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Different vanilloid agonists cause different patterns of calcium response in CHO cells heterologously expressing rat TRPV1

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Abstract

The vanilloid receptor subtype 1 (TRPV1 or VR1) is expressed in nociceptive primary afferents of the C-fiber 'pain' pathway and has attracted considerable attention as a therapeutic target. Here, using rat TRPV1 heterologously expressed in Chinese hamster ovary cells, we show that different agonists show different patterns of modulation of the intracellular Ca^{2+} concentration, monitored in individual cells by fura-2 Ca^{2+} imaging. We identified 5 parameters (potency, maximal response, latency of response, variability of latency of response among individual cells, and desensitization) which behaved differently for different compounds. The potencies of the compounds examined ranged from EC_{50} values of 80 pM to 9 μM. Peak levels of induced $[Ca^{2+}]_i$ were observed either higher (RTX) or lower (anandamide) than for capsaicin. Significant latencies of response were observed for some (e.g. RTX) but not other derivatives, with great variation among individual cells in this latency. Marked desensitization after stimulation was detected in some cases (e.g. anandamide, capsaicin); for others, no desensitization was observed. We conclude that structurally diverse vanilloid agonists induce marked

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diversity in the patterns of Ca^{2+} response. This diversity of response may provide opportunities for pharmacological exploitation.
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Introduction

The detection of painful stimuli occurs primarily at the peripheral terminals of specialized sensory neurons called nociceptors. These remarkable sensory cells respond to a broad range of physical (e.g. heat, cold, and pressure) and chemical (e.g. acid, irritants, and inflammatory mediators) stimuli. The capsaicin (vanilloid) receptor (TRPV1), an excitatory ion channel expressed by C-fiber and some Aδ-fiber sensory neurons (Caterina et al., 1997), is one of the most important systems for the detection and integration of pain-producing chemical and thermal stimuli (Caterina and Julius, 2001; Szallasi and Blumberg, 1999).

TRPV1 represents a potential therapeutic target for a broad range of diseases, including neurogenic bladder, benign prostatic hypertrophy, interstitial cystitis, urinary tract infection, pharyngitis, mucositis, pancreatitis, enteritis, cellulitis, postherpetic neuralgia, peripheral neuropathy, arthritis and bony fractures (Robbins, 2000). Several TRPV1 agonists, including capsaicin (Ellison et al., 1997; Kingery, 1997; Kost and Straus, 1996; Low et al., 1995; Ross et al., 2001) and its ultrapotent analogue resiniferatoxin (Cruz et al., 1997; Kim and Chancellor, 2000; Lazzeri et al., 2000; Silva et al., 2000), are currently in clinical trials.

The evidence for potential therapeutic utility of vanilloids has motivated medicinal chemical efforts (Appendino et al., 2003; Appendino et al., 2002; Walpole et al., 1993c; Walpole et al., 1993a; Walpole et al., 1993b; Wrigglesworth et al., 1996). In addition to the usual concerns of potency and metabolic stability, an on-going issue has been the acute pungency associated with TRPV1 activation. The identification of the ultrapotent capsaicin analogue resiniferatoxin (RTX) (Szallasi and Blumberg, 1989) helped show that such issues were solvable. Capsaicin and RTX both desensitize sensory neuronal functions in vivo. However, resiniferatoxin was at least 1000 times more potent in many systems while being only modestly more pungent than capsaicin. The molecular mechanisms underlying partial dissociation between desensitization and pungency remain unclear but are of great importance.

As vanilloids with novel properties have been described by us (Lee et al., 2002; Lee et al., 2004) and by others (Bevan et al., 1992; McDonnell et al., 2002; Ross et al., 2001; Wahl et al., 2001), they have typically been functionally characterized in vitro by assay of $^{45}Ca^{2+}$ influx or measurement (fluorometric) of intracellular Ca^{2+} concentration, and their affinities for receptor binding have been determined by competition of [^{3}H]RTX binding. Here, we show that different compounds induce distinct patterns of modulation of TRPV1 activity as determined by Ca^{2+} imaging. The compounds differ in potency, in extent of maximal response, in latency of response, in variation in latency of response among individual cells, and in desensitization. These results emphasize the complexity of vanilloid biochemical pharmacology, the potential opportunities for dissection of this complexity through medicinal chemistry, and the importance of considering such variation in the design of in vitro assays for evaluation of compounds.

