CHAPTER 10

The Cytoskeletal Connection to Ion Channels as a Potential Mechanosensory Mechanism: Lessons from Polycystin-2 (TRPP2)

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I. OVERVIEW

Mechanosensitivity of ion channels, or the ability to transfer mechanical forces into a gating mechanism of channel regulation, has been split into two main working (not mutually exclusive) hypotheses. One is that elastic and/or structural changes in membrane properties act as a transducing mechanism of channel regulation. The other hypothesis involves tertiary elements, such as the cytoskeleton which, itself by dynamic interaction(s) with the ion channel, may convey conformational changes including those ascribed to mechanical forces. This hypothesis is supported by numerous instances of regulatory changes in channel behavior by alterations in cytoskeletal structures/interactions. However, only recently, the molecular nature of these interactions has slowly emerged. Recently, a surge of evidence has emerged to indicate that transient receptor potential (TRP) channels are key elements in the transduction of a variety of environmental signals. Herein, we summarize recent work, which in brief, define the molecular linkage and regulatory elements of polycystin-2 (PC2), a TRP-type (TRPP2) nonselective cation channel whose mutations cause autosomal dominant polycystic kidney disease (ADPKD). We provide evidence for the involvement of cytoskeletal structures in the regulation of PC2 and assess how these connections are the transducing mechanism of environmental signals to its channel function. We conclude and propose that the actin network, which attaches to the PC2 channel, is a novel osmosensitive device, where the three-dimensional structure of the actin gel apposed to the channel, elicits its regulation. Thus, ''environmental forces'' such as hydroosmotic pressure control PC2 channel activity, by conveying a sensory mechanism to the channel, and through the integrity of the cytoskeleton. Taken together, our findings strongly support the hypothesis that the channel-cytoskeletal interface is a functional unit, with general and important implications in mechanosensitivity.

II. INTRODUCTION

All cells are continually exposed to external physical forces to which they adapt. Therefore, a large part of the cell's physiology is placed in detecting and responding to environmental stresses, including (osmotic, hydrostatic) pressure, shear, flow, stretch, and compression. Conversion of mechanical sensation into intracellular signals that modifies the cellular response is called "mechanotransduction." Long before, molecular physiological techniques identified mechanosensitive molecular devices in most cells, it was accepted that mechanosensitivity was only observed in specialized sensory organs. Thus, mechanosensitivity had only been identified in crayfish stretch receptors (Brown *et al*., 1978), Pacinian frog muscle spindle (Katz, 1950) and specialized skin cells as the Paciniam corpuscle (Mendelson and Loewenstein, 1964), and the stretch receptor in crickets (Coillot and Boistel, 1969). Specialized organs responsible for hearing and touch are also expected to mediate their function through sensory receptors. However, many other organs such as the brain, spinal cord, bladder, and joints are subjected to mechanical forces, which require mechanotransduction. Vascular and alveolar distension, and the mechanical response of several organs to pressure, such as the urinary bladder, the intestines, the placenta, and the kidney also require mechanosensitive responses. Changes in cerebrospinal fluid production in the brain, for example, may raise intracranial pressure, causing anatomical deformities as hydrocephalus. In the eye, all tissues are continuously subjected to variations in intraocular pressure whose dysfunction may cause glaucoma (Vittitow and Borras, 2002; Kalapesi *et al*., 2005), corneal edema, iris ischemia, and changes in the trabecular meshwork lens opacity (Johnstone and Grant, 1973; Borras, 2003; Kalapesi *et al*., 2005). Other examples of exposure to excessive mechanical force such as pressure or compression include peripheral nerve entrapments, obstructive nephropathy, hypertensive hypertrophic cardiomyopathy, hypertensive glomerulosclerosis, and compression of vertebral fractures and disc herniation. The blood vessel endothelium and tubular epithelia of the mammalian nephron are also subjected to shear, stretch, and tension, and skin and mucosal epithelia are subjected to continuous stretch. Striated skeletal muscle is also under tension, whose compression affects sarcomere contraction. Therefore, mechanically active environments impose various forms of pressure from both inside their plasma membrane (i.e., osmotic, cytoskeletal) and externally, including forces such as stretch, and when intracellular pressure may rise, for instance in hypoosmotic shock or due to elevated extracellular pressure such as in increased flow and shear.

Cells respond to environmental stress by changing their morphologic appearance (Ives *et al.*, 1986; Dartsch and Hammerle, 1986; Dahlin *et al.*, 1987) activating signaling pathways and the production of a number of second messengers (Skinner *et al.*, 1992; Matsuo and Matsuo, 1996; Matsuo *et al.*, 1996; Okada *et al.*, 1998; Yokoyama *et al.*, 1999; Mikuni-Takagaki, 1999; Mallouk and Allard, 2000; Endlich *et al.*, 2002; Ji *et al.*, 2002) and/or by affecting gene and protein expression (Terakawa *et al.*, 2002; Voisin and Bourque, 2002; Borras, 2003; Kelly *et al.*, 2006; Zhou *et al.*, 2006). It should be expected, therefore, that several not mutually exclusive adaptive mechanisms are present in most cells to help cope with environmental stress. However, such mechanisms at the cellular level have just began to be defined. Mechanosensing molecular devices in cells described to date include mechanically gated ion channels, plasma membrane-bound enzymes such as phospholipases A2 and C, cytoskeletal structures, and receptor complexes with cell–cell and cell–matrix adhesion properties, such as cadherins, selectins, and integrins (Ingber, 1997; Ko and McCulloch, 2000; Stamenovic and Ingber, 2002). Among these, the cytoskeleton provides underlying support to the plasma membrane and forms part of the linkage to the extracellular matrix (Ingber, 1997). Integrins are transmembrane receptors, which link to the extracellular matrix components of the cytoskeleton such as actin and intermediate filaments. Integrin receptors are accepted as sensory transducers of mechanical stress via the extracellular matrix, through the plasma membrane, into the cytoskeleton to elicit the activation of intracellular signaling pathways (Ingber, 1997). Inhibition of integrins affect mechanotransduction (Yoshida *et al.*, 1996; Mobasheri *et al.*, 2002). The interdependence of the plasma membrane and cytoskeleton in adaptation to applied forces has also been recognized. Membrane-bound enzymes and proteins such as phospholipase A2, phospholipase C, and tyrosine kinases have been implicated in mechanosensory function. Thus, membrane stretch, including by osmotic swelling, induces release of prostaglandins and cAMP, affecting the hydrolytic production of phospholipids (Kreisberg *et al.*, 1982; Skinner *et al.*, 1992; Yokoyama *et al.*, 1999; Ko and McCulloch, 2000). *In vitro* studies on stretched mesangial cells, cardiac myocytes, and fetal lungs, as well as flow and shear on human umbilical vein endothelial cells show activation of phospholipase C, generating diacylglycerol (DAG) and the ensuing molecules in the phosphatidylinositol pathway. In addition to this, the cytoskeleton itself is capable of modifying the cellular environment. Physical stress has been shown to induce changes in actin polymerization and thus mechanotransduction by providing additional sites for actin–myosin interaction, thereby enhancing force generation in response to increased intravascular pressure (Zhelev and Hochmuth, 1995; Cipolla *et al.*, 2002).

Another class of molecular mechanosensory devices in cells implicates mechanosensitive channels, which are phenomenologically defined as channel

structures whose gating and activation is identified in response to forces acting at the plasma membrane. The widespread discovery of mechanosensitive channels arose with the advent of the patch clamping techniques, which allowed the identification of channel phenotypes *in situ* (Hamill *et al*., 1981). The technical problems came about with this milestone arose and its paradigmatic shift in membrane physiology. Reportedly, the first observation of mechanosensitive ion channel activity is attributed to Guharay and Sachs (1984), who noted that channel activity increased with suction while trying to form patch clamp seals in cultured chick skeletal muscle. The authors found that the channel's open probability increased by the applied vacuum pressure (Guharay and Sachs, 1984). Their results provided the first direct evidence for cells to respond to applied pressure with changes in ionic conductance. The conclusion of this original report was that the cytoskeleton controls mechanically gated ion channels by tonically repressing their activity (Guharay and Sachs, 1984). Thus, patch clamping studies in particular those of cell-attached and excised patches had an intrinsic caveat in that the very acquisition of the patch had associated with it a consequent rearrangement of intrinsic cytoskeletal structures. This problem, which may also plays a relevant role in the very definition of mechanosensitive channel adaptation was clearly manifested in phenomenological differences between the altered channel activity of cell-attached patches, which appear quite different from data obtained in whole-cell studies (Zhang and Hamill, 2000). Thus, an intrinsic uncertainty lies in the fact that every patch has an unknown albeit relevant attached cytoskeleton. Serious attempts at avoiding this "contaminating" factor have provided strong evidence for mechanosensitive channels whose function is intrinsically associated with structural changes to the membrane itself (Hamill and Martinac, 2001, see below). To what extent channels sensitive to membrane stretch are intrinsically (i.e., under physiological conditions) regulated by cytoskeletal components instead is to date, a largely open question. Nonetheless, a number of mechanosensitive channels have been identified across various cell types ranging from prokaryotes, such as bacteria and archea, to eukaryotic cells in mammalian organs, including the central and peripheral nervous system, myocytes, blood vessel endothelium, renal epithelia, hair cells, and fibroblasts (reviewed in Hamill and Martinac, 2001). Stretch-regulated channels have been particularly described in a number of excitable cells, including snail neurons, mammalian astrocytes, atrial myocytes, dystrophic muscle from mdx mice and toad gastric smooth muscle, and possibly many other cells (reviewed in Morris, 1990; Hamill and Martinac, 2001). To date, only a few channels have been molecularly identified on the basis of specific responses to stress forces (Sukharev and Corey, 2004). Mechanosensitive channels, whose gating has been associated to activation by changes in membrane

tension, appear to share functional similarities (Hamill and Martinac, 2001), including weakly selective cation perm-selectivity (Morris, 1990), and permeability to divalent cations, allowing significant Ca^{2+} influx during stretch (Sackin, 1994). In prokaryotes, a stretch-activated mechanosensitive channel was first evidenced by experiments on *Escherichia coli*, where the MscL channel was identified, which open at pressures just under those which would disrupt membranes (Gu *et al.*, 1998; Martinac, 2004). Imposed vacuum pressure is often one of the identifying forces in determining the presence of mechanosensitive channel activity. Nonetheless, other forces such as osmotic pressure are equally relevant in defining the activating mechanism of mechanosensitive channels. In cultured trabecular meshwork cells, for example, high conductance Ca^{2+}-activated K^+ channels are activated in response to either membrane stretch or hypotonic shock (Gasull *et al.*, 2003). Several instances of claimed mechanosensitivity have arisen from studies where "membrane stress" has been achieved by osmotic shock. Due to the universality of anisoosmotic cell responses, and in particular cell volume regulation, which implicates cytoskeletal structures, the true nature of mechanosensitivity requires further exploration. Interestingly, both mechanosensitive channel activation and inactivation have been reported (Morris and Sigurdson, 1989), which brings to the issue of the techniques with which mechanosensory channel function is described and further explored. To date, at least three families of channels have been identified as functionally linked to mechanosensory function, by this being understood, that the phenotypes underlying the channels later identified, were of a mechanical nature. The two-pore domain potassium channels TREK and TRAAK are a group of four-transmembrane domain channels preferentially found in the CNS (Patel *et al.*, 1998; Patel and Honore, 2001). These channels, whose intrinsic properties are regulated by stretch of the plasma membrane, are likely implicated in the response to various "environmental" forces including mechanical or osmotic stress, intracellular pH, or temperature. As expected from mechanosensitive channels, and indeed associated with its activation by membrane stretch, TREK-1 is also modulated by osmotic cell swelling (Patel *et al.*, 1998). Studies also showed that apart from stretch or membrane tension, TRAAK and TREKs appear to be activated by arachidonic acid metabolites and other ligands (Fink *et al.*, 1998; Maingret *et al.*, 2000), which interestingly enough is a common mechanism of activating TRP channels as well (Minke, 2001). The DEG/ENaC channel family has been implicated in mechanosensitive channel activity, despite the fact that to date, no clear evidence is available as to whether channels of this family indeed respond to stretch activation (Sukharev and Corey, 2004). The first member of this family was originally identified after the long search for the first member, a highly-Na^+ selective,

and amiloride-sensitive cation channel of tubular epithelia (ENaC) of the distal nephron (Canessa *et al.*, 1993). Interestingly, although not previously sought after as a mechanosensitive channel, ENaC was found to share homology with genes associated with neural degeneration, the nematode degenerins (DEGs; Canessa *et al.*, 1993). Subsequently, ENaC activity has been found to be mechanosensitive, at least in response to osmotic changes, when heterologously expressed in *Xenopus* oocytes. Further confirmation of the intrinsic mechanosensitivity by ENaC has been postulated in reconstituted channels in a lipid bilayer (Awayda *et al.*, 1995). The encompassed conclusions have not been without controversy (Rossier, 1998), and the true mechanosensitive nature of ENaC remains as open question. However, ENaC function has been previously observed to require cytoskeletal components, namely actin, and Benos' group provided strong evidence for ENaC to bind actin directly (Mazzochi *et al.*, 2006). This, in combination with the fact that the epithelial Na^+ channel complex also contains at least another pore, Apx, and cytoskeletal proteins such as spectrin suggests that *in vivo* conditions may trigger activating responses, which are much more complicated than originally expected. Other members of the DEG/ENaC family, the DEGs are nematode gene products of several genes (Hamill and McBride, 1993; Goodman and Schwarz, 2003), which include those which encode cytoskeletal proteins (i.e., tubulins, MEC-7, -12), structural channel proteins (MEC-4, MEC-10, DEG-1, and UNC-105) and matrix proteins (MEC-1, MEC-5, and MEC-9). This superfamily also includes, the acid-sensitive channels of vertebrate neurons (ASICs; also known as BNCs and BNaCs), and *Drosophila* PPKs (Sukharev and Corey, 2004). Channels of this superfamily are likely involved in touch and other mechanosensations (Welsh *et al.*, 2002; Goodman and Schwarz, 2003), although direct proof is still lacking. By far the most appealing superfamily of sensory channels discovered in recent years is that of the TRP channels (Section II.C), with clear connections to a number of sensory responses, including mechanosensitivity.

A. The Channel-Cytoskeleton Connection

Due to the techniques used to assess mechanosensitivity in ion channels, it is inherently clear that except for few exceptions (Perozo *et al.*, 2002; Maroto *et al.*, 2005) many mechanosensitive channels likely work in concert with cytoskeletal structures. Actin-based networks are implicated in such diverse cellular functions as phagocytosis, regulation of cellular shape, locomotion, and hormone action (Painter and McIntosh, 1979; Stendahl *et al.*, 1980; Hall, 1984; Stossel, 1984; Smith, 1988; Stossel, 1993). Thus, it is not surprising that a

wide number of channel species have been linked to the cytoskeleton. Disregarding epiphenomenological aspects of cell conductance, such as trafficking and vesicle fusion of channel-containing membranes, most channels, whose function has been looked in detail, associate directly and/or indirectly, to cytoskeletal proteins. Most studies have employed drugs that selectively stabilize or destabilize either actin filaments or microtubules, with resulting effects on specific ion channel activity, either as changes in whole-cell conductance or single-channel activity by the patch clamping techniques. However, direct proof, namely the anchoring and binding of specific cytoskeletal proteins to individual channel proteins is only beginning to emerge. A body of earlier evidence demonstrated that various cytoskeletal components, including actin and actin-associated proteins, anchor, colocalize, and regulate both the spatial stability as well as the function of ion transport proteins. Ankyrin and spectrin, colocalize with the band 3 anion exchanger (Drenckhahn *et al.*, 1985), the α-subunit of the Na^+, K^+-ATPase in epithelial cells (Morrow, 1989), rat brain voltage-sensitive (Edelstein *et al.*, 1988), and epithelial Na^+ channels (Smith *et al.*, 1991). Direct cytoskeletal connections have been found to a number of identified channel structures including ligand-gated channels such as the NMDA (Lei *et al.*, 2001; Yuen *et al.*, 2005), AMPA (Kim and Lisman, 2001), and acetylcholine receptors (Bloch *et al.*, 1997; Mitsui *et al.*, 2000; Shoop *et al.*, 2000), voltage-gated Na^+ (Srinivasan *et al.*, 1988, 1992; Undrovinas *et al.*, 1996), K^+ (Mazzanti *et al.*, 1996; Jing *et al.*, 1997; Nakahira *et al.*, 1999) and Ca^{2+} (Johnson and Byerly, 1994; Lader *et al.*, 1999; Johnson *et al.*, 2005) channels, and Cl^- channels as the GABA(A) (Wang *et al.*, 1999; Luccardini *et al.*, 2004) and glycine (van Zundert *et al.*, 2002, 2004) receptors. Channels in nonexcitable cells are also linked to the cytoskeleton, including amiloride-sensitive cation channels such as ENaC (Berdiev *et al.*, 1996; Ismailov *et al.*, 1997; Mazzochi *et al.*, 2006) and Apx (Prat *et al.*, 1996; Zuckerman *et al.*, 1999), and Cl^- channels such as CFTR (Prat *et al.*, 1994, 1995; Ismailov *et al.*, 1997), and CLC channels (Ahmed *et al.*, 2000; Dhani *et al.*, 2003). Direct interactions include the binding of key cytoskeletal components such as actin itself to channels like ENaC (Mazzochi *et al.*, 2006) and CFTR (Chasan *et al.*, 2002), or tubulin, as it has been observed for TRPV1 (Goswami *et al.*, 2004) and GABA and glycine receptors (Kirsch *et al.*, 1991; Coyle *et al.*, 2002). Spectrin, for example, has been observed to bind to voltage-gated Na^+ channels in the brain (Srinivasan *et al.*, 1988) and may be a structural component of the epithelial channel complex containing both ENaC and Apx in cells from the distal nephron (Zuckerman *et al.*, 1999). Linker proteins as α-actinin have been found to bind directly to the C-terminal end of the glutamate receptor (Wyszynski *et al.*, 1997) and to L-type Ca^{2+} channels (Sadeghi *et al.*, 2002). Similar interactions also occur between the α-actinin and voltage-gated K^+ channels (Maruoka *et al.*, 2000), and TRPP2 (PC2; Li *et al.*, 2005).

