Intact Microtubules Preserve Transient Receptor Potential Vanilloid 1 (TRPV1) Functionality through Receptor Binding

Received for publication, December 13, 2011, and in revised form, January 10, 2012. Published, JBC Papers in Press, January 17, 2012, DOI 10.1074/jbc.M111.332296

Barbara Storti,†§, Ranieri Bizzarri,†§, Francesco Cardarelli,§, and Fabio Beltram‡§

From the †National Enterprise for nanoScience and nanoTechnology (NEST), Scuola Normale Superiore and Istituto Nanoscienze-CNR, Piazza S. Silvestro 12, 56127 Pisa and the §Center for Nanotechnology Innovation, @NEST, Istituto Italiano di Tecnologia, Piazza San Silvestro 12, 56127 Pisa, Italy

Background: TRPV1-microtubule interaction is important in transducing nociception stimuli by cytoskeletal rearrangement.

Results: Intact microtubules are essential in maintaining TRPV1 functionality.

Conclusion: Our data were recast in a model of TRPV1 aggregation and binding.

Significance: Our findings reveal the presence of a feedback cycle that regulates the interplay between nociception and cytoskeletal integrity.

The transient receptor potential cation channel subfamily V member 1 (TRPV1) is a protein currently under scrutiny as a pharmacological target for pain management therapies. Recently, the role of TRPV1-microtubule interaction in transducing nociception stimuli to cells by cytoskeletal rearrangement was proposed. In this work, we investigate TRPV1-microtubule interaction in living cells under the resting or activated state of TRPV1, as well as in presence of structurally intact or depolymerized cytoskeletal microtubules. We combined a toolbox of high resolution/high sensitivity fluorescence imaging techniques (such as FRET, correlation spectroscopy, and fluorescence anisotropy) to monitor TRPV1 aggregation status, membrane mobility, and interaction with microtubules. We found that TRPV1 is a dimeric membrane protein characterized by two populations with different diffusion properties in basal condition. After stimulation with resiniferatoxin, TRPV1 dimers tetramerize. The tetramers and the slower population of TRPV1 dimers bind dynamically to intact microtubules but not to tubulin dimers. Upon microtubule disassembly, the interaction with TRPV1 is lost thereby inducing receptor self-aggregation with partial loss of functionality. Intact microtubules play an essential role in maintaining TRPV1 functionality toward activation stimuli. This previously undisclosed property mirrors the recently reported role of TRPV1 in modulating microtubule assembly/disassembly and suggests the participation of these two players in a feedback cycle linking nociception and cytoskeletal remodeling.

Communication of cells with the external environment includes a large array of complex phenomena that depend on the activity of specialized membrane proteins and on the interaction between membrane transporters and the cytoskeleton. Important details of the interaction mechanisms between cytoskeletal and membrane proteins are still unknown. Cytoskeletal components were found to be downstream effectors of some membrane transporters (2). The regulatory activity of cytoskeletal components on membrane transporters was also investigated (3). The capsaicin receptor (TRPV1) is part of the transient receptor potential vanilloid family. It is a nonselective voltage-dependent cation channel involved in pain signaling (4). Identification of the molecular mechanisms involved in the activation of TRPV1 is relevant to the discovery and design of drugs capable of controlling TRPV1 activity and thereby pain stress in humans (5, 6). Besides sensory neurons, TRPV1 is expressed in many other cell types (e.g. endothelial cells). TRPV1 is a polymodal receptor that integrates a number of physical and molecular stimuli. Modulators of TRPV1 activity include noxious heat (7), low pH (8), capsaicin and capsaicin analogues like resiniferatoxin (RTX) (9). Single particle electron cryomicroscopy investigations suggested that TRPV1 assembles into a tetrameric complex at the cell membrane (10). This finding is consistent with biochemical studies in living cells that demonstrated a tetrameric stoichiometry for functional TRPV1 (11–13).

TRPV1 was recently shown to bind to microtubule β-tubulin in a Ca⁺²⁺-regulated fashion (14). In vitro experiments highlighted the role of TRPV1 in stabilizing microtubules (hereafter denoted as μTs). Activation of TRPV1 was demonstrated to yield fast disassembly of μTs, albeit at agonist (capsaicin or RTX) concentrations much larger than saturation (15). Apparently, the interaction between TRPV1 and β-tubulin is mediated by the two small amino acid stretches 710–730 and 770–797, positively charged at physiological pH (pI = 11.2–12.6) and matching the negatively charged C-terminal sequence of β-tubulin (16). This finding is also supported by biochemical experiments carried out on TRPV1 deletion mutants within the α subunit of TRPV1.

This work was supported in part by the Italian Ministry for University and Research under the framework of FIRB Projects RBLAG03ER8R, RBPR05JH2P, and PRIN 2008IU4MLB_002.

This article contains supplemental “Experimental Procedures” and Figs. S1–S12.

