A mutation in TRPC6 channels abolishes their activation by hypoosmotic stretch but does not affect activation by diacylglycerol or G protein signaling cascades

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Wilson C, Dryer SE. A mutation in TRPC6 channels abolishes their activation by hypoosmotic stretch but does not affect activation by diacylglycerol or G protein signaling cascades. Am J Physiol Renal Physiol 306: F1018–F1025, 2014. First published March 5, 2014; doi:10.1152/ajprenal.00662.2013.—Canonical transient receptor potential-6 (TRPC6) channels have been implicated in the pathogenesis of kidney disease and in the regulation of vascular smooth muscle tone, podocyte function, and a variety of processes in other cell types. The question of whether their gating is intrinsically mechanosensitive has been controversial. In this study we have examined activation of two alleles of TRPC6 transiently expressed in CHO-K1 cells: the wild-type human TRPC6 channel, and TRPC6-N143S, an allele originally identified in a family with autosomal dominant familial focal and segmental glomerulosclerosis (FSGS). We observed that both channel variants carried robust cationic currents that could be evoked by application of membrane-permeable analogs of diacylglycerol (DAG) or by the P2Y receptor agonist ATP. The amplitudes and characteristics of currents evoked by the DAG analog or ATP were indistinguishable in cells expressing the two TRPC6 alleles. By contrast, hypoosmotic stretch evoked robust currents in wild-type TRPC6 channels but had no discernible effect on currents in cells expressing TRPC6-N143S, indicating that the mutant form lacks mechanosensitivity. Coexpression of TRPC6-N143S with wild-type TRPC6 or TRPC3 channels did not alter stretch-evoked responses compared with when TRPC3 channels were expressed by themselves, indicating that TRPC6-N143S does not function as a dominant-negative. These data indicate that mechanical activation and activation evoked by DAG or ATP occur through fundamentally distinct biophysical mechanisms, and they provide support for the hypothesis that protein complexes containing wild-type TRPC6 subunits can be intrinsically mechanosensitive.

canonical transient receptor potential-6 (TRPC6) channels; glomerular filtration; TRP channel; focal and segmental glomerulosclerosis
vations suggest that the observable modes of TRPC6 activation are cell-type dependent and may depend on whether or not stomatin-prohibitin family proteins (1) or other TRPC family subunits (17, 29) are also present in the same complex.

In the present study we provide additional evidence that the gating of TRPC6 channels is mechanosensitive. Specifically, we show that the N143S mutation of TRPC6, originally identified in a family with a genetic form of focal and segmental glomerulosclerosis (FSGS) (30), has responses to 1-oleoyl-2-acetyl-sn-glycerol (OAG) and activation of cell surface GPCRs indistinguishable from wild-type channels (30, 31). However, this particular mutation causes TRPC6 channels to be completely unresponsive to hypoosmotic stretch. This indicates that TRPC6 activation by mechanical stimuli occurs through biophysical mechanisms that are distinct from those used by chemical stimuli (OAG and G protein-mediated responses). These data also suggest that TRPC6 channels can respond directly to a mechanical stimulus.

**METHODS**

**Cell culture and transfection.** CHO-K1 cells obtained from American Type Culture Collection (Manassas, VA) were used in this study because of earlier reports indicating that they do not express endogenous mechanosensitive currents prior to transfection (23). Several other cell lines that we tested, including several samples of HEK293 and HEK293T cells, could not be used because they exhibit endogenous stretch-activated currents large enough to preclude analysis of heterologously expressed channels. Constructs encoding NH2-terminal FLAG-tagged wild-type and mutant human TRPC6 channels were generously provided by Dr. Johannes Schlöndorff of Beth Israel Deaconess Medical Center and Harvard Medical School (Boston, MA). The preparation and properties of these constructs were described previously (31). A construct encoding wild-type TRPC3 was provided by Dr. John Wood of University College, London. Cells were transiently transfected using the Trans IT transfection reagent (Mirus Bio, Madison, WI) according to the manufacturer’s directions. Cells were cotransfected with an expression construct encoding GFP, which allowed us to select transfected cells for electrophysiological analysis on the basis of fluorescence. The efficiency of transfection was further confirmed by immunoblot analysis using rabbit anti-TRPC6 (Alomone Laboratories, Jerusalem, Israel) and by confocal microscopy using an antibody directed against the FLAG tags (Cell Signaling). Methods for confocal analysis have been described previously (18).