The actin cytoskeleton may modify mechanosensitive channels by attaching and modifying directly the plasma membrane, and/or structural dynamics of channel function, directly (reviewed in Morris, 1990; Hamill and McBride, 1993; Sackin, 1994). Actually, the original description of mechanosensitive channels strongly implicates the actin cytoskeleton as a modulator of mechanosensitivity (Guharay and Sachs, 1984). Following the application of pressure or suction to a membrane patch, there is a lag response noted in channel activation and deactivation, suggesting that an elastic component must exist to "transduce" membrane tension toward the channels. This lag response is likely due to the viscoelastic actin network relaxing with time, transferring the membrane tension to the mechanosensitive channels (Guharay and Sachs, 1984). Thus, cytoskeletal dynamics may be an important component of the delay of channel activation, likely acting as a stabilizing or restraining force on stretch-regulated channel function (Guharay and Sachs, 1984; Small and Morris, 1994; Laitko *et al.*, 2006).

B. Actin Filaments and Their Disruption: Effect of Cytochalasins

It is important therefore to consider how the actin cytoskeleton is assembled, and how it can be disrupted. The formation of actin filaments (F-actin) from actin monomers (G-actin) is generally viewed as a condensation polymerization in which G-actin monomers, condense, in a rate-limiting step, to form nuclei which then rapidly elongate to form F-actin in equilibrium with G-actin at its critical monomer concentration (Bray, 1992). Normally, for every actin monomer added to the polymer, one G-actin-bound ATP is hydrolyzed to F-actin-bound ADP. This hydrolysis of ATP allows actin to polymerize in a "head-to-tail orientation," where association and dissociation at either end occur at different rate constants (Wegner, 1976; Cartier *et al.*, 1984; Korn *et al.*, 1987). The degree of polymerization is therefore a steady state condition, which requires the constant supply of actin monomers and nucleotides. Interestingly, ATP hydrolysis by F-actin modifies elastic properties to the filament, which in turn, may render actin networks of distinct mechanical properties (Janmey *et al.*, 1990b). Most of the work related to the actin cytoskeletal control of ion channels, whether mechanosensitive or not, has relied on natural toxins, which affect actin polymerization. It is imperative; therefore, that a clearer understanding of their modes of action, and effect(s) in cells are better understood.

Cytochalasins are fungal metabolites, which were originally described by their inhibiting effect on a wide variety of cellular movements (Bray, 1992). It is interesting that cytochalasin B (CB), one of the most popular cytochalasins in changing cell motility, was originally claimed to have no effect on

several cell functions (Wessells *et al.*, 1971), including a lack of effect on actin filament disruption (Wessells *et al.*, 1971; Forer *et al.*, 1972). Conversely, cytochalasins have been observed to cause effects, which may not be directly associated with cytoskeletal derangement but are nonetheless important when claiming a change in a particular cellular function. CB has been found, for example, to bind and inhibit glucose transport (Fay *et al.*, 1990), affect ascorbic acid uptake in fibroblasts (Fay *et al.*, 1990), inhibit protein synthesis by releasing mRNA (Ornelles *et al.*, 1986), and induce mitochondrial contraction (Lin *et al.*, 1973). Soon after the discovery of the cytochalasin-induced inhibition of cellular motility, it was observed that these compounds actually affect the rate of actin polymerization (Brenner and Korn, 1979; MacLean-Fletcher and Pollard, 1980). Early experiments demonstrated that high concentrations (100 μM) of CB lower by 30–50% the viscosity of actin filaments (Spudich and Lin, 1972). Hartwig and Stossel (1976) also showed that CB inhibits F-actin gelation by a high molecular weight actin cross-linking protein, later to be identified as filamin A. Thus, CB inhibition of actin gelation has been used as a criterion to disrupt the cell's cytoskeleton. Nevertheless, the final outcome of this interaction lies on the concentration and incubation times to which cells are exposed to cytochalasins. Substoichiometric concentrations of CB strongly inhibit network formation by actin filaments themselves (Brenner and Korn, 1979), and suggest that this is a direct effect on actin, rather than an effect on actin cross-linking proteins (Brenner and Korn, 1979). Similar data were obtained independently using different cross-linking proteins (Hartwig and Stossel, 1979). To date it is accepted that cytochalasins have distinct and multiple effects on actin polymerization (Bray, 1992). CB reduces the rate of actin polymerization, in a process, which involves inhibition of monomer addition to the barbed, or fast growing, end of actin filaments, which is favored for elongation (Brenner and Korn, 1979; Brown and Spudich, 1979; Lin and Lin, 1979; Lin *et al.*, 1980). Similar effects have been observed for cytochalasin D (CD; Brown and Spudich, 1979; Lin *et al.*, 1980). Addition of polymers to the barbed end of F-actin is fundamentally different from the addition of G-actin. This is based on the fact that this reaction involves the binding of one actin in the monomer conformation to two actin molecules in the polymer conformation. Thus, a key element in determining the effect of a given cytochalasin is its putative interaction with G-actin. While binding of CB to G-actin has never been demonstrated, Goddette and Frieden, found that CD actually binds to monomeric actin with 1:1 stoichiometry and a dissociation constant of 18 μM (Goddette and Frieden, 1986a,b). This stoichiometry and affinity can be changed in the presence of low Mg^{2+} (Goddette and Frieden, 1986a,b). Thus, it has been concluded that CD induces dimer formation (Goddette and Frieden, 1986a). These studies are highly relevant, as the presence of CD

actually induces actin polymerization by an enhancement of the initial rate of nucleation. The large decrease in final extent of F-actin is also attributed to the formation of such dimers (Goddette and Frieden, 1986b). Therefore, binding of CB is restricted to the polymeric form of actin, and restricted even further to sites exposed at the barbed end of the filaments. In contrast, CD treatment may actually induce a pool of short actin oligomers. The outcome of the interaction between cytochalasins and actin networks depends on the toxin species, the concentration, and the time of incubation. Direct effects on actin occur at low CB concentrations, comparable to those which inhibit cell movements, and at least the relative potencies of CB and CD on cell movements (Atlas and Lin, 1978) and on actin are similar (Brenner and Korn, 1979; Brown and Spudich, 1979; Lin *et al*., 1980; MacLean-Fletcher and Pollard, 1980). However, CE inhibits cell movements (Atlas and Lin, 1978) at much lower concentrations than it inhibits actin polymerization (Lin *et al*., 1980), binds to actin (Lin *et al*., 1980), or inhibits network formation (MacLean-Fletcher and Pollard, 1980). Species-specific effects are also relevant. CB, for example, failed to inhibit actin polymerization in the sperm acrosomal reaction (Sanger and Sanger, 1975). It can be concluded therefore, that cytochalasins modify the cell's architecture, and subsequently cell motility and other cell functions by a number of interrelated effects, including the alteration of the steady state interaction between G- and F-actin, and by their respective interactions with supramolecular actin networks (Bray, 1992). Cytochalasins reduce the viscosity of actin gels, by both decreasing the average filament length through a change in the steady state between net polymerizing and depolymerizing ends, and by inhibiting the reannealing of spontaneous breaks in F-actin. It is important to know, however, that the capping effect of cytochalasins is not shared by other actin depolymerizing toxins, such as latrunculin A, which only elicits a tight 1:1 binding interaction with G-actin (Bray, 1992). The steady state pool of actin organization, namely the actin network in the presence of cytochalasins might also shift the interaction with proteins that either block actin polymerization and, conversely, by proteins that block the net depolymerizing ends of actin filaments. This steady state of F-actin pool *in vivo* would be therefore strongly influenced, by the disruptive effect of cytochalasin itself, by localized ionic gradients created by channel function, and by proteins such as profilin which specifically interact with monomeric actin (Markey *et al*., 1978; Reichstein and Korn, 1979). On the basis of the dynamic steady state of the actin cytoskeleton, and in particular cortical cytoskeleton, it is necessary that each instance, in which cytochalasins are used to assess mechanosensitive channel function, is carefully evaluated. Important parameters to consider include in the endogenous state of the actin cytoskeleton, the concentration of the drugs used, and most importantly, the time of incubation with the drug.

Agents that disrupt cytoskeletal organization, such as the fungal toxins cytochalasin or colchicine, which disrupt actin filaments and microtubules, respectively, have been shown to regulate stretch-activated ion channel activity (Guharay and Sachs, 1984; Patel *et al.*, 1998; Wan *et al.*, 1999). In *Lymnaea* neurons, Johnson and Byerly (1993) originally determined that agents that modify cytoskeletal organization also alter Ca^{2+} channel activity. The cytoskeletal disrupters, colchicine and CB were both found to speed Ca^{2+} channel decline in ATP, whereas the cytoskeletal stabilizers, taxol and phalloidin, were found to prolong Ca^{2+} channel activity without ATP. In addition, cytoskeletal stabilizers reduced Ca^{2+}-dependent channel inactivation (Johnson and Byerly, 1994). Thus, it was concluded that both channel metabolic dependence and Ca^{2+}-dependent inactivation might be controlled by cytoskeletal interactions. Indeed, the state of cortical cytoskeleton organization is important in the control of voltage-gated Ca^{2+} channels. In cultured neonatal mouse cardiac myocytes (NMCM), for example, we determined that CD disruption of the actin cytoskeleton blunts L-type Ca^{2+} currents (Lader *et al.*, 1999). This phenomenon, which is largely prevented by addition of the actin stabilizer phalloidin, could be mimicked in NMCM genetically deficient in the actin-severing protein gelsolin (Lader *et al.*, 1999). Whole-cell and single-channel recordings were obtained in retinal bipolar neurons of the tiger salamander (Maguire *et al.*, 1998). In that study, we showed that acute (20–30 min) disruption of endogenous actin filaments with CD instead activated voltage-gated K^+ currents in these cells, which was largely prevented by intracellular perfusion with phalloidin. Interestingly, direct addition of actin to excised, inside-out patches activated and/or increased single K^+ channels. This is an important control experiment, as it strongly supports a direct cytoskeletal interaction, rather than a membrane-induced change in channel function. The above evidence is thus indicative of a more general and quite appealing mechanism by which cytoskeletal structures control feedback mechanisms in voltage-gated cation channels. Both activation and inhibition can be elicited by dynamic changes in cytoskeletal conformations. Insofar as mechanosensitive channel function is concerned, in *Lymnaea* neurons, for example, treatment with CB, CD, or *N*-ethylmaleimide enhances mechanosensitive channel activity (Small and Morris, 1994; Wan *et al.*, 1999). This was viewed as evidence of an effect by cytoskeletal structures on channel function. Nonetheless, it is important to consider that only when the channel phenotypes are identified at the molecular level, we will know the precise nature of the interaction. In Cos-7 cells transfected with either the mechanosensitive TRAAK or TREK-1 CD reduces delay time in activation and enhances peak amplitude of K^+ channel activity (Patel *et al.*, 1998; Maingret *et al.*, 1999; Patel *et al.*, 2001).

Colchicine has also been shown to enhance TRAAK channel activity (Patel *et al.*, 1998). Thus, cytoskeletal connections may also have important consequences to the kinetic properties of mechanosensitive channels.

In epithelial cell physiology, cytoskeletal integrity was early on implicated in transepithelial ion transport (Pratley and McQuillen, 1973) and the response to vasopressin (Hardy and DiBona, 1982). However, the role of actin filament organization on apical epithelial Na^+ channel activity was unknown at the time. The first direct demonstration that cytoskeletal dynamics provide a functional interface with Na^+ channels, came from work in our laboratory, where we tested whether the effect of CD on Na^+ channel activation was indeed mediated by a cytoskeletal connection and not a change in the membrane elasticity. In this study, we observed that addition of exogenous actin plus ATP, and actin–gelsolin complexes of known size to excised inside-out patches (Cantiello *et al.*, 1991), mimicked the effect of CD. We first found that Na^+ channels immunocolocalize with F-actin from the cortical cytoskeleton suggesting that actin is always present in proximity to apical epithelial Na^+ channels in A6 cells. Addition of the actin filament disrupter, CD (5 μg/ml) to cell–attached patches, induced Na^+ channel activity within 5 min of addition. It is important to note that contrary to previous reports where other cytochalasins and exposure times were applied (Guharay and Sachs, 1984), our data strongly suggested a direct cytoskeletal-gating mechanism. CD also increased Na^+ channel activity in excised patches, further suggesting the remaining presence of cytoskeletal structures interacting with the channel. The first indication that indeed it was the cytoskeletal connection, and not changes in the plasma membranes arose from the fact that addition of short actin filaments (>5 μM) to excised patches also induced Na^+ channel activity. Further, the effect of actin on Na^+ channel activity was reversed by addition of the G-actin-binding protein DNase I, and completely prevented by treatment of the excised patches with this protein (Cantiello *et al.*, 1991). Addition of the actin cross-linking protein, filamin A, reversibly inhibited both spontaneous and actin-induced Na^+ channels. Conversely, addition of short actin filaments in the form of actin-gelsolin complexes in molar ratios <8:1 was also effective in activating Na^+ channels. This cytoskeletal interaction was found essential for the regulation by vasopressin and the PKA-induced activation of epithelial Na^+ channels ascribed to both Apx (Prat *et al.*, 1993a,b), and ENaC (Berdiev *et al.*, 1996). The Na^+ channel complex of renal cells copurifies with ankyrin, fodrin, and actin itself (Smith *et al.*, 1991). Thus, it is likely that actin either binds directly to the channel, or that actin may first interact with actin-binding proteins, which in turn regulate-by binding or other indirect interaction-ion channel function. In favor of the former possibility,

studies by Benos' group provided strong evidence with regard to the role of actin on the heterotrimeric $\alpha\beta\gamma$-rENaC (Ismailov *et al.*, 1997). Functional reconstitution of rENaC in lipid bilayers, where the components were restricted to this epithelial Na^+ channel, indicated that actin itself modified rENaC channel kinetics. Further, only short actin filaments, but neither G- nor F-actin, were able to regulate rENaC channel activity. Evidence for a direct interaction between ENaC and actin has only surfaced recently, confirming this hypothesis (Mazzochi *et al.*, 2006). Interestingly, ENaC has been claimed to respond to membrane stretch both in *Xenopus* oocytes (Awayda and Subramanyan, 1998), and in reconstituted lipid bilayers (Awayda *et al.*, 1995). The fact that this channel complex interacts with spectrin (Zuckerman *et al.*, 1999) and binds directly to actin (Mazzochi *et al.*, 2006), forwards the likely possibility that a cytoskeletal connection is at the center of epithelial Na^+ channel putative mechanosensitivity, despite early controversy in this regard (Rossier, 1998). Although impossible to generalize presently, other ion channels not associated with either mechanosensitivity or excitable membranes have followed the path of the epithelial Na^+ channels. Independent studies demonstrated that membrane-resident CFTR, a cAMP-activated channel of nonexcitable cells, is functionally regulated by actin filament organization (Prat *et al.*, 1994, 1995), in a process, which is independent of PKA stimulation (Prat *et al.*, 1995). Interestingly, actin was found to bind and functionally and directly interact with CFTR (Chasan *et al.*, 2002), providing further support to the idea that channel function can indeed be controlled by cytoskeletal dynamics. It is important to indicate, however, that this regulation has to be explored in detail, and no assumptions can be made, unless the state of the cytoskeleton in the expression system is clearly defined. Failure of eliciting amiloride-sensitive Na^+ currents in *Xenopus* oocytes, Apx was postulated to be a regulatory protein instead of a channel protein (Staub *et al.*, 1992). However, expression of Apx in filamin A actin-binding protein (ABP-280)-deficient, and thus cytoskeletally deranged human melanoma cells clearly showed that Apx indeed induces a cation-selective conductance (Prat *et al.*, 1996; Cantiello, 1999). These human melanoma cells also proved efficient in further confirming the important role of actin filament organization in the control of CFTR function (Prat *et al.*, 1999), and most relevantly, the response to cell volume regulation (Cantiello *et al.*, 1993). In this study, we observed that ABP-280-deficient human melanoma cells, failed to respond to hypoosmotic shock with K^+ channel activation and the regulatory volume decrease (RVD) observed in most cells. Genetic rescue by expression of ABP-280, however, restored RVD, indicating that the basal K^+ permeability of control cells is tonically inhibited, but rapidly activates by osmotic stress, in the presence of organized actin networks (Cantiello and Prat, 1996).