Materials and methods

Materials

Capsaicin was from Sigma (St. Louis, MO, USA), RTX and anandamide were from Biomol (Plymouth Meeting, PA, USA). MSK195, *N*-[2-(3,4-dimethylbenzyl)-3-(pivaloyloxy)propyl]-2-[4-(2-aminoethoxy)-3-methoxyphenyl]acetamide, was initially described as compound 13 (Lee et al., 2002). JYL79, *N*-[2-(3,4-dimethylbenzyl)-3-(pivaloyloxy)propyl]-*N*′-(4-hydroxy-3-methoxybenzyl)thiourea, was initially described as compound 23 (Lee et al., 2001). SU200, *N*-(4-*tert*-butylbenzyl)-*N*′-(4-hydroxy-3-methoxybenzyl)thiourea, was initially reported as compound 1i (Wrigglesworth et al., 1996) and was prepared as described therein.

Cell culture

The selected stable CHO cell clone expressing TRPV1 (Tet-Off induced CHO-TRPV1 cells) was the generous gift of James E. Krause and Daniel N. Cortright (Neurogen Corp., Branford, CT). These cells were cultured in maintaining media (F12 supplemented with 10% FBS (USA sourced), 25 mM HEPES, pH 7.5, 250 μg/ml geneticin (all from Life Technologies Inc., Rockville, MD, USA) and 1 mg/L tetracycline (Calbiochem, La Jolla, CA, USA).

Intracellular Ca^{2+} imaging

Cells (CHO-TRPV1 Tet-off cells) were plated on 25 mm round glass coverslips in maintaining media (F12 supplemented with 10% FBS, 25 mM HEPES, pH 7.5, 250 μg/ml geneticin and 1 mg/L tetracycline). The next day, the media was changed to inducing media (maintaining media without tetracycline but containing 1 mM sodium butyrate) to induce TRPV1 expression. Experiments were performed approximately 24 hours after induction. For fura-2 loading, the cells were incubated in DPBS containing calcium and magnesium (Life Technologies Inc., Rockville, MD, USA), 1 mg/ml BSA (Sigma, St. Louis, MO, USA), and 5 μM fura-2-AM (Molecular Probes, Eugene, OR, USA) for 2 hours at 20 °C. The cells were then kept in maintaining media at room temperature until the measurements, which were carried out in DPBS containing 1 mg/ml BSA. The fluorescence of individual cells treated with a single dose of the agonists was measured with an InCyt Im2 fluorescence imaging system (Intracellular Imaging Inc., Cincinnati, OH, USA). This procedure was used to avoid the possible desensitization that might occur if increasing doses were given to the same cells in a cumulative fashion. The cells within a field were illuminated alternately at 340 and 380 nm. Emitted light >510 nm was measured. To determine the approximate intracellular Ca^{2+} concentrations, the system was calibrated using a fura-2 calibration kit according to the manufacturer's instructions (Molecular Probes, Eugene, OR, USA). Data were analyzed with the Incyt 4.5 software and further processed with Excel (Microsoft), GraphPad Prism 2.0 (Graphpad Software) and Origin 6.0 (OriginLab Corp., Northhampton, MA) software.

Determination of the EC_{50} and the maximal intracellular Ca^{2+} concentration

The TRPV1 agonist was added at 1 min to fura-2 loaded CHO-TRPV1 cells and the cells were monitored continuously for an additional 10 min. The maximal averaged response ($[Ca^{2+}]_i$) was fitted as

a function of the concentration of the applied agonist according to the Hill equation to yield an EC_{50} value (half maximally effective concentration). Maximal values were compared to those induced by a maximally effective dose of capsaicin in the same experiment and differences were evaluated by t-test (significant difference if $p < 0.05$). Experiments were performed three times, each on different days with 28–49 cells measured simultaneously each time.

Latency

The recorded raw data were analyzed from individual experiments in which the compound was applied at a concentration near the previously determined EC_{50} value (yielding 35–60% of maximal effect). The responses of the individual cells were evaluated from three independent experiments (34–49 cells in each experiment). At each time point, cells in which the increase in $[Ca^{2+}]_i$ was greater than 10% of the maximal average increase in $[Ca^{2+}]_i$ for the entire population of cells during the time period were scored as responders at that specific time. The latencies were then calculated from the combined results of the three experiments.

Desensitization

The cells were treated with the agonists (at a concentration yielding 35–60% of the maximal response) for 20 min. Starting at 1 min after addition, the responses of the individual cells were averaged, then the $[Ca^{2+}]_i$ measured at 21 min (desensitized) was compared to the maximal $[Ca^{2+}]_i$ during the first 11 min (peak level, control). Experiments were done 3–4 times and the data were analyzed to calculate the mean, S.E.M. and statistical significance (compared to the control, significant difference if $p < 0.05$).

