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Adenosine A₃ receptor activation inhibits pronociceptive N-type Ca²⁺ currents and cell excitability in dorsal root ganglion neurons

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Abstract

Recently, studies have focused on the antihyperalgesic activity of the A₃ adenosine receptor (A₃AR) in several chronic pain models, but the cellular and molecular basis of this effect is still unknown. Here, we investigated the expression and functional effects of A₃AR on the excitability of small- to medium-sized, capsaicin-sensitive, dorsal root ganglion (DRG) neurons isolated from 3- to 4-week-old rats. Real-time quantitative polymerase chain reaction experiments and immunofluorescence analysis revealed A₃AR expression in DRG neurons. Patch-clamp experiments demonstrated that 2 distinct A₃AR agonists, CI-IB-MECA and the highly selective MRS5980, inhibited Ca²⁺-activated K⁺ (K_{Ca}) currents evoked by a voltage-ramp protocol. This effect was dependent on a reduction in Ca²⁺ influx via N-type voltage-dependent Ca²⁺ channels, as CI-IB-MECA-induced inhibition was sensitive to the N-type blocker PD173212 but not to the L-type blocker, lacidipine. The endogenous agonist adenosine also reduced N-type Ca²⁺ currents, and its effect was inhibited by 56% in the presence of A₃AR antagonist MRS1523, demonstrating that the majority of adenosine's effect is mediated by this receptor subtype. Current-clamp recordings demonstrated that neuronal firing of rat DRG neurons was also significantly reduced by

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Conflict of interest statement

D.S. is a co-founder of BioIntervene, Inc, that licensed related intellectual property from Saint Louis University. The remaining authors have no conflicts of interest to declare.

Appendix A. Supplemental digital content

Supplemental digital content associated with this article can be found online at <http://links.lww.com/PAIN/A733>.

A₃AR activation in a MRS1523-sensitive but PD173212-insensitive manner. Intracellular Ca²⁺ measurements confirmed the inhibitory role of A₃AR on DRG neuronal firing. We conclude that pain-relieving effects observed on A₃AR activation could be mediated through N-type Ca²⁺ channel block and action potential inhibition as independent mechanisms in isolated rat DRG neurons. These findings support A₃AR-based therapy as a viable approach to alleviate pain in different pathologies.

Keywords

Dorsal root ganglion neurons; Pronociceptive Ca²⁺ currents; Adenosine A₃ receptors; Action potential

1. Introduction

Pain control is a vast, unmet medical need with a high societal cost impact.¹⁸ Current treatments (opioids, nonsteroidal anti-inflammatory drugs, antidepressants, or anticonvulsants) are frequently inadequate or associated with adverse side effects.^{7,18,43} Therefore, new therapeutics for managing patient pain are being developed.

Voltage-dependent Ca²⁺ channels (VDCCs) are crucial mediators of neuropathic pain, as confirmed by the fact that α 2 δ ligands, ie, gabapentinoids, are a first-line treatment for this type of pain.^{13,55} Activation of VDCCs at a presynaptic level induces neurotransmitter release through the peripheral and central nervous system, including sensory neurons in dorsal root ganglia (DRG), which are considered the primary site of nociception. It is noteworthy that the selective N-type VDCC blocker ω -conotoxin GVIA (ω -CTX) inhibits dorsal root stimulus-evoked excitatory postsynaptic currents in lamina I dorsal horn neurons by 60%,²³ suggesting that these channels exert a primary role in mediating neurotransmitter release during nociception. Furthermore, aberrant expression and/or activity of N-type VDCCs is associated with neuropathic pain,²⁰ and ziconotide, a derivative of ω -CTX, was FDA approved in 2000 (Prialt) for intrathecal treatment of severe and refractory chronic pain.^{2,28,38} However, severe adverse effects are associated with a direct Ca²⁺ channel block; thus, a modulation of Ca²⁺ influx would be preferable.

Adenosine is an endogenous neuromodulator acting on 4 metabotropic receptors: the Gi-coupled A₁ and A₃ adenosine receptors (ARs) and the Gs-coupled A_{2A} and A_{2B} subtypes.¹⁵ A₁AR activation inhibits pain behaviours in different models of acute or chronic pain.¹⁰ Unfortunately, the therapeutic utility of A₁AR agonists is limited by their adverse cardiovascular effects. Of note, exciting preclinical observations designate the A₃AR as a powerful antihyperalgesic mediator in different in vivo rodent models of experimental neuropathic pain,^{14,29,30,33,34,37,51,56} suggesting the use of A₃AR agonists for chronic pain treatment without cardiovascular implications.

A₃ARs are expressed in DRG neurons, with species-specific differences being described.^{44,53} However, cellular mechanisms mediating pain relief on A₃AR activation remain largely unexplored. A₃AR-selective ligands currently available allow for the dissection of A₃AR-based molecular effects on DRG neuronal excitability.³⁰ The recently synthesized and highly

selective A₃AR agonist MRS5698, and the more water-soluble congener MRS5980, proved effective in preventing allodynia and hyperalgesia associated with traumatic nerve injury, chemotherapy, and bone cancer in rodents.^{14,29,34} These second-generation, highly selective A₃AR agonists are at least several orders of magnitude more selective than the early generation exemplified by IB-MECA and CI-IB-MECA.^{29,34,51}

Several early electrophysiological studies reported that adenosine and its analogues inhibit Ca²⁺-dependent plateau potentials and VDCC activation in isolated rat DRG neurons.^{11,19,36} However, AR subtypes were initially poorly characterized, with only A₁AR and a “generic” A₂ subtype being described based on their ability to modulate intracellular cAMP accumulation. Nevertheless, MacDonald and coworkers³⁶ argued that neither A₁AR nor A₂AR seems to be responsible for Ca²⁺ current inhibition in rat DRG neurons, leading to the possibility that a hypothetically different AR could be involved. Building on this observation, the aim of this study was to fill a major gap by investigating whether the A₃AR modulates membrane currents and excitability in isolated rat DRG neurons.

2. Material and Methods

2.1. Cell cultures

Animal experiments were performed according to Directive 2010/63/EU of the European Parliament and of the European Union Council (September 22, 2010) and to the Italian Law on Animal Welfare (DL 26/2014). The protocol was approved by the University of Florence Institutional Animal Care and Use Committee and by the Italian Ministry of Health. All efforts were made to minimize animal suffering and to use a minimal number of animals needed to produce reliable scientific data. Sprague-Dawley rats (3–4 weeks old, Envigo, Udine, Italy) of both sexes were housed in a temperature- and humidity-controlled vivarium (12-hour dark/light cycle, free access to food and water) and sacrificed by cervical dislocation. Primary DRG neurons were isolated and cultured as described.^{16,40} Briefly, ganglia were bilaterally excised and enzymatically digested using 2 mg/mL of collagenase type 1A and 1 mg/mL of trypsin (both compounds from Sigma-Aldrich, Milan, Italy) in Hank's balanced salt solution (25–35 minutes at 37°C). Cells were then pelleted and resuspended in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated horse serum, 10% heat-inactivated fetal bovine serum, 100 U/mL penicillin, 0.1 mg/mL streptomycin, and 2-mM L-glutamine for mechanical digestion. After centrifugation (1200g, 5 minutes), neurons were suspended in the above medium, enriched with 100 ng/mL of mouse nerve growth factor and 2.5 mM of cytosine-β-D-arabino-furanoside free base, and then plated on 13-mm or 25-mm glass coverslips coated by poly-L-lysine (8.3 mM) and laminin (5 mM). Dorsal root ganglion neurons were cultured for 1 to 2 days before being used for experiments. In a set of experiments, DRG cultures were maintained in the absence of nerve growth factor. However, no difference was found in any of the effects tested in the present research, and data were pooled.

2.1.1. Electrophysiology—Whole-cell patch-clamp recordings were performed as previously described.^{4,5} The following solutions were used: standard, K⁺-containing, extracellular solution (mM): NaCl 147; KCl 4; MgCl₂ 1; CaCl₂ 2; HEPES (4-(2-

hydroxyethyl)-1-piperazineethanesulfonic acid) 10; and D-glucose 10 (pH 7.4 with NaOH). Standard K⁺-based pipette solution (mM): K-gluconate 130; NaCl 4.8; KCl 10; MgCl₂ 2; CaCl₂ 1; Na₂-ATP 2; Na₂-GTP 0.3; EGTA 3; and HEPES 10 (pH 7.4 with KOH). The calculated liquid junction potential for K⁺-gluconate pipettes was 15.0 mV. All voltage values reported here have been adjusted accordingly. For K⁺-replacement experiments, extracellular K⁺ was substituted by equimolar Cs⁺ and the following, Cs⁺-based, pipette solution used was (mM): CsCl 120; Mg₂-ATP 3; EGTA 10; and HEPES 10 (pH 7.4 with CsOH). Ca²⁺ currents were isolated by using Cs⁺-based pipette solution and by adding 1-μM tetrodotoxin (TTX), 200-nM A887826 to a 5-mM Ca²⁺-containing and Cs⁺-substituted extracellular solution to block TTX-sensitive and TTX-insensitive Na⁺ channels, respectively. As reported in Results, ramp experiments revealed that Cl-IB-MECA-inhibited Ca²⁺ currents in DRG neurons were completely prevented in the presence of extracellular Cd²⁺. The effect of Cd²⁺ was not different when applied at 100 μM, 500 μM, or 1 mM concentrations (see Supplementary Fig. 2, available at <http://links.lww.com/PAIN/A733>). So, when studying the effect of this compound on Ca²⁺ currents evoked in isolation, 100-μM Ni²⁺ was added to the extracellular solution to exclude T-type Ca²⁺ channels, which do not seem to be involved in A₃AR-mediated effect. For this reason, the hypothetical, additional, involvement of T-type Ca²⁺ channels in A AR-mediated Ca²⁺ channel modulation was not investigated in the present research and will be addressed in a separate work. Na⁺ currents were isolated by using Cs⁺-based pipette solution and by adding 100-μM Cd²⁺ and 100-μM Ni²⁺ to a 5-mM Ca²⁺-containing and Cs⁺-substituted extracellular solution.

