Chapter 2

TRP-Mediated Cytoskeletal Reorganization: Implications for Disease and Drug Development

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Abstract

So far the major focus of Transient Receptor Potential (TRP) channels in the context of pathophysiological disorders was centered exclusively on the ionic conductivity mediated by these channels. However, recently the importance of non-ionic functions of TRP channels in different pathophysiological disorders has emerged. Recently several physical and functional interactions of TRP channels with cytoskeletal components have been characterized. These interactions play important roles in executing the non-ionic functions and regulations of TRP channels per se. In the membranous environment, TRP channels form dynamic signaling complexes that include components like microtubule and actin cytoskeleton, other scaffolding and key regulatory components. TRP channels can also regulate the integrity and dynamics of different cytoskeletal systems in complex manner. In many cases, these regulations seem to be independent of Ca^{2+} influx mediated by these channels and thus have immense significance in the context of pathophysiological disorders. In this review, I highlight the importance of TRP channel interactions and multidirectional regulations with cytoskeletal components in detail. This aspect opens up new avenues to target TRP signaling complexes by pharmacological manners. The strategies to target TRP complexes rather than targeting TRP channel solely might be useful for several clinical purposes.

Key words: TRPV, Cytoskeleton, Signalplex, Ion channels, Tubulin, Actin

Abbreviations

4αPDD	4α-phorbol-didecanoate
CIRB domain	Calmodulin- and IP_3R-binding region
CMT2	Charcot–Marie-Tooth disease type 2
DRG neurons	Dorsal root ganglion neurons
eGFP	Enhanced green fluorescence protein
EGTA	Ethylene glycol tetraacetic acid
FRET	Fluorescence Resonance Energy Transfer
5'I-RTX	5'-iodoresiniferatoxin
HUVEC	Human umbilical vein endothelial cell
TRP Channels	Transient receptor potential channels
PC2	Polycystin-2
TRPN	NomPC-like TRP channel

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TRPC	Transient receptor potential canonical
TRPML	Transient receptor potential mucolipin
MAPs	Microtubule-associated proteins
MBP	Maltose-binding protein
NF200	Neurofilament heavy chain 200 KDa
OAG	1-oleoyl-2-acetyl-sn-glycerol
PKC	Protein kinase C
PKD	Polycystic kidney disease
PKCε	Protein kinase Cε sub type
RTX	Resiniferatoxin

1. Introduction

Though the importance of cytoskeleton in different signaling events and cellular functions are well established, the entire arrays of cytoskeletal organization at the sub-membranous region and complexity of the cytoskeleton at the lipid environment have not yet been understood (1, 2). While the presence and function of cortical actin cytoskeleton just beneath the plasma membrane are well established, the events and mechanisms by which membrane proteins and sub-membranous microtubule cytoskeleton regulate each other and execute multiple cellular functions are just emerging (3). In that context, it is important to mention that so far several proteomic studies indicate that components from microtubule cytoskeleton physically interact with several transmembrane proteins such as ion channels, pumps, and receptors (4–7). These reports are also in full agreement with the fact that tubulin, actin, and other cytoskeletal proteins are selectively enriched in several biochemical preparations which represent different membrane fractions (8–11). Even the presence of tubulin and actin is observed in very specialized subset of total membrane fraction, namely in the lipid rafts and/or in the post-synaptic density fraction of the synaptic membranes (9, 12–14). Taken together, both membrane-associated actin and tubulin represent membrane cytoskeleton which is highly relevant for several signaling and provide important platform on which the functions of the membrane proteins are dependent. Surprisingly, the dynamics, biochemical characteristics, regulations, and functions of the sub-membranous microtubule cytoskeleton are still poorly understood (15, 16). However, for simplicity it can be said that a small portion of the microtubule cytoskeletal components are selectively present in the membrane fraction. These components constitute different dynamic complexes that are primarily formed by the transmembrane proteins. Within these complexes, the cytoskeletal components can interact directly or indirectly with the membrane proteins.

Transient Receptor Potential (TRP) channels are a group of newly discovered non-selective cation channels that can be activated by several physical and chemical stimuli and are involved in several physiological functions (17–20). Dysfunction and mis-regulation of TRP channels have been linked with several pathophysiological and genetic disorders (21). So far, research with TRPs has focused mainly on the ionic conductivity mediated by these channels. In spite of several reports demonstrating that TRP channels share co-localization with several cytoskeletal proteins at highly specific sub-cellular locations, the importance of TRP-cytoskeleton cross-talk in the context of structure–function and regulation has been neglected for a long time. Only in recent time, the importance of cytoskeletal proteins in the multi-dimensional regulation of TRP channels has come to light. So far, a handful of reports suggest that cytoskeletal proteins play an important role in the context of structure–function and regulation of TRP channels. In the same context, a number of studies have indicated that TRP channels physically interact with actin and microtubule cytoskeleton as well as with many other proteins at the plasma membrane. Therefore the significance of these interactions is manifested by the functional aspects of TRP channels. All these studies also indicate that cytoskeletal, vesicular and other membrane regulatory proteins interact with TRP channels and form scaffolds at the plasma membrane which can be described as dynamic functional complexes central to many physiological functions. Improper structure–function and regulation of these complexes seem to be intimately associated with the development of diseases and pathophysiological conditions. Therefore these complexes represent potential, specific and novel pharmacological targets.

It is noteworthy to mention that so far crosstalk between TRP channels with cytoskeleton has not been investigated properly though the indication of such crosstalk was reported long back. For example, it has been demonstrated that capsaicin-responsive DRG neurons are devoid of neurofilament 200 kDa protein (Capsaicin-responsive dorsal root ganglion (DRG) neurons cannot be labeled with a monoclonal antibody (RT97) that detects NF200 kDa) though the reason for such specific regulation is still not known (22, 23). Nevertheless, understanding of such fine regulations between TRP channels and cytoskeleton has tremendous importance in the case of several pathophysiological disorders and diseases. In this review I highlight the interaction of different cytoskeletal proteins with TRP channels at several levels and also how these complexes are regulated.

