CHAPTER TEN

TRANSIENT RECEPTOR POTENTIAL CHANNELS IN MECHANOSENSING AND CELL VOLUME REGULATION

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Abstract

Transient receptor potential (TRP) channels are unique cellular sensors responding to a wide variety of extra- and intracellular signals, including mechanical and osmotic stress. In recent years, TRP channels from multiple subfamilies have been added to the list of mechano- and/or osmosensitive channels, and it is becoming increasingly apparent that Ca^{2+} influx via TRP channels plays a crucial role in the response to mechanical and osmotic perturbations in a wide range of cell types. Although the events translating mechanical and osmotic stimuli into regulation of TRP channels are still incompletely understood, the specific mechanisms employed vary between different TRP isoforms, and probably include changes in

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the tension and/or curvature of the lipid bilayer, changes in the cortical cytoskeleton, and signaling events such as lipid metabolism and protein phosphorylation/dephosphorylation. This chapter describes candidate mechanosensitive channels from mammalian TRP subfamilies, discusses inherent and technical issues potentially confounding evaluation of mechano- and/or osmosensitivity, and presents methods relevant to the study of TRP channel regulation by mechanical and osmotic stimuli and involvement in cell volume regulation.

1. INTRODUCTION

Transient receptor potential (TRP) channels are unique cellular sensors, the important roles of which include the detection of mechanical forces and of changes in cell volume or intra- or extracellular osmolarity. Based on sequence homology, mammalian TRP channels are divided into six subfamilies: TRPC (canonical), TRPV (vanilloid), TRPM (melastatin), TRPP (polycystin), TRPML (mucolipin), and TRPA (ankyrin). Most TRPs are polymodal channels activated by multiple physical and chemical stimuli through distinct molecular mechanisms (for reviews, see Liedtke, 2005; Liedtke and Kim, 2005; Nilius and Voets, 2005; Nilius *et al.*, 2007; Pedersen *et al.*, 2005; Ramsey *et al.*, 2006; Voets *et al.*, 2005). The first evidence for an involvement of TRP channels in mechanosensation came from mutations in the *Caenorhabditis elegans osm-9* gene, which encodes a TRPV-like channel. Worms with mutations in this channel were shown to exhibit defects in the avoidance reaction to high osmolality and nose touch (Colbert *et al.*, 1997). In recent years, pivotal roles of mammalian TRP channels in mechano- and osmosensing have been demonstrated. This chapter briefly describes these channels and the evidence linking them to mechano- and osmosensing and discusses methods relevant to the study of TRP channel regulation by mechanical and osmotic stimuli and the potential involvement of TRP channels in the regulation of cell volume.

2. GENERAL MECHANISMS OF MECHANO- OR OSMOSENSING BY MEMBRANE PROTEINS

Membrane transport proteins appear to sense mechanical forces and/or changes in osmolarity by a number of fundamental mechanisms. These are outlined in 1 and discussed later, focusing on the mechanisms relevant to ion channels in general and TRP channels in particular (for reviews on general mechanisms of mechanotransduction, see Hamill and Martinac, 2001; Kung, 2005; Nicolson, 2005; Perozo, 2006). Any channel embedded in a lipid bilayer is exposed to negative and positive pressures created by the bilayer. Under equilibrium conditions, the conformational energy of the channel

matches this energy profile. Any change of this equilibrium can result in modulation of channel activity (Fig. 10.1A). A number of mechanisms have been shown to be involved in mechano-/osmosensing by plasma membrane ion channels. First, a change of the forces acting within the lipid membrane causes conformational changes in the channel protein. In this manner, forces acting at the crucial lipid-protein interface may gate a channel upon membrane stretch or bending (Fig. 10.1B). Second, channel displacement through a tether, for example, a reorganization of the cortical cytoskeleton, can cause a mismatch between the conformation energy of the channel and the intrinsic lipid tension, leading to changes in channel gating (Fig. 10.1C). Third, changes in the membrane content of specific lipids (e.g., phosphatidylcholines, lysophosphatidylcholines, or arachidonic acid and its metabolites) can alter the membrane structure around a channel protein in a concave or a convex manner, depending on the shape of the relevant lipid components, thereby inducing energetic mismatch and changes in channel gating (Fig. 10.1D). The two pore potassium channels, TREK-1 and TRAAK, provide interesting examples of gating by changes in membrane lipid composition. A phospholipid sensor controls mechanoactivation of TREK-1 and phosphatidylinositol (4,5)-biphosphate [PtdIns(4,5)P₂] sensitizes its activation, and TRAAK is activated by convex bending of the membrane in a manner that can be mimicked by the introduction of membrane components causing convex membrane curvature (Chemin *et al.*, 2005; Maingret *et al.*, 1999; Patel and Honore, 2001; Patel *et al.*, 1998). Fourth, mechanical or osmotic stress may trigger intracellular signaling cascades involving, for example, lipid metabolism, which may affect channel gating and/or plasma membrane insertion/retrieval (Fig. 10.1E). Fifth, mechanical or osmotic stress also elicits numerous changes in protein phosphorylation/dephosphorylation events, several of which have been shown to play a major role in the regulation of mechano- or osmosensing by membrane transport proteins, including TRPs (for specific examples, see later) (Fig. 10.1F). A discussion of the mechanisms by which such signaling events are initiated by mechanical or osmotic stress is beyond the scope of this chapter, but important mechanisms likely involve integrin clustering, cytoskeletal reorganization, and changes in intracellular ionic strength and/or concentrations of macromolecules or specific ions (for a more general description, see Hoffmann and Pedersen, 2006; Kung, 2005). Although not further discussed here, since there is yet no evidence of such a mechanism for TRP channels, it is also notable that at least some membrane transporters appear to respond directly to volume-dependent changes in intracellular ionic strength by ionic strength-dependent interactions of a specific intracellular domain, the cystathionine β -synthase domain, with the lipid bilayer (Biemans-Oldehinkel *et al.*, 2006).