Cells were transferred to a 1-mL recording chamber mounted on the platform of an inverted microscope (Olympus CKX41, Milan, Italy) and superfused at a flow rate of 2 mL/minute by a 3-way perfusion valve controller (Harvard Apparatus, Holliston, MA). Borosilicate glass electrodes (Harvard Apparatus) were pulled with a Sutter Instruments puller (model P-87) to a final tip resistance of 1.5 to 3 MΩ. Data were acquired with an Axopatch 200B amplifier (Axon Instruments, Union City, CA), low-pass filtered at 10 kHz, stored, and analysed with pClamp 9.2 software (Axon Instruments). All the experiments were performed at room temperature (RT: 20–22°C). Series resistance (Rs), membrane resistance (Rm), and membrane capacitance (Cm) were routinely measured by fast hyperpolarizing voltage pulses (from –60 to –70 mV, 40-ms duration). Only cells showing a stable Cm and Rs before, during, and after drug application were included in the analysis. Immediately after seal breaking-through, cell's resting membrane potential was determined by switching the amplifier to the current-clamp mode. A voltage-ramp protocol (800-ms depolarization from +65 to –135 mV; holding potential, or V_h, of –75 mV) was used to evoke a wide range of overall voltage-dependent currents before, during, and after drug treatments. TRPA1- or TRPV1-mediated currents were detected as inward currents activated in –60 mV (corresponding to –75 mV after liquid junction potential correction) clamped cells on superfusion of the respective agonists allyl isothiocyanate (AITC) and capsaicin, respectively, as already shown elsewhere.^{16,40} Voltage-dependent Ca²⁺ channels' currents were evoked in Cs⁺-replacement conditions by a 0-mV step depolarization (200 ms, V_h = –65 mV) once every 30 seconds, a time that allowed complete recovery from eventual Ca²⁺ channel in-activation and minimized Ca²⁺ current run down. The current-to-voltage relationship of Ca²⁺ currents was obtained by eliciting 10 depolarizing voltage steps (200-

ms duration, 10-mV increments, 5-second interval) from -50 to $+50$ mV starting from a V_h of -65 mV. Na^+ currents were evoked in Cs^+ -replacement conditions by a 0-mV step depolarization (40 ms, $V_h = -90$ mV) once every 5 seconds.

Averaged currents were normalized to cell capacitance and expressed as pA/pF. Cell capacitance was used to estimate neuronal diameter by assuming an approximated spherical cell shape according to the calculated C_m for all biological membranes of $1 \mu\text{F}/\text{cm}^2$ and to the equation of the sphere surface: $A = 4 \pi r^2$.²⁸

Current-clamp recordings were performed as described.⁵ A ramp current protocol consisting in 1-second injection of 30-pA positive current from the resting membrane potential (only cells showing a V_m of at least -50 mV were chosen) was used to evoke action potential (AP) firing in a typical DRG neuron once every 30 seconds. After at least 3-minute recording of a stable baseline, Cl-IB-MECA (100 nM) was applied for 8 to 10 minutes. Action potential firing was quantified by counting the number of APs evoked by a ramp current (1 second). When the Cl-IB-MECA was applied in the presence of MRS1523 (100 nM) or PD173212 (1 μM), the A_3AR antagonist or the N-type VDCC blocker was added at least 10 minutes before the agonist. Current-clamp recordings were filtered at 10 kHz and digitized at 1 kHz. All current-clamp values reported in Table 1 are the average of at least 3 episodes and have been corrected for liquid junction potential. All AP parameters refer to the first AP generated by the ramp. Resting V_m was calculated as the averaged membrane voltage measured 200 ms before ramp current injection. The rate of AP depolarization and hyperpolarization was measured as the first derivative of membrane potential over time (mV/ms). Action potential threshold was defined as the point at which the derivative first exceeded 30 mV/ms. Action potential amplitude was calculated as the difference between the peak reached by the overshoot and the threshold. Action potential time to peak was measured as the time between the threshold and the voltage peak reached by the AP. Action potential half-width was measured as the time to reach half the AP amplitude (ms). The current needed to produce the first spike was defined as “current threshold” and was measured as the minimal amount of current (pA) injected during the ramp protocol leading to the first AP initiation. The AP duration measured the difference between the time of reaching the threshold potential during the rising phase and the time when the repolarizing potential crossed the threshold value again. The fast afterhyperpolarization was measured as the difference between the voltage threshold and the minimum potential reached after the AP peak.

2.2. Quantitative real-time polymerase chain reaction analysis

Total RNA was isolated using Nucleospin RNA (Macherey–Nagel Duren, Duren, Germany) with DNase treatment according to the manufacturer’s instructions. The expression of A_3ARs was evaluated by quantitative real-time polymerase chain reaction (RT-PCR) using gene-specific fluorescently labeled TaqMan MGB probe (minor groove binder). For the RT-PCR of A_3ARs , a predeveloped assay was used: Adora3 Rn_00563680_m1. The amount of target mRNA was normalized to the endogenous reference, GAPDH Rn 01749022_g1, and to a homogenate of the rat brain taken as a positive, according to the 2^{-C_t} method. Data are the result of 3 independent experiments performed in triplicate.

2.3. Immunocytochemical analysis

Primary DRG cultures grown on 13-mm diameter coverslips were fixed with 4% paraformaldehyde in 0.1 M phosphate-buffered saline for 20 minutes at RT. Rabbit polyclonal A₃AR-selective primary antibody (Alomone Labs, Jerusalem, Israel; <https://www.alomone.com>) was diluted 1:200 in bovine serum dilution buffer (450-mM NaCl, 20-mM sodium phosphate buffer, pH 7.4, 15% fetal bovine serum, and 0.3% Triton X-100) and incubated for 2.5 hours at RT. Cells were then washed 3 times with phosphate-buffered saline and incubated for 1 hour at RT with a donkey anti-rabbit secondary antibody (diluted 1:500 in bovine serum dilution buffer) conjugated to AlexaFluor 488 (Life Technologies, Invitrogen, Milan, Italy). Coverslips were mounted with Vectashield mounting medium (Vector Laboratories, Burlingame, CA) containing 4',6-diamidino-2-phenylindole (DAPI) to visualize cells nuclei, digitized, and acquired by using an Olympus BX40 microscope equipped with *CellSens Dimension* Software (Olympus, Hamburg, Germany). Control experiments were performed by incubating fixed cells with the secondary antibody alone to exclude nonspecific binding.

2.4. Intracellular Ca²⁺ measurement

Intracellular cytosolic Ca²⁺ dynamic ([Ca²⁺]_i) was evaluated in fura-2-loaded DRG neurons as described.⁸ Briefly, 10⁴ cells were plated on round glass coverslips (25-mm diameter) and seeded for 1 to 2 days in a complete medium. Cells were loaded with 4-μM fura-2AM (Molecular Probes-Invitrogen Life technologies, San Giuliano Milanese, Italy) for 45 minutes at 37°C and then washed with the K⁺-containing standard extracellular solution described above. Coverslips were mounted in a perfusion chamber and placed on the stage of an inverted reflected light fluorescence microscope (Zeiss Axio Vert. A1 FL-LED) equipped with fluorescence excitation (385 nm) based on LED. Before electrical field stimulation, cells were incubated for at least 5 minutes with different solutions containing the following molecules: control (standard extracellular solution), 1-μM TTX + 200-nM A887826, 30-nM CI-IB-MECA, and 1-μM verapamil + 0.5-μM PD173212. Fura-2 fluorescence was recorded with a Tucsen Dhyana 400D CMOS camera (Tucsen Photonics, Co, Ltd, Fuzhou, China) with a frame rate of 40 Hz and a resolution of 1024 × 1020 pixels². Ca²⁺ dynamic was measured by single-cell imaging analysis at 35°C.⁸ Images were recorded using Dhyana software SamplePro and dynamically analyzed with the open-source community software for bio-imaging Icy (Institute Pasteur, Paris, France). Ca²⁺ transients were induced by electrical field stimulation at 0.1-Hz frequency, 100-mV voltage, and 50-ms width duration. Preliminary experiments were performed to optimize stimulation parameters in control condition (standard extracellular solution). Frequency, voltage, and duration were chosen to obtain a high number of responder cells, defined as “spiking” cells, without signs of membrane electroporation and stable fura-2 fluorescence. A signal-to-noise ratio of at least 5 arbitrary units was considered as Ca²⁺ transient. Spiking DRG neurons were identified as cells showing at least 5 Ca²⁺ transients in 1 minute. In spiking cells, the following parameters were evaluated as the mean of at least 3 different Ca²⁺ transients: the ratio between the fluorescence maximal variation induced by electrical field stimulation and basal fluorescence ($\Delta F/F$, measured as arbitrary units) and the decay time of Ca²⁺ transient (tau, τ). Tau was calculated according to the following equation: $Y = y_0 + A \times e^{(-X/\tau)}$.