C. The Superfamily of TRP Channels

The TRP superfamily comprises a large group of related cation channels that display a wide diversity of activating modes and cation selectivity (Montell, 2001; Vennekens *et al.*, 2002; Voets and Nilius, 2003). All members of this superfamily share the same structural features: six putative transmembrane (6TM) domains, S1–S6, with a characteristic pore region between transmembrane segments S5 and S6, which is typical of voltage-gated channels (Phillips *et al.*, 1992; Birnbaumer *et al.*, 1996). The sequence of this region is highly conserved across the superfamily. TRP proteins also display moderate homology with the extended family of voltage and cyclic nucleotide-gated channels in particular the transmembrane domains of the pore region (Phillips *et al.*, 1992). However, the voltage sensor present in the S4 domain of voltage-gated cation channels is missing in TRP channels (reviewed in Montell, 2001; Minke and Cook, 2002). Furthermore, a 25-amino acid "TRP domain" with unknown function is also present in the C-terminus of most, but not all TRP channels. There are large variations in the amino and carboxyl cytoplasmic tails present in different TRP channels, which may partially reflect their functional diversity. For example, the N-termini of TRPC and TRPV channels contain several ankyrin repeats, whereas the TRPC and TRPM C-termini contain proline-rich motifs. Interestingly, some TRP channels also contain distinct putative motifs for binding of calmodulin (CaM), dystrophin, and the PDZ-scaffolding protein ENAD in their C-terminal tails (reviewed in Minke and Cook, 2002). These motifs may be relevant in regulation of channel function by such mechanisms as Ca^{2+}-induced channel inactivation and cytoskeletal control of channel function. Evidence is mounting to suggest that TRP channels oligomerize with homolog or heterologous partners enabling channel complexes with distinct functional features (Birnbaumer *et al.*, 1996; Xu *et al.*, 1997; Tsiokas *et al.*, 1999; Lintschinger *et al.*, 2000). An emerging consensus is that most TRP channels play important roles in various sensory physiologies (Clapham, 2002, 2003; Voets and Nilius, 2003). TRP channels participate in numerous sensory transduction responses, including hearing, vision, and thermosensation, as well as recently identified responses to a variety of physical stimuli including heat, cold, osmolarity, stretch, shear flow, and pressure (Montell, 2001; Montell *et al.*, 2002a; Voets and Nilius, 2003). From the evolutionary point of view, the TRP channel superfamily is highly conserved throughout animal phylogeny. There are at least 28 mammalian members identified thus far, which have been classified into multiple subfamilies (Montell, 2001; Montell *et al.*, 2002a; Voets and Nilius, 2003).

In terms of their function, TRP proteins are nonselective cation channels with diverse cation perm-selectivity properties, including a high Ca^{2+}

selectivity in some, but not all TRP channels (Minke and Cook, 2002). The first two TRP channels were identified in *Drosophila* as light sensitive channels with either very high (P_{Ca}:P_{Na}>100:1) or moderate (P_{Ca}:P_{Na}, ~4:1) Ca^{2+} permeability. The low Ca^{2+} selective channel is encoded at least in part by the TRP-like (TRPL) gene, which shares ~40% homology with the canonical TRP (Phillips *et al.*, 1992; Niemeyer *et al.*, 1996). Subsequently, a number of new members have also been isolated from various eukaryotic species, which are associated with different sensory stimuli, including cold and heat, osmotic challenges, and other receptor stimulatory responses (Montell, 2001; Voets and Nilius, 2003). The TRP family was originally classified (Harteneck *et al.*, 2000) into short (TRPC), osm-9-related (TRPV, vanilloid), and long (melanostatin-related, TRPM) channels, based on the protein length, and thus potential regulation of their cytoplasmic tails (Harteneck *et al.*, 2000). The TRPC (canonical) subfamily is the closest to the *Drosophila* TRP. The osm-9-like gene encodes a TRP protein associated with osmotic responses in *Caenorhabditis elegans* (Strotmann *et al.*, 2000). TRP have now been extended to other 6TM transmembrane proteins (TRPP, TRPML) with weaker homology but potentially similar topological features and regulatory roles in cell function. Thus, a new encompassing and comprehensive nomenclature for the TRP superfamily has recently been adopted, where there are group-1 comprising five subfamilies (TRPC, TRPV, TRPM, TRPN, and TRPA) and more distant group-2 comprising two subfamilies (TRPP and TRPML). The recently discovered epithelial Ca^{2+} channels CaT1 and ECaC, have now been renamed TRPV6, and TRPV5, respectively (Peng *et al.*, 1999; Yue *et al.*, 2001; Hoenderop *et al.*, 2002). TRPV5–6 may represent a major contributing factor to the apical Ca^{2+} absorption step in transporting epithelia. The Ca^{2+} permeable nonselective cation channels PC2 and PCL (Mochizuki *et al.*, 1996; Chen *et al.*, 1999) have been incorporated to the TRPP subfamily of TRP proteins (Montell *et al.*, 2002b). The PC2 topologically similar protein, mucolipin-1 (TRPML subfamily), which is genetically linked to mucolipidosis type IV is also a cation channel (Raychowdhury *et al.*, 2004). Mucolipin homologs have also been implicated in sensory functions (Di Palma *et al.*, 2002). The widespread distribution of TRP channels among excitable and nonexcitable cells and the fact that most TRPs permeate Ca^{2+} has forwarded the working hypothesis that TRP channel function underlies the ubiquitous "capacitative" Ca^{2+} response (Birnbaumer *et al.*, 1996; Golovina *et al.*, 2001; Putney *et al.*, 2001). This was originally supported by the finding that signals as PLC activation, lead to the opening of the *Drosophila* TRP. Hydrolysis of phosphatidylinositol-4,5-bisphosphate (PIP2) by PLC generates second messengers such as inositol 1,4,5 trisphosphate (IP3) and DAG. This leads to cascades involved in the production of polyunsaturated fatty acids and elicit Ca^{2+} store-activation responses (Xu *et al.*, 1997;

Chorna-Ornan *et al.*, 2001). A number of TRP channels are regulated by various branches of this metabolic pathway (Minke, 2001; Minke and Cook, 2002). However, TRP channels appear to be activated by a wider variety of stimuli, chiefly among which is mechanical stimulation (see below). TRP channels are also regulated by a number of specific intracellular ligands mostly associated with their cytoplasmic domains. These include putative PKC (TRPC, TRPV), and other kinases PKA (TRPP1-TRP2, TRPV1–2, 5–6-CaT1, ECaC1), and PI3K (TRPC2–3, 5–7, TRPV1, 4, 6), Ins3P receptor and CaM (all TRPC), and PDZ domain-containing proteins (TRP, TRPC4, 5), including INAD and NINAC myosin III (reviewed in Montell, 2001; Li *et al.*, 2002). Other putative ligands for intracellular regulation include cytoskeletal proteins, such as dystrophin-like motifs (TRPC1), and interactions with troponin-I, and tropomyosin-1 (PC2 or TRPP2). Other expected ligands for intracellular regulation may involve directly or indirectly trimeric and small G-proteins, ATP, InsP3, and DAG, and arachidonic acid ligands such as arachidonic acid (AA) itself. AA byproducts of potential DAG kinases and lipases reactions include anandamides (TRPV1), and HPETE (TRPV), which are linked to regulation of the vanilloid receptors (reviewed in Benham *et al.*, 2002). Interplay among activation mechanisms is apparent by the fact that cell swelling, a common activating factor of various TRP channels also activates phospholipase A2 (PLA2). This activity increases arachidonic acid production (Basavappa *et al.*, 1998), and downstream metabolites, such as 5′,6′-epoxyeicosatrienoic acid, which has been shown to activate TRPV4 (Watanabe *et al.*, 2002). Further, inhibition of either PLA2 or cytochrome P450 strongly inhibited the hypotonicity-induced TRPV4 channel currents (Vriens *et al.*, 2004).

D. TRP Channels and Mechanosensation

Much attention has been placed on potential roles of TRP channels in mechanosensory responses, particularly in view of the fact that they are targets of various environmental cues such as sound, light, pressure, and osmotic imbalance (Clapham, 2002, 2003; Corey, 2003; Voets and Nilius, 2003). TRP channel members may thus be considered novel mechanosensitive channels (Birnbaumer *et al.*, 1996; Clapham, 2003; Corey, 2003). Two models have been proposed for the signal transduction mechanism, and gating of mechanosensitive channels. Some mechanosensitive channels (Hamill and Martinac, 2001) appear to be gated by direct changes in membrane tension, which is generated in the lipid bilayer on osmotic imbalance. A study identified the mechanosensitive channel of *Xenopus* oocytes, as TRPC1, which may not require cytoskeletal components, but dynamic

changes in membrane structure to elicit activation (Maroto *et al.*, 2005). Nonetheless, the phenomenon known as adaptation of mechanosensitive channels may implicate cytoskeletal elements (Hamill and McBride, 1992; Small and Morris, 1994; Laitko *et al.*, 2006). An alternative model may rely more on the tethering by cytoskeletal proteins which play a key role in transducing mechanical deformation (Corey, 2003). The first mechanosensory TRP channel to be described in flies was "no mechanoreceptor potential C" NompC (TRPN1), the defining member of the TRPN subfamily (Walker *et al.*, 2000). Mechanoelectrical responses in bristle sensory neurons occur rapidly on deflection of the bristle hair shaft and result from the opening of the NompC. Another *Drosophila* TRP known as "painless" (Tracey *et al.*, 2003) is also involved in mechanosensation. The painless mutant is required for mechanical nociception (Tracey *et al.*, 2003). "Painless" contains eight tandem ankyrin repeats in the N-terminal domain. A distinguishing feature of NompC is the presence of 29 ankyrin repeats between the N-terminus and the first transmembrane segment (Walker *et al.*, 2000). The role of this large tandem array of ankyrin motifs is not known, but it is proposed to form the gating spring that leads to opening of the channel pore (Howard and Bechstedt, 2004). Other sensory responses implicate the *C. elegans* osmotin-like protein-9 (OSM-9) channel. OSM-9 was originally thought to be a mechanosensitive channel because osm-9 mutants are defective in osmotic avoidance and in sensitivity to nose touch. OSM-9 is now considered a member of the TRP vanilloid (TRPV)-related subfamily of TRP channels, which contains three ankyrin repeat domains at its N-terminal intracellular domain.

One particular subfamily of TRP members (vanilloid, TRPV) is emerging as quintessentially sensory channels, implicated in mechanosensation (Montell *et al.*, 2002a; Clapham, 2002, 2003; Voets and Nilius, 2003). Vertebrate TRPV channels are sensitive to various forms of physical and chemical stimuli (O'Neil and Heller, 2005), whose response effects an increased Ca^{2+} permeability. TRPV1–4 channels are moderately Ca^{2+}-selective, while TRPV5 and TRPV6 are highly selective Ca^{2+} channels (den Dekker *et al.*, 2003). Much of this information was originally obtained from studies in lower organisms. The *Drosophila* genome, for example, harbors two genes for TRPV-like channels. One gene (*iav*) encodes inactive (IAV), a protein that is related to OSM-9 and the second gene (*nan*) encodes Nanchung (NAN). Both genes encode channels implicated in hearing in *Drosophila* (Kim *et al.*, 2003; Gong *et al.*, 2004), which when expressed functionally *in vitro* are activated by hypoosmolarity (Kim *et al.*, 2003; Gong *et al.*, 2004). This feature is very similar to the osmosensitivity observed in cells expressing the mammalian TRPV4 (Liedtke *et al.*, 2000; Strotmann *et al.*, 2000; Wissenbach *et al.*, 2000). Other TRPV channels including TRPV2 share

some gating characteristics of TRPV4, as TRPV2-expressing cells, respond to both hypotonic cell swelling and application of membrane stretch, with increased Ca^{2+} influx (Muraki *et al*., 2003). TRPV4 osmosensitivity was originally surmised by its activation by hypotonic cell swelling (Liedtke *et al*., 2000; Strotmann *et al*., 2000). The molecular mechanism(s) of the TRP-mediated osmosensing response, is far from been clearly understood, however, exposure to hypotonic media on both sides of the membranes failed to activate TRPV4, suggesting that cell swelling induced by hypotonicity in the media, affected the activation signal. This was hypothesized to reflect a response to membrane stretch instead. However, direct application of pressure to the cell failed to activate the channel at room temperature (Strotmann *et al*., 2000). This could be reversed at higher temperatures (Guler *et al*., 2002; Watanabe *et al*., 2002). Thus, other physical parameters sensitize the channel, including hypotonic media and application of shear stress (Gao *et al*., 2003; O'Neil and Heller, 2005). TRP channels from the TRPN and TRPA subfamilies are additional candidates for mechanosensation, in particular for transduction of sound. Interestingly, TRPA1 was first cloned from mammalian fibroblasts (Jaquemar *et al*., 1999) and originally thought to be instead a thermo-sensitive and ligand-gated channel (Story *et al*., 2003; Jordt *et al*., 2004). Evidence indicates that TRPA1 is also a mechanosensitive channel (Corey *et al*., 2004). Knockdown of TRPA1 expression in zebrafish hair cells markedly impaired the transduction channel electrical activity of the otocyst to sound vibrations (Corey, 2003; Corey *et al*., 2004). Similarly, knockdown of TRPA1 in cultured mouse utricle hair cells inhibited electrical activity associated with hair-cell transduction (Corey, 2003; Corey *et al*., 2004).

E. Cytoskeletal Connections in TRP Channels

The widespread display of sensory functions in which TRP superfamily members are involved underlies the relevant role of associated proteins, which may help sense, or otherwise transduce physical forces into their regulation. Montell *et al*. have extensively reported on the connections made by TRPs, to the anchoring cytoskeleton (Wes *et al*., 1999; Xu *et al*., 2001; reviewed in Montell, 2005). In brief, TRPC binds directly to a scaffold protein, "inactivation no afterpotential-D" (INAD), which consists of five protein interaction modules referred to as postsynaptic density/discs-large/zonula occludens (PDZ) domains (Huber *et al*., 1996; Shieh and Zhu, 1996). In addition, this "core complex" forms a large macromolecular assembly known as the signalplex (Xu *et al*., 1998), containing other signaling proteins such as the "neither inactivation nor afterpotential-C" (NINAC) myosin III (Wes *et al*., 1999). In addition, the mechanosensitive properties of TRP

channels have been associated with the cytoplasmic tails of the protein. The N-terminus of most TRP channels (but notably not TRPP subfamily members) contains several ankyrin repeat domains. Ankyrin repeat motifs are present in tandem copies, which are considered to associate with the cytoskeleton and thus mediate protein–protein interactions of a tethered mechanism required for mechanical gating. To support this contention, studies indicate that ankryn motifs contain elastic properties (Sotomayor *et al.*, 2005), whose reversible spring behavior may be relevant in mechanosensation (Lee *et al.*, 2006). TRPV4, for example, has three ankyrin repeat domains (Liedtke *et al.*, 2000), a fact that may help explain its implication in vertebrate mechanosensation, in that, it can sense hypoosmotic stress (Alessandri-Haber *et al.*, 2003). However, a direct correlation between the lengths of this potential tethering TRPV channels, most known for their mechanosensitive properties, contains three to five ankyrin repeat motifs. Cloning of the *nompC* gene revealed a TRP channel protein (NompC, TRPN) with 29 N-terminal ankyrin repeats. TRPN is only distantly related to other TRP families (Corey, 2003). Corey *et al.* (2004) showed that TRPA1 (also called ANKTM1), which contains 17 ankyrin motifs, constitutes, or is a component of, the mechanosensitive transduction channel of vertebrate hair cells. Despite the fact that the activation mechanism elicited by mechanical force is unknown, the spring-like structure of the ankyrin repeats is consistent with a ''tethered channel'' model (Howard and Bechstedt, 2004; Sotomayor *et al.*, 2005; Lee *et al.*, 2006). Given that TRP channels are most likely tetramers (Hoenderop *et al.*, 2003; other stoichiometric interactions have been postulated as well; Flockerzi *et al.*, 2005), the ankyrin motifs in each monomer may also help the channel subunits to assemble. Arniges *et al.* (2006) provided evidence that the ankyrin motifs are implicated role in the multimeric assembly of TRPV4 channels.

Specific cytoskeletal connections in TRP channels may actually be a distinguishing feature among different TRP subfamilies, thus providing wider variety of regulatory connections. Goel *et al.* (2005) identified several proteins that interact with the TRPC5 and TRPC6 channels, which are chiefly localized to specialized postsynaptic dendritic spines in the rat brain, where they may play a critical role in synaptic responses to neurotransmitters. Twenty-eight proteins were identified in the TRPC5 immunoprecipitate from rat cerebral cortex, including the prominent actin, and other cytoskeletal proteins including spectrin, nonmuscle myosin, α-actinin, and tubulin. The interaction between TRPC5 and TRPC6 with α-actinin, actin, and drebrin, was confirmed by immunoprecipitation followed by Western blot analysis. Remarkably, the α-3 subunit of the Na^+, K^+-ATPase, the main component of the Na^+ pump was also identified as an interacting partner with both TRPC5- and TRPC6-channel proteins (Goel *et al.*, 2005).

The Na^+ pump is known to bind ankyrin, which in turn binds to spectrin, linking the transporter to the underlying cytoskeleton. Thus, the Na^+ pump may immunoprecipitate with TRPC5 via an interaction with spectrin or may directly bind to the ankyrin-like repeats found in the channel proteins. A role of other TRP channel members in mechanosensory function has been dismissed based on the fact that no discrete consensus sequences—namely ankyrin repeats are found in TRPP2 (PC2), for example (Delmas, 2005). However, clear cytoskeletal connections have been established between PC2 and the various components of the cytoskeleton. Originally, Gallagher *et al.* (2000) determined that Hax-1, a cytoskeletal protein that interacts with the F-actin-binding protein cortactin, interacts with PC2. Chen and collaborators have extensively explored PC2 interacting proteins and found that both cytoskeletal proteins troponin-I (Li *et al.*, 2003b) and tropomyosin-1 (Li *et al.*, 2003a) directly bind to PC2, further strengthening a link between the cytoskeleton and the PC2 channel. We have expanded on these findings, to demonstrate that the cytoskeletal connections of PC2 are a key component of a novel sensory mechanism based on dynamic changes in the actin cytoskeleton attached to the channel.

