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Artificial sweeteners and salts producing a metallic taste sensation activate TRPV1 receptors

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Riera CE, Vogel H, Simon SA, le Coutre J. Artificial sweeteners and salts producing a metallic taste sensation activate TRPV1 receptors. *Am J Physiol Regul Integr Comp Physiol* 293: R626–R634, 2007. First published June 13, 2007; doi:10.1152/ajpregu.00286.2007.—Throughout the world many people use artificial sweeteners (AS) for the purpose of reducing caloric intake. The most prominently used of these molecules include saccharin, aspartame (Nutrasweet), acesulfame-K, and cyclamate. Despite the caloric advantage they provide, one key concern in their use is their aversive aftertaste that has been characterized on a sensory level as bitter and/or metallic. Recently, it has been shown that the activation of particular T2R bitter taste receptors is partially involved with the bitter aftertaste sensation of saccharin and acesulfame-K. To more fully understand the biology behind these phenomena we have addressed the question of whether AS could stimulate transient receptor potential vanilloid-1 (TRPV1) receptors, as these receptors are activated by a large range of structurally different chemicals. Moreover, TRPV1 receptors and/or their variants are found in taste receptor cells and in nerve terminals throughout the oral cavity. Hence, TRPV1 activation could be involved in the AS aftertaste or even contribute to the poorly understood metallic taste sensation. Using Ca^{2+} imaging on TRPV1 receptors heterologously expressed in the human embryonic kidney (HEK) 293 cells and on dissociated primary sensory neurons, we find that in both systems, AS activate TRPV1 receptors, and, moreover, they sensitize these channels to acid and heat. We also found that TRPV1 receptors are activated by $CuSO_4$, $ZnSO_4$, and $FeSO_4$, three salts known to produce a metallic taste sensation. In summary, our results identify a novel group of compounds that activate TRPV1 and, consequently, provide a molecular mechanism that may account for off tastes of sweeteners and metallic tasting salts.

multisensory taste; pain; calcium imaging

IN MANY FOODS, ARTIFICIAL sweeteners (AS) represent a major dietary supplement. Their consumption is involved with weight management, prevention of dental decay, and for diabetics the control of blood glucose. Saccharin, aspartame, and acesulfame-K are among the most commonly used AS. Cyclamate, although currently not approved for use in the United States, is used in more than 50 countries worldwide. Unlike sucrose, these compounds are not perceived to be sweet at all concentrations. In fact, for all these compounds, as concentration increases, the taste perception shifts from pleasant (sweet) toward unpleasant (bitter/metallic) (10, 12, 41). This shift has been explained by the activation at low concentrations of sweet tastant-sensing G protein-coupled receptors (GPCRs) T1R2/T1R3 (23) and at higher concentrations, the activation of the bitter tastant sensing GPCRs, T2R43, and T2R44 for saccharin and acesulfame-K (21). Other taste sensations related to the aftertaste elicited by artificial sweeteners have been attributed to their diffusion into taste receptor cells where they can alter intracellular signaling pathways (37, 38, 40, 57). A recent psychophysical study showed that it is sometimes difficult to distinguish between the bitterness caused by quinine and the irritating sensation produced by capsaicin, the principal pungent ingredient in chili peppers that activates TRPV1 receptors (24). This result suggests that some of the bitter taste sensation may involve the activation of capsaicin-sensitive TRPV1 receptors.

TRPV1 receptors or their variants are present in taste receptor cells (TRCs) and in sensory neurons in the mouth (15), where they are activated by acidic and thermal stimuli (5, 30, 51), as well as a wide range of molecules that include vanilloids, alcohols, terpenoids, aldehydes, and lipids. Vanilloids include capsaicin from chili pepper, resiniferatoxin from cactus resin (49), zingerone and gingerol from ginger (7, 28), and eugenol from cloves (55). TRPV1 receptors were also found to be activated by nonvanilloid plant-derived molecules like camphor (54), by the fungal extract scutigeral, and by piperine from black pepper (27, 36, 48). Other TRPV1 ligands include ethanol (1–3%), (52), reducing agents such as dithiothreitol (53), and certain 1,4 dialdehydes (e.g., isovelleral) (47). Endogenous TRPV1 ligands include molecules such as anandamide, *N*-arachidonyl dopamine, and eicosanoids (13, 14, 46, 59). Peptide toxins from tarantula venom also activate TRPV1 receptors (45). As the capsaicin binding site is on the cytoplasmic surface of TRPV1 (17, 18), all of these organic molecules must either be synthesized intracellularly or be able to permeate into the lingual epithelia, where they could diffuse into TRCs (40, 57, 58) or nerve terminals (4, 15) and eventually activate TRPV1 receptors.

TRPV1 receptors have also been shown to be directly activated on the extracellular side by high concentrations of Ca^{2+} and Mg^{2+} (1). At the sensory level, the taste perception of these two minerals is very close and was described as primarily bitter with additional sensations such as salty, metallic, astringent, sour and sweet qualities (25, 43). In addition to AS, metallic taste can be strongly elicited by metal salts containing zinc, iron and copper (22, 25, 56). Consequently, we explored whether TRPV1 receptors would be activated by these metallic salts and thus contribute to some of their gustatory sensations.

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To rationalize the off-tastes of the AS, we tested whether they could act on the somatosensory system by targeting TRPV1 receptors. In summary, we found that TRPV1 receptors are activated by both AS and selected metal salts and therefore can have a role in their irritating and metallic aftertaste perceptions.

MATERIALS AND METHODS

Chemicals. Capsaicin, saccharin, aspartame, acesulfame-K, sodium cyclamate (Fig. 1), capsazepine, sucrose, SB-366791, and ruthenium red were obtained from Sigma-Aldrich (Buchs, Switzerland). Stock solutions in DMSO were prepared: 1 mM capsaicin, 33 mM SB-366791, 33 mM capsazepine, and 1 M saccharin, which were kept at 4°C. When added to cells, the final concentration of DMSO was <0.1% (vol/vol) for capsaicin, SB-366791, and capsazepine and was <0.2% (vol/vol) for saccharin. $CaSO_4$, $MgSO_4$, $CuSO_4$, $ZnSO_4$, and $FeSO_4$ were obtained from Sigma-Aldrich. Bitter tasting molecules caffeine, theophylline, salicin, 6-*n*-propylthiouracil, phenylthiocarbamide, quinine-HCl, and β-glucopyranoside, which were also obtained from Sigma-Aldrich, were dissolved in buffer. All molecules were diluted with buffer to their final concentration.