According to the fitting function, the tau (τ) parameter represented the time necessary for $[Ca^{2+}]_i$ to reach 36.8% of the maximal value. Tau was therefore reported as the decay time value (s). At least 3 Ca^{2+} transients for each different treatment were analysed and averaged. The cell diameter of analyzed DRG neurons was measured in pixels using ImageJ software and transformed in micrometer by a specific calibration scale.

Experiments were repeated in 4 different neuronal preparations. All DRG neurons (identified by transmitted light microscopy) found in an optical field (using 40 $\times$ magnification objective) were analyzed. From 16 to 22 cells were evaluated blindly every experimental day for each experimental treatment.

2.5. Drugs

2-Chloro-*N*⁶-(3-iodobenzyl)-adenosine-5'-*N*-methyluronamide (Cl-IB-MECA), 5-(4-butoxy-3-chlorophenyl)-*N*-[[2-(4-morpholinyl)-3-pyridinyl]methyl]-3-pyridinecarboxamide (A887826), 3-propyl-6-ethyl-5-[(ethylthio)carbonyl]-2-phenyl-4-propyl-3-pyridine carboxylate (MRS1523), *N*-(2-methoxyphenyl)-*N*'-[2-(3-pyridinyl)-4-quinazolinyl]-urea (VUF5574), 8-cyclopentyl-1,3-dipropylxanthine (DPCPX), *N*⁶-cyclopentyladenosine (CPA), adenosine, apamin, and tetraethylammonium (TEA) were purchased from Sigma (www.sigmaaldrich.com). Tetrodotoxin (TTX) and 4-*t*-butyl-*N*-methyl-*N*-aralkyl-peptidylamine (PD173212) were purchased from Alomone Labs. Verapamil was purchased from Calbiochem (Merck, Darmstadt, Germany). The new, highly selective, A_3 receptor agonist (1*S*,2*R*,3*S*,4*R*,5*S*)-4-(2-((5-chlorothiophen-2-yl)ethynyl)-6-(methyl-amino)-9H-purin-9-yl)-2,3-dihydroxy-*N*-methylbicyclo[3.1.0]hexane-1-carboxamide (MRS5980) was synthesized as reported previously.^{12,51} See Supplementary Figure 1 for chemical structure of AR ligands used in the present research (available at <http://links.lww.com/PAIN/A733>). All drugs were stored at $-20^{\circ}C$ as 10^3 to 10^4 times more concentrated stock solutions, dissolved daily in the extracellular solution to the final concentration, and applied by bath superfusion. Tetrodotoxin, apamin, and TEA stock solutions were prepared in distilled water. All other compounds were dissolved in dimethyl sulphoxide. Control experiments performed in the present research demonstrated that the maximal dimethyl sulphoxide concentration used in the present work (0.1%) was inactive in modulating membrane currents in our experimental conditions in DRG neurons.

2.6. Statistical analysis

Data are expressed as mean $\pm$ SEM. Student paired or unpaired *t* tests, and one-way or two-way analysis of variance (ANOVA) followed by Bonferroni analysis were performed, as appropriated, to determine statistical significance (set at $P < 0.05$). In experiments of intracellular Ca^{2+} measurement, 3 different parameters were measured: (1) the ratio between DRG neurons defined as “spiking” (Ca^{2+} transient) and “not spiking” in each experimental condition; (2) F/F ; and (3) tau of Ca^{2+} transient evoked in “spiking” cells. The effect of each treatment in changing the ratio between “spiking” and “not spiking” was evaluated using the χ^2 test (PRIMER); other data (reported as mean $\pm$ SEM) were statistically analysed using one-way ANOVA followed by Bonferroni analysis. Data were analysed using “Origin 10” or “GraphPad Prism” (GraphPad Software, San Diego, CA) software.

3. Results

3.1. Selective A₃AR activation inhibits Ca²⁺ currents in cultured rat dorsal root ganglion neurons

Because no data are available up to now concerning the electrophysiological effect/s of A₃AR on DRG neurons, we first tested the prototypical A₃AR agonist Cl-IB-MECA under “similar physiological conditions,” ie, we applied a voltage-ramp protocol (from +65 to −135 mV, 800-ms duration: see inset of Fig. 1A) in K⁺-containing solutions before, during, and after the superfusion of this compound. As shown in Figure 1A, the application of 100-nM Cl-IB-MECA decreased overall outward currents evoked by the voltage ramp: the effect peaked within 5 minutes and was partially reversed after drug washout. Figure 1B shows that the net Cl-IB-MECA-inhibited current was an outward current activated at potentials positive to −45 mV. Ramp current inhibition measured at +65 mV in the presence of Cl-IB-MECA was statistically significant in 14 cells investigated (Figs. 1C and D: from 799.0 ± 111.9 pA/pF in control to 617.2 ± 92.3 pA/pF in 100-nM Cl-IB-MECA, $P = 0.0005$, the paired Student *t* test), whereas no changes in inward ramp currents at −135 mV were detected (Figs. 1C and D: from −46.6 ± 10.7 pA/pF in control to −42.8 ± 7.5 pA/pF in 100-nM Cl-IB-MECA, $P = 0.3734$, the paired Student *t* test). The Cl-IB-MECA effect was observed in all cells tested, in agreement with high A₃AR levels expression in DRG homogenate as revealed by RT-PCR (Fig. 1E) and immunocytochemical analysis (Figs. 1F and G). Our data are in line with previous work in the literature demonstrating the expression of this receptor subtype on rat DRG neurons, even if species-specific differences have been found.^{44,53} The effect was concentration dependent (Fig. 1H), with an EC₅₀ of 2.0 nM (confidence limits: from 1.2 to 3.2 nM; Fig. 1I), and prevented by 2 different A₃AR antagonists: VUF5574 (100 nM) and MRS1523 (100 nM) (Figs. 1J and K). Another highly selective A₃AR agonist was tested: MRS5980, whose EC₅₀ is described in the subnanomolar range (EC₅₀ = 0.7 ± 0.1 nM⁵²). This compound also concentration dependently inhibited total outward currents evoked by the ramp protocol at +65 mV in 8 cells investigated (Figs. 2A–C), and the effect was prevented by MRS1523 (inset in Fig. 2A).

The effect of both A₃AR agonists was shared by the endogenous ligand adenosine (30 μM: Fig. 2D), which significantly decreased ramp currents at +65 mV in 5 cells tested without modifying the inward component (Fig. 2E). Notably, the adenosine effect was significantly inhibited by VUF5574 (100 nM; n = 6: Fig. 2F; $**P = 0.0054$, the unpaired Student *t* test).

To define A₃AR-responding DRG neurons as nociceptors, cells were tested for their responsiveness to the TRPA1 agonist AITC and the TRPV1 agonist capsaicin. At least 5 minutes after Cl-IB-MECA removal, cells were voltage clamped at −75 mV and the 2 TRP agonists were applied consecutively. As shown in a typical cell in Figure 3A, both compounds activated an inward current in the vast majority of cells investigated (Fig. 3B): 8 of 33 cells tested for TRP response were insensitive to 1 of the 2 TRP agonists, and 1 cell was insensitive to both compounds but still sensitive to Cl-IB-MECA. In Figure 3C, we pooled cell capacitance values measured in those 33 TRP-sensitive cells vs capacitance values measured in the residual cells analyzed in the present research but not tested for TRP responses (TRP not tested: n = 112). Of note, cell capacitance, as a measure of a spherical-

approximated cell soma, was not statistically different in TRP-sensitive (25.1 ± 1.7 pF; $n = 33$, corresponding to a cell diameter of $28.3 \mu\text{m}$: see Methods) vs TRP not tested (25.3 ± 1.2 pF; $n = 112$, corresponding to a cell diameter of $28.4 \mu\text{m}$; the unpaired Student t test, $P = 0.9825$; Fig. 3C) neurons. Our results thus indicate that both groups of cells were composed of neurons with a $25\text{-}\mu\text{m}$ diameter, therefore adhering to the definition of small- to medium-sized DRG neurons defined as “nociceptors.”^{22,48}

