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Determinants of potassium channel assembly localised within the cytoplasmic C-terminal domain of Kv2.1

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Abstract

The C-terminal domain of the voltage-gated potassium channel Kv2.1 is shown to have a role in channel assembly using dominant negative experiments in *Xenopus* oocytes. Kv2.1 channel polypeptides were co-expressed with a number of polypeptide fragments of the cytosolic C-terminus and the assembly of functional channel homotetramers quantified electrophysiologically using the two electrode voltage clamp technique. Co-expression of C-terminal polypeptides corresponding to the final 440, 318, 220 and 150 amino acid residues of Kv2.1 all resulted in a significant reduction in the functional expression of the full-length channel. A truncated version of Kv2.1 lacking the final 318 amino acids of the C-terminal domain ($Kv2.1_{1-535}$) exhibited similar electrophysiological properties to the full-length channel. Co-expression with either the 440 or 318 residue polypeptides resulted in a reduction in the activity of the truncated channel. In contrast, the 220 and 150 residue C-terminal fragments had no effect on $Kv2.1_{1-535}$ activity. These data demonstrate that C-terminal interactions are important for driving Kv2.1 channel assembly and that distinct regions of the C-terminal domain may have differential effects on the formation of functional tetramers.

1. Introduction

The voltage-gated potassium channels comprise a large family of membrane proteins which display a diverse range of kinetic properties. The functional channel is formed from four alpha subunit polypeptides, each of which is thought to consist of six transmembrane segments (S1–S6), a pore forming (H5) region, and cytoplasmic hydrophilic N- and C-terminal domains [1–3]. Functional diversity originates in part by the expression of a large number of different alpha subunit polypeptides, and in part by the interaction of alpha subunit tetramers with cytoplasmic beta subunits [4–11]. Additional functional heterogeneity may arise from the association of alpha subunits as either homo- or heterotetrameric complexes [12–18]. The differential assembly of alpha subunits into tetrameric complexes requires recognition between the relevant polypeptides following translation and probably occurs within the endoplasmic reticulum [19,20]. The specificity of the mechanisms of recognition and assembly allow for the association either of identical subunits or of different subunits from within the same subfamily, but usually preclude the formation of heterotetramers between subunits from different subfamilies [21–24] (the possible ex-

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ception being the formation of Kv1.2/Kv3.1a heteromultimeric channels [25]).

Subfamily specificity during assembly is conferred by a conserved region within the cytosolic N-terminus, termed the tetramerisation (T1) or NAB domain [18,26–28]. In some channels this same domain has been shown to be not only crucial for the preclusion of heterotetramerisation between subfamilies, but also essential for the assembly process itself. Accordingly, deletion mutants of the Kv1.1 potassium channel which lack the N-terminus, are not assembled into functional channels [27]. Similarly, in dominant-negative experiments co-expression of N-terminal polypeptides with full-length alpha subunit in *Xenopus* oocytes is sufficient to inhibit functional expression [26,28]. However, it has also been shown for a number of related channels including Kv1.3 [29] Kv1.4 [23] and Kv2.1 [30], that the expression of functional channels in oocytes is not abolished by the removal of the N-terminus. Hence, in these channels, regions outside the N-terminus must also play a part in stabilising intersubunit associations.

In this report, dominant negative techniques have been applied to investigate the influence of different regions of the C-terminal domain on subunit assembly. Expression was studied in two variants of Kv2.1; the full-length channel, and a C-terminal deletion mutant $Kv2.1_{1-535}$. In the latter case, the final 318 amino acids are deleted, but nevertheless it is able to express functional channels in oocytes with similar electrophysiological characteristics to the full-length channel [30]. By comparing the results for the two channel types we have established the likely regions of interaction between the C-terminus and parent protein.

2. Methods

2.1. Molecular biology

A number of cDNA templates encoding different regions of Kv2.1 were generated by PCR from the full-length clone of Kv2.1 (a gift from Dr A. Brown) and subcloned into pBluescript vectors (Stratagene) using standard molecular techniques. C-terminal constructs $Kv2.1_{414-853}$, $Kv2.1_{536-853}$, $Kv2.1_{634-853}$ and $Kv2.1_{704-853}$ encoded the final 440, 318, 220 and 150 residues of the cytoplasmic C-terminal domain, respectively. The C-terminal truncations of the full-length channel, $Kv.2.1_{1-413}$ and $Kv2.1_{1-535}$, lacked the coding regions for the final 318 and 440 amino acid residues respectively of the full-length channel protein. Ligation products were used to transform *Escherichia coli* XL2-Blue cells (Stratagene). Plasmid DNA was purified from LB cultures using Qiagen plasmid kits and authenticity of constructs was confirmed by DNA sequencing. Capped cRNA was synthesised in vitro from linearised templates using a Ribomax T7 transcription kit (Promega). The final concentration of purified cRNA was determined spectrophotometrically.