**Electrophysiology.** Whole cell recordings from CHO-K1 cells were made under the same recording conditions and using methods that we have used earlier for studies of podocytes (1, 2, 18, 19). Briefly, control bath salines (340 mOsm/100%) contained 150 mM NaCl, 5.4 mM CsCl, 0.8 mM MgCl2, 5.4 mM CaCl2, and 10 mM HEPES, pH 7.4. A 70% bath solution (238 mOsm) was made by dilution of

![Fig. 1. Characteristics of cationic currents in CHO-K1 cells. Examples of conventional whole cell recordings made from a nontransfected cell (A) and a cell expressing wild-type TRPC6 channels (TRPC6-WT) by transient transfection (B). Currents during ramp voltage commands (−80 mV to +80 mV in 2.5 s) are shown under various conditions. Application of hypoosmotic saline (70%) did not result in an increase in cationic currents in the nontransfected cell. After restoring the control saline (100%) and then applying 100 μM 1-oleoyl-2-acetyl-sn-glycerol (OAG), no significant change in current was seen. After washing for 5 min, a subsequent exposure to 200 μM ATP did not increase currents. By contrast, all of these treatments evoked reversible increases in cationic current in the CHO-K1 cell expressing TRPC6-WT. Currents returned to baseline levels within 5 min after washing off each treatment with 100% saline (not shown).](http://ajprenal.physiology.org/)

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this solution. Recording pipette solutions in all experiments contained 10 mM NaCl, 125 mM CsCl, 6.2 mM MgCl₂, 10 mM HEPES, and 10 mM EGTA, pH 7.2. In some experiments, the pipette solution also contained 50 μM guanosine 5'-O-(2-thiodiphosphate) (GDP-βS), a GDP analog resistant to hydrolysis and phosphorylation that competitively inhibits G protein activation by GTP. The bath was perfused at a constant flow rate (0.3 ml/min), and outwardly rectifying currents were periodically evoked by ramp voltage commands (−80 to +80 mV over 2.5 s) from a holding potential of −40 mV. In most experiments, this was done before and after application of 70% bath saline, 200 μM ATP, or 100 μM OAG delivered by bath superfusion. OAG was obtained from Avanti Polar Lipids (Alabaster, AL). Currents were digitized and analyzed with pClamp software (v10.1, Molecular Devices, Sunnyvale, CA). Whole cell currents were quantified at +80 mV. All traces shown were filtered at 200 Hz using the digital Bessel filter implemented in pClamp. Quantitative data were analyzed by one-way ANOVA followed by Tukey’s post hoc test.

