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Caged Capsaicins: New Tools for the Examination of TRPV1 Channels in Somatosensory Neurons

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The vanilloid capsaicin, N-*(4-hydroxy-3-methoxybenzyl)-8-methyl-non-6-enamide, is the pungent ingredient of chili peppers and is used in pain research as an activating ligand of heat-sensitive transduction channels in nociceptive neurons. Here we describe the synthesis and application of two capsaicin derivatives modified at the hydroxy function of the vanillyl motif: α-carboxy-4,5-dimethoxy-2-nitrobenzyl-caged (CDMNB-caged) capsaicin and {7-[bis(carboxymethyl)amino]coumarin-4-yl}methoxycarbonyl-caged (BCMACMOC-caged) capsaicin. These compounds show dramatically reduced pungency, but release active capsaicin upon irradiation with UV light. CDMNB-caged capsaicin can be used to perform concentration-jump experiments, while BCMACMOC-caged capsaicin is membrane-impermeant and can be applied selectively to the intracellular or extracellular sides of a plasma membrane. Both compounds can serve as valuable research tools in pain physiology.*

Introduction

Concentration-jump experiments represent a powerful tool for the exploration of ligand-gated ion channels, since, if ligands can be applied in a sudden step, the immediate response of the channel conveys information that is otherwise difficult to obtain. Details of the gating process, such as activation and inactivation rates, as well as the identification of distinct gating states, become accessible in such experiments. Moreover, the association kinetics of modulators of the gating process can be measured in such nonequilibrium experiments and can open the way to a better understanding of channel regulation. Application of concentration jumps to ion channels in excised membrane patches is technically well established: test solutions at the surface of the membrane patch can be rapidly changed through the use of systems with piezo-controlled perfusion heads, and the channels' responses can be monitored with a time resolution in the submillisecond range.[1] In contrast, it is often not feasible to use such systems for concentration-jump experiments with intact cells or tissue slices, as perfusion capillaries cannot deliver test solutions to the entire cell surface at the same time. Several seconds may elapse before the solution exchange is completed, a delay that obscures all gating events that occur on faster timescales. Moreover, rapid intracellular application of channel ligands is not possible with this method.

A solution to these problems is offered by light-induced release of ligands from inactive precursor molecules (caged ligands).[2] Caged ligands can be released with high temporal and spatial precision, while the ligand concentration released by a light flash can sometimes be quantified.[3] The photochemical properties of a caged compound are mainly determined by the caging group—the residue that renders the channel ligand inactive—and can be optimized for specific experimental requirements. Here we have designed and tested two novel caged compounds that release the vanilloid capsaicin upon illumination with UV light. These compounds are designed to aid in the exploration of capsaicin receptors: the "*transient receptor potential vanilloid receptor*" channels of the TRPV1 type.[4] TRPV1 is a cation channel that mediates pain perception in nociceptive somatosensory neurons.[5] Like other members of the TRP ion channel family, TRPV1 polypeptides possess six transmembrane domains and probably form tetrameric channel proteins. When opened by noxious stimuli—in particular by temperatures exceeding 43 °C or by a drop in pH to below 6.8—the channels conduct a depolarizing cation current, partly carried by Ca^{2+}. As well as by noxious heat and pH, the channels are also opened by the vanilloid capsaicin, the primary pungent compound in chili peppers,[6] which finds use in biophysical studies of native vanilloid receptors as well as for studies of heterologously expressed TRPV1 channels. The compound opens TRPV1 channels at physiological temperature, pH, and voltage by shifting the activation curve for voltage-dependent gating into the physiological range.[7] Recent studies have indicated that capsaicin binds to the intracellular side of the TRPV1 protein to open the channel,[8–10] although it

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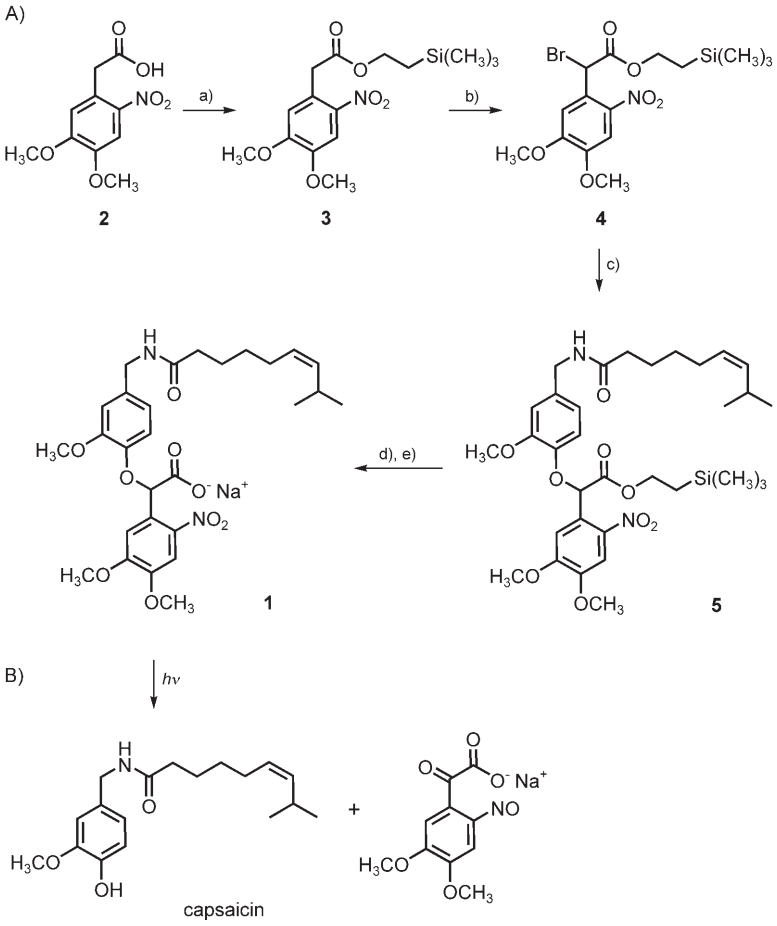
has been suggested that the interaction with intracellular domains alone is not sufficient.[11] Apart from the questions of where capsaicin binds and of how capsaicin binding is translated into channel opening, various other problems pertaining to channel function are also unsolved. They include the subunit composition of native vanilloid receptors, the shape of Ca^{2+} signals during pain perception, and the kinetics of channel regulation by cellular constituents such as calmodulin and protein kinases. For many biophysical experiments, it would be helpful to be able to perform concentration-jump experiments with capsaicin, in which the TRPV1 agonist would be applied rapidly to either the intracellular or the extracellular side of the channel. Caged capsaicin[12] or vanilloid analogues[13,14] have been introduced in several recent publications, and here we report the synthesis and application of two novel caged capsaicins that were developed specifically for electrophysiological experimentation, including the kinetic analysis of TRPV1 channels and the distinction between intra- and extracellular actions of capsaicin.



Scheme 1. A) Synthesis and B) photolysis of **1**. a) TMSE, DMAP, DCC, ethyl acetate, 10 °C, 0.5 h, RT, 12 h, 66%. b) NBS, AIBN, CCl_4, 76 °C, 7 h, 55%. c) Capsaicin, K_2CO_3, DMF, RT, 18 h, 83%. d) TFA/CH_2Cl_2/H_2O (75:24:1), RT, 20 min, 70%. e) Dowex W50-Na^+, 93%.