III. ROLE OF ACTIN CYTOSKELETAL DYNAMICS IN PC2-MEDIATED CHANNEL FUNCTION

A. Role of PC2 in Health and Disease

ADPKD describes a group of genetic disorders with almost identical clinical features, collectively affecting 1:1000 of the world's population. ADPKD is largely (~95%) caused by mutations in the *PKD1* and *PKD2* genes. Clinical manifestation of mutations in either gene are largely similar, both in human and animal models, suggesting the current working hypothesis that the encoded transmembrane proteins, PC1 (polycystin-1, TRPP1) and PC2 (polycystin-2, TRPP2), both recent additions to the superfamily of TRP channels, form a functional complex associated with cell-signaling events. Thus, a partnership between PC1 and PC2 converges into a common signaling cascade which is now thought to involve Ca^{2+} transport. The molecular steps linking this signaling pathway to renal cell function have only recently become apparent. Studies implicate the PC1-regulated and PC2-mediated Ca^{2+} entry, as a sensory mechanism for renal epithelial cell function (Nauli *et al.*, 2003). Further understanding of the molecular steps in this signaling pathway stems from the fact that PC2 is a TRP channel (Montell *et al.*, 2002b). Studies (González-Perrett *et al.*, 2001; Vassilev *et al.*, 2001; Koulen *et al.*, 2002) confirmed the original hypothesis

that the *PKD2* gene product is a cation-selective channel (Mochizuki *et al.*, 1996). Nevertheless, the actual mechanism(s) associated with the onset and development of cyst formation in ADPKD remains largely unknown. It is speculated that cyst formation in ADPKD implicates dysfunctional ion transport and/or abnormal cell growth in target epithelia, likely elicited by dysregulation of, PC2-modified Ca^{2+} signals. Our understanding of the role(s) PC2 plays in renal cystogenesis derives from two issues still being studied and assessed. First, the cellular localization and putative interaction of PC2 with expected partners such as PC1, remains a matter of current interest (Koulen *et al.*, 2002; Luo *et al.*, 2003). PC1 and PC2 cell expression is developmentally disconnected, such that either protein may be independent of each other, and/or associated with other likely partners (Delmas, 2004). Work by Wilson and collaborators indicates that PC1 is part of cell adhesion complexes, implying a role in cell-cell interactions, and cell–matrix adhesion. These complexes entail supermacromolecular structures containing focal adhesion proteins, including talin, vinculin, p130Cas, FAK, paxillin, pp60c-src, and α-actinin in human renal epithelial cells, when cell–matrix interactions prevail (Geng *et al.*, 2000). Conversely, PC2, acting as a TRP channel is capable of interactivity with PC1, as expected (Nauli *et al.*, 2003), but also with other TRP channels (Tsiokas *et al.*, 1999), such that its functional and regulatory properties may differ depending on location, abundance, and complexing with specific partners (Delmas, 2004). Second, different functional profiles as to how PC2 acts as a channel have been depicted from work in different cell systems (González-Perrett *et al.*, 2001; Vassilev *et al.*, 2001; Koulen *et al.*, 2002). Nonetheless, the current hypothesis is that PC2 function is implicated in Ca^{2+} signaling, as evidence suggests the ability of PC2 to mediate Ca^{2+} influx into renal epithelial cells (Luo *et al.*, 2003; Nauli *et al.*, 2003). Interestingly, other forms of renal cystic disease do not implicate directly *PKD* genes, but instead proteins associated with the axonemal machinery and ciliary structures (reviewed in Calvet, 2002, 2003; Cantiello, 2003). Thus, it is possible, that different pathophysiological events likely converge to common points of PC2 regulation. For example, the C-terminus of PC1 structurally interacts (Tsiokas *et al.*, 1997), and regulates PC2 channel function (Xu *et al.*, 2003). This provides a recognizable molecular aspect of the PC1–PC2 complex. A normal PC1–PC2 complex is required for proper sensory function of renal epithelial cells (Nauli *et al.*, 2003), whose primary cilia respond to bending by volume flow and shear stress, with Ca^{2+} entry and cell activation (Praetorius and Spring, 2001; Praetorius *et al.*, 2003). PC2 also interacts with a number of other proteins, including TRP channels (Tsiokas *et al.*, 1999; Delmas, 2004), and cytoskeletal proteins (Gallagher *et al.*, 2000; Li *et al.*, 2003a,b), providing testable hypotheses as to putative mechanisms of PC2 regulation. In the

following sections, we assessed the various aspects of PC2 regulation by the actin cytoskeleton. We determined the role of cytoskeletal dynamics in PC2 regulation, the molecular links that anchor this channel protein to actin networks, and how the three-dimensional structure of such a gel, likely acts as a tethering structure of the channel, in brief, eliciting a novel signal transducer, the PC2 channel–cytoskeleton interface.

B. *Presence of Actin and Associated Proteins and Effect of CD on Channel Activity in hST*

To assess a regulatory role of the actin cytoskeleton on PC2-mediated channel function, enriched human syncytiotrophoblast (hST), apical vesicles were reconstituted in a lipid bilayer system. Experiments were conducted in the presence of a K^+ chemical gradient, with 150 mM in the *cis* compartment and 15-mM KCl in the *trans* compartment, respectively as originally reported (González-Perrett *et al.*, 2001). Experiments, where no (or little) spontaneous activity was originally observed at the beginning of the experiment, were chosen (Fig. 1A). Addition of CD (5 μg/ml; $n = 17$) to the *cis* compartment initiated K^+-permeable ion currents (Fig. 1A–D). CD-activated membrane currents increased eightfold, from 0.023 ± 0.019 pA, to 0.217 ± 0.154 pA ($n = 8$, $p < 0.01$) at $\sim 7.42 \pm 0.28$ min, $n = 5$, after exposure to the drug. Currents were highly cation selective (Fig. 1C), and further characterized as those previously observed as mediated by PC2 (González-Perrett *et al.*, 2001), with a single-channel conductance of 135 pS ($n = 3$, Fig. 1C), and inhibition by La^{3+} and 50 μM amiloride (data not shown). The hST apical membranes that were incubated for 1–3 days at 4°C in the presence of CD (5 μg/ml), to completely collapse the actin networks, only displayed flickery, sporadic channel openings, and very little activity (Fig. 1D). Inmunofluorescence analysis of the hST vesicles (Fig. 1E), indicated that PC2 colocalized with F-actin, which was disrupted by addition of CD. Western blot analysis (Fig. 1F) determined the presence of PC2, actin and the actin-binding proteins, α-actinin, and gelsolin in the hST vesicles. This was confirmed by the colabeling of TRITC-phalloidin and antiactin antibodies, to label F-actin, and the entire actin pool, respectively (Montalbetti *et al.*, 2005b). Although both monomeric and polymeric actin, were observed in the intravesicular compartment, most F-actin displayed stronger labeling in proximity to the membrane. Incubation of hST apical membranes with CD (10 μg/ml, Fig. 1E, bottom) for 1 h at 4°C affected the presence of F-actin in the vicinity of the membrane. An extended incubation with CD (>24 h), further collapsed cytoskeleton where most actin was "detached" from the plasma membrane. These findings provided the first indication that a

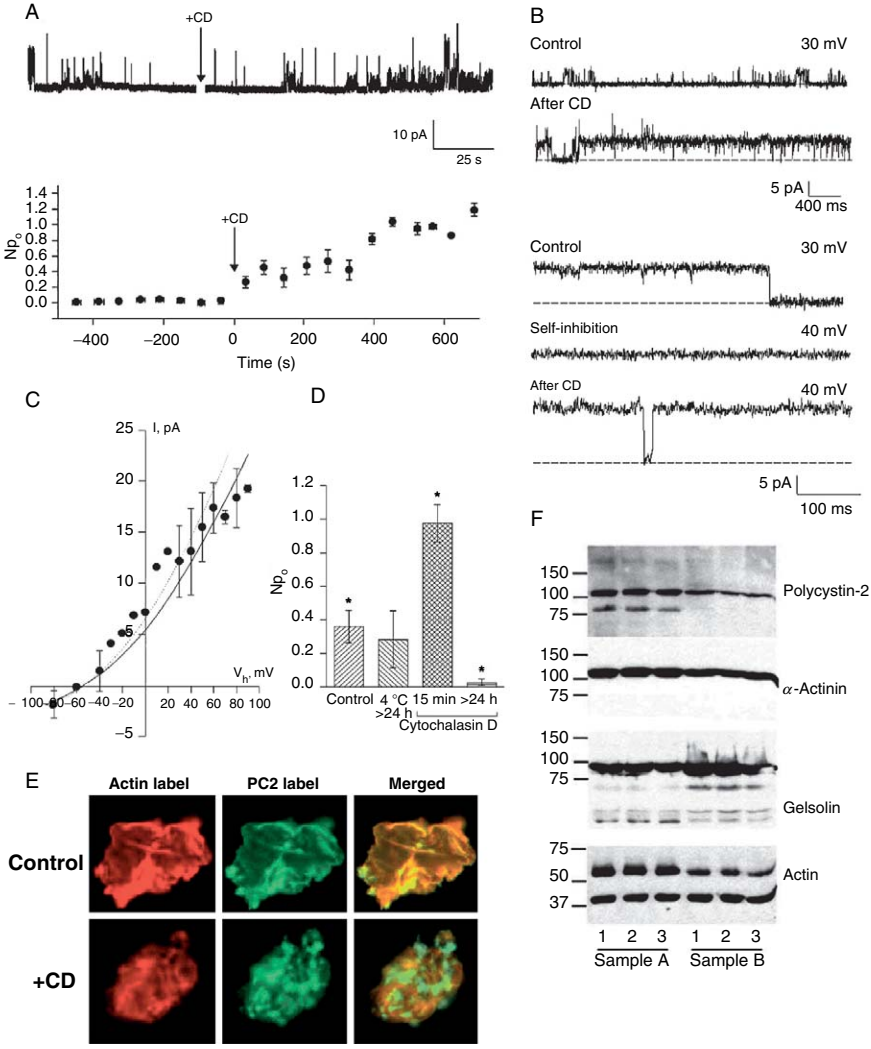

FIGURE 1 Effect of cytochalasin D on PC2 channel activity in hST. (A) Top: spontaneous ion channel activity increased after addition of cytochalasin D (CD, 5 μg/ml) to the *cis* chamber. The hST apical vesicles were reconstituted in the presence of a KCl chemical gradient, with 150 mM KCl in *cis*, and 15-mM *trans* compartments, respectively. Bottom: average channel activity before and after CD addition, is expressed as the number of channels, times the open channel probability ($n = 19$). (B) Expanded tracings of single-channel currents. CD activation preserves the single-channel conductance (135 pS, $n = 3$; $n = 19$). (C) Highly cation-selective single-channel conductance of the CD-activated K^+ permeable channels was 135 pS conductance. Experimental data (filled circles) are indicated as mean $\pm$ SEM ($n = 3$). The solid line is the fitting of data with the GHK equation. Dashed line indicates spontaneous PC2 single-channel conductance

PC2–actin cytoskeleton interface is a functional unit whereby changes in the actin structures control channel function and thus ion transport.

C. *Effect of Gelsolin and Actin on PC2 Channel Activity in hST*

To further test the nature of the changes in endogenous actin filament organization, which mediate the regulation of PC2, the effect of cytoskeletal reorganization on PC2 was further explored by addition of the Ca^{2+}-dependent actin-severing protein, gelsolin (Montalbetti *et al.*, 2005b). To support the physiological relevance of this maneuver, we first confirmed the presence of gelsolin in the hST vesicle preparation by Western blot analysis (Fig. 1F). This was the first direct demonstration of gelsolin in cortical cytoskeleton of hST (Montalbetti *et al.*, 2005b). Addition of gelsolin (40 nM) to control membranes, in which the Ca^{2+} concentration in the *cis* compartment was kept at 10 μM, increased K^+ channel activity in 15 out of 16 experiments (Fig. 2A). The Ca^{2+} concentration used for this study was similar to that used for assessing hST spontaneous K^+ channel activity (González-Perrett *et al.*, 2001). The current–voltage relationship remained as observed for the control channel (Fig. 2B). Membrane currents increased 85-fold, from 0.022 ± 0.016 pA, $n = 14$ to 1.89 ± 1.60 pA, $n = 7$, for control vs gelsolin-treated membranes, respectively ($p < 0.01$). Addition of gelsolin, either in the absence of Ca^{2+} or the presence of Ca^{2+} (10 μM) plus EDTA (10 mM), was without effect on channel activity (0.024 ± 0.018 pA, $n = 10$ vs 0.051 ± 0.027 pA, $n = 6$, $p < 0.2$, Fig. 2C, bottom). However, this lack of gelsolin effect in the absence of *cis* Ca^{2+} reversed (in five out of six experiments) after further addition of Ca^{2+} (10 mM) to the *trans* chamber (Fig. 2D). Under these conditions, membrane currents increased to 1.22 ± 0.69 pA, $n = 5$ ($p < 0.01$) in the presence of *cis* gelsolin (40 nM). The activation observed after addition of Ca^{2+} to the *trans* chamber was mediated by Ca^{2+}

(González-Perrett *et al.*, 2001). (D) Average data for mean currents before, after 10 min addition of CD, and after chronic treatment with the drug (n=8, p<0.05 between control and acute CD treatment). (E) Top: colocalization of actin filaments and PC2 in control hST vesicles. The hST apical membranes were colabeled with TRITC-phalloidin and an anti-PC2 antibody to determine the presence of actin filaments and the channel, respectively. Colocalization of F-actin and PC2 was confirmed in both the plasma membrane and the intravesicular compartment. Bottom: treatment of hST membrane vesicles with CD (10 μg/ml) for 24 h modified the actin cytoskeleton, with extensive detachment from the membrane. (F) Western blot analysis of hST apical membrane vesicles confirmed the presence of PC2 and cytoskeletal components, including actin, and the PC2-associated proteins α-actinin, and gelsolin. Treatment of hST membrane vesicles with CD (10 μg/ml) for either 1 (labeled 2) or 24 h (labeled 3) had little effect on the amount of actin and actin-associated proteins present in the vesicle preparation. Data reproduced from the *Journal of Physiology* (Montalbetti *et al.*, 2005b), with permission.

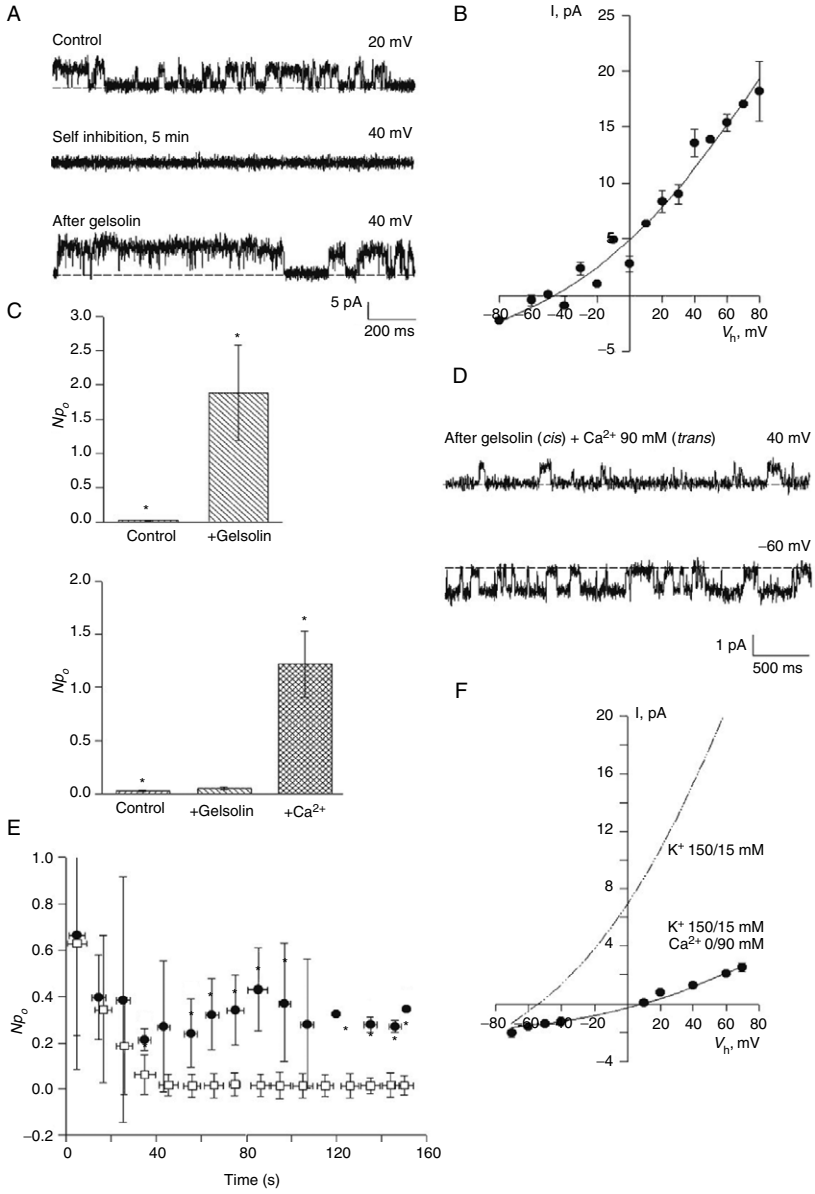

FIGURE 2 Effect of gelsolin on PC2 channel activity in hST. (A) The actin-severing protein gelsolin activated PC2 channel activity in hST membranes in the presence of 10 μM Ca^{2+} ($n = 7$). (B) Current–voltage relationship of cation-selective channel activated by addition of gelsolin is identical to that activated by CD. (C) Top: average data are the mean ± SEM of 13 and

transport through the K^+ permeable channels (Fig. 2D). The regulatory effect of actin network remodeling on PC2 channel function was confirmed by inwardly (Ca^{2+}-carrying) currents in the presence of external Ca^{2+} (90 mM). This is reflected in an increased open probability of the channel (Fig. 2E), while the single-channel conductance was much lower (Fig. 2F) as originally reported (González-Perrett *et al.*, 2001). The data are consistent with a feedback mechanism where the transported Ca^{2+} from the *trans* chamber activates "cytoplasmic" gelsolin (*cis* compartment) thus remodeling the endogenous actin network. This was confirmed by a different strategy, namely, the direct addition of exogenous actin (1 mg/ml) to spontaneously active, control hST apical membranes. Interestingly, addition of monomeric actin inhibited K^+ channel activity in 15 out of 17 experiments (Fig. 3). The mean membrane current decreased by 74.2%, from 0.048 ± 0.011 pA, $n = 5$, to 0.012 ± 0.007 pA, $n = 5$ ($p < 0.03$). This was unexpected, as most channel function activated by short CD treatment is usually mimicked by addition of polymerizing concentrations of monomeric actin (Cantiello *et al.*, 1991; Prat *et al.*, 1994, 1995). However, this inhibitory effect of actin was not observed in the presence of CD (< 15 min), in which channel activity remained high (data not shown). In chronically CD-treated membranes (>24 h), in contrast, PC2 channel activity was reactivated by addition of actin (Fig. 3C). In three out of three experiments, membrane currents increased from 0.0005 ± 0.0001 pA to 0.026 ± 0.014 pA, $n = 3$, $p < 0.05$, for the absence and presence of actin, respectively. These data are most consistent with a scenario in which competition occurs between exogenous (monomeric) actin and the endogenous pool of actin filaments, and likely channel-associated proteins. The CD and gelsolin data are in agreement with a role of actin network remodeling in the regulation of PC2 channel activity.

