Arginine-rich peptides are blockers of VR-1 channels with analgesic activity

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Abstract Vanilloid receptors (VRs) play a fundamental role in the transduction of peripheral tissue injury and/or inflammation responses. Molecules that antagonize VR channel activity may act as selective and potent analgesics. We report that synthetic arginine-rich hexapeptides block heterologously expressed VR-1 channels with submicromolar efficacy in a weak voltage-dependent manner, consistent with a binding site located near/at the entryway of the aqueous pore. Dynorphins, natural arginine-rich peptides, also blocked VR-1 activity with micromolar affinity. Notably, synthetic and natural arginine-rich peptides attenuated the ocular irritation produced by topical capsaicin application onto the eyes of experimental animals. Taken together, our results imply that arginine-rich peptides are VR-1 channel blockers with analgesic activity. These findings may expand the development of novel analgesics by targeting receptor sites distinct from the capsaicin binding site.

Key words: Pain; Nociceptor; Capsaicin; Dynorphin; Non-competitive antagonist; Ionic pore

1. Introduction

Capsaicin, the pungent ingredient of red peppers, exerts its irritant action by exciting sensory nerve endings that signal tissue injury (nociceptors), and whose activation leads ultimately to a conscious sensation of pain [1–3]. Because excitatory effects of capsaicin are followed by long-lasting inactivation, capsaicin is also used as an analgesic molecule for the treatment of diverse pain and inflammatory conditions [4–6]. In recent years the understanding of the molecular mechanisms underlying capsaicin nociceptive and analgesic activity has advanced considerably with the cloning of a capsaicin-operated neuronal receptor referred to as the vanilloid receptor subunit 1 (VR-1) [7,8]. VR-1 is a non-selective cation channel with high Ca^{2+} permeability. Activation of VR-1 by capsaicin leads to an increase in intracellular Ca^{2+} that results in a change of the metabolic state in sensory neurons [7,9]. Ca^{2+} permeability appears also to be involved in capsaicin-induced desensitization of VR-1 channels, the physiological property underlying the analgesic activity exhibited by capsaicin [3,9]. By contrast, high doses of the vanilloid produce a prolonged activation of VR-1 channels leading to neurodegeneration [3,10]. In addition to their sensitivity to capsaicin, VR-1 channels are also operated by thermal stimuli, by inflammatory mediators and by protons [3,7–9].

The central role played by VR-1 channels in the transduction of chemical and thermal stimuli makes this receptor a molecular target for drug intervention [3,6]. This notion has been substantiated by the pain perception impairment exhibited by mice lacking VR-1 channels [11,12]. Despite the wealth of information accrued in recent years on VR-1 function, the number of available compounds that modulate its activity is still quite limited [6]. Structure–activity studies have focused on synthesizing competitive capsaicin antagonists such as capsazepine and analogs [5,13]. Among the non-competitive antagonist family, only ruthenium red is accepted as a VR-1 blocker [14]. Here, we report that arginine-rich hexapeptides are potent blockers of the recombinant homomeric VR-1 channel expressed in *Xenopus* oocytes. Synthetic hexapeptides blocked VR-1 channels with submicromolar efficacy in a modestly voltage-dependent manner. We also found that naturally occurring, arginine-containing peptides such as dynorphins block VR-1 channels at micromolar concentrations. Our findings suggest that arginine-rich peptides act as VR-1 channel blockers. Notably, synthetic hexapeptides displayed analgesic activity as evidenced by the attenuation of the irritant activity induced by topical application of capsaicin onto the eyes of experimental animals. Hence, VR-1 arginine-rich peptide channel blockers may constitute a novel family of analgesic molecules.