Results and Discussion

Synthesis and properties of CDMNB-caged capsaicin

In designing the sodium salt of α-carboxy-4,5-dimethoxy-2-nitrobenzyl-caged (CDMNB-caged) capsaicin **1** (Scheme 1), we tried to optimize the properties that are critical for application of the compound as a tool in concentration-jump experiments: good solubility and stability in Ringer-type saline, absorption at >330 nm, and sufficiently high quantum yield. The CDMNB residue is a novel caging group that is photoactivatable at wavelengths >350 nm and has good solubility in aqueous buffer at physiologically relevant pH values by virtue of the presence of the negatively charged carboxylate group. In **1**, the phenolic hydroxy function of capsaicin, which is important for agonist activity,[6] is blocked by transformation into an ether. The synthesis of 2-bromo-2-(4,5-dimethoxy-2-nitrophenyl)acetic acid 2-(trimethylsilyl)ethyl ester (**4**) required for caging of capsaicin is shown in Scheme 1 A. Dimethoxy-2-nitrophenylacetic acid **2** was esterified with 2-(trimethylsilyl)ethanol (TMSE) to give the ester **3**, which was converted into the bromide **4** by treatment with *N*-bromosuccinimide (NBS). Capsaicin was then coupled with **4** in DMF in the presence of K_2CO_3 to yield **5**, and subsequent removal of the TMSE group with trifluoroacetic acid (TFA) afforded the free-acid form of **1** in 58% overall yield. As observed with other TMSE esters,[15] cleavage of the TMSE ester by treatment with TBAF was less useful. Final purification of the free-acid form of **1** was achieved by reversed-phase HPLC; its transformation into the sodium salt **1**, which showed better solubility properties than the free-acid, was advantageous. The absorption spectrum of **1** in 5% CH_3CN/HEPES buffer (pH 7.2) showed a maximum at 349.5 nm with a molar extinction coefficient (ε) of 4700 $M^{-1}cm^{-1}$.

Irradiation of **1** at 334 or 365 nm in aqueous buffer resulted in the liberation of free capsaicin (Scheme 1 B), the quantum yield (ϕ) of the photocleavage reaction in 5% CH_3CN/HEPES buffer (pH 7.2) being 0.05. The uncaging action cross section ($\varepsilon\phi$), which is proportional to the amount of release for a given photon exposure at 349.5 nm, was about 235 $M^{-1}cm^{-1}$, while the saturation concentration of **1** in 0.01 M HEPES/KOH buffer (pH 7.2) was >1 mM. Since TRPV1 channels show half-maximal activation in the range of 0.1–0.7 μM capsaicin, the solubility of **1** allows rapid release of at least a 1000-fold excess of channel ligand, so the solubility of **1** does not present a limit to kinetic experiments and offers the potential for analysis of capsaicin association rates in TRPV1 channels. HPLC monitoring of **1** in aqueous HEPES buffer at pH 7.2 over a 24 h period revealed 1.7% formation of free capsaicin, which indicates that the caged compound is sufficiently stable in solution to permit experiments over a time range of several hours.

Kinetic experiments with nociceptive neurons

To test **1** on somatosensory neurons, we isolated the neurons from rat dorsal root ganglia (DRGs) and confirmed the expres-

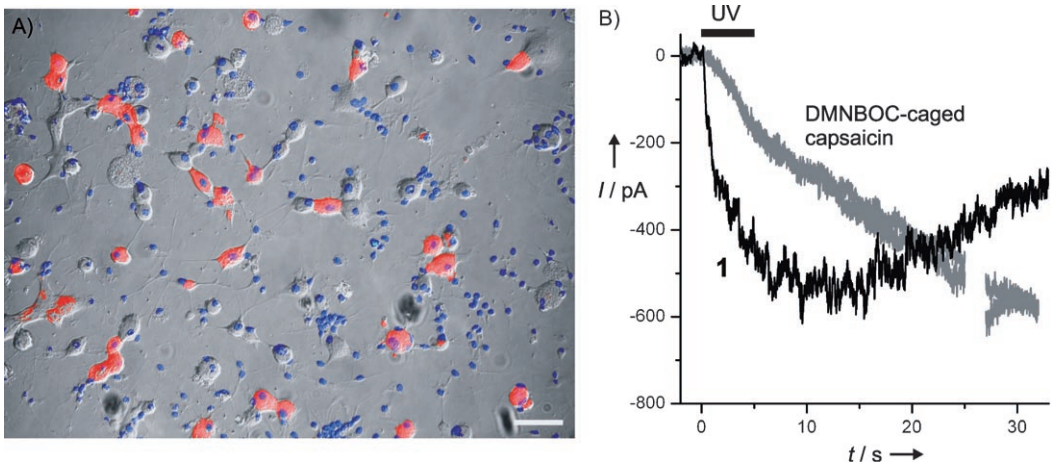


Figure 1. A) Primary culture of rat dorsal root ganglion (DRG) cells. Overlay of Nomarski and fluorescence micrographs showing the culture 24 h after dissection of the DRGs. Cell nuclei appear blue (DAPI stain), while TRPV1 expression is indicated by red immunofluorescence. Bar: 50 μm. About 50% of the medium-diameter neurons express TRPV1; these cells were used for the functional examination of **1**. No TRPV1 immunoreactivity was seen in large-diameter cells and in most small neurons and small, non-neuronal cells. B) Photoinduced currents in two DRG neurons with DMNBOC-caged capsaicin (5 μM, gray trace) and **1** (1 μM, black trace). Whole-cell currents recorded at −70 mV over 5 s UV illumination.

sion of TRPV1 protein by immunocytochemistry. Figure 1A shows a primary culture of DRG neurons one day after preparation. To test the effect of photorelease of capsaicin on the DRG neurons, medium-diameter neurons were examined electrophysiologically in the patch-clamp whole-cell configuration. In a previous study, the caged capsaicin compound with the 4,5-dimethoxy-2-nitrobenzyloxycarbonyl (DMNBOC) caging group had been tested in heterologously expressed TRPV1 channels:[12] photolysis of DMNBOC-caged capsaicin had induced cell activity after a mean delay of 5 s, making this compound unsuitable for kinetic studies. We compared the effects of 5 s illumination of DRG neurons in the presence either of DMNBOC-caged capsaicin (5 μM) or of **1** (5 μM), and the results of this comparison (Figure 1B) demonstrate that the CDMNB cage releases capsaicin much more quickly and more efficiently than the DMNBOC cage. In whole-cell current-clamp experiments, the DRG neurons had resting voltages of −60 to −80 mV and a mean membrane capacitance of (43 ± 18.5) pF (70 cells). Injection of current pulses (100 pA, 200 ms) mostly elicited 1–3 action potentials. The cells responded neither to a 500 ms flash of UV light in the absence of caged capsaicin (Figure 2A, gray trace) nor to the presence of **1** (1 μM) in the bath solution in the absence of UV light. However, nine out of 40 cells (22%) responded with firing of multiple action potentials when a UV flash was applied in the presence of **1** (1 μM, Figure 2A, black trace). Capsaicin-induced currents were recorded from excised, inside-out membrane patches in the voltage-clamp configuration at −70 mV, and control UV flashes were first applied in the absence of caged capsaicin. After perfusion of the bath with **1** (1 μM), 11 out of 30 (37%) neurons responded to a 500 ms UV flash with inward currents ranging from 0.5 nA to 2.5 nA. Current densities varied between 4.5 and 52.5 pA pF^{-1} (mean (21.3 ± 15.9) pA pF^{-1}), illustrating the heterogeneity of TRPV1 expression between individual cells. Concentrations of **1** of more than 2 μM could not be used in these experiments because the caged compound showed a residual activity at higher concentrations. This activity was not due to contamination with free capsaicin but was caused by the caged compound itself (data not shown).