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So far, it is difficult to differentiate among mechanisms 1 through 5 just outlined. First, osmotic cell swelling can be inherently associated with

mechanical stretch and increased membrane tension (mechanism 1). However, in many cell types, cell swelling is associated with unfolding of membrane invaginations, that is, the cortical cytoskeleton is exposed to increased tension and may affect a tethered ion channel (mechanism 2). Also in accordance with this notion, cell volume perturbations elicit substantial rearrangements of the actin-based cytoskeleton (Di Ciano-Oliveira *et al.*, 2006; Pedersen *et al.*, 2001). In contrast, the membrane per se need not be exposed to increased tension until very substantial cell swelling has occurred (Hamill and Martinac, 2001; Maingret *et al.*, 1999; Pedersen *et al.*, 2001). Cell volume changes also elicit a number of signaling events involving the metabolism of membrane components, which may regulate membrane proteins both by altering bilayer curvature per se or by direct interactions of the transporter with the specific lipid messenger in question. Thus, phospholipase A₂ (PLA₂) is activated by cell swelling, leading to a breakdown of phospholipids into arachidonic acid and lysophosphatidic acid, and is conversely inhibited by cell shrinkage, leading to reduced arachidonic acid release (e.g., Basavappa *et al.*, 1998; Pedersen *et al.*, 2006). Moreover, hypotonic cell swelling has been shown to decrease (see Pedersen *et al.*, 2001) and hypertonic cell shrinkage to increase (Nasuhoglu *et al.*, 2002; Yamamoto *et al.*, 2006) cellular PtdIns(4,5)P₂ levels. Also the lipid kinase phosphatidylinositol 3-kinase (PI3K) is reported to be volume sensitive (Yamamoto *et al.*, 2006), although the specific effects of volume perturbations on PI3K activity seem to be variable (see Pedersen *et al.*, 2001). As discussed later, arachidonic acid metabolites, PtdIns(4,5)P₂, and PI3K are involved in the regulation of several mechano- and osmosensitive TRP channels (Rohacs, 2007), and hence could play a role in messenger-dependent mechano- and osmosensing (mechanism 4). Multiple protein

Figure 10.1 Some possible mechanisms of mechano- or osmosensing by TRP channels. (A) Any channel in a plasma membrane is exposed to lateral forces from the bilayer (see energy profile at the left-hand side), which create positive or negative forces (lateral pressure indicated by the arrows) acting on the channel. In equilibrium, the conformational energy of the channel matches this energy profile. Any changes in this equilibrium may cause modulation of channel activity, e.g., gating. (B) Channel gating by changes in bilayer tension, which will alter the tension profile shown in A (referred to in the text as mechanism 1). (C) Channel gating by tethering to cytoskeletal elements, which are reorganized by mechanical or osmotic stress (mechanism 2). (D) Channel gating by changes in membrane curvature (mechanism 3). (E) Channel gating by an intracellular messenger that can be produced by an enzyme acting as a mechano/osmo/volume sensor (e.g., volume-sensitive activation of PLA₂, resulting in the generation of arachidonic acid, which either itself or in the form of its downstream metabolic products gates the channel (mechanism 4). (F) Channel gating as a consequence of regulation by signaling events induced by mechanical or osmotic stress can induce signaling events, e.g., via integrins or kinases, which in turn regulate both the activity of channels resident in the plasma membrane and the membrane insertion/retrieval of channels (mechanism 5). See text for details.

kinases and phosphatases are regulated in a volume- or mechanosensitive manner (see, e.g., Hoffmann and Pedersen, 2006), and TRP regulation by changes in protein phosphorylation/dephosphorylation may also be relevant to their mechano- and osmosensitivity (mechanism 5). One striking example is the modulation of TRPV4 by kinases of the WNK family. WNK4 downregulates TRPV4 via decreased cell surface expression. Because WNK kinases are activated by anisotonic conditions, they form a link to hypotonic TRPV4 activation (Fu *et al.*, 2006). In yeast cells, Yvc1p, a vacuolar membrane protein with homology to TRPV (Denis and Cyert, 2002), mediates the hyperosmolarity-induced Ca^{2+} release, which might be regulated by intracellular phosphorylation/dephosphorylation signaling cascades (Liedtke and Kim, 2005). This cascade involves MAP kinase activation, which is triggered by Ste20, a shrinkage-activated kinase in yeast (for a review, see Strange *et al.*, 2006). Finally, it is clear that at least some ion channels and volume-sensitive transporters respond to ionic strength rather than cell volume per se; however, the mechanisms remain to be fully elucidated and are not further discussed here (Nilius and Droogmans, 2001; Voets *et al.*, 1999).