1 To whom correspondence should be addressed. Tel.: 39-050-509124; Fax: 39-050-509417; E-mail: barbara.storti@sns.it.

2 The abbreviations used are: RTX, resiniferatoxin; RFP, red fluorescent protein; μT, microtubule; tICS, temporal image correlation spectroscopy; N&B, Number and Brightness.
Binding of Intact Microtubules Preserve TRPV1 Functionality

C-terminal region (13). Notably, the C terminus of TRPV1 is involved in several other functions among which stands the structural determinants sensitive to heat and capsaicin (17). The proximity of regulatory stretches and tubulin-binding sites in the same region of TRPV1 is consistent with the identification of the μT cytoskeleton as downstream effector of TRPV1. This interplay suggests that TRPV1 may regulate some specific cell functions (and possibly neuronal functions) through μT cytoskeleton reshaping. Yet, the biological function of the TRPV1-μT interaction may not be restricted to this effect. One can ask, for instance: do μTs modulate TRPV1 activity? Indeed, this μT-TRPV1 interaction may play a role in tuning cell response to TRPV1 activation upon different factors, including noxious stimuli.

In this work, we set out to clarify whether μT integrity affects the biological and biophysical properties of functional TRPV1 expressed in living cells. Several authors reported static descriptions of TRPV1-μT interactions based on in vitro assays or on fixed cells. A membrane receptor is characterized, however, by a set of intertwined molecular properties, such as mobility, binding, and oligomerization status, all of which must be monitored in real time to provide a meaningful picture of the receptor biology and particularly its interactions with other cellular components. Accordingly, our experimental strategy combined fluorescent labeling of TRPV1 and μTs with suitable fluorescent proteins that allowed us to perform a set of high resolution/high sensitivity fluorescence-microscopy studies each tailored to unveil a specific TRPV1 property in living cells. Interactions dynamical and assembly properties were studied under resting or activated state of TRPV1 by agonist stimulation, namely, capsaicin (17). Remarkably, the combination of FRET and tICS imaging allowed us to identify a TRPV1 pool not interacting with μTs.

Fluorescence resonance energy transfer (FRET (18)) was used to probe the binding interaction between TRPV1 and μTs. Changes in TRPV1 oligomerization were quantitatively investigated by a new technique based on fluorescence fluctuation analysis referred to as Number and Brightness (N&B) (19). Thanks to this technique, we can show a clear picture of the membrane organization of TRPV1 in living cells. Fluorescence anisotropy associated with homo-FRET was used in combination with N&B to detect the presence of large receptor aggregates (20). TRPV1 membrane mobility was investigated by temporal image correlation spectroscopy (tICS), a method based on the time correlation analysis of fluorescence fluctuations recorded in the presence of a few slowly moving molecules imaged in a micron-size area (21–23). Remarkably, the combination of FRET and tICS imaging allowed us to identify a TRPV1 pool not interacting with μTs. FRET and tICS integration was made possible by the use of a fluorescent protein mutant recently described by our group (24). All our data were recast into a model accounting for the molecular interplay between TRPV1 and μTs. More importantly, our results clearly demonstrate the role of assembled μTs in preserving the structural functionality of TRPV1, thus showing a specular behavior to the known role of TRPV1 in modulating μT integrity.

**EXPERIMENTAL PROCEDURES**

**Constructs and Transfection**—The transfections of all constructs in CHO K1 cells were carried out using Lipofectamine reagent (Invitrogen) according to the manufacturer’s instructions. In all experiments, cells were maintained at 37 °C in a 5% CO2 atmosphere. TRPV1-YFP construct is a kind gift from Wei Cheng (University of California, Davis), and its activity upon transfection was tested according to Ref. 25. The TRPV1-E0 construct was generated by site-directed mutagenesis of p-TRPV1-EYQ1 (26) using the following primers: Y203T, 5′-CAACCACACTAATGGCCACCAAGCTCGGCGTGAAGAAA-3′; Q222E, 5′-CGCGATCACATGTTCCTGAGTTCTGGACCGCCCGGG-3′. Tubulin-RFP construct is provided from Evrogen (catalog no. FP145).

**FRET and Anisotropy Imaging**—Measurements were carried out with a Leica TCS SP2 inverted confocal microscope (Leica Microsystems AG, Wetzlar, Germany). ×63 (NA 1.4) (for FRET) or ×40 (NA 1.25) (for anisotropy) PlanApo oil immersion objectives were used while setting the confocal pinhole at 1 Airy unit. FRET was performed by the sensitized emission technique under excitation of 405 nm (for donor TRPV1-E0GFP) or 488 nm (for donor TRPV1-YFP). Fluorescence emission of the RFP acceptor was detected at 600–640 nm. FRET analysis was carried out with a homemade plugin of the ImageJ software (supplemental Fig. S1). For anisotropy imaging with TRPV1-YFP, samples were excited at 488 nm, and fluorescence was collected at 520–600 nm at parallel and perpendicular polarization with respect to the exciting beam using the internal polarizer of the microscope. Anisotropy maps were obtained by a homemade plug-in running under ImageJ (supplemental Fig. S1).

**tICS and tICS-FRET**—tICS and tICS-FRET measurements were carried out with an Olympus FluoView 1000-ASW-2.0 (Japan) confocal laser scanning microscope using a ×63 (NA 1.4) PlanApo oil immersion objective while setting the confocal pinhole at 1 Airy unit. For each measurement, a small section of the plasma membrane (about 13 × 13 μm2) was imaged in scanning mode (26 ms/frame or 2 s/frame) by acquiring 300 images. Spectral setups were as follows: tICS, excitation 488 nm and emission 500–600 nm (TRPV1-YFP); tICS-FRET, excitation 405 nm, donor emission 480–560 (TRPV1-E0GFP), and acceptor emission 615–715 (tubulin-RFP). Image autocorrelation was obtained by the ICS temporal plugin of ImageJ; fitting of autocorrelation curves was performed by homemade software running under IgorPro 6 or Origin.