Some important parameters of ion channel function, including desensitization to capsaicin, can be obtained only when the channels of an individual cell are repeatedly activated. To test whether **1** was useful for such experiments, we applied 500 ms flashes firstly in the absence of caged capsaicin, and then repeatedly in the presence of **1** (1 μM) at intervals of 300 s. Figure 2B illustrates how the capsaicin-induced currents progressively decreased with each flash, reflecting the well-characterized desensitization of TRPV1 channels to capsaicin.[16] Capsaicin application by photolysis allows fast photorelease in kinetic experiments with a time resolution in the millisecond range, and to explore the use of **1** for kinetic experiments we released different concentrations of capsaicin by applying light flashes of various durations in the presence of **1** (1 μM). Figure 2C shows a whole-cell current recording during photorelease of capsaicin. Immediately following the onset of the flash, a lag phase (Δt) of (124 ± 58) ms (15 cells) elapsed before current induction, while after this delay the current followed a single-exponential time course. For kinetic analysis, all currents were normalized to the maximal amplitude, measured after current saturation had been achieved by uncaging of capsaicin in a 2 s illumination. Figure 2D shows normalized currents elicited by flashes of 100 and 200 ms duration in two different cells. The time constant of current increase (τ), calculated according to $I(t) = I_{max}(1 - e^{-t/\tau})$ after the end of the lag interval, varied with the flash duration between 600 and 1100 ms (Figure 2E). This dependence reflects the different concentrations of capsaicin released from **1** (1 μM) by flashes of different durations. Higher concentrations of photoreleased capsaicin cause faster onsets of the macroscopic current, as would be expected from the binding kinetics of ligand-gated channels. These data show that **1** can be used to study kinetic details of activation in TRPV1 channels.

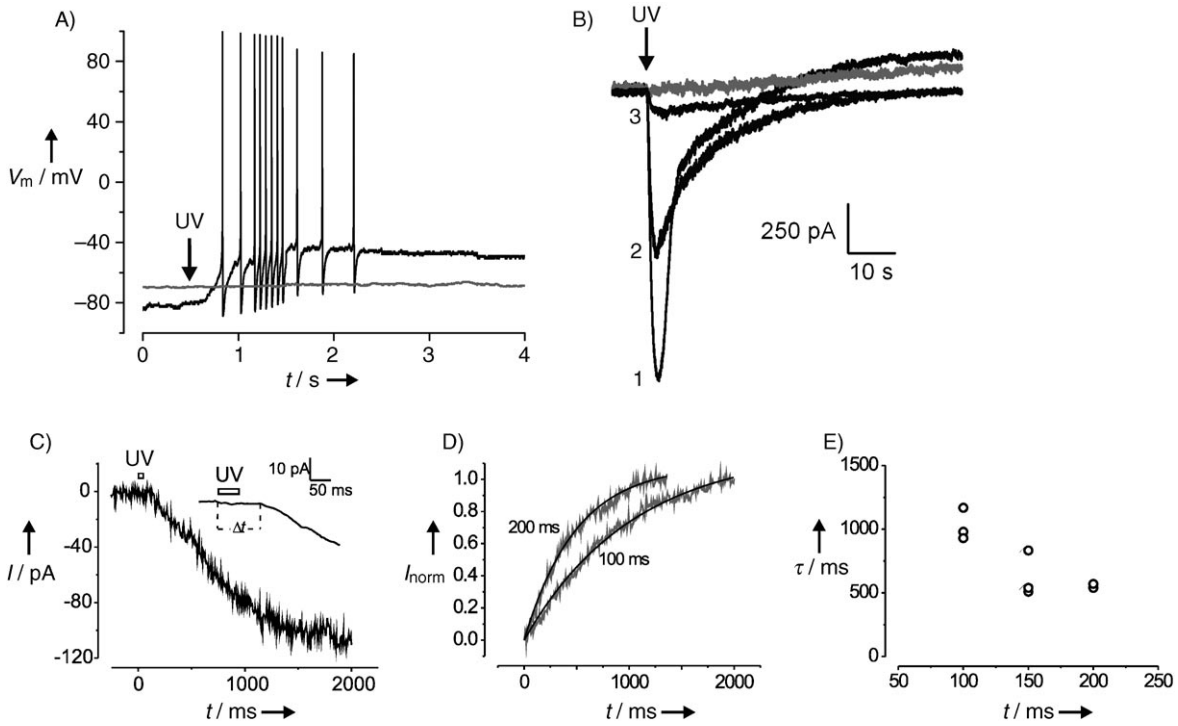


Figure 2. Application of **1** to DRG neurons. A) Whole-cell, current-clamp recording of a medium-diameter DRG neuron in control solution, as well as in solution containing **1** (1 μM). In the absence of the caged compound the membrane voltage did not change upon illumination with UV light for 500 ms (arrow, gray trace). In the presence of caged capsaicin, a light flash of the same intensity and duration induced a burst of action potentials (arrow, black trace) in the same cell. B) Repeated photorelease of capsaicin from **1** (1 μM) reveals desensitization of capsaicin receptors. The interval between flashes was 300 s. Voltage clamp at −70 mV. C) Use of **1** for kinetic experiments. Whole-cell current recording at −70 mV recorded from a DRG neuron upon photorelease of capsaicin from **1** (1 μM) triggered by a 50 ms flash of UV light. The onset of the current occurred with a delay (Δt) of 105 ms (inset). D) Dependence of channel-activation kinetics on flash duration. A longer flash induced a faster increase in the normalized current in a DRG neuron clamped at −70 mV, illustrating that the response kinetics of TRPV1 depend on the concentration of photoreleased capsaicin. E) The time course of whole-cell current increase after photorelease of capsaicin was characterized by a time constant (τ) that monotonically decreased with increasing flash duration. The data were obtained from eight DRG neurons at −70 mV and demonstrate the usefulness of **1** for kinetic examinations of capsaicin-activated ion channels.

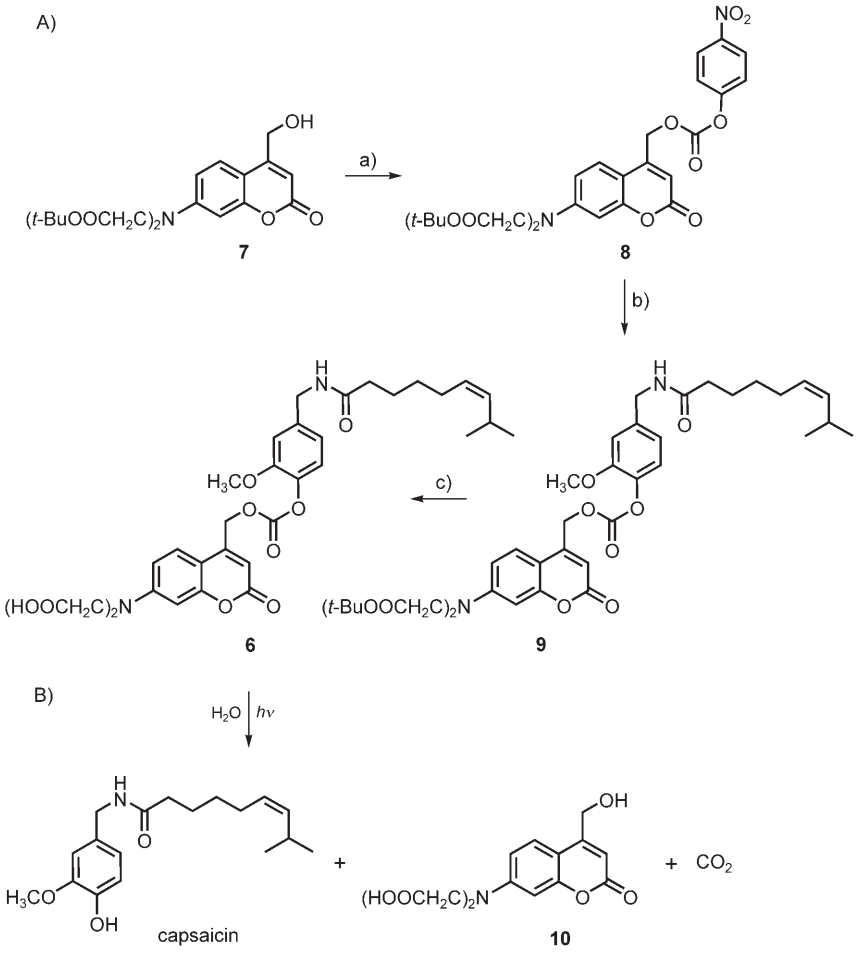
Synthesis and properties of BCMACMOC-caged capsaicin

