Conserved extracellular cysteine residues in the inwardly rectifying potassium channel Kir2.3 are required for function but not expression in the membrane

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Abstract The mouse potassium channel Kir2.3 possesses conserved extracellular cysteine residues at positions 113 and 145. We have investigated the role of these cysteines in structure/function and membrane trafficking. Cysteine to serine mutations resulted in the absence of potassium currents in oocytes and co-expression of these mutants with wild-type channel showed a dominant negative inhibition of wild-type currents. FLAG-tagged channels expressed in oocytes were detected in the cell membrane by anti-FLAG antibody for wild-type and mutant channels. In vitro translation using the reticulocyte lysate system showed that mutation of these residues did not affect processing nor insertion into membranes. Cysteine residues at 113 and 145 are therefore required for function of the Kir2.3 channel but not for processing into the cell membrane; disulfide bonds between subunits are unlikely.

Key words: Potassium channel; Inward rectifier; Cysteine; Disulfide bond; Expression

1. Introduction

Potassium channels are a very large family of integral membrane proteins with diverse physiological functions [1]. They are mainly divided into families comprising the inwardly-rectifying channels (Kir) (which have two transmembrane domains per subunit) such as Kir2.3 [2] studied here and the voltage-gated (Kv) outwardly rectifying channels (with six transmembrane domains per subunit) such as Shaker [3,4].

Both Kv and Kir channels are known to assemble in a subunit-specific manner. For Kir channels, subunit assembly is mediated through interaction between the proximal C-terminus and the second transmembrane domain (M2) of adjacent subunits [5]. This is unlike Kv channels, where interaction between subunits is via the distal N-terminus and first transmembrane domain (S1) of each subunit [6,7]. Both Kv [8] and Kir [9] channels are tetrameric structures, and are thought to assemble into tetramers in the endoplasmic reticulum (ER) [10,11] of cells, before being conveyed to the cell membrane (together with any auxiliary subunits present in the channel such as the β-subunit of Kv channels).

The presence of naturally occurring cysteines in these channels raises the question as to whether disulfide bonds exist between subunits. For the TWIK channel (which comprises two subunits, each with a tandem repeated two-transmembrane domain [12]), it has previously been reported that a disulfide bond between extracellular cysteines on adjacent subunits is required for functional expression of this channel [12], but is not required for channel function once expressed. For the six-transmembrane Shaker channel subunits, intracellular cysteines could potentially be involved in inter-subunit disulfide bonds, but such bonds are in fact neither present nor required for functional expression of Shaker channels, and indeed the formation of such bonds experimentally has no effect on channel function [13].

Kir potassium channels possess two conserved extracellular cysteine residues at positions corresponding to 113 and 145 in Kir2.3 channels (Fig. 1). A possible role for these two cysteines might be in the formation of disulfide bonds between subunits, for example to stabilise the tetrameric structure, or in the formation of bonds within each subunit to maintain subunit conformation. In this study we have investigated whether these two highly conserved cysteines are necessary for transport to the cell membrane and for channel function. A preliminary abstract of this work has appeared [14].

2. Materials and methods

2.1. Molecular biology

The mouse Kir2.3 clone (accesion number U11075, [2]) in pEXO was used for the present studies (clone kindly donated by F. Lesage). Serine was substituted for cysteine at positions 113,140 and 145 using Kunkel's method for site-directed mutagenesis [15]. The mutation to serine (i.e. replacing sulfur in cysteine by oxygen) was chosen to minimise disruption of the protein. All mutations were confirmed by dideoxy sequencing.

A Kir2.3 FLAG-tagged construct was made as follows; the construct also had a heart myosin kinase site (HMK) tag which was not utilised in the present study. The Kir2.3 insert was first excised from the pEXO vector using 5′ *Eco*RI and 3′ *Xho*I (MBI Fermentas). The HMK-FLAG DNA sequence was excised from a pBluescript-HMK-FLAG plasmid construct using the same enzymes, and ligated into pEXO using T_4 DNA ligase (New England Biolabs). An *Eco*RI restriction site was engineered into the Kir2.3 insert just before the stop codon using PCR mutagenesis and Kir2.3 ligated into the pEXO-HMK-FLAG plasmid at the *Eco*RI site. The resultant clone had the last four amino acids of Kir2.3 replaced by N-S-[HMK]-E-L-[HMK]-K-L-[FLAG]. In-frame ligations were confirmed by dideoxy sequencing.

cRNA was transcribed in vitro using T_7 polymerase from the above constructs linearised with *Bam*HI (Ambion MEGASCRIPT). Estimations of cDNA concentrations were made by comparing the ethidium stained bands with standard λ-*Hin*dIII markers (MBI Fermentas) on agarose gels. Estimations of cRNA concentrations were made by comparison with standard cRNA markers (Promega), separated on a formaldehyde agarose gel [16].