Results

In an effort to better understand the potential diversity of response to vanilloids, we have characterized in detail several novel agonists, together with some better studied compounds, namely capsaicin, resiniferatoxin (RTX) and anandamide (see Fig. 1 for structures). SU200 was developed as a capsaicin analog of enhanced potency (Wrigglesworth et al., 1996). JYL79 and MSK195 were designed based on a proposed pharmacophore model derived from RTX (Lee et al., 1999). We measured simultaneously the responses of multiple individual CHO-TRPV1 cells (CHO cells expressing the rat vanilloid receptor subtype 1) by intracellular Ca^{2+} imaging so that we could determine both average behavior and the degree of individual variation among cells.

We first compared the dose response curves for elevation of intracellular Ca^{2+} concentrations ($[Ca^{2+}]_i$) upon treatment with the different compounds. The cells were incubated for 1 min in the absence of agonist; they were then treated with a series of concentrations of the agonist for 10 min and the peak response at each concentration was determined. The EC_{50} values and the maximal $[Ca^{2+}]_i$ values were obtained by fitting to the Hill equation. The EC_{50} values ranged from 10^{-10} M to 10^{-5} M (RTX, 81 ± 20 pM; SU200, 217 ± 161 pM; JYL79, 2.46 ± 1.0 nM; capsaicin, 35 ± 11 nM; MSK195, 52 ± 12 nM; anandamide, 9 ± 0.2 μM, Fig. 2A). These EC_{50} values were all close to those determined from $^{45}Ca^{2+}$ uptake experiments (Lee et al., 2001; Lee et al., 2002; Olah et al., 2001; Wrigglesworth et al., 1996).

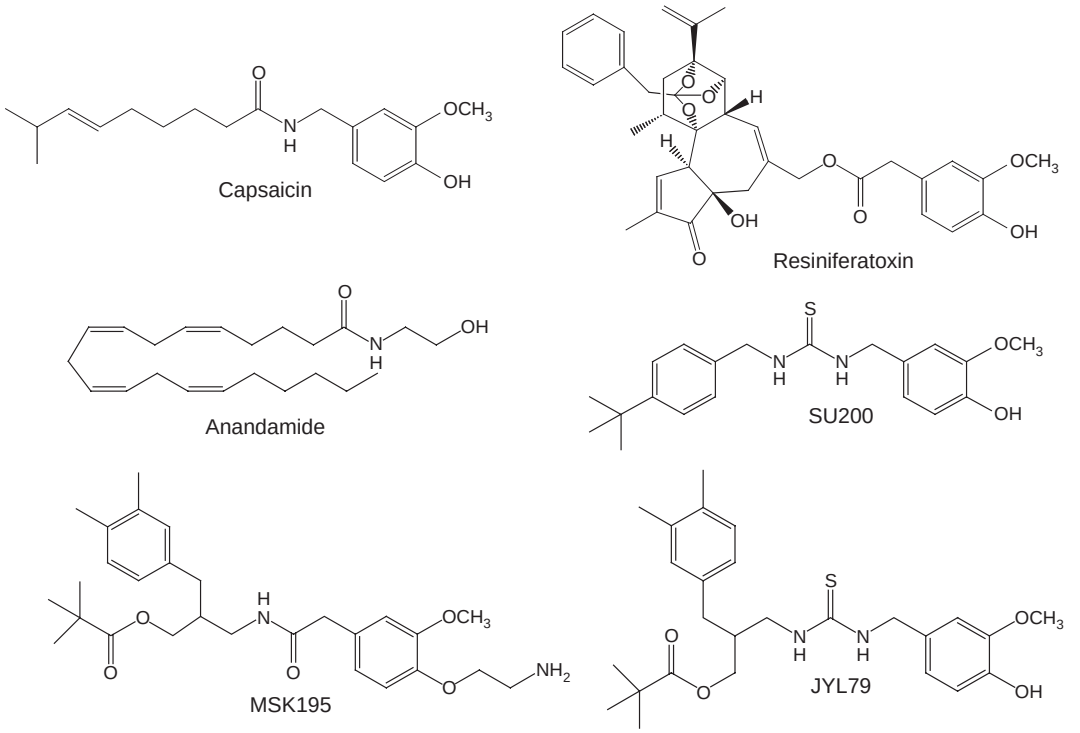

Fig. 1. Structure of TRPV1 agonists.