For studies of the effect of capsaicin on TRPV1 channels, it would be crucial to have a caged capsaicin that could be applied exclusively on one side of the membrane and would display no residual activity in the caged form at micromolar concentrations. Such a compound would have to be membrane-impermeant, at least in the time range needed for the biophysical experiments. To this end we designed {7-[bis(carboxymethyl)amino]coumarin-4-yl}methoxycarbonyl-caged (BCMACMOC-caged) capsaicin (**6**) using our recently developed BCMACM protecting group.[17] Compound **6** was prepared as shown in Scheme 2 by esterification of the alcohol **7**[17] with 4-nitrophenyl chloroformate to provide the carbonate **8** and subsequent treatment of **8** with capsaicin in the presence of 4-dimethylaminopyridine (DMAP) to yield the *tert*-butoxy-protected derivative **9**, which was purified by reversed-phase HPLC on a preparative scale. TFA deprotection of **9** resulted in pure **6** in 25% overall yield.

Compound **6** is easily soluble (>1 mM) in aqueous buffer at pH 7.2 and, under these conditions, is resistant to spontaneous hydrolysis in the dark. Photoactivation of **6** by irradiation with wavelengths of 365 or 405 nm in aqueous buffer produces capsaicin, compound **10**, and CO_2 (see Scheme 2B). The photoreaction is clean. The absorption spectrum shows a maximum at 383 nm with an extinction coefficient (ε) of 18750 $M^{-1}cm^{-1}$, while the fluorescence quantum yield was 0.021, with the fluorescence maximum at 478 nm. The single-photon photochemical quantum yield (ϕ) in buffer (pH 7.2) was 0.12, which gives an uncaging action cross section ($\varepsilon\phi$) of 2250 $M^{-1}cm^{-1}$ at 383 nm, indicating that **6** is about 10 times more sensitive to light than **1** at their respective absorption maxima. Furthermore, we also found that **6** is sensitive to two-photon excitation (data not shown).

Assessment of membrane translocation

To assess the ability of **6** to translocate across lipid membranes, high-sensitivity isothermal titration calorimetry (ITC) uptake and release[18] experiments were performed and evaluated in terms of a simple surface partition equilibrium as described elsewhere.[19] Figure 3A illustrates the raw data obtained from uptake (a) and release (b) experiments performed at 25 °C with small unilamellar vesicles composed of the zwitterionic phospholipid 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC), while Figure 3B shows the normalized reaction heats measured in uptake (circles) and release (squares) titrations as obtained by integration of the calorimetric traces depicted in



Scheme 2. A) Synthesis and B) photolysis of **6**. a) 4-Nitrophenyl chloroformate, DMAP, CH_2Cl_2, RT, 4 h. b) Capsaicin, DMAP, CH_2Cl_2, 20 h, 28% from **7**. c) TFA/CH_2Cl_2/H_2O (75:24:1), RT, 20 min, 90%.

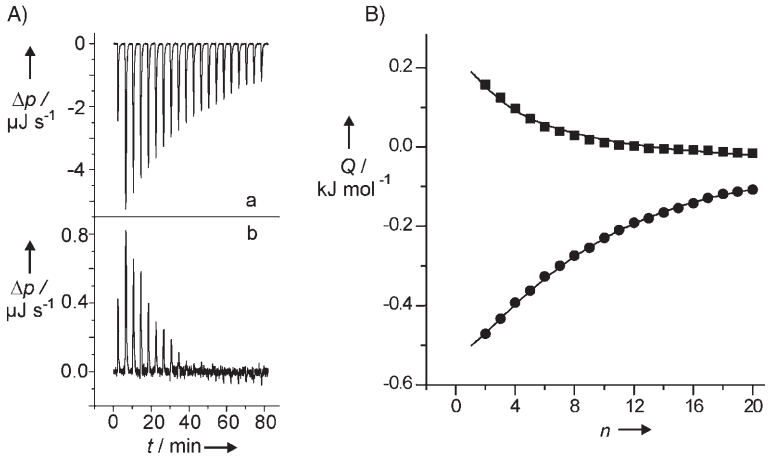


Figure 3. Determination of membrane permeability by ITC. A) ITC raw data obtained at 25 °C. Differential heating power (Δp) versus time (t). a) Uptake: aliquots (10 μL) of POPC vesicles (40 mM) were titrated to **6** (100 μM). b) Release: aliquots (10 μL) of POPC vesicles (20 mM) preloaded on both leaflets with **6** (541 μM) were injected into buffer. B) Evaluation of ITC experiments. Normalized heats of reaction (Q) versus injection number (n). A global fit (——) to both uptake (●) and release (■) experiments yielded the values of the thermodynamic parameters given in the text, demonstrating that **6** cannot cross lipid membranes. The first peak was discounted because it was usually affected by sample loss due to leakage effects during the equilibration phase preceding the actual ITC run.

Figure 3 A. A simultaneous fit (solid lines) to both data sets based on a partitioning model outlined elsewhere[19] yielded a mole ratio partition coefficient of $\tilde{K}_C^{b/i} = 1.4 \times 10^3\ L\,mol^{-1}$, changes in Gibbs free energy of $\Delta G_C^{b/i,0} = -17.9\ kJ\,mol^{-1}$, in enthalpy of $\Delta H_C^{b/i,0} = -16.8\ kJ\,mol^{-1}$, and in entropy of $\Delta S_C^{b/i,0} = 3.8\ J\,mol^{-1}\,K^{-1}$, an effective charge number of $z_C^{eff} = -1.5$, and a lipid accessibility factor of $\gamma = 0.6$. Membrane binding is therefore mainly driven by an exothermic enthalpy change, whereas the entropic contribution is small. Most importantly, $\gamma = 0.6$ indicates that **6** binds to or desorbs from only the outer membrane leaflet, so **6** cannot measurably translocate across phospholipid membranes on the timescales of these ITC experiments.

BCMACOC-caged capsaicin inside and outside the cell

The ITC experiments revealed that **6** has a very low membrane permeability and can thus be used to challenge the TRPV1 channel exclusively from the internal or external side. We tested this compound with homomeric TRPV1 channels expressed in HEK 293 cells, a system often used to study the TRPV1 gating process. Compound **6** was completely inactive when applied in the caged form at 10 μM to the outsides of HEK 293 cells expressing TRPV1 channels, while a UV flash induced whole-cell currents in the range of −1 to −5 nA at −70 mV, declining within about 30 s because of channel desensitization (Figure 4 A). To compare the effects of photoreleased capsaicin on either side of the channels, we applied either 2 μM **6** to the bath solution or, alternatively, 2–10 μM **6** to the pipette solution. Cytosol and pipette solution were then allowed to equilibrate for 10 min before application of a light flash. This protocol for filling cells with caged compounds had been established previously with caged derivatives of various bioactive molecules[20] and with similar coumarinylmethyl cages.[3] Figure 4 B (a–c) shows that the photolysis of intracellular caged capsaicin (Figure 4 B; label: i) elicited only small currents and that subsequent application of extracellular capsaicin (Figure 4 B; label: e) elicited large currents; this demon-