RESULTS

These experiments were carried out on amino-terminal FLAG-tagged TRPC6 channels transiently expressed in CHO-K1 cells along with cotransfected GFP. Results obtained using these constructs have been described by other investigators (30, 31). The present experiments were carried out in CHO-K1 cells transiently coexpressing human TRPC6 channels together with GFP as assessed by imaging with fluorescence microscopy from live cells during the course of the experiment. Currents were monitored during application of ramp voltage commands (−80 mV to +80 mV over 2.5 s). The mechanical stimulus used in these experiments was a membrane stretch evoked by bath application of an external solution that is 70% hypoosmotic to the control external saline, as described previously (1). Currents were also evoked by bath application of 100 μM OAG or 200 μM ATP. The latter compound acts through P2Y purinergic receptors endogenously expressed in CHO-K1 cells (35). In nontransfected cells, the hypoosmotic stimulus evoked very small cationic currents that never exceeded 50 pA when measured at +80 mV (Fig. 1A). The lack of endogenous stretch-activated channels in this cell line is consistent with previous reports (23). Application of OAG or ATP also failed to evoke significant changes in macroscopic currents in nontransfected CHO-K1 cells (Fig. 1A). However, when CHO-K1 cells were transfected with an amino-terminal FLAG-tagged wild-type human TRPC6 construct, hypoosmotic stretch (using the 70% external solution) evoked robust outwardly rectifying cationic currents of several hundred picoamperes in amplitude, which readily reversed upon restoration of normal osmotic saline (Fig. 1B). A subsequent application of either 100 μM OAG or 200 μM ATP in normal osmotic saline in the same cell also evoked reversible activation of the TRPC6 channels (Fig. 1B). In cells expressing wild-type TRPC6 channels we observed that adding 50 μM GDP-βS to the recording pipette completely inhibited responses to 200 μM ATP, but a subsequent hypoosmotic stretch was still able to activate the current (Fig. 2). This result suggests that mechanical activation of TRPC6 does not require G protein signaling. Macroscopic currents through the wild-type TRPC6 channels were completely blocked by pretreatment with 10 μM SKF-96365, an inhibitor of TRP superfamily channels (Fig. 3A) and also by 50 μM La³⁺, another agent that inhibits TRPC6 channels (1) (Fig. 3B).

A distinctly different pattern was observed in CHO-K1 cells expressing amino-terminal FLAG-tagged TRPC6-N143S (Fig. 4). In cells expressing that allele, membrane stretch with 70% hypoosmotic solution had no effect on cationic currents in any of 10 cells tested in experiments based on four different transfections (Fig. 4, A and B). Restoring the control saline in these cells, followed by application of 100 μM OAG (Fig. 4A) or 200 μM ATP (Fig. 4B) resulted in robust increases in cationic currents indistinguishable from those observed in wild-type cells in all of these cells. The results of these experiments are summarized in Fig. 5, in which we show expression of these alleles in CHO-K1 cells as assessed by immunoblot and confocal microscopy (Fig. 5A) and mean
current evoked by the three different stimuli in CHO-K1 cells (Fig. 5, B–D). Not all TRPC6 mutations associated with FSGS show this pattern. Thus the TRPC6-R895C allele, which was also isolated from a family with FSGS (30), exhibits responses to hypoosmotic stretch that are indistinguishable from wild-type (data not shown; unpublished observations).

We also asked whether TRPC6-N143S could inhibit stretch-activation of other TRPC6 or TRPC3 channels coexpressed in CHO-K1 cells—in other words, whether this mutation could function as a dominant-negative for mechanical activation. We observed robust hypoosmotic stretch-activated currents in CHO-K1 cells coexpressing TRPC6-N143S together with wild-type forms of TRPC3 (Fig. 6). Thus the TRPC6-N143S isoform does not function in a dominant-negative manner to block stretch-activation of coexpressed channels of the same TRPC subfamily.

DISCUSSION

The question of whether members of the TRPC family of cationic channels are intrinsically mechanosensitive is relevant to the mechanisms of myogenic responses in vascular smooth muscle (37), cardiac contractility (8), and sensory transduction (29). It has also been quite controversial. TRPC6 channels are expressed in podocyte foot processes, where responses to mechanical stimuli have been proposed to play an important role in physiological (1, 15) and pathophysiological processes (1, 21, 22). Indeed, the TRPC6 mutation characterized in this study was originally discovered in a family with a genetic form of FSGS, a form of podocyte pathology characterized by marked alterations in podocyte shape and function (30).

