Attenuation of thermal nociception and hyperalgesia by VR1 blockers

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Vanilloid receptor subunit 1 (VR1) appears to play a critical role in the transduction of noxious chemical and thermal stimuli by sensory nerve endings in peripheral tissues. Thus, VR1 antagonists are useful compounds to unravel the contribution of this receptor to pain perception, as well as to induce analgesia. We have used a combinatorial approach to identify new, nonpeptidic channel blockers of VR1. Screening of a library of trimers of *N*-alkylglycines resulted in the identification of two molecules referred to as DD161515 {*N*-[2-(2-(*N*-methylpyrrolidinyl)ethyl]glycyl]-[*N*-[2,4-dichlorophenethyl]glycyl]-*N*-(2,4-dichlorophenethyl)glycinamide} and DD191515 {[*N*-[3-(*N*,*N*-diethylamino)propyl]glycyl]-[*N*-[2,4-dichlorophenethyl]glycyl]-*N*-(2,4-dichlorophenethyl)glycinamide} that selectively block VR1 channel activity with micromolar efficacy, rivaling that characteristic of vanilloid-related inhibitors. These compounds appear to be noncompetitive VR1 antagonists that recognize a receptor site distinct from that of capsaicin. Intraperitoneal administration of both trialkylglycines into mice significantly attenuated thermal nociception as measured in the hot plate test. It is noteworthy that these compounds eliminated pain and neurogenic inflammation evoked by intradermal injection of capsaicin into the animal hindpaw, as well as the thermal hyperalgesia induced by tissue irritation with nitrogen mustard. In contrast, responses to mechanical stimuli were not modified by either compound. Modulation of sensory nerve fibers excitability appears to underlie the peptoid analgesic activity. Collectively, these results indicate that blockade of VR1 activity attenuates chemical and thermal nociception and hyperalgesia, supporting the tenet that this ionotropic receptor contributes to chemical and thermal sensitivity and pain perception *in vivo*. These trialkylglycine-based, noncompetitive VR1 antagonists may likely be developed into analgesics to treat inflammatory pain.

Identification and cloning of the VR1 channel represented a significant step in the clarification of the molecular mechanisms underlying transduction of noxious chemical and thermal stimuli by peripheral nociceptors (1). This receptor is a nonselective cation channel with high Ca^{2+} permeability activated by capsaicin and its analogues, as well as by endocannabinoids (1, 2). In addition, VR1 also is gated by noxious heat (>43°C) and low pH (3, 4). Moreover, VR1 channel activity is strongly modulated by the action of inflammatory mediators such as bradykinin and prostaglandins, presumably by PKC or PKA mediated receptor phosphorylation (5, 6). There is evidence that VR1 plays a key role in both nociception and inflammatory pain (7, 8). Therefore, VR1 has been considered a molecular integrator of noxious stimuli in the peripheral terminals of primary sensory neurons involved in pain signaling. This tenet is also supported by results indicating that modulators of VR1 channel activity exhibit analgesic activity (9–11). Thus, the implication of VR1 in pain and hyperalgesia makes this ion channel as a prime therapeutic target for analgesic drugs.

Despite the wealth of information accrued in recent years on VR1 function, the number of available compounds that modulate its activity is still quite limited (12). A significant effort has been focused on developing competitive capsaicin antagonists (11, 13–16). The family of noncompetitive antagonists such as ruthenium red and arginine-rich peptides has been also considered (9, 17). Most of available substances, however, exhibit either unwanted side effects or toxicity or metabolic instability (10, 12, 18). To address this issue, we used a combinatorial-based approach to identify nonpeptide based VR1-specific channel blockers such as oligo N-substituted glycines (also known as peptoids) that exhibit high proteolytic stability (19). We identified two peptoids that appear to be noncompetitive VR1 antagonists. These molecules attenuated *in vivo* thermal nociception and hyperalgesia, and suppressed capsaicin-evoked pain.

Methods

Synthesis of Trialkylglycines-Based Combinatorial Mixtures and Individual Compounds. The library and individual oligo *N*-alkylglycines were prepared by simultaneous multiple solid phase synthesis (20). The mixture positions (X in Fig. 1) were incorporated by coupling a mixture of 22 selected amines with the relative ratios adjusted to yield equimolar incorporation. Briefly, starting from Rink amide resin [0.7 milliequivalent (meq)/g; Rapp Polymere, Germany] the eight-step synthetic pathway involved the initial release of the fluorenylmethoxycarbonyl (Fmoc) protecting group. Thereafter, the successive steps of acylation with chloroacetyl chloride followed by the corresponding amination of the chloromethyl intermediate, using the selected individual amine (O in Fig. 1) or the mixture of amines (X in Fig. 1), was conducted. The purity and identity of the individual oligo *N*-alkylglycine active compounds was determined by analytical HPLC, laser desorption time-of-flight MS, and ^{1}H and ^{13}C NMR (see *Supporting Text*, which is published as supporting information on the PNAS web site, www.pnas.org).