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6 experiments, for control ($n = 13$) and gelsolin-treated ($n = 6$) membranes, respectively. Bottom: in a Ca^{2+}-free solution, however, gelsolin (30 μM) was unable to induce channel activation. Further addition of Ca^{2+} (10 μM) to the *cis* chamber restored stimulation. Average data are the mean ± SEM for the gelsolin effect in the absence ($n = 6$) and presence of Ca^{2+} ($n = 5$) added to the *cis* chamber (30 μM). Control data were the average of 10 experiments. (D) Addition of Ca^{2+} to the *trans* chamber, reactivated channel activity in the presence, but not the absence of *cis* gelsolin. The data indicate that Ca^{2+} transport feeds back into gelsolin activation, and thus reinitiation of hST cation channel activity ($n = 5$). (E) The presence of gelsolin (100 nM) in the *cis* compartment decreases the inhibitory effect of Ca^{2+} transport through cation-permeable channels in hST. Data are the mean ± SEM ($n = 5$), obtained as Np_o of current activity at positive potentials. Asterisks indicate statistical difference at least $p < 0.05$. (F) Ca^{2+} transport through the channel reduces the single-channel conductance and shifts the reversal potential by ~60 mV (−54 to +10 mV). Data reproduced from the *Journal of Physiology* (Montalbetti *et al.*, 2005b), with permission.

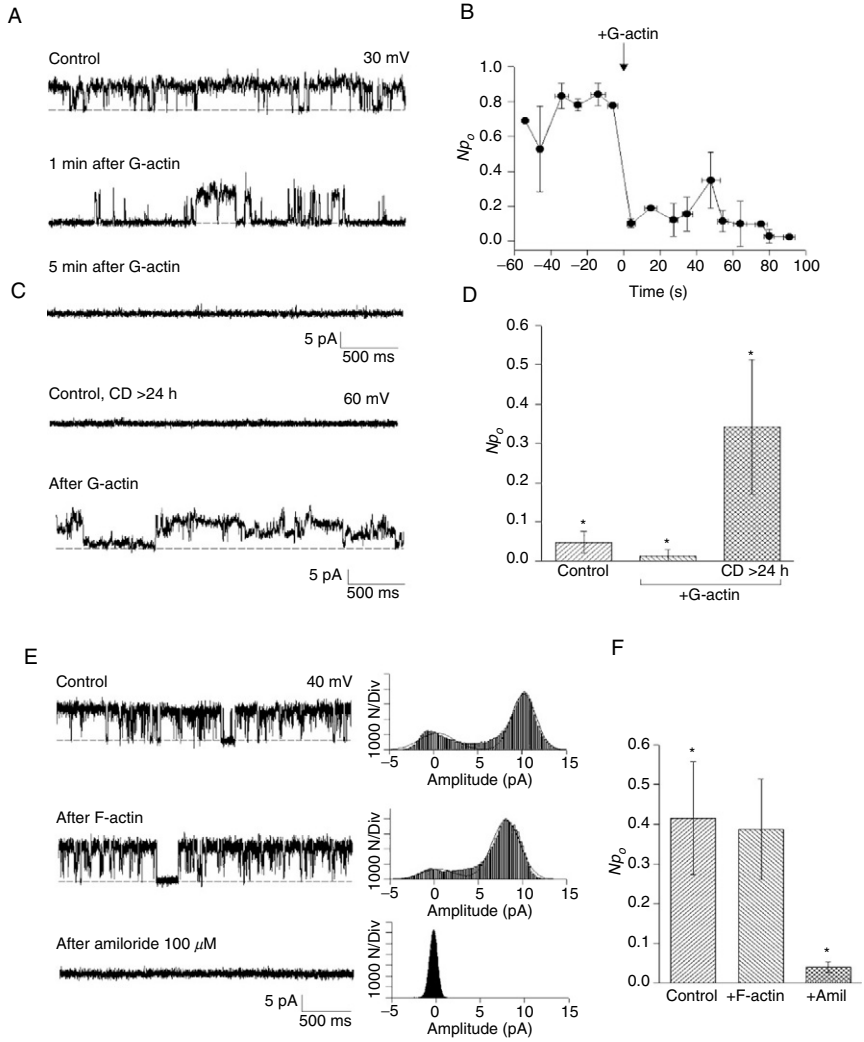

FIGURE 3 Effect of actin on cation channel activity in hST. (A) Representative single-channel tracings of hST apical membranes in asymmetrical KCl. Addition of actin (1 mg/ml) to the *cis* chamber inhibited spontaneously active ion channel currents ($n = 17$). (B) Average channel activity expressed as Np_o, where N is the total number of active channels, and p_o is the channel's open probability indicate that monomeric actin inhibited channel activity within 1 min (arrow; $n = 5$). (C) Addition of actin to chronically CD-treated membranes (>24 h), in contrast, stimulated otherwise largely quiescent membranes. (D) Summarized data are indicated as means ± SEM for control conditions ($n = 7$), and after addition of actin to acutely (15 min, center, $n = 7$) and chronically (24 h, right, $n = 3$) CD-treated membranes. While actin addition was inhibitory to control membranes, the same actin concentration was stimulatory in

IV. IDENTIFICATION OF ACTIN-BINDING PROTEIN INTERACTIONS WITH POLYCYSTIN-2

A. Interaction Between PC2 and α-Actinin Revealed by Yeast Two-Hybrid System

To begin a search for the molecular determinants of the cytoskeleton–PC2 functional interaction, we used the yeast two-hybrid system to screen proteins which interact with the intracellular N-terminus of PC2 (PC2N, amino acids M1-K215; Li *et al.*, 2005). A bait construct, pGBKT7-PC2N, was used to screen a human heart cDNA library. One plasmid isolated from the library represented the C-terminal two-thirds of human muscle-type α-actinins, which forms a complex *in vivo* in cells and tissues (Fig. 4A). The identified α-actinin-2 cDNA is a 3-kb fragment starting at nucleotide C1187 (aa L341), which encodes most of the central domains (including three spectrin-like repeats) and the C-terminus of α-actinin. Given that mammalian α-actinin has four isoforms with high sequence similarity, we further explored whether nonmuscle α-actinin-1 can also bind PC2. Indeed, α-actinin-1 associated with PC2N albeit not as strongly as the α-actinin-2–PC2N interaction (Fig. 4A). We also performed a similar yeast two-hybrid assay to determine whether the C-terminus of PC2 (PC2C, aa 682–968) also interacts with α-actinins (Fig. 4A). Interestingly, while the entire cytoplasmic PC2C showed no interaction with α-actinins 1 or 2, shorter segments within PC2C displayed strong interaction with both α-actinins (Fig. 4A). This finding suggested the possibility that a domain within the first part (aa 682–820) of PC2C may inhibit the PC2C–α-actinin interaction. We narrowed down this interaction to a smaller segment of 58 amino acids (PC2CC, aa 821–878), which is responsible for association with α-actinins. Interestingly, the segment PC2CC, which binds α-actinin, overlaps with the domain that 35 interacts with tropomyosin-1 (Li *et al.*, 2003a). On the other hand, while the spectrin-like domain II of α-actinin-2 seemed to be required for association with the PC2 C-terminal segments PC2CA (aa 821–968) and PC2CC, we found that the domain IV alone was responsible for mediating association with PC2N. Our findings were most consistent with the possibility that the PC2–α-actinin interaction actually entails at least two discrete domains in the channel protein.

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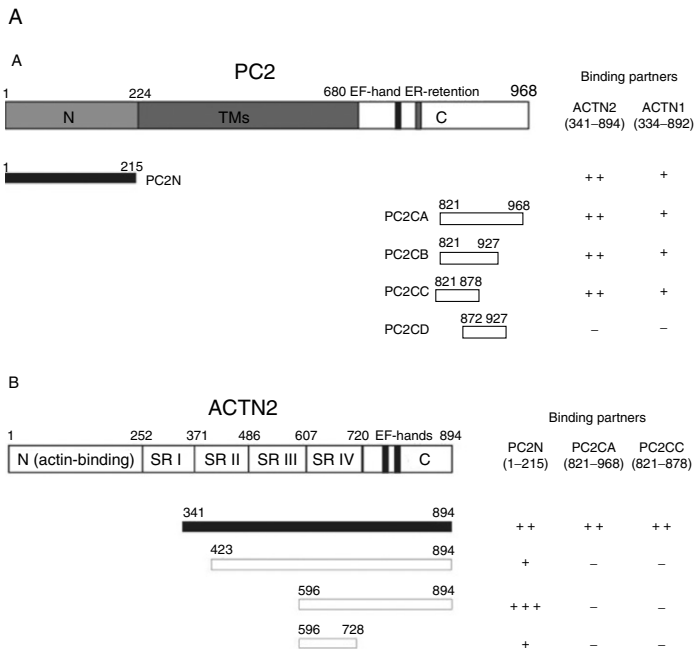
chronically CD-treated membranes ($p < 0.05$ in both cases). (E) Addition of prepolymerized actin (2 h in 150-mM KCl plus 1-mM $MgCl_2$) to the *cis* chamber was without effect on spontaneous ion channel activity (middle tracing). Channel activity was however, readily inhibited by amiloride (100 μM, bottom tracing; $n = 5$). All-point histograms to the right of each tracing indicate current amplitude. (F) Average data for conditions in (E). Data reproduced from the *Journal of Physiology* (Montalbetti *et al.*, 2005b), with permission.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Binding partners
ACTN2 (341–894)
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PC2N
+ +
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PC2CA 821 968
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PC2CB 821 927
+ +
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PC2CC 821 878
+ +
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PC2CD 872 927
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ACTN2
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371
486
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720 EF-hands 894
N (actin-binding)
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Binding partners
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PC2CA (821–968)
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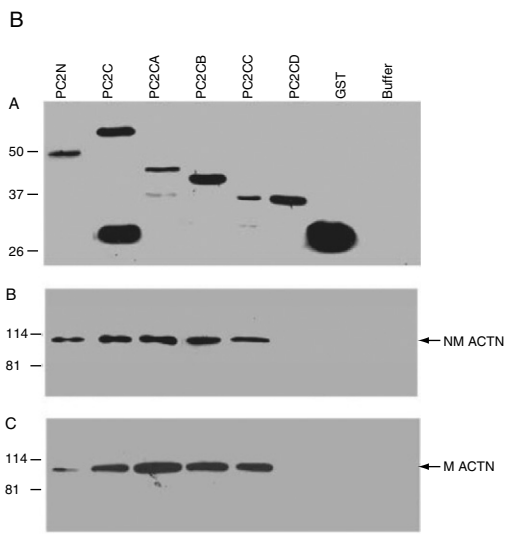
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PC2N
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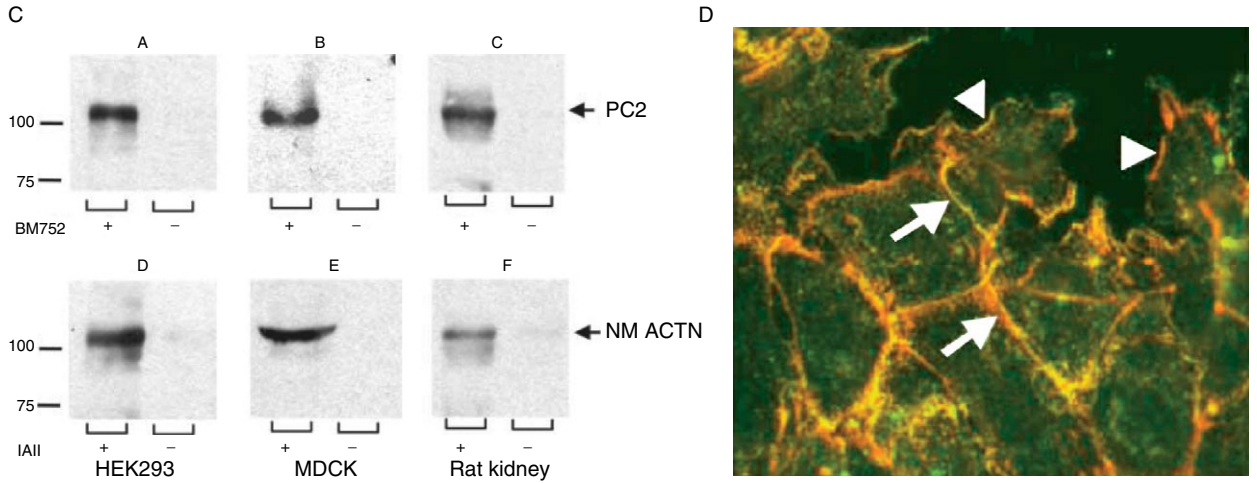


FIGURE 4 Evidence for the interaction between PC2 and α-actinin. Schematics of segments of PC2 and α-actinin (ACTN2) used to reveal interaction by yeast two-hybrid system. Solid bars indicate the interacting candidate (bait) constructs in the initial library screening. (A) Human PC2 segments with marked starting and ending amino acid residues and their association with human α-actinin-1 (ACTN1) and α-actinin-2 (ACTN2) indicated by "+++," "++," "+," and "−" for development of blue color within 1, 3, and 24 h, and no development of blue color within 24 h, respectively. (B) ACTN2 segments and their association with PC2N, PC2CA, and PC2CC. (B) Interaction between PC2 segments and α-actinins as shown by the GST pull-down assay. (A) *E. coli* extracts expressing GST-tagged PC2 polypeptides PC2N, PC2C, PC2CA, PC2CB, PC2CC, PC2CD, or GST alone were visualized by the GST antibody. (B and C) Fusion proteins were incubated with purified nonmuscle α-actinin (NM-ACTN) from chicken gizzard (B) and muscle α-actinin protein (M-ACTN) from rabbit skeletal muscle (C). Glutathione–agarose beads were used to precipitate GST epitope-binding proteins. The resultant protein samples were immunoblotted with α-actinin antibodies BM75.2 (nonmuscle) or EA53 (muscle). Molecular mass markers (in kDa) are shown. (C) Interaction between endogenous PC2 and α-actinin in cultured cells, and the rat kidney. Total cell lysate from HEK293 (A) and MDCK cells (B) and total protein from rat kidney (C) were precipitated with either nonmuscle α-actinin antibody (BM75.2) or nonimmune mouse IgG. Precipitates were detected with anti-PC2 antibody (1A11). In reciprocal co-IP experiments, cell lysates from HEK293 (D) and MDCK cells (E) and total protein from rat kidney (F) were precipitated with 1A11 or nonimmune mouse IgG. The precipitates were detected with BM75.2. (D) Cellular colocalization of PC2 (green, 1A11 antibody) and nonmuscle α-actinin (red, BM75.2 antibody) in subconfluent MDCK and IMCD cells. Triangles and arrows indicate the plasma membrane and cell–cell junction localization, respectively. Data reproduced from *Human Molecular Genetics* (Li *et al.*, 2005), with permission.