As evident from the discussion just given, mechano and osmo/volume/ionic strength sensitivity are related in an inherently complex manner, yet are obviously not identical entities in terms of the biophysical characterization of membrane transporters. A number of technical aspects further complicate their experimental separation. None of the usually applied methods comply with evaluation of one of the described mechanisms in isolation. For the narrow purpose of patch clamp measurements in artificial lipid membranes—devoid of the cortical cytoskeleton—mechanosensitivity may be defined as activation by negative or positive pressure applied through the patch clamp pipette. However, a problem specifically related to analysis of mechano and osmo/volume sensitivity by the whole cell patch clamp technology is that bath hypotonicity is unavoidably coupled with a constantly increasing cell volume (see later), as well as with changes in intracellular ionic strength and concentrations of macromolecules and probably also with mechanical stimulation by fluid shear stress during cell superfusion (for discussion, see Hamill and Martinac, 2001; Nilius *et al.*, 1998; Voets, 1999).

3. TRP CHANNELS IN MECHANO- AND OSMOSENSING

The ability of cells to sense mechanical stimuli is fundamental to such essential physiological functions as embryonic development, hearing, touch sensitivity, and control of kidney function, vascular tone, and muscle stretch (see, e.g., Hamill and Martinac, 2001; Kung, 2005). Multiple TRP channels from various subfamilies have been shown to be sensitive to

various forms of mechanical stress, including fluid shear stress, and increased membrane tension resulting from membrane stretch (Liedtke and Kim, 2005; Nilius *et al.*, 2007; O'Neil and Heller, 2005; Pedersen *et al.*, 2005; Voets *et al.*, 2005). This section briefly reviews the evidence for mechano- or osmo-sensitivity of individual TRP channels.

TRPC1 has been identified as a mechanosensitive cation channel (MscCa) in *Xenopus laevis* oocytes (Maroto *et al.*, 2005). TRPC1 is also mechanosensitive in liposomes, and hence is probably directly activated by membrane stretch (mechanism 1). Very likely, TRPC1 is involved in stretch-induced muscle damage by elevating $[Ca^{2+}]_i$ upon membrane stretch (Allen *et al.*, 2005).

Another TRPC channel, the receptor-activated, nonselective cation channel TRPC6, is activated by mechanically or osmotically induced membrane stretch/deformation in a manner inhibited by the tarantula peptide toxin GsMTx-4, an inhibitor of mechanosensitive ion channels (Spassova *et al.*, 2006). The activation mechanism is phospholipase C independent. Instead, it seems to depend directly on the lateral-lipid tension and lipid-protein mismatch, such that the stretch-induced reduction in membrane bilayer thickness alters the channel conformation to the open state (mechanism 1) (Spassova *et al.*, 2006). Diacylglycerol, a well-known TRPC6 activator, may act similarly by changing membrane curvature, whereas GsMTx-4 may relieve membrane lipid stress and inhibit channel activation (Spassova *et al.*, 2006). MxA, a member of the dynamin superfamily, binds to TRPC6 and connects the channel with the cytoskeleton via the second ankyrin repeat (Lussier *et al.*, 2005). Referring to the description of mechanism 2 given earlier, it has yet to be shown that this coupling to the cytoskeleton may be involved in mechanosensing by TRPC6. Physiologically, the mechanosensitivity of TRPC6 appears to be important in the control of vascular tone in response to increased intravascular pressure (the Baylis effect) (Beech *et al.*, 2004; Inoue *et al.*, 2006; Welsh *et al.*, 2002).

Finally, several TRPCs have been described as stretch-activated channels (SAC) in skeletal muscle (7–8 pS in 100 mM Ca^{2+}), which are involved in the pathophysiology of Duchenne muscular dystrophy. These SACs are inhibited by Gd^{3+} , SKF-96365, and GsMTx4 and are upregulated by IGF-1 (Ducret *et al.*, 2006; Gailly, 2002; Vandebrouck *et al.*, 2002). However, it remains to be analyzed thoroughly whether these channels, as suggested, correspond to TRPC1, TRPC3, and TRPC6 (Gailly, 2002).

A number of TRPV channels are mechano- or volume/osmosensitive. A splice variant of TRPV1, TRPV1b, in which a stretch of 60 amino acids is deleted in the intracellular N-terminal region (Lu *et al.*, 2005), forms stretch-inhibited cation channels, for example, these channels are activated by hypertonic cell shrinkage (Ciura and Bourque, 2006; Naeini *et al.*, 2005). TRPV1b has been shown to mediate the osmosensitivity of arginine/vasopressin-releasing neurons in the supraoptic nucleus (Naeini *et al.*, 2005).

TRPV1 is also required for the response of osmosensory neurons in the organum vasculosum lamina terminalis, the primary osmosensor in the brain. In these neurons, hypertonic exposure activates an inward current, which is absent in *trpv1*−/− mice (Ciura and Bourque, 2006).

TRPV2 has been described as a stretch-activated channel functioning as a mechanosensor in vascular smooth muscle cells and also appears to be activated by osmotic cell swelling (Beech *et al.*, 2004; Muraki *et al.*, 2003). Stretch activation of TRPV2 has been assigned a role in skeletal and cardiac muscle degeneration caused by dystrophin–glycoprotein complex disruption (Iwata *et al.*, 2003). The mechanism of activation of TRPV2 in response to cellular stretch/swelling is still a matter of debate. However, it is known that PI3-kinase promotes TRPV2 membrane insertion (Penna *et al.*, 2006); it has also been discussed whether altered cellular PtdIns(4,5)P₂ levels may underlie TRPV2 mechanosensitivity (Yamamoto *et al.*, 2006). Interestingly, TRPV2 interacts with a protein kinase A (PKA)-dependent signaling module, containing PKA and an A-kinase adapter protein (acyl-CoA-binding domain protein ACBD3), and phosphorylation by PKA may modulate mechanoactivation of TRPV2 (Stokes *et al.*, 2004).

