Bioorganic & Medicinal Chemistry 16 (2008) 9349–9358

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry

journal homepage: www.elsevier.com/locate/bmc

Structure–activity relationships of 1,4-dihydropyridines that act as enhancers of the vanilloid receptor 1 (TRPV1)

Eun Joo Roh [a,†], Jason M. Keller [b,†], Zoltan Olah [b,†,‡], Michael J. Iadarola [b,*], Kenneth A. Jacobson [a,*]

[a] Molecular Recognition Section, Laboratory of Bioorganic Chemistry, National Institute of Diabetes and Digestive and Kidney Diseases, Building 8A, Room B1A-19 LBC, Bethesda, MD 20892-0810, USA
[b] Neurobiology and Pain Therapeutics Section, National Institute of Dental and Craniofacial Research, National Institutes of Health, Bethesda, MD 20892, USA

ARTICLE INFO

Article history:
Received 27 May 2008
Revised 15 August 2008
Accepted 22 August 2008
Available online 26 August 2008

Keywords:
Ion channel
Pain
Receptor
Capsaicin
Dorsal root ganglion

ABSTRACT

Vanilloid agonists such as capsaicin activate ion flux through the TRPV1 channel, a heat- and ligand-gated cation channel that transduces painful chemical or thermal stimuli applied to peripheral nerve endings in skin or deep tissues. We have probed the SAR of a variety of 1,4-dihydropyridine (DHP) derivatives as novel 'enhancers' of TRPV1 activity by examining changes in capsaicin-induced elevations in $^{45}Ca^{2+}$-uptake in either cells ectopically expressing TRPV1 or in cultured dorsal root ganglion (DRG) neurons. The enhancers increased the maximal capsaicin effect on $^{45}Ca^{2+}$-uptake by typically 2- to 3-fold without producing an action when used alone. The DHP enhancers contained 6-aryl substitution and small alkyl groups at the 1 and 4 positions, and a 3-phenylalkylthioester was tolerated. Levels of free intracellular Ca^{2+}, as measured by calcium imaging, were also increased in DRG neurons when exposed to the combination of capsaicin and the most efficacious enhancer **23** compared to capsaicin alone. Thus, DHPs can modulate TRPV1 channels in a positive fashion.

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1. Introduction

Capsaicin **1** is the neuroactive component of hot chili peppers, paprika and related spices, which stimulates peripheral terminals of nociceptive sensory neurons to produce a sensation that mimics painful heat. The receptor for capsaicin is the vanilloid receptor 1 (TRPV1/VR1) located on the nerve endings, axon and soma of a subpopulation of nociceptive (pain-sensing) sensory ganglion neurons.[1] TRPV1 is a member of the transient receptor potential (TRP) ion channel family, named for its homology with drosophila TRP channels and its ability to recognize ligands containing a vanillin moiety, such as capsaicin **1** (Chart 1).[2] The ion flux induced upon activation of TRPV1 depolarizes the afferent nerve ending, transmitting an action potential from the skin to synaptic contacts with spinal cord second order neurons, which then transmit the information to the brain where the perception of pain is registered.[3] Because of its critical role in acute and chronic inflammatory pain, antagonists of TRPV1 are sought as new analgesic agents.[4–10] Selected non-vanilloid antagonists of TRPV1 (the first antagonist **2** and clinical candidates SB-705498 **3** and AMG 517 **4**) and representative activators (an endogenous lipid mediator **5**, and the potent agonist **6**) are shown in Chart 1.[2] The effect of heat on TRPV1 was also reversibly reduced by micromolar nifedipine, a typical dihydropyridine (DHP) that is used clinically as an L-type calcium channel antagonist.[11] A number of compounds have been found that can either specifically block access of putative endovanilloids[12] to the binding site or, by an allosteric effect, prevent opening of the pore-loop (P-loop) domain of the channel.[2] The organometallic derivative ruthenium red is a pore-loop blocker of TRPV1.[8,13] More recently, an ATP binding region within the large intracellular ankyrin repeat amino terminal domain was identified that appears to regulate receptor desensitization,[14] which contrasts functionally with an ATP interaction at the Walker A motif in the C-terminal region that increases TRPV1 activity.[15] Thus, multiple auxiliary, pharmacologically tractable domains are present in TRPV1 that can be targeted to block or modulate ion channel function.

In addition to blocking, the *stimulation* of TRPV1 has also been utilized therapeutically. Chronic topical application of high concentration capsaicin, which deadens pain sensation locally, has

Abbreviations: DHP, 14-dihydropyridine; DMEM, Dulbecco's modified Eagle's medium; DRG, dorsal root ganglion; ER, endoplasmic reticulum; FAB, fast atom bombardment; FBS, fetal bovine serum; NSAID, non-steroidal antiinflammatory drug; RTX, resiniferatoxin; SAR, structure–activity relationship; TLC, thin-layer chromatography; TMS, tetramethylsilane; TRP, transient receptor potential.

* Corresponding authors. Tel.: +1 301 496 2758; fax: +1 301 402 0667 (M.J.I.); tel: +1 301 496 9024; fax: +1 301 480 8422 (K.A.J.).
E-mail addresses: miadarola@dir.nidcr.nih.gov (M.J. Iadarola), kajacobs@helix.nih.gov (K.A. Jacobson).
† These authors contributed equally to this work.
‡ Present address: The Institute of Biochemistry, The Biological Research Center of the Hungarian Academy of Sciences, Temesvári krt. 62, Szeged 6726, Hungary.

0968-0896/$ - see front matter Published by Elsevier Ltd.
doi:10.1016/j.bmc.2008.08.048

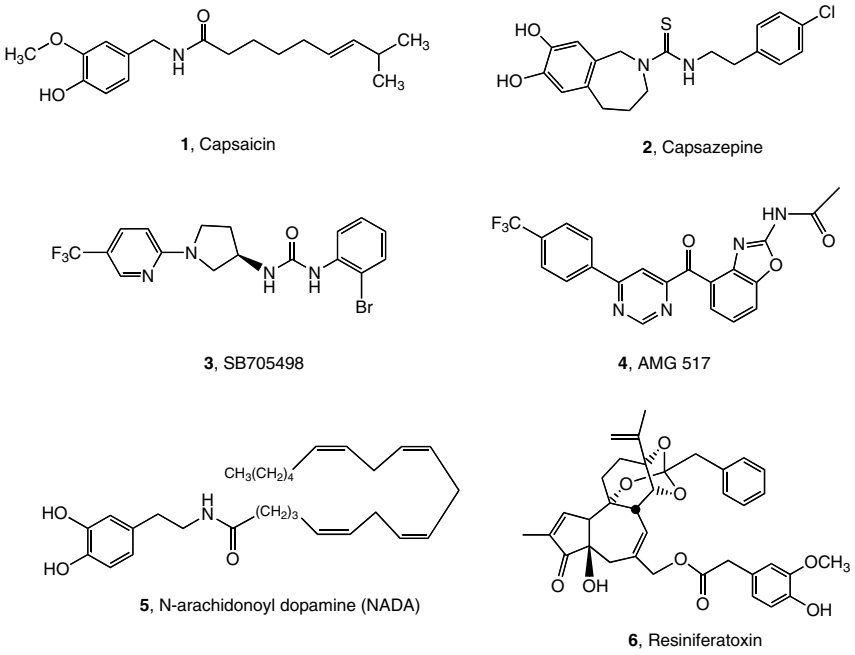


Chart 1. Selected known activating (**1**, **5**, and **6**) and inhibiting (**2**–**4**) ligands of TRPV1.

been available as an over-the-counter treatment for arthritic pain for many years, and higher dose formulations are also being explored.[16] Resiniferatoxin (RTX) **6**, one of the most potent vanilloid agonists, is being used in animals to treat intractable pain conditions resulting from advanced cancer, and potentially in human patients.[17,18] The therapeutic effect in this application results from Ca^{2+} excitotoxicity that occurs selectively in TRPV1-expressing, primary afferent nociceptive neurons when RTX is administered in the vicinity of the cell body or the peripheral or central nerve terminals.[19–21]

While TRPV1 is obviously located on the plasma membrane, another receptor pool ($TPRV1_{ER}$) also occurs intracellularly, where it is localized on the endoplasmic reticulum (ER).[13,19,22–26] Several studies have demonstrated that $TPRV1_{ER}$ is capable of being stimulated by exogenously applied vanilloids, since they readily cross cell membranes.[13,23–26] We have hypothesized that activation of $TPRV1_{ER}$ can contribute to nociceptive signaling due to de novo, intracellularly produced endovanilloids that release Ca^{2+} from the ER following stimulation of $TPRV1_{ER}$.[19,22] The multiplicity of environmental and intra- and intercellular stimuli that are capable of activating TRPV1 suggest that additional insights might be obtained by further pharmacological evaluation of the vanilloid binding site, various sites on the ion channel pore, or other non-vanilloid sites on this receptor/ion channel.[8–10,14,26]

We have discovered that a class of 1,4-dihydropyridine (DHP) derivatives with 6-aryl substitution has a novel action as enhancers of TRPV1 activity in several experimental models. They greatly increase the maximal ion flux stimulated by capsaicin, but exhibit minimal or no intrinsic agonist activity of their own. It is possible that these enhancers, in the presence of endovanilloids, would exhibit agonist-enhancing actions leading to receptor desensitization or nerve terminal inactivation.[3,17,21] The biological activity of this novel class of enhancers represents a new type of pharmacological activity for TRPV1 function and reinforces the idea of dynamic pore modulation.[28] The TRPV1 receptor structure is not known, and therefore the structure–activity relationship (SAR) described in this study is more descriptive than mechanistic.