2.2. Electrophysiology

Wild-type or mutant cRNA (1–5 ng in 50 μl) was injected into

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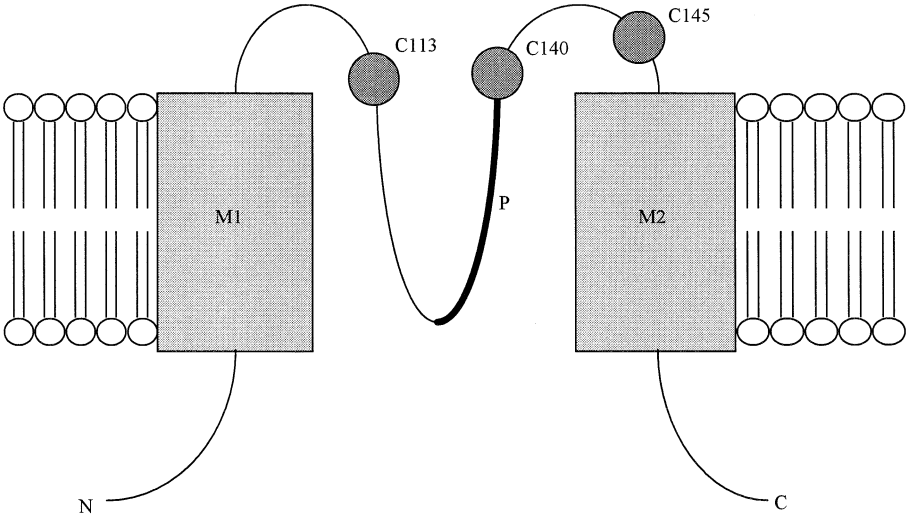

Fig. 1. Membrane topology of Kir2.3 channel subunits. The positions of the extracellular cysteines present in Kir2.3 channel subunits are indicated, along with the pore (P) region of the channel. Residues C113 and C145 are conserved across the Kir family.

Dumont stage V or VI *Xenopus* oocytes. Mixtures of wild-type and mutant cRNA were also injected, keeping the amount of wild-type constant at 1 ng. Oocytes were maintained at 19°C in modified Barths solution (88 mM NaCl, 1 mM KCl, 2.4 mM $NaHCO_3$, 0.82 mM $MgSO_4$, 0.33 mM $Ca(NO_3)_2$, 0.41 mM $CaCl_2$, 7.5 mM Tris-HCl, pH 7.6, 10 000 U/l penicillin, 100 mg/l streptomycin) and membrane currents recorded at room temperature (22–25°C) using a two-electrode voltage clamp (Geneclamp 500, Axon instruments) as previously described [17]. Recording electrodes were filled with 3 M KCl with resistances of 1–1.5 MΩ and 0.5–1 MΩ for voltage and current electrodes, respectively. Oocytes were superfused (1.5 ml/min) with high potassium solution (100 mM KCl, 17 mM NaCl, 10 mM HEPES, 1.8 mM $CaCl_2$, pH 7.2) in a 50 μl chamber. Membrane potential was held at 0 mV and 200 ms test potential steps were applied in 10 mV increments from −100 mV to +50 mV at 0.1 Hz in order to construct current-voltage (*I-V*) relationships. Data were filtered either at 1 kHz or 2 kHz and sampled at 3 kHz or 4 kHz, respectively, using CED software and a CED 1401 plus interface.

2.3. Immunofluorescence

Oocytes were injected with wild-type or mutant Kir2.3 cRNA (1 ng), and 24–48 h later fixed overnight in methanol at −20°C, then cryoprotected overnight at 4°C in 30% sucrose in phosphate buffered saline (PBS) (130 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.4). Sections (20 μm) were cut at −20°C and mounted on poly-A-lysine coated slides (BDH). After exposure to bovine serum albumin (1%) in PBS for 2 h at 4°C (to prevent non-specific binding), slides were incubated overnight in mouse primary M2 anti-FLAG antibody (Stratagene) (20 μg/ml). Slides were then incubated overnight with secondary anti-mouse FITC (fluorescein isothiocyanate) conjugated antibody (Sigma). Sections (mounting medium Vector Laboratories) were examined by illumination at 494 nm and observation of immunofluorescence at 519 nm, using confocal microscopy as previously described [18].

