CMLS, Cell. Mol. Life Sci. 60 (2003) 2228–2240
1420-682X/03/102228-13
DOI 10.1007/s00018-003-3230-5

CMLS Cellular and Molecular Life Sciences

Research Article

Distinct features of recombinant rat vanilloid receptor-1 expressed in various expression systems

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Received 16 June 2003; received after revision 26 July 2003; accepted 31 July 2003

Abstract. In this study, we expressed rat vanilloid receptor 1 (VR1) in various heterologous expression systems using different VR1-encoding vectors, and examined how the VR1 agonists capsaicin and resiniferatoxin affected intracellular calcium. Our results clearly show that the magnitude and kinetics of response as well as the extent of tachyphylaxis differ markedly between systems. Using green fluorescent protein-tagged VR1, we show that much of the VR1 is localized to intracellular membranes. Consistent with this localization, VR1 agonists are able to liberate calcium from intracellular stores in the absence of extracellular calcium. As with other parameters of response, the three expression systems differ in the degree to which, in the absence of extracellular calcium, capsaicin and resiniferatoxin can liberate calcium from the intracellular stores. Our findings emphasize the influence of the expression system on characteristics of the response of VR1 to its ligands and the need for caution in extrapolating such results to other settings.

Key words. Vanilloid receptor 1; capsaicin; resiniferatoxin; heterologous expression system; calcium.

A distinct subpopulation of primary sensory neurons possesses a marked sensitivity to capsaicin [1, 2], the major pungent ingredient of hot peppers, and to other vanilloids [e.g., resiniferatoxin (RTX) isolated from the latex of *Euphorbia resinifera*] [3], which act via the stimulation of vanilloid receptors (VRs) [2, 4, 5]. The molecular description of the first VR on sensory neurons (VR1) [6], consistent with results obtained from previous electrophysiological and pharmacological studies [2, 5], revealed that the sensory neuron VR1 functions as a non-specific, calcium-permeable cation channel.

The activation of VR1 on sensory neurons by vanilloids results in ionic (mostly calcium and sodium) influx, an increase in intracellular calcium concentration ($[Ca^{2+}]_i$), and a subsequent desensitization of the response resulting in tachyphylaxis [2, 5]. In the analysis of these rather complex phenomena, one of the key issues, in addition to the heterogeneity of action of different vanilloids [2, 7], was to describe the dependence of the processes on extracellular calcium concentration ($[Ca^{2+}]_e$). The patch-clamp technique showed unambiguously that the vanilloid-induced membrane currents are only minimally affected by $[Ca^{2+}]_e$ [8–10]. In contrast, in most cellular preparations, the processes of desensitization/tachyphylaxis showed a marked dependence on the availability of calcium in the medium [9, 11, 12].

However, the dependence of the increase in $[Ca^{2+}]_i$ on $[Ca^{2+}]_e$ remains unclear. Several groups have reported that VR1 is exclusively expressed on the surface membrane of sensory neurons and that vanilloids are capable

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of increasing $[Ca^{2+}]_i$ only in the presence of extracellular calcium [13–15]. In contrast, other findings have suggested that VR1 could functionally be expressed not only in the plasma membrane but also in intracellular membranes (e.g., in the membrane of the endoplasmic reticulum, ER) of the neurons; hence, the VR1-mediated cellular responses may also be initiated in the absence of extracellular calcium [16–18].

Molecular characterization of VR1 stimulated multiple efforts to describe how VR1 functions as an ionic channel in cells. Various heterologous expression systems for VR1 have been used to describe molecular and pharmacological features of the receptor channel and cellular mechanisms initiated by the activation of VR1, and also to provide tools for VR1-targeted drug design in pain therapy.

Results obtained in these systems, however, did not resolve the issue of the calcium dependence of $[Ca^{2+}]_i$ responses and, moreover, provided conflicting data both on the localization of VR1 in cells and on the effect of various vanilloids. The ability of capsaicin to increase $[Ca^{2+}]_i$ was reported to be totally dependent on $[Ca^{2+}]_e$ in VR1-expressing Chinese hamster ovary (CHO) [15] and in human embryonic kidney (HEK) 293 cells [6, 19]. In contrast, using cos-7 cells expressing the enhanced green fluorescent protein (eGFP)-tagged VR1 (VR1eGFP), we have previously shown [16, 17] that the application of RTX initiated a calcium rise and disruption of various intracellular organelles (ER and nuclear membranes). We have also described that VR1eGFP was localized in the surface membrane and, unexpectedly, also in the membrane of the ER, a finding that was also supported by others [20].

In this study, therefore, our goal was to investigate and compare the functional properties of VR1 expressed in various heterologous systems using similar technical approaches. We employed the previously mentioned transient expression system in which a GFP-tagged VR1 was transiently expressed in cos-7 cells [16, 17], an inducible expression system in which the level of VR1 was controlled by a tetracycline-regulated repressor protein in CHO cells [21–23], and a stable expression system in which a VR1-encoding ε-tagged plasmid [16] was stably expressed in C6 rat glioma cells. In these cells, we compared the effects of capsaicin and RTX on calcium homeostasis and cellular integrity in calcium-containing and calcium-free solutions to describe the relative contributions of the surface membrane and (the possible) ER-localized VR1s in the establishment of the vanilloid-induced cellular effects, and to analyze vanilloid actions. We conclude that several crucial functional features of VR1 expressed in various systems are different from one another, which suggests a need for careful selection of the given expression system and for caution in interpreting and comparing data obtained in such systems to results with primary sensory neurons.

Materials and methods

Construction of heterologous expression systems for rat VR1

For the transient expression system, cos-7 cells were transfected with 1–2 μg VR1eGFP plasmid or with the control empty vector by the LipofectAMINE (Life Technologies, Gaithersburg, Md.) transfection reagent, using the protocol suggested by the manufacturer (VR1eGFP/cos-7 cells). The plasmid was constructed as described in our previous report [16]. Briefly, VR1-specific mRNA was obtained from rat dorsal root ganglion using RT-PCR, and was cloned to the multiple cloning site of the pEGFP-N3 vector (Clontech, Palo Alto, Calif.) using various restriction enzymes. Transfected cells, cultured for 48 h in Dulbecco's modified Eagle's medium (DMEM; Sigma, St. Louis, Mo.) supplemented with 10% fetal calf serum (FCS), 2 mM glutamine, and antibiotics (all from Sigma) at 35 °C to avoid temperature-induced activation of the VR1 [6, 16], were used for calcium imaging.

The inducible expression system was generated as reported previously [23]. Briefly, cDNA of the rat VR1 was subcloned into pUHG102-3 (Clontech) and was tranfected into CHO cells carrying the pTet Off Regulator plasmid (Clontech) (VR1/CHO cells). In these cells, expression of the pUHG plasmid (hence VR1) is repressed in the presence of tetracycline and is expressed upon removal of the antibiotic. Therefore, cells were routinely cultured in Ham F-12 medium (supplemented with 10% FCS, 2 mM glutamine, and antibiotics, all from Sigma) which contained 1 μg/ml tetracycline (Sigma). Before calcium imaging, cells were seeded on glass coverslips and were switched to tetracycline-free Ham F-12 medium and cultured at 35 °C for the time indicated (usually for 48 h, see below). To evaluate the efficacy of the induction of VR1 expression, Western blot analysis was performed (see below).

For the stable expression system, a previously constructed metallothionein promoter-based pεMTH vector [24] encoding the cDNA of the rat VR1 was used [16]. This vector (2–4 μg cDNA) was transfected into C6 rat glioma cells growing in six-well tissue culture dishes (VR1/C6 cells). Cells were then selected in 10% FCS-supplemented DMEM containing 750 μg/ml G418 (geneticin; Life Technologies) for 12–18 days; then, single colonies were isolated. VR1-overexpressing cells were cultured in supplemented DMEM containing 500 μg/ml G418 at 35 °C. The efficacy of recombinant overexpression in several clones was monitored by Western blotting (see below).