2. Materials and methods

2.1. Peptide synthesis and purification

Arginine-rich hexapeptides (RRRRWW-NH_2, RRWWIR-NH_2, and RYYRRW-NH_2; capital letters denote L-amino acids) were manually synthesized by the solid-phase method using the base-labile 9-fluorenylmethoxycarbonyl (Fmoc) group for protection of the amino function [15]. Peptides were assembled on a TentaGel S RAM resin (0.24 mmol/g; Rapp Polymere, Germany) to produce a peptide with a C-terminal amide moiety using diisopropylcarbodiimide (DIC)/hydroxybenzotriazole (HOBT)-mediated couplings (1 eq. each amino acid of HOBT and 2 eq. DIC). The Fmoc groups were cleaved by 20% piperidine in dimethylformamide for 30 min. Each deprotection or coupling step was verified by the ninhydrin test. Peptides were cleaved from the resin with 70% trifluoroacetic acid/20% dichloromethane/5% water/2.5% ethanodithiol/2.5% chlorotriisobutylsilane for 4 h at room temperature. Fully deprotected peptides were precipitated in *t*-butyl-

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methyl ether, centrifuged, dissolved in acetic acid and freeze-dried. Peptides were purified by HPLC on a Lichrosphere 100 RP 18 (5 μm) column (Waters). Peptide identity was confirmed by MALDI-TOF spectrometry.

2.2. cRNA preparation and microinjection into Xenopus oocytes

All the procedures have been described in detail elsewhere [16,17] and are followed accordingly, unless otherwise indicated. Briefly, capped cRNA for the VR-1 subunit was synthesized from linearized cDNA (kindly provided by Dr. David Julius) using the mMESSAGE mMACHINE™ from Ambion (Texas). cRNA (0.2 mg/ml) was microinjected (50 nl) into defolliculated oocytes (stage V and VI) as described. Oocytes were functionally assayed 2–4 days after cRNA injection.

2.3. Electrophysiological characterization of VR-1 channels expressed in Xenopus oocytes

Whole-cell currents were recorded with a conventional two-microelectrode voltage-clamp amplifier at 20°C [16,17]. All electrophysiological studies were performed in Mg^{2+}-Ringer's solution (in mM: 10 HEPES pH 7.4, 115 NaCl, 2.8 KCl, 0.1 $BaCl_2$, 2.0 $MgCl_2$). Homomeric VR-1 channels were activated by application of 20 μM capsaicin in the absence or presence of increasing concentrations of channel blockers at a holding potential (V_h) of −80 mV. Responses were normalized with respect to that evoked in the absence of channel blockers. Dose–response curves were fitted to a Hill equation [18]:

$$\frac{I}{I_{max}} = \frac{1}{1 + \left(\frac{[\text{blocker}]}{IC_{50}}\right)^n}$$

where IC_{50} denotes the channel blocker concentration that inhibits half of the response obtained in its absence (I_{max}) and n denotes the Hill coefficient which is an estimate of the number of drug binding sites [19]. Experimental data were fitted to the Hill equation with a non-linear least-squares regression algorithm using MicroCal ORIGIN version 5.0 (Microcal, Amherst).

I–V characteristics were recorded using a ramp protocol [17]. Oocytes were depolarized from −80 mV to 20 mV in 5 s (20 mV/s). Leak currents were measured in the absence of agonist in the external bath medium and subtracted from the ionic current recorded in the presence of the ligand. Voltage dependence of channel blockade was studied as described [20].

2.4. Evaluation of in vivo analgesic activity

The topical analgesic activity of VR-1 channel blockers was evaluated in OF-1 mice by comparing the number of scratching movements evoked by topical ocular application of 2.5 μl of a 0.01% of a capsaicin solution (333 μM), before and after treatment with peptides or their vehicle [21,22]. The number of capsaicin-induced scratching movements was counted during 1 min immediately after application of the drug to one eye. Thereafter, the eye was extensively washed with saline solution. After a 15 min period, a 2.5 μl drop of either VR-1 channel blocker at the specified concentrations or saline solution was applied. Five minutes afterwards, a mixture of capsaicin and channel blocker (or, alternatively, saline solution) was applied to the same eye and the number of scratching movements during 1 min after drug instillation was counted. This protocol was performed in both eyes. The normalized number of scratching movements was defined as follows:

$$\text{Normalized scratching movements} = \frac{S_b - S_a}{S_c - S_a}$$

where S_b denotes the number of scratching movements in the presence of channels blocker or saline upon the second capsaicin application, S_c is the number of scratching movements of the first capsaicin application, and S_a represents the number of scratching movements of the second capsaicin instillation upon the application of the local anesthetic 40% oxobuprocaine/10% tetracaine. Capsaicin was dissolved in 15% ethanol and 8.5% Tween-80 (33 mM) and then diluted with saline solution to the final concentration (0.33 mM). Capsazepine was prepared at 1.0 mM in 5% dimethyl sulfoxide. Peptides were dissolved in saline solution. Results are given as mean ± S.E.M.,