2.4. In vitro expression

Wild-type and mutant Kir2.3 ^{35}S-labelled proteins were prepared using the Promega T_7 'TNT-Quick' reticulocyte lysate kit (Promega), with or without canine pancreatic microsomes (Promega) as described in the manufacturer's protocol (except that microsomes were used at half the manufacturer's specification). Reactions were also carried out in the presence or absence of $CuCl_2$ (10 μM). Where required, the microsomal fraction was pelleted by centrifuging for 1.5 h at 100 000 × *g* in a Beckman ultracentrifuge. Labelled proteins were separated on an 8% SDS polyacrylamide gel, in the presence or absence of 2-mercaptoethanol (1.25%) (Sigma). Gels were exposed on a Fuji-Bas imaging plate for 8–12 h and read in a Fuji-Bas phosphor-imager. Bands were compared with Dalton VII protein molecular weight markers (Sigma).

3. Results

3.1. Functional properties of mutant channels

We firstly studied the effects of replacing the extracellular cysteines at positions 113, 140 and 145 with serine on the function of Kir2.3 channels (Fig. 1). We used mutant C140S channels as positive controls, since we have previously shown that C140S mutation leads to functional channels [14]. Upon injection of cRNA (1 ng) for wild-type and C140S mutant, oocytes showed currents with strong inward rectification properties in the high potassium solutions used here (Fig. 2). By contrast, oocytes injected with cRNA (1 ng) for mutants C113S and C145S did not display observable currents and in fact currents were indistinguishable from endogenous currents recorded from uninjected oocytes (Fig. 2). Injection of higher amounts of cRNA (5, 10 or 50 ng per oocyte) also failed to give observable currents for either C113S or C145S mutants (data not shown).

Similar experiments were carried out with FLAG-tagged Kir2.3 constructs. Placing the FLAG tag at the C-terminus of Kir subunits has been shown not to interfere with subunit assembly or functional expression [5]. Indeed, when injected into oocytes, cRNA for FLAG-tagged wild-type and C140S mutant gave currents which were similar to those seen for non-tagged channels (Fig. 3). Also, the FLAG-tagged mutants, C113S and C145S, again failed to display observable currents (Fig. 3), as for the corresponding untagged channels.

In summary, unlike wild-type and C140S mutant, the mutants C113S and C145S did not express currents. Addition of the FLAG-tag to wild-type and mutant channels did not affect the functional properties of these channels.

3.2. Expression of mixtures of mutant and wild-type channels

In order to investigate whether the C113S and C145S mutants were able to associate with wild-type subunits and form functional channels, we co-expressed wild-type cRNA together with either mutant C113S or C145S cRNA. The amount of wild-type cRNA injected was kept constant throughout and the amount of mutant cRNA was varied. When co-expressed with wild-type, C113S mutant subunits exhibited a dominant