Data in the literature demonstrate that adenosine and its analogues inhibit VDCCs in rat DRG neurons^{11,36} with no obvious distinction between A_1 - vs A_3 -mediated effects provided to date. For this reason, we tested the hypothesis that the decreased total outward currents observed in the present work by A_3 AR activation might depend on a decrease of Ca^{2+} entry from VDCCs and, thus, reduced activation of Ca^{2+} -activated K^+ conductances (K_{Ca}). Therefore, we applied Cl-IB-MECA in the presence of the nonselective VDCC blocker Cd^{2+} . First of all, by using our voltage-ramp protocol, we confirmed the activation of K_{Ca} channels in DRG neurons by applying extracellular Cd^{2+} that, per se, induced a $34.0 \pm 13.3\%$ inhibition of total outward currents evoked by the ramp protocol (from $+245.7 \pm 15.6$ pA/pF in ctrl to 167.2 ± 26.3 pA/pF in $100\text{-}\mu\text{M}$ Cd^{2+} at $+65$ mV, $n = 4$, $P = 0.0339$; the paired Student t test; Supplementary Fig. 2C: $34.0 \pm 8.7\%$ current inhibition, available at <http://links.lww.com/PAIN/A733>). The effect of Cd^{2+} was not different when applied at $100 \mu\text{M}$, $500 \mu\text{M}$, or 1 mM concentrations (see Supplementary Fig. 2A–C, available at <http://links.lww.com/PAIN/A733>). Thus, we applied Cl-IB-MECA in the presence of Cd^{2+} to test whether the A_3 AR agonist effect was prevented in conditions of Ca^{2+} entry block. As shown in Figure 4A–C, Cd^{2+} completely prevented Cl-IB-MECA-induced decrease of outward ramp currents. Data were confirmed by applying 2 selective blockers of K_{Ca} channels: apamin (100 nM), which selectively blocks small-conductance K_{Ca} (SK channels), and TEA at low concentrations ($200 \mu\text{M}$), which selectively inhibits big-conductance K_{Ca} (BK channels). Alone or in combination, both compounds significantly or completely prevented Cl-IB-MECA-mediated inhibition of ramp-evoked outward currents (Figs. 4D and E). The above data demonstrate that BK and SK channel activation was necessary for the A_3 AR-mediated ramp-current inhibition in rat DRG neurons.

Two possibilities exist to explain this phenomenon: (1) A_3 AR activation directly inhibits SK and BK channels; or (2) A_3 ARs activation inhibits Ca^{2+} entry from VDCCs, which, in turn, decreases SK and BK channel opening. To test the latter hypothesis, we blocked all K^+ currents by replacing intracellular and extracellular K^+ ions with equimolar Cs^+ . In these experimental conditions, an inward component arose in the ramp protocol peaking around 0 mV (Fig. 4F). This current was identified as a Ca^{2+} current because it was abolished by extracellular Cd^{2+} (Figs. 4F and G). When applying Cl-IB-MECA in Cs^+ -replacement conditions, a significant decrease in inward peak current was observed (Fig. 4H: from -64.3 ± 14.8 pA/pF in control to -41.1 ± 11.4 pA/pF in Cl-IB-MECA, $*P = 0.0431$, the paired Student t test; $n = 5$), thus demonstrating that A_3 AR activation directly inhibited Ca^{2+} currents.

Dorsal root ganglion neurons express different subtypes of Ca^{2+} currents.^{46–48,57} To identify which of them are inhibited by the A_3 AR, we further isolated VDCC-mediated currents by adding the $\text{Na}_v1.1$, 1.2 , 1.3 , 1.4 , 1.6 , and 1.7 blocker TTX ($1 \mu\text{M}$) plus the $\text{Na}_v1.8$ inhibitor

A887826 (200 nM, to block this TTX-resistant Na⁺ channel) to the Cs⁺-containing extracellular solution. Na_v1.8 is known to be expressed at high levels in DRG neurons,⁹ which we confirmed, because in the presence of 1-μM extracellular TTX, 200-nM A887826 completely blocked residual, TTX-resistant, Na⁺ currents (Figs. 5A and B). As shown in Figure 5C, under these experimental conditions, we successfully isolated Ca²⁺ currents activated by a 0-mV voltage step depolarization (200-ms duration), which were completely blocked by 100-μM Cd²⁺ (Figs. 5C–E). Such Ca²⁺ currents were predominantly N-type currents because application of selective N-type blocker PD173212 (500 nM; Figs. 5F and G) achieved a >80% block.^{25,26,50} Residual, PD173212-insensitive (up to 1 μM; Fig. 5H) Ca²⁺ currents in our experimental conditions were blocked by Cd²⁺ (Fig. 5H) and were attributed to Cd²⁺-sensitive L-type and, eventually, R- and/or P/Q-type VDCCs, consistently with a previous report.⁵⁷

Current-to-voltage (I–V plot) relationship of Ca²⁺ currents activated by a series of depolarizing voltage steps (from –50 to +50 mV, 10-mV increment, 200-ms duration, V_h = –65 mV; inset in Fig. 5I) was consistent with Cd²⁺-blocked (Fig. 5I) and PD173212-sensitive (Fig. 5J) N-type VDCC activation.

When Cl-IB-MECA was applied in these experimental conditions, a reversible decrease in total Ca²⁺ currents (both in the peak and steady-state component) was observed within 5 minutes of drug application (Fig. 6A). The I–V plot of such Ca²⁺ conductances (Fig. 6C) demonstrated that the A₃AR agonist significantly inhibited VDCCs from 0 to +20 mV (the paired Student *t* test, *n* = 5). The Cl-IB-MECA effect was significant in 8 cells tested (Fig. 6B), either as peak current amplitude (arrow in the right panel of Fig. 6A or Fig. 6B) or at the steady state (arrowhead in the right panel of Fig. 6A: from 57.4 ± 14.5 to 39.8 ± 10.2 pA/pF; *P* = 0.0174, the paired Student *t* test). However, the A₃AR agonist apparently did not change the Ca²⁺ current kinetics because the time to peak was unaffected (from 15.1 ± 2.2 to 15.7 ± 3.0 ms; *P* = 0.5553, the paired Student *t* test, data not shown). Cl-IB-MECA-mediated inhibition of VDCCs was concentration dependent with a maximal effect observed at 30 nM (Fig. 6E), in accordance with ramp experiments, and completely prevented by the A₃AR antagonist MRS1523 (100 nM) and by the selective N-type channel blocker PD173212 (500 nM) but not by the selective, at least at 1-μM concentration,⁶ L-type blocker lacidipine (1 μM; Figs. 6D and E). The above data demonstrate that A₃AR activation selectively inhibits N-type Ca²⁺ currents in rat DRG neurons.

In the attempt to compare A₃AR- vs A₁AR-mediated effects on VDCCs, we applied the A₁AR-selective agonist CPA. As shown in Figures 6E and F, CPA inhibited Ca²⁺ currents by 24.8 ± 3.2% (*n* = 9) and by 24.1 ± 3.0% (*n* = 7) at 1 and 10 μM concentrations, respectively. Of note, Ca²⁺ current inhibition measured in the presence of 1-μM CPA was significantly smaller compared with the Cl-IB-MECA-mediated effect (44.1 ± 5.6% inhibition in the presence of 30-nM; Cl-IB-MECA vs 24.8 ± 3.2% inhibition in the presence of 1-μM CPA, *P* = 0.0464, one-way ANOVA, Bonferroni posttest, Fig. 6E).

The newly synthesized A₃AR agonist MRS5980 mimicked the Cl-IB-MECA effect in inhibiting Ca²⁺ currents (Figs. 7A and C) but with higher efficacy, showing a maximal VDCC inhibition at 3 nM concentration (Fig. 7B) compared with 30 nM for Cl-IB-MECA.

Furthermore, the MRS5980-mediated effect was prevented in the presence of A₃AR antagonist MRS1523 (100 nM; Fig. 7B).

To explore the contribution of A₃AR-mediated inhibition of VDCCs when the endogenous agonist is present in the extracellular space, we applied adenosine (30 μ M) in the absence or presence of a selective adenosine A₁ or A₃AR antagonist, DPCPX or MRS1523, respectively. As expected from published data,^{11,36} adenosine inhibited Cd²⁺-sensitive Ca²⁺ currents with a maximal effect observed at 30 μ M (Fig. 7E). Of note, the effect induced by 30-mM adenosine was blocked by 39.1 $\pm$ 9.0% in the presence of DPCPX (500 nM) and by 56.6 $\pm$ 9.0% in the presence of MRS1523 (100 nM; Figs. 7E and F). No significant difference was found between DPCPX- and MRS1523-mediated inhibition of adenosine effects (one-way ANOVA, Bonferroni posttest).