Recombinant Rat VR1 Channels Expression in *Xenopus* Oocytes and Channel Blockade. All of the procedures have been described in detail elsewhere (9, 21, 22). Whole-cell currents from rat VR1-injected oocytes were recorded in Mg^{2+}-Ringer's solution (in mM: 10 Hepes, pH 7.4/115 NaCl/2.8 KCl/0.1 $BaCl_2$/2.0 $MgCl_2$) with a two-microelectrode voltage-clamp amplifier at 20°C (9). VR1 channels were activated by application of 10 μM capsaicin in absence or presence of peptoid mixtures or individual compounds at a holding potential (V_h) of −80 mV. Receptor

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Abbreviations: VR1, vanilloid receptor subunit 1; DD161515, [*N*-[2-(2-(*N*-methylpyrrolidinyl)ethyl]glycyl]-[*N*-[2,4-dichlorophenethyl]glycyl]-*N*-(2,4-dichlorophenethyl)glycinamide; DD191515, [*N*-[3-(*N*,*N*-diethylamino)propyl]glycyl]-[*N*-[2,4-dichlorophenethyl]glycyl]-*N*-(2,4-dichlorophenethyl)glycinamide; $[Ca^{2+}]_i$, intracellular Ca^{2+}.

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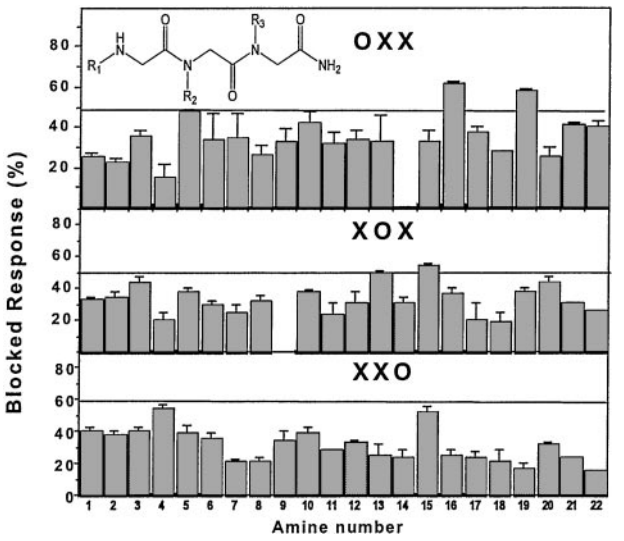

Fig. 1. Screening of an oligo N-substituted glycine combinatorial library to identify VR1 channel blockers. VR1 blockade profile of the 66 library mixtures. Each graph represents the blocking activity for each of the three positions that compose the library. The bars denote the activity of each mixture as a function of the number of the defined amine used to generate the chemical diversity. Library mixtures were assayed at 100 μg/ml. Horizontal bar denotes the cut-off blocking activity (set to 50%). (*Inset*) Generic formula of the trialkylglycine combinatorial library, where R_1, R_2, and R_3 denote the sites where chemical diversity was introduced.

selectivity of identified trialkylglycines was evaluated on heterologously expressed NMDA receptors (rat NR1:NR2A), and on K^+ (mouse Kv1.1), Na^+ (rat SKN4A), and Ca^{2+} (rat α1H T-type) selective channels (see *Supporting Text*).

Culturing of Trigeminal Primary Sensory Neurons, Fluorimetric Ca^{2+} Measurements, and Electrophysiological Recordings. Trigeminal primary neurons from newborn mice (P7) were cultured as described (23). Plated trigeminal neurons appear as round-to-ovoid cells with a variety of diameters. Intracellular Ca^{2+} measurement was performed in single cells loaded with the acetoxymethylester of Fura-2 (Molecular Probes) as described (23). Fluorometric measurements were carried out using a digital imaging system, with a cooled CCD camera (Sensys, Photometrics, Tucson, AZ) and IMAGING WORKBENCH software (Axon Instruments, Foster City, CA).

Action potentials were recorded with an external solution containing (in mM) 124 NaCl, 5 KCl, 1.2 KH_2PO_4, 2 $CaCl_2$, 1 $MgCl_2$, 24 $NaHCO_3$, and 10 glucose (pH 7.4; 95% O_2 + 5% CO_2) at 33 ± 1°C; and pipette solution containing (in mM) 140 KCl, 10 NaCl, 4 Mg-ATP, 0.4 Na-GTP, and 10 Hepes (pH 7.3 adjusted with KOH). Patch-pipettes had a resistance of 3 to 4 MΩ. Membrane voltage was recorded with an EPC8 patch-clamp amplifier (HEKA Electronics, Lambrecht/Pfalz, Germany).

Rat Knee Joint Nociceptor Fiber Preparation. Adult male Wistar rats (300–350 g) were anesthetized with thipentone (100 mg/kg i.p.). The trachea, the left femoral vein, and the femoral artery were cannulated. Arterial blood pressure was continuously monitored. An additional catheter was inserted into the right saphenous artery for close intraarterial injection of substances into the joint area. The right femur was fixed by a special grip and a pool was formed by skin flaps and filled with warm paraffin oil. The saphenous nerve was dissected and fine filaments were subdissected from the peripheral end. Nerve fibers innervating the knee joint were identified by the location of their receptive field, which was determined by the firing response to probing the structures in and around the knee joint with a hand-held glass (24). The recordings were made in multiunit filaments containing between two and five identifiable units. The mechanical stimuli consisted of normal and noxious outward and inward rotation of the knee joint lasting 10 s. Activation of the units by close intraarterial injection of KCl (0.1 mM, 0.1 ml) was used to ascertain that solutions reached the sensory endings in the knee joint.

