the FEBS Journal

Functional reconstitution of mammalian 'chloride intracellular channels' CLIC1, CLIC4 and CLIC5 reveals differential regulation by cytoskeletal actin

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Keywords
chloride intracellular channels; cytoskeleton; ion channel; multiconductance channel; planar bilayer

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(Received 10 September 2007, revised 10 October 2007, accepted 16 October 2007)

doi:10.1111/j.1742-4658.2007.06145.x

Chloride intracellular channels (CLICs) are soluble, signal peptide-less proteins that are distantly related to Ω-type glutathione-*S*-transferases. Although some CLICs bypass the classical secretory pathway and autoinsert into cell membranes to form ion channels, their cellular roles remain unclear. Many CLICs are strongly associated with cytoskeletal proteins, but the role of these associations is not known. In this study, we incorporated purified, recombinant mammalian CLIC1, CLIC4 and (for the first time) CLIC5 into planar lipid bilayers, and tested the hypothesis that the channels are regulated by actin. CLIC5 formed multiconductance channels that were almost equally permeable to Na^+, K^+ and Cl^-, suggesting that the 'CLIC' nomenclature may need to be revised. CLIC1 and CLIC5, but not CLIC4, were strongly and reversibly inhibited (or inactivated) by 'cytosolic' F-actin in the absence of any other protein. This inhibition effect on channels could be reversed by using cytochalasin to disrupt the F-actin. We suggest that actin-regulated membrane CLICs could modify solute transport at key stages during cellular events such as apoptosis, cell and organelle division and fusion, cell-volume regulation, and cell movement.

Chloride intracellular channel (CLIC) proteins are 'structural homologues' of Ω-class glutathione *S*-transferases (Ω-GSTs) [1]. They are widely expressed in multicellular organisms, and can coexist in both soluble and integral membrane forms, because some soluble, signal peptide-less CLICs bypass the classical secretory pathway, and autoinsert directly into membranes [2,3]. Soluble CLIC2 displays minor GST-related enzymatic activities, although it does not seem to be a classical thiol transferase [4], and soluble CLIC4 (p64H1) is associated with apoptosis [5], but in general the cellular roles of the soluble proteins are poorly understood, and more interest has centred on membrane CLICs. The regulatory mechanisms of membranous CLICs *in vivo* and *in vitro* have not been elucidated.

The *Caenorhabditis elegans* CLIC-like protein exc-4 appears to form a charge-compensating ion channel to facilitate the fusion of intracellular vesicles, explaining its essential role to generate a hollow tubule from a single cell [6,7]. Mammalian CLIC1 and CLIC4 are transmembrane components [8,9] of poorly selective intracellular and plasma membrane ion channels, and form similar channels *in vitro* in the absence of any other protein [10,11]. Although the roles of the mammalian channels remain obscure, and CLIC proteins can also modify the behaviour [12] of other, well-established ion channels, the channel activity of CLIC1 does

Abbreviations
CLIC, chloride intracellular channel; GE, gel exclusion; GHK, Goldman–Hodgkin–Katz equation; GSH, reduced glutathione; GST, glutathione *S*-transferase; GST, glutathione *S*-transferase; IMAC, immobilized metal affinity chromatography; *I/V*, current–voltage; TMD, transmembrane domain.

not appear to be an artifact of artificial protein overexpression, because the activity of endogenous CLIC1 increases in dividing, nontransfected Chinese hamster ovary cells [13], and in brain microglia exposed to Alzheimer's Aβ protein [14].

Mammalian CLICs interact extensively with components of the cytoskeleton. For example, rat brain CLIC4 (p64H1) is associated with actin in a multiprotein complex [15], and human CLIC5 (strictly CLIC5A, which is expressed from the same gene as CLIC5B, or p64) was identified in an actin-rich complex from placental microvilli [16]. The proposed topology and membrane organization of CLIC1, CLIC4 and CLIC5 [17] suggest that these interactions could be retained in the membrane forms of the proteins, and the association of membrane CLICs with the actin cytoskeleton may be functionally important. For example, rearrangement of the cytoskeleton and concurrent activation or inhibition of plasma membrane solute transporters are often prominent features of cell-volume regulation, and specific ion channels are known to be functionally associated with the cortical actin cytoskeleton, especially in epithelial cells [18].

We began our studies by reconstitution of recombinant CLIC5 in the planar bilayers, and set out to test the hypothesis that membrane CLICs are regulated by cytoskeletal actin, after functionally reconstituting recombinant human CLIC1, CLIC4 and CLIC5 in planar lipid bilayers. CLIC1 and CLIC4 have previously been characterized at the single-channel level, and, in this study, we confirmed for the first time that membrane CLIC5 also forms ion channels. The ion channels formed by CLIC1 and CLIC5 were directly and reversibly regulated by F-actin, without an intermediate molecule or adaptor protein. In contrast, the ion channels formed by CLIC4 were not regulated by actin under the same conditions.

Results

Bilayer reconstitution of CLIC5

Unlike CLIC1 and CLIC4, CLIC5 has not been reconstituted previously at the single-channel level, but purified recombinant CLIC5 (Fig. 1) gave rise to characteristic ion channel activity (Fig. 2) within 5–10 min of adding 25 ng mL^{-1} protein to bilayers formed from palmitoyl-oleoyl phosphatidylethanolamine, palmitoyl-oleoyl phosphatidylserine and cholesterol (4 : 1 : 1, mol/mol) in the presence of 5 mM reduced glutathione (GSH). No channels were observed using control of immobilized metal affinity chromatography (IMAC) eluates from non-CLIC5-expressing bacteria (five independent preparations), confirming that the activity did not arise from endogenous bacterial proteins.