Cloning and expression of human TRPV1, transient receptor potential ankyrin 1, transient receptor potential melastatin 8. A human TRPV1 expression construct was prepared from a cDNA clone obtained from RZPD (German Resource Center for Genome Research, Berlin, Germany). Following the manufacturer's instructions, the gene was cloned into pcDNA5/FRT (Invitrogen, Carlsbad, CA) and confirmed by sequencing. To obtain the insertion of the TRPV1 expression cassette by Flp recombination, HEK 293 cells containing a recombination site (Flp-In system; Invitrogen) were cotransfected with hTRPV1-pcDNA5/FRT and pOG44 using lipofectamine. Stable clones expressing TRPV1 were selected using hygromycin antibiotic selection, and colonies were expanded to obtain a large stock of TRPV1-expressing cells. TRPV1 protein expression was confirmed by Western blot analysis (positive band at 85 kDa). Human transient receptor potential ankyrin 1 (TRPA1) and transient receptor potential melastatin 8 (TRPM8) cDNA clones were obtained from Origene Technologies (Rockville, MD) and were transiently transfected in HEK 293 cells using lipofectamine 2000 (Invitrogen).

HEK cell culture. HEK 293 cells were grown as monolayers in DMEM (Sigma) supplemented with nonessential amino acids, 10% FBS, 2 mM L-glutamine and maintained at 37°C under 95% O_2-5% CO_2. hTRPV1-expressing cells were cultured with the antibiotic hygromycin. Cells were passaged every 3 or 4 days, and the highest passage number used was 14 for TRPV1-expressing cells and 20 for nontransfected cells. Capsaicin sensitivity in hTRPV1-expressing cells was assessed by imaging intracellular Ca^{2+} concentration (see below) and used as a control for TRPV1 expression.

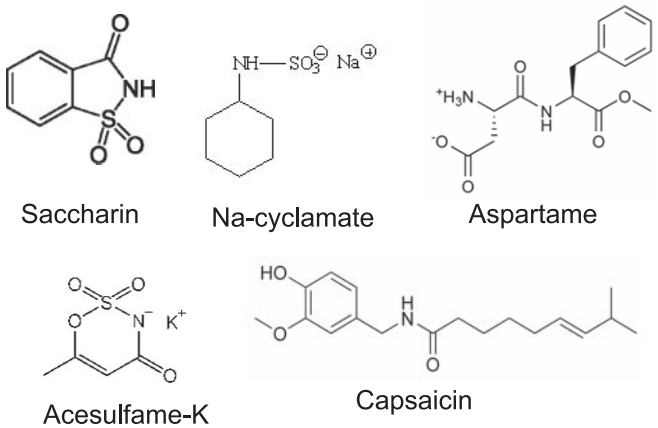

Fig. 1. Chemical structures of the studied artificial sweeteners (AS) diverge from the common vanilloid molecule, capsaicin (Cap). The molecular structure of AS is diverse, comprising dipeptide derivates (aspartame), N-sulfonylamides (acesulfame-K, saccharin), and sulfamate (Na-cyclamate). Capsaicin is a vanilloid (containing the 4-hydroxy-3-methoxybenzyl chemical group). At 25°C, the pK_a values of the corresponding acids are 1.8 for saccharin, 3.2 and 7.9 for the di-acid aspartame, 1.9 for cyclamic acid and 2 for acesulfame.

Measurement of intracellular calcium levels $[Ca^{2+}]_i$ using a fluorescent plate reader. TRPV1-, TRPA1-, TRPM8-expressing cells and nontransfected cells were seeded into 96-well plates (Costar, High Wycombe, UK) coated with poly-D-lysine at a density of ~100,000 cells per well in supplemented DMEM and cultured overnight. Cells were then incubated at room temperature for 20 min with Hank's balanced salt solution (HBSS) supplemented with 2 mM $CaCl_2$ and 20 mM HEPES, containing the cytoplasmic calcium indicator 2 μM Fura-2/AM (Molecular Devices, Sunnyvale, CA) and 0.04% pluronic acid (Molecular Devices). The cells were washed once and resuspended in the same HBSS buffer before being incubated for 25 min at room temperature with either HBSS buffer alone or containing the TRPV1 antagonist capsazepine (10 μM). The 96-well plates were then placed into the plate reader (λ_{ex1} = 340 nm, λ_{ex2} = 380 nm, λ_{em} = 508 nm), and Fura-2 ratios (F340/F380) were recorded to quantify the changes in $[Ca^{2+}]_i$ upon stimulation with AS and metallic salts. Solutions of agonists in HBSS at pH 7.4 were applied at different concentrations after 12 s and were not removed during the recording. The metallic salts acidified the HBSS pH for concentrations superior to 20 mM from pH 7.4 to 5.5.

All experiments were conducted at 37°C except TRPA1 and TRPM8 ligand assays, which were performed at room temperature (22°C), the acid potentiation experiments, which were also performed at 22°C, and the temperature variation experiments (Fig. 4). In the pH variation experiments, the solutions in which pH was adjusted with HCl were applied on the cells immersed in a small volume of buffer at pH 7.4. The final pH of the solutions at 25°C was measured using a pH meter (Methrom, Herisau, Switzerland) in separate vials. In the temperature variation experiments, the agonists' solutions were equilibrated at each temperature and applied on the cells that where incubated in a small volume of HBSS at 22°C.

Responses to molecules were expressed as a percentage of the maximum response evoked by 1 μM capsaicin at 37°C (under these conditions 1 μM was independently assessed to be a saturating concentration). For all experiments, the peak response was taken to be the characteristic value. Data were analyzed using SOFTmax PRO software (Molecular Devices). Dose-response curves and EC_{50} values were calculated using the Hill equation (GraphPad Prism Software, San Diego, CA). The Hill equation was calculated as

$$\%A = \%A_{MIN} + \frac{[\%A_{MAX} + \%A_{MIN}]}{1 + 10^{(\log EC50 - \log C)\cdot n}}$$

where %A is the percentage of activation, C is the concentration of ligand, n is the Hill slope of the curve.