Western blotting

In the cases of the inducible and stable expression systems, the expression of VR1 was evaluated by Western blotting [25]. Cells were harvested in homogenization buffer, subjected to SDS-PAGE according to Laemmli [26] and trans-

ferred to nitrocellulose membranes (BioRad, Vienna, Austria). Membranes were then probed with a goat anti-VR1 primary antibody (Santa Cruz, Santa Cruz, Calif.). A peroxidase-conjugated rabbit anti-goat IgG antibody (Bio-Rad) was used as the secondary antibody, and the immunoreactive bands were visualized by an ECL Western blotting detection kit (Amersham, Little Chalfont, UK) on light-sensitive film (AGFA, Brussels, Belgium).

Representative results of the expression of VR1 in the inducible and stable expression systems are seen in figure 1. Western blot analyses were performed on VR1/CHO cells harvested 0, 12, 24, and 48 h after induction, i.e., after withdrawing the tetracycline from the culturing medium. As seen in figure 1A, the expression of VR1 increased with time after tetracycline removal; therefore, cells after 48 h were used for calcium imaging. In addition, Western blot measurement was also performed on VR1/C6 cells and on C6 cells transfected with the empty pεMTH vector. As seen in Figure 1B, VR1 expression was several times higher in the three representative overexpressing clones than in control C6 cells.

Calcium imaging and analysis

Changes in intracellular calcium concentration ($[Ca^{2+}]_i$) were detected as described in our earlier reports [25, 27]. A calcium-sensitive probe was introduced into the intracellular space by incubating the cells with 5 µM fura-2 AM for 1 h at 37 °C. Before each measurement, the cells were kept at room temperature (22–24 °C) in normal Tyrode's solution (in mM: 137 NaCl, 5.4 KCl, 0.5 $MgCl_2$, 1.8 $CaCl_2$, 11.8 Hepes-NaOH, 1 g/l glucose, pH 7.4) for half an hour to allow homogeneous distribution of the dye. The coverslips, containing the fura-2-loaded cells, were then placed on the stage of an inverted fluorescence microscope (Diaphot;, Nikon, Tokyo, Japan). In the case of the transient expression system, single cells expressing the VR1eGFP fusion protein in cos-7 cells were selected by eGFP green fluorescence illuminating the cells at 488 nm wavelength. For calcium imaging, excitation was altered between 340 and 380 nm using a dual wavelength monochromator (Deltascan; Photon Technology International, New Brunswick, N. J.). The emission was monitored at 510 nm with a photomultiplier at an acquisition rate of 10 Hz per ratio. $[Ca^{2+}]_i$ levels were calculated according to the method of Grynkiewicz et al. [28] from the ratio ($R=F_{340}/F_{380}$) of the fluorescence intensities measured with excitation wavelengths of 340 (F_{340}) and 380 nm (F_{380}) as described earlier [28] ($K_d=76$ nM, $R_{min}=0.42$, $R_{max}=8.6$, $F380_{[0]}/F380_{[Ca]}=15.3$).

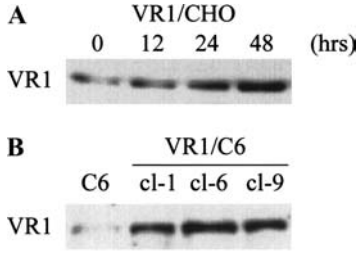

Figure 1. Determination of efficacy of VR1 expression in VR1/CHO and VR1/C6 cells. Cells were harvested in lysis buffer, similar amounts of proteins were subjected to SDS-PAGE, and Western immunoblotting was performed using a goat antibody against VR1 as described in Materials and methods. (*A*) VR1/CHO cells were induced to express VR1 by change to medium without tetracycline, and were subjected to Western blot analysis 0, 12, 24, and 48 h after induction. (*B*) VR1 expression was determined in empty vector transfected C6 cells (C6) and in three individual VR1/C6 clones (cl) with high VR1 levels.

Cells were continuously washed by either normal or calcium-free Tyrode's solution (in the latter case, the $CaCl_2$ was substituted by 1 mM EGTA) using a slow background perfusion system, whereas the agents investigated (capsaicin from Sigma; RTX and capsazepine from Alexis, San Diego, Calif.) were applied through a rapid perfusion system positioned in close proximity to the cell measured. All compounds were applied until the maximal response was elicited.

Analyses of the $[Ca^{2+}]_i$ transients were performed by a PTI analysis program developed by us which measures maximal amplitude of the transient (above the baseline $[Ca^{2+}]_i$ level, in nM), the time to peak value (TTP, time interval between the start of the application of the drug and the peak of the elevation, in s), and the rate of rise value (ROR, slope of the ascending phase measured between the onset and peak of the transient, in nM/s). All data are expressed as the mean ± SE.

Real-time confocal microscopy

Cos-7 cells were plated on glass coverslips and transfected with 1–2 µg VR1eGFP plasmid and, after 48 h in culture, were analyzed with an MRC-1024 Bio-Rad confocal microscope, as described before [16, 17]. To study the two- and three-dimensional distribution of fluorescent signals, each x-y plane was scanned over 1 s at 30-s intervals and at 0.2-µm increments in the z axis mode.

Results

In the VR1eGFP/cos-7 cell transient expression system, capsaicin induced cellular responses more effectively in 1.8 mM extracellular calcium solution

First, we investigated the effect of capsaicin on the intracellular calcium homeostasis of cells in calcium-free and calcium-containing extracellular solutions. Control (the empty pEGFP vector-transfected) cos-7 cells never responded to capsaicin with any type of increase in $[Ca^{2+}]_i$ whether in calcium-free or in 1.8 mM $[Ca^{2+}]_e$ solutions (data not shown). In contrast, as seen in figure 2, capsaicin at 1 µM was able to increase $[Ca^{2+}]_i$ of VR1eGFP/cos-7 cells both in 1.8 and 0 mM $[Ca^{2+}]_e$ solu-