2. Results

2.1. Chemical synthesis

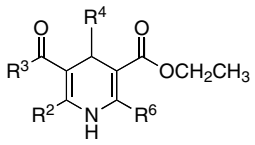
The synthesis of the DHP derivatives (**7**–**32**, Table 1) was carried out by two variations of standard method for a three-component Hantzsch condensation (Scheme 1).[29] Both 3-esters **7**–**11** and 3-thioesters **12**–**32** were prepared. For unsubstituted 6-phenyl DHPs, the precursor was an ethyl 3-aminocinnamate **33**. The β-enaminoesters **38** or thioesters **39** required for the route to synthesize unsubstituted 6-phenyl DHPs (Scheme 1B) were prepared from a dioxinone derivative **41** or Meldrum's acid **43** as shown in Scheme 2. Many of these DHPs and related analogues were prepared for use in previous studies of antagonists of adenosine and P2 receptors.[30–36]

2.2. Biological activity

The effects of compounds in a small, targeted library of DHP derivatives on capsaicin-induced $^{45}Ca^{2+}$-uptake were studied in a stably transfected TRPV1ε-NIH3T3 mouse fibroblast cell line and in primary cultures of dorsal root ganglion (DRG) neurons that express the TRPV1 channel endogenously. The enhancing effect of the DHP derivatives was demonstrable in both systems: the TRPV1ε-NIH3T3 cell line (Fig. 1) and the DRG primary cells (Fig. 2). For the various DHP derivatives with enhancing activity, the concentration–response curves in the presence and absence of capsaicin clearly demonstrate that in this assay the enhancers have no observable activity when added alone (data not shown). Rather, they augment the maximal activity obtained with the vanilloid agonist.

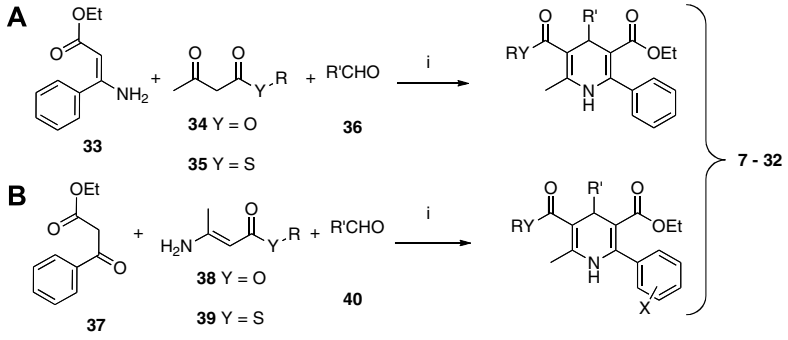
The SAR was probed through a comparison of analogues in which the 3- and 5-ester groups and the substitution at the 1, 2, 4, and 6 positions, including aryl substitutions of the 6-phenyl group, were varied. Some of the members of the DHP library proved to be enhancers of the action of the TRPV1 agonist capsaicin, which led to further synthesis based on chemical leads. DHP derivatives that were substituted at the 4-position with phenyl rings and at the 6-position with small alkyl groups typically did not show enhancing activity (data not shown), and some of the DHP derivatives that were substituted at the 6-position with

Table 1
Activities of the 1,4-dihydropyridine derivatives as enhancers at the TRPV1 receptor ectopically expressed in NIH3T3 cells



Compound	R^2	R^3	R^4	R^6	EC_{50} ± SEM (μM)	Hill Slope ± SEM	E_{max}[a] ± SEM (%)	n[b]
3-Esters								
7[d]	CH_3	CH_3CH_2O	CH_3CH_2	Ph	—[c]	—	383 ± 39	3
8[d]	CH_3	CH_3CH_2O	$(CH_3O)_2CH$	Ph	—	—	114 ± 24	2
9	CH_3	CH_3CH_2O	CH_3CH_2	3-Cl–Ph	17.1 ± 1.2	1.45 ± 0.28	347 ± 24	3
10	CH_3	CH_3CH_2O	CH_3CH_2	3-F–Ph	26.7 ± 1.1	1.98 ± 0.30	403 ± 21	3
11	CH_3	CH_3CH_2O	CH_3CH_2	4-F–Ph	29.2 ± 1.2	1.86 ± 0.42	372 ± 33	3
3-Thioesters								
12[d]	CH_3	CH_3CH_2S	CH_3CH_2	Ph	28.1 ± 1.0	1.55 ± 0.17	435 ± 11	3
13	CH_3	CH_3CH_2S	CH_3CH_2	2-F–Ph	—	—	198 ± 2	2
14[e]	CH_3	CH_3CH_2S	CH_3CH_2	3-F–Ph	21.8 ± 1.2	1.59 ± 0.33	446 ± 39	3
15	CH_3	CH_3CH_2S	CH_3CH_2	4-F–Ph	28.4 ± 1.2	1.82 ± 0.33	409 ± 32	3
16	CH_3	CH_3CH_2S	CH_3CH_2	4-CN–Ph	—	—	129 ± 2	2
17	CH_3	CH_3CH_2S	CH_3CH_2	Cyclohexyl	—	—	87 ± 8	2
18[d]	CH_3	CH_3CH_2S	$CH_3CH_2CH_2$	Ph	22.4 ± 1.2	1.38 ± 0.23	364 ± 16	3
19	CH_3	CH_3CH_2S	$CH_3CH_2CH_2$	3-Cl–Ph	—	—	177 ± 19	2
20	CH_3	CH_3CH_2S	^cPr	4-F–Ph	—	—	175 ± 4	2
21[d]	CH_3CH_2	CH_3CH_2S	CH_3CH_2	Ph	—	—	257 ± 17	3
22[d]	$CH_3CH_2CH_2$	CH_3CH_2S	CH_3CH_2	Ph	—	—	148 ± 3	2
23[d,e]	CH_3	$CH_3OCH_2CH_2S$	CH_3CH_2	Ph	21.3 ± 1.1	1.79 ± 0.17	626 ± 25	3
24	CH_3	$(CH_3)_2CHS$	CH_3CH_2	2-F–Ph	—	—	124 ± 4	2
25	CH_3	$PhCH_2S$	CH_3CH_2	2-F–Ph	—	—	123 ± 1	2
26	CH_3	$PhCH_2CH_2S$	$CH_3CH_2CH_2$	Ph	27.6 ± 1.5	1.14 ± 0.32	360 ± 35	3
27	CH_3	$PhCH_2CH_2S$	CH_3CH_2	2-F–Ph	—	—	117 ± 4	2
28	CH_3	$PhCH_2CH_2S$	CH_3CH_2	3-Cl–Ph	—	—	194 ± 29	2
29	CH_3	$PhCH_2CH_2S$	CH_3CH_2	4-F–Ph	51.6 ± 1.6	1.18 ± 0.25	232 ± 7	3
30	CH_3	$PhCH_2CH_2S$	CH_3CH_2	4-NO_2–Ph	—	—	101 ± 3	2
31	CH_3	$PhCH_2S$	CH_3CH_2	4-NO_2–Ph	—	—	98 ± 3	2
32	CH_3	CH_3CH_2S	CH_3CH_2	2-Naphthyl	—	—	116 ± 19	2

[a] E_{max}, TRPV1 activation at $[Enhancer]_{max}$ (100 μM), expressed as % of control (2 μM capsaicin).
[b] 'n' represents the number of separate assay plate runs, with compounds in duplicate wells per assay.
[c] For compounds where EC_{50} and Hill Slope could not be calculated (i.e., the data could not be curve fitted), only the E_{max} is shown.
[d] K_i value in binding to the human A_3 adenosine receptor, in μM (corresponding compound number in Ref. 27): **7**, 2.27 (**9**); **8**, 15.3 (**14**); **12**, 2.01 (**10**); **18**, 2.17 (**12**); **21**, 0.907 (**21**); **22**, 2.09 (**22**); **23**, 4.58 (**11**).
[e] Compound **23** is MRS1477; **14** is MRS3625.