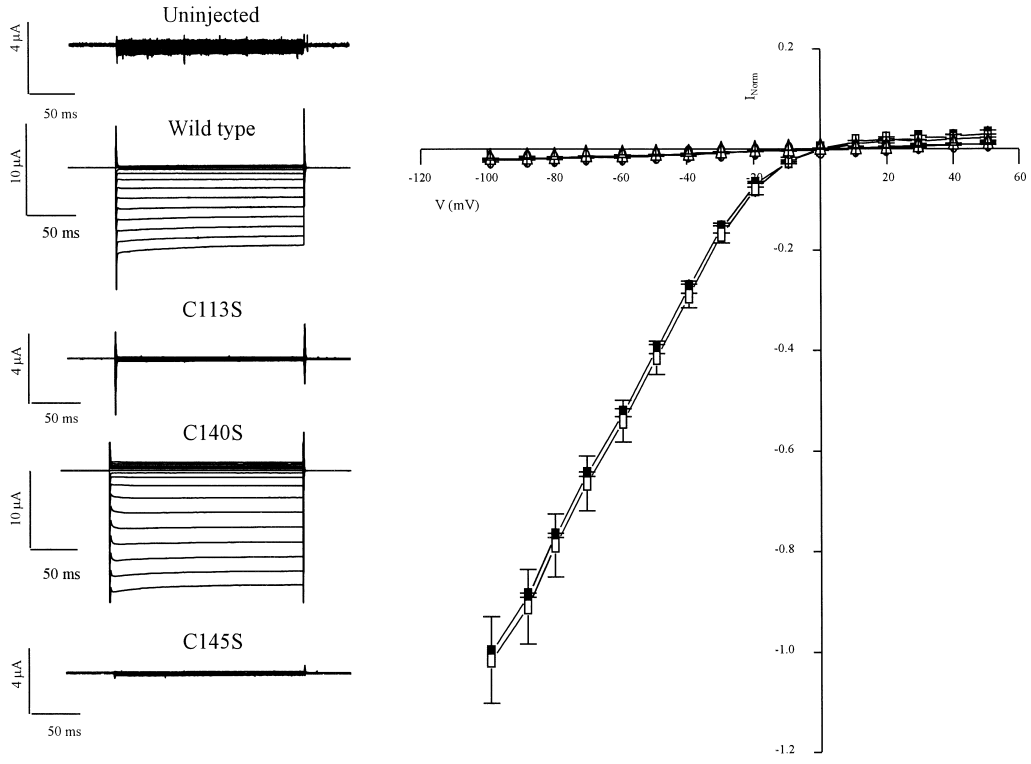

Fig. 2. Current families and *I-V*curves for wild-type and mutant Kir2.3 channels. Current voltage relationships are shown for oocytes injected with 1 ng cRNA for wild-type (■), C113S (●), C140S (□), C145S (○), as well as for uninjected oocytes (△). All currents were normalised to the wild-type current value at −100 mV. Data are presented as mean ± S.E.M. for five oocytes. Sample current recordings for each mutant are also shown.

negative effect on the wild-type current (Fig. 4A,C). A similar result was also obtained for C145S mutant subunits when co-expressed with wild-type (Fig. 4B,D). These reductions in current relative to wild-type alone occurred without otherwise affecting the shape of the *I-V* curves (Fig. 4A,B). Furthermore, the data are in reasonable agreement with the theoretical curve for a tetrameric channel (Fig. 4C,D), given the uncertainties in estimating cRNA quantities and other expected deviations from the theoretical curve [5].

These results suggest that the C113S and C145S mutations in Kir2.3 channels do not affect the association of subunits to form tetrameric channels, but do lead to a loss of channel function through a dominant negative effect. This suggests that the mutant channels are expressed and processed into tetramers, but in a non-functional form.

3.3. Expression of FLAG-tagged channels in oocytes

The next stage was to test directly whether the mutant subunits C113S and C145S were transported to the cell membrane (where they would form non-functional channels, see above) or if they were retained within the cell, for instance as tetramers in the ER. For this, we expressed FLAG-tagged wild-type and C140S mutant cRNA in oocytes as positive controls (uninjected oocytes were used as negative controls). We compared expression with FLAG-tagged C113S and C145S mutants, using anti-FLAG antibodies as a probe (together with secondary fluorescent antibody). We have already shown above that the presence of the FLAG-tag itself did not itself affect channel function. Confocal fluorescent images of anti-FLAG antibody binding showed that in contrast to uninjected oocytes, oocytes injected with either FLAG-tagged wild-type or C140S mutant cRNA showed clear labelling of the cell membrane (Fig. 5), as well as within the cell. This pattern of labelling was also seen for oocytes injected with FLAG-tagged C113S or C145S cRNA (Fig. 5).

The results in this section therefore show that the wild-type and mutant channels studied here were expressed in the cell membrane of *Xenopus* oocytes. They also indicate that cysteines at positions 113 and 145 are not necessary for expression of the subunits of wild-type channels in the oocyte cell membrane.

3.4. In vitro translation studies

In order to study whether the two cysteine residues at 113 and 145 participate in a disulfide bond between subunits, perhaps at an early stage of channel processing within the cell, we used an in vitro reticulocyte lysate transcription-translation system to study expression of the mutants.

For wild-type and for C113S, C140S and C145S mutants, the reticulocyte lysate system always yielded a protein of molecular weight around 60 kDa when run under non-reducing conditions on SDS polyacrylamide gels (Fig. 6), close to the expected molecular weight of a single subunit (59 kDa) [19]. No bands corresponding to dimers or tetramers were seen (data not shown), even in the presence in the reaction mixture of the oxidation-promoting Cu^{2+}, suggesting lack of covalent (e.g. disulfide) bonds between subunits. Indeed there were no differences in the bands when mercaptoethanol was present on the gel, which would have broken these bonds under these reducing conditions. We however cannot rule out intra-subunit disulfide bonds from these experiments.

