CHAPTER 9

TRPCs as MS Channels

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I. OVERVIEW

This chapter reviews recent evidence indicating canonical or classical transient receptor potential (TRPC) channels are directly or indirectly mechanosensitive (MS) and can therefore be designated as mechano-operated channels (MOCs). The MS functions of TRPCs may be mechanistically related to

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their better known functions as store-operated and receptor-operated channels (SOCs and ROCs). In particular, mechanical forces may be conveyed to TRPC channels through the "conformational coupling" mechanism that transmits information regarding the status of internal Ca^{2+} stores. Furthermore, all TRPCs are regulated by receptors coupled to phospholipases that are themselves MS and can regulate channels via lipidic second messengers. Accordingly, there may be several nonexclusive mechanisms by which mechanical forces may regulate TRPC channels, including direct sensitivity to bilayer mechanics, physical coupling to internal membranes, and/or cytoskeletal proteins, and sensitivity to lipidic second messengers generated by MS enzymes. Various strategies that can be used to separate out different MS-gating mechanisms and their possible role in specific TRPCs are discussed.

II. INTRODUCTION

MS ion channels transduce mechanical force into ion flux. To exhibit direct mechanosensitivity, a channel protein must be sensitive to some membrane parameter that changes with mechanical deformation. In many cases, the mechanotransduction step involves a shift in the equilibrium between closed and open channel conformations caused by changes in bilayer mechanics (e.g., lipid packing, bilayer thickness, curvature, and/or lateral pressure profile) or by direct "tugging" on the protein by cytoskeletal and/or extracellular tethers (Hamill and Martinac, 2001; Kung, 2005; Markin and Sachs, 2007; Matthews *et al.*, 2007; Powl and Lee, 2007). However, some channels may be indirectly MS in that they derive their mechanosensitivity from being functionally linked to MS enzymes that regulate the channel via second messenger or phosphorylation. Apart from mechanosensation, MS channels have been implicated in several basic cellular functions, including the regulation of cell volume, cell shape, motility, growth, and cell death. Because abnormalities in MS channels may also contribute to major human diseases, including muscular dystrophy, kidney disease, cardiac arrhythmias, hypertension, and tumor cell invasion ("mechanochannelopathies"), there is great interest in identifying the molecules that form MS channels and discovering agents that can selectively block their activity and/or expression (Chapter 4, Gottlieb *et al.*; Chapter 10, Cantiello *et al.*; Chapter 15, Drew *et al.*; Chapter 16, Lansman; Chapter 17, Maroto and Hamill; Chapter 19, Chapleau *et al.*). In eukaryotic cells, three membrane protein families, epithelial Na^+ channel (ENaC), two pore domain K^+ (TREK), and TRP families have been implicated in forming MS Na^+ (MscNa), K^+ (MscK), and cation/Ca^{2+} (MscCa) channels, respectively (Chapter 3, Bazopoulou and Tavernarakis;

Chapter 6, Drummond; Chapter 7, Chemin *et al.*; Chapter 8, Castiglioni and García-Añoveros; Chapter 10, Cantiello *et al.*). Here, we focus on the TRPCs, which have been implicated in forming the ubiquitous stretch-activated MscCa (Maroto *et al.*, 2005).

III. PRACTICAL ASPECTS OF RECORDING MS CHANNELS

The most direct method to determine if an ion channel is MS is to apply a hydrostatic or osmotic pressure gradient across the membrane patch while monitoring single-channel currents (Hamill *et al.*, 1981; Hamill, 1983; Guharay and Sachs, 1984; Hamill, 2006). This method led directly to the discovery of MscK and MscCa in frog red blood cells and cultured chick myotubes, respectively (Hamill, 1983; Guharay and Sachs, 1984). Subsequently, MscK and MscCa were shown to be widely expressed in sensory and nonsensory animal cells and proposed to function in various physiological processes including regulatory volume decrease (RVD) in response to osmotic swelling (Sachs, 1988; Morris, 1990; Sackin, 1995; Sachs and Morris, 1998; Hamill and Martinac, 2001; Patel and Honore, 2001). In several cases of RVD, it was possible to demonstrate that the same channel (e.g., MscK, MscCa, MscL, and MscS) was activated by cell swelling and membrane stretch (Christensen, 1987; Sackin, 1989; Cemerikic and Sackin, 1993; Levina *et al.*, 1999; Vanoye and Reuss, 1999). However, in other cases, most notably the vanilloid transient receptor potential 4 (TRPV4), the channel was sensitive to cell volume changes without displaying stretch sensitivity (Strotmann *et al.*, 2000). This discrepancy may arise because TRPV4 is not directly MS but instead derives its volume sensitivity from being coupled to one or more MS enzymes (Watanabe *et al.*, 2003; Xu *et al.*, 2003; Vriens *et al.*, 2004; Cohen, 2005a). In particular, one group has proposed that TRPV4 is coupled to an osmotic-sensitive Src protein tyrosine kinase that regulates channel activation by tyrosine phosphorylation (Xu *et al.*, 2003; Cohen, 2005b). Another group (Watanabe *et al.*, 2003; Vriens *et al.*, 2004) has proposed that TRPV4 is coupled to the volume-sensitive phospholipase A_2 (PLA_2; Basavappa *et al.*, 1988; Lehtonen and Kinnunen, 1995) that releases arachidonic acid (AA) from membrane phospholipids, which is then metabolized, via the action of cytochrome P450, into 5′,6′-epoxyeicosatrienoic acid (5′,6′-EET). In support of the latter scheme, it was shown that blocking either PLA_2 or cytochrome P450 inhibits TRPV4 activation, whereas direct application of 5′,6′-EET activates TRPV4 in a membrane-delimited manner (Watanabe *et al.*, 2003; Vriens *et al.*, 2004). The group that carried out the PLA_2 study was unable to reproduce the Src results (Cohen, 2005b), indicating the mechanism(s) that activates TRPV4 may vary with cell type and/or experimental conditions.

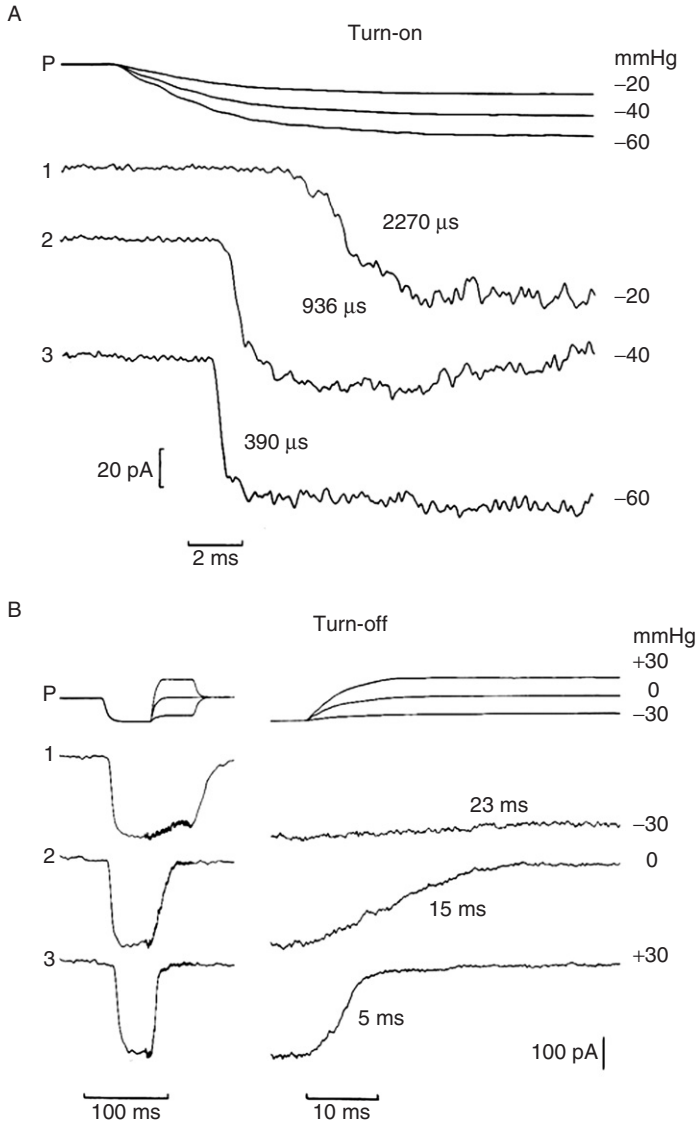

FIGURE 1 Fast turn-on and turn-off of MS channel currents measured in response to suction steps applied with a pressure clamp. A shows in the top trace (labeled P) three superimposed suction steps of −20, −40, and −60 mmHg applied to a cell-attached patch on a *Xenopus* oocyte. The lower three traces show the change in latency and the rate of turn-on of the currents in response to the increasing suction steps. The numbers in microseconds alongside each trace reflect the time from 20% to 80% of the peak current. B shows recordings designed to show the pressure dependence of the current turn-off. In the left-hand panel, the

In any case, the results indicate that while TRPV4 may function as a mechano-effector, it is not directly MS (O'Neil and Heller, 2005). There are added complications with other channels because they can be activated both by membrane stretch and by lipidic second messengers including AA and lysophospholipids (Martinac *et al.*, 1990; Kim, 1992; Hamill and McBride, 1996; Casado and Ascher, 1998; Patel *et al.*, 2001; Chapter 7, Chemin *et al.*). In this case, the issue becomes how to distinguish between direct and indirect mechanisms of mechanosensitivity.