Dorsal root ganglia (DRG) neuron imaging. Dissociated DRG neurons from neonatal (2 or 3 days) rats were obtained frozen in dry ice from Cambrex Bio Science (Walkersville, MD). Cells were thawed and resuspended in neurobasal medium (Invitrogen) supplemented with 2% B27 (Gibco, Gaithersburg, MD), 2 mM glutamine and 100 U/ml penicillin/streptomycin (Sigma). Cells were plated onto coverslips coated with poly-D-lysine (30 μg/ml) and laminin (2 μg/ml) and were cultured with β-nerve growth factor (Sigma) at a concentration of 5 ng/ml. Changes in $[Ca^{2+}]_i$ were measured using ratiometric digital fluorescence imaging (as above). Neurons were loaded at 37°C for 30 min with 5 μM Fura-2-AM and pluronic acid (0.04%) in the supplemented HBSS as above. This was followed by 20-min deesterification of Fura-2-AM in the dark at room temperature. Images of individual neurons were acquired with a cooled, charge-coupled device camera (Cascade II; Photometrics, Tucson,

AZ) mounted to an Axiovert100 inverted microscope. Autofluorescence was negligible and with illumination times of 100–300 ms, F340/F380 remained stable. The projected soma diameters (μm) were measured with a calibrated eyepiece under phase contrast illumination. Traces were recorded from neurons with soma diameters ranging between 18 and 30 μm.

Coverslips with attached neurons were placed in a flow chamber with continuous flow of supplemented HBSS. Chemical stimuli present in HBSS were applied at 30–33°C to the flow chamber for 30 s, and cells were rinsed 2 or 3 min in HBSS between stimuli. For each neuron the average fluorescence ratio F340/F380 was calculated using Metafluor software (Universal Imaging; Molecular Devices). In the SB-366791 antagonism experiments, neurons were first exposed to saccharin (or aspartame) followed by a 2-min wash with HBSS. Subsequently, SB366791 was perfused for 1 min, with saccharin (or aspartame) addition over the last 30 s. After 2-min wash, saccharin (or aspartame) and capsaicin were added sequentially. Neurons were finally washed with buffer, and then KCl (50 mM) was added to verify neuronal activity. Between 225 and 250 neurons were analyzed in 15 separate experiments, and each observation shown in Fig. 5 was made at least in triplicate.

RESULTS

AS activate TRPV1 in a dose-dependent manner but not TRPA1 and TRPM8 receptors. Upon measuring increases in intracellular Ca^{2+}, we found that AS, like capsaicin, activate TRPV1 receptors heterologously expressed in HEK 293 cells (Fig. 2). Such responses are not observed in nontransfected cells (Fig. 2). To ensure that the observed responses were specific to TRPV1 and could not be obtained in other chemosensitive TRP receptors, we recorded from HEK cells with expressed TRPA1 or TRPM8 receptors. As expected, TRPA1- and TRPM8-expressing cells were responsive to their specific ligands, cinnamaldehyde and allyl isothiocyanate for TRPA1 and menthol for TRPM8, respectively (3, 16, 35, 39), but at room temperature they were insensitive to AS (not shown). Moreover, to rule out involvement of bitter taste transduction pathways, we tested 5 mM caffeine, 10 mM theophylline, 10 mM salicin, 1 mM 6-*n*-propylthiouracil, 1 mM phenylthiocarbamide, 0.1 mM quinine-HCl, and 10 mM β-glucopyranoside. None of these bitter tasting molecules elicited a specific response in cells transfected with TRPV1 and only quinine-HCl resulted in equal Ca^{2+} mobilization in both transfected and nontransfected cells (not shown).

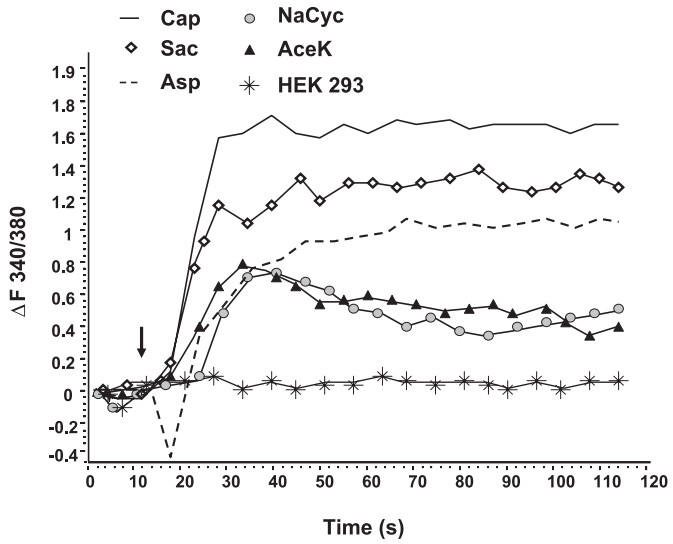

Fig. 2. $[Ca2+]_i$ flux induced by capsaicin and artificial sweeteners stimulation of human embryonic kidney (HEK) cells stably expressing transient receptor potential vanilloid-1 (TRPV1). $[Ca^{2+}]_i$ response induced by 1 μM capsaicin (Cap), 2 mM saccharin (Sac), 8 mM aspartame (Asp), 25 mM sodium cyclamate (NaCyc), and 25 mM acesulfame-K (AceK) were monitored using the FLEX assay in hTRPV1 stably expressing cells. $[Ca^{2+}]_i$ fluxes were measured as changes in fluorescence intensity, before and after the addition of agonists (indicated by arrow). The plotted signal corresponds to the ratio 340/380 nm. None of the tested molecules induced a $[Ca^{2+}]_i$ response in nontransfected cells (HEK 293), or in TRPA1- and TRPM8-expressing cells (not shown) at the used concentrations. One response of HEK 293 cells to 2 mM Sac is shown, the remaining responses were omitted for clarity. Obtained latency values between injection and maximum induced response were (in seconds) Cap (17 ± 1.7), Sac (20.6 ± 3), Asp (32 ± 6), NaCyc (25.3 ± 1.1), and AceK (26.6 ± 3). (n = 3, means ± SE).