TRPV4 was the first TRP channel to be described as a volume-activated, Ca²⁺-permeable cation channel (Liedtke *et al.*, 2000; Nilius *et al.*, 2001; Strotmann *et al.*, 2000). TRPV4 is activated by osmotic cell swelling in a manner that is not due to direct stretch activation (Liedtke *et al.*, 2000; Strotmann *et al.*, 2000), is independent of intracellular ionic strength and not reproduced by the presence of GTPγS in the pipette solution (Nilius *et al.*, 2001), and is not dependent on N-terminal ankyrin repeats (Liedtke *et al.*, 2000). It has been shown that TRPV4 activation by cell swelling is mediated by the arachidonic acid metabolite 5',6'-epoxyeicosatrienoic acid (5',6'-EET) (Vriens *et al.*, 2004, 2005; Watanabe *et al.*, 2003), consistent with the previously demonstrated swelling-induced activation of PLA₂ (Kinnunen, 2000; Pedersen *et al.*, 2006). Phosphorylation of TRPV4 at Tyr²⁵³ in the N-terminal region has also been proposed to play a role in channel activation by hypotonicity, although this finding has been disputed (Cohen, 2005; Xu *et al.*, 2003). Importantly, activation of TRPV4 by cell swelling appears to be modulated by protein–protein interactions. (i) Thus swelling-induced activation of TRPV4 is absent in cystic fibrosis airway epithelial cells, suggesting a functional role of the cystic fibrosis transmembrane regulator (CFTR) in mechanosensing by TRPV4 (Amigues *et al.*, 2004). (ii) TRPV4 activation by hypotonicity in salivary gland epithelial cells appears to depend on the interaction with its binding partner aquaporin 5 (AQP5) rather than on cell swelling directly (Liu *et al.*, 2006). (iii) Pacsin 3, a protein thought to block dynamin-mediated endocytosis, interacts functionally with TRPV4 (Cuajungco *et al.*, 2006), and coexpression with Pacsin 3 increases TRPV4 insertion in the plasma membrane, yet attenuates its activation by cell swelling (D'Hondt and Nilius, unpublished result). This indicates that insertion/retrieval mechanisms may

play a role in the regulation of TRPV4 by cell swelling. Interestingly, a similar mechanism may operate in the shear stress-dependent activation of TRPM7 (see later) (Oancea *et al.*, 2006). (iv) Finally, TRPV4 and the polycystic kidney disease protein 2 (TRPP2) colocalize in the kidney and interact in the primary cilium (Giamarchi *et al.*, 2006), apparently forming a novel mechanosensitive complex.

TRPM3 exists as at least 12 splice variants, for which a unified nomenclature is lacking. A long splice variant (1555 amino acids) of TRPM3 forms a constitutively active channel that is further stimulated by muscarinic acetylcholine receptor activation (Grimm *et al.*, 2003; Lee *et al.*, 2003; Oberwinkler *et al.*, 2005), while a shorter (1325 amino acids) human TRPM3 channel is stimulated by hypotonic cell swelling (Grimm *et al.*, 2003) by mechanisms that remain to be elucidated.

TRPM4 has been described as a mechano-/stretch-sensitive channel involved in the control of pressure-induced smooth muscle cell depolarization and myogenic vasoconstriction in cerebral arteries and isolated vascular smooth muscle cells (Dietrich *et al.*, 2006; Earley *et al.*, 2004; Inoue *et al.*, 2004, 2006; Kraft and Harteneck, 2005). However, the mechanism of activation of TRPM4 under these conditions remains to be elucidated in detail.

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TRPM7 has been considered as a candidate for mechanosensation in a variety of cell types. TRPM7 has been proposed to be directly activated by cell stretch and potentiated by hypotonic cell swelling (Numata *et al.*, 2006). Moreover, shear stress induces a translocation of TRPM7 to the plasma membrane and a rapid increase in TRPM7 currents (Oancea *et al.*, 2006).

TRPA1 has long been considered a paradigm for a mechanosensitive channel. TRPA1 exhibits 14 N-terminal ankyrin repeats (Lee *et al.*, 2006; Nagata *et al.*, 2005; Story *et al.*, 2003), which have been speculated to act as a gating spring in mechanosensing by this channel (Howard and Bechstedt, 2004; Lee *et al.*, 2006; Nagata *et al.*, 2005; Sotomayor *et al.*, 2005). TRPA1 was proposed to be the mechanically gated transduction channel necessary for the auditory response in mammals (Corey *et al.*, 2004; Gillespie *et al.*, 2005); however, studies in *trpa1*^(-/-) mice revealed no obvious deficits in auditory function (Bautista *et al.*, 2006; Kwan *et al.*, 2006). Although not the auditory transduction channel, TRPA1 may well be a mechanosensory TRP channel involved in other physiological functions (Corey, 2006).