IV. DISTINGUISHING DIRECT VS INDIRECT MS CHANNELS

Channels that are directly MS should only be limited by the conformational transitions of the channel protein, and may therefore be activated and deactivated with relatively brief delays (i.e., in the submillisecond or millisecond range). In comparison, channels dependent on enzymatic reactions and/or diffusion of second messenger may be expected to show much longer delays in opening and closing (e.g., $\geq$1 s). Figure 1 illustrates the activation and deactivation of the oocyte MS channel in response to increasing pressure steps. The transition time for the pressure step is limited by the speed of the pressure clamp (McBride and Hamill, 1992; 1995, 1999; Besch *et al.*, 2002). However, once the threshold pressure for activation is reached, the MS current turns on in few hundred microseconds (Fig. 1A). With increasing step size, both the latency and rise time of the MS current decreases consistent with the pressure reaching threshold faster. Similarly, the current turn-off indicates the channels close faster with larger pressure steps (Fig. 1B). The slower time for turn-off compared with turn-on presumably reflects the relatively slower rate of MS channel closure under these conditions (Fig. 1B). Similar brief delays and fast channel opening have been reported for activation and deactivation of the expressed a *T*WIK (tandem of P domains in a weak inward rectifier K^+ channel)-*r*elated *a*rachidonic *a*cid stimulated K^+ channel (TRAAK) (Honoré *et al.*, 2006). In contrast, an MscK expressed in snail neurons, which like TRAAK is a two-pore domain K^+ channel (Vandorpe and Morris, 1992), shows activation delays of up to several seconds (Small and Morris, 1994). However, because the delays can

superimposed suction pulse waveforms are shown in the upper trace and the corresponding current responses are shown in the lower traces. The initial activating suction was −40 mmHg for all three pulses. To turn off the currents the suction was stepped back to three increasing positive pressures. The right-hand panel shows on an expanded timescale the regions of the turn-off that were highlighted in the left panel with the numbers in microseconds representing the turn-off times (20–80%), and indicate that as the turn-off step size increases the channels turn off faster (Reproduced from McBride and Hamill, 1993).

be abolished by mechanical or chemical disruption of the cytoskeleton (CSK), they presumably arise from CSK constraint of the bilayer that prevents rapid transmission of tension to the channel. So far, studies measuring possible delays in pressure activation of TRPs that are suspected of being indirectly MS have not been performed. In the case of TRPV4, which has been functionally linked to PLA_2 (Vriens *et al.*, 2004), it will be interesting to determine whether its apparent lack of stretch sensitivity when measured in the patch was overlooked because of long delays and slow channel activation in response to applied pressure.

A further strategy for discriminating between direct and indirect MS channel mechanisms is to use specific inhibitors to test for involvement of MS enzymes (e.g., *p*-bromophenacyl bromide for PLA_2, 4-amino-5-(4-chlorophenyl)-7-(*t*-butyl)pyrazolo[3,4,d] pyrimidine (PP2) for Src tyrosine kinase, and U73122 for phospholipase C (PLC). In particular, the stretch sensitivity of the MS channel in arterial smooth muscle has been reported to be abolished by the PLC inhibitor U73122 (Park *et al.*, 2003). Furthermore, Ca^{2+} influx in dystrophic muscle that is mediated by a TRPC-dependent SOC and/or MOC (Vandebrouck *et al.*, 2002; Ducret *et al.*, 2006) can be abolished by inhibitors of PLA_2 (Lindahl *et al.*, 1995; Boittin *et al.*, 2006; Section VIII.A.3).

The most unequivocal method for distinguishing direct from indirect mechanosensitivity is to examine whether the detergent-solubilized channel protein retains stretch sensitivity when reconstituted in pure liposomes. So far, this test has been applied to several MS channels in prokaryotes and MscCa expressed in the frog oocyte (Sukharev *et al.*, 1993; Kloda and Martinac, 2001a,b; Sukharev, 2002; Maroto *et al.*, 2005). This approach also offers the potential of definitive evidence on whether lipidic second messengers [e.g., diacylglycerol (DAG), AA, lysophospholipids and 5′,6′-EET] activate the channel by binding directly to the channel protein and/or its surrounding lipid without intermediate steps. Furthermore, the same method may also be applied to determine whether multiprotein component MS-signaling complexes can be functionally reconstituted from their specific elements (e.g., TRPV4, PLA_2, and so on).

Although stretch sensitivity measured in the patch can be used to demonstrate a channel protein is MS at the biophysical level, it cannot prove the channel functions as a physiological mechanotransducer (Hamill, 2006). Indeed, many structurally diverse voltage- and receptor-gated channels [e.g., Shaker, L-type Ca^{2+} channels, *N*-methyl-D-aspartate receptor (NMDAR), S-type K^+ channels], as well as the simple model peptide channels alamethicin and gramicidin A, display stretch sensitivity in patch recordings (Opsahl and Webb, 1994; Paoletti and Ascher, 1994; Martinac and Hamill, 2002; Chapter 11, Morris and Juranka). In order to demonstrate functionality, one also needs to show that blocking the channel (pharmacologically and/or genetically) inhibits a mechanically induced cellular/physiological process.

V. EXTRINSIC REGULATION OF STRETCH SENSITIVITY

Stretch sensitivity is unlikely to be accounted for by a single structural domain analogous to the S-4 voltage sensor-domain shared by voltage-gated Na^+, K^+, and Ca^{2+} channels (Hille, 2001); even the relatively simple peptide channels, gramicidin and alamethicin, which have dramatically different structures and gating mechanisms, exhibit stretch sensitivity (Hamill and Martinac, 2001; Martinac and Hamill, 2002). Furthermore, stretch sensitivity is not a fixed channel property but rather can undergo significant changes with changing extrinsic conditions. For example, mechanical and/or chemical disruption of the CSK can either enhance or abolish the stretch sensitivity of specific channels (Guharay and Sachs, 1984; Hamill and McBride, 1992, 1997; Small and Morris, 1994; Patel and Honore, 2001; Hamill, 2006); changes in bilayer thickness (Martinac and Hamill, 2002), membrane voltage (Gu *et al*., 2001; Chapter 11, Morris and Juranka), or dystrophin expression (Franco-Obregon and Lansman, 2002; Chapter 16, Lansman) can switch specific MS channels from being stretch-activated to stretch-inactivated; specific lipids (Patel and Honore, 2001; Chemin *et al*., 2005), nucleotides (Barsanti *et al*., 2006a and references therein), and increased internal acidosis (Honoré *et al*., 2002; Barsanti *et al*., 2006b) can convert MS channels into constitutively open "leak" channels. The basis for these changes is often because changes in the bilayer, CSK, and/or ECM alter how mechanical forces are conveyed to the channel protein. The practical consequence may be that the specific conditions associated with reconstitution and/or heterologous expression may alter the stretch sensitivity of the reconstituted/expressed channel.

VI. STRATEGIES TO IDENTIFY MS CHANNEL PROTEINS

Once a channel has been functionally identified as stretch sensitive, there are several strategies that can be used to identify the membrane protein. The first strategy of "expression cloning" involves generating a cDNA library from cells expressing the channel, and then screening the library, typically in *Xenopus* oocytes or a mammalian cell line. This strategy has been used to clone several voltage- and receptor-gated channels, including the first vanilloid receptor TRP channel TRPV1 (Caterina *et al*., 1997). However, its application to MS channels has proven problematic because the expression vehicles express their own endogenous MS channels. The second strategy of "functional protein reconstitution" involves detergent solubilizing and reconstituting membrane proteins into liposomes and then screening for stretch sensitivity using patch clamp recording. This strategy has been used to successfully identify/clone a number of MS channel proteins from bacteria and archaea

(Sukharev *et al.*, 1993, 1994; Sukharev, 2002; Martinac, 2007). It was also used to implicate a TRPC in forming MscCa (Maroto *et al.*, 2005).

