Chloride channel activity of CIC-2 is modified by the actin cytoskeleton

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The chloride channel CIC-2 has been implicated in essential physiological functions, including cell-volume regulation and fluid secretion by specific epithelial tissues. Although CIC-2 is known to be activated by hyperpolarization and hypo-osmotic shock, the molecular basis for the regulation of this channel remains unclear. Here we show in the *Xenopus* oocyte expression system that the chloride-channel activity of CIC-2 is enhanced after treatment with the actin-disrupting agents cytochalasin and latrunculin. These findings suggest that the actin cytoskeleton normally exerts an inhibitory effect on CIC-2 activity. An inhibitory domain was previously defined in the N-terminus of CIC-2, so we sought to determine whether this domain might interact directly with actin in binding assays *in vitro*. We found

that a glutathione S-transferase fusion protein containing the inhibitory domain was capable of binding actin in overlay and co-sedimentation assays. Further, the binding of actin to this relatively basic peptide (pI 8.4) might be mediated through electrostatic interactions because binding was inhibited at high concentrations of NaCl with a half-maximal decrease in signal at 180 mM NaCl. This work suggests that electrostatic interactions between the N-terminus of CIC-2 and the actin cytoskeleton might have a role in the regulation of this channel.

Key words: binding *in vitro*, electrostatic interactions, voltage clamp, *Xenopus* oocyte.

INTRODUCTION

CIC-2 belongs to the large CIC family of chloride-channel-forming proteins [1]. It has been implicated in essential neuronal and epithelial cell functions. For example, CIC-2 expression has been implicated in the modification of GABA_A-receptor function in neurons [2]. Further, it has been suggested that CIC-2 might be capable of mediating chloride secretion in neonatal rodent airways and in rabbit gastric mucosa [3,4]. Despite the physiological significance of this protein, the regulation of its channel function remains incompletely understood.

So far the regulation of CIC-2 function has been studied most thoroughly in the *Xenopus* oocyte expression system [5–7]. In *Xenopus* oocytes, CIC-2 is activated by hypotonic shock, membrane hyperpolarization to approx. –120 mV and low extracellular pH [5–7]. Phorbol esters, well-known activators of protein kinase C, inhibit CIC-2 currents [2]. Studies of ‘CIC-2-like currents’ endogenously expressed in certain cell types, such as epithelial T₈₄ cells, pyramidal cell neurons, parotid gland cells and cardiac myocytes reveal similar, but not identical, regulation [2,8–10]. For example, ‘CIC-2 like’ currents in neurons are not stimulated by hypotonicity and the voltage required for activation of ‘CIC-2 like’ whole-cell currents is less hyperpolarized (–80 mV) than in *Xenopus* oocytes [2]. Hence CIC-2 might contribute to chloride currents in resting neurons [2]. Variations in the activation properties of CIC-2 currents in different cell types have been linked hypothetically to differences in the membrane environment, the intracellular concentration of chloride and the extent of association with cytoskeletal proteins [11]. There is extensive evidence linking the regulation of ion transporters with cytoskeletal proteins, especially those transporters that exhibit osmosensitivity [12–18]. Changes in extracellular osmolarity have been shown to initiate transient changes in the integrity of the actin cytoskeleton and it has been postulated that

this might in turn trigger alterations in localization and/or function of ion transporters, including ion channels [12].

Elegant mutagenesis studies of CIC-2 identified a region within the N-terminus of the protein that might comprise the hypotonicity-sensitive and voltage-sensitive ‘gating particle’ (see Figure 2 in [6]). Deletion of the ‘essential’ region within the N-terminus results in a constitutive ‘open phenotype’ that no longer requires hypotonicity or hyperpolarization for activation. Deletion of the ‘modifier’ region causes an intermediate-open phenotype. These studies led to the proposal of a model in which the N-terminus of CIC-2 functions as a ‘ball-region’ in a ball-and-chain model for channel gating [6].

The results of our current work indicate that disruption of the actin cytoskeleton mimics the effect of hypotonicity and induces an ‘open phenotype’ in the CIC-2-associated chloride conductance. Further, our biochemical studies suggest that this effect might be due to the disruption of an electrostatic interaction between the actin cytoskeleton and the N-terminus of CIC-2.

