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Calcium Channels in Primary Cilia

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Abstract

Purpose of review—Primary cilia have become important organelles implicated in embryonic development, organogenesis, health, and diseases. While many studies in cell biology have focused on changes in ciliary length or ciliogenesis, the most common readout for evaluating ciliary function is intracellular calcium.

Recent findings—Recent tools have allowed us to examine intracellular calcium in more precise locations, i.e. the cilioplasm and cytoplasm. Advances in calcium imaging have also allowed us to identify which cilia respond to particular stimuli. Furthermore, direct electrophysiological measurement of ionic currents within a cilium has provided a wealth of information for understanding the sensory roles of primary cilia.

Summary—Calcium imaging and direct measurement of calcium currents demonstrate that primary cilia are sensory organelles that house several types of functional calcium channels. Although intracellular calcium now allows a functional read-out for primary cilia, discussions on the relative contributions of the several channel types have just begun. Perhaps all of these calcium channels are required and necessary to differentiate stimuli in different microenvironments.

Keywords

cilium; imaging; patch-clamping

Introduction

Cilia are sensory organelles that project from the apical membrane in many cell types. Based on their axonemal structure, cilia can be divided into “9+0” or “9+2” types. However, a general classification of cilia is more complex due to the discoveries of a “9+4” axonemal structure in Hensen's node of rabbit (1) and a “3+0” structure in a protozoan (2). Primary cilia are usually considered to be non-motile, solitary structures with a “9+0” axoneme, although some are in fact motile. They were first described in the 1800s (3). In 1997, Schwartz et al. showed that a non-motile primary cilium could easily be deflected by fluid

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Conflicts of interest

None

movement surrounding the cell (4). This is probably the first suggestion of a sensory role for primary cilia; it offered a physiological function for primary cilia in sensing fluid flow. Since then, non-motile cilia have been proposed to have broad and complex sensory roles (**Table 1**).

Primary cilia and intracellular read-out

The most commonly used read-out of ciliary function is the concentration of intracellular calcium. In the 2000s, the laboratories of Spring and Zhou independently demonstrated that primary cilia play a crucial role in sensing mechanical fluid-shear stress (30, 31). Both laboratories showed that bending primary cilia with a micropipette or with fluid flow activates the cilium and increases cytoplasmic calcium in renal epithelial cells. Since then, other laboratories have utilized intracellular calcium as a read-out to understand the functions of primary cilia in vascular endothelial cells (32, 33), renal epithelial cells (34-36), eye trabecular meshwork cells (37), nodal cells (19, 21), hepatocytes (17, 22), osteocytes (38), and chondrocytes (39).

A recent advance is the use of genetically encoded calcium-sensitive proteins targeted to the cilium. This technique allows differentiation of intracellular calcium signaling between cilioplasm and cytoplasm. Independent studies from the Nauli, Jacobs, Inoue, and Sun laboratories have detected calcium changes within primary cilia in response to fluid flow (40-43). As discussed below, Clapham's group argues that these changes do not result from calcium crossing the ciliary membrane (44).

In vascular endothelial cells, a nitric oxide read-out has also been used to understand primary ciliary function (45). Protein modification, cleavage, and proteomic studies have also been used for this purpose (45, 46). Cell morphology may be another potential readout of ciliary function (47).

Direct measurement of ionic currents in the primary cilium

The first direct electrophysiological evidence for the presence of single channel currents in a cilium was provided by Cantiello's group (48). In 2005, they demonstrated a transmembrane cation-conducting channel of 156 pS in artificial bilayers that included cilia from renal epithelial cells. This channel was inhibited by an antibody against polycystin-2 (TRPP2), suggesting that TRPP2 contributed to the channels seen. When normalized to total protein, ciliary membrane was found to have 400 times more channel current, compared to plasma membrane.

In a subsequent study, Cantiello's group learned that vasopressin further increases cationic channel activity in cilia (48). Due in part to electrophysiological evidence, it was proposed that primary cilia have a cAMP-dependent second messenger signaling mechanism whose function includes the modulation of ciliary TRPP2 channel activity (49). TRPP2 activity is thought to modulate calcium level in the cilioplasm. This regulatory pathway may also provide a molecular mechanism for microtubule regulation by calcium channel function, which in turn may help modulate ciliary length and function (50).