VII. GENERAL PROPERTIES OF TRPCs

This section provides an overview of the TRPC subfamily (for reviews see Minke and Cook, 2002; Vazquez *et al.*, 2004a; Montell, 2005; Nilius and Voets, 2005; Parekh and Putney, 2005; Owsianik *et al.*, 2006). The first TRP was discovered in a *Drosophila* mutant that showed a transient rather than a sustained receptor potential in response to light (Cosens and Manning, 1969; Minke *et al.*, 1975; Montell and Rubin, 1989). On the basis of these kinetics, the protein was designated TRP. Subsequently seven mammalian TRP homologues were discovered that together with TRP now make up the TRPC1–7. Other TRP subfamilies include TRPV (vanilloid), TRPA (ankyrin), TRPP (polycystin), TRPM (melastatin), TRPML (mucolipid), TRPN (NompC), and TRPY (yeast), and these together with TRPCs form the TRP superfamily. In addition to MS TRPCs, specific members of the other subfamilies have also been implicated in mechanotransduction so that the MS mechanisms discussed below may be general (Walker *et al.*, 2000; Palmer *et al.*, 2001; Zhou *et al.*, 2003; Nauli and Zhou, 2004; O'Neil and Heller, 2005; Voets *et al.*, 2005, Saimi *et al.*, 2007). The proposed transmembrane topology of TRPCs is reminiscent of voltage-gated channels—sharing six transmembrane-spanning helices (TM1–6), cytoplasmic N- and C-termini, and a pore region between TM5 and TM6—but lacking the positively charged residues in the TM4 domain that forms the voltage sensor. The seven mammalian TRPC channels also share an invariant sequence in the C-terminal tail called a TRP box (E-W-K-F-A-R), as well as 3–4 N-terminal ankyrin repeats. Although the ankyrin repeats may act as gating springs for MS channels (Howard and Bechstedt, 2004; Saimi *et al.*, 2007; see also Chapter 8, Castiglioni and García-Añoveros; Chapter 10, Cantiello *et al.*), their exact role and that of the TRP box remains to be verified (Vazquez *et al.*, 2004a; Owsianik *et al.*, 2006). The TRPCs share very little sequence identity in the region that is C-terminal of the TRP box, except for the common feature of CaM- and IP_3R-binding domains that have been implicated in Ca^{2+} feedback inhibition and activation by store depletion, respectively (Kiselyov *et al.*, 1998; Vaca and Sampieri, 2002). On the basis of sequence homology, the TRPCs have been divided into two major subgroups TRPC3/6/7 (70–80% homology) and TRPC1/4/5 (65% homology). TRPC2 is in a special class because multiple stop codons within its open reading frame make it a pseudogene in humans. However, it does form a functional channel in rodents (Section VIII.B).

A. TRPC Expression

TRPCs are widely expressed in mammalian tissues, with some human cells expressing all six and others expressing only one or two (Riccio *et al.*, 2002; Antoniotti *et al.*, 2006; Goel *et al.*, 2006; Hill *et al.*, 2006). The latter cells may prove useful for dissecting out specific TRPC functions, but it is necessary that selective expression be verified at both transcriptional and protein levels, since low turnover proteins may require little mRNA, and high mRNA levels need not translate into high membrane protein levels (Andersen and Seilhamer, 1997). Another caveat is that TRPC expression patterns may vary during development and with culture conditions (e.g., presence or absence of growth factors). For example, TRPC1 expression is upregulated by (1) serum deprivation where it contributes to increased proliferation of pulmonary arterial smooth muscle cells (Golovina *et al.*, 2001), (2) tumor necrosis factor α where it enhances endothelial cell death (Paria *et al.*, 2003), and (3) vascular injury *in vivo* which contributes to human neoitimal hyperplasia (Kumar *et al.*, 2006); TRPC6 expression in pulmonary arterial smooth muscle cells is enhanced by platelet-derived growth factor and by idiopathic pulmonary arterial hypertension (Yu *et al.*, 2003, 2004).

B. TRPC Activation and Function

Studies of TRPC activation and function are complicated by their polymodal activation and splice variants that display different activation mechanisms (Ramsey *et al.*, 2006). However, all TRPCs are regulated by PLC-coupled receptors (i.e., G-protein–coupled receptors or tyrosine kinase receptors). PLC hydrolyzes a component of the bilayer, PIP_2, into two distinct messengers—the soluble $InsP_3$ that activates the IP_3R in the ER to release Ca^{2+} from internal stores and the lipophilic DAG that may regulate TRPs indirectly via protein kinase C (PKC) or by interacting directly with the TRPCs in a membrane-delimited manner (Delmas *et al.*, 2002; Clapham, 2003; Ramsey *et al.*, 2006). Furthermore, Bolotina and colleagues have shown that a diffusible second messenger produced by depletion of Ca^{2+} stores activates a Ca^{2+} independent phospholipase ($iPLA_2$) that generates lysophospholipids, which are themselves capable of activating SOCs when exogenously applied to inside-out patches (Smani *et al.*, 2003; Bolotina and Csutora, 2005). Therefore, although all TRPCs could be classified as ROCs (but see Janssen and Kwan, 2007), they are more often subdivided into either SOCs, based on their sensitivity to Ca^{2+} store depletion that may or may not depend on PLC–IP_3R signaling, or ROCs

that are activated by DAG or its byproducts, but are insensitive to Ca^{2+} store depletion (Hofmann *et al*., 1999). To be classified as a SOC, the channel should be gated by a variety of procedures that only share the common feature of reducing Ca^{2+} stores (Parekh and Putney, 2005). Unfortunately, there have been conflicting reports for all seven TRPCs on whether they function as SOCs, ROCs, or both. Here, we focus on a further complication that the same mechanisms that make a channel a SOC or a ROC may also contribute to it being MS.

C. TRPC–TRPC Interactions

If all 7 TRPC subunits are expressed in a given cell and 4 subunits are required to form a channel (i.e., homotetrameric and heterotetrameric), then there could be as many as 100 different TRPC channels types. However, the number would be smaller if only certain TRPC–TRPC combination can occur. Two different models for TRPC interactions have been proposed: a homotypic model in which only subunits within each subfamily can interact to form channels with TRPC1/4/5 forming SOCs and TRPC3/6/7 forming ROCs (Hofmann *et al*., 2002; Sinkins *et al*., 2004), and a heterotypic model that also allows interactions between subfamily members, in this case with TRPC1, TRPC3, and TRPC7 proposed to form SOCs (i.e., without TRPC4 and TRPC6) and TRPC3, TRPC4, TRPC6, and TRPC7 proposed to form ROCs (without TRPC1; Zagranichnaya *et al*., 2005). In the heterotypic model, TRPC1's role is limited to SOCs and TRPC4's and TRPC6's roles are limited to ROCs, while TRPC3 and TRPC7 can participate as both SOCs and ROCs (Zagranichnaya *et al*., 2005). Interestingly, both models were generated from studies of the human embryonic kidney cell line, HEK-293, with the homotypic model based on gain-of-function (i.e., from TRPC overexpression) and the heterotypic model based on loss-of-function (i.e., from TRPC suppression). However, one complication with the former approach is that the level of TRPC expression can determine channel function. In particular, it has been shown that low TRPC3 expression result in SOCs, while high expression result in ROCs (Vazquez *et al*., 2003). This effect presumably occurs because high expression promotes homomeric TRPC3 channels, whereas low levels allow for heteromers that include endogenous subunits (Brereton *et al*., 2001; Vazquez *et al*., 2003). Differences in channel function may also arise depending on whether the cell is permanently or transiently transfected, presumably because stable transfection allows time adaptive changes in endogenous protein expression (Lievremont *et al*., 2004).

D. TRPC Interactions with Scaffolding Proteins

TRPCs also interact with a variety of regulatory and scaffolding proteins that may add further diversity and segregation of the channels (Ambudkar, 2006). In particular, it has been shown that several TRPCs assemble into multiprotein and lipid-signaling complexes that result in physical and functional interactions between the plasma membrane and CSK and ER resident proteins. These interactions may also allow for mechanical forces to be conveyed via a tethered mechanism to gate the channel (Howard *et al.*, 1988; Hamill and Martinac, 2001; Matthews *et al.*, 2007; Chapter 3, Bazopoulou and Tavernarakis; Chapter 10, Cantiello *et al.*). Alternatively, the interactions may also serve to constrain the development or transmission of bilayer tension to the TRPC and thereby "protect" it from being mechanically activated (Small and Morris, 1994; Hamill and McBride, 1997). For all TRPCs, the C-terminal coiled-coil domains and N-terminal ankyrin repeats have the potential to mediate protein–CSK interactions. All TRP family members also encode a conserved proline-rich sequence LP(P/X)PFN in their C-termini that is similar to the consensus-binding site for Homer, a scaffold protein that has been shown to facilitate TRPC1 interaction with IP_3R—disruption of which has been proposed to promote SOC activity (Yuan *et al.*, 2003). For example, TRPC1 mutants lacking Homer protein-binding sites show diminished interaction between TRPC1 and IP_3R and the TRPC1 channels are constitutively active. Similarly, coexpression of a dominant-negative form of Homer increases basal TRPC1 channel activity (Yuan *et al.*, 2003). Another protein I-mfa, which inhibits helix-loop-helix transcription factors, also binds to TRPC1 and blocks SOC function (Ma *et al.*, 2003). TRPC1 also expresses a dystrophin domain in its C-terminus (Wes *et al.*, 1995) that may allow interaction with dystrophin, and possibly explain why the absence of dystrophin in Duchenne muscular dystrophic muscle results in TRPC1 channels being abnormally gated open (Section VIII.A.3). TRPC1 also shows a putative caveolin-1-binding domain that may promote its functional recruitment into lipid rafts and increase SOC activity (Lockwich *et al.*, 2000; Brazier *et al.*, 2003; Ambudkar, 2006). TRPC1 has been shown to interact with stromal interaction molecule (STIM), the putative ER Ca^{2+} sensor that can apparently regulate TRPC1 SOC function (Huang *et al.*, 2006). Junctate is another IP_3R-associated protein, and it interacts with TRPC2, TRPC3, and TRPC5 (but not TRPC1) to regulate their SOC/ROC function (Treves *et al.*, 2004; Stamboulian *et al.*, 2005). In pulmonary endothelial cells, TRPC4 is localized to cell–cell adhesions in cholesterol-rich caveolae and has been shown to interact with the spectrin CSK via the protein 4.1 (Torihashi *et al.*, 2002; Cioffi *et al.*, 2005).