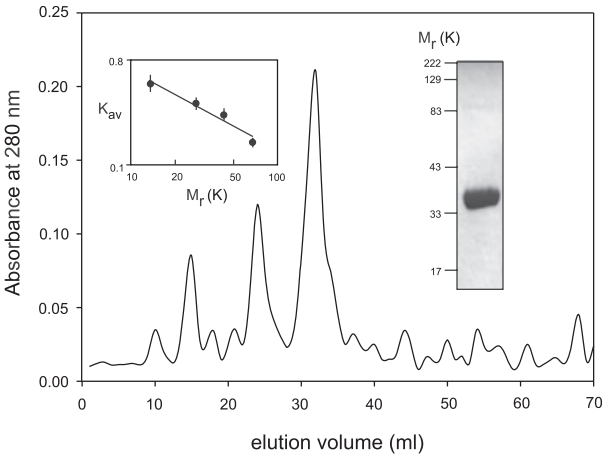

Fig. 1. FPLC and SDS/PAGE analysis of reduced, recombinant CLIC5. The FPLC elution peaks correspond to molecular mass of 125, 63 and 38 kDa (main peak), respectively, calculated from the inset semi-log calibration curve. The protein standards were BSA (67 kDa), ovalbumin (43 kDa), pGEX vector GST (27 kDa) and ribonuclease A (13.7 kDa). The void volume V_0 and the total column volume V_t were 4.5 and 70 mL, respectively, measured using bromophenol blue and blue dextran. The inset Coomassie-stained SDS/PAGE analysis shows a major band at approximately 35 kDa, with no evidence of multimers under denaturing conditions, unlike the properly folded protein during FPLC.

The channels were more complex than previous recordings from CLIC1 and CLIC4, and almost always showed multiple conductance levels (Fig. 2A,B), even after adding reduced amounts of protein (1–5 ng) to minimize channel incorporation. Following recordings at +100 mV with 500 mM KCl in the *cis* chamber and 50 mM KCl in the *trans* chamber, to maximize the single-channel currents (Fig. 2C), CLIC5 amplitudes could be grouped into seven well-defined, nonoverlapping distributions (Fig. 2D). The amplitudes extended from 0.21 ± 0.15 pA (mean ± SD, $n = 7$), close to the minimum amplitude measurable in our system, to a maximum level of 12.5 ± 7.5 pA (mean ± SD, $n = 7$). However, the maximum level was only seen in approximately 10% of our experiments.

Transitions between the various open levels of CLIC5 appeared to be strongly cooperative. For example, we occasionally observed 'direct' transitions between large-amplitude channels and the closed (zero current) level, with no apparent intermediate levels (examples are noted in Fig. 3A). Most of the amplitudes in Fig. 2D (which may represent a combination of substates and cooperative gating) could be fitted to a simple exponential distribution, consistent with an