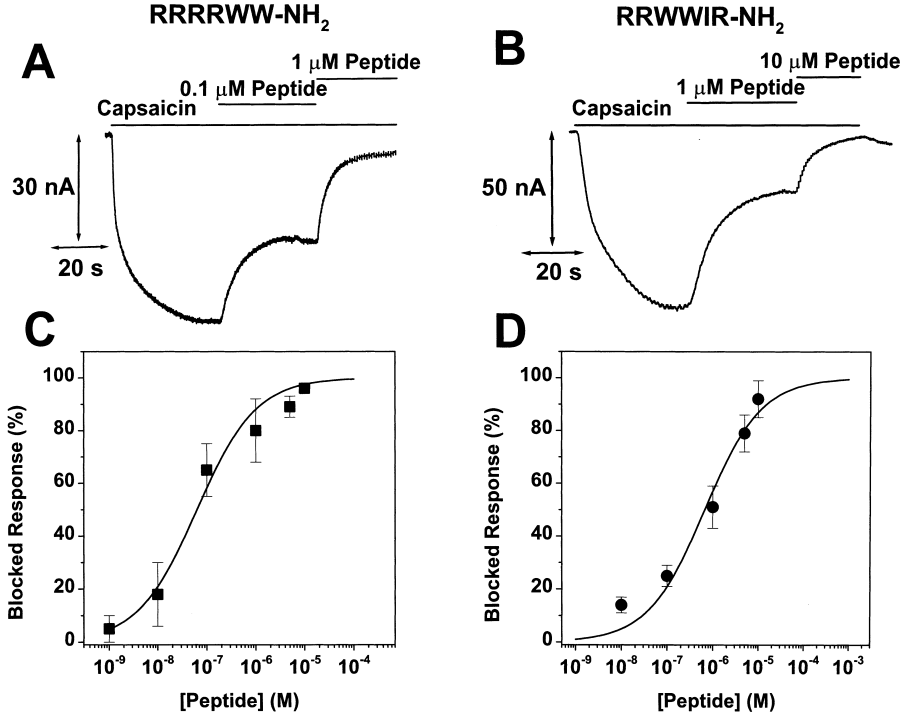

Fig. 1. Arginine-rich hexapeptides inhibit VR-1 channel activity. Block of capsaicin-evoked ionic currents from recombinant VR-1 channels by RRRRWW-NH_2 (A) and RRWWIR-NH_2 (B). The pulse protocols are indicated above the current traces. The length of the solid lines indicates perfusion duration. VR-1 channels were activated by 20 μM capsaicin and recorded at a holding potential (V_h) of −80 mV. Downward deflection indicates inward current. Dose–response curves for (C) RRRRWW-NH_2 and (D) RRWWIR-NH_2 inhibition of capsaicin-activated ionic currents of VR-1 channels expressed in *Xenopus* oocytes. Responses were normalized with respect to that in the absence of hexapeptides. Ionic currents were elicited by 20 μM capsaicin at $V_h = -80$ mV. Solid lines depict the theoretical fits to a Michaelis–Menten binding isotherm. Each point represents the mean ± S.E.M. with N (number of oocytes) ≥ 4.