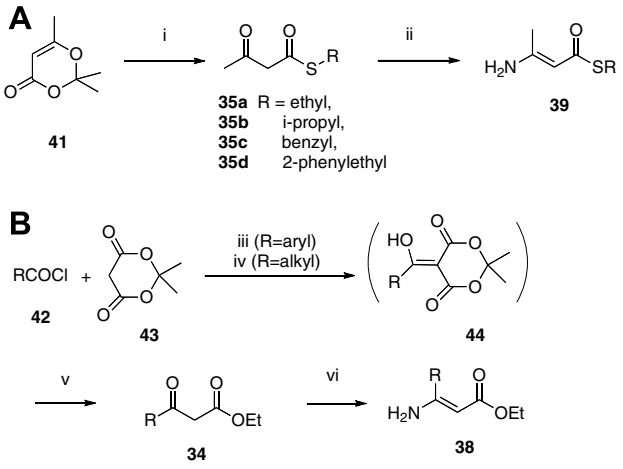


Scheme 1. Synthesis of 1,4-DHP derivatives. Reagents and conditions: (i) EtOH, 24–48 h, 90–95 °C.

phenyl rings and with small alkyl groups at the 2- and 4-positions (**7**–**32**, Table 1) enhanced the effects of capsaicin.

The potency (EC_{50}) and fold of augmentation of E_{max} elicited by 2 μM (ED_{100}) capsaicin in the TRPV1ε-NIH3T3 cell line are shown in Table 1. The EC_{50} values of the enhancers were typically in the range of 15 to 30 μM. The most potent enhancer, using the stably expressing TRPV1ε-NIH3T3 cell line as the basis for the assay, was the 6-(3-chlorophenyl) derivative **9** with an EC_{50} of 17.1 ± 1.2 μM. This compound did not achieve as high an efficacy (augmentation of E_{max}) as compound **23**, which contained 3-methyloxyethylthio ester and 6-phenyl groups. Thus, in the TRPV1ε-NIH3T3 cells, the most efficacious compound was **23**, which had an EC_{50} of 21.3 ± 1.1 μM (Fig. 1), and produced a 5.3-fold increase in calcium influx.

In both the 3-ester and 3-thioester series of DHP derivatives, an ethyl ester was always present at the 5 position. In the ester series, an unsubstituted 6-phenyl group, as in **18**, was associated with relatively high efficacy as an enhancer. Branching at the 4-position as in acetal **8** was not tolerated. Halo substitution of the 6-phenyl ring in **9**–**11** enhanced potency as enhancers, with the pattern 3-Cl > 3-



Scheme 2. Synthesis of β-ketoester **34**, β-ketothioester **35**, and β-amino-α,β-unsaturated ester intermediates **38** and **39**. Reagents and conditions: (i) RSH, toluene, 100–110 °C; (ii) ammonium acetate, EtOH, 95–100 °C; (iii) DMAP, CH_2Cl_2, 0–25 °C, 2–4 h; (iv) pyridine, CH_2Cl_2, 0–25 °C, 2–4 h; (v) EtOH, toluene, reflux, 18 h; (vi) ammonium acetate, EtOH, 95 °C, sealed tube.

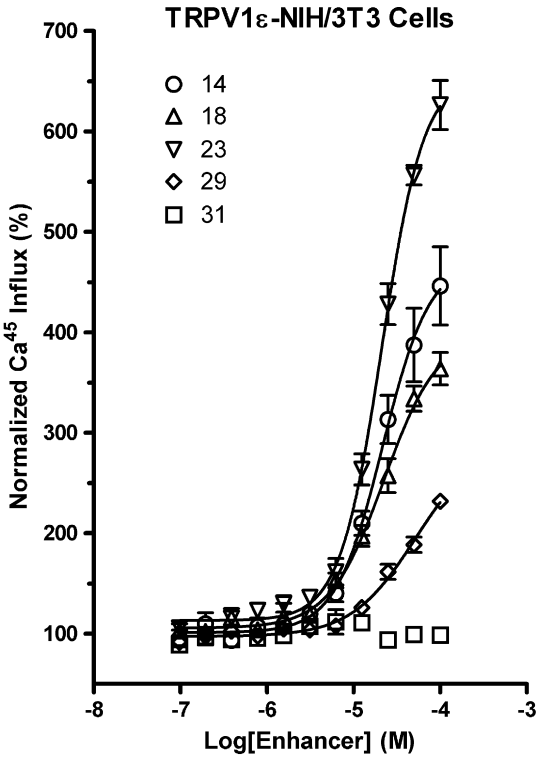


Figure 1. Representative dose–response kinetics of DHPs in the presence of a vanilloid evaluated in NIH3T3 cells ectopically expressing TRPV1. In the presence of 2 μM capsaicin, strong, dose-dependent effects of some DHPs, for example, compounds **14**, **18**, or **23**, were observed. Other DHPs were slightly active in the dose range evaluated (e.g., **29**), while some were neutral even in the presence of capsaicin (e.g., **31**). The *y*-axis is normalized to the effect of capsaicin alone.

F or 4-F. Substitution of the 6-phenyl ring with fluoro **13**–**15** or cyano **16** did not enhance potency over that of unsubstituted 6-phenyl **12**. 2-Fluoro and 4-cyano substitution of the 6-phenyl moiety greatly reduced the enhancing activity. The aromatic character of the 6-aryl group was essential for enhancer activity, since the 6-cyclohexyl analogue **17** was inactive.

Homologation of the 4-ethyl group in **18** did not favor increased potency or efficacy, in comparison to **12**. Compound **19**, the 6-(3-

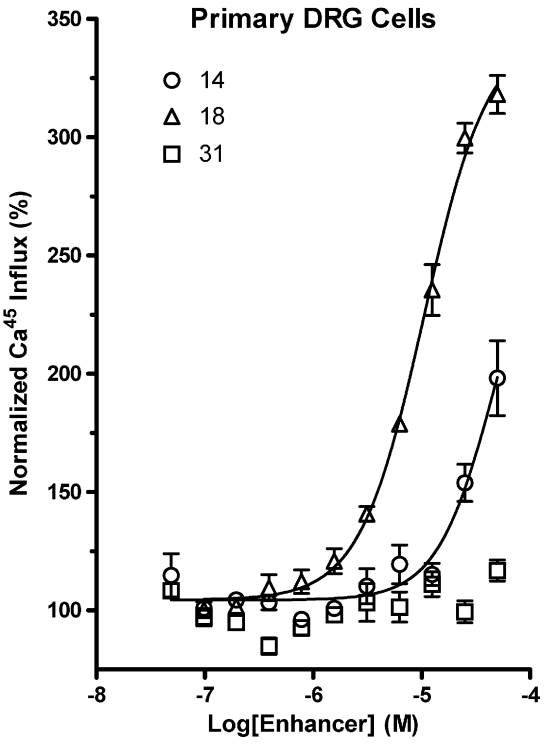


Figure 2. Dose–response kinetics of DHPs evaluated in primary DRG cells. Active DHPs retained similar kinetics in primary DRGs (e.g., **18**), while inactive compounds were also inactive in DRGs (e.g., **31**). Due to DMSO solvent sensitivity, the maximum DHP concentration evaluated in DRGs was 50 μM. The *y*-axis is normalized to the effect of capsaicin alone.

chlorophenyl) analogue of **18**, was greatly reduced in percent activation, while the 4-cyclopropyl-6-(3-chlorophenyl) analogue **20** was less attenuated in activity. Homologation of the 2-substitution from methyl appeared to decrease maximal enhancement (ethyl **21** and propyl **22** analogues in comparison to methyl **12**).

At the 3-thioester position, extension of the ester moiety was well tolerated at TRPV1, as in the highly potent and efficacious 2-methoxyethyl analogue **23**. 3-Phenylalkylthio substitution, such as a 3-(2-phenylethylthio) ester **26**, was tolerated. However, the 3-benzylthio substituted analogue **25** was greatly reduced in maximal enhancement. Substitution of the 6-phenyl ring in the 3-(2-phenylethylthio) series in compounds **27**–**30** produced either a reduction of potency in the 4-fluoro analogue **29** or a marked reduction of efficacy in **27** and **30**.

In the primary DRG cells (Table 2), a 3-ethylthio-4-propyl-6-phenyl derivative **18** was highly efficacious and showed comparatively greater potency than in the TRPV1ε-NIH3T3 cell line, with an EC_{50} of 10.5 ± 1.1 μM (Fig. 2). The enhancers increased the maximal effect on $^{45}Ca^{2+}$-uptake in response to vanilloid agonists in the DRG

Table 2
Activities of DHPs evaluated in primary dorsal root ganglion cells[a]

Compound	EC_{50} ± SEM (μM)	Hill Slope ± SEM	E_{max} ± SEM, % of control
9	17.0 ± 1.2	2.24 ± 0.53	259 ± 6
10	20.5 ± 1.1	2.64 ± 0.59	206 ± 7
11	15.7 ± 1.3	2.01 ± 0.77	175 ± 9
14	—	—	198 ± 16
18	10.5 ± 1.1	1.47 ± 0.16	318 ± 8
26	—	—	231 ± 13
29	—	—	148 ± 10
30	—	—	117 ± 5

[a] Results from a single assay run in quadruplicate wells. The DMSO concentration during the assay did not exceed 0.2%.

cells by typically 2- to 3-fold, and compound **18** produced a 3.2-fold increase in the calcium influx.