Direct electrophysiological measurement in a single cilium is challenging due in part to the tiny size of the cilium (diameter ~200 nm). In addition, the cilium in a typical cell monolayer is oriented perpendicular to (above) the cell surface, which makes the cilium difficult to resolve. Kleene's laboratory circumvented this problem by growing adherent cells on small, spherical beads that could be easily moved within the recording chamber. With this technique, an entire cilium can be pulled into a recording microelectrode from the side of the bead, allowing sensitive, repeatable electrical recordings from the cilium (51). This method has allowed identification of a ciliary transient receptor potential cation channel, subfamily M, member 4 (TRPM4), which can be activated by high cilioplasmic calcium (52). This channel does not conduct calcium, and its physiological significance is not yet understood. However, the concentration of cilioplasmic calcium is expected to influence its activity. In 2013, Clapham's laboratory also developed a means of directly recording from the ciliary membrane and discovered a ciliary PKD1L1/TRPP3 channel, which is described below (53).

Ciliary calcium channels

The primary cilium incorporates several types of calcium-conducting channel in its membrane. These channels may facilitate the cilium's role as a polymodal sensory antenna.

1. Transient receptor potential cation channel, subfamily C, member 1 (TRPC1)

It is generally thought that TRPC1 mediates calcium entry into the cell in response to depletion of endoplasmic calcium stores or activation of receptors coupled to the phospholipase C system (54). Cantiello's laboratory demonstrated by immunocytochemistry that TRPC1 is present in the primary cilia of renal epithelia (48). Interestingly, it has also been reported that TRPP2 can form a channel in combination with TRPC1 (55). Heteromultimers of TRPP2 and TRPC1 were found to have a single-channel conductance, amiloride sensitivity, and ion permeability that are distinct from those of TRPP2 or TRPC1 alone. This channel assembly was found in the renal epithelial cilia and could be activated by a G-protein-coupled receptor. It was further proposed that a TRPP2/TRPC1 complex plays a role in the mechanosensory function to initiate calcium signaling at the base of the cilium (55). This view is consistent with the idea that TRPC1 itself is a stretch-activated calcium channel (56).

2. Transient receptor potential cation channel, subfamily P, member 2 (TRPP2)

Several laboratories have independently shown that TRPP2 (also known as polycystin-2, PC2, or PKD2) contributes to responses to fluid flow in cholangiocytes (17), embryonic node (19, 21, 43), left-right organizer of zebrafish (43), osteocytes (57), renal epithelia (29, 30, 58, 59), smooth muscle cells (60), and vascular endothelia (24).

Fluid-shear force causes bending of primary cilia and activates a polycystin-1/TRPP2 complex (30). TRPP2 conducts calcium and is linked to polycystin-1, which exhibits a remarkable mechanical strength, supporting a role in mechanotransduction (61). The calcium signal through this mechanical fluid activation is then amplified by calcium release from stores modulated by the ryanodine receptor. The mechanosensitivity of the polycystin-1/TRPP2 complex has been confirmed directly; the ratio of polycystin-1/TRPP2

plays an important role in the cell mechanics of mechanosensation (62). This model supports the idea that normal expression levels of polycystin-1 and TRPP2 are important to suppress the cystic kidney phenotype (63-65).

Among calcium channels, TRPP2 probably has the earliest functional role during embryogenesis in establishing the left-right asymmetry of the visceral organs (19-21). TRPP2 regulates embryonic nodal gene expression at the left side of the embryonic endodermal node. TRPP2 functions in mechanosensation by increasing internal calcium in response to the leftward nodal flow. Norris' laboratory further showed that polycystin-1L1 (PKD1L1) is the interacting flow sensor and is required to regulate the TRPP2 channel within the embryonic endodermal node (20).

3. Transient receptor potential cation channel, subfamily P, member 3 (TRPP3)

TRPP3, also called polycystin-L (PKDL) or polycystin-2L1 (PKD2L1), is localized to primary cilia (53, 66) and the centrosome (67). TRPP3 is a calcium channel with a high homology with TRPP2 (68). TRPP3 is regulated by calcium, exhibiting both calcium-dependent activation and calcium-dependent inactivation (69). This indicates that TRPP3 may act as a transducer of calcium-mediated signaling.

While directly recording from the primary cilia of several cell types, Clapham's laboratory found a large, outwardly rectifying, current (53). Because knocking down either PKD1L1 or TRPP3 abolished this current, it is likely due to a PKD1L1/TRPP3 complex. The current is cation-nonselective, with similar permeabilities for calcium and barium ions (53), and is inactivated by high internal ciliary calcium (IC_{50} = 445 nM) (70). Coexpression of PKD1L1 and TRPP3 yielded calcium-permeable channels of 103 pS. It is further proposed that the PKD1L1/TRPP3 heteromeric channel establishes the cilium as a unique calcium compartment within cells that modulates Hedgehog signaling pathways (53).