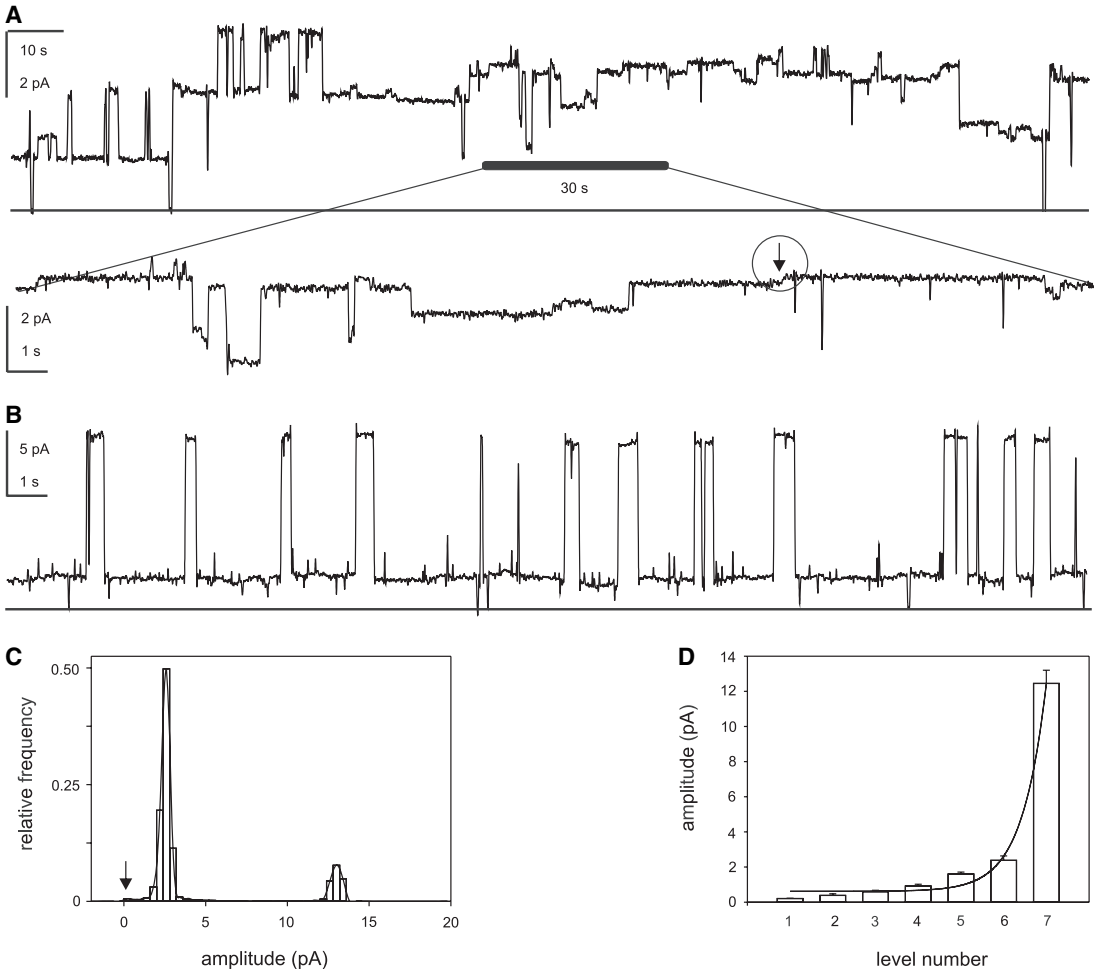

Fig. 2. Bilayer reconstitution of CLIC5 reveals multiconductance channels. CLIC5 was reconstituted in 5 mM GSH with 500 mM KCl in the *cis* chamber and 50 mM KCl in the *trans* chamber, and channel activity was recorded at a holding potential of +100 mV. (A) Contiguous 3 min recording illustrating a range of amplitudes up to approximately 2 pA. The solid line shows the zero-current level. The central portion of the trace is expanded, with an encircled arrow to indicate the smallest measurable current transition. (B) A record from another experiment under the same conditions showing an additional open level at approximately 12 pA. The solid line shows the zero-current level. (C) All-points amplitude histogram of the data in trace (B) (0.4 pA per bin), fitted to two Gaussian distributions (the arrow shows the zero-current level). (D) All the observed CLIC5 amplitudes grouped into seven discreet levels (bars represent mean ± SD, $n = 7$ independent experiments). The curve fits the equation: current (pA) = $[5 \times 10^{-5} \times \exp(1.77 \times \text{level number})] + 0.62$.

initial model in which individual CLIC5 channels contain various numbers of CLIC5 subunits, such that linear increases in circumference result in squared increases in cross-sectional area and a corresponding exponential increase in conductance. This simple model was testable, because it predicted reduced inter-ionic selectivities in 'large-diameter' pores compared with 'small-diameter' pores.

Ionic selectivity of CLIC5

We initially assessed the cation versus anion (K^+ versus Cl^-) selectivities of multilevel CLIC5 channels reconstituted in 500 versus 50 mM KCl based on the 'macroscopic' reversal potential, i.e. the voltage-clamp potential at which the net transbilayer current was zero. Surprisingly, for a putative anion-selective (CLIC) channel, the reversal potential was negative (at least −15 mV in four successive experiments). We then measured channel amplitudes at a range of holding potentials in experiments for which at least two specific, individual channel amplitudes could be clearly distinguished from each other by amplitude histogram analysis (Fig. 3A, compare with Fig. 2B,C), and plotted the corresponding current/voltage (I/V) relationships (Fig. 3B).

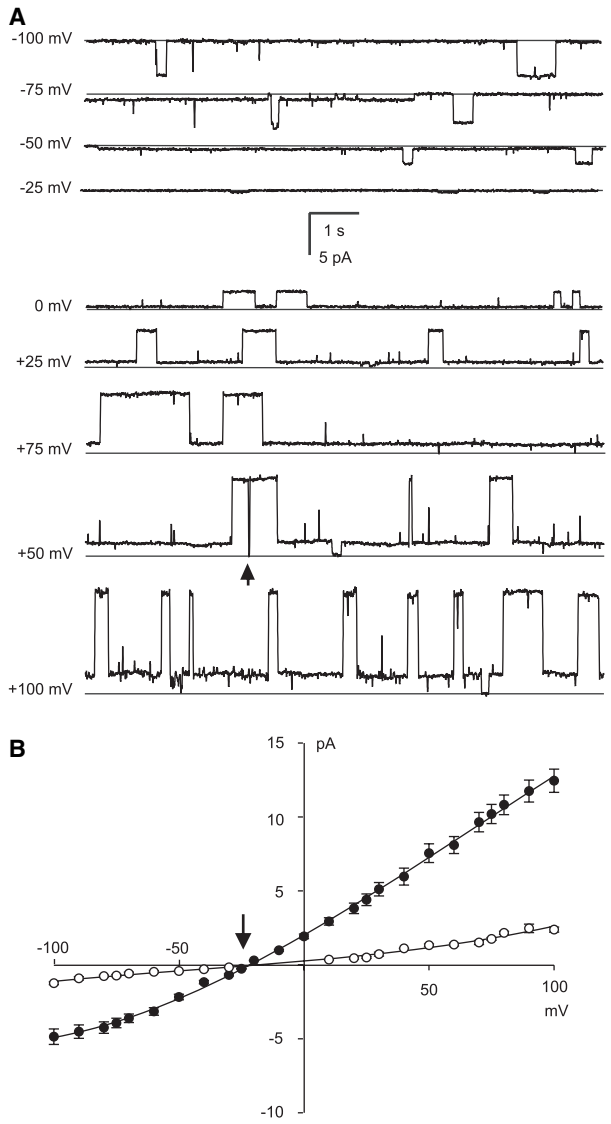

Fig. 3. CLIC5 current/voltage (*I/V*) relationships show mild selectivity for cations versus anions. Channel activity similar to Fig. 2B was analysed by amplitude histogram analysis (as in Fig. 1C) to return two well-defined main open levels (corresponding to levels 6 and 7 in Fig. 1D) under asymmetric ionic conditions (500 mM KCl *cis*, 50 mM KCl *trans*). (A) Example traces from a single experiment at a range of holding potentials, for which the solid lines indicate the closed (zero-current) levels. The arrow below the +75 mV trace indicates a direct transition between the maximum open level and the closed level, similar to various transitions at −100 mV. (B) *I/V* relationships of the large (filled circle) and small (open circle) conductances, shown as means ± SD ($n = 7$ independent experiments). The smooth curves are fitted by linear interpolation (three orders), and the common reversal (equilibrium) potential is marked by an arrow.

The *I/V* relationships in Fig. 3B were assembled from six independent experiments showing the same general pattern of channel activity. Under asymmetrical ionic conditions, positive single-channel currents at a holding potential of 0 mV (i.e. with no electrical driving force) can only be explained by net diffusion of K^+ from the *cis* chamber to the *trans* chamber. The reversal potentials of the two clearly identified conductance levels were indistinguishable, at −20.5 ± 1.5 and −20.6 ± 6.7 mV for the high- and intermediate-amplitude currents, respectively (means ± SD, $n = 6$), suggesting that their selectivities (and their conduction pathways) were identical. This argues against the hypothesis that different conductances result from pores with different diameters, or indeed different proteins, and instead suggests that collections of basically similar CLIC5 channels open and close together in a cooperative manner, and intermediate conductance levels are too brief to be resolved [19,20].