Shown in Fig. 2 are typical responses evoked near maximal concentrations of saccharin (2 mM; Sac), aspartame (8 mM; Asp), Na-cyclamate (25 mM; NaCyc) and acesulfame-K (25 mM; AceK). In the TRPV1-expressing cells, the application of 1 μM capsaicin evoked a transient increase in $[Ca^{2+}]_i$ that was characterized by a relatively rapid initial onset and a peak occurring about 16 s after injection. Like capsaicin, the responses to saccharin and aspartame did not markedly desensitize in 30 s, whereas the responses to NaCyc and AceK desensitized. The initial slope of the fluorescence change for the AS is less steep than that found for capsaicin, and their maximal intensities were both smaller than for capsaicin and different from each other. Moreover, all four of the AS tested have a longer onset latency (time between compound injection and maximum induced response) than 1 μM capsaicin.

The initial decrease shown in the response to 8 mM aspartame arose at this high injected concentration by an increase in light scattering caused by small aggregates that reduced the fluorescence. However, the response increased to normal values once the aspartame diffused throughout the chamber. Moreover, at lower concentrations, the transient decrease in light scattering was not observed.

TRPV1-expressing cells respond to all four AS in a dose-dependent manner (Fig. 3*A*). Responses of Sac (0.1–2 mM), Asp (0.5–9 mM), NaCyc (1–25 mM), and AceK (1–50 mM) were measured as the peak increase in fluorescence, expressed relative to the maximum 1 μM capsaicin response. These data were then fitted using the Hill equation. The highest concentration used for each AS was established either because of curve saturation, as observed for Sac, or a TRPV1-independent Ca^{2+} influx (Asp), or by the contribution of the Na^+ or K^+ counter-ions in NaCyc and AceK, respectively. For NaCyc and AceK, the counter-cation influence was assessed using solutions of NaCl and KCl, respectively, having the equivalent Na^+ or K^+ concentrations. Below 25 mM, NaCl did not elicit a Ca^{2+} influx (Fig. 3*A*), whereas 25 mM KCl induced a significant increase in $[Ca^{2+}]_i$. From this result, it is evident that the influence of the K^+ ion in the AceK prevented an accurate calculation of an EC_{50} value.

To characterize further whether TRPV1 is specifically activated by AS, we challenged the AS activation with 10 μM capsazepine (CPZ), a TRPV1-antagonist. We found that AS activation was essentially eliminated in the presence of CPZ (Fig. 3*B*). In the presence of 10 μM CPZ, we found that the response to 1 μM capsaicin was inhibited by about 70%.

We also observed that natural membrane impermanent sweet tastants, such as sucrose at high concentrations (100 mM)

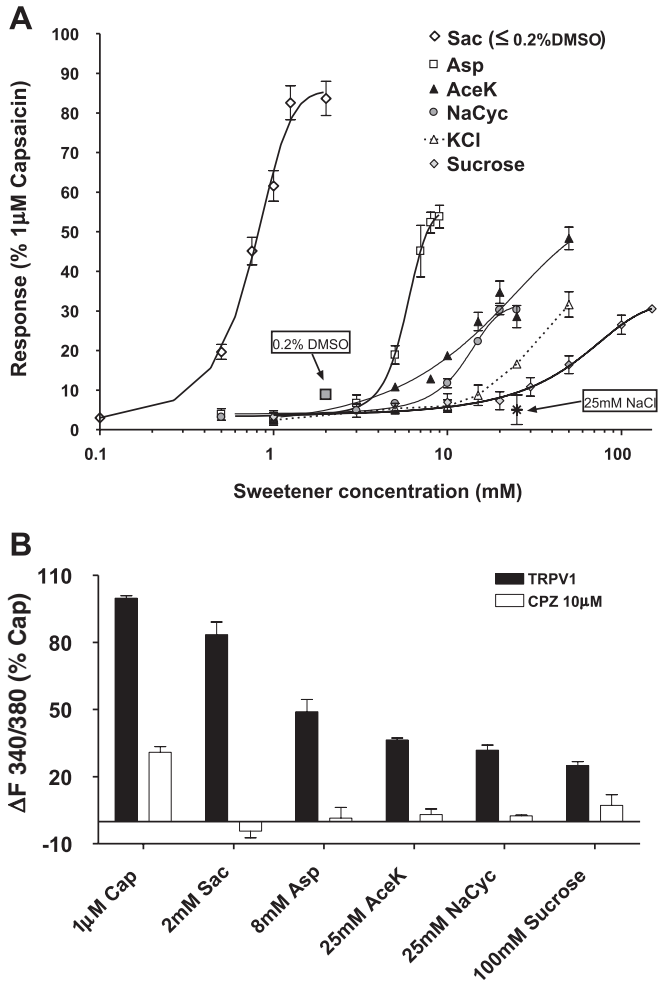

Fig. 3. Artificial sweeteners induce dose-dependent increases in $[Ca^{2+}]_i$ rise in TRPV1 expressing HEK 293 cells. *A*: $[Ca^{2+}]_i$ responses were monitored using Fura-2-AM in hTRPV1 HEK 293 cells before and after the addition of artificial sweeteners and the natural sweet tastant sucrose. Responses of saccharin (Sac; 0.1–2 mM), aspartame (Asp; 0.5–9 mM), Na-cyclamate (NaCyc; 1–25 mM), acesulfame-K (AceK; 1–50 mM), and sucrose (1–150 mM) were measured as a peak increase in fluorescence, normalized responses (means ± SE, $n = 3$ or 4) were expressed relative to the maximum 1 μM capsaicin response at pH 7.4 and fitted by the Hill equation. Obtained half-maximum activation concentrations were (in mM): EC_{50} (Sac) = 0.8 ± 0.03; EC_{50} (Asp) = 4.6 ± 0.001, EC_{50} (NaCyc) = 15.3 ± 3.2 and EC_{50} (sucrose) = 40 ± 28. Hill coefficients were H (Sac) = 2.04 ± 0.4, H (Asp) = 0.48 ± 0.12, H (NaCyc) = 0.14 ± 0.02 and H (sucrose) = 0.01 ± 0.008. NaCl (1–25 mM) and KCl (1–50 mM) were used to assess the influence of Na^+- and K^+-coupled sweeteners. Note that below 25 mM, NaCl did not induce a rise in $[Ca^{2+}]_i$. Acesulfame-K concentration-dependent increase in $[Ca^{2+}]_i$ was not saturating. At concentrations >25 mM, there is unspecific Ca^{2+} entry due to the K^+ cation, as shown by equimolar KCl response. Saccharin was dissolved in DMSO (final concentration ≤0.2%), which did not elicit a change in Ca^{2+} by itself. *B*: normalized maximum induced responses of the artificial sweeteners and the sweet-tasting molecule sucrose without (solid bars) and with (open bars) pretreatment with 10 μM capsazepine (CPZ). CPZ can selectively block Cap and fully inhibit all sweetener-induced responses. Values are presented as means ± SE; $n = 3$ or 4.

selectively evoke increases in intracellular calcium in HEK 293 cells with TRPV1 receptors in a dose-dependent manner (Fig. 3*A*). At higher concentrations non-TRPV1 dependent increases were observed (data not shown). These responses may result from changes in osmolality-induced effects on TRPV1 (26, 29, 52). Sucrose response could be inhibited by 10 μM CPZ (Fig. 3*B*).