Based on earlier studies in vascular smooth muscle and certain heterologous expression systems, it has been argued that mechanical activation of TRPC6 channels is an indirect effect (16, 24, 34). These groups have observed that mechanical stimuli can cause activation of $G_\alpha$-coupled GPCRs, including the AT1R receptor for ANG II, and H1 histamine receptors (24). This in turn leads to activation of phospholipases that generate chemical intermediates such as DAG or 20-HETE, which in turn evoke an essentially chemical form of TRPC6 activation (16). These workers observed that hypoosmotic stretch by itself could cause activation of certain types of GPCRs, and that this effect was suppressed by inverse agonists of those receptors. Stretch activation of TRPC6 was also suppressed by the inverse agonists, as well as by inhibitors of PLA2 and PLC (16, 24). According to the model proposed by these investigators, TRPC6 channels are not directly mechanosensitive. Instead, they become active in response to normal G protein-mediated cascades that are stretch-sensitive. Mechanical stimuli would therefore result in formation of lipid mediators such as DAG that evoke TRPC6 activation. In other words, according to this model, the biophysical mechanism causing TRPC6 activation is fundamentally the same regardless of whether the cell is stimulated mechanically or with a GPCR agonist, and is ultimately mediated by canonical lipid signaling pathways. In support of this model, these workers report that mechanical activation of TRPC6 through this indirect mechanism is resistant to GsMTx4 (16).

On the other hand, other laboratories working on other cell types have observed that mechanical stimulation results in a GsMTx4-sensitive activation of channels containing TRPC6 subunits (1, 8, 29, 33). Two of these studies reported that the mechanosensitivity of TRPC6 channels is strongly influenced by certain TRPC6-interacting proteins, including TRPC3 subunits (29) or stomatin-like hairpin loop proteins such as podo-
cin (1). Both of these interacting proteins are expressed in podocyte cell lines (1, 17). Thus, while mechanical activation of TRPC6 channels can be indirect (as in vascular smooth muscle), it does not have to be (as in podocytes and cochlear hair cells). What is actually observed may therefore depend on the type of cell being studied.

It is this background that underlies the significance of the present observations. Thus, if mechanical effects on TRPC6 were necessarily indirect and mediated by GPCR/phospholipase transduction pathways, then mutations in TRPC6 channels that affect their gating would be expected to alter both types of responses in exactly the same way. On the other hand, if mutations differentially affect responses to mechanical and chemical stimuli, it would indicate that these comprise biophysically distinct modes of channel activation. The data here unequivocally indicate that a mechanical stimulus evokes a distinct mode of TRPC6 activation. First, mechanical activation persists when G protein signaling is blocked, conditions that abolished responses evoked by activation of GPCRs. Second, mechanical activation cannot be detected in TRPC6-N143S, even though this allele shows normal responses to a DAG analog and activation of endogenous G protein-coupled receptors responsive to ATP. It is possible that the mode of activation that dominates in a particular TRPC6 channel will depend on its local membrane microenvironment, for example the cholesterol content of the bilayer domains immediately surrounding the channels, and also the stoichiometry of the TRPC channel complex (1, 15, 29). For this reason, TRPC6 channels could exhibit different primary modes of activation in different cell types, or even in different compartments of the same cell (1).

The TRPC6-N143S mutation was originally isolated from a family with FSGS, and the mutation has an autosomal dominant inheritance pattern with respect to the disease phenotype (30). In earlier studies of this mutation, no gain of function was observed when channel activation was evoked by muscarinic receptor/PLC transduction cascades in a heterologous expression system (30, 31). Consequently, the mechanisms whereby TRPC6-N143S causes FSGS have been unknown; indeed, the group that initially described the TRPC6-N143S mutation has raised the possibility that this mutation is not the cause of FSGS, despite genetic evidence they uncovered that suggests it (31). The present study has extended these observations to show that responses of the TRPC6-N143S channels to purinergic P2Y receptor stimulation are also similar to wild type, even though a component of PLA2 signaling is likely to underlie these responses (35). The available data therefore suggest that a Ca$^{2+}$/H11001 overload mechanism is unlikely to account for the glomerular pathology associated with this particular allele—in marked contrast to the majority of known TRPC6 mutations associated with FSGS (30, 31, 38). It is tempting to consider that the loss of mechanosensitivity of TRPC6 channels that we observe with this allele contributes to the kidney disease (although this is admittedly difficult to reconcile with the apparently dominant mode of disease transmission with this allele). Perhaps the loss of this function could make podocytes more susceptible to detachment in response to other stimuli in the face of mechanical stress, such as hyperfiltration. Such a