After starting experiments with 50 mM KCl in both chambers (with a corresponding reversal potential of 0 mV), addition of 150 mM KCl to the *cis* chamber shifted the reversal potential to −8.7 ± 1.6 mV (mean ± SD, $n = 3$), again indicating a slight preference for cations. Using Eqn (1), the value for P_K/P_{Cl} was 2.0 ± 0.2 (mean ± SD, $n = 6$) or 2.1 ± 0.4 (mean ± SD, $n = 3$) for the two conditions (high- and low-salt gradients), respectively. Under bi-ionic conditions (Fig. 4), with KCl in the *cis* chamber and NaCl in the *trans* chamber, the mean reversal potential averaged over a wide range of salt activities was +7.7 ± 0.3 mV (mean ± SD, $n = 8$ activity ratios). Given that the *cis* versus *trans* activity of Cl^- was equal in each case, regardless of the total ionic activity, these experiments directly compare the relative selectivity of CLIC5 for the two cations. Using Eqn (2), the relative cation permeability ratio P_{Na}/P_K is 1.3 ± 0.04 (mean ± SD, $n = 8$).

In vitro regulation of CLIC channels by F-actin

Given the original association of native CLIC5 with actin [16], we first tested the effect of adding purified platelet actin (Cytoskeleton Inc., Denver, CO, USA) to CLIC5 channels reconstituted in 5 mM GSH with 500 mM KCl in the *cis* chamber and 50 mM KCl in the *trans* chamber, after adding 100 μg mL^{-1} BSA to block nonspecific protein binding sites. G-actin was stirred into each chamber in turn to a final concentration of 250 nM (10 μg mL^{-1}), then polymerized by adding 5 mM $MgCl_2$ and 0.5 mM ATP. The *trans* KCl concentration was also increased to 100 mM to promote polymerization. The critical concentration of actin is comfortably exceeded under these conditions [21]. Finally, 10 μM cytochalasin B was added to the *cis* chamber to disrupt the F-actin. Mean currents were

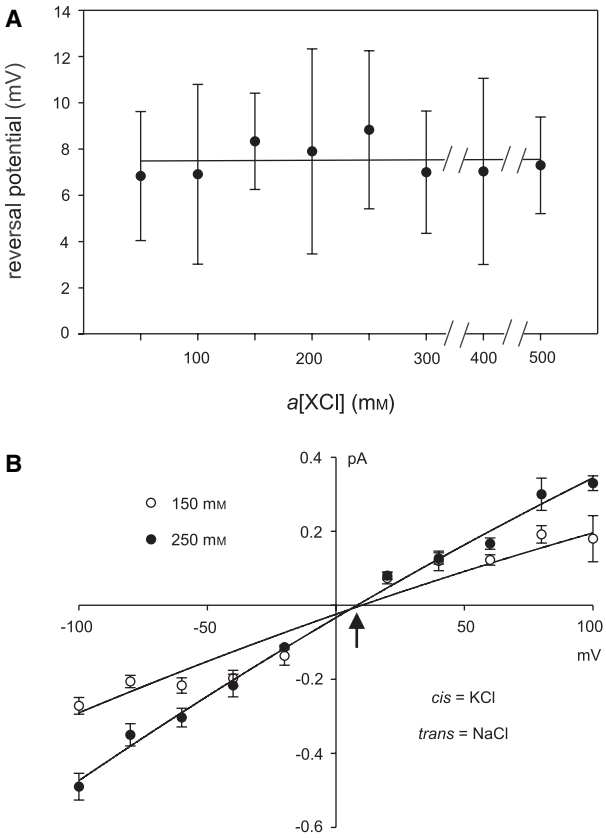

Fig. 4. Bi-ionic reversal potential measurements show poor selectivity between Na^+ and K^+. (A) Mean reversal potentials ± SD (n = 3–6 independent experiments) with KCl *cis* and NaCl *trans*, at the (matching) activities (a[XCl]) indicated. Note the breaks in the plot after a[XCl] exceeds 300 mM. The line (fitted by linear regression) has a gradient of zero, and corresponds to a mean E_r of +7.7 mV, averaged over a range of eight activities. (B) I/V relationships for matching activities as described in (A) for 150 mM (open circles) and 250 mM (filled circles); each point is the mean (± SD) of 3–6 experiments. The smooth lines are best least-squares fits to the GHK current equation (Eqn 3). The fits returned P_{Na}/P_K ratios of 0.70 and 0.74, respectively, and the corresponding reversal potentials (+9.5 and +7.8 mV) are indicated.

calculated from contiguous 60 s recordings for each condition, and typical recordings from a single experiment are illustrated, in the order in which they were obtained, in Fig. 5A–F. Figure 5G summarizes the results obtained from seven independent experiments.

Single-channel currents through CLIC5 were almost completely abolished by polymerizing the *cis* actin (Fig. 5E), and the effect was reversed by disassembling the F-actin with cytochalasin B (Fig. 5F). Addition of G-actin to the *cis* or *trans* chambers in the absence of actin-polymerizing agents did not affect channel activity, nor did polymerization to F-actin on the *trans* side. None of the additions affected CLIC5 in the absence of actin, and neither G-actin nor F-actin modified control bilayers in the absence of CLIC proteins. In additional control experiments carried out in the presence of 10 μM latrunculin B (to inhibit actin polymerization [22]), the mean current of 99 ± 12% with *cis* G-actin (mean ± SD, $n = 3$) remained essentially unchanged even under 'actin-polymerizing' conditions, at 105 ± 16% (mean ± SD, $n = 3$).