Finally, we evaluated the impact of selective A₃AR activation on neuronal excitability. We induced AP firing by injecting a depolarizing ramp current from the resting membrane potential. As shown in Figure 8A, this protocol induced repetitive firing in DRG neurons, which was markedly reduced in the presence of CI-IB-MECA (Figs. 8A and B). The effect was statistically significant in 6 cells tested (Fig. 8C) and prevented in the presence of A₃AR antagonist MRS1523 (100 nM; Figs. 8D–F) but not by the N-type VDCC blocker PD173212 (1 μ M; Figs. 8G–I). Of note, a two-way ANOVA comparison between the effect of CI-IB-MECA when applied alone (Fig. 8J, left column: “in ctrl”) or applied in the presence of PD173212 (Fig. 8J, right column: “in PD173212”) highlighted that: (1) a statistical difference was found between the number of APs recorded in control conditions or in the presence of PD173212 alone (Fig. 8J, black circles: before CI-IB-MECA: 6.5 $\pm$ 1.3 APs in ctrl, n = 6, vs 19.1 $\pm$ 3.4 APs in PD173212, n = 7; P = 0.0011), indicating that N-type VDCC block exerts a proexcitatory effect on cells firing; and (2) no difference was found in the inhibitory effect of CI-IB-MECA when applied in the absence or presence of PD173212 (gray circles: after CI-IB-MECA: 3.3 $\pm$ 1.9 APs in CI-IB-MECA, n = 6, vs 5.6 $\pm$ 1.1 APs in CI-IB-MECA + PD173212; P = 0.7109), indicating that CI-IB-MECA effect on firing is not occluded by N-type VDCC. To corroborate the proexcitatory effect of N-type VDCC block, we applied PD173212 alone in a separate group of cells, and we observed a significant increase in AP firing (Suppl. Fig. 3, available at <http://links.lww.com/PAIN/A733>) accompanied by a significant cell depolarization (Suppl. Fig. 3B and C, available at <http://links.lww.com/PAIN/A733>; and Table 1: from -63.6 ± 2.9 mV in control to -59.7 ± 3.6 mV in 1- μ M PD173212; P = 0.0165, the paired Student t test, n = 7) and lowering of AP threshold (Table 1: from -12.7 ± 1.9 mV in control to -14.1 ± 1.51 mV in 1- μ M PD173212; P = 0.0491, the paired Student t test, n = 7). Furthermore, in accordance with K_{Ca} inhibition and in particular with BK channel block, we measured a significant increase in AP duration (from 3.9 $\pm$ 0.7 ms in control to 4.5 $\pm$ 0.6 ms in 1- μ M PD173212; P = 0.0089, the paired Student t test, n = 7) and a reduction in fast afterhyperpolarization (from 46.0 $\pm$ 2.1 mV in control to 34.8 $\pm$ 4.4 mV in 1- μ M PD173212; P = 0.0168, the paired Student t test, n = 7) on PD173212 superfusion (see Table 1). Other AP parameters (reported in Table 1) were not modified by any treatment. In the attempt to identify the mechanism by which A₃AR inhibits cell firing, we tested the effect of CI-IB-MECA on Na⁺ currents with no obvious differences detected in its presence (Suppl. Figs. 4A and B, available at <http://links.lww.com/PAIN/A733>), either on current amplitude (Suppl. Fig. 4C, available at <http://links.lww.com/PAIN/A733>),

[links.lww.com/PAIN/A733](https://www.pain-sci.com/PAIN/A733)) or time to peak (Suppl. Fig. 4D, available at [http://links.lww.com/PAIN/A733](https://www.pain-sci.com/PAIN/A733)).

3.2. Intracellular Ca^{2+} measurements confirmed that A_3AR activation inhibits electrical field stimulation–evoked Ca^{2+} transients in isolated dorsal root ganglion neurons

Fura-2–loaded DRG neurons analysed by transmitted light and fluorescence showed a round morphology with an average diameter of $27.6 \pm 0.3 \mu\text{m}$ ($n = 318$).

In control conditions (standard extracellular solution), 60% (40 of 68) of the DRG neurons presented electrically evoked Ca^{2+} transients on 0.1-Hz field stimulation. These cells were defined as “spiking” cells. The dynamic of cytosolic Ca^{2+} increase presented a rapid onset and a return to basal Ca^{2+} levels following a monoexponential kinetic. Figure 9A shows typical Ca^{2+} transient traces in control conditions, in the absence of extracellular Ca^{2+} ($0[\text{Ca}^{2+}]_{\text{out}}$), in the presence of TTX + A887826, or in the presence of verapamil + PD173212.

In $0[\text{Ca}^{2+}]_{\text{out}}$, only 4 of 37 (10.8%, Fig. 9B) analysed DRG neurons responded to electrical field stimulation ($P = 0.003$ vs control, the χ^2 test, Table 2). Indeed, as shown in a typical trace in Figure 9A (dotted line), the majority of analysed cells did not show Ca^{2+} transients in $0[\text{Ca}^{2+}]_{\text{out}}$. Figure 9B depicts Ca^{2+} transients in 1 of the 4 spiking cells in $0[\text{Ca}^{2+}]_{\text{out}}$. In those 4 spiking cells, the F/F was slightly reduced, whereas the monoexponential decay phase was significantly reduced (Table 2). We supposed that in the 4 of 37 cells oscillating in $0[\text{Ca}^{2+}]_{\text{out}}$, the electrical field stimulation could induce a Ca^{2+} release from intracellular stores, as already shown,⁴⁵ or, alternatively, that incomplete Ca^{2+} buffering was achieved.

In the presence of TTX + A887826, only 12 of 55 (21.8% Fig. 9B) DRG neurons were spiking ($P = 0.012$ vs control, the χ^2 test), and in the majority of cells, we did not observe any Ca^{2+} increase (Fig. 9A). Figure 9B displays the trace of 1 of 12 responder cells in TTX + A887826. Among them, either F/F or tau was slightly (but not significantly) reduced (Table 2). In finding an explanation to the observation that 12 of 55 cells still oscillated in the presence of TTX plus A887826, we have to consider that A887826 block of TTX-resistant Na^+ currents is dependent on $\text{Na}_v1.8$ channels being in the open state.⁶⁰ In fact, A887826 potently ($\text{IC}_{50} = 8 \text{ nM}$) inhibits TTX-resistant Na^+ currents in rat DRG neurons in a voltage-dependent way, being about 8-fold less potent at relatively hyperpolarized ($V_m < -60 \text{ mV}$) membrane voltages in comparison with -40 mV -clamped cells.⁶⁰ Alternatively, we can envisage that in those 12 spiking cells, field depolarization was sufficient to activate VDCCs, in line with previous observation.³⁶

The block of L+N-type VDCCs by verapamil ($1 \mu\text{M}$) + PD173212 (500 nM) induced a significant reduction in F/F (Table 1, $P = 0.003$, ANOVA followed by the Bonferroni test) without a significant decrease in tau. The number of spiking cells under these experimental conditions was 17 of 37 cells investigated (45.9%, not significant vs control: $P = 0.658$, the χ^2 test).

Preincubation with CI-IB-MECA significantly decreased the number of spiking cells (17 of 64; 23%; $P = 0.012$, the χ^2 test) as compared to control (Fig. 9C). However, Ca^{2+} parameters of spiking cells were not modified by this treatment (Table 2).

4. Discussion

In the present work, we investigated the expression and electrophysiological effects of A_3AR in primary sensory neurons. We report here evidence of A_3AR mRNA expression in the rat DRG, and we demonstrate that A_3AR activation inhibits both pronociceptive N-type VDCC activation and AP firing in isolated DRG neurons. The bulk of evidence in studies of in vivo animal models indicates that adenosine is a powerful antihyperalgesic compound,¹⁰ with a crucial role recognized for A_1AR activation. Of note, the A_3AR has recently gained attention because its activation provides pain relief without adverse side effects.³⁰

In this study, we demonstrated by RT-PCR that A_3AR mRNA is abundant in DRG homogenate and by immunocytochemistry that A_3AR protein is present on both neurons and glia. It should be noted that although it is generally accepted that A_3AR s are expressed either by rat or human DRG neurons, a debate exists concerning their presence in the mouse. Based on mRNA sequencing, it appears that mouse DRG neurons do not express A_3AR .^{44,53}

We then explored the A_3AR functional role in DRG neurons by performing patch-clamp recording in the absence or presence of 2 different receptor agonists: the commercially available CI-IB-MECA and the new highly selective A_3AR agonist MRS5980.^{12,51} Our first approach was to investigate the effects of A_3AR under basal (nonpharmacologically manipulated) conditions by using a voltage-ramp protocol to activate a wide range of voltage-dependent conductances. We found that the prototypical A_3AR agonist CI-IB-MECA reduced total outward K^+ currents evoked by the ramp. It is worth stressing that when tested for TRP responses, the vast majority of neurons responding to CI-IB-MECA were also sensitive to TRPA1 and/or TRPV1 agonists, AITC and capsaicin, respectively, and presented an average cell diameter of 25 μm , thus being considered as “nociceptors.”

When analyzed in detail, the CI-IB-MECA effect on ramp K^+ currents was found to be dependent on Ca^{2+} channel opening because it was prevented by the nonselective VDCC blocker Cd^{2+} . Indeed, in accordance with previous works,^{39,41} we demonstrated that DRG neurons express Ca^{2+} -activated K^+ channels (K_{Ca}) as indicated by the observation that extracellular Cd^{2+} inhibits 34.0% of total outward currents evoked by the ramp. At variance, CI-IB-MECA, that indirectly reduces Ca^{2+} currents through A_3AR activation, inhibits 23.3% of total ramp-evoked outward currents. Among K_{Ca} , SK and BK channels are involved in the CI-IB-MECA effect, as demonstrated by the fact that apamin plus TEA (200 μM) prevented ramp current inhibition.