In the context of mechano- and osmosensing, it is noteworthy that in addition to ankyrin repeats, several TRP subfamilies exhibit other conserved domains potentially linking them to the actin-based cytoskeleton. Thus, direct interactions with PDZ scaffold proteins such as ezrin-binding phosphoprotein 50 (EBP50)/Na⁺/H⁺ exchanger regulatory factor (see Pedersen *et al.*, 2005) link several TRP subfamilies to the ezrin/radixin/moesin proteins (Lockwich *et al.*, 2001), which act as integrators between the F-actin cytoskeleton and

integral membrane proteins (e.g., Bretscher *et al.*, 2002) and which have been shown to be volume sensitive (Darborg *et al.*, 2005; Wu *et al.*, 2004).

Finally, the polycystic kidney disease protein 2 (PKD2 or TRPP2) is involved in mechanosensation in the primary cilia of kidney cells. In yeast, a TRPP2-like channel is involved in mechanosensing (sensing of cell shape) (Palmer *et al.*, 2001). TRPP2 forms a complex with polycystin 1-like (PKD1), which is important for TRPP2 regulation by many stimuli; however, data from PKD1 knockout mice also point to a possible mechanosensory role for TRPP2 in the absence of PKD1 (for an excellent review, see Giamarchi *et al.*, 2006). Trafficking of TRPP2 from the endoplasmic reticulum and the Golgi complex to the plasma membrane, where at least some mechanical forces are detected, is directed by the phosphoproteins phosphofurin acidic cluster-1 and -2 and involves binding of these proteins to TRPP2 promoted by its phosphorylation at Ser⁸¹² by casein-kinase 2 (Köttgen and Walz, 2005; Kottgen *et al.*, 2005). Ca²⁺ influx via TRPP2 translates mechanical deflections of primary cilia into a signaling cascade associated with growth control and differentiation in renal epithelial cells (Nauli and Zhou, 2004; Nauli *et al.*, 2003). A role for TRPP2 in osmosensing has also been proposed (see, e.g., Montalbetti *et al.*, 2005).

4. TRP CHANNELS IN CELL VOLUME REGULATION

Cell volume perturbations occur under physiological and pathophysiological conditions in a wide range of cell types, and the ability to regulate cell volume is fundamental to cell function and survival. Following osmotic cell shrinkage or swelling, most cell types are able to regulate their volume in processes termed regulatory volume increase or regulatory volume decrease (RVD), respectively (Hoffmann and Pedersen, 2006; Lang *et al.*, 1998). Obviously, to establish that a given channel actually contributes to cell volume regulation, it is not sufficient to show that it is activated by cell volume perturbations, as the effect of its activity on cell volume regulation must be assessed. Although, as outlined earlier, a number of TRP channels have been shown to be activated by cell swelling (or swelling-induced stretch), direct evidence that TRP channels contribute to RVD is still sparse. In many cell types, increases in [Ca²⁺]_i contribute to the RVD response after cell swelling, most commonly by the activation of Ca²⁺-sensitive K⁺ channels (see Hoffmann and Pedersen, 2006). This swelling-activated increase in [Ca²⁺]_i reflects opening of stretch-activated channels, at least some of which have now been shown or proposed to be TRP channels (e.g., Arniges *et al.*, 2004; Maroto *et al.*, 2005; Numata *et al.*, 2006), and hence, the role of TRP channels in volume regulation is generally assumed to be to elicit an increase in [Ca²⁺]_i.

Among the mammalian TRP channels, a role in volume regulation has been documented most thoroughly for TRPV4. As noted earlier, TRPV4 is activated, at least in part, as a consequence of the cell swelling-induced release of 5',6'-EET (Vriens *et al.*, 2004, 2005; Watanabe *et al.*, 2003). Direct evidence for a role in volume regulation is available for TRPV4, as *trpv4*^{-/-} mice have a reduced capacity for RVD (Liedtke and Kim, 2005), and exogenous expression of TRPV4 confers osmoregulatory capacity to CHO cells (Becker *et al.*, 2005). Moreover, RVD was reduced markedly in cells with reduced expression of CFTR or AQP5, both of which interact with TRPV4 (Arniges *et al.*, 2004; Liu *et al.*, 2006). In both cases, this defect in RVD was shown to be because of a reduction of the TRPV4-mediated Ca²⁺ entry activated by hypotonic stimulation (Arniges *et al.*, 2004; Liu *et al.*, 2006).

Other TRP channels activated by cell swelling are, as noted previously, TRPV2, TRPM3, probably TRPM7, and TRPP2 (Chen and Barritt, 2003; Grimm *et al.*, 2003; Kraft and Harteneck, 2005; Montalbetti *et al.*, 2005; Muraki *et al.*, 2003; Numata *et al.*, 2006). However, although some mechanisms have been proposed, events leading to the swelling-induced activation of these channels are considerably less well understood than for TRPV4. Nonetheless, for a few of these channels, evidence has been presented for a role in the RVD process based on assessments of cell volume regulation after cell swelling. Hence, knockdown of TRPM7 (Numata *et al.*, 2006), as well as of TRPC1 (Chen and Barritt, 2003), was found to reduce the rate of RVD (although for TRPC1 knockdown, the effect on RVD was marginal).

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5. EXPERIMENTAL PROCEDURES

This section describes the foundations and practical procedures for selected methods useful in the evaluation of mechano- and osmosensitivity, as well as of transporter effects on [Ca²⁺]_i cell volume. Standard patch clamp procedures have been described extensively elsewhere (see, e.g., Hille, 2001; Sakmann and Neher, 1995) and are not detailed here.