We next determined whether CLIC1 and CLIC4 showed similar sensitivities to *cis* F-actin, by reconstituting them in the presence of 5 mM GSH and repeating the experiments (and analysis) carried out on CLIC5. The ion channels formed by CLIC1 were also inhibited or inactivated by *cis* F-actin, and the effect was reversed by cytochalasin B (Fig. 6A,C). Like CLIC5, 10 μM latrunculin B prevented inhibition, with a mean current of 114 ± 27% (mean ± SD, $n = 3$) with *cis* G-actin, compared to 118 ± 22% (mean ± SD, $n = 3$) under 'actin-polymerizing' conditions. In contrast, CLIC4 was unaffected by F-actin (Fig. 6B,D). It should be stressed that, apart from using a different CLIC isoform in each case, our experiments on CLIC1, CLIC4 and CLIC5 were in every other respect identical, so CLIC4 contributes a useful negative control. Thus F-actin regulates CLIC channel activity in an isoform-specific manner.

Discussion

CLIC5 forms poorly selective ion channels

CLIC1 and CLIC4 autoinsert into membranes to form ion channels [3]; however, this property has not been investigated for CLIC5. CLIC5 also associates with cytoskeletal filaments [16], but the functional consequences of such interactions are unknown. We set out to express and purify mammalian CLIC1, CLIC4 and CLIC5, and incorporate the proteins into planar bilayers to test the hypothesis that they form actin-regulated ion channels. CLIC5 channels, like those corresponding to CLIC1 [17], and especially CLIC4 [11], were poorly selective rather than chloride-selective, reinforcing the suggestion that the CLIC nomenclature may need to be revised as more information becomes available. Strikingly, both CLIC1 and CLIC5 are directly and very strongly inhibited by cytoskeletal F-actin, in the complete absence of any other accessory or adapter protein, while CLIC4 is unaffected under similar conditions.

Given that human CLIC5 is 63% identical to human CLIC1, and 75% identical to human (and rat) CLIC4, we anticipated that CLIC5 would also insert spontaneously into planar lipid bilayers to form

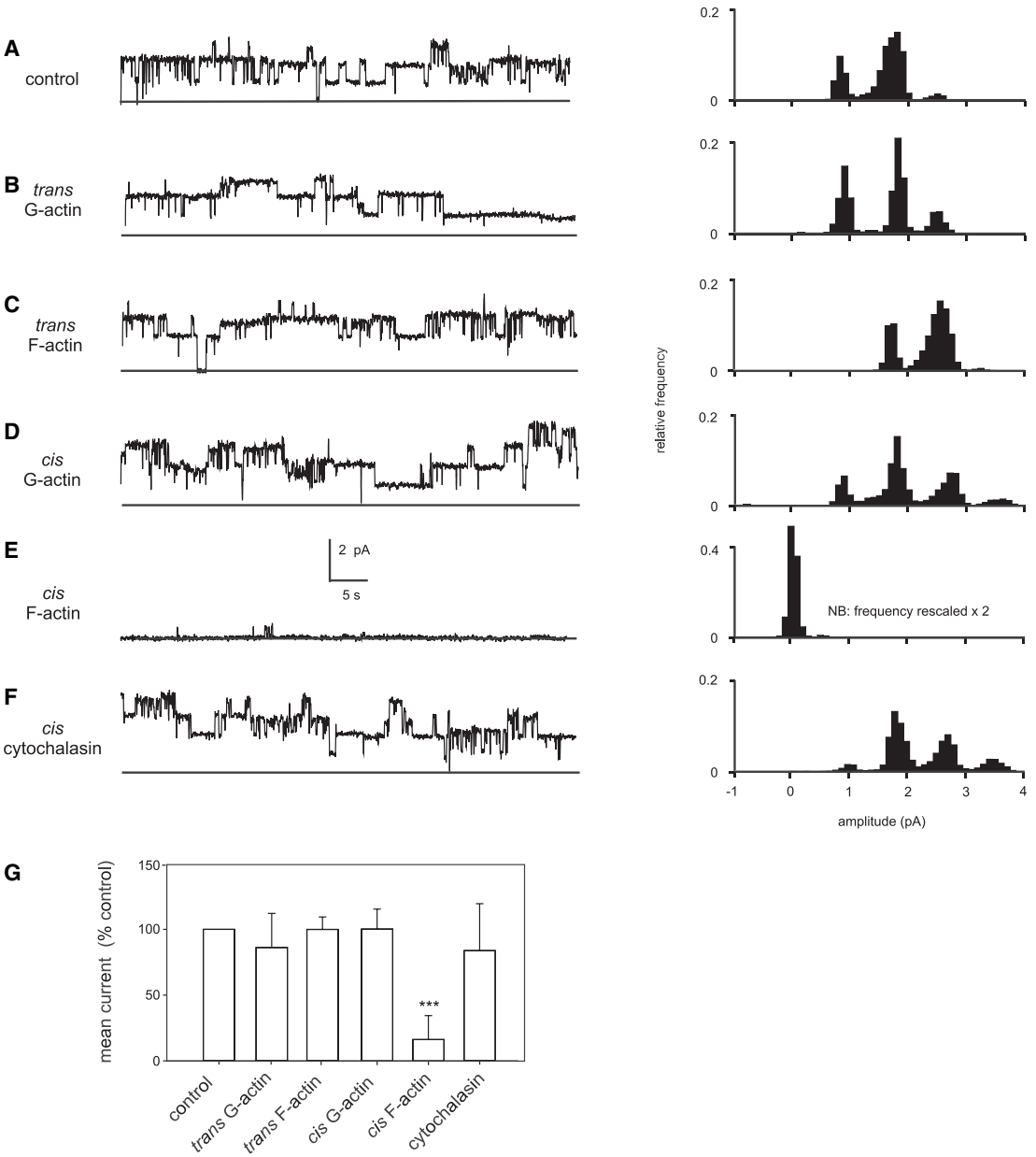

Fig. 5. *cis* F-actin reversibly inhibits CLIC5 channels. (A–F) Successive CLIC5 recordings from a single experiment at a holding potential of +100 mV under asymmetric ionic conditions (500 mM KCl in the *cis* chamber, 50 mM KCl in the *trans* chamber), with the additions shown to the left of each trace. The closed levels are shown as solid lines, and in this experiment the channels correspond mainly to level 4 in Fig. 1D. Each contiguous 60 s recording is accompanied by its corresponding all-points amplitude histogram [binwidth 0.1 pA, note the frequency scale change in (E)]. Actin and cytochalasin B were added at concentrations of 250 nM and 10 μM, respectively. (G) Combined results from seven independent experiments (bars represent means ± SD, $n = 7$). *$P < 0.001$ for the reduction in mean current with *cis* F-actin.