EXPERIMENTAL

Electrophysiology

Stage VI *Xenopus* oocytes were surgically removed and defolliculated by digestion with collagenase as described previously [1]. They were injected with 50 nl of water (as control) or 50 nl of water containing CIC-2 cRNA (1–3 ng/nl cRNA). Approximately 18 h after injection, oocytes were studied by two-electrode voltage-clamp protocols to assess CIC-2 channel activity. The bath solution was standard oocyte solution containing 100 mM NaCl, 1.8 mM CaCl₂, 1 mM MgCl₂, 2 mM KCl and 5 mM Hepes, pH 7.6. Voltage-step protocols were applied before and after treatment with the actin-cytoskeleton-disrupting agents

Abbreviations used: GST, glutathione S-transferase; Nt, N-terminal; Ct, C-terminal.

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Figure 1 Disruption of the actin cytoskeleton causes CIC-2 activation

(A) Two-electrode voltage-clamp protocols (with a holding potential of -60 mV stepping by 40 mV increments from -140 to $+20$ mV) revealed hyperpolarization-activated currents in CIC-2-expressing *Xenopus* oocytes but not water-injected (control) oocytes (left panel). At 20 min after treatment with cytochalasin D ($10 \mu\text{M}$), the activation of currents at both hyperpolarizing and depolarizing voltage steps was detected in CIC-2-expressing oocytes. (B) The current-voltage (I - V) relationship of the currents in (A) are shown. Solid lines correspond to data acquired from CIC-2-expressing oocytes (with or without cytochalasin) and dotted lines show data acquired from control oocytes (with or without cytochalasin). (C) Currents (means $\pm$ S.D.) from control (Ctl) and CIC-2-expressing oocytes before (open bars) and 20 min after (filled bars) cytochalasin treatment at -140 and $+20$ mV. Significant responses to cytochalasin D were observed only in CIC-2-expressing oocytes: $*P < 0.005$ ($n = 5$).

cytochalasin D ($10 \mu\text{M}$; Sigma) or latrunculin B ($1 \mu\text{M}$; Biomol, Plymouth Meeting, PA, U.S.A.) at the holding potential of -60 mV. Every 5 min, voltage was stepped from -140 to $+20$ mV in 40 mV increments to assess chloride selectivity on the basis of the measured reversal potential and the current-voltage relationship of the activated whole-cell current.

Plasmid construction, fusion protein expression and purification

The nucleotide sequence corresponding to residues 31–74 (N-terminal; Nt) of the rat CIC-2 sequence was amplified from clone B12-2 (provided by Professor T. J. Jentsch, Zentrum für Molekulare Neurobiologie Hamburg, Hamburg University, Germany), ligated into pGEX-2T (Pharmacia) and sequenced. Similarly, the nucleotide sequence corresponding to residues 869–907 (C-terminal; Ct) was amplified from the same clone, ligated into pGEX-2T and sequenced. Subsequently, the expression of glutathione S-transferase (GST) fusion proteins (GST-Nt and GST-Ct) was induced in DH5 α host bacteria and the proteins were purified with glutathione-Sepharose beads in accordance with the protocol provided by Pharmacia.

Overlay blots

GST-Nt and GST-Ct fusion proteins were subjected to SDS/PAGE [12% (w/v) gel] and then transferred to nitrocellulose [19]. After transfer, membranes were blocked with 5% (w/v) BSA in TBST buffer containing 150 mM NaCl, 0.5% (v/v) Tween 20 and 0.2% (w/v) NaN_3 in 25 mM Tris/HCl, pH 7.5. Then the membranes were incubated with $1 \mu\text{g/ml}$ biotinylated

actin in TBST buffer containing BSA as above for 3 h. After being washed in TBST buffer, the membrane was incubated for 1 h with avidin-horseradish peroxidase for detection by enhanced chemiluminescence (Amersham Pharmacia Biotech).

Pull-down experiments

GST alone or GST fusion proteins (GST-Nt or GST-Ct) were coupled to glutathione-agarose beads (Pharmacia). Equal volumes of the beads were then incubated with increasing concentrations of F-actin overnight at 4°C . Purified actin (Cytoskeleton Inc., Denver, CO, U.S.A.) was polymerized in accordance with the manufacturer's instructions to produce a stock supply of F-actin. The beads were then spun down and washed three times with PBS containing Tween 20 (0.1%, v/v), pelleted again and then washed once with PBS alone. The beads were resuspended in SDS loading buffer containing 63 mM Tris/HCl, pH 6.8, 10% (v/v) glycerol, 2% (w/v) SDS and 25 mM dithiothreitol; proteins were separated by SDS/PAGE [12% (w/v) gel] [19]. The gels were stained with Coomassie Blue and the relative intensities of the bands were evaluated with NIH Image Software.