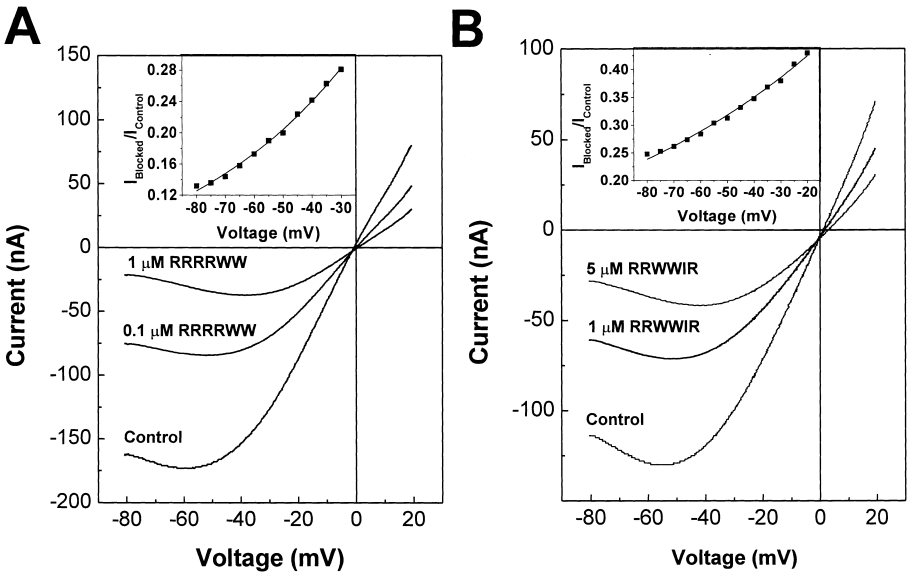

Fig. 2. Voltage-dependent blockade by RRRRWW-NH_2 (A) and RRWWIR-NH_2 (B) of recombinant VR-1 channels expressed in amphibian oocytes. Current–voltage characteristics of ionic currents evoked by 20 μM capsaicin in the absence (control) or presence (RRRRWW-NH_2 or RRWWIR-NH_2) of hexapeptides at the specified concentrations. Each trace is representative of at least three oocytes. Oocytes were held at −80 mV and depolarized to 20 mV in 5 s (20 mV/s) using a ramp protocol. Leak currents were obtained in the absence of capsaicin and subtracted from the agonist-evoked ionic currents. Inset represents the fraction of unblocked response ($I_{blocked}/I_{control}$) as a function of the applied voltage. Solid lines depict the best fit to a model that considers the existence of a single binding site within the pore and a negligible multiple ion occupancy of this site [29]. The values obtained for RRRRWW-NH_2 were $\delta = 0.20$ and IC_{50} (0 mV) = 0.46 μM, and for RRWWIR-NH_2 were $\delta = 0.16$ and IC_{50} (0 mV) = 0.65 μM.

with N (number of animals) ≥10. Treatment administration (channel blockers and saline) was randomized. Scratching movements were evaluated blindly.

3. Results

3.1. Arginine-rich hexapeptides block VR-1 channel activity with high efficacy and potency

Homomeric VR-1 permeation properties closely resemble those described for the *N*-methyl-D-aspartic acid receptor (NMDAR), namely they are non-selective cation channels with remarkable permeability to Ca^{2+} and high sensitivity to Ca^{2+} blockade [9]. These properties suggest the presence of a high affinity Ca^{2+} binding site in the permeation pathway akin to that present in NMDARs [23,24]. The NMDAR Ca^{2+} binding site is a target site for arginine-rich hexapeptides, which block its channel activity with high efficacy and potency [25]. Hence, we reasoned that VR-1 receptors may also be modulated by these molecules. To test this hypothesis we selected the hexapeptides: RRRRWW-NH_2 which shows high efficacy blocking NMDARs, RRWWIR-NH_2 that display lower blocking efficacy and selectivity, and RYYRRW-NH_2 which shows the lowest affinity for NMDARs.