Furthermore, either deletion of the putative 4.1 protein-binding site on the TRPC4 C-terminus of TRPC4 or addition of peptides that competitively bind to that site are able to reduce SOC activity. Another site for TRPC4–CSK interaction involves the PSD-95/disk-large protein/zona occludens 1 (PDZ)-binding domain located at the TRPC4 distal C-terminus that binds to the Na^+/H^+ exchange regulatory factor (NHERF)-scaffolding protein (Tang *et al.*, 2000; Mery *et al.*, 2002). TRPC6 interacts with the stomatin-like protein podocin that may modulate its MOC function in the renal slit diaphragm (Reiser *et al.*, 2005). Interestingly, another stomatin homologue, MEC-2, links the putative MS channel to the microtubular CSK in *Caenorhabditis elegans* neurons (Chapter 3, Bazopoulou and Tavernarakis).

In summary, TRPCs undergo dynamic interactions with various scaffolding proteins that may act to inhibit or promote a particular mode of channel activation. Any one of these interactions may be important in modulating the mechanosensitivity of TRPC by focusing mechanical force on the channel or constraining the channel and/or bilayer from responding to mechanical stretch. It may be that the right combination of TRPC proteins and accessory proteins are needed to produce channels that are not constitutively active but are responsive to store depletion and/or mechanical stimulation.

E. Single TRPC Channel Conductance

Single-channel conductance provides a good identifying fingerprint of specific channels. However, compared with whole-cell current recording studies, there have been relatively few studies of the single-channel currents that are either enhanced or deleted by TRPC overexpression or suppression, respectively. Furthermore, there is no simple way to determine if a channel reflects a homomeric rather than a heteromeric TRPC. Another practical issue for comparisons has been the lack of standardized recording conditions. Nevertheless, a survey of the TRPC single-channel literature indicates the following order for conductance values TRPC3 (65 pS) > TRPC5 (50 pS) > TRPC4 (32 pS) ~ TRPC6 (31 pS) > TRPC1 (~20 pS) for estimates made from cell-attached recordings with 100- to 150-mM Na^+/Cs^+, 1- to 4-mM Ca^{2+}/Mg^{2+} at −40 to −100 mV (Hurst *et al.*, 1998; Kiselyov *et al.*, 1998; Hofmann *et al.*, 1999; Yamada *et al.*, 2000; Liu *et al.*, 2003; Strübing *et al.*, 2003; Bugaj *et al.*, 2005; Maroto *et al.*, 2005; Inoue *et al.*, 2006). The only available estimates for TRPC2 (42 pS) and TRPC7 (60 pS) were made with no divalents (Perraud *et al.*, 2001; Zufall *et al.*, 2005). These numbers may serve as a baseline for the future conductance measurements of the purified/reconstituted TRPCs.

F. TRPC Pharmacology

The pharmacological tools to study TRPCs are limited with the following agents reported to block, stimulate, or have no effect on different TRPCs (Ramsey *et al.*, 2006): SKF-96365 blocks TRPC3 and TRPC6 currents (at ~5 μM), and is considered a ROC more than a SOC blocker; 2APB (2-aminoethoxydiphenyl borate) blocks TRPC1 (80 μM), TRPC5 (20 μM), and TRPC6 (10 μM) but not TRPC3 (75 μM), and is considered more a SOC than a ROC blocker; Gd^{3+} and La^{3+} block TRPC1 and TRPC6, but potentiate TRPC4 and TRPC5 (in micromolars; Jung *et al.*, 2003); flufenamate blocks TRPC3, TRPC5, and TRPC7 (100 μM), but potentiates TRPC6; and tarantula venom peptide, GsmTX4, (Gottlieb *et al.*, 2004) blocks TRPC1 in mammalian cells but not in *Xenopus* oocytes (Hamill, 2006; Chapter 4, Gottlieb *et al.*). Other agents of interest that need to be systematically tested on both SOC and ROC activity include gentamicin, ruthenium red, GsmTX4, and amiloride (Lane *et al.*, 1991, 1992; Rüsch *et al.*, 1994; Flemming *et al.*, 2003; Suchyna *et al.*, 1998, 2004; Jacques-Fricke *et al.*, 2006).

VIII. EVIDENCE FOR TRPC MECHANOSENSITIVITY

Below, we consider the MS role of specific TRPCs. At this time, the main evidence exists for TRPC1 (SOC), TRPC6 (a DAG-activated ROC), and to a lesser extent TRPC4 (an AA-activated ROC). However, as discussed in Section IX, a basic issue is whether the mechanisms that confer SOC and ROC activity on TRPC channels also contributes to there mechanonsensitivity. In this case, all TRPs may end up expressing some degree of mechanosensitivity.

A. TRPC1

TRPC1 was the first identified vertebrate TRP homologue (Wes *et al.*, 1995; Zhu *et al.*, 1995) and initial heterologous expression of human TRPC1 (hTRPC1) in Chinese hamster ovary (CHO) and sf9 cells enhanced SOC currents (Zitt *et al.*, 1996). However, a subsequent study indicated hTRPC1 expression in sf9 cells induced a constitutively active nonselective cation channel that was not sensitive to store depletion (Sinkins *et al.*, 1998). This early discrepancy raises the possibility that store sensitivity (and perhaps stretch sensitivity) may depend on a variety of conditions (e.g., expression levels, presence of endogenous TRPCs, and state of phosphorylation). For example,

TRPC1 has multiple serine/threonine phosphorylation sites in the putative pore-forming region and the N- and C-termini, and at least one report indicates that PKC_{α}-dependent phosphorylation of TRPC1 can enhance Ca^{2+} entry induced by store depletion (Ahmmed *et al.*, 2004). Despite this early discrepancy, many studies now point to TRPC1 forming a SOC (Liu *et al.*, 2000, 2003; Xu and Beech, 2001; Kunichika *et al.*, 2004; for reviews see Beech *et al.*, 2003; Beech, 2005) and in cases where TRPC1 expression has not resulted in enhanced SOC (Sinkins *et al.*, 1998; Lintschinger *et al.*, 2000; Strübing *et al.*, 2001), it has been argued that TRPC1 was not trafficked to the membrane (Hofmann *et al.*, 2002). This does not seem to be the case when hTRPC1 is expressed in the oocyte (Brereton *et al.*, 2000; see Figs. 2 and 3). In any case, any direct TRPC1 involvement in forming the highly Ca^{2+}-selective SOC or Ca^{2+} release-activated current (I_{CRAC}) seems to be reduced by the finding that a novel protein family (i.e., CRAM1 or Orai1) forms I_{CRAC} channels (Peinelt *et al.*, 2006; but see Mori *et al.*, 2002; Huang *et al.*, 2006).

1. Maitotoxin Activates TRPC1 and MscCa

In 1999, xTRPC1 was cloned from *Xenopus* oocytes and shown to be ~90% identical in sequence to the hTRPC1 (Bobanovic *et al.*, 1999). An anti-TRPC1 antibody (T1E3) targeted to an extracellular loop of the predicted protein was generated and shown to recognize an 80-kDa protein. Immunofluorescent staining indicated an irregular "punctuate" expression pattern of xTRPC1 that was uniformly evident over the animal and vegetal hemispheres. Patch clamp studies also indicate that MscCa is uniformly expressed over both hemispheres (Zhang and Hamill, 2000a). This is in contrast to the polarized expression of the ER and the phosphatidylinositol second messenger system, which are more abundantly expressed in the animal hemisphere (Callamaras *et al.*, 1998; Jaconi *et al.*, 1999). These results indicate that neither TRPC1 nor MscCa are tightly coupled to ER internal Ca^{2+} stores and IP_3 signaling. Originally, it was speculated that punctuate TRPC1 expression reflected discrete channel clusters but it could also indicate the channels are localized in microvilli, which make up >50% of the membrane surface (Zhang *et al.*, 2000). In another study, testing the idea that xTRPC1 formed a SOC, Brereton *et al.* (2000) found that antisense oligonuceotides targeting different regions of xTRPC1 sequence did not inhibit IP_3- or thapsigargin-stimulated Ca^{2+} inflow (cf., Tomita *et al.*, 1998). Furthermore, overexpreesion of hTRPC1 did not enhance the basal or IP_3-stimulated Ca^{2+} inflow (Brereton *et al.*, 2000). However, they did see enhancement of a lysophosphatidic acid (LPA)-stimulated Ca^{2+} influx. Interestingly, LPA also enhances mechanically induced Ca^{2+} influx in a variety of other cells (Ohata *et al.*, 2001). On the basis of the apparent lack of TRPC1-related SOC activity, Brereton *et al.* (2000) speculated that TRPC1 might form the endogenous cation channel activated