broadly similar ion channels. The channels were mildly cation- (not anion-) selective, like CLIC4 under similar recording conditions [11], and the value for P_K/P_{Cl} was approximately 2.0 under both high- (500 mM versus 50 mM, Fig. 3) and low- (150 mM versus 50 mM) salt gradients. The multiple conductance levels of CLIC5 followed an approximately exponential distribution (Fig. 2D), consistent with highly cooperative gating of individual unit conductances. We suggest that this reflects arrays of channels, rather than complex substate behaviour in a single large channel, because most incorporations lacked the largest-amplitude openings. This behaviour recalls the tendency of the purified protein to form multimolecular complexes

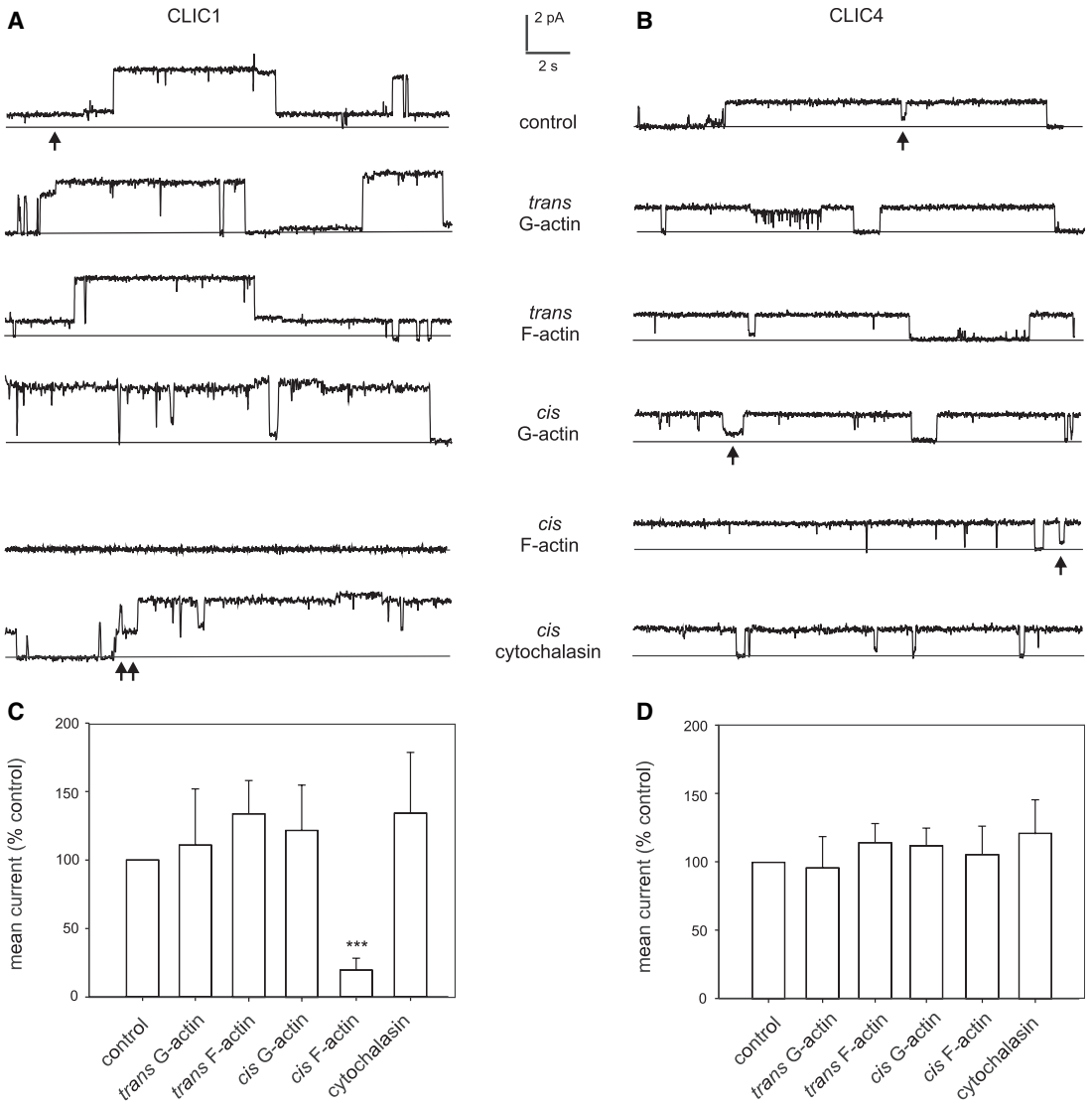

Fig. 6. *cis* F-actin inhibits single-channel currents through CLIC1 but not CLIC4. Examples of recordings from CLIC1 (A) and CLIC4 (B) in experiments carried out under exactly the same conditions used for CLIC5 (Fig. 4). The solid lines show the closed levels, and arrows indicate previously reported substates of approximately 25% and approximately 45% (double arrows for CLIC1). (C,D) Mean CLIC1 and CLIC4 currents measured from 60 s recordings under various conditions. The bars show means ± SD; $n = 8$ for CLIC1 in (C) and $n = 7$ for CLIC4 in (D). $^*P < 0.001$ for the reduction in mean current with *cis* F-actin for CLIC1.

(Fig. 1). Unfortunately, the common multiconductance activity of CLIC5 prevented a detailed investigation of channel gating behaviour, and precluded detailed examination of the channel's conductance/activity relationship.