Behavioral Nociception Assays. Adult male ICR mice were habituated to the test environment for 24 h in Plexiglas chambers prior the nociception assays. Thermal nociception was studied using a hot plate at 52 or 56°C (7, 8). The response latency for paw shaking or licking or jumping was measured. For the tail immersion assay, the animal was gently restrained and the distal two-thirds of the tail were immersed into a water bath at 52°C and the latency to tail flick recorded (7).

Mechanical nociception was evaluated using von Frey hairs as described (25). In brief, mice were placed on a raised wire mesh grid (6 × 6 mm^2 apertures) under plastic chambers. To quantify the mechanical sensitivity of the paw, hairs with different forces (in mN) were applied ten times to the hindpaw in ascending order of force. The frequency and intensity of responses of withdrawal were monitored. The hair was applied for 1–2 s, with an interstimulus interval of 5–10 s.

Capsaicin-Induced Hyperalgesia. Capsaicin (10 μl at 0.06% in 10% ethanol, 10% Tween 80, and 80% saline) was injected intradermally into the heel pad with a 0.3-mm diameter needle attached to a Hamilton syringe (25). The latency and duration times of licking and shaking the paw in response to the injection was recorded. The mechanical sensitivity of the plantar surface of the injected and contralateral paw was tested again 20 min after capsaicin or vehicle injection with von Frey hairs as described (25). Contralateral and ipsilateral paw volumes were measured with a plethysmometer.

Mustard Oil Thermal Hyperalgesia. Thermal sensitivity of mice was assayed in the hot plate at 52°C. Thereafter, mustard oil (10% in mineral oil) was painted onto both hindpaws, the animals were injected either with saline or 0.2 mmol/kg peptoid, and the hot plate test was repeated at the indicated times.

Drug Administration. Trimers of *N*-alkylglycines were dissolved in ethanol, diluted in saline at the desired concentrations (final ethanol concentration was ≤1.0%), and administered i.p. The injected volume was normalized to the animal weight.

Statistical Analysis. All results are given as mean ± SEM, with *n* (number of animals) ≥7. Data were statistically analyzed using the unpaired *t* test or one-way ANOVA test.

Results

A mixture-based combinatorial library made of trimers of *N*-alkylglycines in a positional scanning format was screened to identify blockers of VR1 channel activity (Fig. 1, *Top*, *Inset*). The library consisted of three different sublibraries, each addressing one position of the trialkylglycine. Thus, a single position was defined with 1 of the 22 primary amines used (O in Fig. 1), and the remaining two positions had an equimolar mixture of these amines (X in Fig. 1). Each mixture contained 484 molecules, and the library chemical diversity comprised 10,648 individual trimers.

Peptoid mixtures were assayed for blockade activity of recombinant VR1 channels heterologously expressed in amphibian oocytes. Screening of the complete library identified several mixtures that blocked by ≥50% the capsaicin-evoked ionic current (Fig. 1). Based on the screening data, a family of

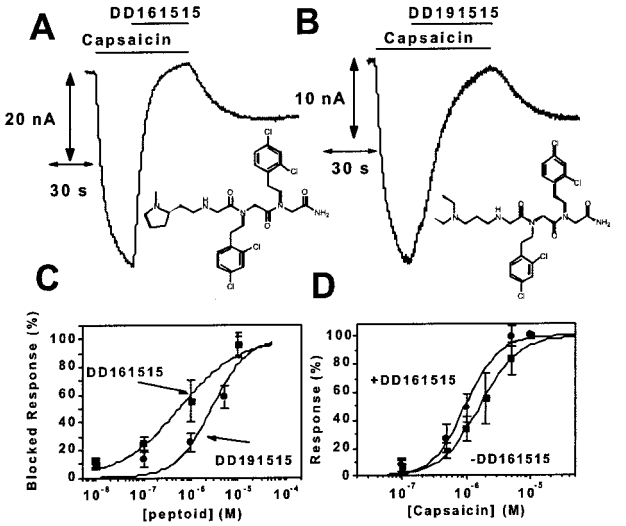

Fig. 2. Trialkylglycines are noncompetitive capsaicin antagonists that block VR1 with high efficacy. Representative blockade activity of the trialkylglycines DD161515 (*A*) and DD191515 (*B*). *Insets* depict the chemical structure of both peptoids. VR1 channels were activated by 10 μM capsaicin and recorded at −80 mV. Peptoids were tested at 10 μM. (*C*) Dose–response curves for compounds DD161515 and DD191515 blockade activity of capsaicin-activated VR1 channels expressed in amphibian oocytes. Responses were normalized with respect to that in the absence of trialkylglycines. Solid lines depict the theoretical fits to a Michaelis–Menten binding isotherm. Each point represents the mean ± SEM with, $n \geq 4$. (*D*) Dose–response curves of VR1 activation by capsaicin in the absence (−DD161515) and presence (+DD161515) of 1 μM peptoid. Data are mean ± SEM, $n = 3$.