5.1. Electrophysiological recordings used to assess mechanosensitivity of TRP channels

5.1.1. Current measurements on TRP channels expressed in mammalian cells

Stretch sensitivity of TRP channels can be measured directly in cells endogenously expressing the channel in question or in an adequate over-expression system. A low DNA concentration should be used for transfection (<2 µg of DNA per well, see also <http://www.mirusbio.com/>) to

reduce the risk of overexpression artifacts. The impact of a particular TRP channel may be studied by using specific modulators, such as 4α PDD for TRPV4 (Liu *et al.*, 2006), specific antisense methods (e.g., Earley *et al.*, 2004), gene silencing methods (see, e.g., Arniges *et al.*, 2004; Numata *et al.*, 2006), and constitutive or conditional knockout models (for a review, see Freichel *et al.*, 2005). Gene silencing has been used as a powerful tool to knock down various TRP channels; however, this approach has to be used cautiously, as off-target gene silencing can present a notable challenge in the interpretation of data from siRNA experiments (Birmingham *et al.*, 2006; Fedorov *et al.*, 2006).

Single-channel recordings to assess channel mechanosensitivity can be performed in the cell-attached and excised inside-out configurations. In these configurations, the amplitude of the single-channel current is measured from the peak-to-peak distance on the amplitude histogram. The open probability (P_o) of the single-channel current can be calculated by dividing the total time spent in the open state by the total time of continuous recording (30–200 s) in patches containing one active channel. To test effects of mechanical stretch, patched membranes are subjected to a pulse of negative pressure applied to the back of the patch pipette by mouth or syringe. The pressure level can be monitored with a manometer or a piezoelectric pressure transducer.

5.1.2. Current measurements on TRP channels reconstituted in liposomes

To study mechanoactivation by membrane stretch directly, avoiding contributions from the cortical cytoskeleton, a liposome preparation may be used. Membrane proteins from an overexpression system (we routinely use HEK or CHO cells) can be reconstituted in liposomes for patch clamp recording (for details, see Hamill *et al.*, 1981; Maroto *et al.*, 2005; Sukharev *et al.*, 1993). To prepare phosphatidylcholine liposomes, phosphatidylcholine is dissolved in chloroform and small aliquots of the lipid are dried under nitrogen, resuspended in 5 mM EDTA, 5 mM EGTA, 1 mM dithiothreitol, 50 mM Tris-HCl, pH 7.4, containing 2% octyl- β -D-glucopyranoside (OG), and bath sonicated for 5 min. A 200- μ l volume (equivalent to 2 mg lipid) is added to the membrane protein to achieve protein:lipid ratios ranging from 1:50 to 1:5000. The mixture is incubated on a platform rocker for 1 h at room temperature. BioBeads are then added to remove the OG, and the suspension is rocked for a further 3 h at room temperature. The liposomes are collected by ultracentrifugation at 90,000 g for 30 min and resuspended. Aliquots of the liposomes are spotted onto glass slides and allowed to dehydrate under vacuum at 4° for 6 h, followed by overnight rehydration (for details, see Sukharev *et al.*, 1993).

For liposome recording, standard patch clamp techniques can be used. The liposomes are placed in a patch clamp chamber containing high $MgCl_2$

solution. Seal resistance in the high MgCl_2 solution should exceed $20 \text{ G}\Omega$. Inside-out patches can be formed by passing the pipette tip briefly through the solution–air interface. As described earlier for cellular systems, channel activation by stretch can be studied by applying negative pressure pulses (for TRPC1, -10 to -200 mm Hg is appropriate) (Maroto *et al.*, 2005; Perozo, 2006).

5.2. Electrophysiological recordings to assess osmosensitivity of TRP channels

5.2.1. Whole cell patch clamp and cell swelling: general considerations

Hypotonic cell swelling in the whole cell patch clamp mode poses the inherent problem that the cell interior will constantly equilibrate with the pipette solution. As a consequence of this, the initial rapid increase in cell volume is followed by a nearly linear continuous increase until the cell ruptures (or extracellular osmolarity is restored), that is, a condition fundamentally different from that resulting from the same transmembrane osmolarity difference in an unpatched cell. In contrast to the cell volume, the current may reach a stationary level; hence, under these conditions, a close correlation between current and cell volume per se does not exist. This behavior can be explained as follows: changes in cell volume over time (dV/dt) are a consequence of the net flux of water across the plasma membrane, which is linearly dependent on the transmembrane osmotic gradient, thus

$$\frac{dV}{dt} = P_w \cdot (c_i - c_o) \quad (10.1)$$

where c_i is the total concentration of solutes of the intracellular medium, c_o is the extracellular medium, and P_w is a constant, here referred to as “water permeation.” P_w is the product of the osmotic water permeability of the membrane (P_f), the cell surface (S), and the partial molar volume of water (V_w).