2.2. Oocyte preparation and electrophysiology

Oocytes from adult female *Xenopus laevis* were defolliculated by treatment with 1 mg/ml collagenase (type 1A; Sigma) in calcium-free Ringer solution (82.5 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, 5 mM HEPES, pH 7.5). Stages IV and V oocytes were injected with 50 nl cRNA and then incubated for at least 48 h at 18°C in modified Barth's solution containing 88 mM NaCl, 1 mM KCl, 2.4 mM $NaHCO_3$, 0.82 mM $MgSO_4 \cdot 7H_2O$, 0.33 mM $Ca(NO_3)_2 \cdot 4H_2O$, 20 mM HEPES, 0.41 mM $CaCl_2$, 1000 U/ml penicillin, 0.1 mg/ml streptomycin, pH 7.5. Whole cell two electrode voltage clamp experiments were performed using a GeneClamp 500 voltage clamp amplifier, linked via a DigiData interface to a computer running pClamp6 software (Axon Instruments). Command voltage steps of 200 ms duration ranging from 10 to 140 mV were employed from a holding potential of −80 mV at a frequency of 0.1 Hz. Current signals were filtered at 2 kHz, digitised current data were stored on hard disk for subsequent analysis using Clampfit software. Passive leak subtractions were performed on-line. Recordings were made using glass microelectrodes filled with 3 M KCl which had resistances in the range of 0.2–1.0 MΩ. A standard Ringer bathing solution was employed; 115 mM NaCl, 2.5 mM KCl, 1.8 mM $CaCl_2$, 10 mM HEPES, pH 7.2, and all experiments were carried out at room temperature (21–23°C). Data are expressed as mean ± S.E.M. Statistical significance of differences between means was determined using a Student's *t*-test; values of $P < 0.05$ were considered significant.