Different VDCC types in DRG neurons have been described: P/Q and N types, encoded by $\text{Ca}_v2.1$ and $\text{Ca}_v2.2$, respectively, which are mainly involved in neurotransmitter release, and R, L, and T types, encoded by $\text{Ca}_v2.3$, $\text{Ca}_v1.1$ to 1.4, and $\text{Ca}_v3.1$ to 3.3, respectively.^{46,47,57} When VDCC-mediated currents were studied in isolation, A_3AR activation selectively inhibited N-type Ca^{2+} channels because the CI-IB-MECA effect was prevented by the ω -

CTX analogue PD173212 but not by the L-type blocker lacidipine. We conclude that A₃AR activation directly inhibits Ca²⁺ entry by VDCC on ramp depolarization and consequently reduces BK and SK channel opening.

It is known that A₁ARs also inhibit VDCCs in DRG neurons.¹¹ Consistently, we observed a $24.8 \pm 3.2\%$ Ca²⁺ current decrease in the presence of CPA. When testing the endogenous agonist effects, adenosine-mediated inhibition of Ca²⁺ currents was either sensitive to the A₃AR blocker MRS1523 or to the A₁AR antagonist DPCPX, demonstrating that these 2 AR subtypes are major mediators of adenosine effects in these cells. Of note, the CPA-mediated Ca²⁺ current inhibition ($24.8 \pm 3.2\%$) was significantly smaller compared with CI-IB-MECA effect ($44.1 \pm 5.6\%$) indicating a prominent functional role of A₃AR vs A₁AR in this cell type.

G_i-coupled receptors, eg, A₃ and A₁ARs, as well as μ -opioid receptors, can inhibit VDCC activation, and the mechanism may occur through the G _{β , γ} subunit binding directly to the channel protein.⁵⁹ Of note, the A₃AR has also been found to activate secondarily G_q proteins,²⁷ which could also modulate VDCCs.²⁴ Here, we describe A₃AR-induced inhibition of VDCCs for the first time in DRG neurons, but we have not determined the G-protein pathway involved. Furthermore, N-type channels are known to promote nociception by providing more than 60% of the neurotransmitter release by DRG neurons to lamina I dorsal horn neurons.^{21,23} Previous observations demonstrate that N-type VDCCs blockers result in analgesia in a range of pain models,^{17,31,55} and ziconotide, the first-choice compound among ω -CTX analogues, is in clinical use as an intrathecal medication for chronic pain in the United States.^{1,2} Of note, a direct block of N-type Ca²⁺ channels, as that achieved by ziconotide or ω -CTXs, is associated with serious side effects (psychological and neuropsychiatric symptoms including depression, cognitive impairment, and hallucinations; anxiety; panic attacks; ataxia; asthenia; headache; and dysesthesia).³⁵ Interestingly, an “indirect” VDCC modulation as that accomplished by A₃AR activation could represent a suitable approach to pain control without adverse side effects.

Beyond VDCC inhibition, we also demonstrated that CI-IB-MECA decreases the number of APs elicited by a depolarizing ramp current injection in isolated DRG neurons. This effect, also shared by μ -opioid agonists,^{3,49,54} could be a further crucial mechanism by which the A₃AR exerts pain relief. The CI-IB-MECA effect was blocked by the A₃AR antagonist MRS1523 but was insensitive to the N-type Ca²⁺ channel blocker PD173212, demonstrating that A₃AR-induced inhibition of cell excitability is not mediated by N-type VDCC inhibition. Indeed, we demonstrated here that a direct block of N-type Ca²⁺ channels by PD173212 induces an opposite effect on cell firing, ie, it increases the number of evoked APs, depolarizes cell membrane, and reduces voltage threshold. This indicates that a reduced Ca²⁺ influx through N-type VDCCs is proexcitatory at a somatic level. Such an effect could be secondary to a reduced activation of K_{Ca} channels as indicated by previous data showing that either BK or SK channel inhibition increases DRG excitability.^{32,41,61} In light of this information, it appears that A₃AR signaling in sensory neurons is complex and could lead to contradictory effects: VDCC inhibition induces, on one hand, a reduction in neurotransmitter release at the presynaptic level, thus providing pain control; on the other hand, it decreases K_{Ca} opening at the somatic level, thus increasing neuronal excitability and

producing a possible proalgesic effect. Indeed, multiple studies demonstrate that A₃AR agonists, applied either centrally^{34,56,58} or peripherally,^{14,29,42} are potent antihyperalgesic compounds.³⁰ This observation supports the notion that the inhibitory effect of CI-IB-MECA on VDCCs at a presynaptic level prevails over the potentially proalgesic A₃AR effect at a somatic level. Furthermore, the potentially proalgesic effect of CI-IB-MECA at a somatic level is masked by another, still unexplored, inhibitory A₃AR effect on cell firing, which could provide a therapeutic advantage of an A₃AR agonist over a direct Ca²⁺ channel blocker. This effect remains unexplained and will be addressed in our future work; our preliminary experiments revealed no obvious differences in Na⁺ currents evoked in the absence or presence of CI-IB-MECA.

Importantly, when we measured the intracellular Ca²⁺ rise after electrical field stimulation, we observed an overall reduction in the excitability of the DRG neuronal population in the presence of the A₃AR agonist. Thus, CI-IB-MECA significantly reduced the number of cells responding to 0.1 Hz stimulation, even if it did not change the dynamics of the Ca²⁺ rise ($\Delta F/F$ or τ) once Ca²⁺ spikes were triggered.

It must be pointed out that the above-mentioned effects may not be the only mechanism by which A₃AR agonists exert antihyperalgesia. Research studies have described a peripheral A₃AR effect on cytokine release during chemotherapeutic-induced neuropathic pain.^{29,56} Additional A₃AR-mediated modulation of nociception could possibly arise from receptor stimulation at a central level, ie, in thalamic nuclei.

We conclude that selective A₃AR stimulation inhibits N-type VDCC opening, leading to a reduction in neurotransmitter release, and reduces electrically evoked excitation in isolated rat DRG neurons. Both these effects, as independent mechanisms, may be important in accounting for A₃AR's pain-relieving effect and support the notion that an A₃AR-based therapy represents an important strategy to alleviate pain in different pathologies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

Selective A₃AR activation inhibits ramp-evoked outward currents in cultured rat DRG neurons. (A) Left panel: original patch-clamp current traces recorded in a representative cell where a voltage-ramp protocol (+65/-135 mV, 800 ms; lower inset) was applied before (ctrl), during, and after CI-IB-MECA (100 nM) superfusion. Upper inset: time course of ramp-evoked currents at +65 mV in the same cell. (B) Net CI-IB-MECA-inhibited current, obtained by subtraction of the ramp recorded in CI-IB-MECA from the control ramp, in the same cell. (C and D) Averaged ramp traces (C) and pooled data at +65 and -135 mV (D) of ramp-evoked currents measured in the absence or presence of CI-IB-MECA in 14 cells investigated. $P = 0.0005$ at +65 mV; $P = 0.3734$ at -135 mV; the paired Student t test, $n = 14$. (E) Real-time polymerase chain reaction experiments demonstrated that A₃AR-coding mRNA is present in rat DRG homogenates. Data were normalized to A₃ receptor expression as a fraction of the house-keeping gene GADPH (glyceraldehyde-3-phosphate dehydrogenase) and have been obtained in 3 independent experiments performed in triplicate. A brain tissue homogenate was taken as the positive control. (F and G) 20× (F) and 40× (G) magnification of A₃AR immunofluorescent labelling (green) of DRG cultures. Cells nuclei were marked with DAPI (blue). Scale bar: 50 μm. (H) Averaged CI-IB-MECA-inhibited currents (pA/pF) vs mV for concentrations: 1 μM, 100 nM, 10 nM, 1 nM, 0.1 nM. (I) Dose-response curve (EC₅₀ = 2 nM) for CI-IB-MECA. (J) Net CI-IB-MECA-inhibited currents (pA/pF) vs mV for CI-IB-MECA, +VUF5574, and +MRS1523. (K) % CI-IB-MECA-inhibited current at +65 mV for MRS1523 ($P = 0.0044$) and VUF5574 ($P = 0.0052$).

inhibited currents at different agonist concentrations (0.1–1000 nM; at least $n = 4$ in each experimental condition). (I) Concentration-response curve of Cl-IB-MECA effect on ramp currents measured at +65 mV (confidence limit: from 1.2 to 3.2 nM). (J and K) Net 100 nM Cl-IB-MECA–inhibited currents (J) and respective pooled data at +65 mV (K) recorded in the absence or presence of 2 different A_3AR antagonists: MRS1523 (100 nM; $n = 5$) and VUF5574 (100 nM; $n = 6$). One-way ANOVA, Bonferroni posttest. ANOVA, analysis of variance; DRG, dorsal root ganglion; RT-PCR, real-time polymerase chain reaction.

Figure 2.