Various other DHP derivatives tested did not enhance the action of capsaicin, and their pharmacological properties will be described later. Thus, the L-type calcium channel blocker nicardipine and the L-type calcium channel activator S(−)-Bay K 8644 were not enhancers in either cell model described above.

In order to confirm the enhancer effect using a more physiologically informative method, we examined **23** using live cell imaging of primary DRG neurons, a subpopulation of which expresses TRPV1. Because repetitive exposures to capsaicin can produce acute desensitization,[2] the comparison was made between cultures exposed to capsaicin only and separate cultures exposed to a combination of capsaicin and **23**. This experiment was designed using a low concentration (200 nM) of capsaicin in order to observe enhancement, yet avoid calcium cytotoxicity. Figure 3A shows the typical response to capsaicin using the dye Fura-4F AM and ratiometric imaging, the left panel at baseline, the right panel after capsaicin alone. Progressively higher intracellular calcium $[Ca^{2+}]_i$ is depicted as a transition from blue to green to red. Comparison of the action of capsaicin to capsaicin + enhancer is depicted graphically in Figure 3B. The traces are the average of 3 separate experiments comprising recordings from a total of 167 neurons. Co-administration of **23** with capsaicin significantly increased the peak Ca^{2+} influx by 13% compared to capsaicin alone. The peak ratio values were 1.518 ± 0.001 for capsaicin alone and 1.701 ± 0.001 in the presence of 10 μM **23**. While this number is lower than the augmentation of E_{max} obtained with the $^{45}Ca^{2+}$-uptake screen, several variables differ between the two methods, notably the dose of capsaicin is lower (200 nM vs 500 nM) and the duration of exposure is much shorter (30 s vs 10 min). The culture contains capsaicin-insensitive neurons as well and these non-TRPV1-expressing neurons do not respond to either capsaicin or **23** when morphology or changes in $[Ca^{2+}]_i$ are used as the endpoints.

DHP derivatives have been adapted through chemical modification to bind to various receptor sites, in addition to the classical L-type calcium channels. Among the receptors to which other DHP derivatives have been shown to bind are the adenosine receptors, and certain DHP derivatives have been engineered to bind with nanomolar affinity to the A_3 adenosine receptor. Among the derivatives investigated here, several were shown to bind to the human (but not rat) A_3 adenosine receptor with a high micromolar affinity.

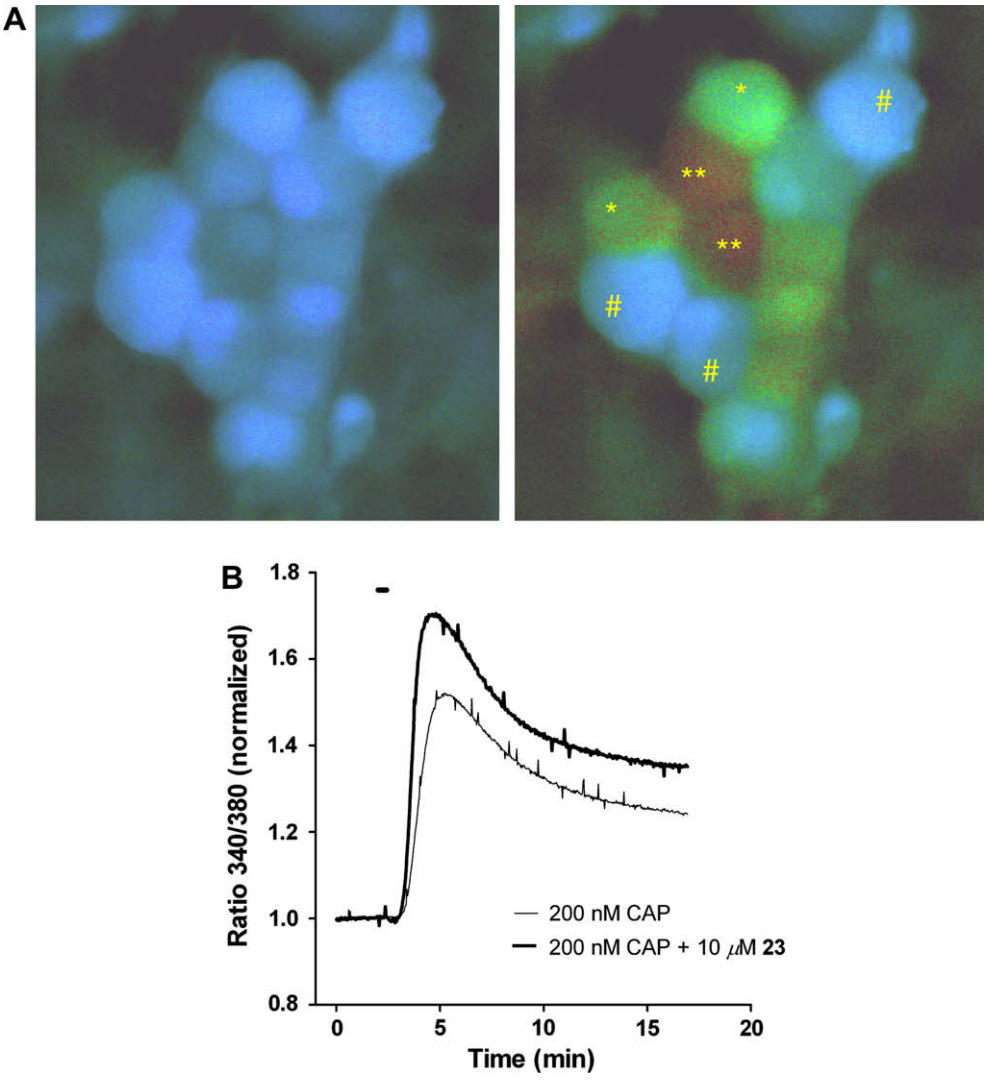


Figure 3. Ratiometric intracellular calcium measurements of primary embryonic DRG neurons in mixed cultures. (A) False color representation of intracellular calcium before (left) and after (right) application of capsaicin using MetaFluor software. Cells marked with '#' are capsaicin-insensitive (i.e., TRPV1-negative) DRG neurons. '*' and '**' mark cells that are, respectively, sensitive or highly sensitive to capsaicin. (B) Cells in a closed perfusion system were exposed to either 200 nM capsaicin or 200 nM capsaicin in the presence of 10 μM **23**. Baseline was recorded for 2 min, and ratios were normalized to the baseline. Each trace is the mean of activated (i.e., capsaicin sensitive) DRG cells from three individual experiments (98 DRGs total for 200 nM capsaicin; 69 DRGs for 200 nM capsaicin + 10 μM **23**). Drugs were added at 2 min for 30 s (indicated by horizontal bar). In cells concomitantly exposed to capsaicin and **23**, intracellular calcium influx increases compared to capsaicin alone.

For example, the L-type calcium channel blocker nicardipine, which was not an enhancer at TRPV1, displayed a K_i value at the A_3 receptor of 3.25 μM.[30] Thus, the TRPV1-enhancing activity does not correlate with A_3 adenosine receptor antagonism. Furthermore, the potent A_3 receptor antagonist 1,4-dihydro-2-methyl-6-phenyl-4-(phenylethynyl)-3,5-pyridinedicarboxylic acid, 3-ethyl 5-(phenylmethyl) ester[33] (MRS1191) had no enhancing effect. Therefore, potency as an A_3 adenosine receptor antagonist or as an L-type calcium channel antagonist did not correlate with TRPV1 enhancement.