Actin co-sedimentation experiments

Polymerized actin ($10 \mu\text{M}$; Cytoskeleton) was incubated with the GST-Nt fusion protein at an equimolar concentration for 30 min

at room temperature. Co-sedimentation experiments were performed at various NaCl concentrations from 0.1 to 0.4 M to evaluate the effect of ionic strength on the interaction. The samples were then centrifuged at 100 000 *g* for 20 min. Supernatants were extracted and the pellets were resuspended with SDS solubilizing buffer. Equal volumes of supernatant and pellet were then analysed by SDS/PAGE and stained with Coomassie Blue. The relative intensities of the bands were measured with NIH Image Software.

RESULTS

CIC-2 is activated by disruption of the actin cytoskeleton in *Xenopus* oocytes

Typically, after expression in *Xenopus* oocytes, CIC-2 mediates chloride currents that are activated in a time-dependent fashion by hyperpolarizing voltage steps of approx. -140 mV (Figure 1A) [5,6]. We used both cytochalasin D (10 μ M) and latrunculin B (1 μ M) to assess the effect of actin-cytoskeleton-disrupting agents on CIC-2 function. These agents are known to disrupt the actin cytoskeleton through different mechanisms [12–18]. Cytochalasin D induces a gradual depolymerization of actin filaments by binding to their fast-growing barbed ends; in contrast with the related compound cytochalasin B, cytochalasin D is unlikely to influence the metabolic status of the oocyte. Latrunculin is chemically unrelated to the cytochalasins and acts by forming complexes with monomeric actin, thus decreasing the pool available for polymerization [20]. Treatment of CIC-2-expressing oocytes with cytochalasin D caused a gradual increase in currents evoked at both hyperpolarizing voltage steps and depolarizing voltage steps. The response was evident at 5 min and maximal at 20–25 min ($n = 5$). Whereas CIC-2 currents typically exhibit an extremely slow rate of activation with hyperpolarizing voltage steps [5,6], cytochalasin treatment consistently ($n = 5$) caused the currents to activate immediately after the application of a similar hyperpolarization step, as shown in Figures 1(A) and 1(B). In control oocytes, not expressing CIC-2, treatment with cytochalasin D resulted in modest, but not statistically significant, changes in whole-cell current. It is therefore likely that the large currents evoked by the treatment of CIC-2-expressing oocytes with cytochalasin are indeed mediated by CIC-2 (Figure 1C). Treatment of oocytes expressing CIC-2 with 1 μ M latrunculin B also caused a significant increase in currents measured at hyperpolarizing (-140 mV) and depolarizing ($+20$ mV) potentials in oocytes expressing CIC-2 of $173 \pm 20\%$ and $175 \pm 30\%$ respectively (means $\pm$ S.E.M.). Hence both cytoskeleton-disrupting agents caused the activation of CIC-2-dependent currents, supporting the notion that CIC-2 is functionally coupled to the cytoskeleton. In further experiments we found that jasplakinolade, a cell-permeable peptide that stabilizes F-actin [20], had no significant effect on CIC-2 currents ($n = 5$), suggesting that the integrity of the actin cytoskeleton rather than its abundance is critical in regulating CIC-2 channel function.

The N-terminus of CIC-2 interacts with actin

Previous mutagenesis studies revealed that a region within the N-terminus of CIC-2, including residues 16–61, is inhibitory to its chloride conductance [6]. Deletions and site-directed mutations within this region, including both putative 'essential' and 'modulating' domains, lead to constitutive CIC-2 channel activity that no longer requires activation by hyperpolarizing voltage steps or hypotonic shock (Figure 2A) [6]. As the 'open' phenotype of these mutants resembles the activation response evoked by cytochalasin D and latrunculin B, we sought to determine

Figure 2 N-terminus of CIC-2 binds actin

(A) The putative topology of CIC-2 is shown. We also show the specific peptides from the N-terminus (residues 31–74) and the C-terminus of CIC-2 (residues 869–907) that were used to generate the GST fusion proteins GST-Nt and GST-Ct. The residues belonging to the 'essential' (24–39) and 'modifier' (39–50) regions in the N-terminus are indicated. (B) Different quantities (1, 5 and 10 μ g) of either GST-Nt or GST-Ct were subjected to SDS/PAGE [12% (w/v) gel]. The predicted molecular mass of GST-Nt is 33 kDa and the molecular mass predicted for GST-Ct is 32 kDa. Binding of biotinylated actin to each fusion protein was assessed with an overlay assay and detected with the use of chemiluminescence. Only GST-Nt showed actin binding in this assay. Western blotting with a polyclonal antibody against GST (α -GST; Pharmacia) confirmed similar loadings of both fusion proteins.