by the marine toxin, maitotoxin (MTX). To test this idea, they compared the properties of MTX-activated conductance in normal and in TRPC1-transfected rat liver cells (Brereton *et al.*, 2001), and found that the endogenous MTX-activated conductance displayed properties different from the enhanced MTX-activated conductance expressed in the hTRPC1-transfected cells. In particular, the endogenous conductance showed a higher selectivity for Na^+ over Ca^{2+} and a higher sensitivity to Gd^{3+} block ($K_{50\%\ block} = 1\ \mu M$ vs 3 μM). These differences were taken to indicate that other endogenous TRPC subunits may normally combine with TRPC1 to form the endogenous MTX-activated conductance, whereas hTRPC1 alone forms the enhanced MTX-activated conductance (Brereton *et al.*, 2001). Unlike with oocytes, it was found that heterologous expression of hTRPC1 in rat liver cells did increase thapsigarin-induced Ca^{2+} inflow.

Evidence from several studies indicates that the oocyte MTX-activated conductance may be mediated by MscCa (Bielfeld-Ackermann *et al.*, 1998; Weber *et al.*, 2000; Diakov *et al.*, 2001). In particular, both display the same cation selectivity, are blocked by 1-mM amiloride and 10-μM Gd^{3+}, are insensitive to flufenamic and niflumic acid, and have a conductance of ~25 pS (measured in symmetrical 140-mM K^+ and 2-mM external Ca^{2+}). Because MTX is a highly amphipathic molecule (Escobar *et al.*, 1998), it may activate MscCa by changing bilayer-membrane interactions, as has been proposed for other amphipaths that can activate MS channels in the absence of membrane stretch (Martinac *et al.*, 1990; Kim, 1992; Hamill and McBride, 1996; Casado and Ascher, 1998; Perozo *et al.*, 2002).

2. TRPC1 and Cell Swelling

To directly test whether TRPC1 might be MS, Chen and Barritt (2003) selectively suppressed TRPC1 expression in rat liver cells and measured their response to osmotic cell swelling. Liver cells express MscCa (Bear, 1990) and previous studies had shown that osmotic swelling of epithelial cells activates an MscCa-dependent Ca^{2+} influx that stimulates Ca^{2+}-activated K^+ efflux accompanied by Cl^-/H_2O efflux and RVD (Christensen, 1987). However, in the TRPC1-suppressed liver cells, hypotonic stress caused a greater swelling and faster RVD than observed in control liver cells (Chen and Barritt, 2003). This opposite response may occur because TRPC1 suppression results in a compensatory overexpression of other TRPCs (or redundant RVD mechanisms) that enhance cell swelling and RVD. It should also be recognized that cell swelling does not always activate MscCa. For example, although hypotonic solution activates a robust Ca^{2+}-independent Cl^- conductance in *Xenopus* oocytes that should contribute to RVD, it fails to activate the endogenous MscCa (Ackerman *et al.*, 1994; Zhang and Hamill, 2000a,b).

3. Abnormal TRPC1/MscCa Activity in Duchenne Muscular Dystrophy

Both TRPC1 and MscCa are expressed in skeletal muscle and both have been implicated in the muscular degeneration that occurs in Duchenne muscular dystrophy (DMD). In particular, muscle fibers from the *mdx* mouse (i.e., an animal model of DMD) show an increased vulnerability to stretch-induced membrane wounding (Yeung and Allen, 2004; Allen *et al*., 2005) that has been linked to elevated $[Ca^{2+}]_i$ levels caused by increased Ca^{2+} leak channel activity (Fong *et al*., 1990) and/or abnormal MscCa activity (Franco and Lansman, 1990; Chapter 16, Lansman). On the basis of the observation that the channel activity was increased by thapsigargin-induced store depletion, it was proposed that the channel may also be a SOC belonging to the TRPC family (Vandebrouck *et al*., 2002, see also Hopf *et al*., 1996). To test this idea, *mdx* and normal muscle were transfected with antisense oligonucleotides designed against the most conserved TRPC regions. The transfected-muscles showed a significant reduction in expression of TRPC1 and TRPC4 but not TRPC6 (all three TRPCs are expressed in normal and *mdx* muscle) and a decrease in the Ca^{2+} leak channel activity. Previous studies indicate that MscCa behaves more like a Ca^{2+} leak channel in *mdx* patches (Franco-Obregon and Lansman, 2002) and in some oocyte patches (Reifarth *et al*., 1999). It has also been reported that SOC and MscCa in *mdx* muscle display the same single-channel conductance and sensitivity to block by Gd^{3+}, SKF96365, 2APB, and GsMTx4 (Ducret *et al*., 2006). These studies implicate TRPC1 as being a subunit of both the SOC and MscCa, which given the presence of a dystrophin domain on the C-terminus of TRPC1 (Wes *et al*., 1995) could explain the shift in gating mode in *mdx* muscle.

4. TRPC1 and Polycystic Kidney Disease

TRPC1 interacts with the putative MS channel TRPP2 when both are heterologously expressed in HEK-293 (Tsiokas *et al*., 1999), and there is evidence that TRPC1 and TRPP2 may form functional heteromers (Delmas, 2004). TRPP2 is a distant member of the TRP family (polycystin subfamily) and has been shown to form a Ca^{2+}-permeable cation channel that is mutated in the autosomal dominant polycystic kidney disease (ADPKD; Nauli *et al*., 2003; Nauli and Zhou, 2004; Giamarchi *et al*., 2006; Chapter 10, Cantiello *et al*.). TRPP2 was originally designated as polycystin kidney disease 2 (PKD2) and shown to combine with PKD1, a membrane protein with a large extracellular N-terminal domain proposed to act as an extracellular sensing antenna for mechanical stimuli. Both TRPP2 and PKD1 are localized in the primary cilium of renal epithelial cells, which is essential for detecting laminar fluid flow (Praetorius and Spring, 2005). However, TRPV4, which is expressed in renal epithelial cells, may also associate with TRPP2

(Giamarchi *et al.*, 2006). It remains to be determined if TRPC1 combines with TRPP2 in renal epithelial cells and whether knock out of TRPC1 and/or TRPV4 blocks fluid flow detection.

5. TRPC1 Is Expressed in Specialized Mechanosensory Nerve Endings

If TRPC1 is a mechanosensory channel, it should be expressed in specialized mechanosensory nerve endings. Glazebrook *et al.* (2005) used immunocytochemical techniques to examine the distribution of TRPC1 and TRPC3–7 in the soma, axons, and sensory terminals of arterial mechanoreceptors, and found that TRPC1, TRPC3, TRPC4, and TRPC5 were expressed in the peripheral axons and the mechanosensory terminals. However, only TRPC1 and TRPC3 extended into the low-threshold mechanosensory complex endings, with TRPC4 and TRPC5 mainly limited to the major branches of the nerve. Although these results are consistent with TRPC1 (and possibly TRPC3) involvement in baroreception, it was concluded that because TRPC1 was not present in all fine terminals that it more likely modulated than directly mediated mechanotransduction. However, it is not clear that all fine endings are capable of transduction. Furthermore, other putative MS proteins (i.e., β and γ ENaC subunits) are expressed in baroreceptor nerve terminals (Drummond *et al.*, 1998) in which case different classes of MS channels (i.e., ENaC and TRPC) may mediate mechanotransduction in different mechanosensory nerves.

6. TRPC1 Involvement in Wound Closure and Cell Migration

The first study to implicate TRPC1 in cell migration was by Moore *et al.* (1998). They proposed that shape changes induced in endothelial cells by activation of TRPC1 were necessary step for angiogenesis. In another study, it was demonstrated that TRPC1 overexpression promoted, while TRPC1 supression inhibited intestinal cell migration measured by wound closure assay (Rao *et al.* (2006). On the basis of the proposal that MscCa regulates fish keratocyte cell migration (Lee *et al.*, 1999) and the identification of TRPC1 as a MscCa subunit (Maroto *et al.*, 2005), the role of TRPC1 was tested on migration of the highly invasive/metastatic prostate tumor cell line PC-3. TRPC1 activity was shown to be essential for PC-3 cell migration and Gd^{3+}, GsMTx4, anti-TRPC1 antibody, and siRNA-targeting TRPC1 were shown to block PC-3 migration by inhibiting Ca^{2+} dynamics required of cell migration (Maroto *et al.*, 2007, submitted for publication).