**N&B Analysis**—We set the Olympus FluoView 1000 to the pseudo-photon-counting data acquisition mode. In this mode, the parameters needed for N&B analysis are the detector offset, the factor S that converts photon counts to digital levels and the readout variance σ02 (supplemental “Experimental Procedures”). For the present analysis these parameters were estimated according to Ref. 27. We obtained S = 3.5, σ02 = 0, and offset = 0.256 × 256 images at 8 bits were collected with a pixel dwell time of 20 μs. A time series of 100 frames with no programmed delay between images was used to reduce statistical error. Low laser power was used to avoid photobleaching. The N&B analysis was performed using the SimFCS software. To
calibrate laser power and scanning conditions required for the measurement of a monomeric protein diffusing within membranes, we measured the brightness of CHO-K1 cells transiently expressing the nonaggregating farnesyl-EGFP adduct (EGFP-F). These conditions were then used for the TRPV1-YFP experiments. We separately measured the difference in apparent brightness between EGFP and YFP by using CHO-K1 cells transiently expressing the two untagged variants (supplemental Fig. S2); we found a YFP/EGFP ratio of brightness very close to 1. Additional experimental details are provided in supplemental “Experimental Procedures”.

RESULTS

Protein Expression and Functionality—Following transient transfection in CHO cells, TRPV1-E0GFP or TRPV1-YFP showed nonhomogeneous localization in the plasma membrane (supplemental Fig. S3) and in the endoplasmic reticulum. Previous reports on fixed cells showed similar localization patterns (28). The ability of cell-expressed TRPV1 to activate inward calcium flux was assessed by stimulation with 20 nM RTX, the latter being a concentration that ensures full saturation of membrane receptors while preserving cell viability (EC50 of RTX is 0.15 nM (29)). RTX was administered while the increase of intracellular calcium was monitored. As expected (29), RTX induced a gradual increase of intracellular Ca2+ that was monitored by the fluorescent indicator calcium crimson. Within 100 s Ca2+ concentration was found to reach its maximum value and to remain constant over time (supplemental Fig. S3). This suggests that after this induction time all channels are activated. Thus, in subsequent experiments involving RTX, we carried out quantitative measurements only after this induction time. The progressive activation of TRPV1 upon RTX suggests a limited receptor activation in basal conditions at 37 °C, consistent with the previously measured parameters of voltage/temperature activation of TRPV1 (~15% of activated TRPV1 at 37 °C).

Binding Interaction between TRPV1 and μT—Tag-RFP (henceforth denoted just as RFP) is a bright red-emitting fluorescent protein that can be used as FRET acceptor with both E0GFP and YFP as donors. Spectral data yield an apparent brightness between EGFP and YFP by using CHO-K1 cells transiently expressing the two untagged variants (supplemental Fig. S2); we found a YFP/EGFP ratio of brightness very close to 1. Additional experimental details are provided in supplemental “Experimental Procedures”.

Membrane Dynamics of TRPV1—This evidence of TRPV1/μT binding in functional cells prompted us to investigate whether the μT assembly affects the membrane dynamics of TRPV1 and thereby its biological role. To this end, we monitored the membrane diffusion of fluorescent TRPV1 in the presence of intact μTs by tICS. Although in most cases we used TRPV1 labeled with YFP because of the slightly larger brightness of YFP over E0GFP, we should like to stress that comparable results were obtained by using TRPV1-E0GFP, consistently with the analogous size of these fluorescent proteins. Note that the tICS data were fitted to an isotropic diffusional two-dimensional model following the membrane localization of TRPV1-YFP (22). Diffusion properties were poorly correlated with the expression level, suggesting that overexpression negligibly contributes to the observed phenotype (supplemental Fig. S4).

At basal conditions, tICS showed that membrane TRPV1-YFP is characterized by two subpopulations with diffusion-dynamics values separated by about 2 orders of magnitude (Table 1). The faster and slower populations of TRPV1 account for 40 and 60% of the correlation amplitude, respectively. No immobile fraction was detected. The accuracy of diffusion constants was ensured by calibrating tICS on size-defined fluorescent nanospheres in solutions of controlled viscosity (supplemental Fig. S5).