We measured a consistent bi-ionic reversal potential (Fig. 4) over a wide range of salt activities, and confirmed that CLIC5 discriminates poorly between K^+ and Na^+ (P_{Na}/P_K approximately 1.3). Overall, the relative selectivity of CLIC5 for physiologically important monovalent ions is: $P_{Na} > P_K > P_{Cl}$, with a ratio of about 1.0: 0.75: 0.37. This indicates minimal inter-ion selectivity. Interestingly, the selectivity ratios were not concentration- (or, more correctly, activity-) dependent, even though, like CLIC1 and CLIC4, CLIC5 was expected to form a multi-ion channel. However, we could only examine relative selectivities at activity ratios extending over less than a single order of magnitude, and not at all at very low activities, so we may have been unable to detect important evidence for nonindependent ion permeation [23].

Recent *in vivo* [7] and *in vitro* [11] experiments identified a single putative transmembrane domain (TMD) near the N-terminus of invertebrate and vertebrate

CLICs. The TMD appears to be both necessary and sufficient for membrane targeting and membrane protein function, and, provided it remains intact, the 'cytosolic' regions of the proteins, from the TMD to the C-terminus, are interchangeable between CLICs [7]. Both approaches (*in vitro* and *in vivo*) implied that the ion channels formed by membrane CLICs must be oligomers, but sequence comparisons suggested that the slightly different ionic selectivity of individual channels does not depend on specific residues in the identified TMD. This led to the suggestion that other parts of the protein, including the channel vestibules, modulate selectivity [11], and key molecular determinants of specific CLIC properties may not become testable until the structures of the membrane proteins are available.

CLICs exist as soluble and membranous proteins [3], and they interact with various other cytosolic proteins [15]. As CLIC function was investigated by their reconstitution in planar bilayers, it is possible that modulation of either channel permeability or selectivity by cytoplasmic and membranous components present inside an intact cell may have been overlooked. However, as the channel properties of CLIC5 have not been reported to date, this reduced system has the advantage of characterizing CLIC5 function in the absence of modulatory factors. Furthermore, the role of actin in regulating channel function is best observed in such a reduced system considering the multiple and contrasting roles that the cytoskeleton performs in intact cells.

Ion channel regulation by F-actin

Every conductance level in multiconductance CLIC5 bilayer recordings was inhibited by F-actin, and the effect was reversed by F-actin disassembly. Direct F-actin regulation was specific to CLIC1 and CLIC5, and did not extend to the very similar protein CLIC4, which served as an excellent negative control for nonspecific binding. Actin modulation of CLIC1 and CLIC5 was specific from the cytosolic side, implying a role of the C-terminus of CLICs in channel regulation. Recently, CLIC1 was shown to be regulated by the cystic fibrosis transmembrane conductance regulator [24], which, in turn, is known to be directly regulated by actin [25]; similarly, CLIC5 is known to colocalize with actin and ezrin [16], indicating a possible *in vivo* modulation of CLIC1 by actin filaments and functional interaction between CLICs and other ion channels.

Ion channels are always tightly regulated in cells, and membrane CLICs appear to be controlled in at least three ways. Firstly, the proteins preferentially assemble into functional ion channels in specific lipid environments [17]. Secondly, mammalian membrane CLICs contain a critical cysteine residue immediately before the putative TMD, and we have suggested that channel complexes could be functionally regulated by the *trans* (extracellular or luminal) redox potential via glutathione-dependent trans-thiolation [17]. Finally, as we show here, CLIC1 and CLIC5 are regulated *in situ* by cytoplasmic F-actin.

Potential roles of CLIC5 and other membrane CLICs

Our results add to the growing list of diverse ion channels regulated by actin [18,25–27]. Further work will be required to determine how F-actin interacts with the proteins, and how it mediates channel inhibition. Direct regulation of cystic fibrosis transmembrane conductance regulator channels by cytoskeletal actin has been attributed to putative actin-binding regions [25], but, apart from noting some suggestive charge differences when flexible [1] 'hinge' regions in the soluble structures are aligned, we could find no structural evidence to support this hypothesis for the CLIC proteins. This does not exclude the possibility of additional structural roles for the proteins. For example, CLIC5 appears to be an important component of many actin-rich structures in cells [28], including the inner ear stereocilia that were found to be defective in CLIC5-deficient mice with impaired hearing and balance [29]. However, it is tempting to speculate that selected membrane CLICs could be specifically activated when cells or organelles undergo specific physiological changes.

Actin is one of the most abundant proteins in the cell, and plays a significant role in many physiological functions. It has numerous binding partners and a high tendency for specific and nonspecific interactions in the cell, including the nucleus [30], where CLICs have been localized and shown to participate in physiological processes such as regulation of the cell cycle [13]. It is known that ion channels do not operate as randomly diffusing moieties in the plasma membrane of cells. They interact with the cytoplasmic proteins, which in turn link them to cytoskeleton or intracellular signalling pathways. Direct or indirect interaction of CLICs with cytoskeletal elements such as actin or dynamin [15,16] is likely to result in their immobilization and clustering in membranes, and in the targeting of these proteins to an appropriate site where they may participate in various physiological processes. The direct interaction of actin with CLIC1 and CLIC5 (but not CLIC4) actin implicate them in diverse CLIC-specific

functions, which could include movement, swelling or division of the cell, endocytosis and exocytosis, intracellular vesicle fusion, and apoptosis. The major challenge in future is to understand the functional significance of these protein interactions in various physiological processes.

A number of key questions remain. Although p64 and other CLICs contain multiple protein interaction sites [3], we could not identify a putative actin-binding site, e.g. a site similar to the actin-binding site in the α subunit of amiloride-sensitive epithelial Na^+ channels [31], nor could we identify (from alignments of the three CLIC proteins) speculative actin-binding residues in CLIC1 and CLIC5 that are absent in CLIC4. With respect to the three-dimensional structure, we do not of course know whether (or to what extent) the CLIC 'cytoplasmic domain' refolds in the membrane forms of the proteins, or indeed how the proteins assemble into subunits. However, the tendency of CLIC5 to form multimolecular assemblies may be encouraging from the perspective of future structural studies of this membrane protein.

