*) 100 μM dexmedetomidine, or 1000 μM clonidine (*shaded columns*) in the absence (−) or presence (+) of 30 μM veratridine, 0.5 μg/ml α-scorpion venom, 5 μg/ml β-scorpion venom, and 1 μM PbTX-3. The influx of ^{22}Na to the cells was then measured. Basal values at 37°C were subtracted from the data. Mean ± SEM ($n = 3$). $^*P < 0.05$, compared with cells that were not treated with dexmedetomidine and clonidine; $^{\#}P < 0.05$, compared with veratridine alone within each untreated and dexmedetomidine- or clonidine-treated cell group; $^{\S}P < 0.05$, compared with veratridine plus PbTX-3 within each untreated and dexmedetomidine- or clonidine-treated cell group

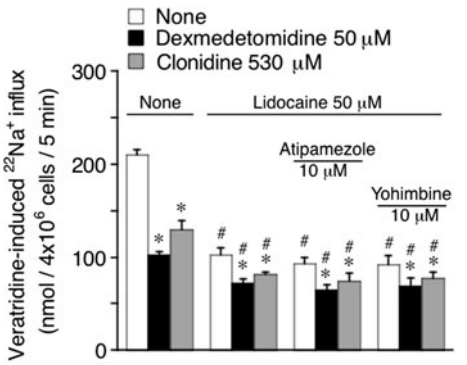

Fig. 5 Enhancement of the inhibition of veratridine-induced $^{22}Na^+$ influx by dexmedetomidine/clonidine in combination with lidocaine, and no antagonism by atipamezole and yohimbine of the lidocaine-enhancing effects of dexmedetomidine and clonidine. Cells were incubated at 37°C for 5 min in 1 ml KRP buffer containing 2 μCi $^{22}NaCl$ and 100 μM veratridine without or with 50 μM lidocaine, 10 μM atipamezole, or 10 μM yohimbine in the absence (*open columns*) or presence of 50 μM dexmedetomidine (*solid columns*) or 530 μM clonidine (*shaded columns*). Basal values at 37°C were subtracted from the data. Mean ± SEM ($n = 3$). $^*P < 0.05$, compared with cells that were not treated with dexmedetomidine and clonidine within each untreated and lidocaine-treated cell group; $^{\#}P < 0.05$, compared with cells that were not treated with lidocaine within each untreated and dexmedetomidine- or clonidine-untreated cell group

dexmedetomidine for the tetrodotoxin-sensitive $Na_V1.7$ current in our present study was similar to that for the tetrodotoxin-resistant Na^+ current in rat DRG neurons (58 μM) and the compound action potential in dog stellate ganglia (71.6 μM) [9, 35]. On the other hand, the IC_{50} of clonidine for the tetrodotoxin-sensitive $Na_V1.7$ current in our present study was greater than that for the tetrodotoxin-resistant Na^+ current (257 μM) in rat DRG neurons, and smaller than that for the Na^+ current in rat spinal dorsal horn neurons (707 μM), although it was similar to that for the Na^+ current in rat sciatic nerve C fibers (450 μM) and in rabbit vagus C-fibers (647 μM) [36–38]. It was reported that intrathecal injection of 100 μg dexmedetomidine or 300 μg clonidine—an antinociceptive dose—produced 0.5–50 μmol in the CSF [9, 39, 40]. This concentration of clonidine in the CSF was much lower than the IC_{50} of clonidine obtained in the present study. On the other hand, the upper end of the CSF concentration of dexmedetomidine was close to the IC_{50} of dexmedetomidine obtained in the present study. This suggests that the antinociceptive effects of dexmedetomidine after intrathecal/epidural administration or peripheral perineural injection are due in part to Na^+ channel blocking.