2. Cross-Talk Between TRP Channels and Cytoskeleton: Co-localization and Genetic Interactions

The importance of cytoskeleton in the context of function and regulation of TRP channels came from the common observation that these channels and specific cytoskeletal proteins are co-expressed in some specialized cells. Often these two groups of proteins are located at distinct subcellular structures also, a topic that has been discussed in detail by us recently (24). As these subcellular structures are characterized by the presence of these specialized proteins and/or by intricate cytoskeletal organization, specific localization strongly suggests that TRP channels either interact with some of the cytoskeletal proteins and/or are involved with the development as well as function of these structures. For example, polycystine channel sub type 2 (PC2) co-localizes with polyglutamylated tubulin at the basal bodies/cilia of ciliated epithelial cells present in mouse trachea (25). This co-localization is also in agreement with the involvement of PC2 channel in ciliary function. PC2 forms a complex with pericentrin and this interaction is also required for primary cilia assembly (25). In the same context, both PC1 and PC2 are present in the primary cilium of kidney cells (26). PC2 channel is also localized at the primary cilia of renal epithelial cells (27). *Xenopus* TRPN1 (NOMPC) localizes to the tip of the microtubule-based cilia in epithelial cells (kinocilial bulb) and tip of the inner-ear hair cells (28). There TRPN co-localizes with cytoskeletal components like actin, tubulin, and Cdh23. In *Drosophila melanogaster*, NOMPC (a member of the TRP channel family) localizes to the tubular body and distal cilium of Campaniform and Chordotonal receptor cells and is involved in these ciliary functions (29). In *Drosophila*, TRPN (=NOMPC) localizes at the distal end of mechanosensory cilia also and co-localizes with EYS (an extracellular protein that marks the proximal end of the sensory cilia) (30). TRPC6 localizes in podocytes where it interacts with podocin and nephrin, components that belong to actin cytoskeleton (31). These examples suggest that TRP channels localize at the specific cytoskeleton-enriched structures and share a special relation with the cytoskeletal components.

Like PC, TRPN and TRPC channels, recent results suggest that TRPV channels share physical and functional interactions with cytoskeletal components. TRPV1 and TRPV4 are reported to be localized at the tip of filopodia in both neuronal and non-neuronal cells when expressed ectopically (24, 32, 33). This is also in agreement with the endogenous localization of TRPV1, TRPV4 and other TRP channels at the spines (33–35). Interestingly, expression of TRPV1 induces filopodia that possess a characteristic bulbous head which contains negligible amount of F-actin but accumulates TRPV1 there (24, 32). This phenotype resembles well with the expression of the non-conventional myosin II, III, V, X

and XV (24, 36–47). These similarities suggest that overexpression of TRPV1 may alter the function of these myosin motors and execute similar dominant-negative effects. Indeed, changes in the expression pattern as well as distribution of certain cytoskeletal proteins including non-conventional myosin motors after the ectopic expression and/or activation of TRP channels have been reported (32, 48, 49). Ectopic expression of TRPV1 in F11 cells results in altered expression as well as reorganization of non-conventional myosins, namely endogenous myosinIIa and myosinIIIa (32). In agreement with this, observation another study has also confirmed that overexpression of TRPC6 in transgenic mice resulted in an increased expression of beta-myosin heavy chain in cardiac tissues (50).

In many cases, the development and the function of these specialized cells/structures are regulated by both TRP channels and these cytoskeletal proteins. In agreement with this, mutations in either TRP channels or specific cytoskeletal proteins often lead to similar, if not same, phenotype as well as pathophysiological disorders and/or syndromes. Taken together, involvements of these two groups of proteins in common functions and occurrence in same cell (even in the same sub-cellular regions) are highly indicative of physical, functional, and genetic interactions (51). As the examples are too many, it is impossible to cover all these in this review. However, some key examples, like multi-dimensional relation of TRP channels with different motor proteins are described here.

For example, as is the case in many ciliary proteins, mutations either in PC1 and PC2 are also involved in polycystic kidney disease (PKD) and result in defective localization, cilia formation and/or loss of flow-induced Ca^{2+} signaling (26, 52). This agrees with the fact that PC channels are regulated by microtubule-based motor proteins such as KIF3a and KIF3b (53). In a similar context, mutations in either kinesin (Kif1b) or TRPV4 result in similar pathophysiology and development of Charcot–Marie-Tooth disease type 2 (CMT2) disease suggesting a strong genetic link between these two (21, 54–57). Mutations in TRP channels as well as in different non-conventional myosin motors are also reported to develop similar pathophysiological disorders and other syndromes like deafness and blindness. For example, both the development and proper function of the stereocilia of hair cells are important for hearing. In normal conditions, the ciliary tips of hair cells contain enriched amounts of endogenous TRP channels as well as several nonconventional myosin motor proteins, indicating that the function of these cells are dependent on these two groups of proteins at the ciliary tips. Indeed, several reports suggest that in the case of deafness, several nonconventional myosin motors (myosin I, IIA, IIIA, VI, VIIA and XV) are important for either development of the stereocilia of hair cells in the inner ear or proper localization of

TRP channels at the tips of these stereocilia (58–60). Reciprocally, mutations and abnormal expression–functions of several TRP channels, namely NompC, TRPML1, TRPML2, TRPML3, TRPV4, TRPV5, and TRPV6 also lead to deafness (30, 61–68).

Like auditory defects, development, polarization of retinal cells and proper trafficking of pigments in the retinal cells are involved in the proper light-sensing mechanisms. In case of blindness, both TRP channels and non-conventional myosins are involved (60). Retrospectively, TRP channel was first discovered by Minke et al. in *Drosophila melanogaster* as the mutant was defective in light-sensing mechanism (69). Indeed, so far several TRP channels have been reported to express in retinal cells. Some of these TRP channels are involved in photo-response and essential for the light sensation as mutation in these TRP channels causes different forms of blindness (70). For example, mutation in TRPM1 is responsible for blindness as it is involved in retinal ON bipolar function (71, 72). In agreement with the involvement in common functions, mutations in myosin motors are also involved in blindness. Mutation in myosin VIIa is involved in the development of "Usher syndrome type 1B" (60). In *Drosophila*, Ca^{2+}-activated myosin V is involved with the closure of the pupil and thus with the light sensation procedure (73). Apart from the genetic interaction, recent reports clearly indicate physical as well as functional interactions between these two groups of proteins. Recently it has been reported that translocation of eGFP-tagged TRP-like channels to the rhabdomeral membrane in *Drosophila* photoreceptors is myosin III dependent (74). A recent proteomic screen has also identified myosin as an interacting protein for TRPC5 and TRPC6 (75). Another study showed that myosin IIa is directly phosphorylated by TRPM7, a cation channel fused to an alpha-kinase (76). In the same notion, a recent proteomic screen has identified the heavy chain of myosin X and cytosolic dynein heavy chain as an interacting protein of TRPC3 (77). All these results suggest that TRP channels and some of the specific cytoskeletal proteins like kinesins and nonconventional myosins are involved in same functions. However, detailed studies are needed to understand these genetic interactions.