B. In Vitro *and* In Vivo *Binding of PC2 with α-Actinins*

To confirm the interaction between PC2 and α-actinins, we used a glutathione *S*-transferase (GST) fusion protein affinity-binding method (Fig. 4B). Polypeptides, including PC2N, PC2C, PC2CA, PC2CB (aa 821–927), PC2CC, and PC2CD (aa 872–927) were fused in frame with a GST epitope and expressed in bacteria (BL21; Li *et al.*, 2005). The PC2 fusion peptides were incubated with purified α-actinin. Using monoclonal α-actinin antibodies, we observed that GST-PC2N, -PC2C, -PC2CA, -PC2CB, and -PC2CC, but not GST-PC2CD or controls (GST alone and buffer without fusion protein lysates), coprecipitated with both nonmuscle and muscle α-actinin (Fig. 4B). These results confirmed that both the PC2 amino terminus and the small C-terminal segment 821–878 of PC2 interact with α-actinin. GST-PC2C showed a clear binding with α-actinins. However, less amount of GST-PC2CA still pulled down slightly more α-actinin, compared with GST-PC2C. This finding is in agreement with the observation from our yeast two-hybrid assay that the PC2C segment (aa 682–820) exhibits an inhibitory effect on the PC2C–α-actinin interaction. To determine whether PC2 also interacts with α-actinin *in vivo*, we coimmunoprecipitated (co-IP, Fig. 4C) both proteins from MDCK cells, and rat kidney (other cell lines and tissues were also tested; Li *et al.*, 2005). Using antibodies against PC2 (1A11), muscle α-actinin (EA53), and nonmuscle α-actinin (BM75.2), we detected the associated proteins by immunoblotting. PC2 was detected in the immunoprecipitate from rat kidney tissue using muscle α-actinin antibody EA53. Reciprocal co-IP using a PC2-specific antibody (1A11) also precipitated muscle α-actinin. These data confirmed that PC2 interacts with muscle α-actinin (Fig. 4C) *in vivo*. Under the same conditions PC2 was immunoprecipitated from MDCK cells using BM75.2 (Fig. 4C). A reciprocal signal corresponding to nonmuscle α-actinin from these cell or tissue lysates was observed in the immunoprecipitate of the same cell/tissue lysates using 1A11. Taken together, these results demonstrate that endogenous PC2 and α-actinins form a complex *in vivo* in cells and tissues. The colocalization of endogenous PC2 and α-actinin was further explored by immunofluorescence in epithelial MDCK cells (Fig. 4D). We found that nonmuscle α-actinin exhibited clear cell surface/periphery localization, notably at cell-cell contacts. Faint staining was also observed along stress fibers and in the perinuclear region. Consistent with recent reports (Scheffers *et al.*, 2002, 2004; Luo *et al.*, 2003), we also found that subconfluent MDCK cells express endogenous PC2 in both the cytoplasm and the plasma membrane and at the cell–cell contacts (Fig. 4D). The fluorescence patterns of α-actinin and PC2 substantially overlapped both in the plasma membrane and at cell–cell junctions, indicating that the two proteins colocalize in these cells.

C. Functional Modulation of PC2 by α-Actinin

α-Actinin has been shown to regulate a number of ion channels, including Ca^{2+} and K^+ channels and NMDA receptors (Krupp *et al.*, 1999; Maruoka *et al.*, 2000; Sadeghi *et al.*, 2002). We thus examined whether α-actinin also modulates PC2 channel function in hST apical membrane vesicles (Fig. 5). Substantial colocalization of PC2 with α-actinin was observed in hST apical membrane vesicles and partially altered by the actin filament-disrupting agent cytochalasin D (10 μg/ml; Fig. 5C). This is in agreement with the overlapping distribution of PC2 and actin filaments in hST where changes in actin filament organization modifies PC2 channel activity (Montalbetti *et al.*, 2005b). After hST vesicles were reconstituted in the lipid bilayer system, addition of α-actinin to the *cis* chamber substantially increased PC2 cation channel activity (Fig. 5A and B). To further assess whether α-actinin regulates PC2 channels by direct interaction with the channel protein, we replicated the experiment with purified PC2 (Li *et al.*, 2005). We found that addition of nonmuscle α-actinin to the *cis* chamber (cytoplasm) elicited a substantial increase in PC2 single-channel activity (Fig. 6A). In average, the mean single-channel currents increased 15-fold by α-actinin. The currents averaged 1.7 ± 0.5 pA, $n = 7$ for controls, and 27.0 ± 5.4 pA, $n = 6$, in the presence of α-actinin ($p < 0.01$). However, α-actinin did not significantly alter the current-voltage relationship of the main (largest) conductance of PC2 (Fig. 6A, right), confirming that the stimulatory effect of α-actinin on PC2 is indeed mediated by the control of its open probability. Interestingly, addition of either monomeric, or F-actin, was without effect on the isolated channel protein (Fig. 6B), strengthening the contention that anchoring proteins are a requirement for the channel to connect to the actin cytoskeleton. This also poses the interesting possibility that other actin-binding proteins already known to interact with PC2, may exert novel regulatory effects, based on competition with α-actinin, and/or direct binding to PC2 (Li *et al.*, 2003a,b; Section IV.A–B).

V. EFFECT OF HYDROOSMOTIC PRESSURE ON PC2 CHANNEL FUNCTION: ROLE OF THE CYTOSKELETON IN OSMOSENSORY FUNCTION

A. Effect of Hydrostatic and Osmotic Pressure on PC2 Channel Regulation

Studies have raised the interesting possibility of a possible role of PC2 in mechanotransduction of environmental signals in renal epithelial cells. PC2 has been localized to the primary cilium of renal cells (Pazour *et al.*, 2002;

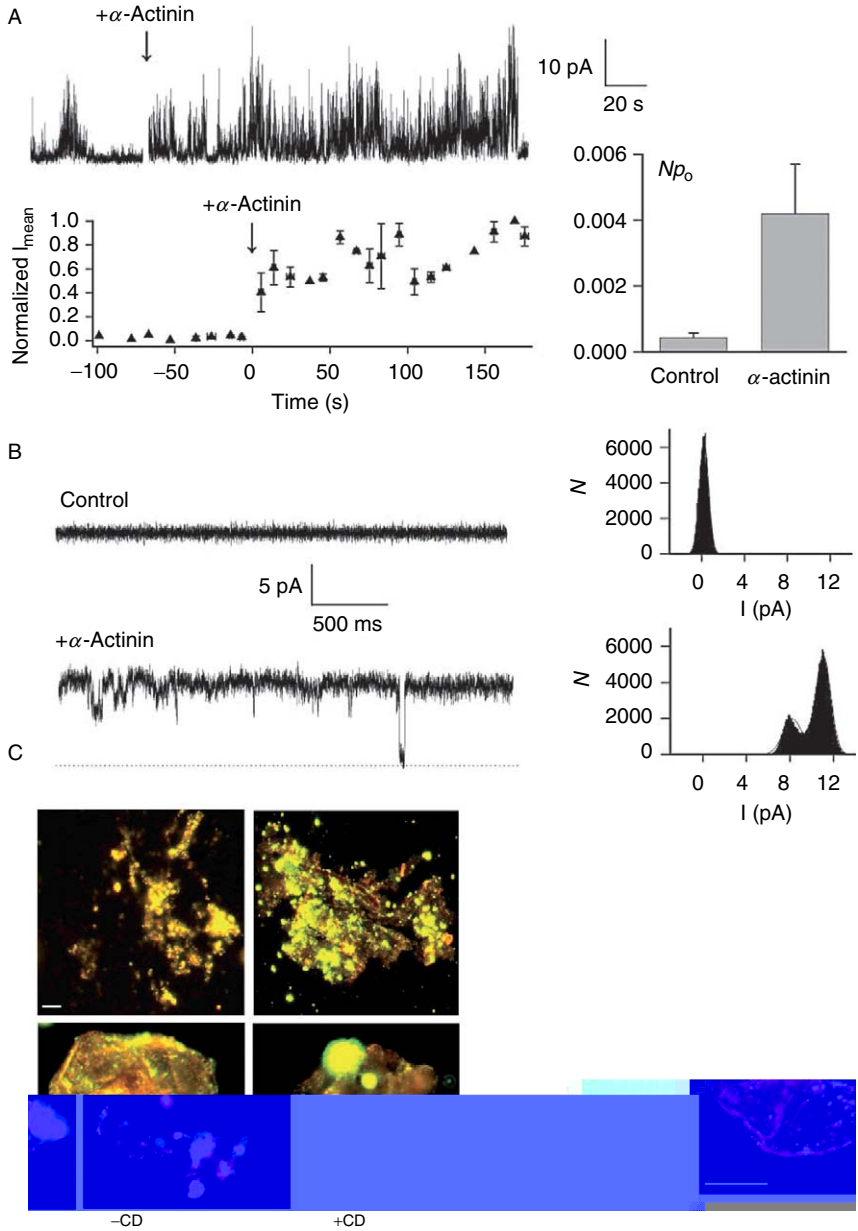


FIGURE 5 Colocalization of, and regulation by, α-actinin with PC2 in hST vesicles. (A) Upper left: representative tracings at 40 mV before and after addition of α-actinin (as indicated). Lower left: average data are indicated as normalized mean currents ($n = 6$).

Yoder *et al.*, 2002), which is thought to be a sensory organelle (Praetorius and Spring, 2001; Pazour and Witman, 2003). We further observed that PC2 is a functional channel in primary cilia (Raychowdhury *et al.*, 2005). The nature of a possible mechanotransduction in PC2 is, however, still unknown. To test a potential role of PC2 in mechanotransduction, we reconstituted hST apical membranes (Montalbetti *et al.*, 2005a). For these studies, we relied on changes in two "environmental" forces, which may convey physical changes to the preparation, namely, changes in osmotic and/or hydrostatic pressure. The experimental setup we used to test whether hydroosmotic pressure might control PC2 function is shown in Fig. 7A, inset. Basal PC2 channel activity was observed in the presence of an osmotic gradient ($\Delta\Pi$), initially imposed in a KCl channel gradient (150- and 15-mM KCl, *cis* and *trans*, respectively, Fig. 7A) as originally reported (González-Perrett *et al.*, 2001). We tested both, changes in osmotic pressure elicited by either compensation of the $\Delta\Pi$ by addition of salt to, and/or imposition of a ΔH by addition of volume to the *trans* compartment (or reduction in *cis* volume or both). Channel activity was first observed in the absence of $\Delta\Pi$ (150-mM KCl in *cis*, and 150-mM NaCl in *trans* compartments, respectively, Fig. 7A). A change in hydrostatic pressure (ΔH), namely a decrease in volume in the *cis* chamber, accompanied by an augment in the *trans* solution, increased PC2 channel activity after rundown. This finding would indicate that changes in the physical parameters of the reconstituted membrane convey a regulatory mechanism to PC2 channel function. To further assess this phenomenon, the osmotic contribution of this activating effect was also assessed. We first determined PC2 channel function in the presence of a KCl chemical gradient (Fig. 7B). Under these conditions, an "outward" (in-to-out) osmotic gradient was imposed. This basal $\Delta\Pi$ was then eliminated by addition of NaCl (150 mM, Fig. 7B) to the *trans* compartment. Channel activity immediately (<5 s) changed after addition of salt, as expected by the elimination of $\Delta\Pi$. This is shown as a decrease in both single-channel conductance (Fig. 7B, center, bottom), and a change in the channel's open probability (p_o, $n = 5$, Fig. 7B, bottom right). Interestingly,

Right: average channel activity is shown as the number of channels times the channel's open probability (Np_o) before and after application of α-actinin ($n = 6$). (B) Representative single-channel tracings (left panels) at 40 mV of reconstituted hST apical membranes in asymmetrical KCl solution. The corresponding all-point histograms are shown on the right. (C) Distribution of endogenous α-actinin and PC2 was also determined in hST apical membrane vesicles. The two proteins substantially colocalized with each other (PC2, green; α-actinin, red). Treatment with cytochalasin D (10 μg/ml for 24 h) showed sporadic detachment of this interaction (merge, yellow). Horizontal bars = 20 μm. Data reproduced from *Human Molecular Genetics* (Li *et al.*, 2005), with permission.

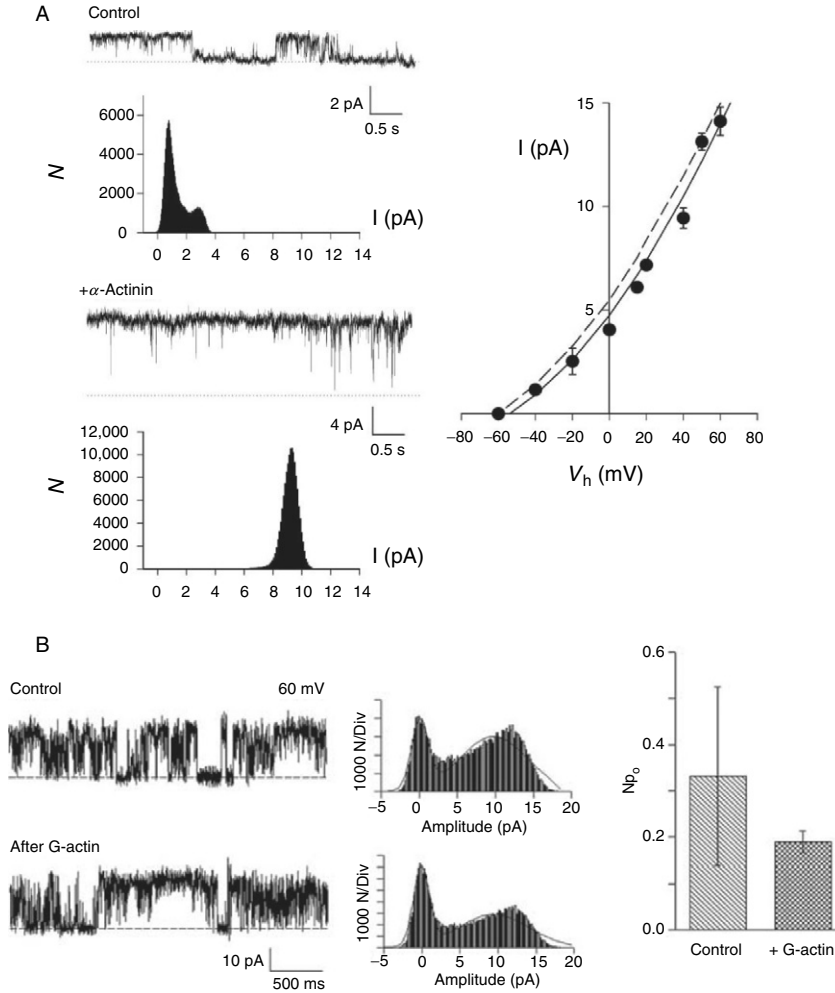


FIGURE 6 Effect of α-actinin and actin on the isolated PC2 channel. (A) Top left: representative single-channel tracings and corresponding all-point histograms (below each tracing) of *in vitro* translated PC2, reconstituted in a lipid bilayer system. The data indicate the increase in single-channel conductance after addition of nonmuscle α-actinin to the *cis* chamber. Data were obtained at 40 mV. Right: current–voltage relationship is shown of the α-actinin-activated channel. Dashed and solid lines correspond to the control condition (González-Perrett *et al.*, 2001) and in the presence of α-actinin ($n = 5$), respectively. (B) Effect of actin on the purified PC2 channel. Left: monomeric actin (1 mg/ml) was added to the *cis* compartment, with no effect on PC2 channel. All-point histograms of current amplitudes are shown on the right. Right: average data ($n = 5$) for control and +actin conditions. Data were not statistically different, $p < 0.4$. Data reproduced from *Human Molecular Genetics* (Li *et al.*, 2005), with permission.

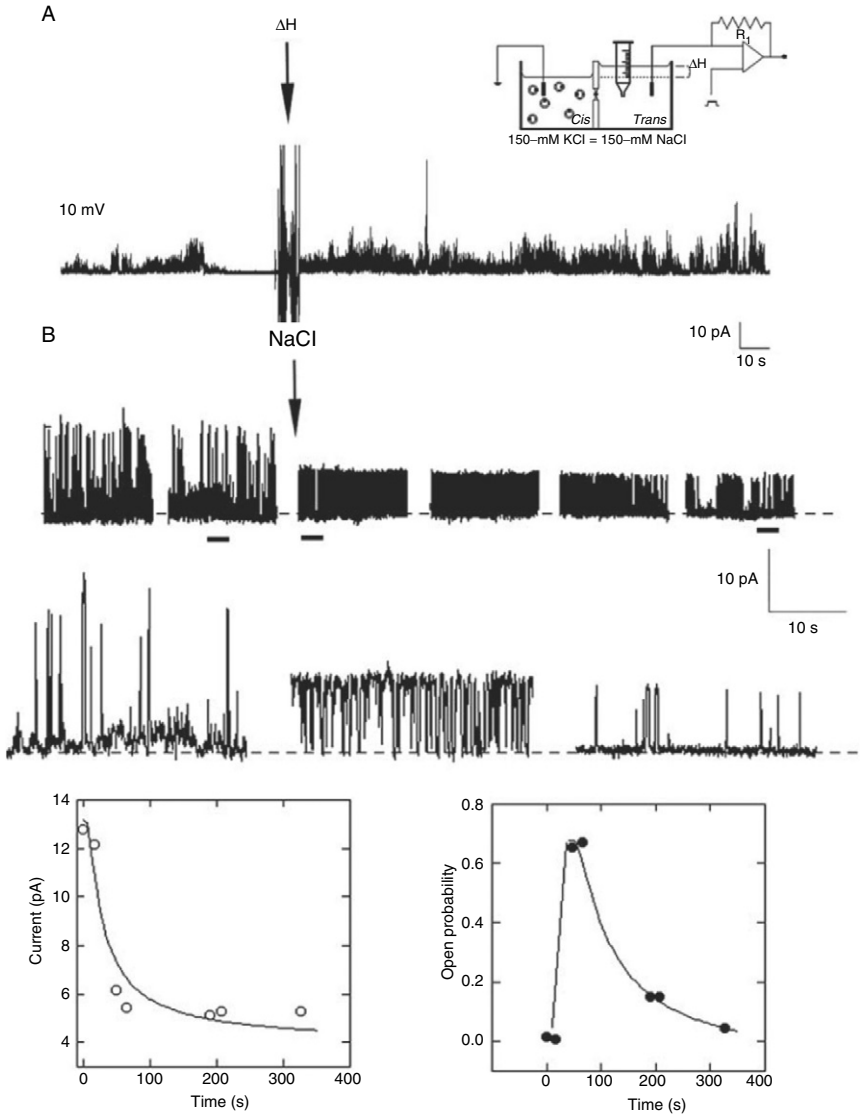


FIGURE 7 Effect of hydroosmotic pressure on hST PC2 channel activity. (A) PC2 channel activity was observed under conditions resembling a cell membrane *in situ*, namely the presence of outward KCl, and inward NaCl chemical gradients (isoosmotic condition). After spontaneous channel rundown, the membrane was exposed to a ΔH by addition of saline solution to the *trans* compartment, which increased the membrane currents, with an onset time varying among the various preparations ($n = 7$). Inset: experimental setup to assess the role of the physical membrane parameters on PC2 channel function. Basal channel activity was originally observed