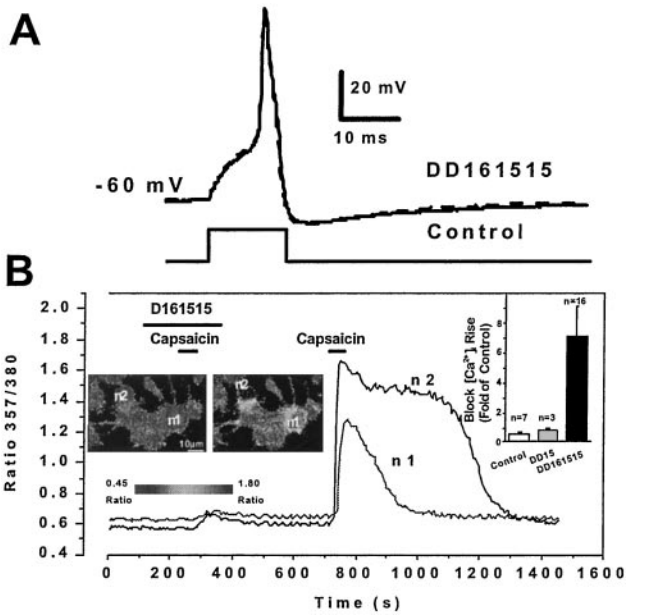

Fig. 3. Effect of trialkylglycines on the action potential and capsaicin-evoked $[Ca^{2+}]_i$ rises in cultured trigeminal neurons. (*A*) Action potentials elicited from trigeminal primary neurons in the absence (control) and presence of 10 μM DD161515 in the external solution. Square pulse under the action potential denotes the stimulation protocol under current clamp. (*B*) The traces show the time course of the fluorescence ratio (F357/F380) changes in the two neurons shown in ratio images (*Left Inset*) of a culture with trigeminal primary sensory neurons (n1, n2), fibroblasts, and glial cells, during exposure to capsaicin in the presence of D161515 (*Left*) and after washing out the peptoid (*Right*). Solid lines depict pulse duration for VR1 agonist and antagonist. (*Right Inset*) Histogram representing the mean ratio between the second and first $[Ca^{2+}]_i$ elevation to capsaicin, in the presence or absence of the indicated compound. Peptoids were tested at 100 μM, and capsaicin was used at 1 μM. Control denotes two consecutive pulses of capsaicin in the absence of peptoid. (Calibration bar of images, 10 μm.)

individual trialkylglycines was synthesized and assayed for VR1 blockade activity. Two compounds [*N*-[2-(2-(*N*-methylpyrrolidinyl)ethyl]glycyl]-[*N*-[2,4-dichlorophenethyl]glycyl]-*N*-(2, 4-dichlorophenethyl)glycinamide and [*N*-[3-(*N*,*N*-diethylamino)-propyl]glycyl]-[*N*-[2,4-dichlorophenethyl]glycyl]-*N*-(2, 4-dichlorophenethyl)glycinamide (referred to as DD161515 and DD191515, respectively) inhibited VR1 channel activity by ≥90% at 10 μM (Fig. 2 *A* and *B*). VR1 block by these two compounds was rapid and reversible. For peptoid DD161515, the IC_{50} was 0.7 ± 0.2 μM and n_H = 0.9 ± 0.2 (mean ± SEM, $n = 7$), and for DD191515 was 2.6 ± 0.6 μM and n_H = 1.1 ± 0.2 ($n = 3$), indicating a higher blockade efficacy of compound DD161515. The Hill coefficient of ≈1.0 is consistent with the occurrence of a single binding site.

These peptoids appear to act as noncompetitive capsaicin antagonists, as suggested by the similar capsaicin concentration required to activate half-maximal response (EC_{50}) in the absence 1.5 ± 0.2 μM ($n = 4$) and in the presence 0.96 ± 0.1 μM ($n = 4$) of 1 μM peptoid DD161515 (Fig. 2*D*). The Hill coefficient of VR1 activation by capsaicin (n_H = 1.5 ± 0.1) was not altered by the presence of the peptoid (n_H = 1.7 ± 0.2). Consistent with this notion, acidification of the extracellular medium or mutation of D646 to asparagine, a residue in the pore forming region of VR1 (26), reduced the peptoid blockade activity (see supporting information). Notably, oligo-*N*-alkylglycines preferentially block VR1 channels, as evidenced by the modest inhibition exerted on NMDA receptors and voltage-gated channels selective for either K^+ or Na^+ or Ca^{2+} (see supporting information). Furthermore, these compounds did not affect the duration nor amplitude of action potentials in trigeminal primary sensory neurons (Fig. 3*A*). Collectively, these findings suggest that trialkylglycines are potent, selective, noncompetitive VR1 antagonists that bind to a receptor site distinct from the vanilloid binding site.