3. Physical Interaction of TRP Channels with Microtubule Cytoskeleton

In the last few years, major progress has been made to elucidate and further characterize the physical interaction of TRP channels with components from microtubule cytoskeleton like tubulin, microtubule associated proteins (MAPs) and different motor proteins (3, 6, 78). So far, direct physical interaction of tubulin has been reported for members belonging to TRPV, TRPC, and TRPP

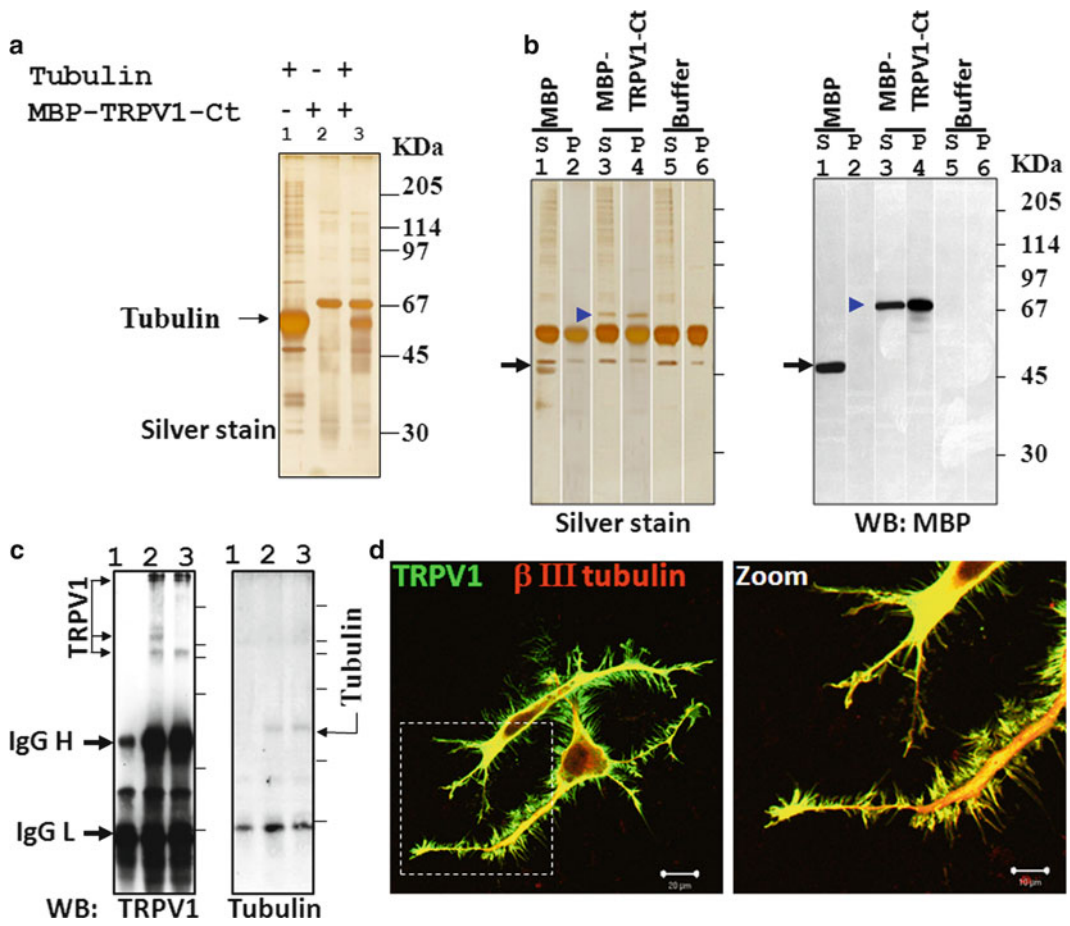

Fig. 1. TRPV1 interacts directly with microtubule cytoskeleton and induces cellular changes upon expression as well as activation. (**a**) MAP-enriched tubulin (lane 1, input) was added to the MBP-TRPV1-Ct coupled with amylase resin (lane 2). A significant fraction of the tubulin interacts with the MBP-TRPV1-Ct. (**b**) The MBP-TRPV1-Ct directly interacts with polymerized microtubules. Purified tubulin dimers were incubated with GTP to form microtubules either in the presence of MBP only (lane 1–2) or in the presence of MBP-TRPV1-Ct (lane 3–4) or in the presence of buffer only (lane 6–7). Polymerized microtubules and bound proteins were subsequently separated from unpolymerized tubulin dimers or unbound proteins by centrifugal separation of pellet (**P**) fraction from supernatant fraction (**S**). Silver-stained gel (*left side*) and anti-MBP western blot analysis reveal specific interaction of MBP-TRPV1-Ct (*blue arrow head*) with polymerized microtubules. MBP alone does not interact with polymerized microtubules and thus do not appear in the pellet fraction. (**c**) Tubulin co-immunoprecipitates with TRPV1. Anti-GFP antibody was used for immunoprecipitation from F11 cells transiently expressing GFP only (lane 1) or GFP-TRPV1 (lane 2). Immunocomplexes were probed for TRPV1 (*left side*) and tubulin (*right side*). Presence of tubulin is detected only in lane 2, but not in lane 1. (**d**) Ectopic expression of TRPV1 (*green*) alters cellular morphology and induces multiple filopodial structure. TRPV1 co-localizes with neuron-specific β-tubulin subtype III (*yellow*) in such filopodial structures. Scale bar 20 mm and 10 mm respectively.

channels (3, 6, 7, 75, 79). Here I discuss the details of these interactions.

Probably the best characterization for the interaction of TRP channels with tubulin has been illustrated by TRPV1, alternatively known as capsaicin receptor (Fig. 1). By proteomic analysis, we identified tubulin as a component present in the complex formed

with the C-terminal cytoplasmic domain of TRPV1 (80). The interaction was subsequently confirmed by several biochemical approaches including co-immunoprecipitation, microtubule co-sedimentation, direct pull-down assay and cross-linking experiments (49, 80). This interaction is direct as both purified tubulin and the C-terminal cytoplamic domain of TRPV1 tagged with maltose-binding protein (MBP-TRPV1-Ct) can form a stable complex. We identified two short regions located within the C-terminus of TRPV1, namely amino acids 710–730 and 770–797 that can retain tubulin interaction independently (49). In contrast, MBP-TRPV1-Nt failed to interact with tubulin in a direct pull-down assay (80). Based on these observations, it was proposed that the tubulin interaction was restricted within the C-terminal cytoplasmic region of TRPV1 only (49, 80). However, recently it has been shown that the N-terminal cytoplasmic region of TRPV1 can also interact with tubulin (81). This difference might be due to the experimental systems and the procedures used. Taken together, this suggests that there might be more than one region located in TRPV1 that can be involved in tubulin interaction. It might also suggest that TRPV1-tubulin interaction is dynamic and might be involved in the conformational changes.

The C-terminal cytoplasmic region of TRPV1 preferably interacts with the β-tubulin and to a lesser extent also with the α-tubulin (49). The cross-linking experiment revealed that MBP-TRPV1-Ct interacts with β-tubulin quickly and the entire amount of β-tubulin forms a high-molecular weight complex with MBP-TRPV1-Ct within a minute. In contrast, the MBP-TRPV1-Ct interacts with α-tubulin slowly and almost half of the β-tubulin fails to form high-molecular weight complex with MBP-TRPV1-Ct even after an hour. This also suggests a stronger binding of TRPV1 to the plus end rather than the minus end of microtubules as the plus ends of microtubule proto-filaments are decorated with β-tubulin. It is therefore tempting to speculate that TRPV1 may act as a microtubule plus-end-tracking protein (+TIP)(6). However, whether TRPV1 can indeed serve as a plus-end-tracking protein remains to be explored.