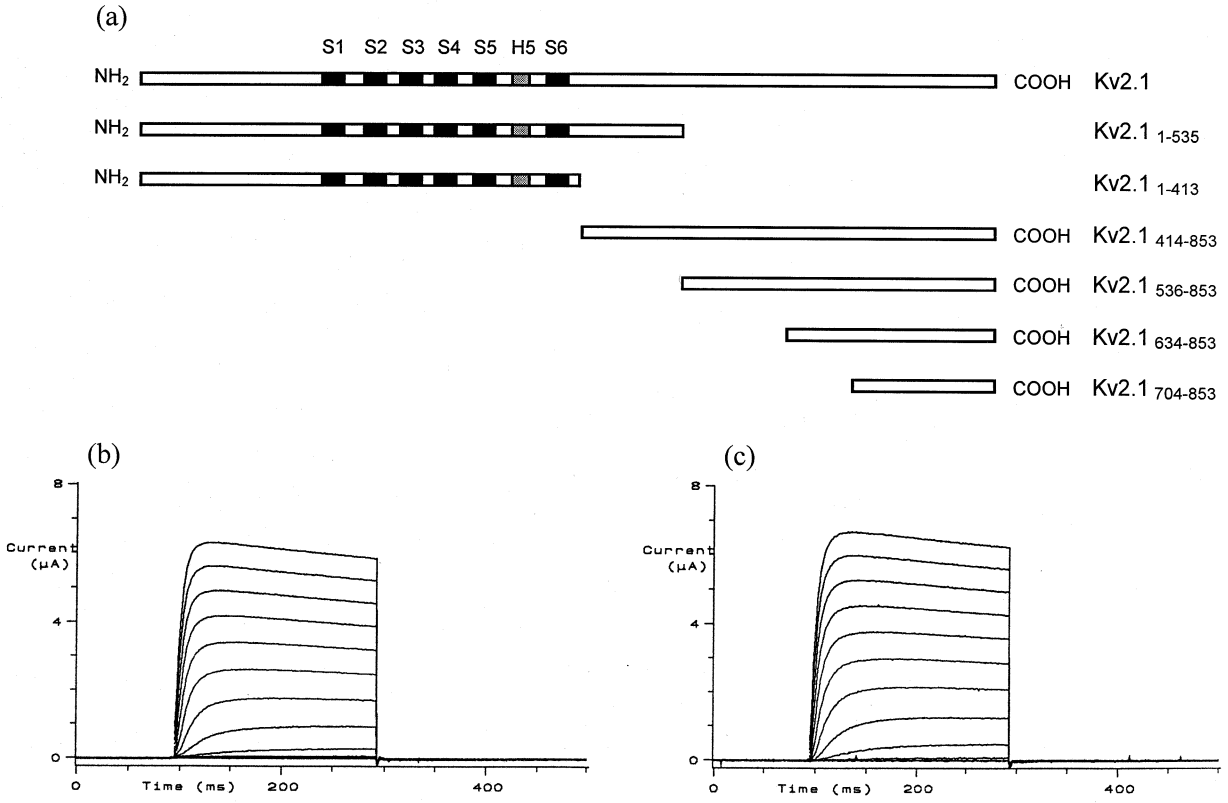

Fig. 1. (a) Showing the relationship between the parent channel, the channel forming C-terminal deletion mutant $Kv2.1_{1-535}$ and the cytosolic C-terminal fragments of Kv2.1. Current recordings from oocytes injected with 2.5 fmol of cRNA encoding either Kv2.1 (b) or $Kv2.1_{1-535}$ (c) demonstrated that these channels shared similar kinetic properties. The C-terminal deletion mutant $Kv2.1_{1-413}$ did not form functional channels when expressed in oocytes even following cRNA injections of 25 fmol (data not shown).

3. Results

Expression in oocytes of full-length Kv2.1 or of the C-terminal truncation mutants $Kv2.1_{1-535}$ and $Kv2.1_{1-413}$ was assessed by analysing current-voltage relationships using the two electrode voltage clamp technique following microinjection of cRNA. Oocytes injected with $Kv2.1_{1-413}$ cRNA, did not demonstrate additional channel activity beyond that present in uninjected controls. In contrast, it was possible to record robust potassium currents in oocytes which had been injected with either full-length Kv2.1 or $Kv2.1_{1-535}$. The influence of C-terminal soluble domain polypeptides co-expressed with Kv2.1 or $Kv2.1_{1-535}$ on functional assembly of either of these channels was monitored by comparing current amplitudes. Measurements were made from oocytes expressing channel protein alone and compared with measurements from cells which had been co-injected with cRNA encoding one of four different C-terminal fragments. These C-terminal polypeptides, designated $Kv2.1_{414-853}$, $Kv2.1_{536-853}$, $Kv2.1_{634-853}$ and $Kv2.1_{704-853}$, represented the final 440, 318, 220 and 150 residues respectively of the C-terminus (Fig. 1a). Approximately 5 fmol of C-terminus cRNA was injected per oocyte, along with approximately 2.5 fmol of the Kv2.1 or $Kv2.1_{1-535}$ cRNA. Expression of C-terminal fragments alone did not alter the background currents in oocytes.

3.1. Full-length Kv2.1

Oocytes injected with Kv2.1 cRNA expressed potassium channels with delayed rectifier properties typical of those described elsewhere for this channel [30]. Thus, 48 h following injection of 2.5 fmol cRNA, outward slowly activating whole cell currents could be elicited by test potentials in the range of −30 mV to +60 mV, which did not inactivate appreciably during the time course of the 200 ms voltage