3. Discussion

The present set of observations delineates a new agonist-enhancing activity present in TRPV1 that can be elicited by DHP compounds. We refer to these agents as enhancers since, at least in the in vitro assays used (the ectopically expressing TRPV1ε-NIH3T3 cell line and primary DRG cultures), no intrinsic activity of the enhancers alone was detected. The enhancing compounds acted only in the presence of a vanilloid agonist. As such, these compounds present a novel approach to selective manipulation of TRPV1 function. The enhancers operate in an activity-dependent fashion and, therefore, are hypothesized to only target nerve endings that are actively engaged in nociceptive transduction. The agonist-dependent actions of the enhancers is consistent with recent observations showing modulation of the TRPV1 pore selectivity filter by vanilloid agonist activation.[28] It is possible that the DHP enhancers increase the elasticity of the pore even further when the pore has been opened initially by vanilloid agonists and allow not only cations, but other large molecules to enter the nociceptive ending.[37,38]

The elucidation of the biological mechanism of the enhancers will be the topic of future studies. The present work explores the initial SAR pattern following the observation of this novel action, and allows the identification of one or more enhancer molecules that could be used as pharmacological tools. The structural requirements for TRPV1 enhancement by DHPs are distinct from the structural requirements of DHPs to interact with several other protein targets,[30] and also delineated from the structure of the DHP derivative nifedipine (a 4-aryl-6-methyl-DHP), which is reported to inhibit TRPV1.[11] Compound **23** was the most efficacious enhancer in the transfected cells, and this enhancement was confirmed using calcium imaging of primary DRG neuronal cultures.

We have recently shown that selective analgesia with vanilloid agonists such as resiniferatoxin (RTX) can be achieved through anatomically localized administration, either by peripheral injection,[21] perineural application,[20,39] direct intraganglionic injection,[17,52] or injection into the spinal CSF[17,18] for treatment of pain unilaterally or at individual or multiple spinal segments. All of these applications depend on excessive opening of TRPV1 to produce a local calcium overload.[19] Potential therapeutic actions of an enhancer may operate through the same calcium overload mechanism. Moreover, the enhancer would be efficacious only at nerve endings where TRPV1 is actively occupied by an endovanilloid, rather than at all susceptible nociceptive nerve endings within the volume of distribution a traditional agonist might reach. Thus, selective, localized actions may be achieved, but without the need for local administration and without blockade or inhibition of the important protective aspects of pain elsewhere in the body,[40] as might be encountered with an antagonist. Consideration of the potential pharmacological actions of the enhancers raises several interesting questions. One is pharmacodynamic: would administration of an enhancer block pain, or would it enhance pain before it is blocked? We currently are investigating these issues using inflammatory hyperalgesia[41] and operant methods to assess pain sensations behaviorally.[42] A second question is whether there is an endogenous ligand at this site. TRPV1 is known to be sensitive to a variety of modulators such as pH, endovanilloids, lipid derivatives and a variety of natural products and venoms (as reviewed[27]), and it is possible that some endogenous compound acts as a TRPV1 enhancer. In such a case, the action of an enhancer of the endogenous activator, such as the present DHP derivatives, might be more event-specific and tissue-specific than a directly acting ligand. Thus, in theory the novel enhancers might be useful clinically either alone or in conjunction with exogenously administered vanilloid activators. Lastly, there is the question of where on TRPV1 the enhancers exert their action; in this regard it is also prudent to consider the possibility that the enhancers might be acting on some other, occult cellular process that produces positive feedback on TRPV1 once it is in the vanilloid agonist-activated state.

Recently, an amino acid sequence required for vanilloid binding was tentatively defined between transmembrane domains 3 and 4,[1] using both domain swapping and mutagenesis. Additional studies emphasize the importance of both the N- and C-terminal regions of TRPV1 as determinants of receptor function.[14,43] The detailed three-dimensional structure of TRPV1 is currently unknown although portions of the structure and the functional roles that these regions play (e.g., to sensitize or promote or inhibit desensitization) are being elucidated.[14,43]

A variety of sites exist that can positively modulate function and potentially interact with DHP enhancers. A brief consideration of the quaternary structure and post-translational modifications is also important. Immunoprecipitation experiments suggest that TRPV1 exists as a homotetramer and, in this regard, exhibits similarities to a wide variety of other cation channels with four individual monomeric subunits or four linked repeating domains, with each monomer or domain having six transmembrane regions with a pore-loop and an ion-conducting pore in the center, faced by transmembrane domain 6.[44] Theoretically, pharmacological enhancement could be exerted through modulation of the quaternary structure. In addition, multiple phosphorylation sites exist on TRPV1 for protein kinase A, protein kinase C and the proline directed kinase CDK5.[45–47] Post-translational phosphorylation of all of these sites can produce sensitization of the receptor and an enhanced sensitivity to noxious chemical and inflammatory agents creating a very broad combinatorial palette from which to produce, maintain, and modulate nociceptive transmission.

Distinct structural features of the DHP pharmacophore are required to produce enhancer activity. In future studies, the effects in three-dimensional space of alterations of charge distribution, hydrophobicity, rotational constraints, π electron clouds, aromaticity, planarity, etc. can be modeled in order to optimize biological activity. Also, it would be desirable to reduce the hydrophobicity of this DHP series. For example, the clogp values of **9**, **14**, and **23** are 5.75, 5.55, and 4.71, respectively. Solubility could be a potentially major factor that limits activity.

The idea of selective suppression of pain is an attractive concept. NSAIDs fulfill this role to some degree, but have the drawback of inhibiting the cyclooxygenases everywhere in addition to sites of tissue injury, and the consequences can produce serious, even fatal side effects in the case of cycloxygenase II inhibitors.[48] The potential for selectivity based on TRPV1 activity status was recently examined by co-administration of capsaicin, to open the TRPV1 channel, with the quaternary local anesthetic QX-314 (*N*-(2,6-dimethylphenylcarbamoylmethyl)triethylammonium bromide), which enters the cell via the open TRPV1 channel.[38] While selectivity for pain-sensing fiber types may be achieved, a major drawback to this approach is the delay in establishing inhibition (~5 min), during which time the algesic actions of capsaicin were behaviorally evident. These can be quite intense: human behavioral and functional brain imaging studies[3,49,50] following intradermal capsaicin administration demonstrate that it potently activates the brain pain network within this time frame and trig-

gers central sensitization. However, in active pain states, an enhancer may obviate the use of a vanilloid agonist.

In conclusion, the finding that a well-described compound class, that is, the DHPs, can modulate TRPV1 channels in a positive fashion represents a useful lead for future pain therapeutics. This study has focused on the SAR of the novel DHP enhancers, which differs considerably from their known action at other target sites, such as L-type calcium channels and adenosine receptors. In future studies it will be desirable to further separate the activity of the TRPV1 enhancers from activity at the purine receptors. The present study revealed that 6-phenyl-, 3-*O*/*S*-ester-, and small alkyl-substitutions at the 2 and 4 positions of the DHP pharmacophore are required for the maximal-enhancing effect. The development of analogues that are more potent and selective in their action at the TRPV1 channel and the validation of this approach in pain models, including in vivo studies, will be required.

4. Experimental procedures

4.1. Chemical synthesis

4.1.1. Materials and instrumentation

Ethyl-3-aminocrotonate, aldehydes, ethylacetoacetate, 2,2,6-trimethyl-4H-1,3-dioxin-4-one, all acid chlorides, 2,2-dimethyl-1,3-dioxane-4,6-dione, ethanethiol, propanethiol, and other general reagents were purchased from Aldrich (Milwaukee, WI), and synthetic intermediates were of analytical grade. Compounds **7**, **8**, **12**, **18**, and **21**–**23** were prepared as reported by Li, et al.[34] All other materials were obtained from commercial sources. Proton nuclear magnetic resonance spectroscopy was performed on a Varian GEMINI-300 spectrometer, and all spectra were obtained in $CDCl_3$. Chemical shifts (δ) relative to TMS are given. Chemical ionization (CI) mass spectrometry was performed with a Finnigan 4600 mass spectrometer, and electron-impact (EI) mass spectrometry with a VG7070F mass spectrometer at 6 kV. High-resolution FAB (fast atom bombardment) mass spectra were taken with a JEOL SX102 spectrometer using nitrobenzoic acid as matrix. clogp values were calculated using ChemDraw Ultra, version 11.0.

4.1.2. General procedure for preparation of substituted 1,4-DHP derivatives

The synthesis of DHP derivatives was carried out by known methods.[29,34] The methods shown in Schemes 1 and 2 were used depending on the substitution on C-6 position of DHP. Equimolar amounts (0.5–1.0 mmol) of the appropriate β-enaminoester (**33**, **38**, or **58**), aldehyde (**36** or **40**), and β-ketoester (**34**, **35**, or **37**) were dissolved in 2–5 mL of absolute ethanol. The mixture was sealed in a Pyrex tube and heated, with stirring, to 80–90 °C for 18–36 h. After the mixture was cooled to room temperature, the solvent was evaporated, and the residue was purified using preparative TLC (silica 60, Aldrich; petroleum ether/ethyl acetate (5:1–10:1)). The products were shown to be homogeneous by analytical TLC and were stored at −20 °C.