A significant understanding about the TRPV1 interaction with tubulin has been derived from sequence analysis of the binding regions. Interestingly, there are two short tubulin-binding stretch sequences that reveal tubulin-binding ability. These two sequence stretches contain highly basic amino acids and contain very high isoelectric points, 11.17 and 12.6 (49). In the context of microtubule interaction, these two regions can act as polycationic stretch sequences that can favor microtubule formation and stabilize them (82–84). Indeed, these two short stretch sequences can also interact with soluble tubulin as well as with polymerized microtubules (49). Interestingly, if assumed to form α-helical conformation, then all the basic amino acids present in these two regions are projected to one side, suggesting potential interactions with negatively charged

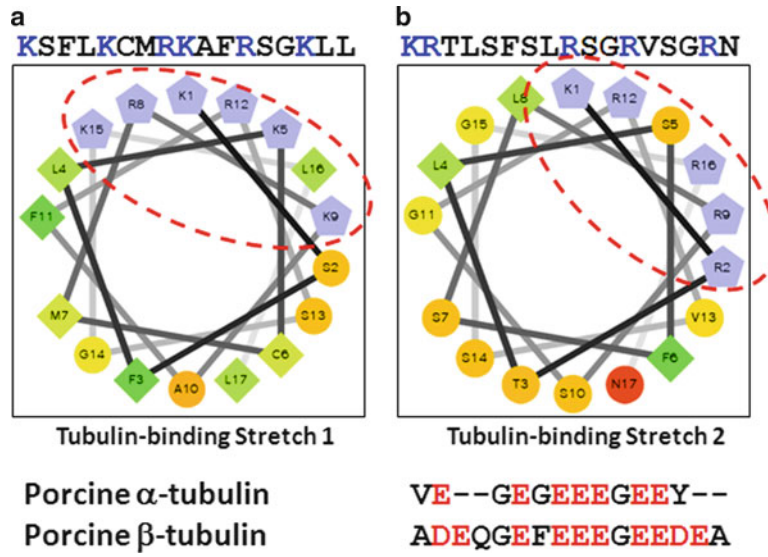

Fig. 2. Tubulin-binding motifs located at the C-terminus of TRPV1 are characterized by the presence of multiple positive charged residues. (**a**) The basic amino acids (indicated in *blue*) are located at one side of the putative helical wheel, where it can interact with the acidic C-terminus of tubulin. (**b**) The extreme C-terminus of both α- and β-tubulin contains highly negatively charged amino acids (indicated in *red*) and is mostly unstructured.

surface. In this context, it is important to note that the C-terminal over-hanging regions of tubulin contain a large number of negatively charged glutamate (E) residues in a stretch characterized as an unstructured region of the tubulin and thus referred to as "E-hook". Due to the presence of several negatively charged residues, the E-hook can be important for the interaction with the TRPV1, especially to these positively charged regions (Fig. 2). In agreement with this, previously we have demonstrated that $\alpha_s\beta_s$-tubulin (protease-digested tubulin dimers that lack approximately 45 amino acids from the C-terminal region including the E-hooks) does not bind to the MBP-TRPV1-Ct (49). More interestingly, in many TRPV channels the distribution of these basic amino acids composing the tubulin-binding regions is conserved even though the overall amino acid conservation is rather limited. This may suggest that tubulin interaction is apparently under high evolutionary pressure and it might be conserved in many TRP channels. Indeed, the C-terminal cytoplasmic region of TRPV4 also reveals interaction with tubulin (33). Similarly, the C-terminal region of the TRPV2 also interacts with purified tubulin (Unpublished observation). However, the exact amino acids of TRPV4 and TRPV2 that are involved in the tubulin interaction have not been determined yet.

It is important to note that different post-translationally modified tubulin, like tyrosinated tubulin (a marker for dynamic microtubules), acetylated tubulin, polyglutamylated tubulin, detyrosinated tubulin, phospho (serine) tubulin and neuron-specific β-III tubulin (all markers for stable microtubules) interact with

MBP-TRPV1-Ct (49). The same phenomenon was also observed with MBP-TRPV4-Ct (33). These results strongly suggest that TRPV channels interact not only with soluble tubulin, but also with assembled microtubules which represent various dynamic states of the microtubules. Indeed, the purified cytoplasmic domain of TRPV1-Ct, TRPV2-Ct, and TRPV4-Ct co-sediment with polymerized microtubules also. In addition to sole binding, MBP-TRPV1-Ct and MBP-TRPV4-Ct exert strong stabilization effect on microtubules. This stabilization effect of the C-terminus of TRPV1 and/or TRPV4 becomes especially apparent under microtubules depolymerizing conditions such as in the presence of nocodazol or increased Ca^{2+} concentrations (33, 80). These observations fit well with the fact that polycations favor microtubule polymerization and stabilization (82).

As TRPV1 represents a non-selective cation channel, the role of increased concentration of Ca^{2+} on the properties of TRPV1–tubulin and/or TRPV1–microtubule complex is of special interest. Tubulin binding to MBP-TRPV1-Ct is sensitive to the presence of Ca^{2+} (80). In contrast, the absence or presence of extra Ca^{2+} has no effect on the interaction of tubulin with MBP-TRPV4-Ct (80). Interestingly, the microtubules formed with MBP-TRPV1-Ct in the presence of Ca^{2+} become 'cold-stable' as these microtubules do not depolymerize further at low temperatures (80). The exact mechanisms by which Ca^{2+} modulates these physio-chemical properties in vitro are not clear. In this regard, it is important to mention that tubulin has been shown to bind two Ca^{2+} ions to its C-terminal sequence (85–88) and thus Ca^{2+}-dependent conformational changes of tubulin may underlie the observed effects of Ca^{2+} (89).

In addition to the interaction with αβ-tubulin dimer with the TRPV channels, there are several reports that suggest that other components of the microtubule cytoskeleton also interact with the TRPV channels. For example, a yeast two-hybrid screen has reported interaction of kinesin 2 and kinesin family member 3B with the TRPV1 (78). Similarly, MAP7 interaction with TRPV4 has been demonstrated (90). This interaction is also mapped down to the C-terminal cytoplasmic domain of TRPV4, especially within the amino acid region 798 to 809. This MAP7 interaction is involved in the surface expression of TRPV4. The biochemical data of direct interaction as well as microtubule stabilization find their correlates in cell biological studies. Ectopic expression of TRPV1 in dorsal root ganglia-derived F11 cells results in co-localization of TRPV1 and microtubules and accumulation of endogenous tyrosinated tubulin (a marker for dynamic microtubules) in close vicinity to the plasma membrane (80). As suggested by its preference to bind to the plus-end-exposed β-tubulin, TRPV1 apparently stabilizes microtubules reaching the plasma membrane and thereby increases the number of pioneering microtubules within the actin cortex. Similarly, TRPV4 co-localizes with microtubules at the plasma membrane (33). Therefore, the stabilization of microtubules in the

plasma membrane induces cellular morphological changes. This also explains at least in part why overexpression of TRPV1 induces massive induction of filopodial structures in neuronal as well as in non-neuronal cells. The mechanism for this is currently under investigation and apparently also includes alterations in the actin cytoskeleton. However, co-localization of TRPV1 with tubulin has been observed all along the filopodial stalk and, of note, including the filopodial tips (32, 91). Tubulin and components attributed to stable microtubules (like acetylated tubulin and MAP2a/b) were also observed within these thin filopodial structures (32).