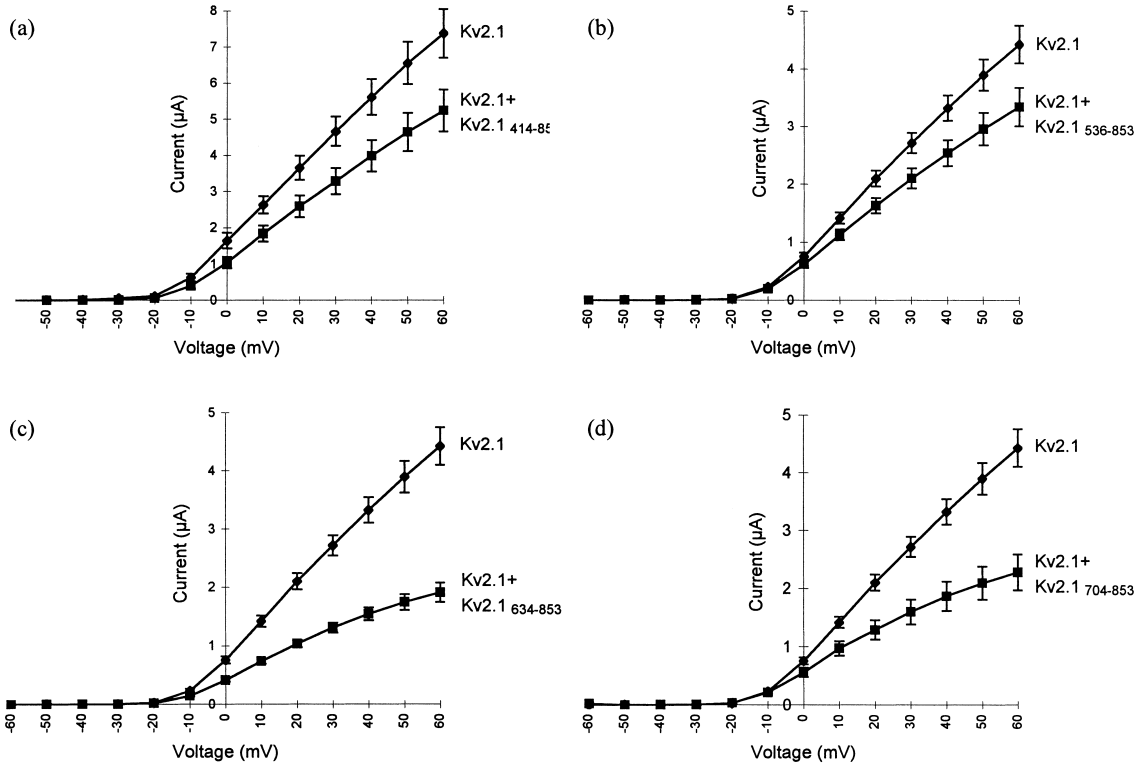

Fig. 2. Current-voltage relationships demonstrating the effects of co-expression of C-terminal fragments on the functional expression Kv2.1. Oocytes injected with cRNA encoding the truncated channel were compared with those co-injected with C-terminal peptides comprising residues (a) $Kv2.1_{414-853}$, (b) $Kv2.1_{536-853}$, (c) $Kv2.1_{634-853}$ and (d) $Kv2.1_{704-853}$. Experimental conditions are described in Section 2.

step (Fig. 1b). The amplitude of the steady state current at +60 mV varied in the range of 4–8 μA between different batches of oocytes. In contrast, currents recorded from uninjected oocytes or those injected with water were very small – typically less than 0.1 μA. Oocytes co-injected with cRNAs encoding both Kv2.1 and the 440 residue C-terminal fragment of Kv2.1 ($Kv2.1_{414-853}$) resulted in whole cell currents which were significantly smaller in amplitude compared with those recorded in oocytes injected with Kv2.1 alone (Fig. 2a). The mean current elicited at a potential of +60 mV was 7.38 ± 0.68 μA ($n = 8$) for oocytes injected with 2.5 fmol of Kv2.1 cRNA alone, reducing to 5.24 ± 0.58 μA ($n = 8$) for oocytes co-injected with 2.5 fmol Kv2.1 cRNA and 5 fmol $Kv2.1_{414-853}$ cRNA. It is possible that such a reduction could be brought about if the translational capacity of the oocyte becomes saturated in the presence of the two transcripts together. To eliminate this possibility whole cell current was titrated against Kv2.1 cRNA up to 10 fmol. There was a linear relationship between the amount of cRNA injected and the functional expression of active channels across the whole of this range, indicating that there was no saturation of translation associated with injections of high concentrations of RNA. The same control was applied to subsequent experiments; on each occasion the results indicated that the translation capacity within the oocyte was not exceeded by the quantities of cRNA injected. The C-terminal fragments $Kv2.1_{536-853}$, $Kv2.1_{634-853}$ and $Kv2.1_{704-853}$ were also capable of producing a significant reduction of Kv2.1 whole cell currents. Thus, while the mean current in oocytes expressing Kv2.1 alone was 4.43 ± 0.33 μA ($n = 10$) at +60 mV, in the presence of $Kv2.1_{536-853}$, $Kv2.1_{634-853}$ or $Kv2.1_{704-853}$ cRNA, this was reduced to 3.34 ± 0.33 μA ($n = 10$), 1.91 ± 0.16 μA ($n = 10$) and 2.28 ± 0.31 μA ($n = 10$) respectively (see Figs. 2b–d and 3). Analysis of individual whole cell currents revealed that there was no difference in activation and inactivation kinetics between oocytes expressing Kv2.1 alone and those co-