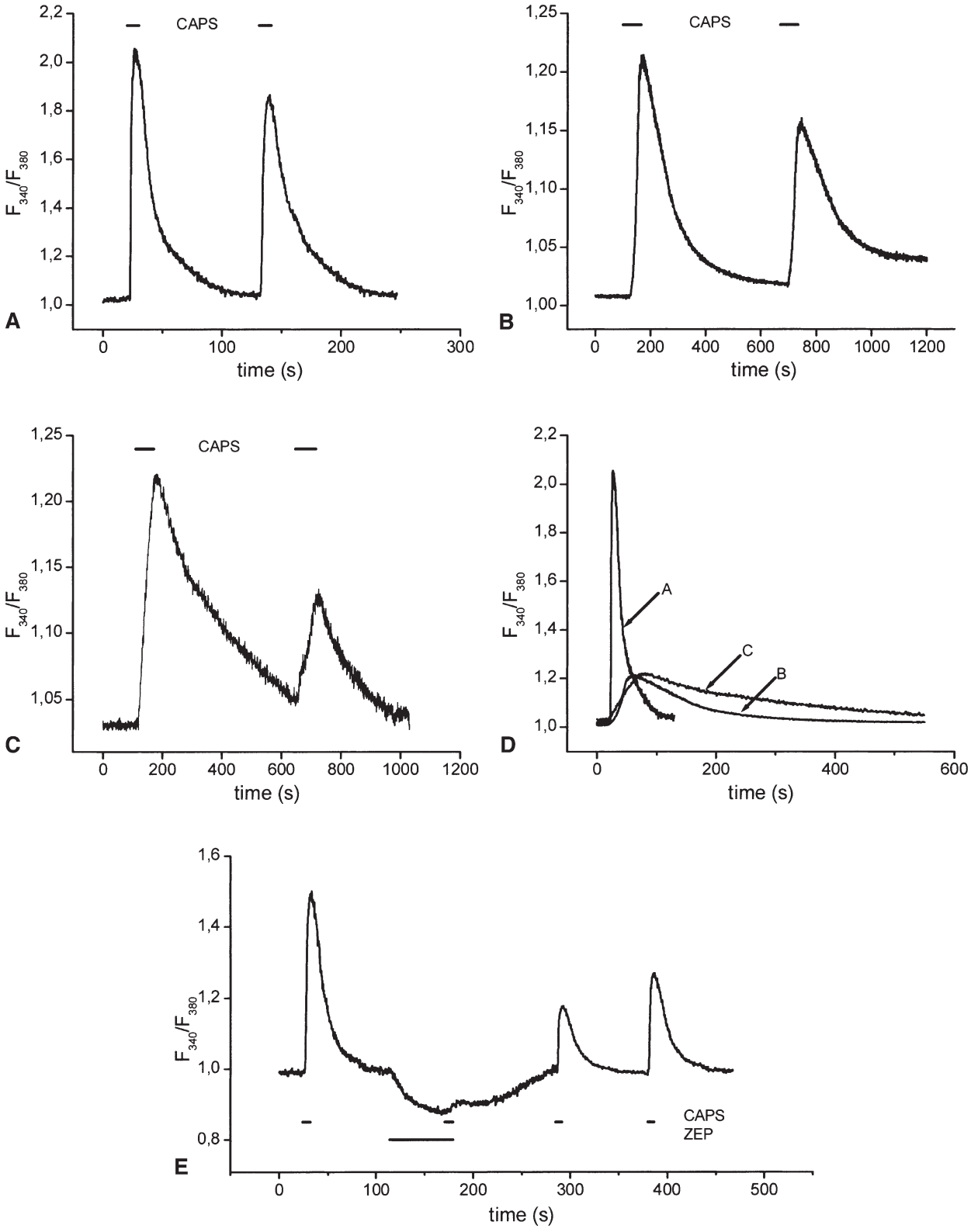

Figure 2. Effect of capsaicin on $[Ca^{2+}]_i$ in VR1eGFP/cos-7 cells. Cells growing on glass coverslips were loaded with 5 µM fura 2-AM and fluorescence ratio (F_{340}/F_{380}) values of excitations at 340- and 380-nm wavelengths were recorded at an acquisition rate of 10 Hz per ratio. The effects of 1 µM capsaicin (CAPS) were measured in 1.8 mM $[Ca^{2+}]_e$ (*A*, *B*) and in calcium-free (*C*) solutions. For better comparison of various parameters of the capsaicin-induced transients in different solutions, the first $[Ca^{2+}]_i$ elevations in A–C (indicated by arrows) are shown using the same time and ratio scales (*D*). The ability of 5 µM capsazepine (ZEP) to significantly yet reversibly inhibit the action of capsaicin in 1.8 mM $[Ca^{2+}]_e$ medium is also shown (*E*). Representative results of multiple determinations are summarized in table 1.

Table 1. Summary of various parameters of $[Ca^{2+}]_i$ transients induced by capsaicin and RTX in VR1eGFP/cos-7 cells.

	Capsaicin (1 µM)			RTX (1 nM)		
	1.8 mM Ca^{2+}		0 mM Ca^{2+}	1.8 mM Ca^{2+}	0 mM Ca^{2+}	
Responding cells (%)	79		46	42	48	
Transient type	fast (69%)	slow (31%)	slow (100%)	slow (100%)	fast (32%)	slow (68%)
Amplitude (nM)	124 ± 29	31 ± 11	28.6 ± 6	68 ± 16	65 ± 22	44 ± 14
Time to peak (TTP, s)	14.9 ± 1	63 ± 10	66 ± 11	133 ± 23	15.7 ± 3	166 ± 21
Rate of rise (ROR, nM/s)	16.6 ± 5	1.1 ± 1	0.4 ± 0.1	1.9 ± 1	7.1 ± 3	0.36 ± 0.2
Tachyphylaxis (% decrease)	24 ±11	40.8±8	44 ±12	100	41 ±11	100
Averaged tachyphylaxis (%)	27.3 ± 9		N/A	N/A	79 ± 13	

Parameters shown in the table were determined as described in Materials and methods. All values are expressed as the mean ± SE of several determinations. N/A, not applicable.

tions. However, there were significant differences in the proportion of responding cells and in the characteristics of the different transients for cells in the different extracellular solutions.

In 1.8 mM $[Ca^{2+}]_e$, 79% of the VR1eGFP/cos-7 cells (n = 42/53) responded with a significant increase in $[Ca^{2+}]_i$ upon capsaicin treatment (table 1). These transients, based on their various kinetic parameters (listed in table 1) could be classified into two groups. In the first group ('fast' transient, fig. 2A, D), the signals were recorded in 69% (n = 29/42) of the capsaicin-responding cells. Among these 'fast' $[Ca^{2+}]_i$ elevations, 66% of the transients (n = 19/29) returned to baseline after the termination of capsaicin application, whereas 3 % (n = 10/29) of the transients showed minimal return (less than 30% decline compared to the maximal level). For the group displaying transients which returned to baseline, we were able to investigate the effect of repeated capsaicin application and to measure the decrease in the amplitude of the transients (i.e., tachyphylaxis). Statistical analyses revealed that, upon repeated capsaicin treatment, the second transients had 24 ± 11% (mean ± SE) less amplitude compared to the maximal value of the first $[Ca^{2+}]_i$ elevation.

In the second group ('slow' transients), the signals were recorded on 31% (n = 13/42) of the capsaicin-responding VR1eGFP/cos-7 cells (fig. 2B, D). The transients had a much reduced maximum amplitude and a slower response (table 1). Similar to the 'fast' transients, approximately two-thirds (62%, n = 8/13) of the 'slow' $[Ca^{2+}]_i$ elevations returned to the baseline. However, within the 'slow' group, we observed a more pronounced tachyphylaxis upon repeated capsaicin application (40.8 ± 8% decrease in amplitude of the second transient compared to the first one; mean ± SE).

We also measured the specificity of the capsaicin response using capsazepine, a competitive antagonist of capsaicin for VR1. As seen in figure 2E, 5 µM capsazepine markedly yet reversibly inhibited the capsaicin-induced $[Ca^{2+}]_i$ responses. Statistical analyses showed that, in contrast to the 27.3 ± 9% average tachyphylaxis on VR1eGFP/cos-7 cells showing any type of responses to capsaicin (table 1), capsazepine decreased the amplitude of the second capsaicin-evoked $[Ca^{2+}]_i$ transient by 91.6 ± 4% (mean ± SE, n = 15) when compared to the peak value of the first elevation induced by capsaicin.

In calcium-free solution, capsaicin was able to evoke $[Ca^{2+}]_i$ transients in only 46% of the VR1eGFP/cos-7 cells (n = 25/54) (table 1). Furthermore, also in contrast to our findings with VR1eGFP/cos-7 cells in 1.8 mM $[Ca^{2+}]_e$, all of these transients were characterized as 'slow' and small (fig. 2C, D, table 1). Although most of the transients (84%, n = 21/25) returned to the baseline after cessation of vanilloid treatment, repeated application of capsaicin resulted in a significant decline in the maximal amplitude (44.5 ± 13% decrease compared to the peak of the first capsaicin-induced transient, mean ± SE).