The mechanism of inhibitory effects of α_2-agonists on $Na_V1.7$: independent of α_2-adrenoceptors

We evaluated whether α_2-agonists could inhibit $Na_V1.7$ via the α_2-adrenoceptor pathway, because the IC_{50} for Na^+ channels was different for dexmedetomidine and clonidine, and the α_2-adrenoceptor selectivity of dexmedetomidine is eight times greater than that of clonidine [41]. α_2-Adrenoceptors are present in the central and peripheral nervous system at autonomic ganglia as well as presynaptic and postsynaptic sites [1, 3]. The previous study analyzed the binding characteristics of [^{3}H]clonidine to membrane fractions prepared from bovine adrenal chromaffin cells, and it showed storable, reversible and high-affinity binding which meant that it could be considered an α_2-adrenoceptor [42]. Although we did not find a similar study in bovine adrenal chromaffin cells, the RT-PCR study showed that rat adrenal chromaffin cells expressed mRNAs for α_{2A}, α_{2B}, α_{2C}-adrenoceptors [43]. We demonstrated that α_2-agonists inhibited $Na_V1.7$ independent of α_2-adrenoceptor, because atipamezole and yohimbine, which are α_2-antagonists, did not prevent the inhibitory effects of dexmedetomidine on veratridine-induced $^{22}Na^+$ influx (Fig. 3c) and the lidocaine-enhancing effects of dexmedetomidine and clonidine (Fig. 5). This finding was similar to that of a previous report, in which yohimbine did not prevent the tetrodotoxin-resistant Na^+ current blocking effects of dexmedetomidine and clonidine [9]. On the other hand, the previous study found that when dexmedetomidine alone, or combined with

lidocaine, was intracutaneously injected into the backs of guinea pigs and the pinprick test was then applied, dexmedetomidine (1 nM–1 μM) itself did not have analgesic effects, though it did enhance the local anesthetic effect of lidocaine; oxymetazoline, a selective agonist of α_{2A}-adrenoceptor, enhanced the effect of lidocaine just like dexmedetomidine did; yohimbine (an antagonist of α_2-adrenoceptor) but not prazosin (an antagonist of α_{2B}- and α_{2C}-adrenoceptors) inhibited the enhancing effect of dexmedetomidine on lidocaine [44]. These results demonstrated that dexmedetomidine enhances the local anesthetic action of lidocaine via α_{2A}-adrenoceptor. In addition, it was reported that perineural α_{2A}-adrenoceptor activation inhibited allodynia, mechanical hypersensitivity, and spinal cord neuroplasticity after nerve injury [45, 46]; also, perineural injection of clonidine at the site of nerve injury increased withdrawal threshold and reduced p38 mitogen-activated protein kinase phosphorylation, which contributes to the development and maintenance of inflammatory and neuropathic pain in sensory neurons [47]. Altogether, this evidence suggests that p38 inactivation may be associated with the analgesic-enhancing effect of α_2-agonists at the spinal cord and peripheral nerve levels. Thus, it is suggested that α_2-agonists produce an analgesic effect at the spinal cord or peripheral nerve level via the inhibition of tetrodotoxin-sensitive/resistant Na^+ channels at high concentrations, as well as the α_2-adrenoceptor pathway at low concentrations.

Because the inhibitory effect of α_2-agonists is not due to an α_2-adrenoceptor-mediated mechanism, it is possible that α_2-agonists directly inhibit Na^+ channels. We explored the site of the Na^+ channel α-subunit at which the α_2-agonists directly bind. The present study shows that the inhibitory effect of α_2-agonists on veratridine-induced $^{22}Na^+$ influx is not reversed by increasing concentrations of veratridine (Fig. 2a, b), suggesting that α_2-agonists do not have a binding site (site 2) in common with veratridine. In addition, α-scorpion venom (site 3 toxin), *β*-scorpion venom (site 4 toxin), or PbTx-3 (site 5 toxin) enhanced veratridine-induced $^{22}Na^+$ influx in the presence or absence of dexmedetomidine and clonidine (Fig. 4). This suggests that α_2-agonists do not interact with these distinct functional segments of the Na^+ channel α-subunit at the very least. We were able to demonstrate that α_2-agonists do not alter the pharmacological properties of $Na_V1.7$, but not that α_2-agonists directly inhibit $Na_V1.7$.

The imidazoline receptor pathway could be considered another mechanism. In addition to their actions at α_2-adrenergic receptors, some α_2-agonists and α_2-antagonists such as dexmedetomidine, clonidine, and atipamezole (which have an imidazole ring in their structure) have some affinity for imidazoline-binding sites [1]. The present study showed that not only dexmedetomidine and clonidine but also atipamezole inhibit Na^+ channel activity in a concentration-dependent manner (Fig. 3a). At a higher dose, yohimbine, which is a nonimidazole α_2-antagonist, also inhibited Na^+ channels, but not in a concentration-dependent manner (Fig. 3b). Although the effect of the imidazoline receptor pathway on the activity of voltage-dependent Na^+ channels is unclear, it is known that voltage-dependent Ca^{2+} channels are inhibited by imidazoline receptor activation in rat cervical ganglion neurons or hippocampal neurons [48, 49]. Thus, it is possible that the inhibitory effects of α_2-agonists and α_2-antagonists that contain an imidazole ring on Na^+ channels are produced via unknown imidazoline receptor pathways, although this will have to be determined through further pharmacological and electrophysiological study of the mechanisms in detail.