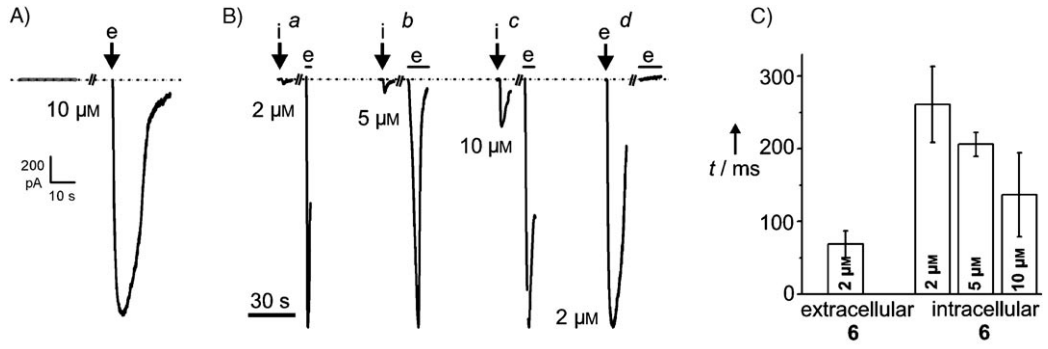


Figure 4. Extracellular (label: e) and intracellular (label: i) application of **6**. A) An HEK 293 cell transfected with TRPV1 was kept in an extracellular medium containing **6** (2 μM), and whole-cell currents were recorded at −70 mV. This did not induce current without UV light (left), but a 1 s flash elicited a transient current (right). B) Three HEK 293 cells were filled with different concentrations of **6**: *a* = 2 μM, *b* = 5 μM, *c* = 10 μM. Whole-cell currents at −70 mV elicited by UV photolysis of intracellular **6** (arrows) were small compared with the subsequent response of the same cells to extracellular free capsaicin (5 μM). Intracellular capsaicin release did not cause desensitization. In contrast, extracellular photolysis of **6** (2 μM, *d*) caused a transient current and strong desensitization, as subsequent application of capsaicin (5 μM) did not produce any current. C) Latencies between the start of the UV flash and the onset of whole-cell currents. Photorelease of extracellular **6** elicited currents with much shorter latency than photorelease of intracellular **6**.

strated that the intracellular release of capsaicin did not cause channel inactivation. In contrast with the small effects of intracellular photorelease, extracellular photorelease triggered large whole-cell currents and complete inactivation (Figure 4B d). Furthermore, the delay between the onset of the flash and current activation was four times longer with intracellular [(261 ± 51) ms, $n = 3$] than with extracellular application [(69 ± 22) ms, $n = 4$] with caged capsaicin (2 μM) in both cases (Figure 4C). Higher intracellular concentrations of caged capsaicin decreased the delay, but even 10 μM intracellular caged capsaicin caused a significantly longer delay [(137 ± 59) ms, $n = 9$] than extracellular caged capsaicin at five times lower concentration. It is unlikely that the photorelease of capsaicin inside the cell is less effective than outside, as our studies with BCMCM-caged cAMP have indicated that photorelease proceeds with similar efficiencies inside and outside cells in the whole-cell configuration. In fact, the concentration of photoreleased cAMP rises more rapidly inside cells than outside, probably as a consequence of the restricted volume of the cytosol (Frings, unpublished), so the low efficiency of TRPV1 activation from the inside suggests that the TRPV1 channel responds to intracellular capsaicin with reduced sensitivity. These results point to profoundly asymmetric control of the TRPV1 channel by its ligand, with more efficient activation by extracellular than by intracellular capsaicin.

Conclusions

Taken together, compound **1** is a caged capsaicin useful for the rapid and controlled activation of TRPV1 channels in cells, but the caged compound shows residual activity, which limits its use to concentrations < 2 μM. Compound **6** does not display measurable activity up to 10 μM and has very high photosensitivity upon one- and two-photon photolysis. In addition, it offers the special advantage of low membrane permeability. These properties make compound **6** especially suitable for concentration-jump experiments to study the asymmetry of capsaicin action on TRPV1 channels.

Experimental Section

Materials: (4,5-Dimethoxy-2-nitrophenyl)acetic acid (**2**) was purchased from Acros Organics (Belgium). 2-(Trimethylsilyl)ethanol (TMSE), 4-dimethylaminopyridine (DMAP), dicyclohexylcarbodiimide (DCC), azobisisobutyronitrile (AIBN), *N*-bromosuccinimide (NBS), 4-nitrophenyl chloroformate, and 6-nitroveratryl chloroformate were obtained from Fluka. Trifluoroacetic acid (TFA) was from Lancaster (UK), capsaicin was obtained from Sigma, and POPC was purchased from Avanti Polar Lipids (USA). The remaining chemicals were of the highest grade commercially available and were used without further purification. 7-[Bis(*tert*-butoxycarbonylmethyl)amino]-4-(hydroxymethyl)coumarin (**7**) was prepared as described previously.[17] TLC plates (silica gel 60 F_{254}) were purchased from Merck, and silica gel for flash chromatography was from J. T. Baker (The Netherlands). CH_3CN from Riedel–deHaen (Germany) was HPLC grade, while water was purified with a Milli-Q-Plus system (Millipore, Germany). All reactions were carried out under N_2. Synthetic and analytical procedures with caged compounds were performed under yellow light provided by sodium vapor lamps.

Instrumentation: ^1H and ^{13}C NMR spectra were recorded with a Bruker DRX 600 or a Bruker AV 300 spectrometer; chemical shifts are given in parts per million (ppm) and *J* values in Hz. Mass spectra were measured by electrospray ionization mass spectrometry in the positive ionization mode with a Finnigan IT/FT-ICR (Thermo Electron) spectrometer. UV/Vis spectra were recorded on a Lambda 9 UV/Vis spectrophotometer (Perkin–Elmer). Fluorescence spectra were taken on a Shimadzu RF-50001PC in tandem with a personal computer and data analysis software package (SPECTRACALC). Analytical reversed-phase HPLC (RP-HPLC) was carried out on a Shimadzu LC-6 A system (flow rate: 1 mL min^{-1}) fitted with a UV detector (operating at 254 and 385 nm) and a fluorescence detector ($\lambda_{exc} = 385$ nm, $\lambda_{em} = 495$ nm) with use of a PLRP-S column (300 Å, 8 μm, 250 mm × 4.6 mm) from Polymer Laboratories (UK). Preparative RP-HPLC was performed on a Shimadzu LC-8 A system (flow rate: 10 mL min^{-1}) with a UV/Vis detector (SPD-6 AV, $\lambda_{exc} =$ 254 nm) on a Nucleogel RP 100–10 (300 × 25 mm) column from Macherey–Nagel (Germany). One-photon photolysis of all employed caged compounds in solution was achieved by use of a high-pressure mercury lamp (HBO 500, Oriel, USA) with controlled light intensity and metal interference transmission filters (334 nm or 365 nm, Oriel, USA). For all experiments, UV and fluorescence

quartz cuvettes with a path length of 1 cm and a cross-sectional area of 1 cm^2 were used. During irradiation, the solutions in the cuvettes were mixed with a magnetic stirrer. All synthetic and analytical procedures were performed in darkness or under yellow light provided by sodium vapor lamps. The melting points are uncorrected.