We have previously shown [16] that the stimulation of VR1eGFP-expressing cos-7 cells by RTX results in specific morphological changes in various intracellular membrane structures. Therefore, using the eGFP-tag to provide a visual measure of cellular events, we also investigated the differences and similarities between the actions of capsaicin in calcium-free and calcium-containing solutions at the level of intracellular organelles by real-time confocal microscopy. As seen in figure 3, similar to the calcium imaging data, there were marked differences in the actions of capsaicin depending on the presence of $[Ca^{2+}]_e$. For VR1eGFP/cos-7 cells in calcium-containing solution (fig. 3, upper row), 1 µM capsaicin effectively induced the vesiculation and disruption of intracellular membrane elements, i.e., ER and nuclear membrane disruption, within 5–10 min (as was expected, the kinetics of vanilloid-induced changes in cell integrity were much slower, in the minute range, than those of the calcium responses, in the second range) [16]. In contrast, in calcium-free solution (fig. 3, middle row), capsaicin induced much less pronounced intracellular alterations. Although disorganization of the ER was prominent, the appearance of charac-

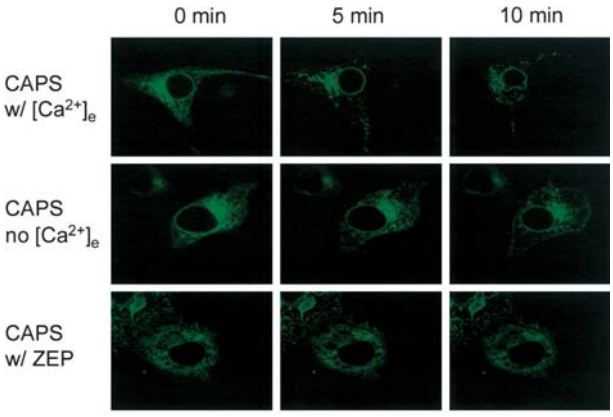

Figure 3. Effect of capsaicin on cellular membrane organization in VR1eGFP/cos-7 cells. Single VR1eGFP/cos-7 cells were identified by their green fluorescence using a confocal microscope. Images were obtained in the z axis mode as described in Materials and methods, and selected time points are represented in the figure. Application of 1 µM capsaicin (CAPS) was initiated at 0 min and was continued throughout the experiment. The action of capsaicin was investigated in 1.8 mM $[Ca^{2+}]_e$ (upper row) and in calcium-free (middle row) solutions, whereas the inhibitory effect of 5 min preincubation with 5 µM capsazepine (ZEP) in 1.8 mM $[Ca^{2+}]_e$ medium is shown in the lower row. Note the more pronounced effect of capsaicin in calcium-containing solution. Representative results of several determinations yielding similar results.

teristic nuclear blebs representing disruption of the nuclear membrane was rare. Preincubation of the VR1eGFP/cos-7 cells with 5 µM capsazepine for 10 min (which alone did not cause any modification of cellular integrity, data not shown) effectively inhibited the cellular changes induced by 1 µM capsaicin (fig. 3, lower row). In addition, capsaicin caused no measurable morphological changes in control cos-7 cells transfected with the empty pEGFP vector (data not shown). Consistent with the calcium imaging data, these findings strongly suggest that the actions of capsaicin on VR1eGFP/cos-7 cells are markedly dependent on the presence of extracellular calcium.

In the VR1eGFP/cos-7 transient expression system, RTX induced cellular responses with very similar potencies in 1.8 and 0 mM $[Ca^{2+}]_e$ solutions

We next investigated the cellular actions of an ultrapotent vanilloid agonist, RTX, on $[Ca^{2+}]_i$ homeostasis and morphological characteristics. As seen in figure 4, 1 nM RTX, like capsaicin, increased $[Ca^{2+}]_i$ both for cells in 0 and 1.8 mM $[Ca^{2+}]_e$ solutions. However, there were significant differences in the RTX-induced transients both as a function of the extracellular calcium and compared to the characteristics of the corresponding capsaicin-induced responses.

For VR1eGFP/cos-7 cells, in 1.8 mM $[Ca^{2+}]_e$ solution, RTX was able to evoke $[Ca^{2+}]_i$ transients in 42% of the cells examined (n = 27/64). However, in contrast to the data obtained with capsaicin (seen in fig. 2), all of these RTX-evoked transients could be classified as 'slow' (fig. 4A, D, table 1). Although 52% (n = 14/27) of the transients returned to the baseline after RTX application, none of the cells showed a $[Ca^{2+}]_i$ response after repeated RTX administration, reflecting maximal tachyphylaxis.

For VR1eGFP/cos-7 cells in calcium-free solution, like those in 1.8 mM $[Ca^{2+}]_e$, RTX was able to evoke $[Ca^{2+}]_i$ transients in approximately half (48%) of the cells examined (n = 33/69). These transients could be classified into two groups based on kinetic analysis (table 1). In the first group ('fast' transients, fig. 4B, D), the signals were recorded on 33% of the RTX-responding cells (n = 11/33) (table 1). On those cells which responded with $[Ca^{2+}]_i$ responses returning to baseline (55% of the 'fast' transients, n = 6/11), repeated application of RTX resulted in a marked tachyphylaxis (40 ± 11% decrease in the peak amplitude compared to that of the first elevation, mean ± SE).

In the second group ('slow' transients, fig. 4C, D), the signals were recorded on 67% of the RTX responding cells (n = 22/33) (table 1). Interestingly enough, although most (82%, n = 18/22) of the 'slow' transients returned to the baseline after the termination of the RTX application, none of the cells showing such $[Ca^{2+}]_i$ signals responded to repeated RTX administration, suggesting maximal tachyphylaxis.

To obtain more data about the possible functional role of the VR1eGFP fusion protein on intracellular calcium stores, we also determined the effect of emptying the intracellular calcium stores on the RTX-induced responses using thapsigargin (TG), an inhibitor of the ER Ca-ATPase molecule [29]. Since the RTX-induced transients in calcium-free solution exerted a marked tachyphylaxis (79.2 ± 13% averaged tachyphylaxis on cells showing any type of responses to RTX, mean ± SE, see table 1), we were unable to measure the effect of TG on repeated RTX-induced transients. Therefore, we investigated the amplitude of the first RTX-evoked transients under control and TG-treated (50 nM, 5 min) conditions and statistically compared the two populations. Whereas 48% of the cells responded with any type of transient $[Ca^{2+}]_i$ elevations to RTX in control calcium-free solution (with an averaged amplitude of 57.4 ± 14 nM, mean ± SE), we could detect only minimal (averaged amplitude of 3.6 ± 2 nM, mean ± SE, n = 20) responses on cells preincubated with TG. We can conclude, therefore, that the emptying of the ER calcium content resulted in a marked (approximately 93%) inhibition of the RTX-induced $[Ca^{2+}]_i$ transients in calcium-free solution.

We also investigated the effect of RTX on morphological characteristics of VR1eGFP-expressing cos-7 cells. As seen in figure 5 (upper and middle rows), 1 nM RTX was able to induce disruption of intracellular membrane components with similar efficiencies in calcium-free and normal solutions (in marked contrast to the more pronounced action of capsaicin in 1.8 mM $[Ca^{2+}]_e$, see fig. 3). How-