To determine whether both populations or just one are involved in μT binding, we carried out tICS experiments in FRET mode; the separate autocorrelation functions of the donor and FRET emissions were calculated from the image would yield an apparent FRET efficiency equal to 0.27 ± 0.03, corresponding to 4–5 nm of separation between TRPV1 and the supposedly single interacting tubulin of μTs, taking into account the size of the β-barrel structure of the fluorescent protein; EGFP-F, farnesyl-enhanced GFP.
sequences of CHO cells expressing both TRPV1-E0GFP and tubulin-RFP (Fig. 2). Note that the E0GFP/RFP pair ensures no emission cross-talk when FRET is imaged at >615 nm under 405-nm excitation. This is very important here because a “pure” FRET signal is essential to obtain a meaningful autocorrelation analysis; a detectable cross-talk emission would hide significant dynamic details of the donor/acceptor binding. The autocorrelation curve of the FRET channel was found to parallel that of the donor TRPV1-E0GFP at longer times that can be associated with the diffusion of the slower receptor component. On the contrary, at shorter times that can be linked to the faster TRPV1 population, the autocorrelation curves are very different (Fig. 2). We conclude that only the slower TRPV1 population interacts with μT.s. From the emission profile, the small offset in the donor autocorrelation curves was attributed to limited E0GFP photobleaching in the acquisition conditions, excluding the presence of an immobile fraction of TRPV1. Control experiments on cells expressing TRPV1-E0GFP or tubulin-RFP alone (supplemental Fig. S6 and Fig. S7) confirmed that the observed pattern is associated only with the interaction between the receptor and μT.s.

Effect of μT Disassembly on TRPV1 Dynamics and Aggregation—TRPV1 dynamics when μT.s are disassembled was also visualized. To this end, we carried out tICS experiments on cells expressing TRPV1-YFP under nocodazole treatment. We found that the diffusion coefficients of both fast and slow components exhibit a 10-fold increase over physiological values (p < 0.0025), although the ratio between the two populations was virtually unaffected (Table 1). Interestingly, autocorrelation curves showed large asymptotic offsets (35% of the global autocorrelation amplitude). We attribute this effect to a significant level of immobile fraction on account of the negligible photobleaching of YFP in our measurement conditions.

We verified that the observed mobility increase of TRPV1 is not linked to an unspecified cytoskeletal effect by performing two control experiments. In the first experiment, the addition of cytochalasin D to cultured cells did not affect either diffusion coefficients or population fractions of membrane TRPV1 measured by tICS (Table 1). In the second experiment (supplemental Fig. S8), we verified that nocodazole administration did not lead to significant changes in the aggregation of EGFP-F, a protein construct targeted to cell membrane and with no specific biological role.

To further investigate the immobile TRPV1 fraction generated upon μT disassembly, we performed static anisotropy measurements on TRPV1-YFP expressed in cells under different stimuli to evaluate whether μT functionality is correlated with TRPV1 aggregation status (Fig. 3). Prior to this analysis, we tested the capability of our microscopy system to distinguish changes in anisotropy by using monomeric and tetrameric GFPs freely diffusing in the cytoplasm (supplemental Fig. S9). At basal conditions, the mean static anisotropy (r) of membrane TRPV1-YFP was 0.17 ± 0.03, a value much smaller than that found for free cytoplasmic YFP (r = 0.27 ± 0.02) (Table 1). Time-resolved emission measurements highlighted that free and TRPV1-bound YFP retain the same fluorescence lifetime (r = 3 ns). In principle, a reduction in r at constant lifetime might be related to an increase in the rotational mobility of the protein. It seems very unlikely, however, that YFP conjugated to TRPV1 can have a greater mobility than free monomeric YFP. A much more convincing explanation for the observed reduction in r should take into account the high efficiency of homo-FRET for YFP conjugates upon oligomerization (31). In fact, YFP is characterized by extensive overlap of its emission and absorption bands; this promotes homo-FRET at distances below 7–8 nm (R0 = 5.11 nm (32)). Homo-FRET does not change the emission lifetime, but it does modify r (20). Apparently, functional TRPV1 molecules adopt a tetrameric configuration with inter-subunit distance around 10 nm (10). The tetramerization domain is located in the C-terminal sequence of TRPV1 (13, 16), resulting in a much shorter distance between YFP tags that are linked to TRPV1 at the C terminus.

Remarkably, administration of nocodazole led to r = 0.13 ± 0.03 for membrane TRPV1-YFP, a value statistically lower than the basal one. Nocodazole did not affect the emission lifetime of YFP. Considering also the immobile fraction observed in tICS analysis, we attributed this r change to the formation of large immobile aggregates, capable of extensive homo-FRET interactions. Consistently, the addition of cytochalasin-D had no effect on r and did not lead to an immobile TRPV1 fraction in tICS measurements. The known homo-FRET anisotropy can fully account for the reduced size

| TABLE 1 | Anisotropy and diffusion parameters derived from tICS measurements on membrane TRPV1-YFP |

| Anisotropy | tICS |
|-----------|------|
|            | No. of cells | Oligomerization status | % AC | D μm²/s | IF | No. of cells |
| EYFP | 0.27 ± 0.02 | 20 | Dimer | 40 ± 12 | (5 ± 3) × 10⁻³ | 0 | 20 |
| Basal | 0.18 ± 0.03 | 15 | ND | 60 ± 10 | (4 ± 1) × 10⁻⁵ | 0 | 20 |
| Nocodazole | 0.12 ± 0.03 | 40 | ND | 25 ± 15⁺ | (9 ± 8) × 10⁻² | 35 ± 15 | 30 |
| Cytochalasin D | 0.17 ± 0.02 | 20 | Dimer | 40 ± 20⁺ | (3 ± 2) × 10⁻⁴ | 0 | 10 |
| RTX | 0.18 ± 0.04 | 15 | Dimer + tetramer | 10 ± 10 | (3 ± 2) × 10⁻³ | 0 | 20 |
|         | 90 ± 10 | 10 | (5 ± 3) × 10⁻⁵ | 0 | 20 |