To determine whether the wild-type and mutant subunits

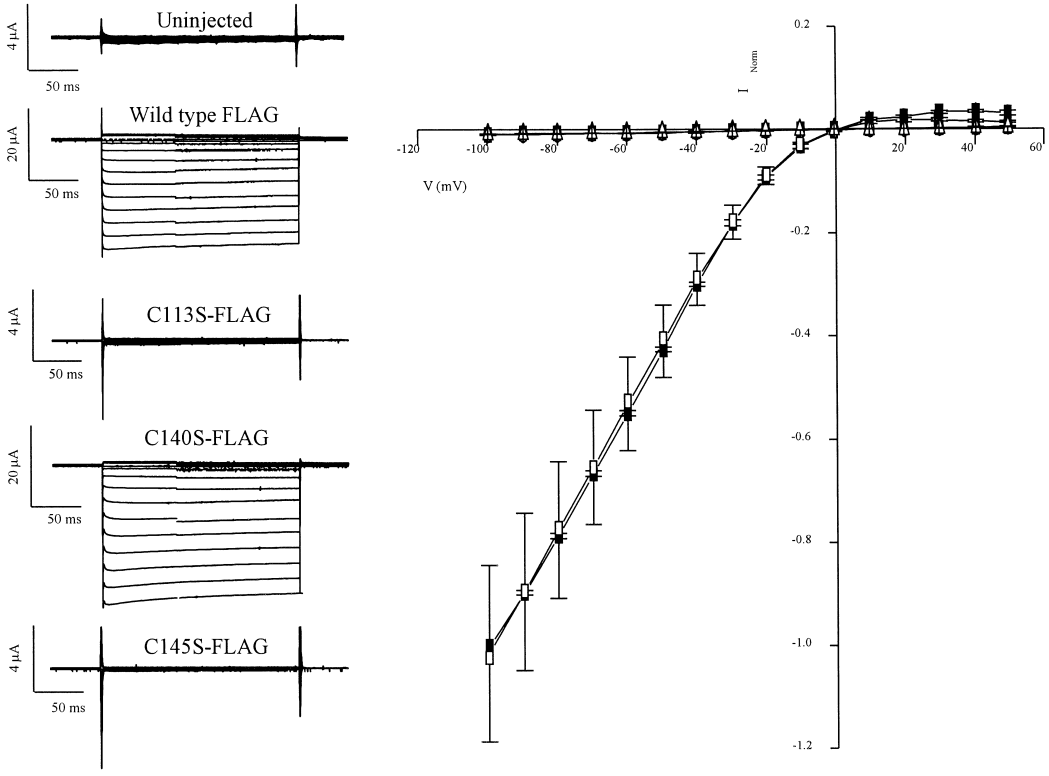

Fig. 3. Current families and *I-V* curves for FLAG-tagged wild-type and mutant Kir2.3 channels. Current voltage relationships are shown for oocytes injected with 1 ng cRNA for FLAG-tagged wild-type (■), C113S (●), C140S (□), C145S (○), as well as for uninjected oocytes (△). All currents were normalised to the wild-type current value at −100 mV. Data are presented as mean ± S.E.M. for 5–7 oocytes. Sample current recordings for each mutant are also shown.

were incorporated into membranes, in vitro translation was also carried out in the presence of microsomal membranes. For wild-type, C113S, C140S and C145S mutants, a single 60 kDa band was present in the pellet (i.e. the membrane fraction) (Fig. 6), with relatively little, if any, labelling in the supernatant fraction. For C145S, the supernatant was also labelled. No size shifts associated with glycosylation in the membrane fraction were seen. This was as expected since there are no consensus sites for glycosylation in the extracellular domains of Kir2.3 (Prosite). Again no bands were observed at higher molecular weight which would correspond to oligomers (data not shown).

In summary, these in vitro translation studies using reticulocyte lysate show that mutation of cysteines at residues 113, 140 and 145 does not prevent insertion of the channel subunits into the membrane and no evidence of disulfide bonds between subunits was seen.

4. Discussion

In this paper, we have shown that mutation of either of the highly conserved extracellular residues C113 or C145 resulted in the absence of currents. Co-expression of these C113S or C145S mutants with wild-type channel showed that these mutant subunits caused a loss of function through a dominant negative effect without affecting the formation of tetrameric channels. Using FLAG-tagged channels expressed in oocytes, we have used anti-FLAG antibody to show that wild-type channels and all mutants studied (C113S, C140S and C145S) were expressed in oocyte membranes. In vitro translation studies using the reticulocyte lysate system showed that mutation of these residues did not affect trafficking within this system nor insertion into membranes, confirming the picture from oocytes.

These data taken together show that mutations of the two conserved cysteine residues at positions 113 and 145 result in loss of function of the channel in oocytes. The dominant negative effect on currents of mixtures of these mutants with wild-type suggests that mutant subunits associate with wild-type subunits to form non-functional tetrameric channels and that these residues are not critical for subunit assembly. This could in principle be due to interference by the mutation at the membrane or within the cell during trafficking of the tetramer from the ER. However, the FLAG-tagged mutant experiments showed that these mutants in fact reached the oocyte cell membrane, indicating normal intracellular trafficking but abnormal function once in the membrane. Normal insertion into membranes was confirmed by the in vitro translation studies using the reticulocyte lysate system and there was no evidence of disulfide bonds between subunits. Clearly cysteines at positions 113 and 145 are not necessary for the correct processing of the channel and insertion into the membrane but are required for channel function. In contrast, mutation of the non-conserved residue C140 did not affect either function or expression in the membrane indicating a non-essential role for this residue in the channel.