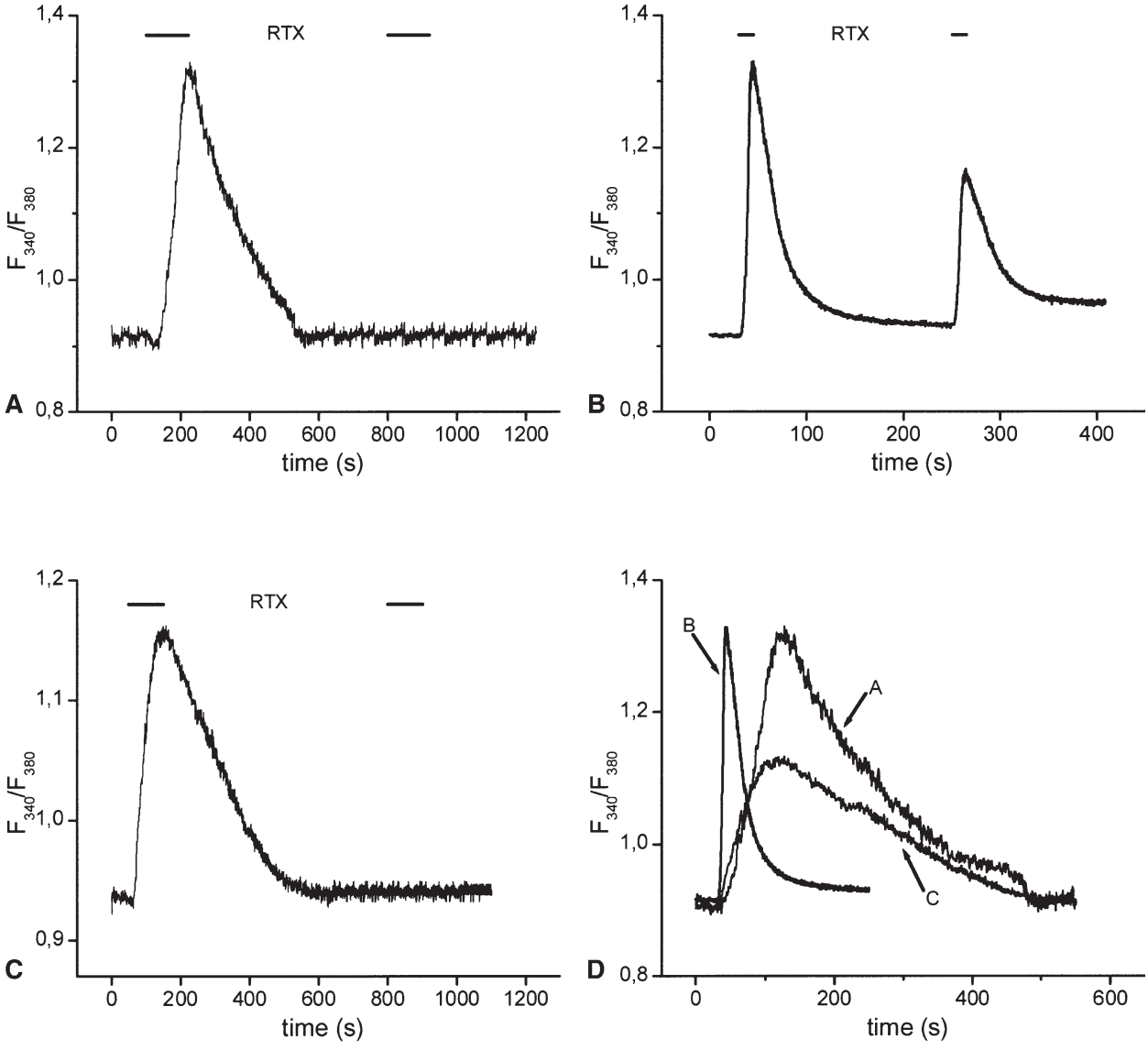

Figure 4. Effect of RTX on $[Ca^{2+}]_i$ in VR1eGFP/cos-7 cells. Cells growing on glass coverslips were loaded with 5 μM fura 2-AM and fluorescence ratio (F_{340}/F_{380}) values of excitations at 340- and 380-nm wavelengths were recorded at an acquisition rate of 10 Hz per ratio. The effects of 1 nM RTX were measured in 1.8 mM $[Ca^{2+}]_e$ (*A*) and in calcium-free (*B*, *C*) solutions. For better comparison of various parameters of the RTX-induced transients in different solutions, the first $[Ca^{2+}]_i$ elevations in panels A–C (indicated by arrows) are also shown using the same time and ratio scales (*D*). Representative results of several determinations are summarized in table 1.

ever, the action of RTX was much slower than that of capsaicin (maximal effects were seen after 15–20 min). Furthermore, we also found that the preincubation of the cells for 5 min with 50 nM TG (hence the emptying of intracellular calcium stores) largely but not completely prevented the disorganization of the intracellular membrane structures induced by RTX (fig. 5, lower row) (the partial extent of this blockade can possibly be explained by the effect of TG alone on $[Ca^{2+}]_i$ [30]). Like capsaicin, RTX was also ineffective on control empty vector-transfected cos-7 cells (data not shown). Consistent with the calcium imaging results, these data again strongly argue for the incorporation of the VR1eGFP into intracellular calcium store structures of cos-7 cells and its functional activity. Furthermore, our findings suggest that the effects of RTX on the VR1eGFP/cos-7 cells are much less dependent on the presence of extracellular calcium than seen in the case of capsaicin.

In the inducible VR1/CHO expression system, RTX but not capsaicin was ineffective when extracellular calcium was removed

In VR1/CHO cells in calcium-containing solution, 1 μM capsaicin induced $[Ca^{2+}]_i$ transients in 66% of cells (n = 66/100) and all of the evoked transients were characterized as ‘fast’ ones (fig. 6A, D, table 2). In striking con-

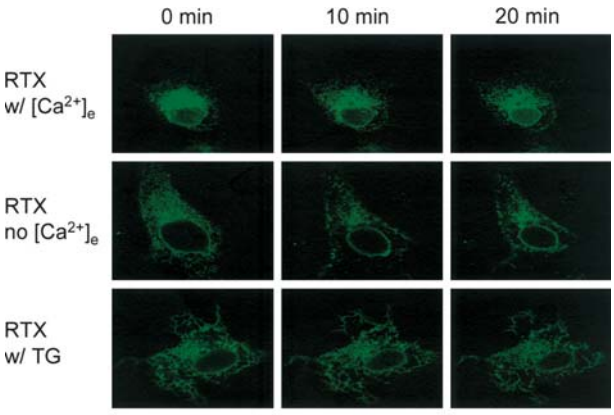

Figure 5. Effect of RTX on cellular membrane organization in VR1eGFP/cos-7 cells. Single VR1eGFP/cos-7 cells were identified by their green fluorescence using a confocal microscope. Images were obtained in the z axis mode as described in Materials and methods and selected time points are represented in the figure. Application of 1 nM RTX was initiated at 0 min and was continued throughout the experiment. The action of RTX was investigated in 1.8 mM $[Ca^{2+}]_e$ (upper row) and in calcium-free (middle row) solutions, whereas the partial inhibitory effect of 5-min preincubation with 50 nM thapsigargin (TG) in calcium-free medium is shown in the lower row. Note the similarly pronounced effects of RTX in both solutions. Representative results of several determinations yielding similar results.

trast, in calcium-free solution, only 25% of the VR1/CHO cells (n = 15/60) responded to capsaicin (table 2) and all of these responses were rather small and 'slow' (fig. 6B, D). In both solutions, most of the capsaicin transients (> 80%) completely returned to baseline (fig. 6A, B); hence, the effect of repeated applications of capsaicin could also be measured. However, in marked contrast to the behavior of VR1eGFP/cos-7 cells (see figs. 2, 4), repeated capsaicin application did not result in tachyphylaxis of the VR1/CHO cells (table 2) in either solution. Instead, especially in 1.8 mM $[Ca^{2+}]_e$ solution, the second application of capsaicin resulted in even higher Ca responses (table 2).

Interestingly (and also contrary to our findings in the VR1eGFP/cos-7 cell transient expression system; see fig. 4, table 1), in the VR1/CHO inducible expression system, 1 nM RTX was completely ineffective in calcium-free solution (table 2). Moreover, even in 1.8 mM $[Ca^{2+}]_e$ solution, only 16% of the cells (n = 8/50) responded to RTX with, in all cases, 'slow' $[Ca^{2+}]_i$ elevations (fig. 6C, D). In addition, we were unable to measure the effect of repeated RTX administration since the $[Ca^{2+}]_i$ levels never returned even close to baseline after the first RTX application.

The VR1 competitive antagonist capsazepine effectively blocked the action of capsaicin and RTX in both solutions. Namely, 2-min preincubation of cells with 5 μM capsazepine completely prevented the effect of the subsequent addition of capsaicin (the reversible action of the inhibitor in calcium-containing solution is seen in fig. 6E). Since the effect of a second application of RTX could not be measured in VR1/CHO cells, we determined the maximal amplitude of the RTX-induced responses in control and capsazepine-pretreated cells. Whereas in control cells, RTX initiated transients with an averaged maximum amplitude of 47 ± 19 nM (mean ± SE, table 2), the vanilloid was able to affect $[Ca^{2+}]_i$ only insignificantly in capsazepine-pretreated cells (3.8 ± 3 nM increase, mean ± SE, n = 8).