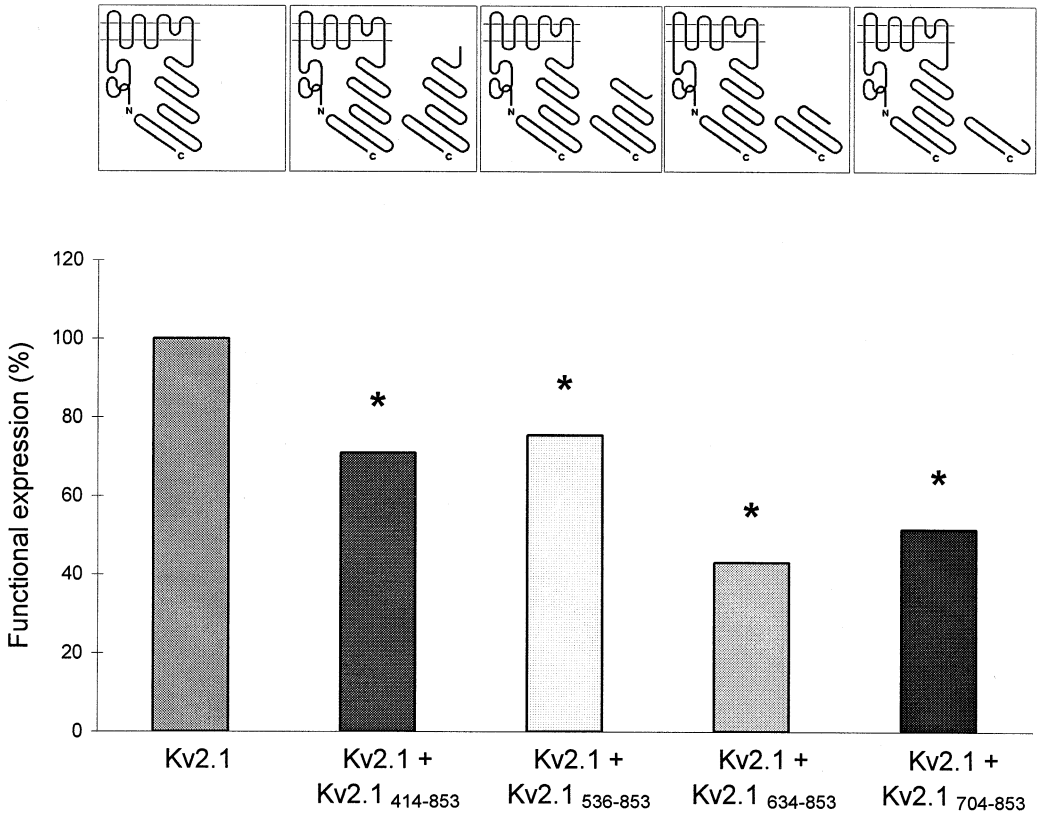

Fig. 3. A comparison of the effects of co-expressing C-terminal fragments with full length Kv2.1, assessed by measuring the magnitude of the current recorded in response to a 200 ms voltage step of +60 mV from a holding potential of −80 mV. The upper panel illustrates the combinations of constructs tested. The corresponding bars on the histogram depict the mean current recorded in oocytes co-expressing these combinations of cRNA displayed as a percentage of the current recorded in cells expressing Kv2.1 alone (*$P < 0.05$).

expressing Kv2.1 the C-terminal peptides, suggesting that the peptides do not act like regulatory beta subunits to modify the gating characteristics of the channel.

In a control experiment, co-injection of Kv2.1 with a two-fold molar excess of cRNA encoding the final 150 C-terminal amino acids of the unrelated voltage-gated ion channel protein Kv1.6 failed to have a significant effect on Kv2.1 functional expression (data not shown). The inhibitory effects of Kv2.1 C-terminal peptides do, therefore, not appear to be due to non-specific interactions.