Artificial sweeteners sensitize the thermal and acid response of TRPV1 receptors. In the absence of capsaicin and in a pH 7.4 buffer, TRPV1 channels are activated at ~42°C (5). In the presence of capsaicin (or at lower pHs), the activation temperature decreases (51). Here, we tested whether AS, like capsaicin, would also increase the thermal sensitivity of TRPV1 (Fig. 4*A*). Saccharin and aspartame were selected to explore their effect on the thermal responses of both transfected (with TRPV1) and untransfected HEK 293 cells, as they are the most potent and structurally different TRPV1 activators (Fig. 3). At

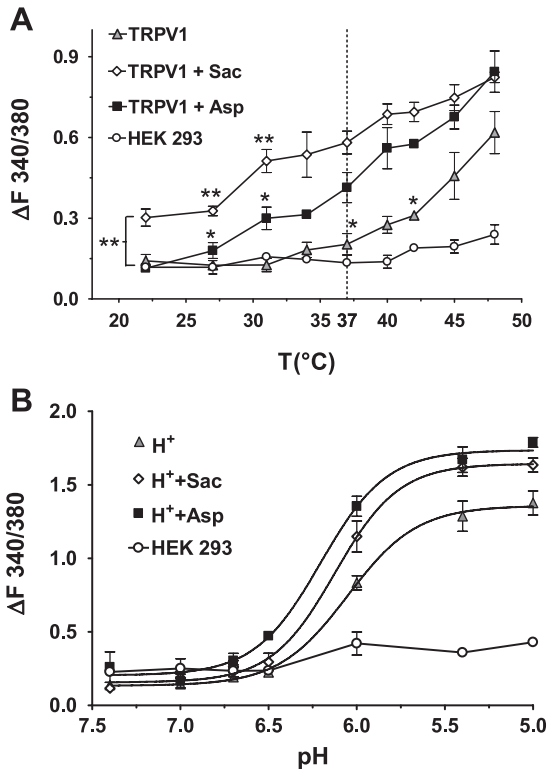

Fig. 4. Saccharin and aspartame shift the threshold and increase the magnitude of heat- and proton-evoked $[Ca^{2+}]_i$ activation in TRPV1-expressing HEK cells. *A*: Ca^{2+} release was induced by a shift of temperature in HEK 293 cells and hTRPV1-expressing cells in the absence or presence of 2 mM Sac or 8 mM Asp. 0.2% DMSO (not shown) had no effect by itself. To quickly shift cells to a desired temperature, buffer heated to a particular temperature, was added to TRPV1 cells incubated at 22°C. Note that both Sac and Asp can lower the threshold of TRPV1 heat activation. Data marked with asterisk and double asterisk indicate significant increase in $[Ca^{2+}]_i$ producing a break in activation ramp (*$P < 0.05$ and **$P < 0.01$, unpaired *t*-test). Note that this break shift from 42°C in TRPV1-expressing cells to 31°C when Sac or Asp are added. Vertical line indicates the observed activation at 37°C. Ca^{2+} signaling in nontransfected cells was basal at room temperature and not affected by the temperature changes. Means ± SE ($n = 5$ or 6). *B*: Buffer with increased acidity (7.4 to 5), adjusted with 1 M HCl was applied either alone or with 2 mM Sac or 8 mM Asp on both TRPV1-expressing cells and HEK 293 cells. One single trace represents the $[Ca^{2+}]_i$ in HEK 293 cells, as the three conditions produced the same fluorescence profile typified by a small increase at pH 6. Concentration-dependent responses were fitted by the Hill equation. Both Sac and Asp increased the efficacy for TRPV1 proton-gated opening by lowering significantly the half-maximal response from pH 6 ± 0.04 to pH (Sac) = 6.13 ± 0.04 and pH (Asp) = 6.2 ± 0.04. Sac and Asp also increase the maximally induced response to 21 ± 3.9 and 27 ± 3.5%, respectively. Hill coefficients were H (H^+) = 2.2 ± 0.63, H (H^+ + Sac) = 2.39 ± 0.43, H (H^+ + Asp) = 2.32 ± 0.37. Values are presented as means ± SE; $n = 4$.

each particular incubation temperature (22°C to 48°C with intervals of 2–5°C), heated buffer was added to TRPV1 cells to induce a temperature jump. In nontransfected cells with either buffer alone or in the presence of 2 mM Sac or 8 mM Asp, no increase in Ca^{2+} was monitored. In transfected cells, an abrupt increase in Ca^{2+} influx was observed at about 42°C, whereupon it monotonically increased with temperature to 48°C. It is seen that in the presence of saccharin or aspartame, there is no obvious break in the fluorescence-temperature relationship; moreover, at virtually every temperature, including 37°C (vertical dotted line), the fluorescence was greater than it was in the absence of these agonists. These data suggest that, like capsaicin, at physiological temperatures, TRPV1 receptors are sensitized by saccharin and aspartame. As controls, we found no temperature-dependent increase in Ca^{2+} was evoked in nontransfected cells with either buffer alone or in the presence of 2 mM Sac or 8 mM Asp.

Another important activator of TRPV1 are protons. Moreover, in the presence of capsaicin and elevated temperatures, the sensitivity to protons increases (5, 51). To further characterize synergies between AS and other known TRPV1 ligands, we tested the influence of pH on the potency of saccharin and aspartame. Indeed, we found that the addition of 2 mM Sac or 8 mM Asp shifted the half-maximal activation of the pH curve from 6 to 6.13 for 2 mM Sac and from 6 to 6.2 for 8 mM Asp. In addition, AS potentiated the maximum induced pH response and increased the Hill coefficients (Fig. 4*B*).