The maximal $[Ca^{2+}]_i$ values evoked by these agonists over 10 min (Fig. 2B) differed. Capsaicin and two of the other agonists elevated the $[Ca^{2+}]_i$ to about 80 nM under these conditions (capsaicin, 83 ± 20 nM; SU200, 83 ± 16 nM and JYL79, 80 ± 20 nM). RTX and MSK195 evoked somewhat higher $[Ca^{2+}]_i$ (RTX, 126 ± 14 nM; MSK195, 113 ± 22 nM; p = 0.16 for RTX and p = 0.37 for MSK195 compared to

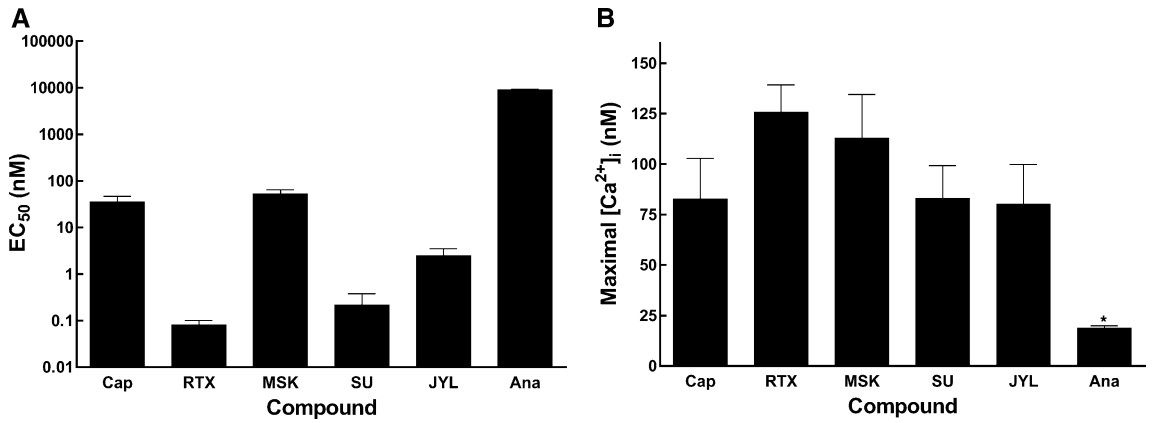

Fig. 2. Potency. CHO-TRPV1 cells were treated with agonists for 10 min. The averaged maximal $[Ca^{2+}]_i$ were plotted as a function of the concentration of the agonist and the plot was fitted according to the Hill equation. The determined EC_{50} values (panel A) and the maximal responses (panel B) are shown from three independent experiments. Bars are mean ± S.E.M., significance (maximal responses compared to capsaicin) $p < 0.05$ (*).

capsaicin, n = 3). Finally, anandamide caused an elevation in $[Ca^{2+}]_i$ to only 18.7 ± 1.3 nM (p = 0.034 relative to capsaicin, n = 3). This latter result fits with the known partial effect of anandamide on rat TRPV1 (Olah et al., 2001; Zygmunt et al., 1999), but is in contrast with other observations (Di Marzo et al., 2001). In that report, anandamide had an EC_{50} of 350 nM and a maximal effect of 68 % of the effect obtained with ionomycin using HEK cells transfected with the rat TRPV1. It was described that the low potency of anandamide in calcium imaging or similar assays could be due to the use of BSA in the assay medium (De Petrocellis et al., 2001). These authors reported that a BSA concentration as little as 0.1 % decreased the effect of anandamide, but not capsaicin, on TRPV1 by more than 80%. However, under our assay conditions similar potencies were obtained with or without BSA in $^{45}Ca^{2+}$ uptake assays (data not shown).

The compounds differed in the rapidity of the $[Ca^{2+}]_i$ response induced upon treatment. To compensate for the different absolute potencies of the compounds, all ligands were applied at concentrations approximating their individual EC_{50} values. Ligands were added at 1 min and responses were quantitated at one minute intervals thereafter. For capsaicin, MSK195, SU200, and anandamide, responses were maximal within 1 min after treatment (capsaicin) or within 70 % of the maximal value for MSK195, SU200, and anandamide (Fig. 3). In contrast, RTX and JYL79 showed almost no response over the first minute with a gradual increase for the next 10 or 5 min, respectively. These differences in response kinetics were not simply related to the potencies of the compounds. For example, SU200 was about 10 times more potent than JYL79 but did not show the slow responses observed for RTX or JYL79.