*Note that the ratio between the autocorrelation amplitude (AC) of fast and slow populations in nocodazole treatment cells (25:40%) is the same of that calculate in basal condition (40:60%).
of $\langle r \rangle$ decrease from physiological state to $\mu T$ disassembly (0.17 versus 0.13) compared with that observed when considering free YFP and membrane-confined TRPV1-YFP (0.27 versus 0.17). Indeed, calculations and experimental evidence show that the plot of static anisotropy versus aggregations follows a nonlinear trend that levels off for oligomerization above 4–6 (33). Note, however, that RTX activation of TRPV1 negligibly affected static anisotropy, suggesting that either minor variations of oligomerization status take place or that the actual changes in $\langle r \rangle$ were hidden by cellular variability.

**Effect of TRPV1 Activation on $\mu T$s—**In this set of experiments, we investigated the effect of RTX to assess whether TRPV1 activation affects its interaction with $\mu T$s. At first, we found that the measured apparent FRET efficiency between TRPV1-YFP and tubulin-RFP is nearly unchanged by administration of RTX in the cell medium (Fig. 1). tICS-FRET experiments again indicated that only the slower diffusing population of TRPV1 binds to $\mu T$s (supplemental Fig. S10). These findings indicate that receptor interaction with $\mu T$s is unchanged upon TRPV1 activation. It is worth noting that RTX does not modify the diffusion coefficient of TRPV1 on the membrane, although the slower fraction becomes predominant (90% of the correlation amplitude). RTX was found not to lead to a significant immobile fraction, thus suggesting that receptor activation is not accompanied by extensive aggregation. This finding was also confirmed by static anisotropy measurements for which $\langle r \rangle = 0.18 \pm 0.04$, a value compatible with what measured for the basal case (Table 1).

Finally, we tested the concomitant effect of nocodazole and RTX by measuring the change in intracellular calcium concentration upon TRPV1 activation (supplemental Fig. S11). Remarkably, nocodazole decreased the maximum amplitude of $Ca^{2+}$ concentration on average by about 40%, affecting appreciably the influx kinetics. It is tempting to correlate the presence of a significant multimerized and poorly mobile TRPV1 fraction, induced by $\mu T$ disassembly, with the reduced response in the receptor activation by RTX.

**Effect of TRPV1 Activation on Oligomerization State—**In the last set of experiments, we monitored the effects of ligand administration on the oligomerization status of TRPV1 by fluctuation spectroscopy (N&B analysis) (19). As benchmark for a monomeric membrane protein, we used transfected EGFP-EGFP (Fig. 4A). We found a homogeneous distribution of the monomer on the membrane (Fig. 4B), brightness = $1.13 \pm 0.03$ counts/molecule (number of cells = 8), corresponding to an average molecular brightness of $e = 0.13 \pm 0.03$ counts/molecule (Fig. 4C). Remarkably, N&B analysis of TRPV1-YFP in basal conditions revealed that the protein was homogeneous in a dimeric form, as witnessed by the Gaussian distribution of molecular brightness centered at $e = 0.25 \pm 0.02$ counts/mol.
Binding of Intact Microtubules Preserve TRPV1 Functionality

FIGURE 3. Confocal steady state intensity and fluorescence static anisotropy images. Cells expressing TRPV1-YFP are shown in different conditions (as reported on the left). The static anisotropy was calculated from cell images acquired between 520 and 600 nm at parallel and perpendicular polarization compared with excitation ($\lambda_{exc} = 488$ nm).

cule (Fig. 4, D–F). The presence of dimer TRPV1 at resting state was recently reported also in Ref. 34 based on patch clamp analysis.

N&B analysis was also performed on the same cell upon RTX administration (Fig. 4G). Notably, TRPV1 dimers rapidly redistribute within the membrane generating a major detectable fraction of tetramers with $e = 0.50 \pm 0.03$ (number of cells = 4) (Fig. 4, H and I, yellow pixels). These findings indicate that, upon activation, TRPV1 oligomerization is characterized by an equilibrium between dimers (smaller fraction) and tetramers (larger fraction).

DISCUSSION

So far, the binding interaction between TRPV1 and $\mu$Ts was inferred from in vitro biochemical assays (14) and co-localization analysis on fixed cells (15). The first step in this study was to verify by a FRET analysis that TRPV1 and tubulin are indeed interacting in the membrane of living cells in physiological conditions. Both in the absence and presence of agonist RTX, we found that TRPV1-E0GFP (or TRPV1-YFP) and tubulin-RFP undergo a well detectable and quantitatively constant energy transfer thus indicating their binding scale proximity. Conversely, $\mu$T disassembly induced by nocodazole led to the complete FRET-signal disappearance. These findings demonstrate the following: 1) TRPV1-tubulin binding takes place only when the latter protein is polymerized in the functional $\mu$T cytoskeleton, and 2) the interaction between TRPV1 and $\mu$T is independent from the channel-activation status.