A possible explanation of our results could be that there is a disulfide bond between the cysteines at positions 113 to 145

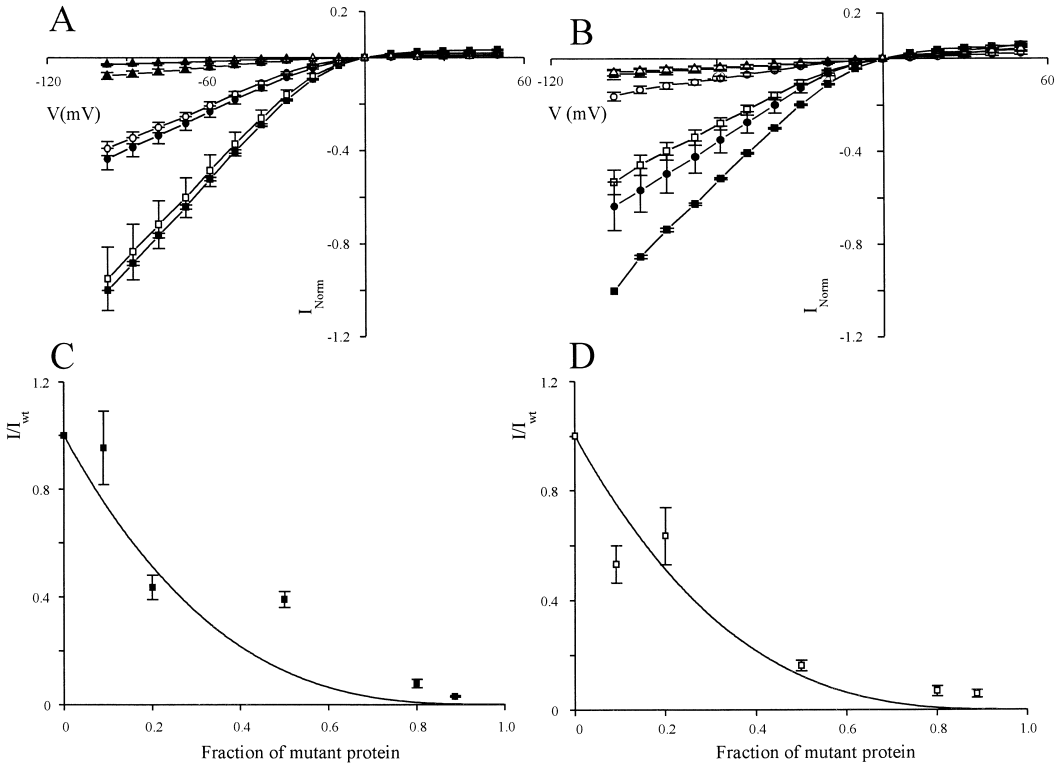

Fig. 4. Co-expression of wild-type and mutant C113S and C145S subunits in oocytes. A: *I-V*curves for mixtures of wild-type and C113S cRNA are shown. wild-type cRNA (■) injected in oocytes was kept constant at 1 ng, and the fraction of mutant cRNA/(mutant+wild-type cRNA) injected was 0.1 (□), 0.2 (●), 0.5 (○), 0.8 (▲) and 0.9 (△). Currents were normalised to the wild-type current at −100 mV. Data are shown for the mean ± S.E.M. of 5–10 oocytes. B: *I-V*curves for mixtures of wild-type and C145S cRNA are shown. wild-type cRNA (■) injected in oocytes was kept constant at 1 ng and the fraction of mutant cRNA/(mutant+wild-type cRNA) injected was 0.1 (□), 0.2 (●), 0.5 (○), 0.8 (▲) and 0.9 (△). Currents were normalised to the wild-type current at −100 mV. Data are shown for the mean ± S.E.M. of 6–27 oocytes. C: A plot of current against the fraction, *p*, of C113S mutant protein injected into oocytes is shown. The experimental data are as shown in (A), and the currents plotted are at −100 mV, normalised to wild-type values. The theoretical curve is for a tetramer, using the equation $I/I_{wild-type} = (1-p)^3$ (modified from [5] to account for the constant wild-type amount injected in the present experiments). D: A plot of current against the fraction of C145S mutant protein injected into oocytes is shown. The experimental data are as shown in (B) and the currents plotted are at −100 mV, normalised to wild-type values. The theoretical curve for a tetramer is also shown as in (C).

within each subunit. Breaking this bond by mutation may introduce an instability in subunit structure which prevents function but still allows the formation of a tetramer in the membrane. However, attempts to break this possible bond with the reducing agent dithiothreitol (DTT) did not affect wild-type Kir2.3 currents in oocytes (unpublished observation), as also happens for the TWIK channel [12]. This suggests that either DTT did not reach this bond, or that breaking the bond had no effect on intact channels once formed, as already suggested for TWIK.