Another striking aspect of the kinetics was the variation in the extent of uniformity of response among individual cells (Fig. 3). Capsaicin, for example, caused virtually all cells to respond immediately to a similar $[Ca^{2+}]_i$ level. At the other extreme, RTX treatment was associated with marked differences in the latencies with which individual cells responded with elevated $[Ca^{2+}]_i$; the gradual time course of the increase in average $[Ca^{2+}]_i$ characterized for the population of cells represented the gradual accumulation of the response of individual cells, each of which showed a sharp increase in $[Ca^{2+}]_i$ but with a variable time before that increase occurred. More precisely, the responses of individual cells to RTX suggested that often there was a limited initial increase in $[Ca^{2+}]_i$ followed by a subsequent sharp increase. Although there is probably a continuum of behavior, MSK195 and SU200 more resembled capsaicin in the pattern of response that they induced; JYL79 more resembled RTX.

The difference in latencies of response between individual cells for compounds such as RTX but not for capsaicin, shown above at compound concentrations corresponding to their ED_{50} values, translated into a striking difference in how cells responded as a function of ligand concentration. As the concentration of capsaicin was increased, the increase in the maximal $[Ca^{2+}]_i$ of the individual cells accounted for the increase in average signal (Fig. 4). In contrast, as the concentration of RTX was increased, the maximal $[Ca^{2+}]_i$ of the individual responding cells remained similar whereas the number of cells which responded accounted for the increase. This observation provides an explanation for the behavior earlier described by Szallasi et al., 1999, who reported that increasing doses of capsaicin increased the average signal for $[Ca^{2+}]_i$, whereas increasing doses of RTX shortened the time until a maximal response for $[Ca^{2+}]_i$ was observed.

It is known that the capsaicin can desensitize TRPV1 in electrophysiological (patch clamp) measurements (Docherty et al., 1996; Piper et al., 1999). For capsaicin, anandamide, and SU200, the initial increase in $[Ca^{2+}]_i$ of the individual cells upon ligand treatment was followed by a partial decrease (Fig. 3). To further explore this desensitization, we characterized the response of cells treated