7. Reconstitution of TRPC1 as an MS Channel

To identify the protein forming the oocyte MscCa, oocyte membrane proteins were detergent solubilized, fractionated by FPLC, reconstituted

in liposomes, and assayed for MscCa activity using patch recording (Maroto *et al*., 2005). A specific protein fraction that ran with a conductivity of 16 mS cm^{-1} was shown to reconstitute the highest MscCa activity and silver-stained gels indicated it displayed the highest abundance of 80-kDa protein. On the basis of previous studies that identified xTRPC1 and hTRPC1 as forming an 80-kDa protein when expressed oocytes (Bobanovic *et al*., 1999; Brereton *et al*., 2000), immunological methods were used to demonstrate that TRPC1 was present in the MscCa active fraction. Furthermore, heterologous expression of the hTRPC1 was shown to greatly increase the MscCa activity expressed in the transfected oocyte, whereas TRPC1-antisense reduced the endogenous MscCa activity (Maroto *et al*., 2005). Figure 2 compares MscCa activity in cell-attached patches on a control oocyte (Fig. 2A) and an oocyte that had been injected with hTRPC1 (Fig. 2B). Despite the almost tenfold increase in current density in the TRPC1-injected oocyte, channel activation and deactivation kinetics in the two patches were similar. However, in some patches, even on the same oocyte, the kinetics of the TRPC1-dependent channels show delayed activation and deactivation kinetics. An example of the slow kinetics is illustrated for a patch that was formed on an oocyte that had been injected with TRPC1 with enhanced green fluorescence protein (eGFP) attached to the C-terminus. Figure 3 shows confocal fluorescence images of the oocyte at low magnification and at high magnification indicating eGFP-TRPC1 concentrated in the surface membrane (Fig. 3).

Figure 4 compares the patch response on a control oocyte and the slow kinetics response of a patch formed on the oocyte displayed in Fig. 3. The basis for the heterogeneity in kinetics of TRPC1 channels may reflect local differences in the underlying CSK and/or bilayer or even the MscCa subunit composition that occurs with TRPC1 overexpression. Maroto *et al*. (2005) also demonstrated that hTRPC1 expression in CHO cells results in increased MscCa activity, consistent with an approximately fivefold greater increase in channel density. The presence of endogenous MscCa activity is consistent with previous reports that indicate CHO cells express TRPC1 along with TRPC2–6 (Vaca and Sampieir, 2002). Although the above results provide compelling evidence that TRPC1 is a structural component on the MscCa, the current increase in TRPC1-transfected oocytes and CHO cells is relatively low compared with that achieved by overexpression of other channel types. This may be because endogenous TRPC1 needs to combine with endogenous TRPCs or other ancillary proteins. On the other hand, the ability to reconstitute MscCa activity following 5000-fold protein to lipid dilution would seem to argue against the requirement of at least ancillary proteins that are not firmly attached to the channel complex.

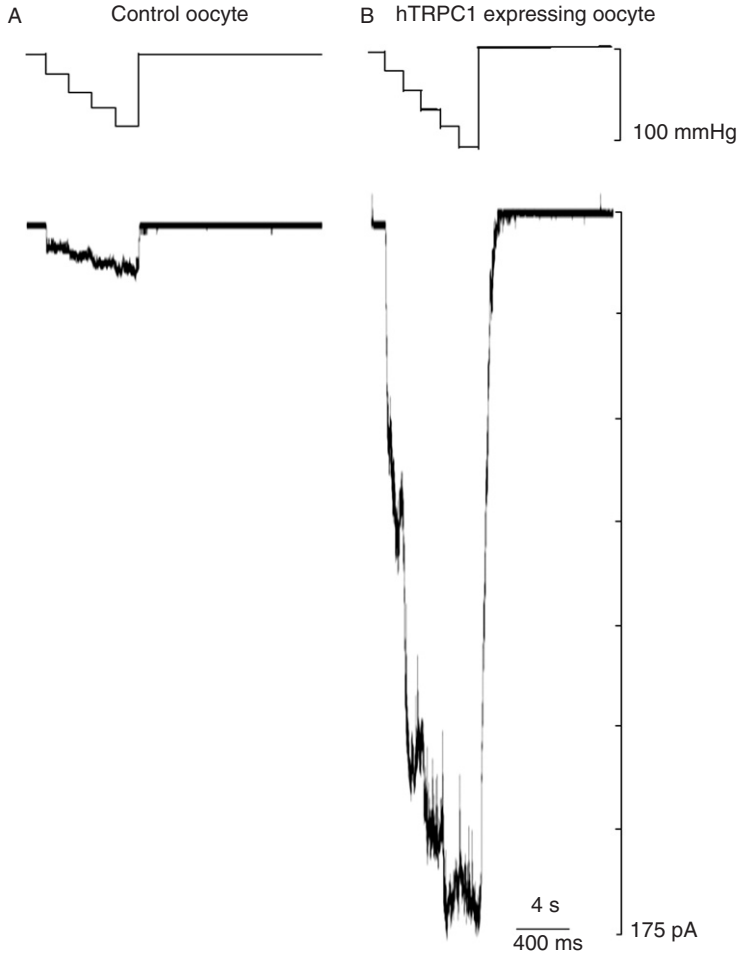

FIGURE 2 MS current activity measured in a control and an hTRPC1-expressing oocyte. (A) Stepwise increase in suction (top trace) applied to a cell-attached patch formed on a control oocyte (i.e., that was injected with 50 nl of water 4 days earlier) induced a current of 12 pA. (B) Similar to A except that the patch was formed on an hTRPC1-expressing oocyte (i.e., injected 4 days earlier with 50 nl of TRPC1 transcripts). In this case, the peak current produced was 175 pA. Examination of the residual channels immediately after the steps indicates the same single-channel currents of ~2 pA. Both recordings were made at a patch potential of −50 mV. (From Maroto *et al.*, 2005).

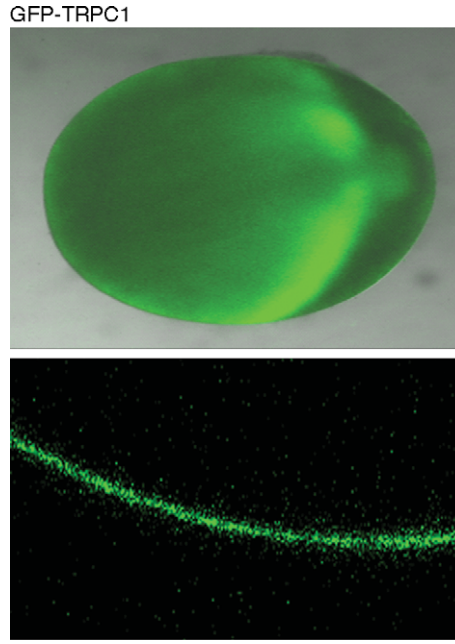

FIGURE 3 Fluorescence images of an oocyte that had been injected 3 days earlier with mRNA construct encoding enhanced green fluorescence protein attached to the C-terminus of *Trpc1*. Upper panel shows whole oocyte. Lower panel shows confocal images focussed on the oocyte edge.

B. TRPC2

At present there is no evidence, direct or indirect, to indicate TRPC2 forms an MS channel. The current view is that it may function as a ROC or a SOC depending on cell type (Vannier *et al.*, 1999; Gailly and Colson-Van Schoor, 2001; Chu *et al.*, 2004; Zufall *et al.*, 2005). For example, TRPC2 has been implicated in pheromone detection in the rodent vomeronasal organ (VNO; Liman *et al.*, 1999) because TRPC2$^{-/-}$ mice lack gender discrimination (Zufall *et al.*, 2005). Because a DAG-activated channel in VNO neurons is downregulated in TRPC2$^{-/-}$ mice and TRPC2 is localized in the sensory microvilli that lack Ca^{2+} stores, it seems that TRPC2 functions as a ROC rather than a SOC at least in VNO neurons (Spehr *et al.*, 2002; Zufall *et al.*, 2005). However, in erythroblasts, and possibly sperm, TRPC2 has been reported to be activated by store depletion. In both cell types, the long splice