As the cysteine residues studied here are not present in the proximal region of the C-terminus or the M2 domain of Kir2.3 channels (regions suggested to be involved in subunit association [5]), it is not surprising that subunits which carry mutations of either C113 or C145 are able to associate with wild-type subunits. The data presented here clearly show that they can associate and therefore suggest that these residues are not involved in subunit association.

Very recent preliminary abstracts have appeared on these conserved extracellular cysteines in the Kir2.1 channel [20,21]. As for Kir2.3 studied here, the cysteine residues C122 and C154 in Kir2.1 (which correspond to 113 and 145 in Kir2.3) are required for function of the Kir2.1 channel, even though the mutant channels can be detected in the cell membrane. Also as for the Kir2.3 channel studied here, an intra-subunit disulfide bond between the conserved cysteines in Kir2.1 seems likely.

The absence of a disulfide bond between subunits of Kir2.3 is in contrast to work on the TWIK1 channel, where functional expression has been shown to be dependent on the formation of a disulfide bond between subunits [12]. Although TWIK1 is a different channel with four transmembrane domains and two pore (P) regions per channel subunit, the cysteine residue shown to be involved in TWIK1 (C69) also lies between the M1 domain and the first P region, like C113 for Kir2.3. The limited sequence homology between these channels probably underlies the differences between these channels in disulfide bond formation properties.

In summary, we have shown that conserved extracellular cysteine residues at positions 113 and 145 are required for function of the Kir2.3 inward rectifier channel but are not required for transport and processing into the cell membrane. There was no evidence for disulfide bonds linking these residues between subunits, although intra-subunit bonds are possible.

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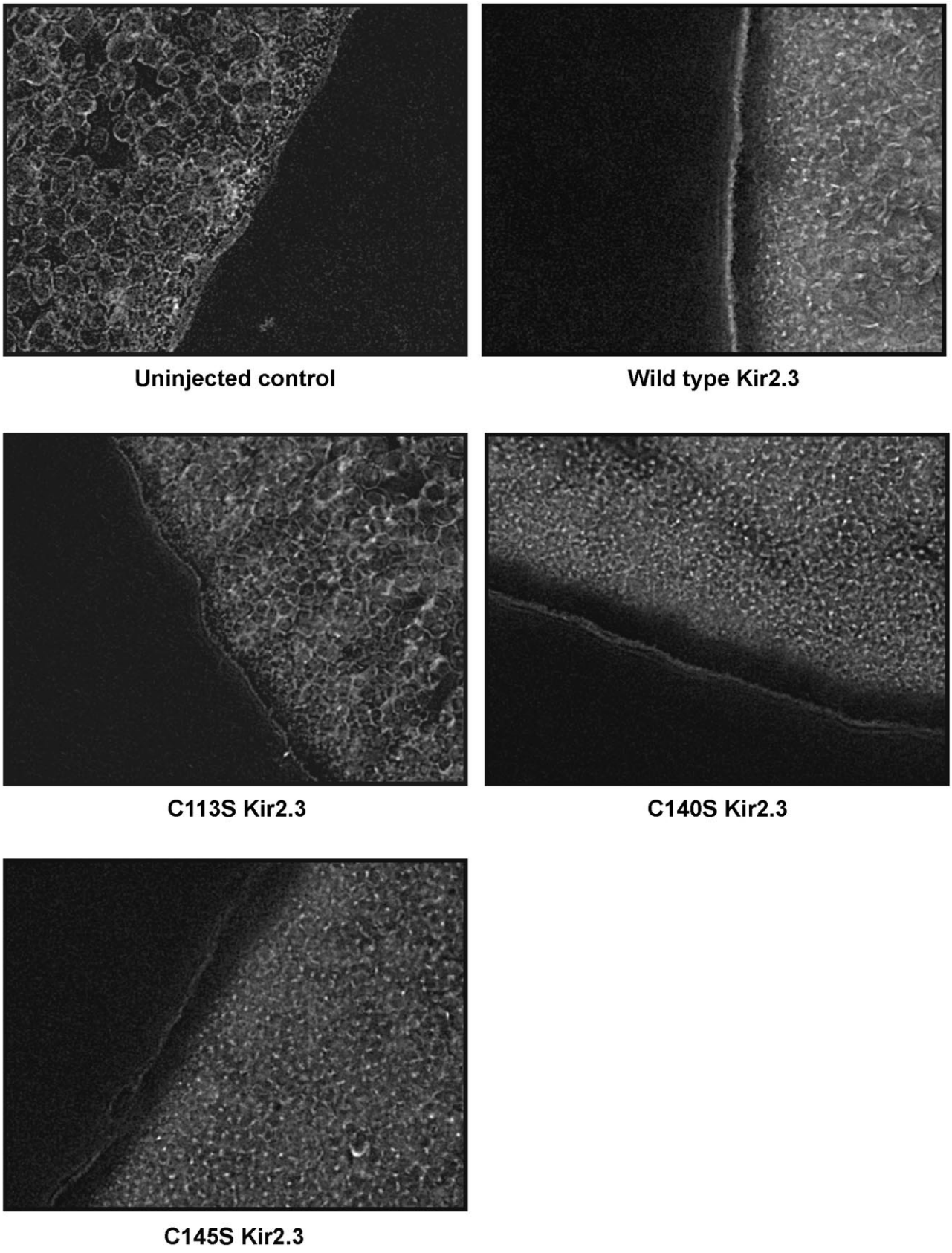

