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CLIC proteins: chameleon proteins at the interface between membranes and the cytoskeleton

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Most proteins exist as either soluble or integral membrane proteins, however, there is a growing class of proteins that can adopt both of these states. Traditionally, these proteins have been mainly limited to bacterial toxins such as colicin and diphtheria toxin. More recently, eukaryotic cytoplasmic proteins have been shown to exhibit these properties. The CLIC family represents a class of proteins that are generally soluble, but can integrate into membranes to form chloride ion channels. CLICs are members of the GST fold family, but, in contrast to the GSTs, they have a reactive cysteine within their putative active site. The CLICs are highly conserved in all chordates and vertebrates, with related proteins in the invertebrates. We have used x-ray crystallography determine the structures of both human and invertebrate CLICs. In particular, human CLIC1 is shown to undergo a dramatic structural transition which is redox regulated. Under oxidising conditions, CLIC1 forms a non-covalent dimer with a radically altered monomer conformation. This novel conformation is stabilised by an intramolecular disulphide bond and the transition is reversed on reduction. Using biophysical and electrophysiological methods, we have shown that both the reduced CLIC1 monomer and the oxidised dimer can auto-insert into artificial lipid bilayers and form anion channels with properties that are indistinguishable from those observed in cells. Our best model for the CLIC1 transmembrane channel state indicates that residues 24 to 46 form a putative transmembrane domain with the first 23 residues on one side of the membrane and C-terminus on the opposite side of the membrane. If this model is correct, then the oxidised CLIC1 dimer must undergo an isomerization of its intramolecular disulphide bond prior to membrane insertion.

Interactions of human profilin-1 and phosphatidylinositol 4,5-bisphosphate in giant unilamellar vesicles

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Human Profilin-I is a small actin binding proteins which also interact with polyphosphoinositides (PPI) (Lassing & Lindberg, 1985) and proline rich motif containing proteins. Profilin is involved in the signaling pathway linking the receptors in the cell membrane to the microfilament system. Profilin is thought to play critical roles in this signaling pathway through its interaction with phosphatidylinositol 4,5-bisphosphate [$PI(4,5)P_2$] (Lu *et al.*, 1996). So far, profilin's interaction with PPI has only been studied in micelles or small vesicles. Profilin binds with high affinity to small clusters of $PI(4,5)P_2$ molecules (Goldschmidt-Clermont *et al.*, 1991). In cells, the organization of PPI lipids is different from micelles, therefore the interaction with profilin might be quite different.

We have used giant unilamellar vesicles (GUV) as a membrane model system to investigate the interactions between $PI(4,5)P_2$ and profilin. BODIPY® TMR $PI(4,5)P_2$C16 (Rhodamine labelled 16-carbon fatty acid containing Phosphatidylinositol 4, 5-bisphosphate) was incorporated into GUV membrane. Confocal images of GUVs were obtained in presence and absence of profilin. These images were analysed using SimFCS software (Prof. Enrico Gratton, Laboratory for Fluorescence Dynamics, Irvine, USA). The diffusion coefficient and the aggregation of PIP2 in the membrane were determined and profilin had a clear effect upon the diffusion coefficient and aggregation of PIP2.

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New twists in old tales. Conotoxins targetted to sodium channels

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The fish-hunting cone snail *Conus radiatus* produces a novel conotoxin, ι-RXIA, which is a member of the recently characterized I_1-superfamily. This superfamily contains eight cysteine residues arranged in a –C-C-CC-CC-C-C- pattern. ι-RXIA is one of several I_1 peptides in which the third last residue is post-translationally isomerized to the D-configuration, and naturally occurring ι-RXIA, with D-Phe44, is significantly more active as an excitotoxin than the L-Pheanalogue both *in vitro* and *in vivo*. We have determined the solution structures of both forms by NMR spectroscopy (Buczek *et al.*, 2007). The structure of ι-RXIA is well defined up to around residue 35 and adopts an ICK structure. The C-terminal region, including Phe44, is disordered. Comparison of the D-Phe44 and L-Phe44 forms indicates that the switch from one enantiomer to the other has very little effect on the structure, even though it is clearly important for receptor interaction based on activity data. Finally, we identify the target of ι-RXIA as a voltage-gated sodium channel. ι-RXIA is an agonist, shifting the voltage dependence of activation of mouse $Na_V1.6$ expressed in *Xenopus* oocytes to more hyperpolarized potentials. Thus, there is a convergence of structure and function in ι-RXIA, as its disulfide pairing and structure resemble those of funnel web spider toxins that also target sodium channels.

Voltage-gated sodium channels are a target for several families of conotoxin. Recently, new representatives of the μ-conotoxin family have been described and their structures determined (Keizer *et al.*, 2003; Bulaj *et al.*, 2005). These suggest a potential of these toxins in the treatment of pain (Green *et al.*, 2007).