As illustrated in Fig. 1A, perfusion of voltage-clamped oocytes expressing VR-1 channels with 20 μM capsaicin elicited a slowly activating inward current that was reduced by ∼50% upon addition of 0.1 μM RRRRWW-NH_2, and by ∼80% when oocytes were exposed to 1.0 μM hexapeptide. Similar blocking activity was obtained for RRWWIR-NH_2, although this hexapeptide appears less potent (Fig. 1B). The inhibitory activity displayed by both arginine-rich hexapeptides was reversible, as evidenced by the recovery of capsaicin-evoked ionic currents after antagonist wash-out (data not shown). The efficacy and potency of VR-1 blockade by these hexapeptides were obtained from the dose–response relationships (Fig. 1C,D). For RRRRWW-NH_2, the IC_{50} was 0.10 ± 0.06 μM and $n = 0.76 \pm 0.10$, for RRWWIR-NH_2 it was 0.40 ± 0.10 μM and $n = 0.80 \pm 0.12$, and for RYYRRW-NH_2 it was 2 ± 1 μM and $n = 0.85 \pm 0.13$, substantiating the higher blockade activity of the former hexapeptide. The Hill coefficient of ∼1.0 is consistent with the occurrence of a single binding site. Peptides appear to be non-competitive antagonists of homomeric VR-1 channels, as evidenced by the virtually identical capsaicin EC_{50} (concentration of capsaicin to activate half the maximal response) in the absence (0.4 ± 0.08 μM, $N = 4$) and in the presence of 0.1 μM RRRRWW-NH_2 (0.3 ± 0.1 μM, $N = 3$) or 0.5 μM RRWWIR-NH_2 (0.6 ± 0.15 μM, $N = 3$).

Taken together, these results indicate that arginine-rich hexapeptides are potent blockers of VR-1 channels and suggest the presence of a negatively charged drug binding site on these receptors similar to that reported for NMDARs, Ca^{2+} channels, and cyclic nucleotide-gated channels [24,26,27].

3.2. Arginine-rich hexapeptides are channel blockers of VR-1 receptors

To investigate the mechanism underlying the blockade activity of arginine-rich hexapeptides and to locate the binding site, we studied the voltage dependence of VR-1 inhibition [28]. *I–V* relationships from −80 mV to 20 mV were obtained using ramp protocols (Fig. 2). VR-1 channel activity displayed a linear *I–V* relation between −40 mV and 20 mV that changed to inward rectification at voltages below −50 mV. The reversal potential for VR-1 channels was 1.3 ± 2.2 mV (mean ± S.E.M., $n = 4$) in Mg^{2+}-Ringer which is in good agreement with previous reports [7]. Arginine-rich hexapeptides blocked VR-1 channel activity primarily at negative membrane potentials without significantly changing the appearance of the *I–V* curve nor the current reversal potentials