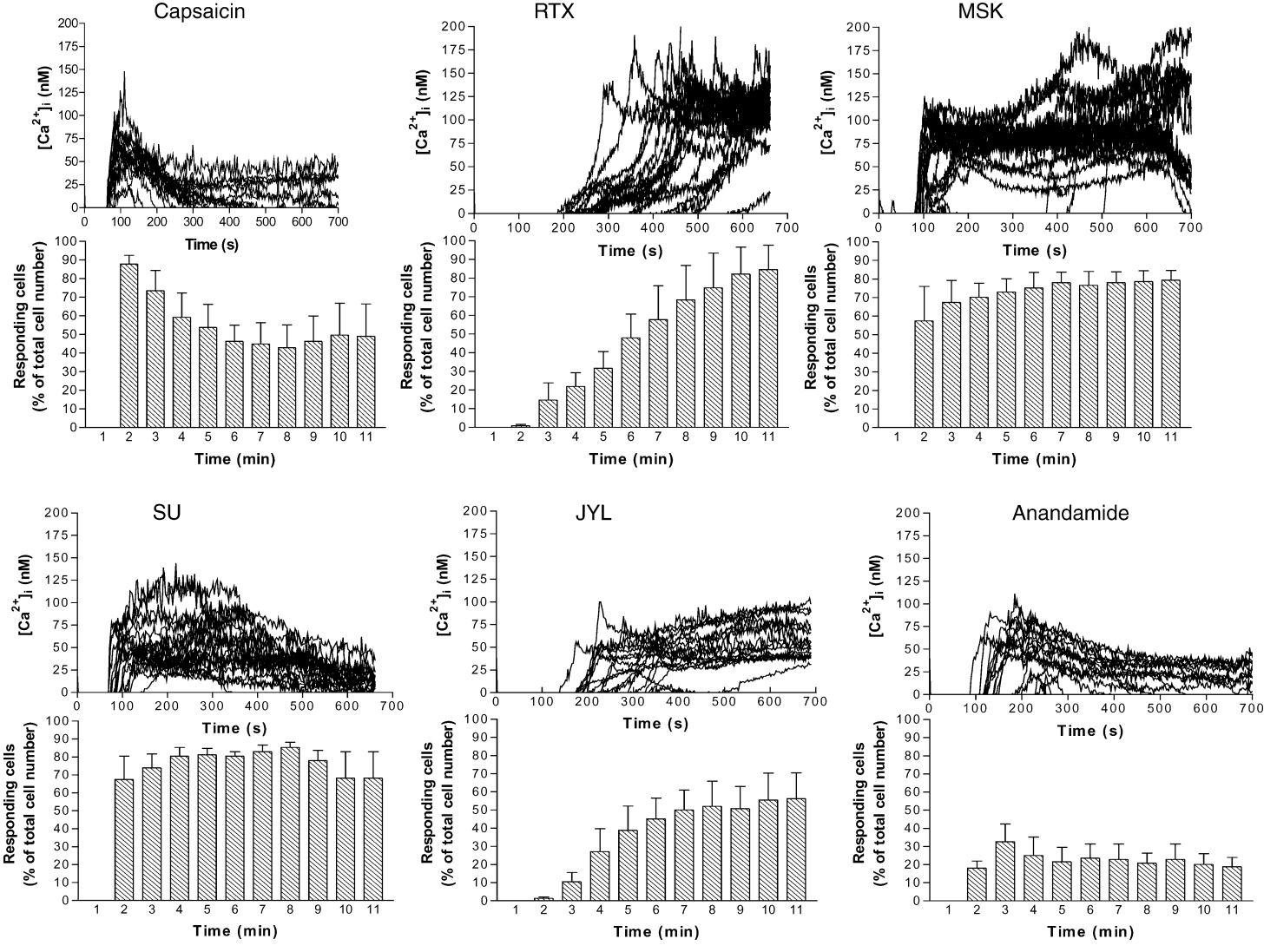

Fig. 3. Latency. Agonists were added to CHO-TRPV1 cells at 1 min. The $[Ca^{2+}]_i$ of the individual cells were recorded for an additional 10 min. The responses of 20 randomly selected cells to TRPV1 agonists are shown on the upper panels. To construct histograms, the responses of the cells were averaged and the maximal $[Ca^{2+}]_i$ was calculated. The cells which have higher $[Ca^{2+}]_i$ than 10% of the determined maximal $[Ca^{2+}]_i$ were counted as responders at the end of each min during the experiment (1–11 min). Finally, the % of the responding cells was calculated (compared to the total cell number) from three independent experiments.