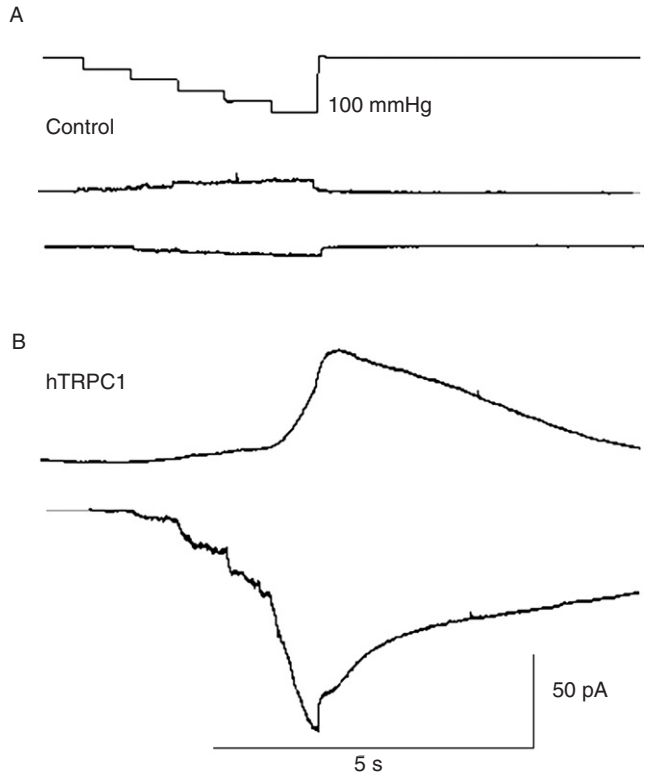

FIGURE 4 Oocyte patches formed on TRPC1-expressing oocytes can show slow turn-on and delayed turn-off in addition to the greatly enhanced currents. (A) Cell-attached patch on a control oocyte showing step responses similar to Fig. 2A. (B) Cell-attached patch on the oocyte shown in Fig. 3 that had been injected with hTRPC1 transcripts. In this case, the pressure-stimulated currents were not only much larger than the wild-type responses but also failed to saturate and exhibited a pronounced delay in both its turn-on and turn-off with the pressure steps. Fast responses similar to Fig. 2B were also seen on this oocyte.

variants of TRPC2 were detected (Yildrin *et al.*, 2003), whereas VNO neurons express the short splice variant (Hofmann *et al.*, 2000; Chu *et al.*, 2002). In sperm, TRPC2 may participate in the acrosome reaction-based inhibition by a TRPC2 antibody *in vitro* (Jungnickel *et al.*, 2001). However, TRPC2$^{-/-}$ mice display normal fertility therefore casting doubt on this role (Stamboulian *et al.*, 2005). In hematopoiesis, erythropoietin modulates Ca^{2+} influx via TRPC2 in possible combination with TRPC6 (Chu *et al.*, 2002, 2004).

C. TRPC3

As with TRPC2, there is no evidence yet for TRPC3 mechanosensitivity. However, TRPC3 does colocalize with TRPC1 in specialized mechanosensory nerve endings, indicating that both may combine to form an MS channel (Glazebrook *et al*., 2005). The growing consensus is that TRPC3 can contribute to both SOC and ROC channels depending on expression levels (Zitt *et al*., 1997; Hofmann *et al*., 1999; Hurst *et al*., 1998; Kamouchi *et al*., 1999; Trebak *et al*., 2002; Putney *et al*., 2004; Vazquez *et al*., 2005; Groschner and Rosker, 2005; Liu *et al*., 2005; Zagranichnaya *et al*., 2005; Kawasaki *et al*., 2006). Suppression of TRPC3 in cerebral arterial smooth muscle while suppressing pyridine receptor-induced depolarization does not alter the pressure-increased depolarization and contraction, which appears to be dependent on TRPC6 (Reading *et al*., 2005). On the other hand, TRPC3 activation appears to depend upon Src kinase that may be MS (Vazquez *et al*., 2004b) and like TRPC6 is directly activated by OAG (Hofmann *et al*., 1999).

D. TRPC4

There is disagreement on whether TRPC4 functions as a SOC and/or ROC (Philipp *et al*., 1996; Tomita *et al*., 1998; McKay *et al*., 2000; Schaefer *et al*., 2000; Plant and Schaefer, 2005). TRPC4 has been suggested to form a ROC activated by AA (Wu *et al*., 2002; Zagranichnaya *et al*., 2005). In particular, using siRNA and antisense strategies to reduce endogenous TRPC4 expression, TRPC4 was shown to be required for the OAG-induced and receptor-operated Ca^{2+} entry as well as the AA-induced Ca^{2+} oscillations but not for SOC function. This AA activation may have implications for the mechanosensitivity of TRPC4 since AA has been shown to activate a variety of MS channels in the absence of applied stretch where it appears to act by directly altering mechanical properties of the bilayer surrounding the channel (Kim, 1992; Hamill and McBride, 1996; Casado and Ascher, 1998; Patel *et al*., 2001). Studies of $TRPC4^{-/-}$ mice indicate TRPC4 is an essential determinant of endothelial vascular tone and endothelial permeability as well as neurotransmitter release from central neurons (reviewed by Freichel *et al*., 2004).

E. TRPC6

The general consensus is that TRPC6 forms a ROC that is activated by DAG in a membrane-delimited fashion and is insensitive to activation by IP_3 and store depletion (Boulay *et al*., 1997; Hofmann *et al*., 1999; Estacion

et al., 2004; Zagranichnaya *et al.*, 2005; Zhang *et al.*, 2006). Although TRPC6 is a member of the TRPC3/6/7 subfamily, it shows distinct functional and structural properties. Functionally, while TRPC6 only forms a ROC, TRPC3 and TRPC7 appear capable of participating in forming both ROCs and SOCs (Zagranichnaya *et al.*, 2005); structurally, whereas TRPC6 carries two extracellular glycosylation sites, TRPC3 carries only one (Dietrich *et al.*, 2003). Furthermore, exogenously expressed TRPC6 shows low basal activity compared with TRPC3 and elimination of the extra glycosylation site that is missing in TRPC3 transforms TRPC6 into a constitutively active TRPC-3-like channel. Conversely, engineering of an additional glycosylation site in TRPC3 markedly reduces TRPC3 basal activity.

1. TRPC6 as a Regulator of Myogenic Tone

TRPC6 is proposed to mediate the depolarization and constriction of small arteries and arterioles in response to adrenergic stimulation (Inoue *et al.*, 2001; Jung *et al.*, 2002; Inoue *et al.*, 2006), and elevation of intravascular pressure consistent with TRPC6 forming a MOC as well as a ROC (Welsh *et al.*, 2000, 2002). The cationic current activated by pressure in vascular smooth muscle is suppressed by antisense-DNA to TRPC6 (Welsh *et al.*, 2000). Furthermore, because the cation entry was stimulated by OAG and inhibited by PLC inhibitor (Park *et al.*, 2003), it was proposed that TRPC6 forms an MS channel that is activated indirectly by pressure according to the pathway:

$$\uparrow \text{intravasular pressure} \rightarrow \uparrow \text{PLC} \rightarrow \uparrow [\text{DAG}] \rightarrow \uparrow \text{TRPC} \rightarrow \uparrow [Ca^{2+}] \rightarrow \uparrow \text{myogenic tone}$$

In this scheme, it is PLC rather than TRPC that is MS. This would imply that since all TRPCs are coupled to PLC-dependent receptors, they may all display mechanosensitivity. However, while there are reports that PLC can be mechanically stimulated independent of external Ca^{2+} (Mitchell *et al.*, 1997; Rosales *et al.*, 1997; Moore *et al.*, 2002), there are also studies that indicate the mechanosensitivity of PLC derives from stimulation by Ca^{2+} influx via MscCa (Matsumoto *et al.*, 1995; Ryan *et al.*, 2000; Ruwhof *et al.*, 2001). In this case, it becomes important to demonstrate that TRPC6 can be mechanically activated in the absence of external Ca^{2+} (e.g., using Ba^{2+}). There is other evidence to indicate TRPC6 may be coupled to other MS enzymes. For example, TRPC6 is similar to TRPV4 in that it is activated by 20-hydroxyeicosatetraenoic acid (20-HETE), which is the dominant AA metabolite produced by cytochrome P-450 w-hydroxylase enzymes (Basora *et al.*, 2003). TRPC6 may also be activated by Src family protein tyrosine kinase (PTK)-mediated tyrosoine phosphorylation (Hisatsune *et al.*, 2004). Indeed, PP2, a specific inhibitor of Src

PTKs, abolishes TRPC6 (and TRPC3) activation and strongly inhibits OAG-induced Ca^{2+} entry (Soboloff *et al.*, 2005). OAG may operate solely through TRPC6 homomers, the cation of vasopressin (VP) may also include the OAG-insensitive TRPC heteromers (e.g., TRPC1 and TRPC6). A further complication is that DAG-dependent activation of PKC appears to stimulate the myogenic channels based on their block by the PKC inhibitor chelerythrine (Slish *et al.*, 2002), whereas PKC activation seems to inhibit TRPC6 channels, which would seem more consistent with direct activation by DAG/OAG (Soboloff *et al.*, 2005).

Despite the above evidence implicating TRPC6 as the "myogenic" channel, TRPC6-deficient mice show enhanced rather than reduced myotonic tone and increased rather than reduced responsiveness to constrictor agonist in small arteries. These effects result in both a higher elevated mean arterial blood pressure and a shift in the onset of the myogenic tone toward lower intravascular pressures, again opposite to what would be expected if TRPC6 was critical for myoconstriction (Dietrich *et al.*, 2005). Furthermore, isolated smooth muscle from $TRPC6^{-/-}$ mice show increased basal cation entry and more depolarized resting potentials, but both effects are blocked if the muscles are also transfected with siRNA-targeting TRPC3. On the basis of this last observation, it was suggested that constitutively active TRPC3 channels are upregulated in $TRPC6^{-/-}$ mice. However, the TRPC3 subunits are unable to functionally replace the lost TRPC6 function that involves suppression of high basal TRPC3 activity (i.e., the TRPC3/TRPC6 heteromer is a more tightly regulated ROC and/or MOC). In summary, although evidence indicates TRPC6 may be a pressure or stretch-sensitive channel and contribute to MOC, the TRPC6 knockout mouse indicates a phenotype that cannot be explained if TRPC6 alone forms the vasoconstrictor channel. It may also be relevant that another study could find no evidence that Gd^{3+}-sensitive MscCa contributes to myogenic tone in isolated arterioles from rat skeletal muscle (Bakker *et al.*, 1999).