4.1.2.1. 3,5-Diethyl-2-methyl-4-ethyl-6-(3-chlorophenyl)-1,4-(+)-dihydropyridine-3,5-dicarboxylate (9). $C_{20}H_{24}ClNO_4$ ^1H NMR ($CDCl_3$) 7.17–7.41 (m, 4H), 5.57 (s, 1H), 4.15–4.24 (m, 2H), 4.03 (t, J = 5.7 Hz, 1H), 3.94 (q, J = 7.1 Hz, 2H), 2.32 (s, 3H), 1.47–1.54 (m, 2H), 1.31 (t, J = 7.2 Hz, 3H), 0.86–0.97 (m, 6H) 348.2 (M-ethyl, base). Anal. ($C_{20}H_{24}ClNO_4$) C, H, N: calcd 63.57, 6.40, 3.71; found 63.29, 6.59, 3.53.

4.1.2.2. 3,5-Diethyl-2-methyl-4-ethyl-6-(3-fluorophenyl)-1,4-(+)-dihydropyridine-3,5-dicarboxylate (10). $C_{20}H_{24}FNO_4$ ^1H NMR ($CDCl_3$) 6.95–7.41 (m, 4H), 5.58 (s, 1H), 4.18–4.24 (m, 2H), 4.03 (t, J = 5.7 Hz, 1H), 3.93 (q, J = 7.1 Hz, 2H), 2.32 (s, 3H), 1.47–1.54 (m, 2H), 1.32 (t, J = 7.2 Hz, 3H), 0.86–0.97 (m, 6H) MS: 332.2 (M-ethyl, base). Anal. ($C_{20}H_{24}FNO_4$) C, H, N: calcd 66.47, 6.69, 3.88; found 66.39, 6.69, 3.91.

4.1.2.3. 3,5-Diethyl-2-methyl-4-ethyl-6-(4-fluorophenyl)-1,4-(+)-dihydropyridine-3,5-dicarboxylate (11). $C_{20}H_{24}FNO_4$ ^1H NMR ($CDCl_3$) 7.26–7.31 (m, 2H), 7.05–7.11 (m, 2H), 5.57 (s, 1H), 4.18–4.24 (m, 2H), 4.02 (t, J = 5.6 Hz, 1H), 3.90–3.97 (m, 2H), 2.32 (s, 3H), 1.48–1.54 (m, 2H), 1.31 (t, J = 7.2 Hz, 3H), 0.96 (t, J = 7.2 Hz, 3H), 0.88 (t, J = 7.5 Hz, 3H) MS: 362.2 (M+H), 332.2 (M-ethyl, base) Anal. ($C_{20}H_{24}FNO_4$) C, H, N: calcd 66.47, 6.69, 3.88; found 66.41, 6.93, 3.76.

4.1.2.4. 3-Ethylthio-5-ethyl-2-methyl-4-ethyl-6-(2-fluorophenyl)-1,4-(+)-dihydropyridine-3,5-dicarboxylate (13). $C_{20}H_{24}FNO_3S$ ^1H NMR ($CDCl_3$) 7.31–7.34 (m, 1H), 7.01–7.19 (m, 3H), 5.62 (s, 1H), 4.16 (t, J = 5.4 Hz, 1H), 3.87–3.94 (m, 2H), 2.87 (q, J = 7.5 Hz, 2H), 2.25 (s, 3H), 1.47–1.57 (m, 2H), 1.22 (t, J = 7.4 Hz, 3H), 0.82–0.92 (m, 6H) MS: 348.2 (M-ethyl, base). Anal. ($C_{20}H_{24}FNO_3S$) C, H, N: calcd 63.64, 6.41, 3.71; found 63.47, 6.39, 3.64.

4.1.2.5. 3-Ethylthio-5-ethyl-2-methyl-4-ethyl-6-(3-fluorophenyl)-1,4-(+)-dihydropyridine-3,5-dicarboxylate (14). $C_{20}H_{24}FNO_3S$ ^1H NMR ($CDCl_3$) 7.34–7.38 (m, 1H), 7.01–7.12 (m, 3H), 5.72 (s, 1H), 4.18 (t, J = 5.7 Hz, 1H), 3.93–4.01 (m, 2H), 2.94 (q, J = 7.2 Hz, 2H), 2.33 (s, 3H), 1.54–1.60 (m, 2H), 1.26–1.32 (m, 3H), 0.89–0.99 (m, 6H). HRMS calcd for $C_{20}H_{25}FNO_3S$ 378.1539; found 378.1525.

4.1.2.6. 3-Thioethyl-5-ethyl-2-methyl-4-ethyl-6-(4-fluorophenyl)-1,4-(+)-dihydropyridine-3,5-dicarboxylate (15). $C_{20}H_{24}FNO_3S$ ^1H NMR ($CDCl_3$) 7.27–7.32 (m, 2H), 7.06–7.12 (m, 2H), 5.69 (s, 1H), 4.18 (t, J = 6.0 Hz, 1H), 3.93–4.00 (m, 2H), 2.94 (q, J = 7.4 Hz, 2H), 2.33 (s, 3H), 1.53–1.60 (m, 2H), 1.29 (t, J = 7.2 Hz, 3H), 0.89–1.02 (m, 6H) MS: 348.2 (M-Et) base, 316.2 (M-EtS). Anal. ($C_{20}H_{24}FNO_3S$) C, H, N: calcd 63.64, 6.41, 3.71; found 63.49, 6.43, 3.58.

4.1.2.7. 3-Ethylthio-5-ethyl-2-methyl-4-ethyl-6-(4-cyanophenyl)-1,4-(+)-dihydropyridine-3,5-dicarboxylate (16). $C_{21}H_{24}N_2O_3S$ ^1H NMR ($CDCl_3$) 7.69 (d, J = 8.1 Hz, 2H), 7.43 (d, J = 8.1 Hz, 2H), 5.71 (s, 1H), 4.19 (t, J = 5.9 Hz, 1H), 3.94–3.99 (m, 2H), 2.95 (q, J = 7.5 Hz, 2H), 2.32 (s, 3H), 1.56–1.61 (m, 2H), 1.29 (t, J = 7.4 Hz, 3H), 0.89–1.02 (m, 6H) MS: 355.2 (M-Et), 323.2 (M-EtS). HRMS calcd for $C_{21}H_{24}N_2O_3NaS$ 407.1403; found 407.1385.

4.1.2.8. 3-Ethylthio-5-ethyl-2-methyl-4-ethyl-6-cyclohexyl-1,4-(+)-dihydropyridine-3,5-dicarboxylate (17). $C_{20}H_{31}NO_3S$ ^1H NMR ($CDCl_3$) 5.81 (s, 1H), 4.08–4.27 (m, 3H), 3.72–3.86 (m, 1H), 2.90 (q, J = 7.2 Hz, 2H), 2.30 (s, 3H), 1.78–1.81 (m, 4H), 1.15–1.43 (m, 14H), 0.77 (t, J = 7.7 Hz, 3H) 336.3 (M-ethyl, base). HRMS calcd for $C_{20}H_{31}NO_3SNa$ 388.1922; found 388.1914.

4.1.2.9. 3-Ethylthio-5-ethyl-2-methyl-4-propyl-6-(3-chlorophenyl)-1,4-(+)-dihydropyridine-3,5-dicarboxylate (18). $C_{21}H_{26}ClNO_3S$ ^1H NMR ($CDCl_3$) 7.17–7.41 (m, 4H), 5.74 (s, 1H), 4.19 (t, J = 5.9 Hz, 1H), 3.98 (q, J = 7.2 Hz, 2H), 2.92 (q, J = 7.5 Hz, 2H), 2.32 (s, 3H), 1.26–1.55 (m, 7H), 0.89–1.17 (m, 6H) MS: 364.2 (M-propyl), 346.2 (M-EtS). Anal. ($C_{21}H_{26}ClNO_3S$) C, H, N: calcd 61.83, 6.42, 3.43; found 61.56, 6.47, 3.33.

4.1.2.10. 3-Ethylthio-5-ethyl-2-methyl-4-cyclopropyl-6-(4-fluorophenyl)-1,4-(+)-dihydropyridine-3,5-dicarboxylate (19). $C_{21}H_{24}FNO_3S$ ^1H NMR ($CDCl_3$) 7.28–7.33 (m, 2H), 7.07–7.13 (m, 2H), 5.74 (s, 1H), 3.94–4.03 (m, 3H), 2.95 (q, J = 7.2 Hz, 2H), 2.35 (s, 3H), 1.29 (t, J = 7.2 Hz, 3H), 1.01 (t, J = 7.2 Hz, 3H), 0.25–0.45 (m, 5H) 348.2 (M-cyclopropyl), 328.2 (M-EtS). Anal. ($C_{21}H_{24}FNO_3S$) C, H, N: calcd 64.76, 6.21, 3.60; found 64.85, 6.22, 3.54.