Because VR1 channels modulate intracellular Ca^{2+} ($[Ca^{2+}]_i$) rises in neurons (1, 3–5), we evaluated whether the peptoid DD161515 prevented the capsaicin-induced Ca^{2+} influx in cultured trigeminal neurons. To minimize the contribution of vanilloid-evoked VR1 desensitization, we used a two consecutive pulse protocol interspersed with washing periods. As illustrated in Fig. 3*B*, capsaicin stimulation of neuronal cultures in the presence of DD161515 did not result in a $[Ca^{2+}]_i$ increase. Peptoid blockade of Ca^{2+} influx was reversible, as evidenced by the conspicuous increment of $[Ca^{2+}]_i$ elicited by a second, consecutive capsaicin pulse after washing out DD161515. At 100 μM, peptoid DD161515 blocked ≥90% of the capsaicin-evoked $[Ca^{2+}]_i$ in ≈70% of the capsaicin-responsive neurons, whereas it was less efficient (≤50%) in the remaining population of capsaicin-sensitive neurons (Fig. 3*B*, *Right Inset*). DD161515 inhibition of Ca^{2+} influx was specific because peptoids that do not block VR1 channel activity, such as DD15 (*N*-[2,4-dichlorophenethyl]glycinamide), did not affect the vanilloid-evoked Ca^{2+}-influx in trigeminal neurons (Fig. 3*B*, *Right Inset*). Therefore, these results indicate that newly identified VR1 blockers inhibit Ca^{2+} entry through the receptor.

We next investigated whether trialkylglycines block VR1 channels present in polymodal endings of nociceptor nerve fibers mediating pain, we evaluated the inhibition by compound DD161515 of sensory discharges evoked by noxious stimulation of the knee joint. In the median articular nerve of the rat, about 25% of the single unit, and 80% of the multiunit filaments that discharged in response to noxious rotation of the knee joint were additionally excited by close intraarterial injection of capsaicin (Fig. 4*A*). A dose of 10 μM capsaicin and prolonged intervals

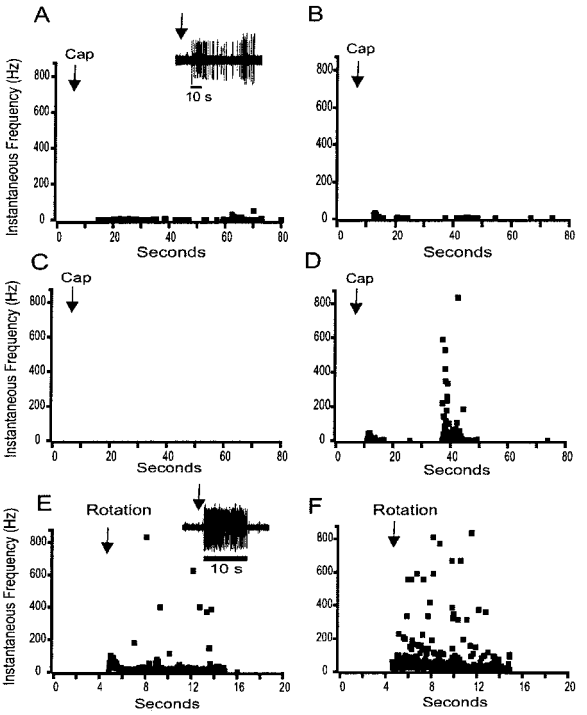

Fig. 4. Inhibition by the peptoid of capsaicin-evoked neural activity in knee joint nociceptor fibers. (*A–D*) Instantaneous frequency of the nerve impulse discharge evoked by intraarterial injections of 100 μl capsaicin, 10 μM (arrows) before (*A*) and 15 min (*B*), 35 min (*C*), and 55 min (*D*) after the administration of 100 μl of a 4 mM (0.4 μmol) solution of the peptoid D161515. (*E* and *F*) The impulse discharge elicited by a 10-s knee joint rotation (starting at the arrow) applied before injection of capsaicin and peptoid and 35 min after administration of the peptoid (immediately before *C*), respectively. (*Insets*) A sample record of the multiunit impulse activity evoked by capsaicin (*A*) and by the mechanical stimulation (*E*).