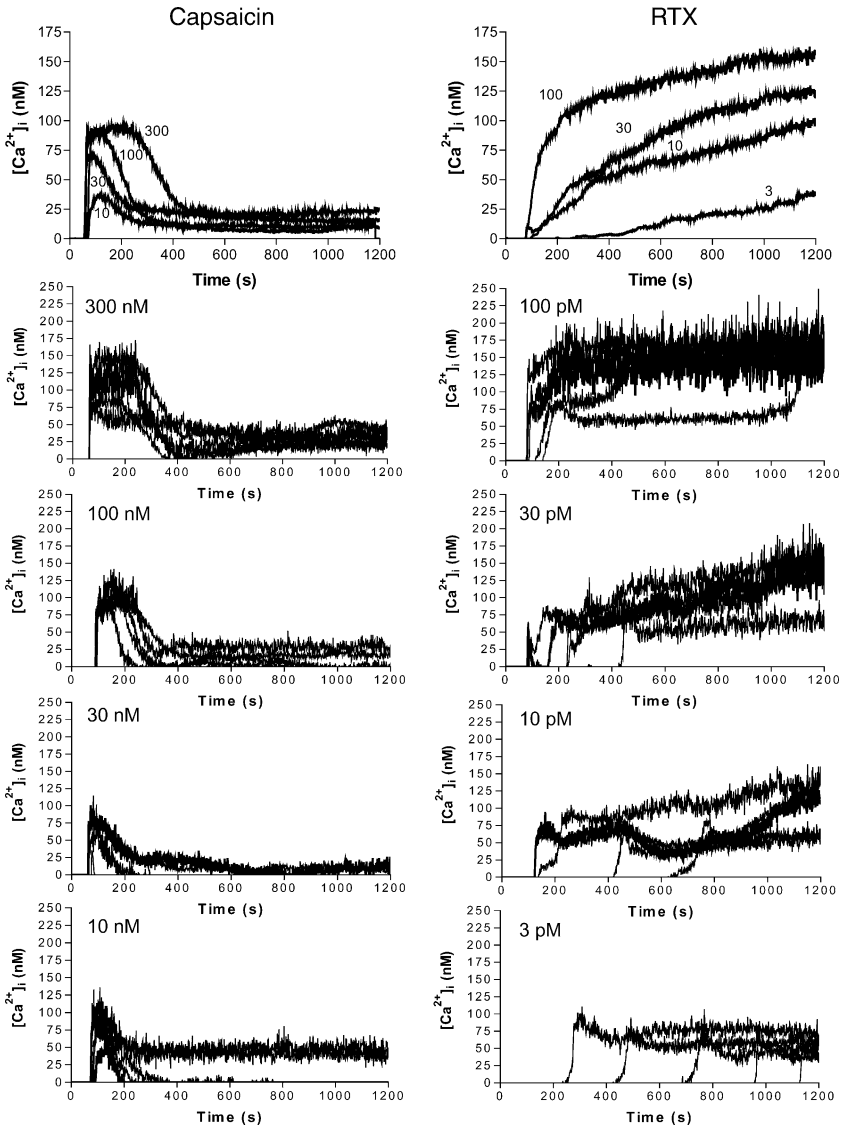

Fig. 4. Kinetics of responses. Agonists were added to CHO-TRPV1 cells at 1 min. The $[Ca^{2+}]_i$ of the individual cells were recorded for 20 min. The averaged responses of individual cells are shown on the upper panels. The individual responses of 5 randomly selected responding cells to TRPV1 agonists are shown on the lower panels. The applied capsaicin and RTX concentrations are indicated in pM for RTX and in nM for capsaicin.

with the agonists for a longer period of time (20 min) (Fig. 5). Compounds differed both in the extent and the kinetics of desensitization. RTX and JYL79 (applied at their ED_{50} values) showed no detectable decrease in the $[Ca^{2+}]_i$ (desensitization) during this period, in contrast to the results with capsaicin, SU200, and anandamide. Among these latter compounds, the rate of decrease was fastest in the case of anandamide, slower in the case of capsaicin and SU200. For JYL79 and MSK195, the elevation in $[Ca^{2+}]_i$ at 20 min was similar to the maximal response during the first 10 min of treatment