Additional information about the interaction between TRPV1 and $\mu$Ts was obtained from the analysis of TRPV1 diffusion in cell membranes by tICS. At basal conditions, TRPV1 is split into two subpopulations characterized by rather different membrane-mobility values (Table 1) hereafter denoted as $D_s$ (slow) and $D_f$ (fast) components. tICS analysis indicated that $D_s$ and $D_f$ account for about 60 and 40% of the autocorrelation amplitude, respectively. Both these components were shown to correspond to TRPV1-YFP dimers based on brightness analysis. Thus, the observed fractions correspond to two actual TRPV1-YFP subpools. The predominant presence of dimeric TRPV1 at basal state was recently described (34).

TRPV1 activation by RTX led to the emergence of TRPV1-YFP tetramers, as shown by brightness analysis. (Note: brightness changes due to direct RTX influence on the fluorophore (YFP) could be ruled out because YFP emission lifetime was unaffected by receptor stimulation.) Although an accurate quantification of the tetramer pool is difficult, we attempted an estimate by linking it to the augmented slower component in the autocorrelation curve measured by tICS under RTX treatment (90% of the overall autocorrelation decay, Table 1). We shall denote this TRPV1 pool by $T_s$. Tetrameric TRPV1 was investigated by cryomicroscopy (10) and biochemical experiments (11–13), but to our knowledge this is the first study carried out in living cells.

Dynamic FRET experiments in tICS modality showed that the slower diffusing components are the only pool that interacts directly with $\mu$Ts (Fig. 2). This was assessed under both basal condition and RTX stimulation and suggests that a direct link exists between slow membrane diffusivity and interaction with $\mu$T. Two nonexclusive situations can be envisaged: (i) a strong, kinetically slow binding interaction between $\mu$Ts and TRPV1 and (ii) membrane confinement induced by $\mu$Ts on TRPV1. In any case, we wish to stress that the activated tetrameric form of TRPV1 was always found to be binding $\mu$Ts, and we believe this suggests that interaction between these two partners is an essential part of receptor functionality.

Indeed depolymerization of $\mu$Ts modifies receptor characteristics. First, the FRET-tICS analysis showed the disappearance of FRET between TRPV1-YFP and tubulin, demonstrating that the receptor interacts only with intact $\mu$Ts. Moreover, in the absence of RTX activation, TRPV1-YFP membrane mobility increased by 1 order of magnitude, although two mobile populations with fairly different diffusion coefficients could still be observed. Such a mobility increase is not associated with an unspecific effect of nocodazole and/or $\mu$T depolymeriza-
tion, because the mobility of a model noninteracting membrane protein (EGFP-F) was not affected under the same conditions. Also, tICS traces collected under nocodazole treatment indicated a non-negligible fraction of immobile aggregated receptor (30% of the autocorrelation amplitude). This finding is supported by static anisotropy measurements; T depolymerization leads to significant reduction of r following an increase of homo-FRET; the latter effect appears related to an increase of the oligomerization status of the receptor. The large fraction of immobile receptor prevents the determination of the oligomerization status of the mobile receptor, because of the large and spatially extended bleaching (supplemental Fig. S12, G–I). It is worth noting that concomitant RTX and nocodazole administration led to extensive cell apoptosis in the time scale of tICS analysis, thus preventing diffusion measurements. Instead, selective cytochalasin administration was found not to affect to any extent the oligomerization status, in keeping with the observed absence of interactions between TRPV1 and the actin cytoskeleton (supplemental Fig. S12, A–F).

These complex experimental findings can be cast within an equilibrium model that must include all receptor forms and their interaction with T (Fig. 5). First of all, we note that at 37 °C, at a membrane potential of 70 mV (typical of CHO resting state), and in absence of agonist, only a minor fraction of TRPV1 (15%) is in the open form. This can be estimated from the simple two-state equation for a voltage-gated channel (35) by inserting TRPV1 parameters and is in line with the known channel thermal activation around 42 °C (36). This observation is consistent with our results; without RTX activation TRPV1 is dimeric, and under ligand binding it mostly converts to a functional tetramer. The dimeric form is split into two subpopulations: one is fast diffusing and does not interact with T, and the other is slow and bound to T. Ligand-induced TRPV1 tetramerization preserves this T binding. The result-

FIGURE 4. N&B analysis of TRPV1-YFP oligomerization state. A, transiently transfected EGFP-F is taken as a benchmark of a monomeric protein diffusing in membranes (scale bar, 10 μm). Cells were imaged with 0.5% laser power at 488 nm and 20 μs/pixel. B, selection of the pixels with apparent brightness corresponding to monomers (B = 1.13 cpm) (ε = 0.13 counts/molecule = molecular brightness (see supplemental “Experimental Procedures”)). Monomers are distributed uniformly throughout the cell. C, brightness histogram. A Gaussian distribution centered at brightness (B) = 1.13 counts/molecule identifies the monomer; only a few pixels are outside the distribution of the monomeric brightness. D, N&B analysis is performed on a cell transiently expressing TRPV1-YFP under physiological conditions (scale bar, 10 μm) with the same experimental settings described above. E, analysis reveals a homogeneous distribution of a dimeric form of TRPV1-YFP throughout the cell, with an average apparent brightness of 1.26 counts/molecule (ε = 0.26 counts/molecule), F, brightness histogram. A Gaussian distribution centered at 1.26 counts/molecule identifies the dimer. Only a few pixels are outside the distribution of the dimeric brightness. These pixels tend to concentrate at the cell border. G, upon stimulation with RTX, TRPV1-YFP rapidly redistributes within the membrane (compare the same cell in D and G). H, brightness-versus-intensity plot showing the dimeric region with brightness centered at 1.26 counts/molecule (red box) and a clear shift of molecular brightness toward the tetrameric form (brightness centered at 1.52 counts/molecule) at higher intensities (yellow box). I, selection of pixels with brightness corresponding to dimers (red) and tetramers (yellow).
Binding of Intact Microtubules Preserve TRPV1 Functionality