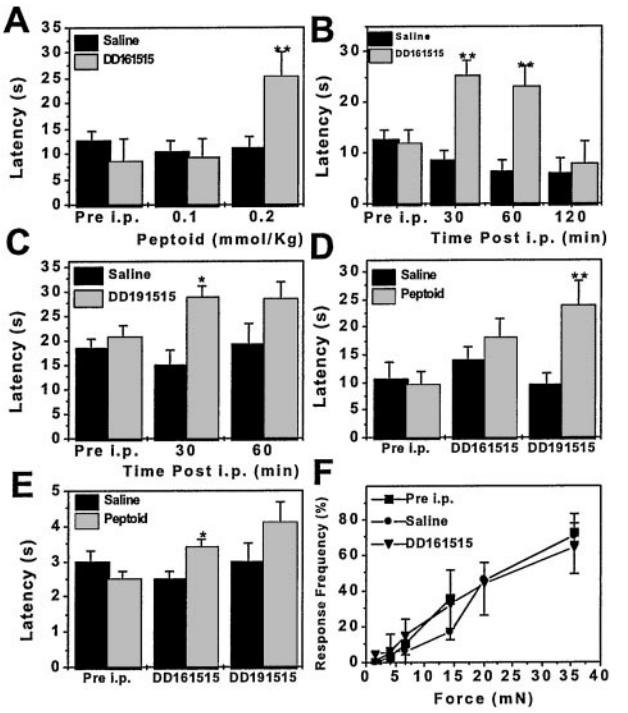

Fig. 5. Identified trialkylglycines attenuate thermal nociception. Response latencies in the hot plate at 52°C (*A–C*) and 56°C (*D*), and the tail immersion at 52°C (*E*) tests. (*F*) Response to mechanical stimulation of the hindpaw with von Frey hairs of six intensities. Mice were monitored before (Pre i.p.) and 30 min after i.p. injection of vehicle (saline) or 0.2 mmol/kg of peptoid (unless explicitly indicated in the figure). All data are given as mean ± SEM with $n \geq 9$ (hot plate 52°, *A–C*), $n \geq 7$ (hot plate 56°C), $n \geq 8$ (tail immersion), and $n \geq 9$ (von Frey hairs). *, $P < 0.05$; **, $P < 0.01$—for saline versus peptoid with the one-way ANOVA test and two tailed-unpaired *t* test.

between injections (10–15 min) were used to prevent the well known inactivation of sensory nerve fibers caused by repeated application of the vanilloid. The capsaicin-evoked impulse discharge was remarkably reduced by peptoid DD161515. As illustrated in Fig. 4*C*, intraarterial injection of DD161515 (0.4 μmol, $n = 3$), followed by washing with saline, gradually abolished (mean time ≈30 min) the impulse discharge of a subsequent capsaicin administration (Fig. 4 *B* and *C*). This inhibitory action was reversible, with a mean recovery time of 1 h (Fig. 4*D*). In contrast, the impulse discharge evoked by mechanical stimulation was virtually unaffected by administration of 0.4 μmol of peptoid DD161515 (Fig. 4 *E* and *F*). Intraarterial administration of a higher dose of peptoid (1.6 μmol, $n = 2$), along with no washing with saline after its injection (which increases the exposure time of nerve endings to the peptoid), resulted in a rapid (≈5 min), complete and sustained (>1 h) inhibition of the capsaicin-evoked nerve responses. In these conditions, however, a gradual attenuation of mechanically stimulated discharges by the peptoid was also observed, suggesting an unspecific effect of the peptoid at high concentrations (data not shown). Collectively, these findings imply that newly identified peptoids may exhibit *in vivo* antinociceptive or/and analgesic activity.

To address whether VR1 antagonists have antinociceptive properties, we conducted behavioral nociception tests in animals before and after i.p. injection of the vehicle (1% ethanol in saline) and the trialkylglycine DD161515. First, we determined whether these compounds modified the response to noxious heat explored with the hot plate test. As illustrated in Fig. 5*A*, compound DD161515 significantly attenuated thermal nociception at 52°C (vehicle: 11 ± 2 s hot plate latency, $n = 8$) when administered at 0.2 mmol/kg (25 ± 3 s, $n = 7$, $P < 0.01$). This antinociceptive activity was transient, exhibiting a maximum activity at 30 min postinjection and disappearing 2 h afterward (Fig. 5*B*). Similar results were obtained with the tail immersion test at 52°C, where peptoid DD161515 prolonged the withdrawal latency by 40% (vehicle: 2.5 ± 0.2 s, $n = 13$; DD161515: 3.4 ± 0.2 s, $n = 17$, $P < 0.05$; Fig. 5*E*). Peptoid DD191515 also displayed an analgesic effect (Fig. 5*C*). Overall, the thermal antinociceptive activity of DD161515 at 52°C was higher than that exhibited by compound DD191515, consistent with its stronger blockade efficacy of VR1 channel activity. In contrast, whereas peptoid DD161515 did not significantly decrease nociception at 56°C, DD191515 exhibited conspicuous antinociceptive activity at this temperature, as demonstrated by the 2.5-fold increase in the hot plate latency time (vehicle: 9.5 ± 2.1 s, $n = 7$; DD191515: 23.7 ± 4.4 s, $n = 7$, $P < 0.01$; Fig. 5*D*). It is noteworthy that these compounds reduced thermal nociception without affecting mechanical sensory transduction. As displayed in Fig. 5*F*, i.p. administration of 0.2 mmol/kg DD161515 did not modify the withdrawal threshold in the von Frey hairs test. These results imply that identified trialkylglycines exhibit *in vivo* thermal antinociception, substantiating the notion that blockade of VR1 channels modulates thermal sensory transduction *in vivo* (7).