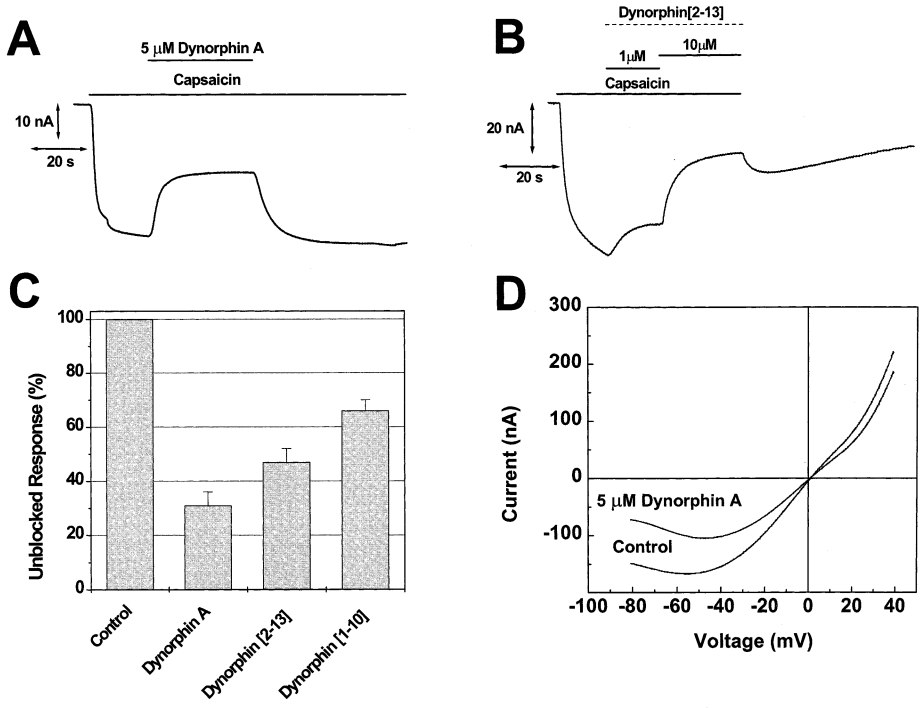

Fig. 3. The naturally occurring, arginine-rich dynorphin peptides block VR-1 channel activity. Block of capsaicin-evoked ionic currents from recombinant VR-1 channels by dynorphin A (A) and dynorphin [2–13] (B). The pulse protocols are indicated above the current traces. The length of the solid lines indicates perfusion duration. C: Comparative blockade activity exerted by 5 μM dynorphin peptides. Responses were normalized with respect to that in the absence of peptides. VR-1 channels were activated by 20 μM capsaicin and recorded at a holding potential (V_h) of −80 mV. Downward deflection indicates inward current. Each point represents the mean ± S.E.M. with $N \geq 3$. D: Current–voltage characteristics of ionic currents evoked by 20 μM capsaicin in the absence (control) or presence (dynorphin A) of 5 μM dynorphin A. Other conditions as described in Fig. 2.

(3.0 ± 3.1 mV for RRRRWW-NH_2 and 4.0 ± 2.6 mV for RRWWIR-NH_2). These results suggest that VR-1 blockade by arginine-rich hexapeptides is weakly voltage-dependent, and imply that the peptide binding sites sense the pore electrostatic field. To further substantiate this observation, we obtained the fraction of unblocked response ($I_{blocker}/I_{control}$) as a function of the membrane potential (Fig. 2, insets). The fraction of unblocked response–voltage relationship is related to the location of the blocker binding site within the membrane electrostatic field [20,28–31]. For both peptides, experimental data exhibited a weak dependence on the applied membrane voltage, consistent with a rather external location of the drug binding site. Indeed, considering the occurrence of a single binding site within the pore electric field and a negligible multiple ion occupancy of this site [29], the inferred electrical distance of the peptide binding site from the mouth of the channel, δ, was 0.2 for RRRRWW-NH_2 and 0.16 for RRWWIR-NH_2 (Fig. 2, insets). This observation implies that the drug binding site is located near or at the entryway of the aqueous pore, and suggests that arginine-rich peptides act as VR-1 channel blockers.

3.3. Naturally occurring arginine-rich peptides block VR-1 channel activity

A question that arises is: do naturally occurring, arginine-rich peptides modulate VR-1 channel activity? Dynorphins are neuropeptides containing a significant number of basic residues in their amino acid sequence suggesting that they may modulate VR-1 channel activity. As displayed in Fig. 3A, dynorphin A ([1–17]: YGGFLRRIRPKLKWDNQ-NH_2) reversibly blocked capsaicin-activated ionic current in oocytes expressing recombinant VR-1 receptors. Similarly, two shorter versions of dynorphin A, dynorphin [2–13] (GGFLRRIRPKLK-NH_2) and dynorphin [1–10] (YGGFLRRIRP-NH_2), comprising the core structure of the longer sequence, blocked VR-1 channel activity with similar efficacy, dynorphin [1–10] being the least potent. Notice that dynorphin blockade was readily washable (Fig. 3A,B). The blocking activity of dynorphin peptides was comparable to that characteristic of arginine-rich hexapeptides, although they displayed lower blockade efficacy (Fig. 3C). The estimated IC_{50} was 3 μM for dynorphin A, 5 μM for dynorphin [2–13] and 10 μM for dynorphin [1–10].

Dynorphins inhibited VR-1 channel activity in a voltage-dependent manner, showing higher blockade efficacy at negative membrane potentials than at depolarizing voltages (Fig. 3D). As for synthetic hexapeptides, the extent of voltage dependence was weak, compatible with a location of their binding site close to or at the vestibule of the channel pore. This finding, in conjunction with the positive charge present in these peptides, suggests that dynorphins may recognize the same receptor binding site as arginine-rich hexapeptides. This notion was further supported by the finding that the IC_{50} value of RRRRWW-NH_2 was increased to 3 ± 1 μM ($N = 3$) and of RRWWIR-NH_2 was shifted to 6 ± 1 μM ($N = 3$) in the presence of 1 μM of dynorphin A.