3.2. *Kv2.1$_{1-535}$*

Oocytes injected with 2.5 fmol cRNA encoding the mutant channel Kv2.1$_{1-535}$, which has a 318 residue C-terminal deletion, expressed functional potassium channels as reported previously [30]. The amplitude of these Kv2.1$_{1-535}$ currents were similar to those of the full-length channel, as were the characteristics of activation, inactivation and voltage dependence (Fig. 1c). The effects of co-expression of the Kv2.1$_{1-535}$ channel with the cytosolic C-terminal polypeptide Kv2.1$_{414-853}$ were similar to those observed with the full-length Kv2.1 – with a reduction of the amplitude of the current elicited at +60 mV from 3.72 ± 0.43 μA ($n = 10$) for Kv2.1$_{1-535}$ alone to 2.27 ± 0.19 μA when co-expressed with Kv2.1$_{414-853}$ ($n = 10$, Fig. 4a). Interestingly, co-expression with the 318 amino acid C-terminal fragment Kv2.1$_{536-853}$ (which represents the portion of the protein missing in the truncated channel), also led to a major reduction in the mean current from 4.43 ± 0.61 to 2.02 ± 0.54 μA ($n = 10$, Fig. 4b). In contrast, there was no significant difference between currents meas-

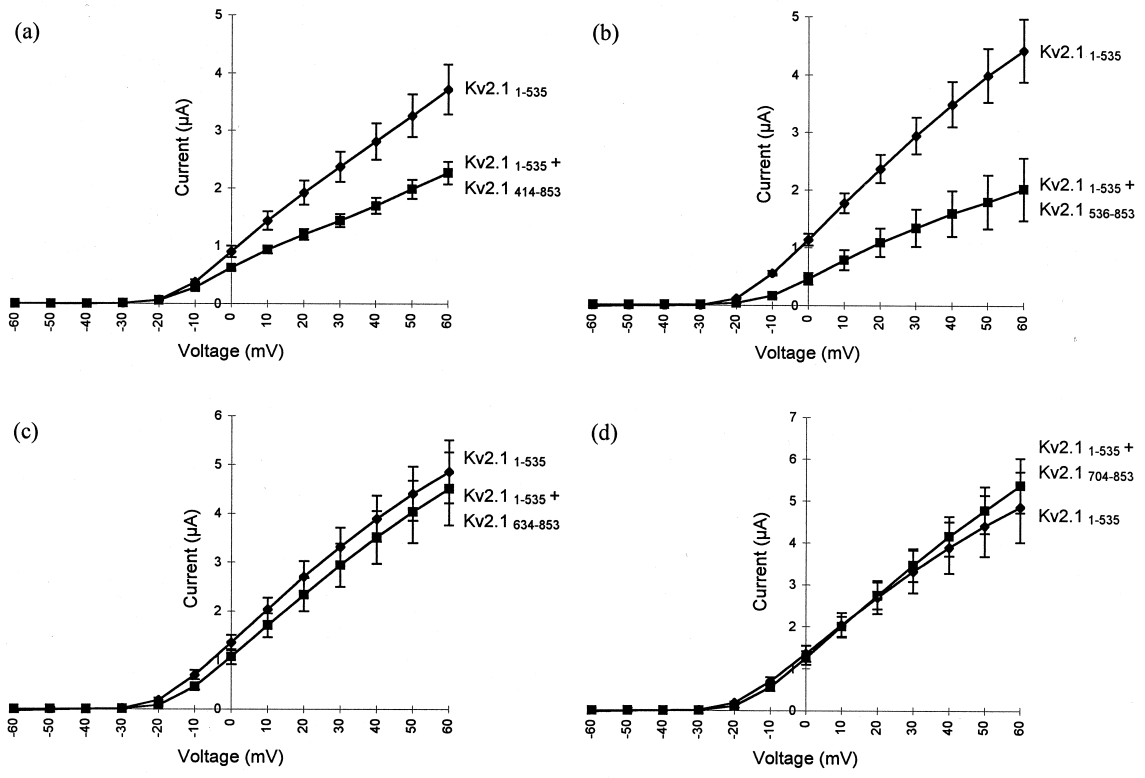

Fig. 4. Current-voltage plots comparing oocytes expressing the truncated channel $Kv2.1_{1-535}$ alone or in conjunction with the C-terminal fragments (a) $Kv2.1_{414-853}$, (b) $Kv2.1_{536-853}$, (c) $Kv2.1_{634-853}$ and (d) $Kv2.1_{704-853}$.

ured in cells expressing $Kv2.1_{1-535}$ channel alone (4.86 ± 0.65 μA, $n = 10$) or with either cytosolic C-terminal polypeptides $Kv2.1_{634-853}$ (5.38 ± 8.85 μA, $n = 10$, Fig. 4c) or $Kv2.1_{704-853}$ (4.51 ± 0.74 μA, $n = 10$, Fig. 4d). These data are summarised in Fig. 5. As was the case with the full-length channel protein, co-expression of the truncated channel with a C-terminal polypeptide from Kv1.6 failed to affect significantly functional expression.