It should be expected that pharmacological blockade of VR1 channel activity may also reduce pain perception (4, 5). To investigate attenuation of pain-related behavior by peptoids, we evaluated capsaicin-induced pain and neurogenic inflammation

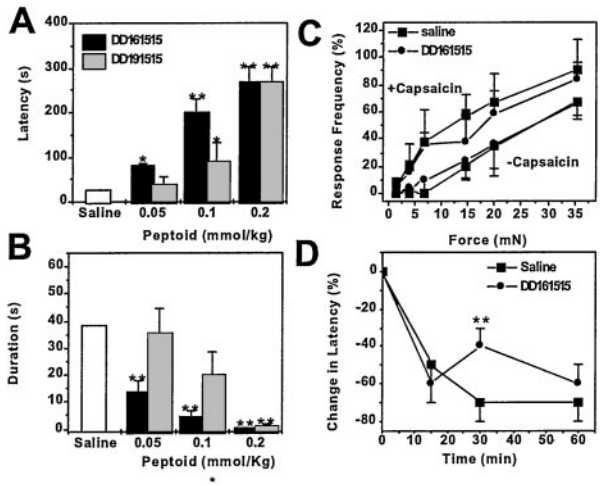

Fig. 6. Selected trialkylglycines ablate capsaicin-evoked inflammation and thermal hyperalgesia. Latency to first action (*A*) and duration of action (*B*) of the behavioral reaction (licking and shaking paw) evoked by intraplantar injection of capsaicin in the absence (saline) or presence of trialkylglycines. (*C*) Responses to mechanical stimulation of the hindpaw with von Frey hairs. Mice were tested 60 min after i.p. injection of saline or 0.2 mmol/kg DD161515 (−Capsaicin). Thereafter, capsaicin (6 μg) was intradermally administered into one hindpaw of the same animals, which were tested 30 min later (+Capsaicin). (*D*) Change in hot-plate latency after painting the plantar hindpaw with 10% mustard oil ($n = 7$). All data are given as mean ± SEM with $n \geq 9$ (*A* and *B*) or $n \geq 7$ (*C* and *D*). *, $P < 0.05$; **, $P < 0.01$—for saline versus peptoid with the one-way ANOVA test and two tailed-unpaired *t* test.

in mice by injecting 6 μg of capsaicin into the hindpaw (Fig. 6; ref. 25). Intraplantar injection of capsaicin evoked a licking and shaking of the paw in mice. The latency and duration of this behavior were, respectively, 20 ± 8 s ($n = 12$) and 37 ± 10 s ($n = 12$) (Fig. 6 *A* and *B*). Intraperitoneal administration of 0.2 mmol/kg DD161515, 30 min before capsaicin intraplantar injection, resulted in a remarkable 13-fold increase of the latency time (269 ± 31 s, $n = 7$; Fig. 6*A*). The licking and shaking in peptoid-treated animals were so brief that they reduced the duration response virtually to zero (Fig. 6*B*). This effect was dose-dependent, becoming evident at doses of 0.05 mmol/kg (80 ± 5.4 s, $n = 7$), and reaching maximum activity at 0.2 mmol/kg (Fig. 6 *A* and *B*). In addition, peptoid DD161515 also prevented the neurogenic inflammation secondary to intradermal administration of capsaicin (Table 1). Similar results were obtained for DD191515, although this compound showed weaker analgesic activity requiring higher doses to attenuate capsaicin-induced pain behavior (Fig. 6 *A* and *B*). As expected, mice sensitized with capsaicin showed intense mechanical hyperalgesia in the treated paw (Fig. 6*C*). However, reduced withdrawal thresholds to punctuate mechanical stimuli were not modified by DD161515 (Fig. 6*C*), indicating that the peptoid do not alter the capsaicin-induced mechanical hyperalgesia. This result appears consistent with the lack of effect of the peptoid on the impulse discharge evoked by mechanical stimulation of nociceptor nerve endings (Fig. 4 *E* and *F*).

Table 1. Trialkylglycines reduce capsaicin-induced neurogenic inflammation

	Paw volumes, mm³	
	Contralateral	Ipsilateral
Saline DD161515	15.1 ± 1.4	20.6 ± 2.0*
0.05 mmol/Kg	12.8 ± 1.2	15.3 ± 1.1
0.2 mmol/Kg	13.0 ± 1.3	15.2 ± 1.6

Peptoids or saline (1% ethanol) were injected intraperitoneally. Capsaicin (6 μg) was intradermally administered into the ipsilateral hindpaw of the animals. Paw volumes were measured with a plethysmometer. Data are given as mean ± SEM with $n \geq 9$. *, $P < 0.001$ for contralateral vs. ipsilateral paw with the one-way ANOVA test.

We also evaluated whether compound DD161515 attenuates the thermal hyperalgesia provoked by application of mustard oil to the plantar hind paw. As shown in Fig. 6*D*, 30 min after tissue damage animals treated with saline exhibited a decrease in latency of 70% (10.9 ± 1.2 s basal vs. 3.8 ± 0.8 s mustard oil, $n = 7$), whereas mice injected with 0.2 mmol/kg DD161515 showed a significantly lower change of withdrawal latency (10.9 ± 0.9 s basal vs. 7.2 ± 0.9 s mustard oil, $n = 7$). Thus, trialkylglycines attenuated capsaicin-evoked pain and neurogenic edema, as well as thermal hyperalgesia induced by tissue irritation, without modifying the mechanical hypersensitivity. Altogether, these results suggest that VR1 channels contribute to chemical and thermal hyperalgesia *in vivo*.