Syntheses

*2-(Trimethylsilyl)ethyl (4,5-dimethoxy-2-nitrophenyl)acetate (**3**)*: A solution of **2** (6.51 g, 27 mmol), DMAP (122 mg, 1 mmol), and TMSE (4.24 mL, 29.7 mmol) in ethyl acetate (150 mL) was cooled to 10 °C and DCC (5.86 g, 28.4 mmol) was added in portions with stirring. The mixture was allowed to warm to room temperature and stirred for 12 h. It was then filtered, the solvent was evaporated, and the resulting residue was purified by flash chromatography on a silica gel column (elution with hexane/ethyl acetate 7:1 *v/v* and hexane/ethyl acetate 4:1 *v/v*) to provide **3** (6.05 g, 65.6 %) as a yellow solid. m.p. 67–68 °C; TLC: R_f 0.48 (THF/hexane 1:2 *v/v*); ^{1}H NMR (600 MHz, [D$_6$]DMSO): δ = 0.00 (s, 9H), 0.89–0.94 (m, 2H), 3.86 (s, 3H), 3.87 (s, 3H), 4.00 (s, 2H), 4.08–4.14 (m, 2H), 7.16 (s, 1H), 7.69 ppm (s, 1H); ESI MS: 340.11 $[M-H]^-$; elemental analysis calcd (%) for $C_{15}H_{23}NO_6Si$ (341.44): C 52.77, H 6.79, N 4.10; found: C 53.01, H 6.78, N 4.13.

*2-(Trimethylsilyl)ethyl α-bromo-(4,5-dimethoxy-2-nitrophenyl)acetate (**4**)*: A mixture of **3** (3.414 g, 10 mmol), NBS (2.636 g, 14.8 mmol), and AIBN (0.140 g, 0.85 mmol) in CCl_4 (110 mL) was heated at reflux for 24 h and then allowed to cool. After filtration the filtrate was evaporated and the resulting residue was purified by flash chromatography on a silica gel column (elution with hexane and hexane/ethyl acetate 4:1 *v/v*) to provide **4** (2.3 g, 54.7 %) as a yellow oil. TLC: R_f 0.68 (THF/hexane 1:2 *v/v*); ^{1}H NMR (600 MHz, [D$_6$]DMSO): δ = −0.02 (s, 9H), 0.88–0.95 (m, 2H), 3.90 (s, 3H), 3.91 (s, 3H), 4.20 (t, J = 8.0 Hz, 2H), 6.34 (s, 1H), 7.37 (s, 1H), 7.71 ppm (s, 1H); ^{13}C NMR (150 MHz, [D$_6$]DMSO): δ = −1.39, 16.71, 45.78, 56.44, 56.56, 64.67, 108.78, 114.63, 126.01, 139.46, 148.91, 153.09, 167.01 ppm; ESI MS: 441.98 and 443,97 $[M+Na]^+$, 457.95 and 459.95 $[M+K]^+$; elemental analysis calcd (%) for $C_{15}H_{22}BrNO_6Si$ (420.33): C 42.86, H 5.28, N 3.33; found: C 42.84 , H 5.27, N 3.39.

*2-(Trimethylsilyl)ethyl (4,5-dimethoxy-2-nitrophenyl)-{2-methoxy-4-[(8-methylnon-6-enoylamino)methyl]phenoxy}acetate (**5**)*: A mixture of **4** (470 mg, 1.12 mmol), capsaicin (305.4 mg, 1 mmol), and K_2CO_3 (309 mg, 2.24 mmol) in DMF (8 mL) was stirred for 18 h at RT. DMF was evaporated in vacuo, the residue was dissolved in $CHCl_3$, washed with H_2O, and then dried, the $CHCl_3$ was evaporated, and the residue was purified by preparative RP-HPLC. The capsaicin ether was eluted by use of a linear gradient of 40–95 % B in 80 min (eluent A, H_2O; eluent B, CH_3CN). The fraction with a retention time of 75.2 min was collected, evaporated in vacuo, and lyophilized to give **5** (537.2 mg, 83.3 %) as a resin-like, yellow solid. TLC: R_f 0.48 (THF/hexane 1:1 *v/v*); ^{1}H NMR (600 MHz, [D$_6$]DMSO): δ = −0.03 (s, 9H), 0.88–0.94 (m, 8H), 1.29 (quint, J = 7.5 Hz, 2H), 1.50 (quint, J = 7.4 Hz, 2H), 1.93 (q, J = 7.6 Hz, 2H), 2.11 (t, J = 7.2 Hz, 2H), 2.20 (sextet, J = 6.6 Hz, 1H), 3.75 (s, 3H), 3.86 (s, 3H), 3.87 (s, 3H), 4.17–4.20 (4H, m), 5.28–5.38 (m, 2H), 6.32 (s, 1H), 6.71 (d, J = 7.8 Hz, 1H), 6.90 (s, 1H), 7.03 (d, J = 7.2 Hz, 1H), 7.39 (s, 1H), 7.68 (s, 1H), 8.21 ppm (br s, 1H); ESI MS: 645.3 $[M+H]^+$, 667.33 $[M+Na]^+$.

*(4,5-Dimethoxy-2-nitrophenyl)-{2-methoxy-4-[8-methylnon-6-enoylamino)methyl]-phenoxy}acetic acid (free-acid form of **1**)*: Compound **5** (420 mg, 0.65 mmol) was dissolved in TFA/CH_2Cl_2/H_2O (75:24:1; 10 mL) and the system was stirred for 20 min at RT. TFA and CH_2Cl_2 were removed in vacuo, and the residue was dissolved in CH_3CN (12 mL) and H_2O (6 mL) and purified by preparative RP-HPLC. The free acid of **1** was eluted by use of a linear gradient 30–95 % B in 80 min (eluent A, 0.1 % TFA; eluent B, CH_3CN). The fraction with a retention time of 53.5 min was collected, evaporated in vacuo, redissolved in dioxane/H_2O, and lyophilized to give the free acid of **1** (255 mg, 69.8 %) as a yellow powder. m.p. 54–55 °C; TLC: R_f 0.27 (THF/hexane 1:1 *v/v*); ^{1}H NMR (600 MHz, [D$_6$]DMSO): δ = 0.91 (d, J = 6.6 Hz, 6H), 1.28 (quint, J = 7.5 Hz, 2H) 1.50 (quint, J = 7.5 Hz, 2H), 1.93 (q, J = 7.0 Hz, 2H), 2.10 (t, J = 7.3 Hz, 2H), 2.20 (sextet, J = 6.6 Hz, 1H), 3.76 (s, 3H), 3.85 (s, 3H), 3.87 (s, 3H), 4.17 (d, J = 5.9 Hz, 2H), 5.28–5.38 (m, 2H), 6.33 (s, 1H), 6.70 (dd, J = 7.2 and 1.6 Hz, 1H), 6.90 (d, J = 1.8 Hz, 1H), 7.01 (d, J = 8.3 Hz, 1H), 7.36 (s, 1H), 7.66 (s, 1H), 8.21 (t, J = 5.9 Hz, 1H); ESI MS: 545.19 $[M+H]^+$, 583.14 $[M+K]^+$; elemental analysis calcd (%) for $C_{28}H_{36}N_2O_9 \cdot H_2O$ (562.62): C 59.78, H 6.81, N 4.98; found: C 59.83, H 6.34, N 5.10.

*Sodium (4,5-dimethoxy-2-nitrophenyl)-{2-methoxy-4-[(8-methylnon-6-enoylamino)methyl]phenoxy}acetate (**1**, CDMNB-caged capsaicin)*: The free-acid form of **1** (25 mg, 0.044 mmol) was converted into its sodium salt (24.6 mg, 0.041 mmol, 92.6 %) by dissolution in CH_3CN (10 mL) and H_2O (90 mL), stirring with a cation exchanger (Dowex 50 W X2 charged with sodium ions), filtration, and lyophilization. ^{1}H NMR (600 MHz, D_2O): δ = 0.77 (d, J = 6.6 Hz, 6H), 1.12 (m, 2H) 1.39 (m, 2H), 1.74 (q, J = 6.6 Hz, 2H), 2.03 (m, 2H), 3.68 (s, 3H), 3.77 (s, 3H), 3.82 (s, 3H), 4.01–4.10 (m, 2H), 5.11–5.15 (m, 1H), 5.20–5.24 (m, 1H), 6.40 (s, 1H), 6.50 (d, J = 7.2 Hz, 1H), 6.78–6.82 (m, 2H), 7.21 (s, 1H), 7.48 ppm (s, 1H); ESI MS: 545.16 $[M+H]^+$, 567.14 $[M+Na]^+$; elemental analysis calcd (%) for $C_{28}H_{35}N_2NaO_9 \cdot 2H_2O$ (602.61): C 55.81, H 6.52, N 4.65; found: C 56.03, H 6.30, N 4.72.