4.1.2.11. 3,5-Diethyl-2-methyl-4-ethyl-6-phenyl-1,4-(+)-dihydropyridine-3,5-dicarboxylate (23). $C_{21}H_{27}NO_4S$ ^1H NMR ($CDCl_3$) 7.37–7.41 (m, 1H), 7.07–7.26 (m, 3H), 5.87 (s, 1H), 4.20 (t, *J* = *5.4* Hz, 1H), 3.91–3.95 (m, 2H), 3.55 (m, 2H), 3.14 (m, 2H), 2.32 (s, 3H), 1.59 (m, 2H), 1.31–1.38 (m, 6H), 0.90–0.95 (m, 6H). Mp 118–119 °C. Anal. ($C_{21}H_{27}NO_4S$) C, H, N: calcd C, 64.75; H, 6.99; N, 3.60; found C, 64.81; H, 7.05; N, 3.43.

4.1.2.12. 3-Isopropylthio-5-ethyl-2-methyl-4-ethyl-6-(2-fluorophenyl)-1,4-(+)-dihydropyridine-3,5-dicarboxylate (24). $C_{21}H_{26}FNO_3S$ ^1H NMR ($CDCl_3$) 7.37–7.41 (m, 1H), 7.07–7.26 (m, 3H), 5.71 (s, 1H), 4.19 (t, *J* = *5.4* Hz, 1H), 3.94–4.00 (m, 2H), 3.65–3.70 (m, 1H), 2.31 (s, 3H), 1.56–1.61 (m, 2H), 1.31–1.38 (m, 6H), 0.89–0.99 (m, 6H). HRMS calcd for $C_{21}H_{26}FNO_3SNa$ 414.1515; found 414.1516.

4.1.2.13. 3-Thiobenzyl-5-ethyl-2-methyl-4-ethyl-6-(2-fluorophenyl)-1,4-(+)-dihydropyridine-3,5-dicarboxylate (25). $C_{25}H_{26}FNO_3S$ ^1H NMR ($CDCl_3$) 7.07–7.41 (m, 9H), 5.74 (s, 1H), 4.17–4.23 (m, 3H), 3.92–4.00 (m, 2H), 2.34 (s, 3H), 1.20–1.22 (m, 2H), 0.87–0.98 (m, 6H). MS: 440.1 (M+H), 462.1 (M+Na) HRMS calcd for $C_{25}H_{26}FNO_3SNa$ 462.1515; found 462.1514.

4.1.2.14. 3-(2-Phenylethylthio)-5-ethyl-2-methyl-4-propyl-6-phenyl-1,4-(+)-dihydropyridine-3,5-dicarboxylate (26). $C_{27}H_{31}NO_3S$ ^1H NMR ($CDCl_3$) 7.21–7.41 (m, 10H), 5.84 (s, 1H), 4.17–4.22 (m, 1H), 3.89–3.97 (m, 2H), 3.13–3.19 (m, 2H), 2.88–2.93 (t, *J* = 7.2 Hz, 2H), 2.33 (s, 3H), 1.31–1.58 (s, 4H), 0.88–0.97 (m, 6H). HRMS calcd for $C_{27}H_{31}NO_3NaS$ 472.1922; found 472.1932.

4.1.2.15. 3-(2-Phenylethylthio)-5-ethyl-2-methyl-4-ethyl-6-(2-fluorophenyl)-1,4-(+)-dihydropyridine-3,5-dicarboxylate (27). $C_{26}H_{28}FNO_3S$ ^1H NMR ($CDCl_3$) 8.27 (d, *J* = 8.7 Hz, 2H), 7.47–7.50 (m, 2H), 7.23–7.29 (m, 5H), 5.64 (s, 1H), 4.21 (t, *J* = 5.7 Hz, 1H), 3.93–4.14 (m, 2H), 3.17–3.22 (m, 2H), 2.83–2.94 (m, 2H), 2.34 (s, 3H), 1.53–1.60 (m, 2H), 0.90–1.03 (m, 6H) HRMS calcd for $C_{26}H_{28}FNO_3SNa$ 476.1672; found 476.1685.

4.1.2.16. 3-(2-Phenylethylthio)-5-ethyl-2-methyl-4-ethyl-6-(3-chlorophenyl)-1,4-(+)-dihydropyridine-3,5-dicarboxylate (28). $C_{26}H_{28}ClNO_3S$ ^1H NMR ($CDCl_3$) 7.17–7.41 (m, 9H), 5.75 (s, 1H), 4.18 (t, *J* = 5.4 Hz, 1H), 3.96 (q, *J* = 7.2 Hz, 2H), 3.13–3.19 (m, 2H), 2.87–2.93 (m, 2H), 2.33 (s, 3H), 1.55–1.59 (m, 2H), 0.88–0.98 (m, 6H). HRMS calcd for $C_{26}H_{29}ClNO_3S$ 470.1557; found 470.1573.

4.1.2.17. 3-(2-Phenylethylthio)-5-ethyl-2-methyl-4-ethyl-6-(4-fluorophenyl)-1,4-(+)-dihydropyridine-3,5-dicarboxylate (29). $C_{26}H_{28}FNO_3S$ ^1H NMR ($CDCl_3$) 7.06–7.33 (m, 9H), 5.71 (s, 1H), 4.18 (t, *J* = 5.7 Hz, 1H), 3.93–4.18 (m, 2H), 3.14–3.19 (m, 2H), 2.90 (t, *J* = 7.8 Hz, 2H), 2.33 (s, 3H), 1.53–1.60 (m, 2H), 0.99 (t, *J* = 7.1 Hz, 3H), 0.91 (t, *J* = 7.4 Hz, 3H) HRMS calcd for $C_{26}H_{28}FNO_3SNa$ 476.1672; found 476.1671.

4.1.2.18. 3-(2-Phenylethylthio)-5-ethyl-2-methyl-4-ethyl-6-(4-nitrophenyl)-1,4-(+)-dihydropyridine-3,5-dicarboxylate (30). $C_{26}H_{28}N_2O_5S$ ^1H NMR ($CDCl_3$) 8.27 (d, *J* = 8.7 Hz, 2H), 7.49 (d, *J* = 8.7 Hz, 2H), 7.23–7.32 (m, 5H), 5.67 (s, 1H), 4.21 (t, *J* = 5.7 Hz, 1H), 3.96–4.01 (m, 2H), 3.15–3.22 (m, 2H), 2.89–2.94 (m, 2H), 2.23 (s, 3H), 1.56–1.62 (m, 2H), 1.01 (t, *J* = 7.2 Hz, 3H), 0.93 (t, *J* = 7.5 Hz, 3H). HRMS calcd for $C_{26}H_{29}N_2O_5S$ 481.1797; found 481.1789.

4.1.2.19. 3-(2-Benzylthio)-5-ethyl-2-methyl-4-ethyl-6-(4-nitrophenyl)-1,4-(+)-dihydropyridine-3,5-dicarboxylate (31). $C_{25}H_{26}N_2O_5S$ ^1H NMR ($CDCl_3$) 8.26 (d, *J* = 9.0 Hz, 2H), 7.49 (d, *J* = 9.0 Hz, 2H), 7.22–7.37 (m, 5H), 5.69 (s, 1H), 4.15–4.21 (m, 3H), 3.94–3.99 (m, 2H), 2.35 (s, 3H), 1.28–1.62 (m, 2H), 0.88–1.02 (m, 6H). HRMS calcd for $C_{26}H_{29}N_2O_5S$ 489.1460; found 489.1461.

4.1.2.20. 3-Ethylthio-5-ethyl-2-methyl-4-ethyl-6-(2-naphthyl)-1,4-(+)-dihydropyridine-3,5-dicarboxylate (32). $C_{24}H_{27}NO_3S$ ^1H NMR ($CDCl_3$) 7.77–7.87 (m, 4H), 7.51–7.53 (m, 2H), 7.38–7.42 (dd, *J* = 1.5, 8.4 Hz, 1H), 5.86 (s, 1H), 4.23 (t, *J* = 5.4 Hz, 1H), 3.94 (m, 2H), 2.96 (q, *J* = 7.5 Hz, 2H), 2.36 (s, 3H), 1.58–1.66 (m, 2H), 1.22–1.30 (m, 3H), 0.83–0.99 (m, 6H). HRMS calcd for $C_{24}H_{27}NO_3SNa$ 432.1609; found 489.1596.

4.1.3. General procedure for preparation of β-amino-α,β-unsaturated esters

A β-ketoester (3 mmol) and ammonium acetate (4.5 mmol) were mixed in 5 mL of absolute ethanol and refluxed at 80 °C for 24 h. The solvent was removed and the residue was chromatographed to give the desired compounds in moderate yields.

4.1.4. Synthesis of β-ketoesters and β-ketothioesters

β-Ketoesters **34** and β-ketothioesters **35** were prepared by the reaction of 2,2,6-trimethyl-4H-1,3-dioxin-4-one **41** and an alcohol or a thiol. Equimolar amounts of 2,2,6-trimethyl-4H-1,3-dioxin-4-one and an alcohol or a thiol were heated with a small volume of toluene at 100 °C in a sealed tube overnight. After the mixture was cooled to room temperature, the solvent was removed under reduced pressure and the residue was purified by chromatography in satisfactory yields.