Conclusion

Dexmedetomidine and clonidine inhibited $Na_V1.7$, which was the tetrodotoxin-sensitive Na^+ channel isoform, independent of α_2-adrenoceptor in cultured bovine adrenal chromaffin cells. Combining dexmedetomidine or clonidine with lidocaine produced additive-type inhibition of $Na_V1.7$. These findings may indicate new pharmacological actions of α_2-agonists, thus leading to an improved understanding of the treatment of pain.

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References

1. Khan ZP, Ferguson CN, Jones RM. Alpha-2 and imidazoline receptor agonists. Their pharmacology and therapeutic role. Anaesthesia. 1999;54:146–65.
2. Axelsson K, Gupta A. Local anaesthetic adjuvants: neuraxial versus peripheral nerve block. Curr Opin Anaesthesiol. 2009;22:649–54.
3. Eisenach JC, Kock MD, Klimscha W. α_2-Adrenergic agonists for regional anesthesia. A clinical review of clonidine (1984–1995). Anesthesiology. 1996;85:655–74.
4. Unnerworthtall JR, Kopajtic TA, Kuhar MJ. Distribution of alpha 2 agonist binding sites in the rat and human central nervous system: analysis of some functional, anatomic correlates of the pharmacologic effects of clonidine and related adrenergic agents. Brain Res Rev. 1984;7:69–101.
5. Erne-Brand F, Jirounek P, Drewe J, Hampl K, Schneider MC. Mechanism of antinociceptive action of clonidine in nonmyelinated nerve fibres. Eur J Pharmacol. 1999;383:1–8.
6. Gaumann D, Forster A, Griessen M, Habre W, Poinsot O, Delia Santa D. Comparison between clonidine and epinephrine