In the VR1/C6 stable expression system, both capsaicin and RTX were effective only in calcium-containing extracellular solution

In VR1/C6 cells measured in calcium-containing solution, the effect of capsaicin was very similar to that seen in VR1/CHO cells under similar conditions (table 3). Namely, all of the cells investigated (n = 99) responded to 1 μM capsaicin application with significant and 'fast' $[Ca^{2+}]_i$ transients (fig. 7A, C, table 3) which returned to baseline after cessation of capsaicin administration. Also similar to the VR1/CHO cells, the VR1/C6 cells never showed any tachyphylaxis upon repeated capsaicin application (table 3). However, in marked contrast to data obtained in the VR1/CHO inducible system, in the VR1/C6 cells, capsaicin was unable to evoke any significant change of $[Ca^{2+}]_i$ in calcium-free solution (n = 56).

The characterization of the effect of RTX in the VR1/C6 cells resulted in strikingly similar data to those obtained in the VR1/CHO inducible system (table 3). Namely, 1 nM RTX was only effective in calcium-containing solution and only in a much smaller portion of the cells than capsaicin (28%, n = 19/68). In addition, all of the RTX-evoked transients were small and 'slow' ones (fig. 7B, C, table 3). As seen in figure 7B, as was expected, the repeated application of RTX resulted in no further change in $[Ca^{2+}]_i$. However, since these transients only partially (if at all) returned toward the baseline after the first RTX application, we were unable to properly and, most importantly, statistically measure the phenomenon of tachyphylaxis.

The effect of capsazepine was tested in this system as well. A 2-min preincubation of the cells with 5 μM capsazepine, as in the inducible system, completely yet reversibly abolished the effect of capsaicin (fig. 7D). Since the effect of a second application of RTX could not be measured in these cells, we determined the maximal amplitude of the RTX-induced responses in control and capsazepine-pretreated cells. Whereas in VR1/C6 cells (not treated with the inhibitor), RTX was able to induce Ca response transients with an averaged maximum amplitude of 29.2 ± 4 nM (mean ± SE, table 3), the RTX was completely ineffective after capsazepine pretreatment (n = 10).

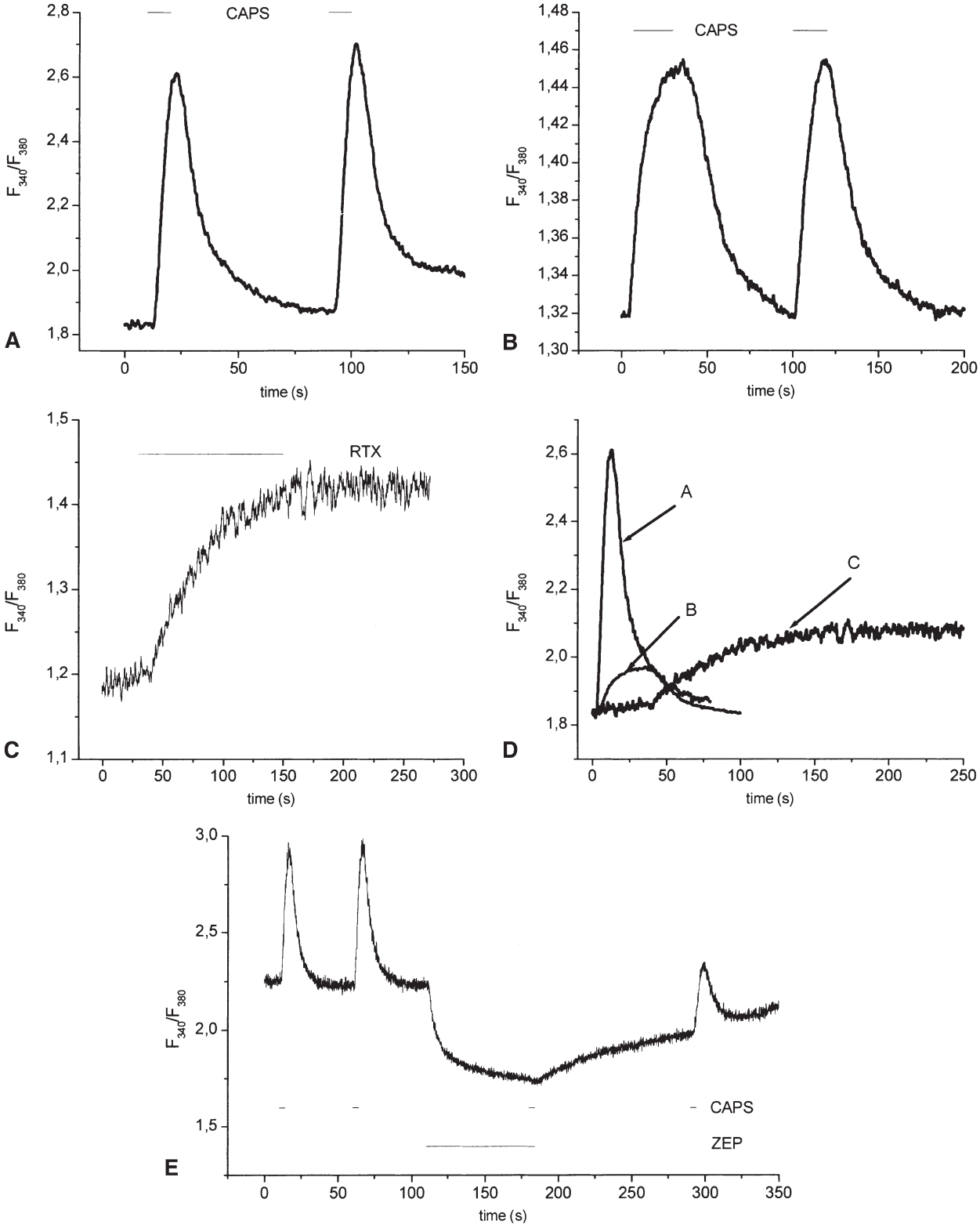

Figure 6. Effects of capsaicin and RTX on $[Ca^{2+}]_i$ in VR1/CHO cells. Cells growing on glass coverslips were loaded with 5 µM fura 2-AM and fluorescence ratio (F_{340}/F_{380}) values of excitations at 340- and 380-nm wavelengths were recorded at an acquisition rate of 10 Hz per ratio. The effects of 1 µM capsaicin (CAPS) in 1.8 mM $[Ca^{2+}]_e$ (*A*) and in calcium-free (*B*) solutions, and the effect of 1 nM RTX in 1.8 mM $[Ca^{2+}]_e$ medium (*C*) are represented. For better comparison of various parameters of the capsaicin- and RTX-induced transients in different solutions, the first $[Ca^{2+}]_i$ elevations *A*–*C* (indicated by arrows) are also shown using the same time and ratio scales (*D*). The effect of 5 µM capsazepine (ZEP) to significantly yet reversibly inhibit the action of capsaicin in 1.8 mM $[Ca^{2+}]_e$ medium is shown in *E*. Representative results of several determinations are summarized in table 2.

Table 2. Summary of various parameters of $[Ca^{2+}]_i$ transients induced by capsaicin and RTX in VR1/CHO cells.

	Capsaicin (1 µM)		RTX (1 nM)
	1.8 mM Ca^{2+}	0 mM Ca^{2+}	1.8 mM Ca^{2+}
Responding cells (%)	66	25	16
Transient type	fast (100%)	slow (100%)	slow (100%)
Amplitude (nM)	183 ± 29	28 ± 4	47 ± 19
Time to peak (TTP, s)	12.9 ± 3	49 ± 14	144 ± 28
Rate of rise (ROR, nM/s)	59 ± 12	7.5 ± 1	3.5 ± 1
Tachyphylaxis (% decrease)	–23 ± 13*	4.5 ± 6	N/A

Parameters shown in the table were determined as described in Materials and methods. RTX was ineffective in 0 mM $[Ca^{2+}]_e$ solution. The negative tachyphylaxis, represented by the asterisk, reflects an increase in the amplitude of the second transient evoked by capsaicin compared to the first one (see text for further details). All values are expressed as the mean ± SE of several determinations. N/A, not applicable.