FIGURE 5. Scheme of structural conversion between all the receptor forms and their interaction with μT. There is a dynamic equilibrium between the μT interactive dimer form (blue dimers linked to red string) and dimer forms noninteracting with μT (blue dimers only) both not active. RTX leads to formation of an active tetrameric form thus displacing the equilibrium between dimers toward the form interactive with μT. μT depolymerization leads to an equilibrium shift because of formation of large aggregates of receptor (pictorially drawn as a blue heptamer). This finding reveals the role of μT in overseeing the correct aggregation status of the receptor.

REFERENCES

1. Scott, J. D., and Pawson, T. (2009) Cell signaling in space and time. Where proteins come together and when they are apart. Science 326, 1220–1224
2. Defilippi, P., Olivo, C., Venturino, M., Dolce, L., Silengo, L., and Tarone, G. (1999) Actin cytoskeleton organization in response to integrin-mediated adhesion. Microsc. Res. Tech. 47, 67–78
3. Mills, J. W., and Mandel, L. J. (1994) Cytoskeletal regulation of membrane transport events. FASEB J. 8, 1161–1165
4. Numazaki, M., Tominaga, T., Takeuchi, K., Murayama, N., Toyooka, H., and Tominaga, M. (2003) Structural determinant of TRPV1 desensitization interacts with calmodulin. Proc. Natl. Acad. Sci. U.S.A. 100, 8002–8006
5. Patwardhan, A. M., Scotland, P. E., Akopian, A. N., and Hargreaves, K. M. (2009) Activation of TRPV1 in the spinal cord by oxidized linoleic acid metabolites contributes to inflammatory hyperalgesia. Proc. Natl. Acad. Sci. U.S.A. 106, 18820–18824
6. Andreev, Y. A., Kozlov, S. A., Koshelev, S. G., Ivanova, E. A., Monastyrnaya, M. M., Kozlovskaya, E. P., and Grishin, E. V. (2008) Analogic compound from sea anemone Heteractis crispa is the first polypeptide inhibitor of vanilloid receptor 1 (TRPV1). J. Biol. Chem. 283, 23914–23921
7. Caterina, M. J., Schumacher, M. A., Tominaga, M., Rosen, T. A., Levine, J. D., and Julius, D. (1997) The capsaicin receptor. A heat-activated ion channel in the pain pathway. Nature 389, 816–824
8. Jordi, S. E., Tominaga, M., and Julius, D. (2000) Acid potentiation of the capsaicin receptor determined by a key extracellular site. Proc. Natl. Acad. Sci. U.S.A. 97, 8134–8139
9. Gavva, N. R., Khlonsky, L., Qu, Y., Shi, L., Tamir, R., Edenson, S., Zhang, T. J., Viswanadhan, V. N., Toth, A., Pearce, L. V., Vanderah, T. W., Porrecia, F., Blumberg, P. M., Lile, J., Sun, Y., Wild, K., Louis, J. C., and Treanor, T. J. (2004) Molecular determinants of vanilloid sensitivity in TRPV1. J. Biol. Chem. 279, 20283–20295
10. Moiseenkova-Bell, V. Y., Stanciu, L. A., Serysheva, I. I., Tohe, B. J., and Wesnells, T. G. (2008) Structure of TRPV1 channel revealed by electron cryomicroscopy. Proc. Natl. Acad. Sci. U.S.A. 105, 7451–7455
11. Kedei, N., Szabo, T., Lile, J. D., Treanor, J. J., Olah, Z., Iadarola, M. J., and Blumberg, P. M. (2001) Analysis of the native quaternary structure of vanilloid receptor 1. J. Biol. Chem. 276, 28613–28619
12. Kuzhikandathil, E. V., Wang, H., Szabo, T., Morozova, N., Blumberg, P. M., and Oxford, G. S. (2001) Functional analysis of capsaicin receptor (vanilloid receptor subtype 1) multimerization and agonist responsiveness using a dominant negative mutation. J. Neurosci. 21, 8697–8706
13. Zhang, F., Liu, S., Yang, F., Zheng, J., and Wang, K. (2011) Identification of a tetrameric assembly domain in the C terminus of heat-activated TRPV1 channels. J. Biol. Chem. 286, 15308–15316
14. Goswami, C., Dreger, M., Jahnel, R., Bogen, O., Gillen, C., and Huch, F. (2004) Identification and characterization of a Ca2+-sensitive interaction of the vanilloid receptor TRPV1 with tubulin. J. Neurochem. 91, 1092–1103
15. Goswami, C., Dreger, M., Otto, H., Schwappach, B., and Huch, F. (2006) Rapid disassembly of dynamic microtubules upon activation of the capsaicin receptor TRPV1. J. Neurochem. 96, 254–266
16. Goswami, C., Huch, T. B., and Huch, F. (2007) Identification and characterization of novel tubulin-binding motifs located within the C terminus of TRPV1. J. Neurochem. 101, 250–262
17. Vlachova, V., Teisinger, J., Susnikova, K., Lyeftenko, A., Ettrich, R., and Vyklicky, L. (2003) Functional role of C-terminal cytoplasmic tail of rat vanilloid receptor 1. J. Neurosci. 23, 1340–1350
18. Jares-Erijman, E. A., and Jovin, T. M. (2003) FRET imaging. Nat. Biotechnol. 21, 1387–1395
19. Digman, M. A., Dalal, R., Horwitz, A. F., and Gratton, E. (2008) Mapping the number of molecules and brightness in the laser scanning microscope. Biophys. J. 94, 2320–2332
20. Tramier, M., and Coppey-Moisan, M. (2008) Fluorescence anisotropy imaging microscopy for homo-FRET in living cells. *Methods Cell Biol.* **85**, 395–414