Discussion

VR1 channels have been implicated in pain detection, making them prime therapeutic marks for target-oriented novel analgesic molecules (4, 7, 8). We screened a restricted, oligo N-substituted glycine-based combinatorial library to find previously uncharacterized VR1 blockers. Oligomers of N-substituted glycines provide a class of small, non-natural molecules that are proteolytically stable and have potent biological activities (27). A major advantage of using short oligomers is that low molecular mass molecules (≤600 Da) usually display acceptable tissue penetration properties and better pharmacological conformities (28–30). The salient contribution of this work is the identification of two VR1 antagonists that modulate nociceptive responses to heat and substantially attenuated capsaicin-evoked pain, thermal hyperalgesia and neurogenic edema *in vivo*, without modifying the threshold to noxious mechanical stimuli. Both peptoids are a previously uncharacterized class of molecules that efficiently antagonize VR1 function, thus attenuating Ca^{2+} influx through this receptor. Their blockade efficacy rivals with that exhibited by the vanilloid-like molecule capsazepine and VR1 channel blockers such as ruthenium red and arginine-rich peptides. Structurally, trialkylglycine-based VR1 blockers do not have a recognizable vanillyl-like motif, suggesting that they do not compete for the vanilloid binding site. Indeed, our findings indicate that identified peptoids bind to a different receptor site than capsaicin. Structure–function studies should uncover the molecular determinants that define this drug binding site on VR1 channels.

The role of VR1 channels in nociception and pain perception has been a matter of intense investigation. Genetic ablation of these channels proved their role in inflammatory pain signaling and thermal and chemical hyperalgesia but not in mechanical hyperalgesia (7, 8). Our findings substantiate this observation in that they show that pharmacological suppression of VR1 activity abolished vanilloid-evoked inflammation and attenuated hyperalgesia to thermal stimuli secondary to tissue irritation. Intriguingly, the contribution of VR1 subunits to thermal nociception is more controversial. Whereas the group of Caterina *et al.* (7) found robust deficits in thermally evoked pain in their VR1-null animals, Davis *et al.* (8) did not observe significant alterations in the responses to acute noxious thermal stimuli in their strain of VR1-knock out mice. This discrepancy may arise from the different mouse strains used in these studies. Our data, however, are in agreement with the findings of Caterina *et al.* and support that VR1 is a sensor for commonly encountered noxious thermal stimuli (4). As shown, both peptoids significantly prolonged the mean latency of the response to heating the plate at ≥52°C. Interestingly, peptoid DD161515 specifically attenuated the response at 52°C, whereas compound DD191515 reduced the

heat-evoked response at both 52°C and 56°C. These results suggest that the analgesic effect of DD161515 is mediated by modulation of the low-threshold heat channel (>43°C)—i.e., VR1 (4). In contrast, peptoid DD191515 might also block a high-threshold heat channel such as VRL-1 (>52°C; ref. 31) or, alternatively, a yet unrecognized protein. As in VR1-null mice, we also observed larger heat-evoked behavioral deficits in the hot plate than in the tail immersion, consistent with the interpretation that VR1 does not contribute equally to all forms of thermal nociception (4, 7). The *in vivo* analgesic activity exerted by these compounds appears to result primarily from a reduced responsiveness of sensory nerve endings in peripheral tissues. Collectively, these results highlight a major role of VR1 in the onset of nociceptive responses to heat and in primary hyperalgesia elicited by tissue damage, thus underscoring its interest as a therapeutic target for treating specific types of pain.

The extent of antinociceptive and analgesic activities of newly identified trialkylglycines blockers was comparable to that displayed by vanilloid-like molecules (10, 11). It should be noted, however, that doses ≥0.05 mmol/kg for attenuation of peripheral nociceptor activity and antiinflammatory effects and as high as 0.2 mmol/kg were required to decrease thermal nociception and hyperalgesia. These therapeutic amounts contrast with the peptoids micromolar blocking efficacy of VR1 channel activity *in vitro*, suggesting a poor absorption/delivery and/or extensive metabolism. The short duration of the response (≤1 h) appears consistent with this notion. In analogy to vanilloids, the hydrophobic nature of both peptoids (logP > 5.0) could hamper their *in vivo* effectiveness (29, 30). The route of administration, which is influenced by the physicochemical characteristics of the molecules, may have also contributed to the low bioavailability observed. For example, i.p. and transdermal administration appears to be better suited for hydrophilic molecules than for hydrophobic compounds. Indeed, vanilloid-like molecules with improved water solubility exhibit enhanced absorption/delivery and metabolic stability resulting in higher antinociception (10, 11). Accordingly, chemical modifications aimed at reducing the hydrophobicity of both trialkylglycines, without altering the pharmacophore identity or significantly increasing their molecular mass, should enhance their therapeutic index. Thus, these peptoids may be considered leads for drug development. At variance with vanilloid-like molecules, trialkylglycines should not exhibit excitatory nor cytotoxic properties because they do not activate the VR1 channel.

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