A very recent study by Zhou's group indicates that TRPP3 in the neuronal primary cilium regulates neuronal excitability and susceptibility to pentylenetetrazol-induced seizure in mice (66). The group showed that TRPP3 interacts with the β2 adrenergic receptor and that the receptor-channel complex regulates cAMP response element-binding protein. This functional interaction plays a crucial role in chronic seizure disorder and epilepsy.

4. Transient receptor potential cation channel, subfamily V, member 4 (TRPV4)

The primary cilium forms a calcium microdomain that is influenced by calcium entry through TRPV4 (40). It was shown that TRPV4 mediates flow-induced ciliary calcium increases. Thus, it is proposed that TRPV4 has a role in mechanotransduction in the primary cilium. Although TRPV4 is involved in the flow-induced calcium transient, interestingly the association of TRPC1 with TRPV4 prolongs the flow-induced calcium influx (71). It has also been shown that TRPV4 interacts with TRPP2 to form a channel complex. The TRPP2/TRPV4 complex has distinct biophysical, pharmacological, and regulatory profiles compared to either TRPP2 or TRPV4 channels (72). Furthermore, TRPV4 interacts with OCRL, an inositol polyphosphate 5-phosphatase. This interaction is required for primary cilia to sense changes in pressure and subsequently regulates calcium influx in response to pressure stimulation (37).

Aside from mechanosensing, the TRPP2/TRPV4 complex forms a thermosensitive molecular sensor in primary cilia (29). Consistent with this view, it has been shown that prolonged cold preservation of an organ could cause irreversible pathological changes in the primary cilia (73). LaRusso's group has also shown that the ciliary TRPV4 can sense changes in osmolality (22). It is proposed that localization of TRPV4 in primary cilia is required to sense tonicity in the microenvironment.

5. Other calcium channels in the primary cilia

Nauli's laboratory has recently shown that the L-type calcium channel modulates cystic kidney phenotype, hydrocephalus, and left-right asymmetry defects (74, 75). L-type calcium channel knockdown in zebrafish facilitates the formation of these ciliopathic phenotypes. The L-type calcium channel is present in renal epithelial cilia, as judged by immunocytochemistry (41). Although it is unclear if this channel has any role in the mechanosensation mediated by primary cilia, it does play an important role in an agonist-induced calcium response measured in the cilia (41). This further reiterates the importance of calcium signaling in the chemosensory roles of primary cilia. The primary cilium also regulates L-type calcium channel expression through Wnt signaling (75). Suppressed Wnt signaling prevents CaV1.2 expression, ultimately resulting in the phenotypes of polycystic kidney disease.

Surprisingly, proteomic screening of mammalian primary cilia has not shown strong evidence of other calcium channels (76, 77). The screening shows mainly signaling proteins. However, recent functional screening indicates that the following calcium channels may regulate ciliogenesis: Itpr3, Cacna1d, Cacnb2 and Catsper4 (78). Unfortunately, it is unclear if any of these channels are localized to primary cilia.

Activation of ciliary calcium channels

A critical remaining issue is to identify stimuli that cause the various ciliary calcium channels to open. Studies intended to relate these channels to ciliary mechanosensitivity have revealed the difficulty of the problem. In cells derived from renal epithelium, it is clear that deflection of the cilium leads to an increase in calcium in the cell body (30, 31). This response requires the cilium, external calcium, and two calcium-conducting channels (TRPP2 and TRPV4) (29, 30, 79). These channels are found on the cilium (although not exclusively), so it has seemed plausible that ciliary deflection might open ciliary calcium channels. Calcium in the cilium might then initiate the cytoplasmic response (although probably not by simple diffusion (70)). To test whether flow increases ciliary calcium in renal epithelial cells, Su et al. targeted a calcium-sensitive fluorescent protein to the cilium and deflected the cilium with fluid flow. This caused a slow increase in ciliary calcium (42). Whether cytoplasmic calcium also changed was not determined. The researchers took care to test for a possible artifact: changes of fluorescence due to movement of the cilium relative to the focal plane during flow. With a calcium-insensitive fluorophore, there was no significant change in fluorescence during flow, suggesting that the calcium increase initially observed was not due to a motion artifact. Cilia in this study were viewed from above (end-on).

Using a similar targeted sensor in renal epithelial cells, but viewing the cilia from the side, Jin et al. also reported flow-induced increases in ciliary calcium and, under some but not all conditions, also measured an increase in cytoplasmic calcium (41). The ciliary response almost always peaked before the cytoplasmic response. Using a FRET-based calcium sensor, Lee et al. also reported flow-induced calcium increases in osteocyte primary cilia and in the cytoplasm (40). On an individual cell basis, about 60% of calcium peaks occurred in the primary cilium prior to the cytoplasm of the same cell. However, the authors could not conclude whether the ciliary response preceded the cytoplasmic response.