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Dimers are forever: New developments in the mechanism of the Na^+,K^+-ATPase

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The Na^+,K^+-ATPase (or sodium pump) was the first ion pump to be discovered (Skou, 1957) and it is one of the most fundamentally important enzymes of animal physiology. The electrochemical potential for Na^+, which the enzyme maintains, is used as the driving force for numerous secondary transport systems, *e.g.*, voltage-sensitive Na^+ channels in nerve. The mechanism of the Na^+,K^+-ATPase is universally described in biology textbooks by the Albers-Post cycle, which represents the catalytic subunit of the enzyme as a monomer undergoing a cyclical sequence of conformational changes, ion binding and release steps and ATP phosphorylation/dephosphorylation reactions. Although this mechanism is consistent with the vast majority of experimental data, for many years research groups around the world have discovered reproducible results which are inconsistent with this mechanism: 1) multiple ATP binding affinities; 2) phosphorylation of half of the ATP binding sites; 3) simultaneous presence of two intermediate states of the cycle; and 4) two-step release of K^+ ions. These inconsistencies indicate that the widely accepted Albers-Post model cannot be the full truth. The results of stopped-flow kinetic experiments and theoretical simulations (Clarke & Kane, in press) indicate that the enzyme exists as a functional dimer within the membrane. To explain these results as well as previous inconsistencies with the Albers-Post model, we propose a new mechanistic model in which the enzyme cycles at a low rate with ATP hydrolysis by one catalytic subunit or at a high rate with ATP hydolysis by two catalytic subunits simultaneously within a dimer, depending on the concentration of available ATP. Thus, we propose a bicyclic model with two gears to replace the classical monomeric Albers-Post cycle.

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TRPC1 is increased in *mdx* muscle, binds to caveolin-3 and is regulated by Src kinase: implications for Duchenne muscular dystrophy

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Duchenne muscular dystrophy (DMD) is an X-linked genetic disease caused by the absence of dystrophin, a membrane anchoring protein. We have shown that calcium entry through stretch activated channels (SACs) contributes to muscle damage in the *mdx* mouse, an animal model of DMD (Yeung *et al.*, 2005). Transient receptor potential canonical 1 (TRPC1) forms SACs in mammalian cells (Maroto *et al.*, 2005) and interacts with caveolin-1 in smooth muscle cells (Lockwich *et al.*, 2000). Caveolin-3 (Cav-3), which is structurally homologous to caveolin-1, is increased in *mdx* muscle (Vaghy *et al.*, 1998). The aim of this study is to investigate the expression levels and interaction of Cav-3 and TRPC1 in *mdx* muscle. TRPC1 and Cav-3 co-localized, co-immunoprecipitated and had increased expression levels in *mdx* muscle (immunohistochemistry, Western blot). Fluorescence Energy Resonance Transfer (FRET) was used to confirm the interaction of the two proteins, in C2C12 myblasts co-transfected with TRPC1-CFP and Cav-3-YFP plasmids. Fluorescence Lifetime Imaging Microscopy (FLIM) showed a shortening of the donor lifetime (TRPC1-CFP) when cells were co-transfected with both plasmids (from 2.7ns to 2.1ns; $p < 0.001$), confirming the interaction between TRPC1 and Cav-3. As Src kinase can activate channels from the TRPC family (Kawasaki *et al.*, 2005), we investigated the role of this kinase on TRPC1 activity and binding properties with Cav-3. Incubation of C2C12 cells with hydrogen peroxide (H_2O_2), a reactive oxygen species (ROS), increased the levels of both the active form of Src kinase (Western blot, $p < 0.01$) and the total tyrosine phosphorylation in those cells (immunohistochemistry, $p < 0.01$). Src activation and tyrosine phosphorylation were partially prevented when cells were incubated with PP2, a Src kinase inhibitor, prior to H_2O_2 treatment. Ratiometric calcium imaging (Fura Red) of C2C12 cells transfected with TRPC1-CFP and Cav-3-YFP revealed that calcium influx was increased upon H_2O_2 treatment only in cells expressing both TRPC1 and Cav-3 plasmids. Furthermore, the calcium influx was prevented when those cells were pre-incubated with PP2. These results suggest that Cav-3 is necessary for TRPC1 activity, which is triggered by activation of Src kinase by ROS. As ROS are known to be increased in *mdx*/DMD, we suggest that targeting the ROS-Src-TRPC1 pathway could lead to the development of new therapeutic approaches for the treatment of DMD.

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In the presence of ATP, acidosis markedly inhibits ClC-1 skeletal muscle chloride channels

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Skeletal muscle acidosis during exercise has long been thought to be a cause of fatigue but recent studies have shown that acidosis maintains muscle excitability and opposes fatigue by decreasing the sarcolemmal chloride conductance. ClC-1 is the primary sarcolemmal chloride channel and has a clear role in controlling muscle excitability but recombinant ClC-1 has been reported to be activated by acidosis. Following our recent finding that intracellular ATP inhibits ClC-1, we investigated here the interaction between pH and ATP regulation of ClC-1. We found that in the absence of ATP, intracellular acidosis from pH 7.2 to 6.2 inhibited ClC-1 slightly by shifting the voltage dependence of common gating to more positive potentials, similar to the effect of ATP. Importantly, the presence of physiological concentrations of ATP greatly potentiated the effect of acidosis on common gating, causing a marked inhibition of ClC-1 channel activity. Adenosine had a similar effect to ATP at pH 7.2 but acidosis did not potentiate this effect, indicating that the phosphates of ATP are important for this cooperativity, possibly due to electrostatic interactions with protonatable residues of ClC-1. A protonatable residue identified by molecular modelling, His847, was found to be critical for both pH and ATP modulation and may be involved in such electrostatic interactions. These findings are now consistent with, and provide a molecular explanation for, acidosis opposing fatigue by decreasing the chloride conductance of skeletal muscle *via* inhibition of ClC-1. The modulation of ClC-1 by ATP is a key component of this molecular mechanism.