Sensory neurons are selectively activated by artificial sweeteners. To address whether AS would stimulate native TRPV1-containing cells, we imaged $[Ca^{2+}]_i$ of acutely dissociated DRG neurons in their presence. We choose neurons with small- to medium-diameter somas (<30 μm), as they were likely nociceptors that contain TRPV1 (5). We found that the application of 500 nM capsaicin evoked significant increases in $[Ca^{2+}]_i$ for 89 of the 225 neurons tested (Fig. 5).

As shown in Fig. 5*B*, 2 mM saccharin evoked $[Ca^{2+}]_i$ transients, albeit exclusively in the DRG neurons that were capsaicin sensitive. The capsaicin-insensitive population of neurons only responded to 50 mM KCl (Fig. 5, *A* and *B*). Similar results were found for 8 mM aspartame (Fig. 5*C*). Cells that responded to Sac, Asp, and NaCyc also responded to subsequent application of 500 nM Cap, whereas capsaicin-insensitive cells did not respond to any of these AS (Fig. 5*B*).

The activation of capsaicin-sensitive neurons by capsaicin or AS was blocked by preincubation with 10 μM SB-366791, a highly specific TRPV1 antagonist (9) (Fig. 5, *A–C*). However, after washing SB-366791 away, ligand-evoked responses could be recovered, and moreover, the capsaicin-sensitive neurons were again responsive to subsequent applications of saccharin (or aspartame) and capsaicin (Fig. 5, *B* and *C*). Neurons that did not respond to either sweeteners or capsaicin were found to be responsive to KCl.

The capsaicin-sensitive neurons also responded to AceK (Fig. 5*D*). All neurons responding to KCl were activated by AceK, even those insensitive to capsaicin, which is likely due to the depolarizing activity of K^+. However, capsaicin-sensitive cells responded faster and with a larger amplitude than the other cells, indicating the importance of the acesulfame anion for TRPV1 activation process.

Selected metal salts directly gate TRPV1 expressed in HEK 293 cells. Another category of molecules that exhibit an unpleasant "off-taste" are the salts $CuSO_4$, $ZnSO_4$, and $FeSO_4$ (25). Here, we tested whether like AS, they could activate TRPV1 receptors. All three salts increased $[Ca^{2+}]_i$ levels in TRPV1-expressing cells but did not in nontransfected cells (Fig. 6*A*). The increase in intracellular calcium was not dose-dependent, most likely because these salts will form complexes in solutions like HBSS, and consequently, they do not behave as completely dissociated ions (34). For example, with increasing concentrations, the responses to $CuSO_4$ first augmented,

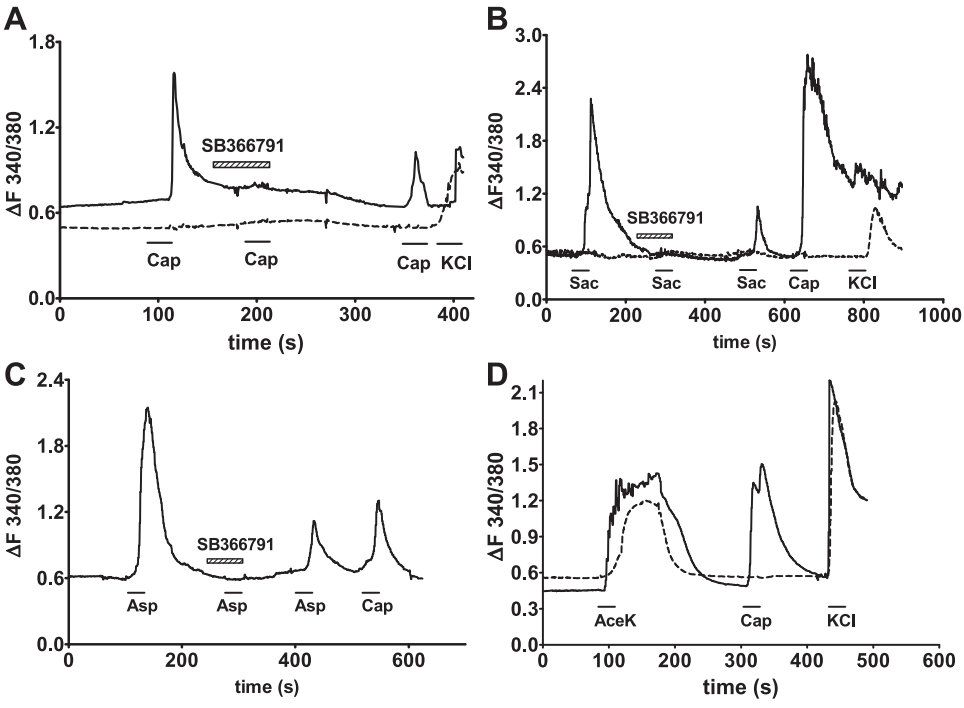

Fig. 5. Artificial sweeteners selectively activate capsaicin-sensitive dorsal root ganglia (DRG) neurons. $[Ca^{2+}]_i$ transients are represented as an increase in fluorescence ratio (F340/F380; basal amplitude = 0.5 ± 0.09) of Fura-2 loaded DRG neurons (225–250 cells from at least three separate cultures were analyzed) evoked by Cap, Sac, Asp, AceK, and NaCyc. Compounds were applied for 30 s, as shown by horizontal bars in the figure, followed a 2-min wash with physiological solution (HBSS). SB366791 applications are shown as indicated by horizontal dashed bars. Successive molecules applications were 500 nM Cap, 10 μM SB366791, 500 nM Cap + 10 μM SB366791, 500 nM Cap and 50 mM KCl (*A*); 2 mM Sac, 10 μM SB366791, 2 mM Sac + 10 μM SB366791, 2 mM Sac, 500 nM Cap and 50 mM KCl (*B*); 8 mM Asp, 10 μM SB366791, 8 mM Asp + 10 μM SB366791, 8 mM Asp, 500 nM Cap, and 50 mM KCl (*C*); 25 mM AceK, 500 nM Cap, and 50 mM KCl (*D*). Typical traces from single neuron represent $[Ca^{2+}]_i$ transients from capsaicin-sensitive (solid line) and capsaicin-insensitive (dashed line) cells.