3.4. Arginine-rich peptides display analgesic activity

VR-1 channels appear involved in the transduction of chemical and thermal nociception. Thus channel blockers of

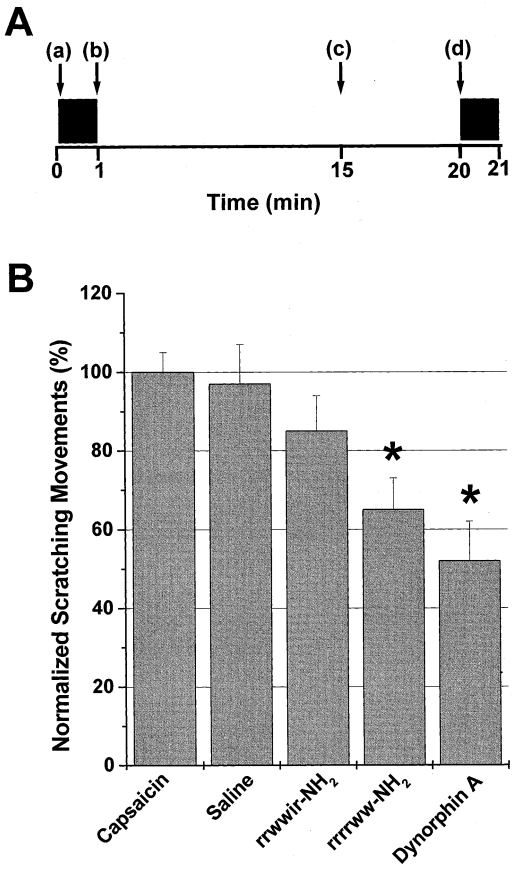

Fig. 4. Arginine-rich peptides ameliorate the pungent activity of capsaicin. A: Experimental protocol to evaluate the potential in vivo analgesic activity of VR-1 blockers. 2.5 μl of 333 μM capsaicin is topically applied (a) onto the eye of a mouse and the pungent activity is evaluated by counting the scratching during the first minute after treatment. The eye is washed with 1 ml saline (b) and allowed to rest for 15 min. Thereafter, 2.5 μl of saline or blocker is applied (c). After 5 min incubation to allow blocker diffusion, capsaicin alone or mixed with blocker is applied (d) onto the eye, and the scratching activity is counted during the first minute after vanilloid application. B: Comparison of scratching activity evoked by topical application of capsaicin onto the eyes of animals. Responses were normalized with respect to the number of scratching movements counted in the first application of capsaicin (29.5 ± 3.1), and that evoked upon treatment with the local anesthetic (10 ± 3) before the second application of the vanilloid. The scratching movements counted upon the second addition of capsaicin in the absence (saline) or presence of peptides were: 29 ± 3.5 for saline, 26.5 ± 2.1 for rrwwir-NH_2, 23 ± 1.8 for rrrrww-NH_2, and 20 ± 2.1 for dynorphin A. Peptide concentration was 10 mM. Note that to rule out the susceptibility of the hexapeptides to proteolytic degradation, we used the D-amino acid peptide counterparts (denoted with lowercase letters) of RRRRWW-NH_2 and RRWWIR-NH_2. D-Amino acid peptides blocked VR-1 channels with similar efficacy as their L-amino acid counterparts (data not shown). Scratching activity was assessed blindly. Values are given as mean ± S.E.M., with $N \geq 10$ animals. *$P < 0.05$, Student's t-test.