Fig. 5. Anti-FLAG antibody binding to oocytes using fluorescent confocal microscopy. FLAG-tagged Kir2.3 cRNA (1 ng) was injected into oocytes and fluorescent images are shown for FLAG-tagged wild-type, C113S, C140S and C145S mutants, as well as for an uninjected oocyte. For each injected channel cRNA, 4–7 oocytes (from two batches) were examined and clear membrane staining was seen in 8, 4, 6 and 6 sections, respectively for wild-type, C140S, C113S or C145S.

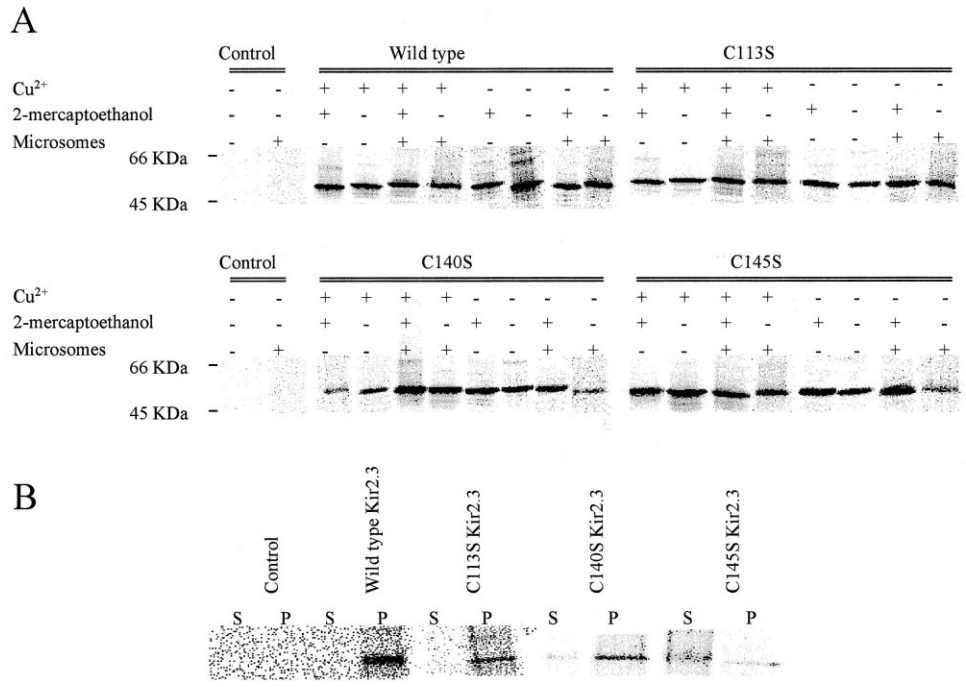

Fig. 6. In vitro translation of wild-type and mutant Kir2.3 subunits using the reticulocyte lysate system. A: Autoradiographs of SDS polyacrylamide gels for control (DNA absent), wild-type and C113S, C140S and C145S mutants. In vitro translation was carried out in the presence or absence of microsomal membranes and/or of Cu^{2+} as shown. Gels were run in the presence or absence of 2-mercaptoethanol as shown. B: In vitro translation in the presence of microsomal membranes. Autoradiographs of SDS polyacrylamide gels for control (DNA absent), wild-type and C113S, C140S and C145S mutants are shown for the membrane fraction (P, pellet) and the supernatant (S).

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