2. TRPC6 as a Regulator of the Kidney Slit Diaphragm

Autosomal dominant focal segmental glomerulosclerosis (FSGS) is a kidney disease that leads to progressive renal kidney failure characterized by leakage of plasma proteins like albumin into the urine (proteinuria). Mutations in TRPC6 were associated with familial FSGS and implicated in aberrant Ca^{2+} signaling that leads to podocyte injury (Reiser *et al.*, 2005; Winn *et al.*, 2005). Furthermore, two of the mutants were demonstrated to be gain-of-function mutations that produce larger ROCs than the wild-type TRPC6 expressed in HEK-293 cells. Ultrafiltration of plasma by the renal glomeruli is mediated mainly by the podocyte, which is an epithelial cell that

lies external to the glomerular basement membrane (GBM) and lines the outer endothelium of the capillary tuft located inside the Bowman's capsule. The podocyte covers the GBM and forms interdigitating foot processes that are connected by slit diaphragms—ultra-thin membrane structures that form at the center of the slit a zipper-like structure with pores smaller than albumin (Kriz, 2005; Tryggvason and Wartiovarara, 2005). The podocyte-specific proteins, nephrin and podocin, are localized in the slit diaphragm and the extracellular domains of nephrin molecules of neighboring foot processes interact to form the zipper structure. Podocin, a member of the stomatin family, is a scaffolding protein that accumulates in lipid rafts and interacts with the cytoplasmic domain of nephrin (Durvasula and Shankland, 2006). Both nephrin and podocin have been shown to be mutated in different familial forms of FSGS. Furthermore, TRPC6 interacts with both nephrin and podocin and a nephrin deficiency in mice leads to overexpression and mislocalization of TRPC6 in podocyte as well as disruption of the slit diaphragm (Reiser *et al.*, 2005). Mechanical forces play an important role in ultrafiltration both in terms of the high transmural distending forces arising from the capillary perfusion pressure as well as the intrinsic forces generated by the contractile actin network in the foot process that control, in a Ca^{2+}-dependent manner, the width of the filtration slits. As a consequence, TRPC6 may act as the central signaling component mediating pressure-induced constriction at the slit. In summary, two quite diverse physiological functions, myogenic tone and renal ultrafiltration, implicate TRPC6 as an MS channel. However, whether TRPC6 acts as a direct mechanosensor as in the case of TRPC1 or is indirectly MS like TRPV4 remains to be determined.

IX. CONCLUSIONS

At least three basic mechanisms referred to as "bilayer," "conformational coupling," and "enzymatic" may confer mechanosensitivity on TRPCs. The bilayer mechanism should operate if the TRPC, in shifting between closed and open states, undergoes a change in its membrane-occupied area, thickness, and/or cross-sectional shape. Any one of these changes would confer mechanosensitivity on the channel. A bilayer mechanism may also underlie the ability of lipidic second messengers (e.g., DAG/OAG, AA, lysophospholipid and 5′,6′-EET) to directly activate TRPCs by inserting in the bilayer to alter its local bilayer packing, curvature, and/or the lateral pressure profile. The only unequivocal way to demonstrate that a bilayer mechanism operates is to show that stretch sensitivity is retained when the purified channel protein is reconstituted in liposomes. At this stage, one can go onto measure

channel activity as a function of changing bilayer thickness (i.e., by using phospholipids with different acyl length chains) and local curvature/pressure profile (i.e., by using lysophospholipids with different shapes; Perozo *et al.*, 2002; Martinac, 2007; Markin and Sachs, 2007; Powl and Lee, 2007).

The second mechanism involves conformational coupling (CC) that has been evoked to account for TRPC sensitivity to depletion of internal Ca^{2+} stores. CC was originally used to explain excitation–contraction (E–C) coupling, involving the physical coupling between L-type Ca^{2+} channel (i.e., dihydropyridine receptors, DHPR) in the plasma membrane and ryanodine receptors (RyR1) that release Ca^{2+} from the sarcoplasmic reticulum (SR; Protasi, 2002). Subsequently, a retrograde form of CC was discovered between the same two proteins that regulate the organization of the DHPR into tetrads and the magnitude of the Ca^{2+} current carried by DHPR (Wang *et al.*, 2001; Paolini *et al.*, 2004; Yin *et al.*, 2005). Another form of CC was demonstrated associated with physiological stimuli that do not deplete Ca^{2+} stores yet activate Ca^{2+} entry through channels referred to as excitation-coupled Ca^{2+} entry channels to distinguish them from SOC (Cherednichenko *et al.*, 2004). Interestingly, RyR1 is functionally coupled to both TRPC1-dependent SOC and TRPC3-dependent SR Ca^{2+} release (Sampieri *et al.*, 2005; Lee *et al.*, 2006).

A key issue for all forms of CC is whether the direct physical link that conveys mechanical conformational energy from one protein to another can also act as a pathway to either focus applied mechanical forces on the channel or alternatively constrain the channel from responding to mechanical forces generated within the bilayer. Another possibility is that reorganization or clustering of the resident ER protein (i.e., STIM) that senses Ca^{2+} stores may alter channel mechanosensitivity by increasing the strength of CC coupling (Kwan *et al.*, 2003).

Some insights into these possibilities can be provided by the process of "membrane blebbing," which involves decoupling of the plasma membrane from the underlying CSK and has been shown to either increase or decrease the mechanosensitivity of MS channels depending on the channel (Hamill and McBride, 1997; Hamill, 2006). Since membrane blebbing would also be expected to disrupt any dynamic interactions between TRPC and scaffolding proteins, it should alter TRPC function. In one case it has been reported that Ca^{2+} store depletion carried out after but not before formation of a tight seal is effective in blocking the activation of SOC channels in the frog oocyte patches (Yao *et al.*, 1999). Presumably, this occurs because the sealing process physically decouples the channels from ER proteins that sense internal Ca^{2+} stores. Tight seal formation using strong suction can also reduce MscCa mechanosensitivity and gating kinetics possibly by a related mechanism (Hamill and McBride, 1992). On the other hand, it has been reported that

I_{CRAC} is retained following cell "ballooning" (i.e., a form of reversible membrane blebbing), indicating that the coupling between the channel and the Ca^{2+} sensor STIM may be relatively resistant to decoupling (Bakowski *et al.*, 2001). In any case, in order to directly demonstrate a role for CC in mechanosensitivity, one needs to show that stretch sensitivity can be altered in mutants in which TRPC–ancillary protein interactions are disrupted (Section VII.D).

The third mechanism of mechanosensitivity relates to functional coupling between TRPCs and MS enzymes. Apart from the PLA_2 and Src that are MS and have been implicated in conferring mechanosensitivity on TRPV4 (Vriens *et al.*, 2004; Cohen, 2005a,b), there is growing evidence that PLC is also MS with reports indicating that mechanosensitivity is either dependent on external Ca^{2+} and Ca^{2+} influx (Matsumoto *et al.*, 1995; Ryan *et al.*, 2000; Ruwhof *et al.*, 2001; Alexander *et al.*, 2004) or Ca^{2+} independent (Mitchell *et al.*, 1997; Rosales *et al.*, 1997; Moore *et al.*, 2002). In either case, these studies indicate that mechanical forces transduced by MscCa and/or by MS enzymes may modulate the gating of all TRP channels. It remains to be determined what are the physiological and/or pathological effects of this MS modulation? The methods discussed in this chapter, including the applications of pressure steps to measure the kinetics of MS enzyme–channel coupling and the use of membrane protein liposome reconstitution for identifying specific protein–lipid interactions, should play an increasing role in understanding the importance of the different MS mechanisms underlying TRPC functions.

Note Added in Proof

Spassova, M. A., Hewavitharana, T., Xu, W., Soboloff, J., and Gill, D. L. (*Proc. Natl. Acad. Sci. USA* **103,** 16586–16591) have reported that overexpression of hTRPC6 in mammalian cells results in increased OAG- and swelling-activated whole cell currents and increased stretch-activated channel activity in inside-out patches. The TRPC6 activity was blocked by GsmTX4 but was insensitive to block by the PLC inhibitor U73122 (c.f., Park *et al.*, 2003). Furthermore, they found that the long delays associated with stretch activation of TRPC6 channels could be reduced by treatment of cells with cytochalasin D. These results are consistent with TRPC6 being directly MS and a common bilayer mechanism underlying OAG- and stretch-activation of TRPC6.

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