these ionotropic channels may exhibit analgesic activity. We approached this issue and measured whether or not arginine-rich peptides ameliorated the irritant activity produced by topical application of capsaicin to the eyes of mice [21,22]. The cornea, conjunctiva, and anterior uvea are densely innervated by polymodal nociceptive fibers that are directly excited by capsaicin [32–34]. When applied topically to the eye of experimental animals, the vanilloid evokes immediate pain as evidenced by the increment in the number of scratching movements of the treated eye (29.5 ± 3.1 scratching movements). Intriguingly, the ocular discomfort produced by capsaicin application was not significantly reduced by application of 1.0 mM of the competitive capsaicin antagonist capsazepine (26.5 ± 3.5 scratching movements), substantiating the proposed existence of vanilloid receptors insensitive to this drug [3,33,35,36]. Treatment with 10 mM of the L-amino acid hexapeptides RRRRWW-NH_2 and RRWWIR-NH_2 did not significantly reduce the number of vanilloid-evoked scratching movements (data not shown). By contrast, capsaicin-induced ocular irritation was decreased by 40% after application of 10 mM of the proteolysis-resistant all-D-amino acid hexapeptide rrrrww-NH_2 (counterpart of L-amino acid peptide RRRRWW-NH_2), and by 15% upon the treatment with the all-D-amino acid peptide rrwwir-NH_2 (Fig. 4). This analgesic activity was comparable, albeit lower, to that displayed by dynorphin A (≥50% attenuation at 10 mM). The observed attenuation of capsaicin-induced ocular irritation was not due to VR-1 desensitization since instillation of a second capsaicin pulse did not alter the number of scratching movements (29.0 ± 3.5). Moreover, the in vivo analgesic activity of rrrrww-NH_2 was concentration-dependent as evidenced by its disappearance at lower peptide concentrations (27.5 ± 2.3 scratching movements at 1 mM). Accordingly, arginine-rich peptides appear to be VR-1 channel blockers with analgesic activity.

4. Discussion

We have found that small positively charged peptides are blockers of VR-1 channels. These peptides blocked capsaicin-operated ionic currents with micromolar efficacy in a weakly voltage-dependent manner. The blockade activity of arginine-rich peptides resembles that reported for ruthenium red, a widely accepted non-competitive antagonist of VR-1 channels [14]. The inhibitory activity of arginine peptides exhibit a Hill coefficient of ~ 1, suggesting the presence of a single binding site on the receptor. The weakly voltage-dependent blockade exerted by synthetic peptides and dynorphins suggests that a small fraction of the electrostatic field (δ) is sensed by their blocking site [28]. Indeed, the estimated electrical distance, $\delta \leq 0.2$, indicates that arginine-rich peptides traverse partway through the membrane electrostatic field to reach its binding site [20,28,29]. Accordingly, arginine-rich peptides appear to be non-competitive antagonists of VR-1 channels that act as channel blockers. Their binding site seems to be located near or at the entryway of the permeation pathway. A question that ensues is: how might the binding site be structured? Analysis of amino acid sequence of the P-loop reveals the presence of four negatively charged residues (E636, D646, E648, E651) that could structure one or more negatively charged rings akin to that described for Ca^{2+} channels and cyclic nucleotide-gated channels [26,27]. Interestingly, D646 and E648 are next to the GMG motif, which is similar to the highly conserved signature sequence GYG motif of voltage-gated potassium-selective channels [37] substantiating the hypothesis that this residue may be an important molecular determinant of both blocker sensitivity and ion selectivity. Accordingly, the molecular composition of the VR-1 P-loop is compatible with the presence of a binding site for positively charged molecules. Structure–function studies on VR-1 chan-

nels will ultimately identify the molecular determinants that define the permeation properties of this novel channel family.

A salient contribution of these results is that channel blockers such as arginine-rich peptides display analgesic activity against capsaicin-evoked ocular irritation. The vanilloid, applied topically to the eyes of mice, produced immediate signs of pain that were significantly attenuated by the topical application of the synthetic D-amino acid hexapeptide. The extent of analgesic activity was comparable to that exhibited by dynorphin A, a naturally occurring opioid peptide that activates κ opioid receptors [38,39]. Interestingly, the finding that dynorphin A blocks VR-1 channels with micromolar efficacy implies that its analgesic activity may be partially due by direct blockade of VR-1 channels. Further experimental work is required to understand dynorphin A-mediated nociception.

Taken together, our findings indicate that arginine-rich peptides are channel blockers of VR-1 with in vivo analgesic activity. These peptides may prove useful as leads for the development of novel analgesic therapies, and their receptor binding site may represent a target for discovery of non-peptide drugs.

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