Apart from the TRPV members, few TRPC members have been reported to interact with tubulin. TRPC5 and TRPC6 have sown to form signaling complexes that contain tubulin (75). In a similar manner, TRPC1 also interacts with tubulin and this interaction is involved with the surface expression of the channel (79). A proteomic study has also reported interaction of few microtubule cytoskeletal proteins, namely microtubule-associated protein 2 (MAP2) and cytosolic dynein heavy chain with TRPC3 (77). In addition, physical interaction of TRPC5 with stathmin, a factor that causes disassembly of microtubule cytoskeleton has been demonstrated (92). The interaction with stathmin is important for the neurite extension, growth cone function and also for synaptogenesis. Interestingly, TRPC5 interaction with stathmin plays an important role in the regulation of neurite and filopodial length also. In agreement with other TRP channels, PC1 and PC2 channels also interact with different tubulins (like α-tubulin, β-tubulin, γ-tubulin, acetylated α-tubulin) and the kinesin motor proteins KIF3A and KIF3B (53, 93).

4. Regulation of Microtubule Cytoskeleton by TRP Channels

In the last few years, significant progress has been made to elucidate the regulation of microtubule cytoskeleton by TRP channels. This is not surprising as activation of TRP channels initiates Ca^{2+} signaling as well as many other signaling events. Indeed recent reports suggest that activation of TRP channels is not an all-or-none event and thus these channels can regulate the cytoskeleton by both Ca^{2+}-dependent as well as Ca^{2+}-independent mechanisms (94). Recently we have demonstrated that TRPV channels regulate cytoskeleton in many different manners. For example, activation of TRPV1 by specific agonists like Resiniferatoxin (RTX) or Capsaicin leads to rapid destabilization of microtubules (48, 95). Notably, TRPV1 activation predominantly affects the dynamic microtubules and not the stable microtubules. This conclusion has been drawn mainly due to the observation that majority of the tyrosinated tubulins (marker for dynamic microtubules) but not the acetylated or polyglutamylated tubulins (markers for stable microtubule)

appear as soluble tubulin after the activation (48). Similarly, activation of TRPV4 also results in disassembly of microtubules (33). Though the exact molecular factors and pathways involved in this microtubule disassembly are not known, involvement of Cam-Ca^{2+} complex can be speculated (96–98). This is due to the fact that catalytic amounts of Cam-Ca^{2+} complex are known to cause severe microtubule disassembly. However, this TRPV-induced microtubule disassembly can be achieved even in a Ca^{2+}-independent manner, especially under certain conditions. For example, TRPV1 mediated microtubule disassembly can also be achieved in the presence of Ca^{2+} chelators like EGTA and strong inhibitor of TRPV1, like 5'I-RTX (49, 91). Even expression of the N-terminal fragment of TRPV1 (ΔTRPV1-Nt) can cause microtubule disassembly in response to some specific components like estrogen (91). These results strongly suggest that Ca^{2+}-independent pathways are also involved in the microtubule disassembly. However, further studies are needed to dissect these different signaling events in detail.

5. Interaction of TRP Channels with Actin Cytoskeleton

Similar to microtubule cytoskeleton, a large number of studies suggest that TRP channels interact with actin cytoskeleton, both physically and functionally. Often TRP channels physically interact with G-actin and other components associated with actin cytoskeleton. For example, members of TRPV, TRPC and PC channels are reported to form molecular complexes that contain actin and/or related components (33, 53, 93). The interaction of TRP channels with actin cytoskeleton is functionally important and relevant for several reasons. First, actin cytoskeleton is located just beneath the plasma membrane and thus has enough physical proximity to interact with the TRP channels. Next, in many cases, TRP channels are present in specialized subcellular structures like at the spines, filipodial tips, etc., that are characterized by the presence of bundled actin cytoskeleton (32–34). In addition, there are TRP channels (like TRPV4) that are involved in mechanosensation, a complex process and thus are supposed to bridge lipid bi-layer with sub-membranous cytoskeleton (7, 99, 100).

So far TRPV4 represents the best characterized TRP channel in terms of multi-dimensional interaction with actin cytoskeleton. Based on the fluorescence resonance energy transfer (FRET) performed in live cells, it was demonstrated that actin and TRPV4 share a close proximity, possibly a physical interaction between these two (101). This physical interaction is logical as both TRPV4 and actin cytoskeletons are functionally involved in mechanosensation as well as in mechanical pain (102). In agreement with that, recently we have demonstrated that the C-terminus of TRPV4

interacts directly with soluble actin as well as with polymerized actin filaments (33). In addition, presence of α2 integrin, an actin-binding protein in the signaling complex formed by TRPV4 has also been reported (103). These interactions are also in agreement with the fact that TRPV4 is enriched in structures like cilia, filopodia, focal adhesion points, dendritic spines and in lamellipodia, where it can regulate the dynamics of actin cytoskeleton (33, 104–110). In the same notion, involvement of TRPV4 in the intercellular junction formation in keratinocytes has been demonstrated (111). In spite of these studies, the exact location on TRPV4 where actin or other actin cytoskeletal proteins bind with it has not been determined. However, we demonstrated that soluble tubulin competes with soluble actin for binding on MBP-TRPV4-Ct suggesting that both actin and tubulin may bind to the same site located on TRPV4-Ct (33). This also suggests that TRPV4 may have a complex regulatory mechanism that switches it from actin cytoskeleton to microtubule cytoskeleton or vice versa.

In contrast with TRPV4, interaction of other TRPV members with soluble actin cytoskeleton is not well established. As TRPV1 localizes in the actin cytoskeleton-enriched structures like at the filopodial structures and at the dendritic spine, it is expected that TRPV1 interacts with actin cytoskeleton (32, 112). However, so far the direct physical interaction of TRPV1 with actin has not been established. In contrary, it has been shown that the same immune complex of TRPV1 that contains tubulin actually lacks actin (80). Even purified MBP-TRPV1-Ct does not interact with soluble actin in a condition where it interacts with tubulin (49). These results apparently suggest that TRPV1 may not interact directly with actin cytoskeleton. However, it might be possible that TRPV1-actin complex is extremely dynamic in nature, needs full-length TRPV1 (or even tetrameric structures) and difficult to extract in soluble phase. Therefore, bio-chemical methods may not be suitable to confirm the interaction and further live cell imaging studies are needed.