whether actin binds to the N-terminus of CIC-2 in experiments *in vitro*. Because the C-terminus of CIC-2 has not been implicated in the regulation of CIC-2 channel function, we predicted that there would be no specific binding of actin to this region of the protein.

We expressed and purified GST fusion proteins corresponding to either the N-terminus of CIC-2 (GST-Nt) or the C-terminus of CIC-2 (GST-Ct) (Figure 2A). First we measured the actin-binding properties of both proteins with an overlay assay (Figure 2B). In brief, different quantities of each fusion protein (1, 5 and 10 μ g) were subjected to SDS/PAGE and transferred to nitrocellulose. The blots were blocked and incubated with biotinylated actin; bound actin was detected by chemiluminescence. As shown in Figure 2(B), biotinylated actin bound GST-Nt protein in a concentration-dependent manner. Some binding of actin to the GST-Ct fusion peptide was detected but this was greatly decreased.

To assess the F-actin-binding activity of the two fusion proteins quantitatively, we performed 'pull-down' studies. The GST fusion proteins and GST alone were coupled to glutathione–

Figure 3 Binding of F-actin to the N-terminus of CIC-2 is saturable

(A) Bound F-actin and fusion proteins were separated by SDS/PAGE [12% (w/v) gel] and detected by staining with Coomassie Blue. The amount of actin bound in relation to the amount of GST fusion protein was quantified with NIH Image and Prism software. (B) The dependence on F-actin concentration for association with GST-Nt (Δ), GST-Ct ($\circ$) or GST alone ($\square$) was assessed and was fitted with a single-site binding algorithm ($r^2 = 0.9$) to yield an apparent K_d of $0.8 \mu\text{M}$ and a B_{max} (pixels corresponding to actin label compared with pixels corresponding to the GST label) of 0.407 . Over the same range of actin concentrations, we observed no saturable binding to either GST-Ct or to GST alone.

agarose beads and equal volumes of the beads were incubated with increasing concentrations of F-actin overnight at 4°C . The proteins bound to the beads were then analysed by SDS/PAGE and detected by Coomassie Blue staining (Figure 3A). The amount of actin bound in relation to the amount of GST fusion protein was quantified and analysed with the use of a single-site binding algorithm (Figure 3B). Over this wide range of concentrations, the binding of F-actin to the N-terminus fusion protein was saturable, with an apparent K_d of $0.80 \mu\text{M}$ and a molar stoichiometry of 1:2 [the value of B_{max} (pixels corresponding to actin label divided by pixels corresponding to GST label) was 0.41]. Over the same range of actin concentrations we observed no saturable binding to the C-terminus fusion protein or to GST alone.

Sequence inspection (with the ExPASy-Compute pI/Mw tool) revealed that the N-terminus peptide was quite basic relative to the C-terminus peptide, with a pI of 8.43 compared with 4.7 . To determine whether the electrostatic forces mediated binding between GST-Nt and F-actin, we assessed the effect of changes in ionic strength on co-sedimentation of these proteins. As shown in Figure 4(A), in the absence of F-actin, all of the GST-Nt remained soluble in the supernatant containing 0.15 M NaCl (Figure 4A). In the presence of F-actin and 0.15 M NaCl, GST-Nt was detectable in the pellet. We found that the binding of GST-Nt to F-actin was profoundly sensitive to ionic strength; binding was essentially abolished at 0.4 M NaCl (Figure 4B). The ratio of GST-Nt to F-actin varied with increasing concentration of NaCl and could be described by a monoexponential function, predicting half-maximal binding at 0.18 M NaCl.