The newly synthesized, highly selective, A₃AR agonist MRS5980 and the endogenous ligand adenosine mimic CI-IB-MECA effect in inhibiting ramp-evoked outward currents in isolated rat DRG neurons. (A) Averaged traces of ramp-evoked currents measured before or after MRS5980 application (100 nM) in 8 cells investigated. Inset: pooled data of ramp-evoked current at +65 mV in the presence of MRS5980 alone or during coapplication with the A₃AR antagonist MRS1523 (100 nM; *n* = 5). The paired Student *t* test. (B) Net MRS5980-inhibited current in 8 cells tested. (C) Comparison between net CI-IB-MECA- or MRS5980-inhibited ramp currents measured at +65 mV at different agonists concentrations. (D) Original ramp current traces recorded in a representative cell before (ctrl), during, or after adenosine (30 μM) superfusion. Inset: time course of ramp-evoked currents at +65 mV in the same cell. (E) Pooled data of ramp-evoked currents at +65 mV or -135 mV before or after the application of adenosine in 5 cells investigated. *P* = 0.0088 at +65 mV; *P* = 0.0738 at -135 mV, the paired Student *t* test, *n* = 5. (F) Averaged adenosine-inhibited currents, obtained by subtraction of the adenosine ramp from the control ramp, recorded in the absence (*n* = 5) or presence of A₃AR antagonist VUF5574 (100 nM, *n* = 6). ***P* = 0.0054 at +65 mV, the unpaired Student *t* test. DRG, dorsal root ganglion.

Figure 3.

Dorsal root ganglion (DRG) neurons responding to Cl-IB-MECA are nociceptors sensitive to the TRPV1 and TRPA1 agonists capsaicin and allyl isothiocyanate. (A) Original current trace recorded in a -75 mV-clamped cell after 10-minute washout of a previous Cl-IB-MECA application. Scale bars: 200 pA; 1 minute. (B) Pooled data of AITC- and capsaicin-activated currents in 33 cells investigated. (C) Pooled data of cell capacitance measured in AITC- and capsaicin-sensitive cells ("TRP-sensitive" neurons: $n = 33$) or in cells not exposed to capsaicin or AITC challenge ("TRP non-tested" neurons, $n = 112$). $P = 0.8045$, the unpaired Student t test. AITC, allyl isothiocyanate.

Figure 4.

A₃AR activation in DRG neurons inhibits Ca²⁺-activated K⁺ currents by reducing Ca²⁺ influx from voltage-dependent Ca²⁺ channels. (A) Original ramp current traces recorded in 1 mM Cd²⁺-containing extracellular solution before (ctrl) or during Cl-IB-MECA (100 nM) application in a representative cell. Inset: time course of ramp-evoked currents at +65 mV in the same cell. (B) Pooled data of ramp-evoked currents measured at +65 mV in the absence or presence of Cl-IB-MECA in Cd²⁺-containing extracellular solution in 7 cells investigated. $P = 0.2653$, the paired Student *t* test. (C) Averaged time course of ramp-evoked currents at +65 mV in the presence of Cl-IB-MECA ($n = 14$), its vehicle (Veh; 0.1% DMSO; $n = 3$), or Cl-IB-MECA in Cd²⁺ ($n = 7$). (D) Original ramp current traces recorded in apamin (100 nM) + tetraethylammonium (TEA, 200 μ M) before (apamin + TEA) or during Cl-IB-MECA (100 nM) application in a representative cell. Inset: time course of ramp-evoked currents at +65 mV in the same cell. (E) Averaged Cl-IB-MECA-inhibited currents in the absence ($n = 14$) or presence of apamin alone ($n = 4$) or during coapplication with TEA ($n = 4$). (F) Original ramp current traces recorded before (ctrl) and during 1-mM Cd²⁺ application in a representative cell where extracellular and intracellular K⁺ were replaced by

equimolar Cs^+ . Note that in these experimental conditions, a Cd^{2+} -sensitive inward current appears, which presents an I–V relationship typical of Ca^{2+} currents. Inset: time course of ramp-evoked current measured at the inward peak in the same cell. (G) Net Cd^{2+} -blocked Ca^{2+} current evoked by the ramp protocol in the same cell. (H) Averaged ramp-evoked currents recorded in Cs^+ -replacement conditions before (ctrl) or during Cl-IB-MECA (100 nM) application in 5 cells tested. $*P = 0.0431$, the paired Student t test. (I) Averaged Cl-IB-MECA-inhibited Ca^{2+} currents in Cs^+ -replacement experiments. DMSO, dimethyl sulphoxide; DRG, dorsal root ganglion.

Figure 5.

The major component of Ca²⁺ currents recorded in DRG neurons is carried by N-type VDCC opening. (A) Time course of Na⁺ currents recorded in Cs⁺-replacement conditions in the presence of extracellular TTX (1 μM), Cd²⁺ (500 μM), and Ni²⁺ (100 μM) and activated every 5 seconds by a 0-mV step depolarization (40-ms duration, V_h = -90 mV: lower inset in (B)) before or during the application of Nav1.8 blocker A887826 (200 nM). (B) Original current traces recorded before and after 3-minute A887826 application. Scale bars: 3 nA; 10 ms. (C) Original current traces evoked in a typical DRG neuron by a 0-mV step depolarization (200 ms; V_h = -65 mV: lower inset) in the presence of extracellular TTX (1 μM), Ni²⁺ (100 μM), and A887826 (200 nM) before (ctrl) or during the application of 100-μM Cd²⁺. Scale bars: 1 nA; 50 ms. (D) Averaged time courses of Ca²⁺ currents, measured at the inward peak, before and during the application of the nonselective VDCC blocker 100-μM Cd²⁺ (n = 4). (E) Pooled data of Ca²⁺ current inhibition in the presence of 100-μM, 500-

μM , or 1-mM Cd^{2+} , $n = 4$. (F) Original current traces evoked in a typical DRG neuron by a 0-mV step depolarization (200 ms; $V_h = -65$ mV) in the presence of extracellular TTX (1 μM), Ni^{2+} (100 μM), and A887826 (200 nM) before (ctrl) or during the application of PD173212 (0.5 μM) and, subsequently, 500- μM Cd^{2+} . Scale bars: 1 nA; 50 ms. (G) Averaged time courses of Ca^{2+} currents before and during the application of selective N-type Ca^{2+} channel blocker PD173212 (500 nM, $n = 4$). (H) Pooled data of Ca^{2+} current inhibition in the presence of 0.5- μM PD173212, 1- μM PD173212, or 0.5- μM PD173212 + 500- μM Cd^{2+} . (I and J) Original current traces (left panels), and respective averaged I–V plots (right panels), of Ca^{2+} currents evoked by a series of 10 depolarizing voltage steps (from -50 to $+50$ mV, 200-ms duration, $V_h = -65$ mV: see lower inset) before or during 500- μM Cd^{2+} (I) or 0.5- μM PD173212 (J) application. Scale bars: 1 nA; 50 ms. DRG, dorsal root ganglion; VDCC, voltage-dependent Ca^{2+} channel. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$, paired Student t-test.

Figure 6.

CI-IB-MECA inhibits N-type Ca^{2+} currents in DRG neurons. (A) Left panel: time course of peak Ca^{2+} currents (I_{Ca}) evoked by a 0-mV step depolarization in a typical DRG neuron. Note that CI-IB-MECA (30 nM) inhibits Ca^{2+} currents that were completely abolished by a subsequent 500- μM Cd^{2+} application. Right panel: original current traces recorded in the same cell at significant time points. Scale bars: 0.5 nA, 100 ms. Arrow indicates peak Ca^{2+} currents, and arrowhead indicates steady-state Ca^{2+} currents. (B) Pooled data of peak I_{Ca} measured in the absence or presence of CI-IB-MECA in 8 cells investigated. $P < 0.0001$, the paired Student t test. (C) Left panel: original Ca^{2+} current traces evoked by a series of depolarizing voltage steps (inset) before (ctrl) or after CI-IB-MECA (30 nM) application in a representative cell. Right panel: averaged I-V plot of Ca^{2+} currents measured at the peak in 5 cells tested. $*P = 0.0106$; $**P = 0.0066$; $\$P = 0.0105$, the paired Student t test. Scale bars: 0.5 nA, 50 ms. (D) Averaged time courses of peak Ca^{2+} current, expressed as % of baseline values, measured before or after the application of CI-IB-MECA in different experimental groups. (E) Pooled data of CI-IB-MECA-or CPA-inhibited peak Ca^{2+} currents at different agonist concentrations, or in 30-nM CI-IB-MECA during coapplication with the A_3AR antagonist MRS1523 (1523, 100 nM) or with the selective N-type and L-type Ca^{2+} channels blockers PD173212 (PD, 0.5 μM) and lacidipine (LAC, 1 μM), respectively. $*P < 0.05$; $***P < 0.0001$ vs 30-nM CI-IB-MECA; $\#P < 0.05$ vs 10-nM CI-IB-MECA, one-way ANOVA, Bonferroni posttest. (F) Left panel: time course of peak I_{Ca} evoked by a 0-mV step depolarization in a typical DRG neuron in the absence or presence of N^6 -cyclopentyladenosine (CPA: 1–10 μM). Right panel: original current traces recorded in the same cell at significant time points. Scale bars: 1 nA, 100 ms. ANOVA, analysis of variance; DRG, dorsal root ganglion.

Figure 7.