Recently, few TRPC members have also been reported to interact with actin cytoskeleton. In a proteomic screen it has been shown that the signaling complex formed by TRPC5 and TRPC6 contains actin and other actin cytoskeletal associated proteins, namely spectrin and myosins (75). Among all, spectrin seems to be a conserved interacting protein for many TRP channels, especially for TRPC members. In agreement with that, a recent study demonstrated that the C-terminal cytoplasmic part of hTRPC4, specifically amino acid residues 686 to 977 interact with αII- and βV-spectrin in a yeast two-hybrid assay (113). Within this region, the amino acids residues 730–758 of hTRPC4 are critical for the interaction with spectrin (113). This interaction was further confirmed by glutathione S-transferase pulldown and co-immunoprecipitation experiments. This interaction with spectrin is

important for the surface expression of TRPC members. Further deletion studies confirmed that amino acids 730–758 of hTRPC4 are critical for the interaction with spectrin. This region contains a coiled-coil domain and is juxtaposed to the Ca^{2+}/calmodulin- and IP_3R-binding region (CIRB-domain) suggesting that the interaction with the cytoskeletal components can have influence on other regulation as well. It is likely that spectrin interacts with TRPC5 also as the same sequence which is important for interaction is present in TRPC5. A recent proteomic study has also reported the interaction of several actin cytoskeletal proteins, namely, spectrin α-chain, spectrin β-chain as well as cofilin-1 as interacting proteins of TRPC3 (77). In the same notion, it has been demonstrated that TRPC4 also interacts with SESTD1, a previously uncharacterized protein that contains a lipid-binding SEC14-like domain and a spectrin-type cytoskeleton interaction domain (114). TRPC4 also interacts with 4.1 protein indicating that TRP channels and cytoskeletal proteins indeed form complex membrane scaffolds. This interaction is also due to a small sequence located at the C-terminus of the TRPC4 which is enriched with positively charged residues. Due to this ionic interaction, association of TRPC4 with membrane cytoskeleton is sensitive to high salt (115). Interaction of PC channels with actin cytoskeleton and associated components like monomeric actin, the actin-related components α-actinin and gelsolin have also been demonstrated (53, 93). However, more studies are needed to identify the entire spectrum of interacting proteins belonging to actin cytoskeleton.

6. Regulation of Actin Cytoskeleton by TRP Channels

Being permeable to Ca^{2+}, activation of TRP channels has the potential to regulate actin cytoskeleton. Indeed several reports suggest that TRP cannels regulate actin cytoskeleton by various manners. Interestingly, the nature of regulation and exact effect of TRPs-mediated regulation of actin cytoskeleton depend on few factors, mainly on the identity of the TRP channels and the cellular system. Best characterization of TRP-mediated regulation of actin cytoskeleton has been demonstrated for TRPV channels, namely for TRPV4. This also fits well with the involvement of TRPV4 in several cellular functions that are also known to require active participation of the actin cytoskeleton. It is noteworthy to mention that TRPV4 is a key molecule involved in mechanical force mediated biological processes. For example, TRPV4 activity is central to cytoskeleton-dependent/mediated regulatory volume decrease of cells, a process where actin-binding proteins contribute to cell volume regulatory ion channel activation (116–120). In the same notion, a recent study demonstrated that disruption

of the actin cytoskeleton increases the intracellular mobility of TRPV4-GFP and results in loss of co-localization of TRPV4 with actin (121). Recently it has been reported that TRPV4 regulates the morphology of human umbilical vein endothelial cell (HUVEC) (122). Activation of TRPV4 in this cell line causes rapid retraction and condensation of cells. In a similar manner, a prolonged activation also causes detachment of cells from the plates. Interestingly, these effects can be blocked by the ruthenium red, a TRP channel blocker. This suggests that TRPV4 activation alters the cytoskeletal integrity and dynamics and affects focal adhesion points as well as microtubules (122). Indeed, recent reports also suggest that TRPV4 activation regulates the morphology and migration of neuroendocrine (GN11) cells (123). These effects are in full agreement with what has been seen in F11 cells that express TRPV4 (33). This cell retraction due to TRPV4 activation is partly due to the loss of microtubules which disrupt the balance between anterograde force mediated by microtubule cytoskeleton and the retrograde force mediated by acto-myosin components.

In a similar manner, functional consequence of TRPV1 on the actin cytoskeleton has been shown in some systems. However, the effect of TRPV1 on actin cytoskeleton seems to be different depending on the cellular systems. For example, TRPV1 activation may enhance actin polymerization in some cellular systems whereas other cellular systems may remain unaffected or reveal depolymerization. Indeed it has been shown that capsaicin treatment increases the actin cytoskeleton, and also increases the actin filament content in neutrophils (124). Similarly, the effect of TRPV1 activation on the actin cytoskeleton has been demonstrated in sperm cells also. It has been shown that inhibition of TRPV1 by capsazepine during capacitation leads to the inhibition of actin polymerization in the acrosomal region (125, 126). Another study also demonstrated that activation of TRPV1 in premature spermatozoa promotes actin cytoskeletal depolymerization and a loss of acrosome structure integrity (125, 126). In case of F11 cells, the dorsal root ganglion-derived cells, activation of TRPV1 results in rapid microtubule disassembly but does not cause disassembly of actin or neurofilament cytoskeleton (48).

Other TRP channels also reveal functional interactions with actin cytoskeleton. For example, TRP1 in human platelets (hTRP1) couples with IP3 receptor and this coupling is controlled by actin cytoskeleton as stabilization of the cortical actin cytoskeleton with pharmacological means prevents this coupling (127). This result suggests that the sub-membranous actin filaments act as negative clamp which prevents constitutive coupling between TRP1 and IP3. In the same manner, PC2 channels regulate the morphology of BeWo cells that represent Human Trophoblast Choriocarcinoma (128). In this cell line, PC2 co-localizes at the cytokinetic midbody where the dynamics of actin cytoskeleton is important for the final

step of the cell division. In the case of neurons, TRPC1 regulates growth cone dynamics by a fine balance of LIM kinase and slingshot phosphatase activity which in turn regulates ADF/cofilin (129). This process is involved in growth cone attraction and repulsion. Taken together these results suggest that TRP-mediated signaling events are involved in reorganization of actin cytoskeleton. These reorganization effects in turn control many of the cellular functions like acrosomal reaction, fertilization, and functional aspects of neurons and immune cells.