Table 3. Summary of various parameters of $[Ca^{2+}]_i$ transients induced by capsaicin and RTX in VR1/C6 cells.

	Capsaicin (1 µM)	RTX (1 nM)
	1.8 mM Ca^{2+}	1.8 mM Ca^{2+}
Responding cells (%)	100	28
Transient type	fast (100%)	slow (100%)
Amplitude (nM)	117 ± 19	29.2 ± 4
Time to peak (TTP, s)	12.1 ± 8	75 ± 13
Rate of rise (ROR, nM/s)	25.7 ± 5	7.3 ± 2
Tachyphylaxis (% decrease)	–9.5 ± 5*	N/A

Parameters shown in the table were determined as described in Materials and methods. Capsaicin and RTX were both ineffective in 0 mM $[Ca^{2+}]_e$ solution. The negative tachyphylaxis, represented by the asterisk, reflects an increase in the amplitude of the second transient evoked by capsaicin compared to the first one (see text for further details). All values are expressed as the mean ± SE of several determinations. N/A, not applicable.

In the VR1eGFP/cos-7 cell transient and the VR1/CHO inducible expression systems, the membrane-incorporated VR1 may be partially open in resting cells

Comparison of different characteristics of control and VR1-expressing cells in 1.8 mM $[Ca^{2+}]_e$ solution revealed another interesting phenomenon. Namely, in the VR1eGFP/cos-7 cell transient and the VR1/CHO inducible expression systems, but not in the VR1/C6 stable expression system, the VR1 channel at the surface membrane may be partially open. We found that the resting $[Ca^{2+}]_i$ of empty eGFP vector-transfected cos-7 cells (n = 15) was 60.2 ± 5 nM, whereas this value in VR1eGFP/cos-7 cells (n = 53) was 148 ± 24 nM (all values are the mean ± SE). Similarly, the resting $[Ca^{2+}]_i$ in VR1/CHO cells was much higher (185 ± 29 nM, mean ± SE, n = 100) than that of the control CHO cells (76 ± 15 nM, mean ± SE, n = 25). However, in the VR1/C6 stable expression system, we did not detect such differences among the resting $[Ca^{2+}]_i$ of control and VR1/C6 cells; in control cells, the resting $[Ca^{2+}]_i$ was 46 ± 5 nM (mean ± SE, n = 17) whereas in VR1-expressing cells, it was 52.2 ± 8 nM (mean ± SE, n = 99).

The involvement of VR1 in the elevated resting $[Ca^{2+}]_i$ of the VR1eGFP/cos-7 cells and the VR1/CHO cells was supported by analyzing the effect of capsazepine. As seen in figure 2E and figure 6E, 5 µM capsazepine, beside inhibiting the action of 1 µM capsaicin, was alone able to markedly decrease the resting $[Ca^{2+}]_i$ in the VR1eGFP/cos-7 cell transient expression system and in the VR1/CHO inducible system. Statistical analysis revealed that this decline was 78 ± 13 nM (mean ± SE, n = 15) in VR1GFP/cos-7 cells and 110 ± 13 nM (mean ± SE, n = 8) in VR1/CHO cells. In marked contrast, similar pretreatment of VR1/C6 cells with the inhibitor resulted in an only 7.8 ± 2 nM (mean ± SE, n = 17) decrease in resting $[Ca^{2+}]_i$ (fig. 7D), yet effectively inhibited the action of capsaicin. Finally, 2-min incubation of control (empty vector-transfected) cos-7, CHO, or C6 cells by 5 µM capsazepine never caused more than a 10 nM decrease in resting $[Ca^{2+}]_i$ (data not shown).

Discussion

In the current study, our goal was to compare the functional characteristics of recombinant rat VR1 expressed in different (transient, inducible, stable) systems. Our results clearly demonstrate that the vanilloid sensitivity and extracellular calcium dependence of VR1-mediated $[Ca^{2+}]_i$ responses are markedly different in the various systems.

Similar to previous results [16, 17], here we showed that the GFP-tagged VR1 expressed in cos-7 cells was localized both to surface membrane and to intracellular (calcium store) structures (figs. 3, 5). The intracellular VR1 was functional (figs. 2, 4) in that both capsaicin and RTX were able to increase $[Ca^{2+}]_i$ and initiate subcellular disorganization even in the absence of $[Ca^{2+}]_e$, and that emptying of intracellular calcium stores by TG interfered