admixture to lidocaine in brachial plexus block. Anesth Analg. 1992;75:69–74.
7. Wood JN, Boorman JP, Okuse K, Baker MD. Voltage-gated sodium channels and pain pathways. J Neurobiol. 2004;61:55–71.
8. Wada A. Roles of voltage-dependent sodium channels in neuronal development, pain, and neurodegeneration. J Pharmacol Sci. 2006;102:253–68.
9. Oda A, Iida H, Takahashi S, Osawa Y, Yamaguchi S, Dohi S. Effects of α_2-adrenoceptor agonists on tetrodotoxin-resistant Na^+ channels in rat DRG neurons. Eur J Anaesthesiol. 2007;24: 934–41.
10. Wada A, Yanagita T, Yokoo H, Kobayashi H. Regulation of cell surface expression of voltage-dependent $Na_v1.7$ sodium channels: mRNA stability and posttranscriptional control in adrenal chromaffin cells. Front Biosci. 2004;9:1954–66.
11. Catterall WA, Goldin AL, Waxman SG. International Union of Pharmacology. XLVII. Nomenclature and structure–function relationships of voltage-gated sodium channels. Pharmacol Rev. 2005;57:397–409.
12. Wada A, Uezono Y, Arita M, Yuhi T, Kobayashi H, Yanagihara N, Izumi F. Cooperative modulation of voltage-dependent sodium channels by brevetoxin and classical neurotoxins in cultured bovine adrenal medullary cells. J Pharmacol Exp Ther. 1992;263:1347–51.
13. Cestèle S, Khalifa RB, Pelhate M, Rochat H, Gordon D. α-Scorpion toxins binding on rat brain and insect sodium channels reveal divergent allosteric modulations by brevetoxin and veratridine. J Biol Chem. 1995;270:15153–61.
14. Cestèle S, Catterall WA. Molecular mechanisms of neurotoxin action on voltage-gated sodium channels. Biochimie. 2000;82: 883–92.
15. Klugbauer N, Lacinova L, Flockerzi V, Hofmann F. Structure and functional expression of a new member of the tetrodotoxin-sensitive voltage-activated sodium channel family from human neuroendocrine cells. EMBO J. 1995;14:1084–90.
16. Toledo-Aral JJ, Moss BL, He ZJ, Koszowski AG, Whisenand T, Levinson SR, Wolf JJ, Silos-Santiago I, Halegoua S, Mandel G. Identification of PN1, a predominant voltage-dependent sodium channel expressed principally in peripheral neurons. Proc Natl Acad Sci USA. 1997;94:1527–32.
17. Belcher SM, Zerillo CA, Levenson R, Ritchie JM, Howe JR. Cloning of a sodium channel α subunit from rabbit Schwann cells. Proc Natl Acad Sci USA. 1995;92:11034–8.
18. Goldin AL. Resurgence of sodium channel research. Annu Rev Physiol. 2001;63:871–94.
19. Dib-Hajj SD, Black JA, Waxman SG. Voltage-gated sodium channels: therapeutic targets for pain. Pain Med. 2009;10: 1260–9.
20. Dib-Hajj SD, Cummins TR, Black JA, Waxman SG. From genes to pain: $Na_V1.7$ and human pain disorders. Trends Neurosci. 2007;30:555–63.
21. Yamamoto R, Yanagita T, Kobayashi H, Yuhi T, Yokoo H, Wada A. Up-regulation of functional voltage-dependent sodium channels by insulin in cultured bovine adrenal chromaffin cells. J Neurochem. 1996;67:1401–8.
22. Maruta T, Yanagita T, Matsuo K, Uezono Y, Satoh S, Nemoto T, Yoshikawa N, Kobayashi H, Takasaki M, Wada A. Lysophosphatidic acid-LPA_1 receptor-Rho-Rho kinase-induced up-regulation of $Na_V1.7$ sodium channel mRNA and protein in adrenal chromaffin cells: enhancement of $^{22}Na^+$ influx, $^{45}Ca^{2+}$ influx and catecholamine secretion. J Neurochem. 2008;105:401–12.
23. Correa-Sales C, Rabin BC, Maze M. A hypnotic response to dexmedetomidine, an α_2 agonist, is mediated in the locus coeruleus in rats. Anesthesiology. 1992;76:948–52.
24. Shirasaka T, Kannan H, Takasaki M. Activation of a G protein-coupled inwardly rectifying K^+ current and suppression of Ih contribute to dexmedetomidine-induced inhibition of rat hypothalamic paraventricular nucleus neurons. Anesthesiology. 2007; 107:605–15.
25. Shiraishi S, Yokoo H, Yanagita T, Kobayashi H, Minami S, Saitoh T, Takasaki M, Wada A. Differential effects of bupivacaine enantiomers, ropivacaine and lidocaine on up-regulation of cell surface voltage-dependent sodium channels in adrenal chromaffin cells. Brain Res. 2003;966:175–84.
26. Waxman SG, Dib-Hajj S, Cummins TR, Black JA. Sodium channels and their genes: dynamic expression in the normal nervous system, dysregulation in disease states. Brain Res. 2000; 886:5–14.
27. Waxman SG. Transcriptional channelopathies: an emerging class of disorders. Nat Rev Neurosci. 2001;2:652–9.
28. Waxman SG, Dib-Hajj S. Erythermalgia: molecular basis for an inherited pain syndrome. Trends Mol Med. 2005;11:555–62.
29. Nassar MA, Stirling LC, Forlani G, Baker MD, Matthews EA, Dickenson AH, Nassar MA. Nociceptor-specific gene deletion reveals a major role for Nav1.7 (PN1) in acute and inflammatory pain. Proc Natl Acad Sci USA. 2004;101:12706–11.
30. Yeomans DC, Levinson SR, Peters MC, Koszowski AG, Tzabazis AZ, Gilly WF, Wilson SP. Decrease in inflammatory hyperalgesia by herpes vector-mediated knockdown of Nav1.7 sodium channels in primary afferents. Hum Gene Ther. 2005;16: 271–7.
31. Hong S, Morrow TJ, Paulson PE, Isom LL, Wiley JW. Early painful diabetic neuropathy is associated with differential changes in tetrodotoxin-sensitive and -resistant sodium channels in dorsal root ganglion neurons in the rat. J Biol Chem. 2004;279: 29341–50.
32. Craner MJ, Klein JP, Renganathan M, Black JA, Waxman SG. Changes of sodium channel expression in experimental painful diabetic neuropathy. Ann Neurol. 2002;52:786–92.
33. Fertleman CR, Baker MD, Parker KA, Moffatt S, Elmslie FV, Abrahamsen B, Ostman J, Klugbauer N, Wood JN, Gardiner RM, Rees M. SCN9A mutations in paroxysmal extreme pain disorder: allelic variants underlie distinct channel defects and phenotypes. Neuron. 2006;52:767–74.
34. Cox JJ, Reimann F, Nicholas AK, Thornton G, Roberts E, Springell K, Karbani G, Jafri H, Mannan J, Raashid Y, Al-Gazali L, Hamamy H, Valente EM, Gorman S, Williams R, McHale DP, Wood JN, Gribble FM, Woods CG. An SCN9A channelopathy causes congenital inability to experience pain. Nature. 2006;444: 894–8.
35. McCallum JB, Boban N, Hogan Q, Schmeling WT, Kampine JP, Bosnjak ZJ. The mechanism of α_2-adrenergic inhibition of sympathetic ganglionic transmission. Anesth Analg. 1998;87: 503–10.
36. Erne-Brand F, Jirounek P, Drewe J, Hampl K, Schneider MC. Mechanism of antinociceptive action of clonidine in nonmyelinated nerve fibres. Eur J Pharmacol. 1999;383:1–8.
37. Butterworth JF, Strichartz GR. The α_2-adrenergic agonists clonidine and guanfacine produce tonic and phasic block of conduction in rat sciatic nerve fibers. Anesth Analg. 1993;76: 295–301.
38. Wolff M, Heugel P, Hempelmann G, Scholz A, Mühling J, Olschewski A. Clonidine reduces the excitability of spinal dorsal horn neurones. Br J Anaesth. 2007;98:353–61.
39. Eisenach JC, Shafer SL, Bucklin BA, Jackson C, Kallio A. Pharmacokinetics and pharmacodynamics of intraspinal dexmedetomidine in sheep. Anesthesiology. 1994;80:1349–59.
40. Castro MI, Eisenach JC. Pharmacokinetics and dynamics of intravenous, intrathecal, and epidural clonidine in sheep. Anesthesiology. 1989;71:418–25.
41. Ma D, Rajakumaraswamy N, Maza M. α_2-Adrenoceptor agonists: shedding light on neuroprotection? Br Med Bull. 2005;71:77–92.