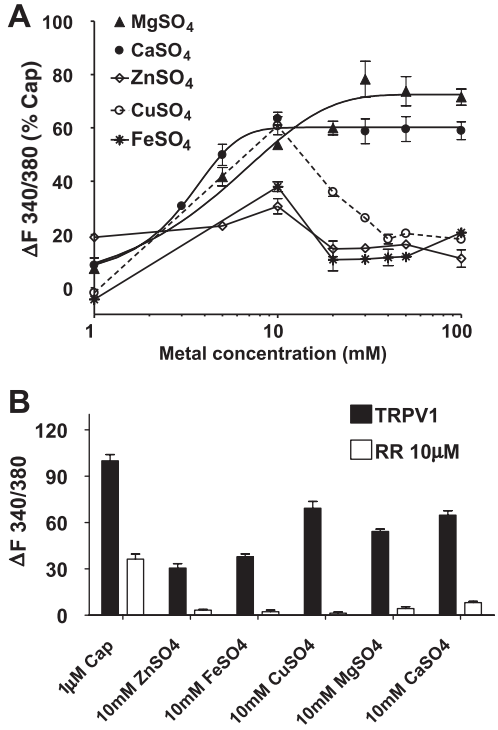

Fig. 6. Multivalent metal salts induce dose-dependent increases in $[Ca^{2+}]_i$ rise in TRPV1 expressing in HEK 293 cells. *A*: $[Ca^{2+}]_i$ responses (% 1 μM Cap) were monitored using Fura-2-AM in hTRPV1 HEK 293 cells before and after the addition of a concentration range (1–100 mM) of $CaSO_4$, $MgSO_4$, $CuSO_4$, $ZnSO_4$, and $FeSO_4$. Note that only $CaSO_4$ and $MgSO_4$ exhibit a dose-dependent effect. The other ions display their maximum activation at 10 mM. None of the metal salts induced a $[Ca^{2+}]_i$ rise in TRPA1 and TRPM8 expressing cells. *B*: Normalized maximum-induced activation of the metals without (solid bars) and with (open bars) pretreatment with 10 μM ruthenium red (RR). RR can selectively block Cap and fully inhibits all metal-induced responses. Values are presented as means ± SE; $n = 3$ or 4.

reached a maximum at 10 mM, and then monotonically decreased. The same pattern was observed for $FeSO_4$ and $ZnSO_4$ even though they induced a smaller Ca^{2+} increase. Using whole cell patch-clamp, Ahern et al. (1) showed in TRPV1 receptors expressed in HEK 293 cells that $CaSO_4$ and $MgSO_4$ salts activate the receptor in a concentration-dependent manner, most likely because these salts are almost completely dissociated. Using measurements of intracellular calcium, we have obtained similar results to Ahern et al. (1) with calcium and magnesium salts (Fig. 6*A*). To further demonstrate that these salts activate TRPV1, we found that they all were completely inhibited by 10 μM ruthenium red, a TRPV1 antagonist (Fig. 6*B*). We also found that with exception of 10 mM $CuSO_4$, 10 μM CPZ also inhibited the responses to the others salts (not shown). Metal salts were also applied on TRPA1- and TRPM8- expressing cells but no response was monitored.

DISCUSSION

The low caloric artificial sweeteners saccharin, aspartame, acesulfame-K, and cyclamate are widely used as a food supplement. However, increasing concentrations of all these compounds shift their sensory attributes from sweet, via activation of T1Rs, to an unpleasant long-lasting taste sensation comprising bitter and metallic components. Although the bitter component can be partially accounted for by the activation of specific T2Rs, the molecular mechanisms of these other taste sensations are poorly understood. To this point, we found that AS and salts that produce a metallic taste are effective agonists for the human TRPV1 receptor. As capsaicin-sensitive TRPV1 receptors are expressed in nociceptive sensory neurons and taste cells, our results not only present a novel class of ligands for TRPV1 channels but also provide a molecular connection that can rationalize the "off tastes" associated with the artificial sweeteners.

Artificial sweeteners activate TRPV1 receptors. We have obtained unequivocal evidence that the four most commonly used AS (saccharin, aspartame, acesulfame-K and sodium cyclamate) activate TRPV1 receptors. In particular, we found all four AS activated TRPV1 receptors in a dose-dependent manner (Fig. 3). In nontransfected HEK 293 cells, AS either did not evoke a response or evoked responses that could be accounted for by an increase in KCl concentration (see below for AceK). Further evidence that AS activate TRPV1 channels is that their responses were largely inhibited by CPZ (Fig. 3*B*). Moreover, like capsaicin, AS were found to sensitize TRPV1 responses to heat (Fig. 4*A*) and acidic stimuli (Fig. 4*B*). Indeed, the sensitization to thermal stimuli suggests that TRPV1 receptors would be active at physiological temperatures in the mouth (34–37°C) (Fig. 4).

In primary sensory neurons, we found that neurons that are responsive to capsaicin (nociceptive) are also responsive to AS (Fig. 6). Moreover, the AS-evoked responses were inhibited by SB366791, a selective TRPV1 antagonist (9).

For heterologously expressed TRPV1 channels, acesulfame-K was able to induce an increase of $[Ca^{2+}]_i$ superior to and independent from the K^+-induced activation (Fig. 3*A*). This behavior correlates well with the Ca^{2+} imaging experiments on DRG neurons in which one subpopulation of neurons was sensitive to capsaicin and Ace-K, whereas the remaining neurons responded only to Ace-K and KCl. One rationalization of these experiments is that increasing KCl will depolarize the neuron and activate voltage-dependent Ca^{2+} channels (VDCC) that are widely expressed in all sensory neurons (20). VDCC typically respond to 50 mM KCl stimulation, which evokes extracellular Ca^{2+} influx.

Do AS diffuse across the plasma membrane to activate TRPV1 receptors? Having established that AS activate TRPV1 receptors, we inquired whether, like capsaicin, the AS bind to an intracellular binding site (17, 18). This is of interest because at pH 7.4, the AS are far from their pK_as and are predominantly in their anionic form (Fig. 1). Therefore, in the absence of any specific transport pathway, AS would be expected to be relatively (to the uncharged form) membrane impermeable because of the large energy barrier involved in transferring a charge from water into the low dielectric environment of a membrane (2).