4,5-Dimethoxy-2-nitrobenzyl 2-methoxy-4-[(8-methylnon-6-enamido)-methyl]phenyl carbonate (DMNBOC-caged capsaicin): Capsaicin was caged with 4,5-dimethoxy-2-nitrobenzyl chloroformate by the published procedure of Zemelman et al.[12] ^{1}H NMR (300 MHz, [D$_6$]DMSO): δ = 0.92 (d, J = 6.7 Hz, 6H), 1.30 (quint, J = 7.5 Hz, 2H) 1.52 (quint, J = 7.5 Hz, 2H), 1.94 (q, J = 7.0 Hz, 2H), 2.14 (t, J = 7.2 Hz, 2H), 2.21 (m, 1H), 3.75 (s, 3H), 3.89 (s, 3H), 3.91 (s, 3H), 4.25 (2H, J = 5.9 Hz, d), 5.31–5.36 (m, 2H), 5.55 (s, 2H), 6.83 (d, J = 8.2 Hz, 1H), 7.03 (s, 1H), 7.14 (d, J = 8.1 Hz, 1H), 7.20 (s, 1H), 7.74 (s, 1H), 8.30 ppm (t, J = 5.7 Hz, 1H); UV: λ_{max} (ε): 340 nm (5670) in HEPES buffer/CH_3CN (95:5); ESI MS: 545.24 $[M+H]^+$; elemental analysis calcd (%) for $C_{28}H_{36}N_2O_9$ (544.60): C 61.75, H 6.66, N 5.14; found: C 62.10, H 6.60, N 4.98.

*{7-[Bis(*tert*-butoxycarbonylmethyl)amino]-coumarin-4-yl}methyl 4-[(8-methylnon-6-enamido)methyl]-2-methoxyphenyl carbonate (**9**)*: DMAP (35 mg, 0.29 mmol) and 4-nitrophenyl chloroformate (70 mg, 0.35 mmol) were added with stirring to a suspension of **7** (100 mg, 0.24 mmol) in CH_2Cl_2 (3 mL). The reaction mixture was stirred for 4 h at RT, after which DMAP (35 mg, 0.29 mmol) and capsaicin (105 mg, 0.35 mmol) were added to the intermediately formed active ester. The reaction mixture was stirred for 20 h, evaporated, and purified by preparative RP-HPLC. Compound **9** was eluted by use of a linear gradient 30–95 % B in 80 min (eluent A, H_2O; eluent B, CH_3CN). The main fraction was collected, concentrated in vacuo, redissolved in CH_3CN/H_2O, and lyophilized to give **9** (50.1 mg, 27.8 %) as a yellow solid. m.p. 65 °C; TLC: R_f 0.31 ($CHCl_3$/MeOH 95:5 *v/v*); t_R = 18.54 min (analytical HPLC, 30–95 % B in A in 20 min, eluent A, H_2O; eluent B, CH_3CN); ^{1}H NMR (300 MHz, [D$_6$]DMSO): δ = 0.92 (d, J = 6.7 Hz, 6H), 1.30 (m, 2H), 1.42 (18H, s), 1.52 (m, 2H), 1.94 (m, 2H), 2.14 (m, 2H), 2.21 (m, 1H), 3.78 (s, 3H), 4.20 (4H, s), 4.26 (d, J = 5.9 Hz, 2H), 5.34 (m, 2H), 5.50 (s, 2H), 6.12 (s, 1H), 6.50 (d, J = 2.1 Hz, 1H), 6.60 (dd, J = 8.9, 2.1 Hz, 1H), 6.84 (d, J = 8.1 Hz, 1H), 7.04 (s, 1H), 7.18 (d, J = 8.1 Hz, 1H), 7.54 (d, J = 8.9 Hz, 1H), 8.31 ppm (t, J = 5.9 Hz, 1H); ^{13}C NMR (75 MHz, [D$_6$]DMSO): δ = 22.5, 24.8, 27.7, 28.7, 30.3, 31.6, 35.1, 41.7,

53.5, 55.7, 65.2, 81.1, 98.2, 106.6, 109.2, 111.7, 119.0, 121.8, 125.3, 126.5, 137.3, 138.1, 139.4, 149.7, 150.3, 151.5, 152.2, 153.4, 155.0, 160.3, 168.7, 172.1 ppm; ESI MS: 751.31 $[M+H]^+$.

*{7-[Bis(carboxymethyl)amino]-coumarin-4-yl}methyl 4-[(8-methylnon-6-enamido)methyl]-2-methoxyphenyl carbonate (**6**; BCMACMOC-caged capsaicin)*: Compound **9** (50.1 mg, 0.67 mmol) was stirred in a TFA/CH_2Cl_2/H_2O mixture (74:25:1, 5 mL) at RT for 20 min. The solvents were evaporated, and the residue was coevaporated twice with ether, dissolved in CH_3CN/H_2O, and lyophilized to give the desired product (40.4 mg, 89.8%). m.p. 120 °C (decomp.); TLC: R_f 0.34 ($CHCl_3$/MeOH/1% TFA 95:5:1 *v/v*); t_R = 18.06 min (analytical HPLC, 30–95% B in A in 25 min, eluent A, 0.1% TFA/H_2O; eluent B, CH_3CN); ^1H NMR (300 MHz, [D_6]DMSO): δ = 0.92 (d, J = 6.7 Hz, 6H), 1.30 (m, 2H), 1.52 (m, 2H), 1.94 (m, 2H), 2.14 (m, 2H), 2.21 (m, 1H), 3.78 (s, 3H), 4.25 (m, 6H), 5.34 (m, 2H), 5.50 (s, 2H), 6.11 (s, 1H), 6.50 (d, J = 2.1 Hz, 1H), 6.62 (dd, J = 8.9, 2.1 Hz, 1H), 6.84 (d, J = 8.1 Hz, 1H), 7.04 (s, 1H), 7.18 (d, J = 8.1 Hz, 1H), 7.53 (d, J = 8.9 Hz, 1H), 8.31 ppm (t, J = 5.9 Hz, 1H); ESI MS: 639.25 $[M+H]^+$; elemental analysis calcd (%) for $C_{33}H_{38}N_2O_{11}\cdot 2H_2O$ (674.70): C 58.75, H 6.27, N 4.15, found: C 58.32, H 6.06, N 4.14.

Solubility: The solubilities of **1** and **6** in HEPES buffer (10 mM HEPES and 120 mM KCl, adjusted to pH 7.2 with 2 N KOH) and that of DMNBOC-caged capsaicin in CH_3CN/HEPES buffer (10 mM HEPES and 120 mM KCl adjusted to pH 7.2 with 2 N KOH) (5:95) were estimated by analytical RP-HPLC at room temperature.

Hydrolytic stability: Freshly prepared solutions of **1** and **6** in HEPES buffer (pH 7.2) were left in the dark at room temperature and monitored over a period of 24 h by analytical RP-HPLC.