Fig. 4. TRPC6-N143S channels do not respond to hypoosmotic stretch. After restoring normal 100% saline, CHO-K1 cells expressing this allele exhibited robust responses to 100 μM OAG (A) or 200 μM ATP (B).
stress could arise from some independent pathological process—a “second hit”—that would not normally be sufficient to cause clinically measurable disease (21). In this regard, constitutive TRPC6 knockout mice do not exhibit proteinuria at rest, and even exhibit at least partial protection against proteinuria evoked by sustained application of agents such as ANG II (9). However, on certain genetic backgrounds, there is compensatory upregulation of TRPC3, which results in a paradoxical hypertension (38), although hypertension is not seen universally (9). These complications have made it somewhat difficult to interpret results from existing TRPC6 knockout mice, as they may retain some of the functions that would normally be carried out by TRPC6 channels in wild-type mice.

TRPC3 channels exhibit very similar gating properties to TRPC6 both with respect to activation by OAG and also in response to mechanical stimuli (14, 29). Given that, it is significant that we did not observe a dominant-negative effect of the TRPC6-N143S mutation on mechanical activation of TRPC3. We should also note that the loss of mechanosensitivity does not occur with all of the TRPC6 mutations associated with FSGS. For example, mechanical responses were indistinguishable from wild-type in the TRPC6-R889C mutation.

**Fig. 5.** Summary of results with TRPC6-WT and TRPC6-N143S channels expressed in CHO-K1 cells. A: confocal micrograph showing expression of the two alleles in cells (red) along with GFP. There does not appear to be any discernible difference in the distribution or amounts of heterologously expressed proteins detected using an antibody against the NH2-terminal FLAG tags on these channels. Scale bar denotes 18 μm. B: mean ± SE of cationic currents measured at +80 mV evoked by hypoosmotic stretch, 100 μM OAG, or 200 μM ATP in nontransfected CHO-K1 cells. None of the treatments evoked a discernible change in currents (N = 10 cells per group). C: all three treatments evoked currents significantly (P < 0.05) greater than baseline in CHO-K1 cells expressing TRPC6-WT. We consistently observed larger effects with OAG and ATP than with membrane stretch in this expression system (N = 10 cells per group). D: in CHO-K1 cells expressing TRPC6-N143S, the currents measured in 70% hypoosmotic saline were not different from baseline measurements in control 100% saline. However, treatments with OAG and ATP again evoked robust responses (N = 10 cells per group). Responses to OAG and ATP are similar in TRPC6-WT and TRPC6-N143S, consistent with previous reports on these alleles based on G protein signaling cascades in other expression systems (2, 29).

**Fig. 6.** Coexpression with TRPC6-N143S does not eliminate stretch-evoked activation of TRPC3. A: typical response to hypoosmotic stretch in a CHO-K1 cell transiently expressing TRPC3. B: typical response to stretch in a cell expressing TRPC3 (as in A) plus an equal amount of expression vector encoding TRPC6-N143S. C: summary of results from multiple cells (N = 3 cells per group). Ordinate is the mean change in current evoked in a single cell by switching from 100% to 70% external saline. These data make it clear that TRPC6-N143S does not exert a dominant-negative effect on stretch-evoked activation of TRPC3.
tion (data not shown). Therefore, it is possible that different TRPC6 mutations can cause glomerular disease by different mechanisms.

In summary, we have shown that the N143S mutation in TRPC6 channels completely eliminates their activation by hypoosmotic stretch in CHO-K1 cells, although their activation by analogs of DAG or by activation of endogenous G protein-coupled receptors is indistinguishable from wild type. Therefore, mechanical activation of TRPC6 channels proceeds by biophysical mechanisms that are distinct from those used in G protein signaling cascades, and these observations strongly suggest that wild-type TRPC6 channels are intrinsically mechanosensitive.

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AUTHOR CONTRIBUTIONS

Author contributions: C.W. performed experiments; C.W. analyzed data; C.W. and S.E.D. prepared figures; C.W. and S.E.D. approved final version of manuscript; S.E.D. conception and design of research; S.E.D. interpreted results of experiments; S.E.D. drafted manuscript; S.E.D. edited and revised manuscript.

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