4. Discussion

Dominant negative experiments in *Xenopus* oocytes have previously been used to demonstrate that the functional expression of *Shaker*B, Kv1.1 and Kv1.5 channels (as determined by current amplitude recordings) can be reduced by the co-expression of their respective N-terminal domains [26,28]. The interpretation of these experiments is that during the post-translational assembly of subunits into tetrameric ion channels, sufficient N-terminal domain polypeptide is able to co-assemble with full-length channel protein to result in the formation of a significant number of non-functional channels. Using the same approach, we have been able to demonstrate that in the case of Kv2.1, the cytoplasmic C-terminus is also important for assembly of functional channels. For these experiments four different polypeptide fragments representing truncations of the C-terminal domain were tested: $Kv2.1_{414-853}$, $Kv2.1_{536-853}$, $Kv2.1_{634-853}$ and $Kv2.1_{704-853}$. These represent the final 440, 318, 220 and 150 amino acids of the alpha subunit respectively. Co-expression of any of the four C-terminal polypeptides with full-length Kv2.1 channel protein in *Xenopus* oocytes produced a significant reduction in expression of channels, as adjudged by a decrease in amplitude of detectable current. Although there are a number of theoretical explanations for these observations, the most likely is that the C-terminal domain of Kv2.1 is capable of associating with the full-length channel polypeptide so as to produce inactive heteromultimeric assemblies, comprising full-length channel polypeptide