DISCUSSION

The results of this study suggest that the actin cytoskeleton can exert an inhibitory effect on CIC-2 chloride channel function. We found that disruption of the actin cytoskeleton by cytochalasin D

and latrunculin B in *Xenopus* oocytes led to the activation of CIC-2 function. The activity of other 'osmosensitive' ion transporters have also been shown to be affected by manipulations in cytoskeletal integrity; multiple mechanisms have been invoked to explain this regulation. Alterations in transporter activity might occur via direct effects of actin on function or indirectly via an actin-associated protein [12,22]. Similarly, the mechanisms by which the actin cytoskeleton regulates CIC-2 function are likely to be complex and involve the interplay of several pathways. Our studies *in vitro* suggest that the N-terminus of CIC-2 can bind to F-actin via electrostatic interactions, thereby implying a direct effect on gating. However, it remains possible that, *in vivo*, the actin cytoskeleton can regulate CIC-2 function indirectly through actin-binding proteins.

Previous mutagenesis studies identified the N-terminus as the regulatory domain of CIC-2 [6,7]. As mentioned previously in *Xenopus* oocyte studies, the activation of CIC-2 currents requires hyperpolarizing voltage steps, hypotonicity or extracellular acidity. Grunder et al. [6] analysed many mutations within the N-terminus of CIC-2 and defined a region within which deletions and certain site-directed mutations abolished regulation, leading to a constitutive 'open phenotype'. However, the effect of specific deletions and amino acid substitutions within this 'inactivation domain' could not readily be explained on the basis of the changes in the biophysical properties of this region. For example, deletions within this region, varying from 2 to 45 residues in length, could confer an open phenotype. The introduction of either two consecutive hydrophobic or two negatively charged residues could also effectively disrupt inactivation. Further, the nature of the interaction between the 'inactivation domain' and its 'docking site' on the helical region spanning between transmembrane domains 7 and 8 of CIC-2 remains unclear [7]. The authors [6,7] suggest that the interaction between the 'inactivation domain' and the putative docking site on the intracellular loop between transmembrane domains 7 and 8 might not be sufficient to account for native gating, because the insertion of

Figure 4 Binding of F-actin to the N-terminus of CIC-2 is affected by ionic strength

(A) GST-Nt (10 μ M) was incubated with (+) or without (–) F-actin in the presence of 0.15 M NaCl. After centrifugation, protein in the pellet (P) or in the supernatant (S) was separated by SDS/PAGE and detected by staining with Coomassie Blue. GST-Nt was detected in the pellet (with F-actin) but exclusively in the supernatant when no actin was present. (B) Co-sedimentation studies were performed twice in the presence of various concentrations of NaCl. The amount of GST-Nt found in the pellet (expressed as a percentage of pelleted actin) was markedly decreased as the concentration NaCl increased from 0.15 to 0.4 M. Means and ranges are shown in the graph. A monoexponential decay function fitted the data ($r^2 = 0.95$) to yield the concentration of NaCl supporting half-maximal binding, namely 0.18 M (Prism Software). Inset: High salt reduces co-sedimentation of GST-Nt with F-actin. Protein in the pellet (P) or in the supernatant (S) analysed by SDS/PAGE and Coomassie Blue staining.

these regions into CIC-1, a related CIC channel that is insensitive to hypotonicity, failed to confer CIC-2-like gating properties.

The N-terminus of CIC-2 does not possess any canonical protein–protein binding motifs such as SH2, SH3 or PDZ motifs. Furthermore, CIC-2 does not possess typical actin-binding motifs such as those present in actinin. However, the present studies *in vitro* suggest that the N-terminus of CIC-2 can interact with actin (K_d 0.8 μ M) with an affinity for actin similar to that of dystrophin [23]. Further, because the interaction between the N-terminus peptide and F-actin is modified by ionic strength, the interaction might occur via electrostatic interactions. Electrostatic forces seem to mediate several physiologically important interactions, such as binding between the N-terminus of band 3 and aldolase, phosphofructokinase or glyceraldehyde [24–26]. Further, the acidic C-terminus of band 3 can interact specifically with carbonic

anhydrase [27]. Moreover, dystrophin, the membrane protein that is defective in Duchenne muscular dystrophy, has been shown to interact with actin via a series of basic repeats within its rod domain; this interaction is sensitive to increasing ionic strength [23]. This interaction is thought to be important for normal arrangement of the actin cytoskeleton. There are therefore several other examples of membrane proteins that can form physiologically significant associations with the actin cytoskeleton via electrostatic interactions.

Although direct proof *in vivo* is lacking, our results suggest that the inhibitory interaction between the ‘inactivation’ domain and its docking site might be stabilized through associations with the actin cytoskeleton. Future studies will be required to assess whether the actin cytoskeleton interacts directly or indirectly with CIC-2 *in situ*.

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