Experimental procedures

Preparation of CLIC proteins

Selected CLICs were expressed as His-tagged proteins in *Escherichia coli* and purified by a combination of IMAC and gel-exclusion (GE) chromatography, as detailed previously for rat CLIC4 [11] and human CLIC1 [17]. In this study, we also expressed a cDNA encoding human CLIC5 (CLIC5A, MGC:53405, IMAGE:4611102, MRC Geneservice, Cambridge, UK). Like CLIC1 and CLIC4, the relevant cDNA was cloned by PCR into pHIS-8, a modified pET-28a(+) vector encoding an N-terminal octahistidine tag and a thrombin cleavage site. The insert was verified by DNA sequencing (MWG Biotech, Ebersberg, Germany), and soluble CLIC5 was recovered from transformed *E. coli* BL21 (DE3) cells by Ni^{2+}-NTA affinity chromatography after isopropyl thio-β-D-galactoside-induced overexpression. The tag was cleaved by thrombin, and the enzyme and the free tag were scavenged using benzamidine–Sepharose 4B beads and Ni^{2+}-NTA resin (Amersham, Chalfont St Giles, UK). CLIC5 aggregated and precipitated in buffers containing 5 mM dithiothreitol, but not in those containing 5 mM β-mercaptoethanol or 5 mM GSH when the protein was diluted, e.g. when added to bilayers. Further purification by GE FPLC using Superdex 200 (Pharmacia, Uppsala, Sweden) in the presence of 5 mM β-mercaptoethanol showed a major peak consistent with the monomeric protein, and additional peaks suggestive of dimers and tetramers of the soluble protein (shown in Fig. 1, along with an example of Coomassie-stained SDS/PAGE carried out under reducing conditions in a 10% w/v acrylamide gel). The yield of (monomeric) CLIC5 was 5.0 ± 0.80 mg L^{-1} (mean ± SD, $n = 5$), and the protein was stored in aliquots at −70 °C in buffer containing 5 mM β-mercaptoethanol.

Ion channel reconstitution

CLIC proteins were incorporated into voltage-clamped planar lipid bilayers formed from palmitoyl-oleoyl phosphatidylethanolamine, palmitoyl-oleoyl phosphatidylserine and cholesterol (4 : 1 : 1, mol/mol), as previously described for CLIC1 [11] and CLIC4 [17]. Briefly, the lipids were dispersed in *n*-decane, and membranes were cast across a 0.3 mm hole separating two solution-filled chambers designated *cis* (the side of subsequent protein addition, which corresponds to the cell cytosol) and *trans* (the external side, which corresponds to the luminal side of intracellular organelles). The *cis* chamber was voltage-clamped at selected holding potentials relative to the *trans* chamber, which was grounded, using agar salt bridges and $Ag/AgCl_2$ wires connected to an Axopatch 200B patch-clamp amplifier (Axon Instruments, Foster City, CA, USA). Liquid junction potentials were routinely offset to 0 mV. Transmembrane currents were low-pass-filtered at 25–250 Hz (8-pole Bessel response) and digitally recorded (PCLAMP software, Axon Instruments). The contents of the chambers were adjusted to provide 500 mM KCl in the *cis* chamber and 50 mM KCl in the *trans* chamber, each containing 10 mM Tris/HCl (pH 7.4) and 5 mM GSH. Purified, soluble CLIC proteins were stirred into the *cis* chamber at up to 25 ng mL^{-1}, and, following the appearance of channels (within 5–10 min), the solution was replaced by perfusion (10 volumes) to limit further incorporation. Test bilayers had a capacitance of 310 ± 20 pF (mean ± SD, $n = 20$) and remained stable for at least 45 min, with no channel-like activity in the absence of added protein.

Single-channel analysis

We adopted the standard electrophysiological convention (i.e. upgoing currents represent net cation flux from *cis* to *trans* in bilayers, and outward positive currents in voltage-clamped cells). The data were analysed using PCLAMP (Axon Instruments) and SIGMAPLOT (SPSS, Chicago, IL, USA). We measured unit or mean channel currents, and generated all-points amplitude histograms and *I/V* relationships. Relative ionic permeabilities were analysed using appropriate forms of the Nernst equation or the Goldman–Hodgkin–Katz (GHK) voltage equation. The permeability ratio of anions to cations (P_A/P_C) was determined from:

$$P_A/P_C = [n \cdot \exp(E_r/k) - 1]/[n - \exp(E_r/k)] \quad (1)$$

where n is the *cis:trans* salt activity ratio, E_r is the reversal (equilibrium) potential, and $k = RT/zF$ (26 mV under our

conditions). Cation permeabilities relative to K^+ (P_C/P_K) were determined under bi-ionic conditions from:

$$(P_C/P_K) = a[K^+]_{cis}/a[C^+]_{trans} \cdot \exp(-zFE_r/RT) \quad (2)$$

where a is the activity coefficient of the relevant salt. E_r was estimated by regression analysis (up to three components) from I/V plots. Selected I/V relationships were refitted to the GHK current equation by calculating the transmembrane currents carried by specific ions (I_s):

$$I_s = \frac{P_s \cdot z_s^2 \cdot E_r F^2/RT \cdot \{[S]_i - [S]_o \cdot \exp(-z_s FE_r/RT)\}}{\{1 - \exp(-z_s FE_r/RT)\}} \quad (3)$$

where P_s is the permeability of ion s. Differences between means were taken to be significant if $P < 0.05$.

Acknowledgements

HS was supported by a University of Edinburgh College of Medicine & Veterinary Medicine Scholarship, and by the Overseas Research Students award scheme. We thank Sutherland Maciver (Centre for Integrative Physiology, University of Edinburgh, UK) for helpful discussions.

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