21. Bates, I. R., Hébert, B., Luo, Y., Liao, J., Bachir, A. I., Kolin, D. L., Wiseman, P. W., and Hanrahan, J. W. (2006) Membrane lateral diffusion and capture of CFTR within transient confinement zones. *Biophys. J.* **91**, 1046–1058

22. Kolin, D. L., and Wiseman, P. W. (2007) Advances in image correlation spectroscopy. Measuring number densities, aggregation states, and dynamics of fluorescently labeled macromolecules in cells. *Cell Biochem. Biophys.* **49**, 141–164

23. Wheeler, D., Sneddon, W. B., Wang, B., Friedman, P. A., and Romero, G. (2007) NHERF-1 and the cytoskeleton regulate the traffic and membrane dynamics of G protein-coupled receptors. *J. Biol. Chem.* **282**, 25076–25087

24. Albertazzi, L., Arosio, D., Marchetti, L., Ricci, F., and Beltram, F. (2009) Quantitative FRET analysis with the EGFP-mCherry fluorescent protein pair. *Photochem. Photobiol. Sci.* **9**, 1307–1319

25. Cheng, W., Yang, F., Takanishi, C. L., and Zheng, J. (2007) Thermosensitive TRPV channel subunits coassemble into heteromeric channels with intermediate conductance and gating properties. *J. Gen. Physiol* **129**, 191–207

26. Abbruzzetti, S., Bizzarri, R., Luin, S., Nifosì, R., Storti, B., Viappiani, C., and Beltram, F. (2010) Photoswitching of E222Q GFP mutants. “Concerted” mechanism of chromophore isomerization and protonation. *Photochem. Photobiol. Sci.* **9**, 1307–1319

27. Ossato, G., Digman, M. A., Aiken, C., Lukacsovich, T., Marsh, J. L., and Gratton, E. (2010) A two-step path to inclusion formation of huntingtin peptides revealed by number and brightness analysis. *Biophys. J.* **98**, 3078–3085

28. Gallego-Sandin, S., Rodriguez-Garcia, A., Alonso, M. T., and Garcia-Sancho, J. (2009) The endoplasmic reticulum of dorsal root ganglion neurons contains functional TRPV1 channels. *J. Biol. Chem.* **284**, 32591–32601

29. Tóth, A., Wang, Y., Kedei, N., Tran, R., Pearce, L. V., Kang, S. U., Jin, M. K., Choi, H. K., Lee, J., and Blumberg, P. M. (2005) Different vanilloid agonists cause different patterns of calcium response in CHO cells heterologously expressing rat TRPV1. *Life Sci.* **76**, 2921–2932

30. Wlodarczyk, J., Woehler, A., Kobe, F., Ponimaskin, E., Zeug, A., and Neher, E. (2008) Analysis of FRET signals in the presence of free donors and acceptors. *Biophys. J.* **94**, 986–1000

31. Sharma, P., Varma, R., Sarasij, R. C., Ira, Goussset, K., Krishnamoorthy, G., Rao, M., and Mayor, S. (2004) Nanoscale organization of multiple GPI-anchored proteins in living cell membranes. *Cell* **116**, 577–589

32. Patterson, G. H., Piston, D. W., and Barisas, B. G. (2000) Förster distances between green fluorescent protein pairs. *Anal. Biochem.* **284**, 438–440

33. Runnels, L. W., and Scarlata, S. F. (1995) Theory and application of fluorescence homotransfer to melittin oligomerization. *Biophys. J.* **69**, 1569–1583

34. Wang, S., and Chuang, H. H. (2011) C-terminal dimerization activates the nociceptive transduction channel transient receptor potential vanilloid 1. *J. Biol. Chem.* **286**, 40601–40607

35. Voets, T., Droogmans, G., Wissenbach, U., Janssens, A., Flockerzi, V., and Nilius, B. (2004) The principle of temperature-dependent gating in cold- and heat-sensitive TRP channels. *Nature* **430**, 748–754

36. Patapoutian, A., Peier, A. M., Story, G. M., and Viswanath, V. (2003) ThermoTRP channels and beyond. Mechanisms of temperature sensation. *Nat. Rev. Neurosci.* **4**, 529–539