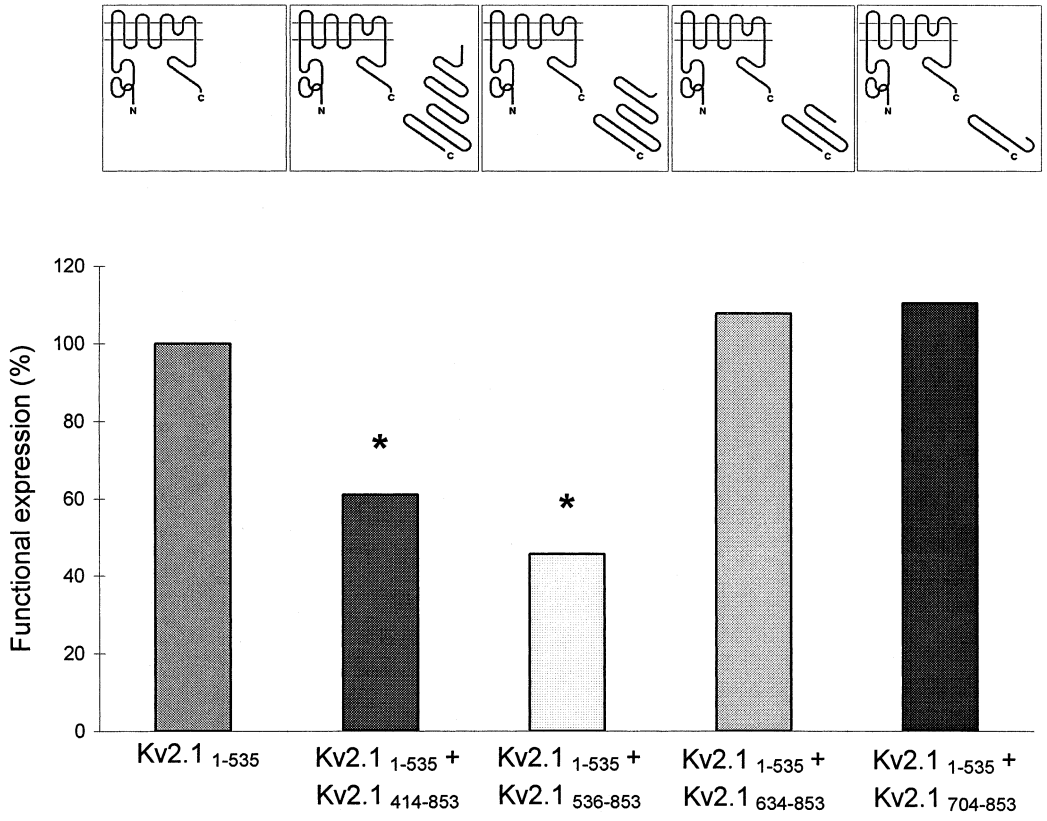

Fig. 5. The effects of the expression of C-terminal fragments of Kv2.1 on the functional expression of the C-terminal truncation of Kv2.1; $Kv2.1_{1-535}$. Oocytes were co-injected with cRNA encoding the constructs depicted in the upper panel. The histogram shows the mean current recorded in response to a +60 mV pulse in cells receiving co-injections of cRNA displayed as a percentage of the current recorded in cells expressing $Kv2.1_{1-535}$ alone ($^*P < 0.05$).

and terminal polypeptide fragments. Irrespective of the precise mechanism of the observed inhibitions, it is clear that the C-terminal domain of the protein must be capable of adopting the native conformation required for the specific quaternary contacts which drive association with the full-length channel polypeptide.

The results of the experiments performed using the truncated mutant channel protein $Kv2.1_{1-535}$ (which lacks the final 318 amino acids from its C-terminus) provide valuable clues as to the likely regions which participate in intersubunit recognition during subunit assembly. Co-expression of either C-terminal polypeptides $Kv2.1_{634-853}$ and $Kv2.1_{704-853}$ with the $Kv2.1_{1-535}$ channel did not affect channel expression (Fig. 4c,d). However, polypeptides $Kv2.1_{414-853}$ and $Kv2.1_{536-853}$ both led to a significant reduction in the formation of functional $Kv2.1_{1-535}$ channels. Since the two shorter C-terminal peptides are able to exert effects only on the full-length protein, the structural entity with which the final 220 residue subdomain interacts must contain, at least in part, contributions from elements present within the final 318 residues (i.e. residues 536–853) of the C-terminal domain. The observation that only C-terminal fragments incorporating residues 414–633 bind to the truncated channels demonstrates that elements within this region are capable of interacting strongly with elements of the alpha subunit which lie within the first 535 amino acids of the channel polypeptide. Furthermore, since the fragment $Kv2.1_{536-853}$ shares no overlap with any of the elements present in the $Kv2.1_{1-535}$ channel, it is evident that the interaction of a subdomain located between residues 536 and 633 with the channel cannot be driven by the association of like regions.

The functional diversity of voltage-gated potassium channels is in part a consequence of the co-as-

sembly of alpha subunits as both homo- and hetero-oligomeric complexes [8,12–16]. A number of studies in recent years have attempted to identify the regions of the channel polypeptide responsible for intersubunit recognition and assembly, yet the details of these processes remain poorly understood. It is clear that the correct assembly of potassium channel subunits is contingent upon a molecular recognition step which favours the association of subunits from within the same subfamily whilst precluding association between different families [18]. It now seems likely that conserved regions located within the cytoplasmic N-terminus of the Kv1, Kv2, Kv3 and Kv4 subfamilies (T1 or NAB domains) may be fundamental in defining the subfamily specificity of multimerisation [18,24,26–28]. For some channels, e.g. Kv1.1, the dominant molecular signals orchestrating both subfamily-specific intermolecular recognition and assembly are located on identical or overlapping regions of the N-terminal domain [18,27]. However, for others, e.g. Kv1.3 [29], Kv1.4 [23], Kv1.5 [28], it appears that although the N-terminus does function in intersubunit recognition, it is not required for the assembly of subunits into functional channels. These two complementary processes may therefore be distinct at the molecular level. In the case of the Kv2.1 channel, it has been shown by ourselves and others that deletion of a region of the NAB domain does not abolish channel expression [30,31]. The present experiments demonstrate that some important determinants for homomeric subunit assembly for this channel are contained within the final 440 amino acids of the C-terminus. Whilst our data show that C-terminal fragments representing the final 318 residues of the full-length polypeptide can clearly influence functional expression, the fact that the Kv2.1 is able to form channels even when these 318 C-terminal residues are deleted indicates that the crucial determinants for assembly are retained in the core of the protein, within the first 535 residues. Indeed, there is evidence to suggest that residues within the pore region may influence the assembly of Kv2.1 [32]. Our observation that the 440 residue C-terminal deletion mutant is inactive, suggests that elements located in the proximal region of the C-terminus, between residues 414 and 535 are indispensable for the functional expression of this channel.

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