7. Regulation of TRP Channels by Cytoskeletal Components

Recently there are a handful of reports that suggest a feedback regulation of TRP channels by the components of the cytoskeleton. Interestingly these feedback regulations by cytoskeleton can occur at the cellular level as well as at the single molecular level. At the cellular level, there were few studies which demonstrated that the functions of certain TRP channels are dependent on the status of the cellular cytoskeleton. Especially it has been shown that pharmacological modulation of different cytoskeletons results in altered influx of ions via these TRP channels. For example, a microtubule stabilizer drug, namely Taxol, reduces TRPV4-dependent currents while the microtubule-disrupting agents like Colchicine and Vincristine as well as actin cytoskeleton regulating drugs like Phalloidin (a stabilizer) or Cytochalasin B (a destabilizer) do not alter the TRPV4-mediated current (90). In agreement with this, recently we also have demonstrated that pharmacological stabilization of microtubules by applying Taxol results in reduction in the Ca^{2+} influx in response to 4α-phorbol-didecanoate (4αPDD) (an agonist of TRPV4) as measured by Ca^{2+} influx assay in whole cells (33). Interestingly, the degree of reduction in Ca^{2+} influx is much robust in the case of second time application of 4αPDD. Notably, this reduction is independent of the expression or availability of the TRPV4 at the plasma membrane, suggesting that dynamics of the microtubule cytoskeleton can regulate the ion channel function. This hypothesis is also supported indirectly by the whole-cell recordings measuring the TRPV4 activation conducted by heat activation. This is due to the fact that activation of single TRPV4 ion channel in response to heat is possible in whole cell recordings but not in a cell-free inside-out patch clamp experiments, suggesting that in the latter some cellular factor is missing (108). Therefore it can be speculated that components from microtubule cytoskeleton like α- or β-tubulin as well as MAPs might be important for the channel function at the single ion channel level. Similarly, involvement of actin cytoskeleton in the regulation of TRPV4 at the cellular level has been demonstrated. Pharmacological disruption

of actin by latrunculin-A results in loss of sensing hypotonicity and the onset of regulatory volume decrease (121). Tubulin interaction seems to control the Ca^{2+} homeostasis via TRPC members present in the ER. It is important to mention that microtubule-based motor proteins indirectly regulate the calcium-selective store-operated currents, a function where TRPC channels are involved (130). This is due to the fact that either stabilization or destabilization of microtubules by pharmacological drugs like Taxol or nocodazole results in altered distribution of cellular organelles as well as availability of the TRPC channels by modulating the endoplasmic reticulum-to-plasma membrane coupling events (130, 131). Ionic conductivity mediated by TRPC7 is also regulated by the status of the actin cytoskeleton as disruption of actin cytoskeleton by Cytochalasin-B results in inhibition of OAG-activated and TRPC7-mediated currents (132).

In agreement with all these reports, it has been demonstrated that the activity of the mechanosensitive ion channels in cultured sensory neurons appears to depend largely on the status of the cytoskeleton. Thus, disruption of actin or microtubule cytoskeleton by pharmacological agents greatly reduces the activity of mechanosensitive channels (133). In this regard, it is important to mention that most of these studies involve Ca^{2+} imaging and/or whole cell patch clamp as the read out systems and thus analyzed the effect of cytoskeletal alteration on a population of TRP channels in general. These studies give a partial mechanistic view of cytoskeletal involvement only and do not address the multidirectional regulation of cytoskeleton on the ion channels, especially at the level of single molecules. This is simply due to the fact that availability of the ion channels at the plasma membrane and normal trafficking of these ion channels are limited if the cytoskeleton is disrupted. Therefore it remains to be established whether the modulation of TRP channels can occur through direct interaction with the cytoskeleton.

As mentioned, recent reports suggest that cytoskeletal components can regulate the properties, especially the ionic conductivity and related behavior of TRP channels, at the single channel level also. This is mainly due to the fact that interactions of cytoskeletal components with TRP channels affect other interactions and thus modulate the sensitization–desensitization properties as well as channel opening probabilities. In most cases, sensitization–desensitization of TRP channels can be modulated through phosphorylation–dephosphorylation events. For example, recently we have demonstrated that phosphorylation of MBP-TRPV1-Ct in vitro at S800 position by PKCε is significantly reduced due to the interaction of tubulin to the MBP-TRPV1-Ct (91). This result strongly suggests that microtubule dynamics is an important regulator for the ionic conductivity mediated by TRPV1. In this respect, it is important to mention that S800 is a key position that regulates the

sensitization–desensitization of TRPV1 (134). The phosphorylation–dephosphorylation of TRP channels can also be regulated by the Ca^{2+}-dependent and/or independent kinases as well as by the Ca^{2+} influx through the channel itself. Kinases like PKCε and other PKCs are also involved in the sensitization–desensitization of TRP channels. Physical interaction and involvement of Ca^{2+}-binding/sensing proteins like calmodulin and CamKII in the desensitization of TRP channels have been reported (33, 135–141). For example, TRPV1 and TRPV4 interact with Calmodulin and are regulated by CamKII and these interactions are involved in the regulation of ion channels. So it is becoming prominent that TRP channels are modulated by Ca^{2+}-dependent as well as Ca^{2+}-independent mechanisms and the Ca^{2+}-independent regulation of TRP channels is just emerging. An example of Ca^{2+}-independent regulation of TRP channel is the regulation of TRPC by Homer. It has been shown that TRPC mutants lacking the homer-binding site become constitutively active (142). Even, point mutations in the ankyrin repeat region (supposed to be involved in protein–protein interaction) of TRPV4 results in constitutively active or inactive channel (21, 54–56). These examples strongly indicate that other scaffolding proteins and cytoskeletal components can regulate TRP channel though the experimental evidences are still limited.

However, in recent time, very few studies have addressed this problem and attempted to establish a direct modulatory role of the cytoskeleton. The best examples of such studies were performed on TRPP channels (53, 93). Montalbetti and co-workers performed single-channel electrophysiological experiments of polycystin channel 2 (PC2) on reconstituted lipid bilayers. This system arguably eliminates all factors except the channel-associated complex. Interestingly, monomeric actin, the actin-related components α-actinin and gelsolin, tubulin including acetylated α-tubulin, and the kinesin motor proteins (KIF3A and KIF3B) are present in these membranes, possibly due to the direct interaction with PC2 channels (53, 93). Disruption of actin filaments with cytochalasin D or with the actin-severing protein gelsolin activates the channel. This activation can be inhibited by the addition of soluble monomeric G-actin with ATP, which induces actin polymerization. This indicates that actin filaments, but not soluble actin, are an endogenous negative regulator of PC2 channels. Also microtubules regulate PC2 channel function only in opposing manner. Depolymerization of microtubules with Colchicine rapidly inhibits the basal level of PC2 channel activity, whereas polymerization and/or stabilization of microtubules from soluble tubulin with GTP and Taxol stimulates the PC2 channel activity (93). Involvement of the microtubule cytoskeleton in the regulation of PC2 channel has also been described in vivo in primary cilia of renal epithelial cells (27). In that system, addition of microtubule destabilizer (Colchicine) rapidly abolished channel activity, whereas the addition of microtubule stabilizers (Taxol) increased channel activity (27). Similar results

were obtained using reconstituted lipid bilayer system, which reveals that both spontaneous activity and the opening probability of TRPP3 ion channels are increased by the addition of α-actinin, demonstrating that this channel can be indeed modulated by cytoskeleton (27). Certainly more studies are needed to explore such regulations at the single level.