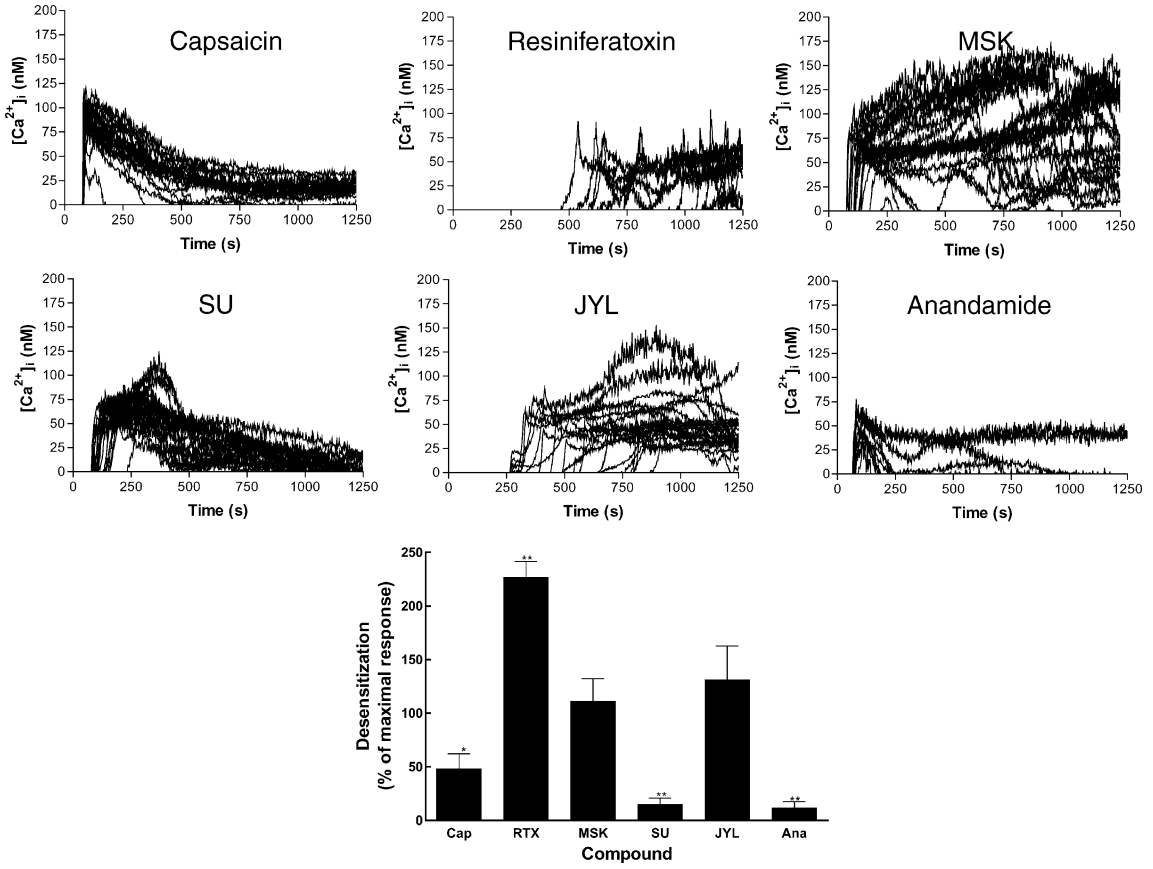

Fig. 5. Desensitization. Agonists were added to CHO-TRPV1 cells at 1 min and the $[Ca^{2+}]_i$ was recorded for an additional 20 min. The responses of 20 randomly selected cells to the agonists are shown on the upper panels. The maximal $[Ca^{2+}]_i$ during the first 10 min treatment (control) was compared to the response at 21 min (desensitized) in 3–4 independent experiments (lower panel). Bars are mean ± S.E.M., significance (desensitized versus control) levels are $p < 0.05$ (*) and $p < 0.01$ (**).

(the metric we used for expressing desensitization). For RTX, the response at 20 min was 2-fold greater (Fig. 4). Since in these experiments we applied the agonists in a concentration which causes about half maximal activation in 10 min, the observed 200% elevation for RTX means that the $[Ca^{2+}]_i$ of the cells by 20 min reached a level corresponding to the full activation of the cells achievable by saturating concentrations of RTX.

Discussion

Here we used intracellular Ca^{2+} imaging to characterize the effects of various vanilloid analogues and of anandamide on individual CHO cells heterologously expressing the vanilloid receptor subtype 1 (TRPV1) of the rat (CHO-TRPV1). We showed that multiple aspects of the $[Ca^{2+}]_i$ response differed depending on the specific ligand. Because compounds differed in maximal response, in rate of response, in the extent of variation of individual cells in the latency of response, and in extent of desensitization,

screening assays that evaluate only potency and whether compounds are agonists or antagonists sacrifice much of the potential information embedded in the kinetics of calcium response.

TRPV1 is currently being vigorously pursued as a therapeutic target (Caterina and Julius, 2001; Robbins, 2000; Szallasi and Blumberg, 1999; Szallasi and Di Marzo, 2000). Initial in vitro screening of candidate molecules has focused on binding potency (IC_{50}) from [^{3}H]RTX binding competition experiments and the effects on channel functions (EC_{50} values, level of agonism or antagonism) from $^{45}Ca^{2+}$ uptake, fluorometric determinations of intracellular Ca^{2+} and patch clamp measurements. It is not yet clear how important aspects of the diversity of in vivo behavior of vanilloids, such as partial dissociation between desensitization / defunctionalization and pungency, correspond to the above parameters. We know that these in vitro assays do not flag the dissociation of pungency from TRPV1 potency for RTX, for example. The identification of the diversity of response at the cellular level, coupled with identification of the structure activity relations responsible for the different aspects of response, should ultimately provide the tools to refine in vitro screening strategies.

So far, TRPV1 appears to be the sole receptor identified for vanilloid analogues (Caterina et al., 2000). However, much uncertainty surrounds the physiological regulators of TRPV1. Several mechanisms have been suggested so far, including activation by heat (Caterina et al., 1997), by acid (Tominaga et al., 1998), by anandamide (Zygmunt et al., 1999), by N-arachidonoyl dopamine (NADA) (Huang et al., 2002; Toth et al., 2003), by other N-acyldopamines (De Petrocellis et al., 2004), by products of lipoxygenases (Hwang et al., 2000), by phosphorylation by protein kinase C (Premkumar and Ahern, 2000), or by association with PIP_2 (Prescott and Julius, 2003). The interplay between ligand recognition, the action of physiological regulators on TRPV1, and feedback responses of these regulators to the changing intracellular ionic environment consequent to TRPV1 activation remains relatively unexplored. The approach of monitoring individual cells and continuing this monitoring over longer time periods should provide further insights into the range of distinct behaviors that can be elicited through creative drug design.

Conclusion

Structurally diverse vanilloid agonists induce marked diversity in the patterns of Ca^{2+} response. This diversity of response may provide opportunities for pharmacological exploitation. The approach of monitoring individual cells and continuing this monitoring over longer time periods should provide further insights into the range of distinct behaviors that can be elicited through creative drug design.

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