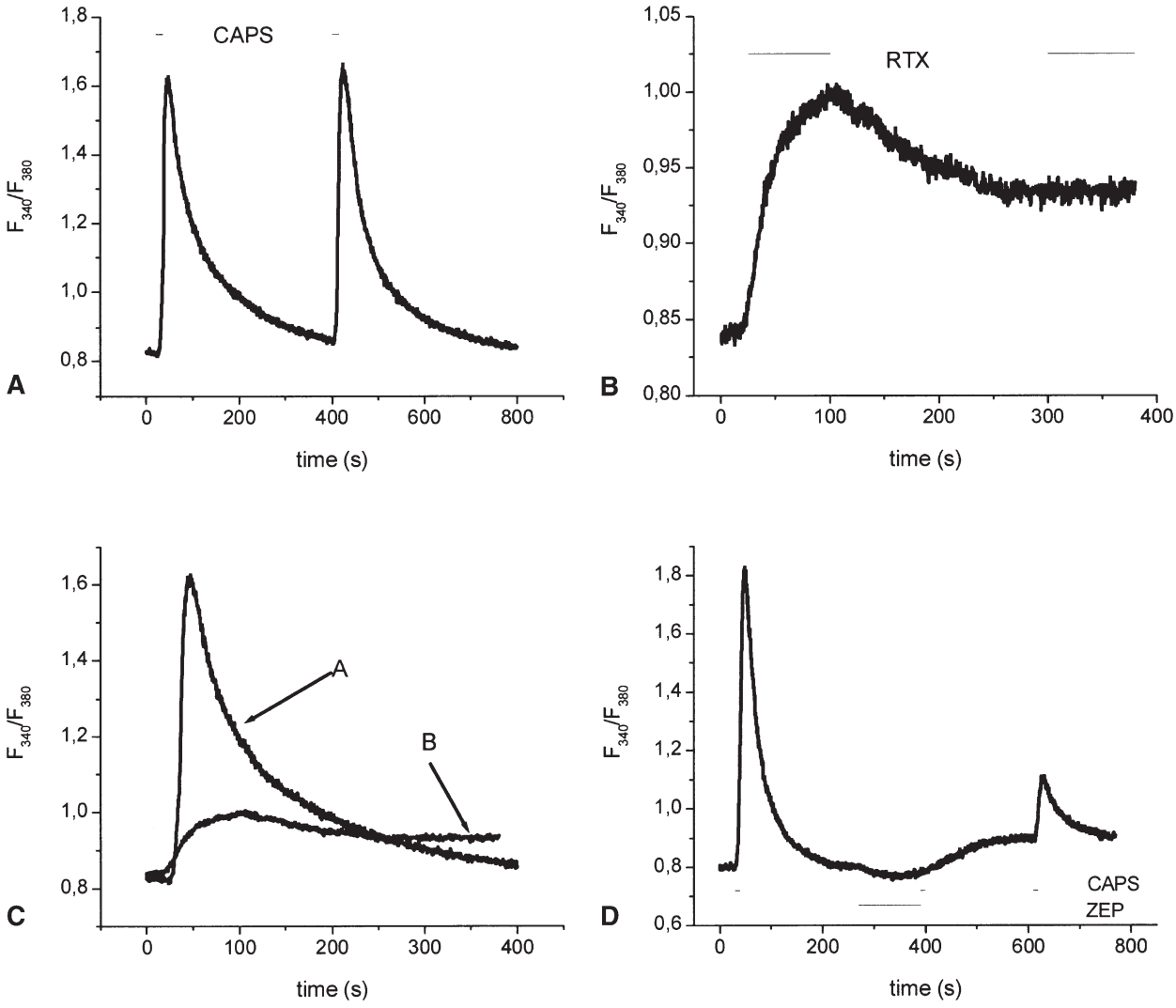

Figure 7. Effects of capsaicin and RTX on $[Ca^{2+}]_i$ in VR1/C6 cells. Cells growing on glass coverslips were loaded with 5 µM fura 2-AM and fluorescence ratio (F_{340}/F_{380}) values of excitations at 340- and 380-nm wavelengths were recorded at an acquisition rate of 10 Hz per ratio. The effects of 1 µM capsaicin (CAPS) (*A*) and 1 nM RTX (*B*) in 1.8 mM $[Ca^{2+}]_e$ solution are represented. For better comparison of various parameters of the capsaicin- and RTX-induced transients, the first $[Ca^{2+}]_i$ elevations in *A* and *B* (indicated by arrows) are shown using the same time and ratio scales (*C*). The effect of 5 µM capsazepine (ZEP) to significantly yet reversibly inhibit the action of capsaicin in 1.8 mM $[Ca^{2+}]_e$ medium is shown in *D*. Representative results of several determinations are summarized in table 3.

with these actions. There were, however, marked differences in the effectiveness of the two vanilloids to affect intracellular calcium depending on the calcium content of the medium. Whereas the action of capsaicin strongly depended on the $[Ca^{2+}]_e$, RTX was equally effective in calcium-containing and -free solutions. These data were in good accordance with recent data on human recombinant VR1 expressed in HEK 293 cells [31], where RTX but not capsaicin was able to increase intracellular calcium in calcium-free medium (see also below).

In the VR1eGFP/cos-7 cells, we observed a remarkable heterogeneity among the vanilloid-induced transients, i.e., both fast and slow transients were recorded for capsaicin in high calcium and for RTX in calcium-free solutions (table 1). Since these phenomena were exclusively seen in the VR1eGFP/cos-7 transient expression system, one explanation is that, due to the transient nature and variable degree of transfection of the VR1eGFP/cos-7 cells, the level of VR1 in the individual cells could be heterogenous, allowing the same vanilloid on different cells having different VR1 expression levels to cause different calcium responses. This hypothesis is consistent with previous molecular biological and functional data that VR1, both in sensory neurons and in recombinant systems, is expressed in various multimeric structures [21] which may result in heterogeneous responses to the same vanilloid [32].

Among the three systems we studied, the most homogenous vanilloid induced responses were observed in the sta-

ble (VR1/C6) system. In these cells, similar to findings in sensory neurons [13–15], capsaicin and RTX were effective only in calcium containing extracellular medium (fig. 7, table 3). In addition, the capsaicin- or RTX-evoked responses were characteristically very similar in all of the cells examined, i.e., capsaicin evoked fast whereas RTX induced slow $[Ca^{2+}]_i$ elevations, also reflecting well the previously described difference in pharmacokinetics of the two vanilloids [2, 33]. These data suggest that the expression of VR1 in VR1/C6 cells is well controlled by the recombinant vector and uniform circumstances can be obtained for VR1 characterization.

The vanilloid-evoked calcium responses in the inducible system (VR1/CHO cells) were generally very similar to those obtained in VR1/C6. In 1.8 mM $[Ca^{2+}]_e$ solution, both capsaicin and RTX induced uniform, fast and slow calcium responses, respectively. However, there were also differences between the inducible and stable expression systems. Namely, in VR1/CHO cells, capsaicin (but not RTX) was able to induce calcium transients even in calcium-free solution. However, under such conditions, capsaicin was effective in only a much lower percentage of the cells examined (25% compared to 66% in high calcium solution; table 2) and, furthermore, the capsaicin-evoked responses were much slower and smaller than seen in calcium-containing medium. Naturally, further experiments are needed to unambiguously clarify the putative localization of some portion of expressed VR1s to intracellular membrane structures (due to the nature of commercially available antibodies against VR1, which were developed to target intracellular domains of VR1, permeabilization of cells is required for immunocytochemistry and this may affect the localization). However, our data, consistent with recent findings by Tóth et al. [22] that TG pretreatment of VR1/CHO cells completely abolished the capsaicin-induced $[Ca^{2+}]_i$ elevations, strongly argue for additional (yet much less than seen in VR1eGF/cos-7 cells) intracellular incorporation of functional VR1 in this system.

A major 'unexpected' result, both in the VR1/C6 and VR1/CHO cell systems when compared to previously described data in sensory neurons [reviewed in ref. 2] was the complete lack of tachyphylaxis after repeated capsaicin applications under our conditions (figs. 6, 7, tables 2, 3). Since the complex mechanism of tachyphylaxis in sensory neurons [1, 2, 11, 12] may also involve the decrease in VR1 density upon repeated or prolonged vanilloid administration [2], we suppose that the stable or relatively stable nature of these expression systems may contribute to the lack of tachyphylaxis. In addition, since the sophisticated calcium handling and signal transduction (kinase, phosphatase) systems described in the host cells [34, 35] are distinct from those of the sensory neurons, the regulation of VR1 by such systems could be markedly different. Nevertheless, the phenomenon that repeated applications of capsaicin result in almost identical calcium responses in VR1/C6 and VR1/CHO cells may even possess an attractive feature for pharmacological studies characterizing inhibitory or sensitizing actions of agents on VR1.

Capsaicin and RTX, although their actions are qualitatively similar, have distinct spectra of action, resulting in differences in their relative potencies for different responses [1, 2]. RTX is generally regarded as an ultrapotent analog of capsaicin; however, there are some responses where it shows only slightly greater potency or, in contrast to the action of capsaicin, ineffectiveness. This latter phenomenon seems to be true for the inducible and stable recombinant expression systems; namely, in VR1/CHO and VR1/C6 cells, RTX was able to evoke only small and slow transients and, of great importance, in a markedly lower percentage of cells than did capsaicin (tables 2, 3). These findings were in good accord with previously published data that the relative potency of capsaicin was much greater on VR1 ectopically expressed in HEK 293 cells than on the native channel in sensory neurons [36], whereas RTX was more potent in activating VR1 in cultured neurons than in the recombinant expression system [37].

Naturally, identifying those factors that may contribute to differences (subcellular localization, calcium dependence, vanilloid sensitivity) seen in the various expression systems is of great importance. Based on both our presented data and the literature, the characteristics of the recombinant vector [16–18, 20, 38], the functional features (e.g., calcium handling) of the host cells [6, 8, 16, 17, 19, 38]; the type of expression (i.e., transient or stable) [10], the level of glycosylation and heterogenous stochiometry [21, 38], and the sensitivities of the methods to record the calcium signals may all contribute to the distinct properties of the systems. The importance of the techniques used for analysis is emphasized by the fact that most authors who either described calcium-dependent [13–15] or -independent [16–18] VR1-mediated responses and, in addition, exclusive surface membrane or simultaneous surface and intracellular membrane localizations, found very similar patterns on sensory neurons expressing the native VR1. In any case, although the relative contributions of different parameters to define VR1 functional characteristics in heterologous expression systems remain to be clearly established, our findings strongly argue for the influence of the heterologous expression system on the determination of VR1 cellular functions and suggest caution in extrapolating such findings to other systems such as primary sensory neurons.

Acknowledgement. The authors are indebted to Ms. I. Varga for helpful technical assistance. This work was supported by Hungarian research grants: OTKA T030246, OTKA F035036, NKFP 00088/2001, OMFB 00200/2002. Tamás Bíró is a recipient of the György Békéssy Postdoctoral Scholarship of the Hungarian Ministry of Education.

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