8. Importance of Cytoskeletal Reorganization in Pathophysiological Disorders: New Pharmacological Challenges, Future Prospects and Concluding Remarks

The direct importance of TRP channels in Ca^{2+} signaling has been the major focus for a long time and still being investigated by many. However, in the last few years another aspect of TRP channels has emerged: the importance of Ca^{2+}-independent signaling event via TRP channels. In this context, the signaling complexes formed by the TRP channels and the cytoskeletal components at the submembranous compartment are of high importance. Being transmembrane proteins, TRP channels play a central role as scaffolds at the sub-membranous regions on which other components are sequestered, interact among themselves and finally form the functional signalplexes (Fig. 3). Interestingly, cytoskeletal components are not only present in these signalplexes, but also play significant roles to "fix" the entire signalplex in the context of cellular space and time. Therefore, both TRP channels and cytoskeletal components are involved in the "scaffolding" of the signalplexes. As the formation of these signalplexs needs TRP channels as transmembrane proteins only, the formation of the signalplexes is dependent on the availability of the TRP channels at the membrane but is largely independent of their Ca^{2+} channel activity. Interestingly, these components also take part in the regulation of ion channel opening—closure properties to a large extent.

The coordinated regulation of different cytoskeletons and vesicular trafficking by TRP channels has immense implication in the context of pharmacological treatment of pathophysiology and several disorders. For example, TRPV1 plays an inhibitory role in urothelial cancer cell invasion and metastasis by altering the microtubule cytoskeleton (143). This type of understanding may allow targeting the cytoskeleton of cancer cells via specific TRP channels or vice versa. In that manner, sprouting of neuronal cells, function of immune cells and sperm cells, etc., can be effectively modulated by targeting TRP-cytoskeletal complex per se. This strategy can be effective as expression, localization, function and regulation of TRP channels are specific yet versatile in nature. For example, the complex of TRP channel with β-tubulin III can be specifically targeted in neurons and/or in some specific cancer cells where the expression of β-tubulin III is reported. Thus, different properties of the individual TRP-signalplexes can be used for the pharmacological and clinical purposes.

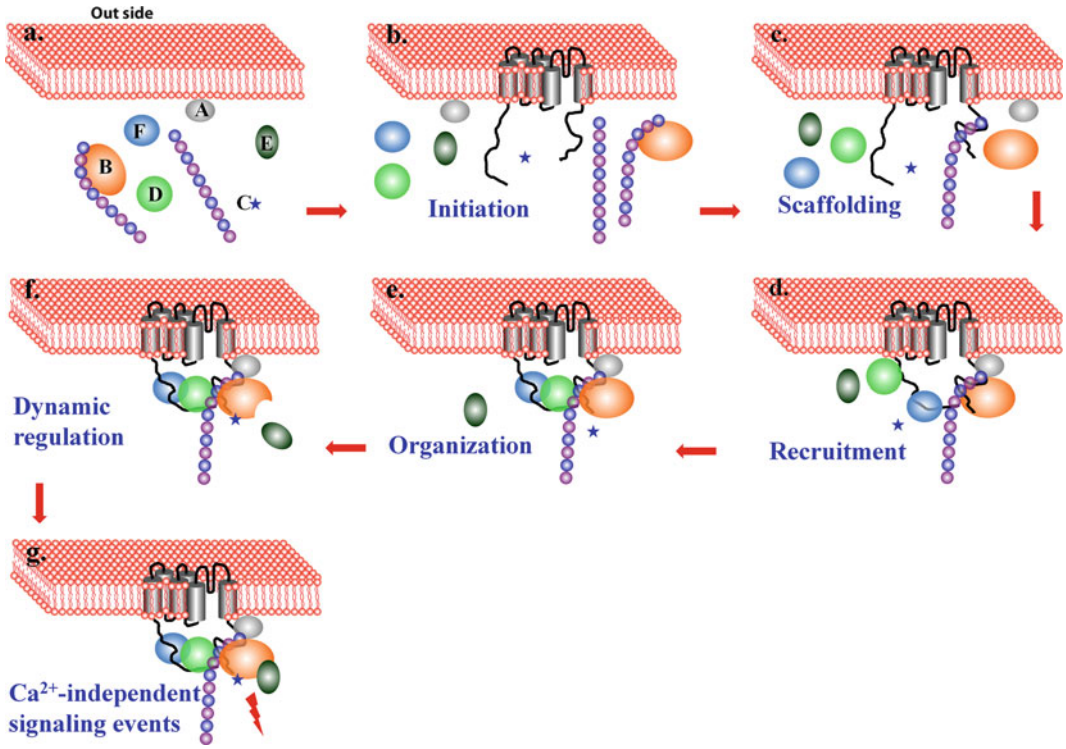

Fig. 3. Ca^{2+}-independent signaling events mediated by TRP channels. As TRP channels are transmembrane in nature, interaction of submembranous cytoskeleton and other scaffolding proteins with these channels initiate formation of scaffolds at the plasma membrane on which several other regulatory factors can associate and get involved in complex signaling events. As this scaffolding act of TRP channels is independent of their ion channel activity, such signaling events are dependent on the presence of TRP channels but independent of the TRP channel-mediated Ca^{2+} influx. These Ca^{2+}-independent signaling events can be described in several distinct steps like initiation (step b), scaffolding (step c), recruitment (step d), organization (step e), dynamic regulation (step f) and signaling events (step g). Though several membrane-associated factors (like A), microtubule-associated factors (like B) and other cytosolic factors (like C, D E and F) are present in the cell, these components cannot form novel signaling complex as these components are either not available at the submembranous region or not properly sequestered there (As indicated in step a). Interaction of TRP channels with cytoskeleton initiates some conformational changes (step b) and results in the formation of novel scaffolds on which some of these key components can sequester (step c). This sequestration of these key factors facilitates recruitment and sequestration of several other cytosolic and membrane-associated factors on the existing TRP complex (Step d). All these associated factors adjust and fine-tune their organization by further conformational changes (step e). The components sequestered in this complex can regulate each other and the entire signaling complex becomes dynamic (f). These complexes can also be further regulated by transient events like by kinase or phosphatase activity (such as by C) and result in some signaling events (step g).

Understanding the molecular mechanism of both Ca^{2+}-dependent and Ca^{2+}-independent signaling events has importance in basic research and also has pharmacological as well as clinical interests. This is especially due to the fact that application of common microtubule-based chemotherapeutics like Taxol and Vinca drugs in cancer patients is known to induce strong neuropathic pain (144–152). Though certain signaling events are involved in these chemotherapeutics-induced pain and hyperalgesia development,

the molecular mechanisms behind these pathophysiological symptoms are still largely unknown (102, 153). However, changes in the microtubule orientation, structure and other changes have been reported. In that context, physical and functional interactions of TRP channels with microtubule cytoskeletal proteins is significant. A better understanding of these physical and functional interactions may allow targeting these pathophysiological disorders in a more systemic manner. This may be more useful for the application of microtubule-based chemotherapeutics also. In future more studies must be conducted in these aspects.

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