It is assumed that the osmotic gradient driving the water flux is approximated by the difference in total concentration of solutes and that the diffusion rate from pipette to cell is the same for all solute species in the pipette. Because of the combination of the solute exchange with the pipette and the variation in cell volume, c_i changes according to

$$\frac{dc_i}{dt} = \frac{1}{V} \left(k_d \cdot (c_p - c_i) - c_i \cdot \frac{dV}{dt} \right) \quad (10.2)$$

Figure 10.2 Cell volume and intracellular osmolyte concentration during exposure to hypotonic bath solutions in whole cell patch clamp experiments. If a cell is exposed to a hypotonic solution, water enters the cell and induces a change in cell volume, dV/dt (A). This increase is reflected by the initial change in volume (B, solid line). During the experiment, solutes will enter the cell from the patch pipette, which in turn induces additional water entry. Therefore, the cell volume will increase continuously (B). The intracellular osmolyte concentration, c_i (dotted line), decreases rapidly and reaches a stationary constant value while the volume is constantly increasing. See text for details.

where the constant k_d describes the rate of diffusion of solutes from pipette to cell and c_i and c_p reflect the global concentrations of solutes in the cell and in the pipette, respectively. Thus, hypotonic cell swelling in the whole cell patch clamp mode can be described by a system of two differential equations with the variables c_i and V . Figure 10.2 shows a numerical solution of this system of differential equations. The initial slope of the volume change mainly reflects the influx of water across the plasma membrane, which is faster than the solute exchange via the patch pipette. The subsequent nearly linear increase in volume is a consequence of the exchange of solutes via the patch pipette. Importantly, this model predicts that c_i decreases significantly during an osmotic challenge and reaches a plateau value, despite the continuous increase in cell volume. For further details, see Nilius (2004) and Voets *et al.* (1999).

5.2.2. Practical procedures

Similar to stretch sensitivity, volume/osmosensitivity of TRP channels may be assessed either in cells endogenously expressing the channel in question or after channel expression in a cell type with low endogenous TRP levels such as HEK 293 cells. For measurements of the volume sensitivity of TRP channels in the whole cell patch clamp mode in mammalian cells, we

generally employ an isotonic solution containing (in mM) 105 NaCl, 6 CsCl, 5 CaCl₂, 1 MgCl₂, 10 HEPES, 90 D-mannitol, 10 glucose, buffered pH 7.4 with NaOH (320 ± 5 mOsm). Cell swelling can thus be induced at constant extracellular ionic strength by omitting the desired amount of mannitol from this solution. The pipette solution for such experiments is generally composed of (in mM) 20 CsCl, 100 Asp, 1 MgCl₂, 10 HEPES, 4 Na₂ATP, 10 BAPTA, and an equivalent amount of CaCl₂ to buffer [Ca²⁺]_i at an required level (for calculation of free intracellular divalent concentrations, we refer to the CaBuf program <ftp://ftp.cc.kuleuven.ac.be/pub/droogmans/cabuf.zip>).

5.3. Assessing the contribution of TRP channels to cell volume regulation: simultaneous measurements of [Ca²⁺]_i and cell volume changes

5.3.1. General considerations

Changes in [Ca²⁺]_i and cell volume can conveniently be assessed simultaneously in a cell population using the fluorescent Ca²⁺-sensitive probe Fura-2 in conjunction with large-angle light scattering. Ratiometric measurements of [Ca²⁺]_i using Fura-2 are widely used and described extensively elsewhere (Gryniewicz *et al.*, 1985; Tsien, 1989). Large-angle light scattering is a noninvasive and sensitive technique for evaluating volume changes in both adherent and suspended cells, exploiting the fact that the light-scattering properties of cells are altered upon cell volume changes. While smaller particles scatter light in all directions, large particles scatter predominantly in the near-forward direction. A cell scatters light both as a large particle and as a collection of small particles reflecting its internal composition, hence both forward and large-angle light scattering are volume sensitive, although the latter appears to be the more sensitive method (Latimer, 1982; McManus *et al.*, 1993; Meyer and Brunsting, 1975). Generally, large-angle light scattering is inversely related to cell volume, consistent with a dependence on the cellular concentration of scattering particles (Fischbarg *et al.*, 1989; Latimer, 1982; McManus *et al.*, 1993). The only real disadvantage to the light-scattering technique is that absolute cell volumes cannot be obtained in this manner; however, for evaluation of RVD rates, this is rarely a problem.

5.3.2. Practical procedures

Cells are seeded 24 h prior to experiments on 10 × 50-mm, HCl- and ethanol-washed coverslips to a confluency of 70 to 90% at the time of the experiments. Cells are loaded with Fura-2 by preincubation with 2 μM Fura-2 acetoxymethyl ester (Fura-2-AM) in standard isotonic medium for 20 min at 37° followed by a wash and a 15-min postloading incubation to ensure proper intracellular cleavage of the acetoxymethylester groups to obtain Fura-2.

We employ a PTI RatioMaster spectrophotometer equipped with a standard photomultiplier (PMT) system, excitation and emission monochrometers for wavelength selection, a 75-W xenon lamp, and a temperature-controlled cuvette house. During experiments, the cuvette is perfused continuously with preheated (37°) standard Ringer's solutions at a speed of about 0.7 ml/min, increasing to 3.5 ml/min during solution changes for rapid complete exchange in the cuvette. The coverslip with the cells is placed at a 50° angle relative to the excitation light, and scattered light is collected at an angle of 90° relative to the excitation light. Excitation and emission wavelengths for light scattering need to be optimized for each cell type used. Generally, the best light-scattering signal is obtained after excitation in the wavelength range of 570 to 600 nm. The emission wavelength is set about 5 nm red shifted to the excitation wavelength to protect the PMT from excitation light. To the extent that the cells swell as near-perfect osmometers, and in the absence of volume regulation, the light-scattering signal will be a linear function of the osmolarity. Practically, this is achieved by very rapid solution changes, such that volume regulation is negligible in the time window studied.