Photochemical quantum yields: The differential photochemical quantum yields (ϕ) were determined for **1** at 334 nm and for **6** at 365 nm in 5% CH_3CN/0.01 M HEPES/KOH buffer (pH 7.2) by the relative method as previously described[21] with use of (*E*,*E*)-1,4-diphenylbuta-1,3-diene in *n*-hexane (ϕ = 0.11) as standard for **1** and (6,7-dimethoxycoumarin-4-yl)methyl diethyl phosphate (ϕ = 0.08)[22] in 5% CH_3CN/0.01 M HEPES/KOH buffer (pH 7.2) as standard for **6**. Identical absorbances for the references and **1** or **6** were used during photolysis. For kinetic investigations the irradiated solutions of **1**, **6**, and (6,7-dimethoxycoumarin-4-yl)methyl diethyl phosphate were analyzed by analytical HPLC and those of (*E*,*E*)-1,4-diphenylbuta-1,3-diene by UV spectroscopy (λ = 328 nm).

Fluorescence quantum yields: The fluorescence quantum yield (ϕ_f) of **6** was determined at 25 °C in CH_3CN/HEPES buffer (5:95, pH 7.2), by the relative method[23] versus quinine sulfate in 0.1 N H_2SO_4 as a standard (ϕ_f = 0.545). At the excitation wavelength used, the absorbance values of the standard and the investigated compound were identical.

Isothermal titration calorimetry: In the ITC uptake experiment, aliquots (10 μL) of POPC small unilamellar vesicles (SUVs, 40 mM) were injected into a 1.4 mL sample cell containing **6** (100 μM). In the release experiment, aliquots (10 μL) of POPC SUVs (20 mM) prepared in the presence of **6** (541 μM) were titrated into pure buffer. In both experiments, an initial 5 μL injection was performed; this was excluded from evaluation because the first injection usually suffers from sample loss during the mounting of the syringe and the equilibration preceding the actual titration. Measurements were done at 25 °C after gentle vacuum degassing of both samples. Baseline subtraction and peak integration were accomplished by use of Origin 5.0 as described by the manufacturer (MicroCal Software, Northampton, MA).

Preparation of dorsal root ganglion neurons: Adult female Wistar rats were killed by CO_2 exposure, which was followed by cervical transsection. The spinal column was prepared and opened sagittally with scissors, and 30–40 dorsal root ganglia were dissected from the lumbar, thoracic, and cervical regions. Ganglia were cut into small pieces and incubated in collagenase solution (0.3% collagenase in DMEM, C-9891, Sigma, Germany, 2 mL) for 1 h at 37 °C. All experiments with animals were performed in accordance with the Animal Protection Law and the guidelines and permission of the University of Heidelberg.

After centrifugation (200 *g*, 20 min) the supernatant was discarded, and trypsin solution (0.25% trypsin in MEM, T-1426, Sigma, Germany, 2 mL) was added to the pellet. The pellet was resuspended by trituration with a truncated, fire-polished Pasteur pipette (3 mm opening). The ganglia were kept in trypsin solution for 30 min at 37 °C and were then centrifuged for 20 min at 200 *g*, the supernatant was discarded, and culture medium [DMEM + 10% FCS, 10106–169 (Gibco Life Technologies, Germany) and 1% Antibiotics/Antimycotics, 15240–062 (Gibco Life Technologies, Germany), 4 mL] was added to the pellet. The pellet was triturated, and then filtered through a 150 μm nylon mesh (Type 1110, Bückmann, Germany). This step allows the elimination of a substantial amount of myelin debris and nondissociated fragments of ganglia, which are retained on the nylon mesh. The pooled cells were plated on cover slips, successively coated with poly-L-lysine (P-1399, Sigma, Germany, 100 μg mL^{-1}) and laminin (L-2020, Sigma, Germany, 20 μg mL^{-1}), and cultured for 1–2 days at 37 °C in culture medium under an atmosphere containing CO_2 (5%). Nerve growth factor (NGF-β from rat, N-2513, Sigma, Germany) was added 1 h after plating at a final concentration of 50 ng mL^{-1}. Cells were used within 24–48 h after plating. To identify cells expressing TRPV1, primary cultures of somatosensory neurons were grown on laminin-coated cover slips and fixed by exposure to paraformaldehyde (4%) for 15 min, followed by a wash in PBS (130 mM NaCl, 7 mM Na_2HPO_4, 3 mM NaH_2PO_4, pH 7.4) and preincubation in PBS containing Chemiblocker (Chemicon, USA, 5%) and Triton X-100 (Sigma, Germany, 0.5%) for 20 min. Polyclonal TRPV1 antiserum (#PC420, Oncogene, Germany; dilution 1:1000) was then applied for 1 h in the same solution but without Triton X-100, followed by 20 min exposure to the secondary antiserum (Alexa-Fluor 568, goat anti-rabbit; Molecular Probes, Netherlands; dilution 1:2000). Cell nuclei were stained by 10 s exposure to DAPI solution (#C7590, Molecular Probes, Netherlands, 0.3 μM). Cover slips were then washed in PBS and embedded in AquaPolyMount (Polysciences, Germany) for fluorescence microscopy.

Patch-clamp recordings with caged capsaicin: Cultured cells were transferred to the recording chamber with standard extracellular solution (140 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 2.5 mM $CaCl_2$, 10 mM HEPES, 10 mM glucose, pH 7.4). Whole-cell currents and action potentials were recorded at room temperature from cells 20–30 μm in diameter, which were most probably polymodal nociceptors.[24] Transient expression of TRPV1 channels in HEK 293 cells was driven by insertion of the HindIII/NotI fragment into pcDNA3.1 (Invitrogen, Germany). Red fluorescent protein was used as a cotransfection marker. This vector was created by subcloning the KpnI/NotI fragment of pDsRed (Clontech, USA) into pcDNA3.1 (Invitrogen, Germany). The TRPV1-DNA (Genbank accession number AF029310) was a generous gift from Dr. Makoto Tominaga, Okazaki Institute for Integrative Bioscience, Okazaki, Japan. Patch electrodes (3–10 MΩ) were prepared from borosilicate glass by use of a horizontal puller (P-97, Sutter Instruments, USA) and filled with pipette solution (140 mM KCl, 5 mM NaCl, 1 mM $MgCl_2$, 10 mM

HEPES, 10 mM EGTA; pH 7.4). Patch-clamp recordings were performed with an EPC-7 amplifier (HEKA, Germany). In the current-clamp mode, the cells usually had membrane potentials of −60 to −80 mV. For action-potential recordings, a small command current was applied to set the potential near −70 mV. Voltage-clamp recordings were carried out at a holding potential of −70 mV. Cell membrane capacitance and series resistance were compensated. Data were collected with an ISO2 patch-clamp system (MFK, Germany) and were digitized at 10 kHz for current-clamp recordings and at 200 Hz for voltage-clamp measurements.

Na-CDMNB-caged capsaicin, DMNBOC-caged capsaicin, and BCMACMOC-caged capsaicin were prepared from stock solutions (10 mM in 100% DMSO, 41639, Fluka, Germany). For photorelease experiments, test solution (standard extracellular solution containing 2 μM or 10 μM caged capsaicin, 1 mL) was added to standard extracellular solution (1 mL) in the recording chamber. Photorelease experiments were started 10–30 s after application of the test solution. Caged capsaicin solution was exposed to the filtered output of a mechanically shuttered mercury arc lamp (AMKO, Germany) attached to the epifluorescence port of a Nikon Diaphot 300 microscope. Wavelengths <335 nm were cut off with a UV filter (WG335, AMKO, Germany). The uncaging beam was reflected by a 400 nm dichroic mirror (Nikon) and directed through an oil immersion objective (Nikon Fluor 40×, NA 1.3). Control measurements were carried out in caged-capsaicin-free standard extracellular solution (1 mL). Mean values are given ± standard deviation and number of experiments (*n*).

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