4.1.5. Synthesis of β-ketoesters via Meldrum's acid

The preparation of ethyl-2-fluorobenzoylacetate **34** (R = 2-F-Ph) is provided as an example. 2,2-Dimethyl-1,3-dioxane-4,6-dione (Meldrum's acid, **42**) and 2-fluorobenzoyl chloride were dissolved in dry CH_2Cl_2. At 0 °C, 4-(dimethylamino)pyridine was added. The reaction mixture was stirred at 0 °C for 1 h and then at room temperature for an additional hour. The reaction mixture was washed with 1 N HCl and water and then dried with anhydrous Na_2SO_4. Removal of the solvent left the desired product, which was directly used for the next reaction without purification. This acylated Meldrum's acid and ethanol were mixed in toluene. This mixture was heated at 90 °C in a flask with an effective reflux condenser for 24 h. The solvent and the excess ethanol were removed and the residue was purified by column chromatography to give the desired product.

4.2. Pharmacological methods

4.2.1. Cell culture

A TRPV1ε-NIH3T3 cell line, ectopically expressing the C-terminally ε-epitope tagged vanilloid receptor 1, was cultured as described previously.[13,19] Cells were seeded in DMEM + 10% FBS one day before use and cultured at 35 °C in a humidified incubator. Mixed primary cultures from the DRG of rat embryos were prepared and characterized similar to the procedures employed in earlier Ca^{2+}-transport and imaging studies.[13,19,22] Briefly, cells were harvested from E15 rat embryos, enzymatically dissociated, and then plated onto 96-well microtiter plates pre-coated with poly-D-lysine and mouse laminin. DRGs were maintained in culture at 35 °C for 5–6 days before assaying.

4.2.2. Vanilloid-induced ^{45}Ca-uptake

Assays were performed using an automated high-throughput liquid handling workstation (Biomek FX, Beckman–Coulter) equipped with a 96-well pipeting head. Compounds were initially screened for calcium transport activity using the NIH3T3 cells stably expressing TRPV1ε. The ED_{100} for capsaicin activation of TRPV1 in TRPV1ε-NIH3T3 cells was determined to be ~2 μM at pH 7.5.

Comparisons to the parental NIH3T3 cell line established the specificity of the capsaicin activation.[13,22] Assays with DRG cells were similar, but due to their increased sensitivity, the optimal capsaicin concentration for DRG activation was determined to be ~0.5 μM.

Immediately before assay, cells were adapted to room temperature (20 °C) for 5 min in 25 mM Tris–HCl (pH 7.5) buffered Hank's balanced salt solution, supplemented with 1 mM sodium ascorbate, 10 μM $CaCl_2$ and 0.8 mM $MgCl_2$ (HCM). $^{45}Ca^{2+}$-uptake was performed for 10 min at 20 °C in HCM using 0.5 μCi/well $^{45}Ca^{2+}$ (MP Biomedicals) as radioactive tracer in 100 μL/well final volume. To stop $^{45}Ca^{2+}$-uptake, the assay mix was removed, and the cells were rapidly changed back into HCM, washed five additional times with 100 μL/well HCM, and then lysed in 100 μL/well RIPA buffer (50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 1% Triton X-100, 0.5% deoxycholate, 0.1% SDS, and 5 mM EDTA) for 30 min. Aliquots (80 μL) of the solubilized cell extracts were transferred to an OptiPlate-96 (PerkinElmer, Inc.) containing 120 μL/well Microscint-40 (PerkinElmer, Inc.), sealed, mixed on a plate shaker for 15 min, and then counted in a microplate liquid scintillation counter (TopCount NXT, PerkinElmer, Inc.).

4.2.3. DMSO solvent sensitivity

In primary DRG cultures, we found a noticeable concentration-dependent increase of capsaicin-induced calcium influx from the DMSO solvent (Fig. S1, Supporting Information). During our initial screening in the TRPV1ε-NIH3T3 cells, DHP stock solutions were formulated to a concentration of 5 mM, and the DMSO concentration in the assay buffer was kept constant at 2% (since the highest DHP concentration evaluated was 100 μM). This DMSO concentration did not affect results with the TRPV1ε-NIH3T3 cells, but it was found to greatly increase the baseline capsaicin activation in DRGs. Therefore, for the DRG assays, we reformulated more concentrated stock solutions (25 mM) from the remaining DHP powder. Due to limited quantities, we were unable to re-assay all DHPs in the primary DRG cells. The maximum DHP concentration evaluated in DRGs was 50 μM, with a corresponding DMSO concentration of 0.2% in the assay buffer.

4.2.4. Dose–response curves

Data was analyzed using GraphPad Prism software. Raw counts from each assay were normalized as percentages to in-plate controls that contained only capsaicin (CPM $[X]_{DHP}$/CPM capsaicin-only × 100). Capsaicin-only activation (E_{max}) was designated as 100% (mean of four wells). Data were plotted as mean ± SEM (%) versus log[Enhancer] (M), and then fit using a standard non-linear dose–response algorithm supplied by the GraphPad software ('Bottom to Top, variable slope'; $Y = \text{Bottom} + (\text{Top} - \text{Bottom})/1 + 10^{((\log EC_{50} - X) \times \text{Hill Slope})}$. The EC_{50}, Hill Slope and ΔE_{max} were extracted from data that could be curve fitted, typically with $r^2 \geqslant 0.90$, in the concentration range evaluated. For compounds that showed activity at only the higher concentrations, and therefore were not closely sigmoidal in this range, only the ΔE_{max} at the highest concentration ($[Enhancer]_{max}$) is shown (see Fig. S2, Supporting Information, for plots of all assay runs in TRPV1ε-NIH3T3 cells).

4.2.5. Intracellular Ca^{2+} measurements

Adapted from published methods with a few changes.[51] Primary DRG neurons (prepared as described above) were plated onto poly-ornithine/laminin (Sigma) coated 25-mm diameter coverslips and cultured 4–6 days before measurement. Cells were loaded with 2.5 μM of the high-K_d calcium indicator Fura-4F AM ester (Molecular Probes) in the presence of 0.0025% pluronic in normal perfusion medium (in mM: 2.5 $CaCl_2$, 10 glucose, 10 Hepes, 3 KCl, 0.6 $MgCl_2$, 130 NaCl, pH adjusted with 1 M Tris base to 7.4, osmolality adjusted with sucrose to 325 mosmol/L) for 20 min at room temperature, followed by a wash and further incubation in perfusion medium for an additional 20 min. The coverslip was inverted and mounted onto a closed flow-through perfusion chamber (Warner Instruments) with a nominal bath volume of 358 μL, then perfused continuously at a rate of 0.5 mL/min using a peristaltic pump (Minipuls 3, Gilson). All experiments were performed at room temperature.

Fluorescence data were acquired with MetaFluor software (Molecular Devices) via a CCD camera (ORCA-ER, Hamamatsu) connected to an upright microscope (BX60, Olympus) using a 20× water-immersion objective. The ratio of fluorescence emission (510 nm) in response to 340/380 nm excitation, controlled by a filter changer (Lambda 10-2, Sutter Instruments), was acquired continuously at 0.5 Hz. Drugs were administered via an in-line manual sample injector (Rheodyne) equipped with a 500 μL sample loop. During experiments, the perfusion medium was supplemented with 1 mM ascorbate. Capsaicin (1 mM EtOH stock) was diluted to 200 nM, and **23** (25 mM DMSO stock) was diluted to 10 μM. Capsaicin-only experiments were supplemented with an equivalent amount of DMSO (0.04%). Cells were recorded for 17 min total: baseline for 2 min, followed by a 15-min recovery. Drugs were administered for 30 s beginning at 2 min (designated as a horizontal bar in Fig. 3B).

Activated cells were individually identified and their corresponding 340/380 ratios measured using the MetaFluor analysis software. Data from activated cells on six separate coverslips (3 with capsaicin only; 3 with capsaicin plus enhancer), from a total of 98 cells analyzed in capsaicin only conditions and 69 cells analyzed when capsaicin plus enhancer were administered, were normalized to baseline and plotted as a function of time. The traces were normalized to baseline by dividing through with the mean of the first 2 min. Mean calculations and statistical analysis were performed using Excel and SigmaPlot software, and plots were generated using GraphPad Prism software. Peak ratio was designated as the mean of 10 consecutive data points from the peak in the calcium response, determined qualitatively.

Acknowledgments

This research was supported in part by the Intramural Research Programs of the NIDDK and NIDCR, National Institutes of Health. We thank Dr. William Trenkle for advice on the chemical synthesis of compound **23** and Dr. Herman Yeh for the assistance with the NMR characterization.

Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2008.08.048.

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