Figure 10.3A and B show an experiment evaluating the linearity of the light-scattering response to osmolarity changes in Ehrlich-Lettre ascites (ELA) murine tumor cells. With these cells, the optimal light-scattering signal in our setup is obtained at 589 nm excitation, and emission is measured at 595 nm. Figure 10.3A is a representative trace, and it is seen that the light-scattering signal is directly related to extracellular osmolarity, that is, inversely related to cell volume. Therefore, data are calculated as the inverse of the light-scattering signal relative to that obtained in the initial, isotonic condition (I_0), that is, $1/(I/I_0)$, or I_0/I . Figure 10.3B shows I_0/I from the experiment in Fig. 10.3A as a function of extracellular osmolarity. Figures 10.3C and 10.3D show the light-scattering signal, and I_0/I , for an

Figure 10.3 The use of large-angle light scattering to monitor cell volume changes. (A) Light scattering as a function of extracellular osmolarity in Ehrlich Lettre ascites cells. Cells were seeded 24 h prior to experiments on 10×50 -mm coverslips to a confluency of about 70% at the time of the experiments. Cells were mounted in a temperature-controlled cuvette in a PTI RatioMaster spectrophotometer and were perfused continuously with preheated (37°) Ringer's solutions, which were changed rapidly to the osmolarity indicated by increasing the perfusion rate from 0.7 to 3.5 ml/min. Excitation was measured at 589 nm excitation, and emission is measured at 595 nm. (B) Data from A were converted to relative cell volumes by calculating the inverse of the light-scattering signal relative to that obtained in the initial, isotonic condition (I_0), i.e., $1/(I/I_0)$, or I_0/I . (C and D) Raw data and I_0/I for an experiment assessing RVD in about 90% confluent ELA cells after a 35% reduction in extracellular osmolarity. The experiment was carried out as described in A. It may be noted that the magnitude of the light-scattering signal, as well as of the relative changes in light scattering, is strongly cell density dependent, hence only populations of equal confluency should be compared.

experiment assessing RVD in ELA cells after a 35% reduction in extracellular osmolarity. RVD may be calculated as the slope of the initial, linear part of the relative cell volume traces following maximal cell swelling (for further examples, see Pedersen *et al.*, 2002).

The simultaneous assessments of Fura-2 fluorescence are carried out by measuring at 510 nm after excitation at 340 and 380 nm. Practically, this is achieved by running continuous cycles of excitation and measurement, such that cells are excited at 589 nm and emission is measured at 595 nm, followed by excitation at 340 and 380 nm, respectively, and emission measurement at 510 nm. Fura-2 data are evaluated as the 340/380-nm ratio after background subtraction and may be converted to $[Ca^{2+}]_i$ values by *in vitro* calibration as described previously (Grynkiewicz *et al.*, 1985). In our hands, Fura-2 loading has no effect on the light-scattering measurements.

5.4. Combined patch clamp and cell volume measurements

5.4.1. General considerations

A combination of volume measurements and patch clamp can be used on larger cells (e.g., epithelial or endothelial cells) adhering to a coated coverslip. The general problem of using whole cell patch clamp in conjunction with hypotonic cell swelling has already been discussed in detail. It should be carefully taken into account that the cell volume of the patched cells increased constantly and never reached a steady state and evaluated how much this unavoidable pitfall influences the conclusions drawn by the experimentalist.

5.4.2. Practical procedures

We have used a method for simultaneous monitoring of the cell height and whole cell currents. This method is useful for flat and adhering cells (e.g., endothelial or epithelial cells), which do not change the surface area attached to the coverslip during swelling. It is not usable for small, round, nonadhering cells, such as HEK cells. Currents are measured in the normal whole cell patch clamp configuration. We measure simultaneously the cell surface area (CSA) of the patched single cells, which is quantified from digital images recorded with a video camera (Model CF 6, Kappa, Gleichen, Germany) (Voets *et al.*, 1999). Cell borders are traced manually, and the surface of the traced region is determined using IMAGE TOOL 1.25 software (University of Texas Health Science Center, San Antonio, TX) (for more details concerning the measurement of cell height without patch clamping, see Van Driessche *et al.*, 1993). CSA is calculated as the average of three independent tracing procedures. Simultaneously, cell thickness, T_c , is monitored constantly as described previously (Van Driessche *et al.*, 1993). Briefly, gelatin-coated coverslips containing nonconfluent endothelial cells are incubated for about 30 min with 4 μ l/ml Red Neutravidin-labeled

microbeads (F-8775, Molecular Probes), followed by a 15-min washing with microbead-free solution. Visualization of the microbeads is performed using a xenon lamp and the XF40/E filter set (Omega Optical, Brattleboro, VT). Fluorescent images at different vertical positions are recorded with the CF 6 video camera, digitized, and displayed on a video monitor. Vertical displacement is achieved using a low-voltage piezoelectric translator (PIFOC P-721; Physik Instrumente, Waldbronn, Germany). T_c is calculated as the vertical distance between beads on the gelatin surface and on the cell surface, and image analysis is performed online (for details, see Voets *et al.*, 1999).

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