42. Sakurai S, Wada A, Izumi F, Kobayashi H, Yanagihara N. Inhibition by α_2-adrenoceptor agonists of the secretion of catecholamines from isolated adrenal medullary cells. Naunyn Schmiedebergs Arch Pharmacol. 1983;324:15–9.
43. Takeda M, Phillips JK, Dubey R, Polson JW, Lipski J. Modulation of ACh-induced currents in rat adrenal chromaffin cells by ligands of α_2 adrenergic and imidazoline receptors. Auton Neurosci. 2001;88:151–9.
44. Yoshitomi T, Kohjitani A, Maeda S, Higuchi H, Shimada M, Miyawaki T. Dexmedetomidine enhances the local anesthetic action of lidocaine via an α-2A adrenoceptor. Anesth Analg. 2008;107:96–101.
45. Lavand'homme PM, Ma W, De Kock M, Eisenach JC. Perineural α_{2A}-adrenoceptor activation inhibits spinal cord neuroplasticity and tactile allodynia after nerve injury. Anesthesiology. 2002;97:972–80.
46. Romero-Sandoval A, Eisenach JC. Perineural clonidine reduces mechanical hypersensitivity and cytokine production in established nerve injury. Anesthesiology. 2006;104:351–5.
47. Liu B, Eisenach JC. Perineural clonidine reduces p38 mitogen-activated protein kinase activation in sensory neurons. Neuroreport. 2006;17:1313–7.
48. Chung S, Ahn DS, Kim YH, Kim YS, Joeng JH, Nam TS. Modulation of N-type calcium currents by presynaptic imidazoline receptor activation in rat superior cervical neurons. Exp Physiol. 2010;95:982–93.
49. Zheng JQ, Weng XC, Gai XD, Li J, Xiao WB. Mechanism underlying blockade of voltage-gated calcium channels by agmatine in cultured rat hippocampal neurons. Acta Pharmacol Sin. 2004;25:281–5.