Recently, Hill and Schaeffer (11) found that trinitrophenol, an anion that does not penetrate HEK 293 cell membranes, activates TRPA1 receptors but not TRPV1 receptors. However, in a series of papers, DeSimone (8) and Naim and colleagues (37, 38, 40) addressed this issue and have demonstrated that at neutral pH, saccharin (presumably in its anionic form) diffuses across liposomes and TRCs, where it accumulates and may affect intracellular pathways. The question of anion transport across membranes remains puzzling (8). However, Thomae et

al. (50), who recently investigated the permeation of weak acids across lipid bilayers, may have provided a rationale for this conundrum. They found that small amphiphilic anions do indeed permeate across bilayers but at a much slower rate than the uncharged form of the acid. They wrote, "The anions, therefore, controlled the total permeation already at 1–2 pH units above their pK_a. These results indicate that in contrast to the expectations of the pH-partition hypothesis, lipid bilayer permeation of an acidic compound can be completely controlled by the anion at physiological pH." In this regard, we note that the pK_as of the AS would be increased by their partitioning into the low dielectric interfacial region of the bilayer (32, 44). Because these results suggest that anions can diffuse into the cytoplasm, they are consistent with an intracellular binding site for the AS. Whether it is the same as that of capsaicin will require further experimentation. How the membrane-impermeable sucrose molecules activate TRPV1 is not at all clear, but one possibility is that at 37°C, where the energy difference between open and closed states is small, the change in osmotic pressure (26) will be sufficient to open the channels. In summary, although TRPV1 channels can be opened by a variety of mechanisms, our data are most consistent with one in which AS activate it via intracellular binding sites.

How the activation of TRPV1 by AS may account for their "off- and aftertastes". From psychophysical studies, it was found that the sensory attributes of AS change and shift from pleasant sweet to an unpleasant bitter and/or metallic taste sensation with increasing concentration. In addition, when AS are compared with sucrose, they exhibit a longer time for the taste sensation to be extinguished, thus giving them a lingering aftertaste. In the following, we discuss the possibilities of how TRPV1 receptors may influence these phenomena.

Chemosensation in the mouth can originate from the activation or inhibition of the corresponding signaling pathways of the TRCs, their associated neurons, and also primary somatosensory neurons expressing TRPV1 receptors in their nerve terminals, which are distributed throughout the oral cavity. Initially, we consider how AS may affect the taste system via transduction pathways in TRCs. The elegant work of Naim and colleagues (40, 57, 58) provides a rationale for the lingering aftertaste of AS that does not require the activation of TRPV1 channels. They showed that AS, like saccharin, can diffuse across the plasma membrane of the TRCs and accumulate, at relatively high concentrations, in the cytoplasm where they may interact with and subsequently delay signal-termination components located downstream of the sweet or bitter responding GPCRs (depending on the concentration). This inhibition in delay in signal termination would therefore extend the taste response, thereby giving rise to the sweet and bitter aftertaste associated with AS. Also, along these non-TRPV1 receptor mechanisms, we note that amphipathic molecules have been shown to open a variety of initially closed ion channels and thus may open channels in TRCs (6, 33). Finally, in *Xenopus laevis* melanophores, it has been shown that saccharin can activate the melatonin receptor, which also is expressed in rat circumvallate papille taste buds (57).

In regard to mechanisms by which AS may activate TRPV1 channels to produce taste sensations, let us now consider the TRPV1 splice variant (TRPV1t) that was identified and characterized by Lyall and colleagues (29–31) in rodent TRCs. These researchers have shown that TRPV1t, like TRPV1, is activated by capsaicin, and temperature and is modulated by ethanol. In addition, upon obtaining whole nerve chorda tympani recordings from rats and wild-type and trpv1$^{-/-}$ mice, they found that TRPV1t activation is involved in the tonic salt response obtained in the presence of inhibitors of ENaCs (e.g., benzamil, amiloride). In this regard, AS activation of TRPV1t could influence the taste sensation in a manner that is dependent on what other taste receptors are present in TRCs containing TRPV1t. To this point, several studies have described the off-tastes of AS as being metallic. Helgren et al. (10) reported that about 25% of a European population characterized an off-taste to saccharin as metallic or bitter. Schiffman et al. (42) found a high variability in the intensity and quality of acesulfame-K that seemed to arise from its bitter and metallic side tastes. Recently, Lim and Lawless (25) characterized metallic taste as the taste of multivalent cations, such as iron, calcium, magnesium, and zinc. This activation by multivalent cations points to a possible role of TRPV1t in metallic taste since Ahern et al. (1) showed that TRPV1 receptors can be activated by high concentrations of calcium and magnesium that bind to sites on the extracellular surface. In this regard, we have found that physiologically relevant concentrations of $CuSO_4$, $ZnSO_4$, and $FeSO_4$ will also activate TRPV1 receptors (Fig. 6*A*) and suggest that the activation of TRPV1t (or other TRP channels in TRCs) by these compounds (as well as Ca^{2+} and Mg^{2+}) could also contribute to metallic taste.

A third candidate AS pathway that would influence taste sensation in a receptor-mediated manner would be TRPV1 channels on the terminals of neurons from the geniculate ganglion. In this regard, using mRNA and immunocytochemistry, Katsura et al. (19) identified TRPV1 channels on the somas geniculate ganglion (GG) neurons. However, it is not known whether these receptors are functional or even on the nerve terminals surrounding TRCs in fungiform papillae. If this were the case, however, then the AS would have to diffuse across the tight junctions between TRCs into the extracellular space between TRCs for them to contact the GG nerve terminals. Interestingly, this may account for the long latency seen with AS.

Our findings that AS activate the somatosensory system through capsaicin-sensitive dissociated primary sensory neurons (Fig. 5) lead to an interesting conclusion: if they can diffuse into the lingual epithelium and activate capsaicin-sensitive trigeminal neurons, then this could contribute to their taste sensation and, moreover, may rationalize the bitter and metallic latencies associated with AS. However, we are unaware of any sensory study describing that AS have a pungent component. Nevertheless, a recent paper from Lim and Green (24) explored at the perceptual level whether bitter and burning/irritating attributes are related. They found, under several conditions, that the bitter taste sensation of quinine and the burning/irritating sensation from capsaicin are extremely similar and even nondistinguishable. We suggest that AS at high concentrations activate T2Rs and TRPV1, which may confuse the sensations. This would provide an explanation for the unclear bitter/metallic aftertaste.

In summary, we found two novel stimulants of TRPV1 receptors: AS and metallic salts. We hypothesize that these interactions may account for of the aversive off-tastes of these compounds.

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