the decay kinetics of p_o increase (Fig. 7B, bottom right) followed a time constant several seconds long, which is inconsistent with the establishment of $\Delta\Pi$. A similar phenomenon was observed by addition of KCl (data not shown), suggesting an osmotic response (Montalbetti *et al.*, 2005a). To further assess the nature of this activation process, osmotically uncompensated ($\Delta\Pi > 0$) membranes were subjected to a hydrostatic gradient (ΔH), in the presence of a KCl chemical gradient (150 and 15 mM, *cis* and *trans*, respectively; Fig. 8A). Cation channel activity was first allowed to spontaneously inactivate. We then imposed a ΔH, which induced channel reactivation, which was delayed in average by 9 s (Fig. 8A, right). The delay activation is again inconsistent with a "compliance" effect on membrane elasticity induced by ΔH, which should be immediate. This stimulatory effect was thought to implicate instead changes in the cytoskeletal structures present in the vicinity of the channels. To test the hypothesis that a PC2–cytoskeletal interaction is actually required for the hydroosmotic activation of the channel, we first reconstituted the purified channel protein instead (Fig. 8B). PC2, exposed to ΔH (in the presence of a KCl chemical gradient), was unable to respond with an increased channel activity. This is consistent with the finding that addition of actin alone was unable to activate the isolated PC2 channel, further suggesting the requirement of actin-associated proteins such as α-actinin (Section IV, above). To confirm this hypothesis, hST vesicles were first treated with the actin cytoskeleton disrupter cytochalasin D (10 μg/ml) for 24 h to collapse the intravesicular cytoskeleton prior to reconstitution. CD-treated apical hST vesicles displayed low channel activity (Fig. 8C). Establishment of ΔH was without a stimulatory effect, such that channel activity disappeared even in the presence of a hydroosmotic gradient. Thus, the stimulatory effect

in osmotically challenged ($\Delta\Pi$) membranes, which were then subjected to a compensatory change in osmotic pressure ($\Delta\Pi = 0$), by addition of either KCl or NaCl, to the *trans* compartment. Conversely, a hydrostatic gradient (ΔH) was imposed under $\Delta\Pi$ and $\Delta\Pi = 0$ conditions, by means of either a decrease in *cis* volume, and/or in addition of volume to the *trans* compartment. Thus, changes in $\Delta\Pi$ were either used instead, or in addition to ΔH, to assess hydroosmotic changes in PC2 channel activity. (B) Effect of osmotic compensation on hST PC2 cation channel activity. A reduction of the imposed osmotic gradient (150-mM vs 15-mM KCl in *cis* and *trans* compartments, respectively) by addition of NaCl (150 mM) to the *trans* compartment, increased PC2 channel activity. Top: both the single-channel conductance and the open probability of the channel were modified by the osmotic compensation (addition of *trans* NaCl, arrow). Expanded tracings below indicate each region of the top tracing expanded under a horizontal bar. Bottom: kinetic changes in single-channel currents and open probability associated with the compensatory osmotic decrease. While the single-channel currents remained lower than their respective controls after elimination of the chemical gradient, (left) the open probability rapidly increased, followed by a slower decrease to control levels (right, $n = 3$). Data reproduced from *Pflügers Archiv* (Montalbetti *et al.*, 2005a), with permission.

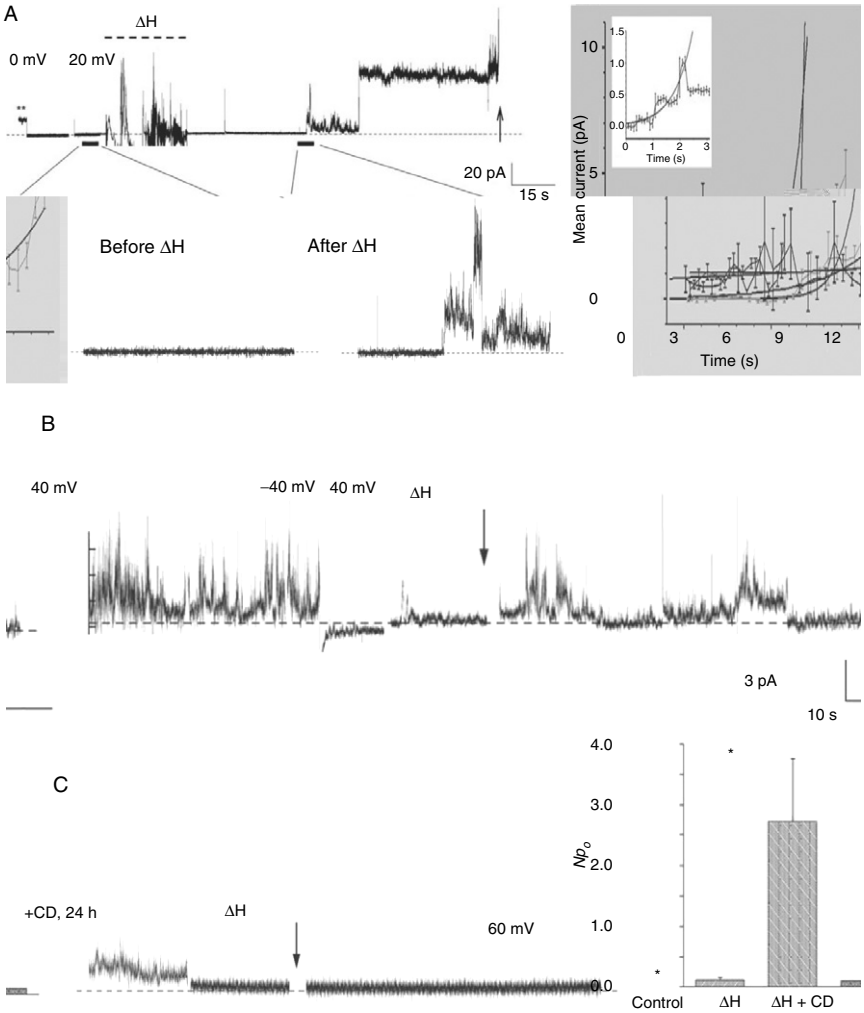


FIGURE 8 Effect of hydrostatic pressure on PC2 cation channel activity in hST. (A) Left: PC2 channel activity was observed under basal ($\Delta\Pi > 0$) conditions in the presence of an outward KCl chemical gradient. Asterisks indicate spontaneous channel activity. After spontaneous channel rundown, the membrane was exposed to ΔH by addition of saline solution to the *trans* chamber, which increased the membrane currents, with a lag time. The upward arrow indicates breaking of the membrane ($n = 3$). Right: average currents from three different experiments are shown. The kinetics of channel activation was fitted with a single exponential in two, and a straight line in one experiment. Inset shows activation kinetics after addition of NaCl to the *trans* compartment, for comparison. (B) The effect of hydroosmotic pressure was also determined in the reconstituted purified PC2 channel. Channel activity was first observed in the presence of a KCl chemical ($\Delta\Pi > 0$) gradient. Channel activity was voltage-inactivated as

of ΔH observed under control conditions requires an organized actin cytoskeleton to respond. The combined studies indicate that the PC2-cytoskeleton interface is a critical part of the transduction mechanism for channel regulation.

VI. THE CHANNEL–CYTOSKELETON INTERFACE: STRUCTURAL–FUNCTIONAL CORRELATES

A. Mechanosensitivity and the Lipid Bilayer

Mechanosensitivity of ion channels has been associated with two main likely transducers of mechanical force. These are either changes in the properties of the membrane lipid bilayer, or the more complicated, and thus less described in detail, interactions with channel-associated proteins, such as the cytoskeletal components (Sackin, 1994). It is thus not surprising that a clearer molecular picture has only been developed for the specific components of the lipid bilayer, forwarding the well-established description of channel regulation by changes in the physical properties of the membrane (Hamill and McBride, 1993; Hamill and Martinac, 2001; Markin and Sachs, 2004). Several types of deformation have been postulated for mechanosensitive channels to undergo transitions from closed to open as transduced directly by restructuring of membrane lipids (Markin and Sachs, 2004). A channel can change its in-plane area, such that an increase in the in-plane area induces stretch activation, while the opposite elicits stretch inactivation (Morris and Sigurdson, 1989). Another type of mechanosensitivity occurs if the channel changes its shape, transducing tension to the bilayer, which combined, can result in a torque, manifested as a tendency of the membrane to bend, and change its shape (Volkov *et al.*, 1998). The channel's open probability would then be sensitive to this torque (Petrov and Usherwood, 1994; Markin and Sachs, 2004). Yet another type of deformation may entail changes in length of the channel complex without a change in shape, such that as a result, a hydrophobic mismatch between the channel and the surrounding lipid bilayer occurs. A stretched bilayer would decrease its

reported (González-Perrett *et al.*, 2002), by switching the holding potential to −40 mV. Further exposure of the membrane to ΔH did not comparatively further increased PC2 channel activity ($n = 3$). (C) Left: PC2 channel activity from CD-treated (>24 h) hST membranes was observed in the presence of a KCl chemical ($\Delta\Pi > 0$) gradient. Imposition of ΔH had no effect on the membrane currents. Right: mean data ± SEM for the control and ΔΠ conditions, and for the CD-treated membranes ($n = 7$). Data reproduced from *Pflügers Archiv*. (Montalbetti *et al.*, 2005a), with permission.

thickness, changing the lipid–channel interaction, which in turn would transfer this mismatch to the energetic equilibrium between open and closed states of the channel under tension. In the context of TRP channel function, however, which form mechanosensitive transducers, and have potential links to the cytoskeleton, the possibility exists for more than one form of mechanotransduction to take place. Maroto *et al.* (2005) showed that TRPC1 is a component of the amphibian mechanosensitive channel. In those studies it was observed that changes consistent with membrane stretch, but not cytoskeletal rearrangement might be responsible for this response. Future experiments may be required to assess the functional role of the N-terminal ankyrin domains in this response. A similar question applies to TRPA1 also implicated in mechanosensory function (Corey *et al.*, 2004), which contains an even longer ankyrin structure, consistent with a tethering mechanism of gating and regulation.

B. Cytoskeletal Interactions with PC2

Our studies demonstrate that changes in the actin cytoskeleton play an important role in conveying regulatory properties to PC2 (TRPP2). These studies clearly define specific molecular interactions between the cytoskeletal components and the ion channel, such that PC2 channel function is largely mediated by the state of the actin cytoskeleton. Most of our studies were conducted in hST apical membranes, where endogenous PC2 is abundantly expressed and functional (González-Perrett *et al.*, 2001). We originally observed that early disruption of the attached cytoskeleton by CD activates PC2 channel function in the hST. We expanded this evidence to place it in the context of endogenous regulation by physiological components of the actin cytoskeleton. The CD effect on PC2 was mimicked by the actin-severing protein gelsolin, in the presence, but not the absence of Ca^{2+}. Thus, the stimulatory effect of the Ca^{2+}–gelsolin complex but not gelsolin alone indicates that cleavage of endogenous actin filaments by Ca^{2+}–activated gelsolin is the triggering mechanism, in agreement with the acute effect of CD. Further, we also found that the activation of gelsolin can also be elicited by *trans* Ca^{2+}, suggesting that Ca^{2+} transport through the channel is itself a feedback mechanism, involving channel-associated proteins. Interestingly, we found that addition of monomeric actin to control membranes had a largely inhibitory effect under control conditions. This was surprising in view that actin rapidly polymerizes under our experimental conditions. However, we observed in contrast a stimulatory effect after complete collapse of the endogenous cytoskeleton induced by a chronic CD incubation period (>24 h). This suggested to us a PC2 regulatory mechanism by which competition of

actin monomers with either the channels themselves or tightly associated proteins, allow binding and likely changes in the actin conformations not observed for the monomer itself. The most likely scenario is that actin monomers compete with PC2-associated prepolymerized actin filaments. Regulation of cation channels in hST by gelsolin and Ca^{2+} further suggests an involvement of organized actin (F-actin) in the vicinity of the channel responsible for the currents reported in the present study.

C. In Search of the Molecular Link

The above experiments provided a clear picture that a dynamic remodeling of cortical cytoskeleton in proximity to the membrane affects and indeed modulate the channel properties of PC2. Insofar as this is concerned it was also clear that despite this phenomenon had been evidenced in numerous channels with different outcomes, actin binding to the channel protein was not in itself the molecular mechanism of regulation. Interestingly, PC2 does not display ankyrin repeats or any obvious consensus domains in the protein that would allow tight interaction between the channel complex and the cytoskeleton. However, studies have suggested a potential mechanosensory for the PC1–PC2 complexes, which are expressed in primary cilia of cultured renal epithelial cells (Pazour *et al.*, 2002; Yoder *et al.*, 2002), might function in transducing environmental information (Nauli *et al.*, 2003). Interestingly, proteins necessary for the assembly or function of primary cilia including cystin, polaris, inversin, and kinesin II also cause polycystic kidney diseases (Ong and Wheatley, 2003). Primary cilia are microtubular organelles, which seem to exclude structural actin. Recent studies do indicate, however, that PC2 also interacts with elements of the actin cytoskeleton. Hax-1, a cytoskeletal protein that interacts with the F-actin-binding protein cortactin, was observed to interact with PC2 (Gallagher *et al.*, 2000). Moreover, Chen and collaborators found that two cytoskeletal proteins, troponin-I (Li *et al.*, 2003b) and tropomyosin-1 (Li *et al.*, 2003a) directly bind to PC2, further strengthening a link between cytoskeletal dynamics and the PC2 channel.

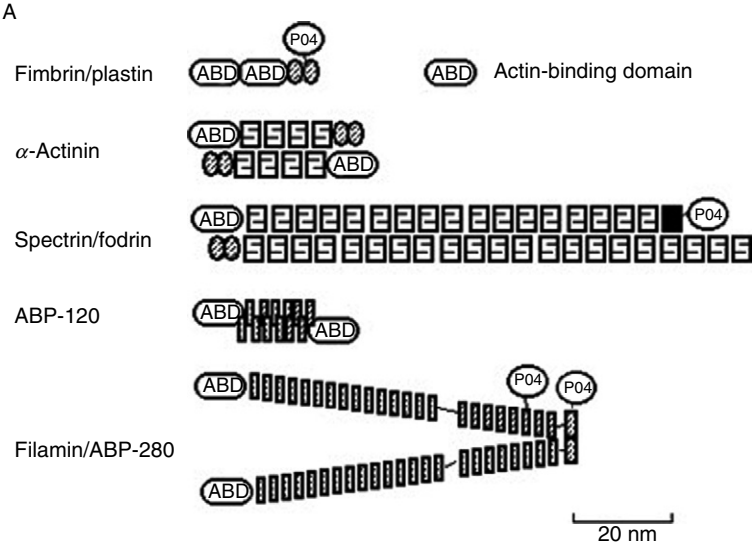
α-Actinin, is a widely distributed actin-bundling protein, which is prominently located in cell–cell and cell–matrix adhesion complexes, which associate with integrin receptors at cell–matrix focal contacts and the cell-cell adhesion belt (Otey *et al.*, 1993; Nieset *et al.*, 1997). Alpha-actinin is present in a number of renal cell types, including epithelial and blood vessel cells. Evidence for the role of α-actinin in renal disease includes the finding that experimental nephritic syndrome can be induced by upregulation of α-actinin (Kaplan and Pollak, 2001), and that mutations in α-actinin-4 cause familial focal segmental glomerulonephritis (Kaplan *et al.*, 2000). α-Actinin has also

been reported to exhibit tumor suppresser activity. Nonmuscle α-actinin overexpressing mouse NIH 3T3 fibroblasts, for example, display a significant reduction in cell motility, while cells with reduced expression of α-actinin have an increased cell motility (Gluck and Ben Ze'ev, 1994). A number of reports have established that α-actinin also regulates the activity of a number of channels. The muscle actinin isoform, α-actinin-2, for example, directly binds and modulates channel gating and current density of the voltage-gated K^+ channel Kv1.5 (Maruoka *et al*., 2000). Alpha-actinin-2 was also found to bind the NR1 and NR2B subunits of the NMDA-type glutamate receptor and regulate its channel function (Shieh and Zhu, 1996; Rycroft and Gibb, 2004). It was revealed that muscle type α-actinin regulates L-type Ca^{2+} channel function (Sadeghi *et al*., 2002). Our present data demonstrate that α-actinin binds directly to, and connects PC2 to the actin cytoskeleton (Li *et al*., 2005). These data demonstrate that PC2 physically and functionally interacts with α-actinins. This interaction was documented by a variety of methods, including the yeast two-hybrid system, that helped identify it, and *in vitro* biochemical assays, immunofluorescence, and coimmunoprecipitation in cultured renal cells and tissues, which confirmed its widespread distribution and physiological relevance of this interaction. Further, a functional interaction exists between α-actinin and PC2, whereby its channel activity can be substantially increased in the presence of this actin-bundling protein but the absence of any other cytoskeletal elements. It is thus likely that we have determined one of the key elements that anchor PC2 to the actin cytoskeleton. It is, however, interesting to note that α-actinin is also an amphipathic protein (Meyer and Aebi, 1989), which has been reported to bind specific phospholipids (Greenwood *et al*., 2000; Fraley *et al*., 2003; Corgan *et al*., 2004). This association decreases α-actinin bundling activity through competitive block of the interaction between its actin-binding domain (ABD) and the actin filament. In the presence of lipids the binding affinity between α-actinin and F-actin changes (Meyer and Aebi, 1989), such that cytoskeletal proteins interaction to PC2 may also be regulated by lipid bilayer components, which likely affect this interaction.