To further understand the role of ciliary calcium in an *in vivo* model, Yuan et al. studied ciliary calcium in live zebrafish embryos, and in particular in the left-right organizer (43). This organizer (analogous to the embryonic node in mouse) initiates left-right asymmetry during embryonic development. A calcium-sensitive fluorophore was targeted to the cilia, and a calcium-insensitive ciliary fluorophore allowed correction for motion artifacts. Ciliary calcium oscillations were observed, particularly along the left side of the organizer. The oscillations were substantially reduced when ciliary motility was impaired, suggesting that the oscillations may be a consequence of ciliary motility. The oscillations were reduced when TRPP2 expression was decreased. In some cells, the increase in ciliary calcium preceded a cytoplasmic calcium wave. In those cells, accumulation of ciliary calcium may be required for generation of a robust cytoplasmic calcium signal. In a recent study by Delling et al., however, deflection of cilia in cells from murine embryonic node caused no detectable elevation of intraciliary calcium, and there was no difference in cytoplasmic calcium oscillation between left-side and right-side embryonic node (44).

Delling et al. recently concluded that flow-induced changes in the fluorescence of ciliary calcium sensors are often artifactual (44). In contrast to Su et al. (42), Delling et al. (44) did observe a small motion artifact, which was shown to be significant when the cilia were viewed from above. After correcting for this ciliary motion, no ciliary Ca^{2+} responses to flow were detected in a variety of primary cilia or in the kinocilia of the inner ear. When no ciliary calcium response is observed, one must consider whether the calcium indicator is sensitive across the range of cilioplasmic calcium concentrations expected. Resting ciliary calcium can be as high as 742 nM (70). In the study that found no ciliary calcium responses, the sensitivity of the indicator to calcium was carefully measured (44). Given that reported sensitivity, the fluorescent signal would already reach ~90% at 742 nM, and any additional increase in ciliary calcium would cause only a slight increase in fluorescence. However, increasing cilioplasmic calcium by membrane permeabilization in the presence of external calcium (>1 mM) showed directly that the calcium sensor was responsive in the cell (44).

Delling et al. also note that even a true increase in ciliary calcium should not be taken to imply the opening of ciliary channels. Instead, it was shown that calcium can first be generated in the cytoplasm and then simply diffuse into the cilium (44). The rates of flow used to increase ciliary calcium create a shear stress on the plasma membrane of 0.3 to 1.0 dyn/cm^2 (41, 42). Such a shear stress can increase cytoplasmic calcium (35, 36, 40, 58, 59). While most organelles are separated from the cytoplasm by enclosing membranes, there is direct passage between cilioplasm and cytoplasm at the ciliary base (necklace). Thus some increases in ciliary calcium may occur via diffusion of calcium from the cell body (44). This

cannot account for a situation where ciliary calcium increases but cytoplasmic calcium does not (41). In some circumstances, there is no increase in cytosolic calcium unless the cilium is present (79).

Summary/Conclusion

Advances in microscopic technique have allowed us to differentiate cytoplasmic and ciliary calcium signaling. The ability to patch-clamp one cilium has permitted us to study single calcium currents from an excised or intact primary cilium. While current studies have provided important information on primary cilia, it may be unsettling to know the complexity of the cilium. Perhaps the question becomes why the cell needs so many different calcium channels in this tiny organelle. It is still a matter of some judgment whether deflection of a primary cilium causes calcium to enter across the ciliary membrane. At this point, our questions greatly outnumber our answers.

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Key Points

- Different calcium channels in a cilium may have different cellular functions.
- Simultaneous measurement of single currents and concurrent imaging of cilioplasmic and cytoplasmic calcium are probably the best ways to resolve the controversies in fluid-flow-induced intracellular calcium changes.
- Although the mechanisms of calcium signaling are controversial, there is a consensus that primary cilia are mechanosensory organelles.

Table I

Sensory roles of solitary non-motile primary cilia

Function	Disease Relevance	Reference
Chemosensor	Nephrocystin; diabetes; obesity; polycystic liver	(5-9)
Developmental regulator	Abnormal development; cancer	(10-12)
Gravitational sensor	Potential osteoporosis or chondroporosis	(13)
Light sensor	Retinitis pigmentosa, blindness	(14, 15)
Mechanosensor	Polycystic kidney, liver, pancreas	(16-18)
Nodal flow sensor	Situs inversus; situs ambiguus; situs isomerism	(19-21)
Osmosensor	Unknown; detect changes in composition and tonicity of ductal bile	(22)
Shear-stress sensor	Hypertension; aneurysm; atherosclerosis	(23-26)
Smell sensor	Anosmia; hyposmia	(27, 28)
Thermosensor	Abnormal thermal hyperalgesia	(29)