The inhibitory effect of Cl-IB-MECA on N-type Ca^{2+} currents in DRG neurons is mimicked by the newly synthesized A_3AR agonist MRS5980 and by adenosine. (A) Left panel: time course of peak I_{Ca} in a typical DRG neuron before and after MRS5980 (30 nM) and Cd^{2+} (500 μM) application. Right panel: original current traces recorded in the same cell at significant time points. Scale bars: 2 nA; 100 ms. (B) Pooled data of MRS5980-inhibited peak Ca^{2+} current at different agonist concentrations or in the presence of 30-nM MRS5980 + 100-nM MRS1523. One-way ANOVA; Bonferroni posttest. (C) Averaged time courses of peak I_{Ca} before or during Cl-IB-MECA (30 nM) or MRS5980 (3 nM) application. (D) Left panel: time course of peak I_{Ca} in a typical DRG neuron before and after adenosine (30 nM) and Cd^{2+} (500 μM) application. Right panel: original current traces recorded in the same cell at significant time points. (E) Pooled data of adenosine-inhibited peak Ca^{2+} currents at different agonist concentrations or in the presence of 30- μM adenosine + the A_3AR antagonist MRS1523 (100 nM) or the A_1AR antagonist DPCPX (500 nM). No significant difference was found between any of the experimental groups (one-way ANOVA, Bonferroni posttest). (F) Averaged time courses of peak Ca^{2+} current amplitude before or after adenosine (30 μM) application alone or in the presence of DPCPX (500 nM) or MRS1523 (100 nM). ANOVA, analysis of variance; DRG, dorsal root ganglion.

Figure 8.

CI-IB-MECA inhibits AP firing in DRG neurons. (A) Original AP traces evoked in a typical DRG neuron by a 1-second depolarizing ramp current injection (lower inset) recorded before (black trace) or after (red trace) 5-minute CI-IB-MECA (100 nM) application. (B) Time course of AP number in the same cell. (C) Pooled data of AP number measured before or after 5-minute CI-IB-MECA application in 6 cells tested. $P = 0.0091$, the paired Student t test. (D) Original AP traces evoked by a 1-second depolarizing ramp current injection recorded before (black trace) or after (green trace) 5-minute CI-IB-MECA (100 nM) application in the presence of MRS1523 (100 nM). (E) Time course of AP number in the same cell. (F) Pooled data of AP number measured before or after 5-minute CI-IB-MECA application in the presence of MRS1523 in 5 cells tested. $P = 0.5809$, the paired Student t test. (G) Original AP traces evoked by a 1-second depolarizing ramp current injection recorded before (black trace) or after (purple trace) 5-minute CI-IB-MECA (100 nM) application in the presence of PD173212 (1 μM). (H) Time course of AP number in the same cell. (I) Pooled data of AP number measured before or after 5-minute CI-IB-MECA application in the presence of PD173212 in 6 cells tested. The paired Student t test. Scale bars: 50 mV; 500 ms. (J) Comparison between the effect of CI-IB-MECA on AP firing when applied alone ("in ctrl," $n = 6$) or when applied in the presence of 1-μM PD173212 ("in PD173212," $n = 7$). Treatment (CI-IB-MECA): $F_{(1,22)} = 11.46$, $P = 0.0027$; time (before/after): $F_{(1,22)} = 14.27$, $P = 0.0010$; interaction: $F_{(1,22)} = 5.416$, $P = 0.0029$; 2-way ANOVA, Bonferroni posttest. Note that there is a statistical difference (** $P = 0.0011$) between the AP

number recorded before Cl-IB-MECA application in the ctrl group (“in ctrl”) or in the PD173212 group (“in PD173212”) but not between the AP number recorded after Cl-IB-MECA application in the ctrl group or in the PD173212 group ($P = 0.7109$). ANOVA, analysis of variance; AP, action potential; DRG, dorsal root ganglion.

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Figure 9.

A₃ receptor stimulation reduces electrically evoked and TTX-sensitive intracellular Ca²⁺ transients in isolated DRG neurons. (A) Typical time courses of Ca²⁺ transient induce in DRG neurons by 0.1-Hz electrical field stimulation in control condition (ctrl: black trace), extracellular free-Ca²⁺ solution (0[Ca²⁺]_{out}: dotted trace), 1-μM tetrodotoxin + 200-nM A887826 (TTX: yellow trace), or verapamil (VERA; 1 μM) + PD173212 (PD; 500 nM). (B) Time courses of spiking cells recorded in 0[Ca²⁺]_{out} (dotted trace) or in TTX + A887826 (gray trace). (C) Numbers of spiking or not spiking DRG neurons in control conditions or after preincubation with 30-nM Cl-IB-MECA. Statistical analysis was performed using the χ^2 test. DRG, dorsal root ganglion.

Table 1

Action potential parameters measured in DRG neurons under the current-clamp mode.

	Ctrl (n = 6)	Cl-IB-MECA (n = 6)	Ctrl (n = 7)	PD173212 (n = 7)	PD173212 (n = 8)	PD173212 + Cl-IB-MECA (n = 8)
Resting Vm (mV)	-61.2 ± 2.3	-60.1 ± 2.1	-63.6 ± 2.9	-59.7 ± 3.6 [*]	-53.3 ± 4.0	-54.1 ± 2.6
AP time to peak (ms)	685.1 ± 100.7	684.6 ± 105.2	530.7 ± 61.9	504.2 ± 70.5	465.0 ± 73.54	480.1 ± 84.65
AP half width (ms)	1.7 ± 0.2	2.6 ± 0.6	2.2 ± 0.6	4.4 ± 2.3	3.8 ± 2.0	2.6 ± 0.6
AP amplitude (mV)	65.0 ± 6.3	57.7 ± 7.9	60.6 ± 7.6	58.0 ± 12.4	57.6 ± 6.2	52.5 ± 5.5
Current threshold (pA)	16.0 ± 5.3	20.7 ± 7.4	4.0 ± 1.6	4.3 ± 2.0	3.3 ± 1.1	3.8 ± 1.1
AP threshold (mV)	-15.7 ± 1.8	-14.5 ± 1.4	-12.7 ± 1.9	-14.1 ± 1.5 [‡]	-14.1 ± 2.6	-13.8 ± 2.4
Current threshold (pA)	16.0 ± 5.3	20.7 ± 7.4	4.0 ± 1.6	4.3 ± 2.0	3.3 ± 1.1	3.8 ± 1.1
AP threshold (mV)	-15.7 ± 1.8	-14.5 ± 1.4	-12.7 ± 1.9	-14.1 ± 1.5 [‡]	-14.1 ± 2.6	-13.8 ± 2.4
AP duration (ms)	3.7 ± 0.4	3.2 ± 0.7	3.9 ± 0.7	4.5 ± 0.6 [‡]	4.3 ± 0.5	4.2 ± 0.8
fAHP (mV)	41.9 ± 2.9	39.5 ± 4.1	46.0 ± 2.1	34.8 ± 4.4 [§]	37.4 ± 3.1	34.3 ± 4.0

The following experimental groups were analyzed: Control: Ctrl vs Cl-IB-MECA (100 nM; n = 6); Ctrl vs PD173212 (1 μM; n = 7); and PD173212 vs PD173212 + Cl-IB-MECA (n = 8). All AP parameters are referred to the first action potential (AP) generated by the ramp. Resting Vm was calculated as the averaged membrane voltage measured 200 ms before ramp current injection. Action potential time to peak was measured as the time between the threshold and the voltage peak reached by the AP. Action potential half-width was measured as the time to reach half the AP amplitude. Action potential amplitude was calculated as the difference between the peak reached by the overshoot and the threshold. The current needed to produce the first spike was defined as “current threshold” and was measured as the minimal amount of current (pA) injected during the ramp protocol leading to the first AP initiation. Action potential threshold was defined as the point at which the derivative first exceeded 30 mV/ms. Action potential duration measured the difference between the time of reaching the threshold potential during the rising phase and the time when the repolarizing potential crossed the threshold value again. The fast afterhyperpolarization (fAHP) was measured as the difference between the voltage threshold and the minimum potential reached after the AP peak.

The paired Student *t* test, n = 7.

^{*} *P* = 0.0165.

[‡] *P* = 0.0491.

[‡] *P* = 0.0089.

[§] *P* = 0.0168 vs respective ctrl.

AP, action potential; DRG, dorsal root ganglion.

F/F and decay time (τ) measured in spiking DRG neurons during electrical field stimulation, according to treatments.

Table 2

Treatment	No. of spiking cells out of analyzed cells	F/F (A.U.) (spiking)	τ (decay time) (s) (spiking)
Control	40 out of 68	3.8 $\pm$ 0.47	1.24 $\pm$ 0.087
0[Ca ²⁺] out	4 out of 37	2.2 $\pm$ 0.81	0.51 $\pm$ 0.082 *
TTX + A887826	12 out of 55	1.6 $\pm$ 0.50	0.98 $\pm$ 0.159
Verapamil + PD173212	17 out of 37	1.6 $\pm$ 0.38 †	0.96 $\pm$ 0.101
CI-IB-MECA	17 out of 64	5.6 $\pm$ 0.77	1.33 $\pm$ 0.106 ‡

The decay time (τ) was calculated according to the following equation: $Y = y_0 + A \times e^{(-X/\tau)}$.

One-way ANOVA followed by the Bonferroni posttest.

* $P=0.006$ vs control.

† $P=0.03$.

‡ $P=0.004$ vs 0[Ca²⁺].

ANOVA, analysis of variance; A.U., arbitrary unit; DRG, dorsal root ganglion.