D. Elastic Properties of Actin Networks

F-actin networks are a major constituent of the cellular cytoskeleton, which determines, to a large degree, the mechanical properties of cells (Janmey *et al*., 1990a; Xu *et al*., 2000). However, the rheological properties of *in vitro* F-actin networks are quite different from those of cells, often by several orders of magnitude (Gardel *et al*., 2004a,b). This stresses the

A



B

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  Human T-plastin (208) VVNIGAEDLRAGKPHLVLGLLWQIIKI (234)
  Chicken fimbrin (210) VVNIGSQDLQEGKPHLVLGLLWQIIKV (236)
Chicken α-actinin (107) LVSIGAEEIVDGNVKMTLGMIWTIILR (133)
    Human fimbrin (477) LVGIAGHDLNEGNPTLTLALIWQLMRR (503)
  Human dytrophin (95)  LVNIGSTDIVDGNHKLTLGLIWNIILH (121)
   Filamin/ABP280 (121) LVSIDSKAIVDGNLKLILGLIWTIILH (147)
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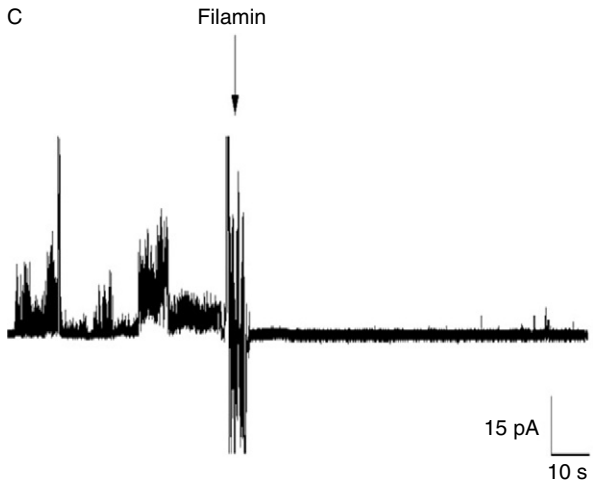

importance of actin-binding proteins and their regulatory mechanisms in the mechanical stability of cells. Actin assembly in the presence of actin cross-linking proteins such as filamin, for example, acquires mechanical properties resembling those of a gel (Brotschi *et al.*, 1978; Stossel, 1982), the rigidity of which is proportional to the concentration of actin-binding protein added (Hartwig and Stossel, 1981; Stossel, 1982). Thus, cross-linked F-actin is more resistant to deformation and water flow in response to stress than an equivalent concentration of pure actin (Ito *et al.*, 1987). During the last 30 years at least 30 proteins have been identified (Kreis and Vale, 1999), which either cross-link or bundle F-actin in various cell domains. Several proteins that cross-link actin filaments also promote bundling of actin filaments (Shizuta *et al.*, 1976; Bretscher and Weber, 1980; Bretscher, 1981; Blanchard *et al.*, 1989). It is expected that this abundance in likely similar protein function generate a redundancy and complementation in cellular responses. Fascin, α-actinin, and the filamins, may all have overlapping roles, and complementary functions (Tseng *et al.*, 2001), enabling wider variety to the mechanical responses of cellular actin networks. The perpendicular branching of actin by filamins, for example, increases the isotropy of F-actin by preventing bundle formation (Hartwig and Stossel, 1981). This suggests that, *in vivo*, F-actin may be at any given time, cross-linked by a variable number of actin-binding proteins, which are essential in determining then specific elastic properties of the actin cytoskeleton. It is interesting to note, however, that little is known about the consequences of the role of the dynamic changes in cytoskeletal structures as they interact with, and potentially regulate ion channels. Even when a number of actin-binding proteins may share similar ABD, their expected role in cytoskeletal organization, and thus cell function may be quite distinct and varied (Weeds, 1982; Matsudaira, 1991; Weeds and Maciver, 1993; Otto, 1994). The consensus sequence of the ABD of a number of ABPs is shared for both actin bundling-, and actin cross-linking-proteins (Matsudaira, 1991). This generic domain, contains a highly conserved actin-binding tandem repeat (Matsudaira, 1991) (Fig. 9A–B). Thus, the ultimate role of ABPs in cell

FIGURE 9 Features of actin-binding proteins (ABPs), and effect of filamin on PC2 from hST. (A) The consensus sequence of the ABD of a number of ABPs is shared for both actin bundling- and actin cross-linking proteins. This generic domain is present as a tandem repeat, of which the most conserved region in repeat A is shown in (B) (Matsudaira, 1991). The ultimate role of ABPs in channel regulation, thus largely depend in the ABP's topology and ability to interact with more than one actin filament. Thus, a filamin (ABP-280) homodimer, would convey a three-dimensional structure to the cross-linked actin network, most consistent with a gel, which is largely different from the expected bundling role of α-actinin (Matsudaira, 1991). (C) Consistent with the importance of the three-dimensional structure of the actin gel in PC2 channel regulation, filamin had a strong inhibitory effect, which is exactly opposite to the effect observed for α-actinin (Fig. 6). Interestingly, a similar finding has been observed for epithelial Na^+ channels (Cantiello, 1995).

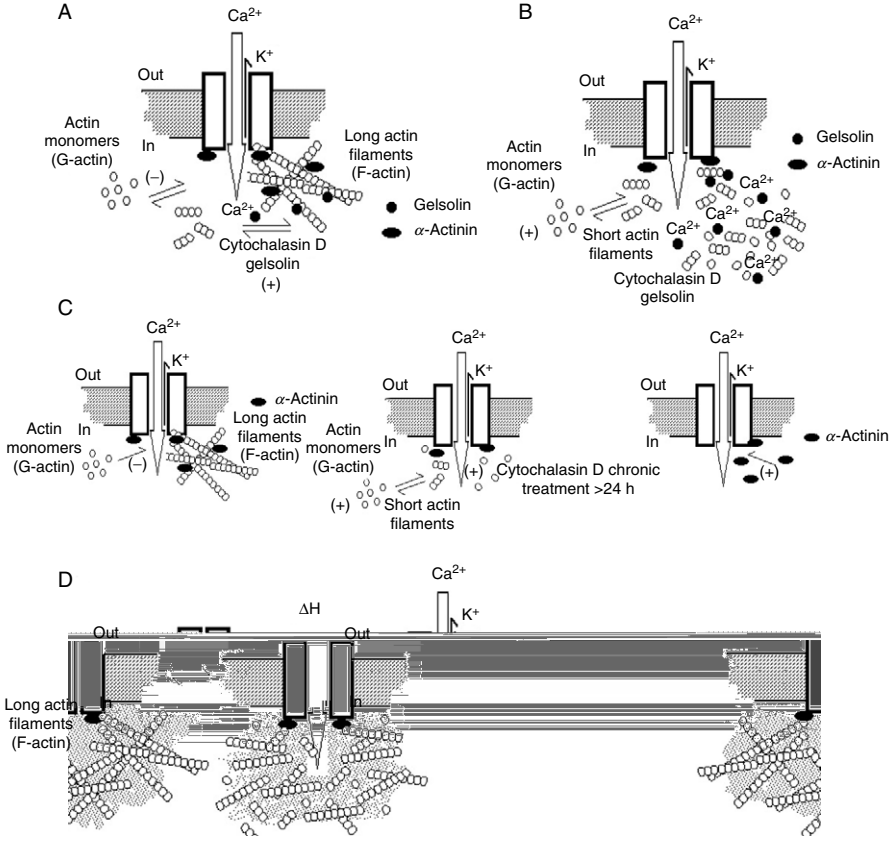

FIGURE 10 Cytoskeletal regulation and osmosensory control of PC2. The encompassed evidence, mostly from endogenous hST-PC2 and the purified channel protein, allows a comprehensive understanding of the role the dynamic changes in the actin cytoskeleton convey on PC2 channel function. (A) Under basal conditions, α-actinin (and/or other actin-binding proteins) link the PC2 channel, to an endogenous cross-linked network of actin filaments. (B) Endogenous F-actin depolymerization by actin-severing proteins, such as gelsolin (likewise by toxins such as CD) activates PC2 channel function, which in turn is modulated the Ca^{2+} transport through the channel, which elicits a feedback mechanism mediated by the cytoskeleton. (C) This is in agreement with the inhibitory effect of monomeric actin on the cytoskeletally associated channel and the lack of a direct functional effect of actin on the isolated channel. In contrast, chronically CD treated membranes (low channel activity) to completely collapse the cytoskeleton can be functionally restored to almost control levels of channel activity by addition of G-actin, which likely replenishes the exhausted pool of endogenous actin. Conversely, the molecular anchor between actin networks and PC2 is clearly demonstrated to be α-actinins, which in turn directly modulate, and may be competed by other actin-associated proteins with similar binding domains. (D) The stimulatory effect of ΔΠ and ΔH in cytoskeletally competent

function, largely depends in the ABP's topology and ability to interact with more than one actin filament. As indicated above, a filamin (ABP–280) homodimer, would convey a three-dimensional structure to the cross-linked actin network, most consistent with a gel, which is largely different from the expected bundling role of α-actinin (Matsudaira, 1991). The fact that the three-dimensional structure of the filamin-cross-linked actin gel would be largely different from that observed with α-actinin, should also be reflected in distinct functional interactions with PC2. We found that addition of filamin (ABP–280) has a strong inhibitory effect on PC2 channel regulation, exactly opposite to the effect observed for α-actinin (Fig. 9). Interestingly, a similar finding has been observed for epithelial Na^+ channels (Cantiello, 1995). Thus, our data are consistent with the hypothesis that the three-dimensional structure of the actin gel is a key element in controlling channel regulation (Fig. 10). A membrane-cytoskeleton functional interface may be critical for the cation-dependent signaling pathway(s) normally associated with various cell functions, including cell cycle, vesicle trafficking, and ion transport. As an example, Ca^{2+} signals which play essential role in cell function, may be under the control of a feedback mechanisms linking Ca^{2+} transport to the remodeling of the cytoskeleton, and in particular channel function, as described in our studies (Montalbetti *et al.*, 2005b, data herein). A universal cell response known as cell volume regulation is elicited by an osmotic stress. This response, often associated with the activation of ion channels and other solute transporters often entails a combined response associated with both, changed in the structure of the cytoskeleton, and changes in the geometry and properties of the lipid bilayer underlying the plasma membrane. These two widely distributed responses are both linked to the mechanotransduction of physical forces to ensure the phenomenologically defined mechanosensitivity of ion channels which respond to such forces. Placed in the context of the PC2–cytoskeletal interface (Fig. 10), it can be expected that the channel, tethered by the α-actinin (or another anchoring protein) to the cytoskeleton, will respond and thus elicit nonlinear responses mediated by the actin networks. Accepting the strong linkage and regulatory function of cytoskeletal components on PC2 function, we speculated and herein

membranes is consistent with a dynamic contribution of the PC2-adjacent actin cytoskeleton, which remains under "swelling equilibrium." This actin network serves as an interface, which provides a sensory mechanism whereby changes in hydroosmotic pressure modulate this actin–PC2 interaction, likely by the anchoring effect of α-actinin, which also control channel function. F-actin disorganization (osmotic shock, CD treatment) uncouples this physical interaction, rendering channels, first active, and an unresponsive to environmental changes.

determined that the PC2-connected actin network is a key regulatory component of the channel.

E. Sensory Role of the Actin Cytoskeleton in PC2 Channel Function

Studies have turned attention to the potential sensory transducer properties of PC2 in renal epithelial and other target cells (Nauli *et al.*, 2003; Cantiello *et al.*, 2004; Nauli and Zhou, 2004; Pazour, 2004). A functional PC1–PC2 channel complex has been implicated in the mechanosensory transduction associated with cilia bending and Ca^{2+} influx in renal epithelial cells (Nauli *et al.*, 2003). It is noteworthy, that ciliary structures are largely microtubular, rather than actin filamental organelles (Pazour and Witman, 2003). Consistent with our contention of the relevance of cytoskeletal structures in PC2 channel regulation, preliminary evidence from our laboratories would suggest that indeed microtubular organization also regulate PC2 in hST apical membranes (Montalbetti *et al.*, 2006). However, little is known as to how the PC1–PC2 channel complex, or more particularly PC2 senses internal and/or external environmental responses, particularly in the plasma membrane. Physical forces such as hydrostatic (ΔH) and osmotic ($\Delta \Pi$) pressure may play an important role in signal transduction elicited by such ''environmental forces,'' which control ion transport, and thus hydroelectrolytic homeostasis. The spontaneous cation channel activity observed in hST vesicles is normally elicited in the presence of an osmotic gradient ($\Delta \Pi$) imposed by a KCl chemical gradient to the plasma membrane (González-Perrett *et al.*, 2001, 2002). Thus, changes in the properties of the membrane may control PC2 channel activity in hST. Further, the elastic pressure arising from osmotically induced membrane deformation may be compensated by hydrostatic pressure differences imposed to the membrane. Both physical changes, osmotically induced changes in cytoskeletal structures in the vicinity of the channels and elastic changes in membrane compliance, can act as regulators of PC2 channel function and thus ion transport. To determine the potential regulatory role of hydroosmotic forces in PC2 function we determined that a compensatory hydrostatic gradient in osmotically challenged hST membranes increases PC2 channel activity. We found (Montalbetti *et al.*, 2005a) that either a decrease in *cis* volume, and/or increase in volume to the *trans* side of the reconstitution chamber, was sufficient to stimulate PC2 channel activity in hST membranes. Interestingly, this phenomenon was no longer observed after pretreatment of the hST membranes with the actin filament-disrupting agent CD. These data strongly suggested to us that a sensory mechanism of endogenous PC2 in human placenta entails a structural-functional interaction between the channel and adjacent cytoskeletal proteins. In this

hypothetical model of sensory transduction, a "swelling equilibrium" in the actin gel (Hodson and Earlman, 1993) associated with the PC2 channel is established under basal (osmotic) conditions, such that any displacement from such equilibrium entails changes in the cortical actin network of the apical hST membrane (Fig. 10). Similarly, a physical challenge such as a change in ΔH, which modifies the elastic compliance of the membrane, also regulates PC2 function. This phenomenon is mimicked by compensatory osmotic changes, such as $\Delta\Pi > 0$ to $\Delta\Pi = 0$, whose equivalent would be an isoosmotic cell volume increase associated with cell volume regulation (MacKnight, 1988; Strange, 1994), a phenomenon also requiring cytoskeletal structures (Ziyadeh *et al*., 1992; Cantiello and Prat, 1996). The requirement of the cytoskeleton in the transmission of force to PC2 was confirmed by our finding that ΔH did not regulate the isolated PC2 channel (Fig. 8). This is further supported by the lack of effect in the CD chronically treated hST vesicles, the requirement of α-actinin to link the channel to the cytoskeleton, and the fact that addition of actin alone is also without effect on the isolated protein. A regulatory pathway of PC2 channel function by the cytoskeleton may be considered a novel sensory mechanism linking physical forces to the swelling equilibrium of the adjacent actin networks, rather than the membrane or channel itself.

VII. PERSPECTIVE AND FUTURE DIRECTIONS

The present studies and conclusions tried to provide a comprehensive understanding of the steps involved in the regulation by the cytoskeleton of a prototypical TRP channel, namely polycystin-2 (TRPP2). Our studies provide a comprehensive analysis of the molecular elements, which underlie the PC2–actin cytoskeleton interface. Although membrane compliance and elastic properties may affect channel function, as it is postulated for most mechanosensitive ion channels, our studies suggest that PC2 channel regulation is instead controlled by physical changes imposed to the cortical actin cytoskeletal structures that link the ion channel to the plasma membrane. The evidence strongly suggest a mechanical interaction, where environmental forces target a true gating mechanism by linking PC2 to the actin cytoskeleton via α-actinin. Both, changes in cytoskeletal dynamics and direct binding of actin-associated proteins convey forces that translate into conformational changes in the channel protein. This has to be viewed in a broader scope, as most ion channels studied to date, are indeed either linked or can be regulated by the various components of the cellular cytoskeleton. We are tempted to postulate, that based on the prevalence of this interaction, most ion channels may display, under specific conditions, some component

of mechanosensitivity. This is further strengthened by the prevalence of putative cytoskeletal-binding domains in the various TRP channels, whose multimeric structure, makes them ideal structures to be modulated and controlled by cytoskeletal interacting proteins. Among these, α-actinins, are rather interesting because not only are known actin-bundling proteins, but also amphipathic proteins that directly interact with lipid components of the plasma membrane. In this regard, two points are noteworthy. First, even in conditions under which the cytoskeleton is apparently disrupted, actin-associated proteins may elicit a response, which may be misconstrued as cytoskeleton independent. Genuine efforts in addressing whether ion channels remain "mechanosensitive" can be unwittingly biased toward the remaining presence of actin-binding proteins. In the context of heterologous expression of TRP channel isotypes, for example, the possibility exists, for heterocomplexes to convey unexpected regulatory properties not observed in the native protein. Thus, a careful analysis of the isolated channel protein (not without experimental problems itself) may help provide a more complete picture of this pervading and likely general mechanism of channel regulation.

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