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A Small Domain in the N Terminus of the Regulatory α-Subunit Kv2.3 Modulates Kv2.1 Potassium Channel Gating

María Dolores Chiara, Francisco Monje, Antonio Castellano, and José López-Barneo

Departamento de Fisiología Médica y Biofísica, Facultad de Medicina, Universidad de Sevilla, E-41009 Sevilla, Spain

Recent work has demonstrated the existence of regulatory K^+ channel α-subunits that are electrically silent but capable of forming heterotetramers with other pore-forming subunits to modify their function. We have investigated the molecular determinant of the modulatory effects of Kv2.3, a silent K^+ channel α-subunit specific of brain. This subunit induces on Kv2.1 channels a marked deceleration of activation, inactivation, and closing kinetics. We constructed chimeras of the Kv2.1 and Kv2.3 proteins and analyzed the K^+ currents resulting from the coexpression of the chimeras with Kv2.1. The data indicate that a region of 59 amino acids in the N terminus, adjacent to the first transmembrane segment, is the major structural element responsible for the regulatory function of Kv2.3. The sequence of this domain of Kv2.3 is highly divergent compared with the same region in the other channels of the Kv2 family. Replacement of the regulatory fragment of Kv2.3 by the equivalent of Kv2.1 leads to loss of modulatory function, whereas gain of modulatory function is observed when the Kv2.3 fragment is transferred to Kv2.1. Thus, this study identifies a N-terminus domain involved in Kv2.1 channel gating and in the modulation of this channel by a regulatory α-subunit.

Key words: molecular diversity; brain potassium channels; regulatory α-subunit; structure–function relationships; gating; modulation; heteromeric channels

Voltage-gated K^+ channels (Kv channels) are multi-subunit transmembrane proteins necessary for action potential repolarization and regulation of repetitive firing (Hille, 1992). Although these channels are functionally diverse, they share a common structure that consists of four homologous α-subunits, each one with six transmembrane segments flanked by intracellular N- and C-terminal domains (Rudy, 1988; Pongs, 1992; Jan and Jan, 1994). Diversity of K^+ channels arises from the existence of multiple genes encoding pore-forming α-subunits grouped in several families (Chandy and Gutman, 1993). Molecular diversity is further increased by the ability of different α-subunits to coassemble as heterotetramers (Isacoff et al., 1990; Ruppersberg et al., 1990; Covarrubias et al., 1991; Li et al., 1992; Sheng et al., 1993; Wang et al., 1993).

A new mechanism to generate K^+ channel diversity has recently been proposed after the cloning of "regulatory α-subunits," which cannot produce functional channels by themselves but are able to coassemble with other Kv α-subunits to form heteromers with specific functional characteristics. One of the first silent α-subunits recognized as regulatory was identified in our laboratory and designated as Kv2.3 because of its high sequence similarity and functional interaction with Kv2.1 channels (Castellano et al., 1996, 1997). Hugnot et al. (1996) independently cloned the same protein and called it Kv8.1. Coexpression of Kv2.3 (or Kv8.1) and Kv2 channels (Kv2.1 or Kv2.2) results in macroscopic K^+ currents with slowed kinetics and altered voltage dependence (Castellano et al., 1996, 1997; Salinas et al., 1997a). Besides Kv2.3, other silent α-subunits with a possible regulatory function have been identified (Post et al., 1996; Patel et al., 1997; Salinas et al., 1997b; Kramer et al., 1998). Most of the regulatory subunits studied so far seem to interact specifically with Kv2 channels. These channels are broadly distributed in the mammalian brain, and the Kv2.1 type appears to be particularly important in regulating neuronal excitability because it is expressed in virtually every nerve cell, being a major contributor to the delayed rectifier K^+ current in hippocampal neurons (Trimmer, 1991; Drewe et al., 1992; Hwang et al., 1992, 1993; Murakoshi and Trimmer, 1999). Kv2.3 is specifically expressed in the brain and, like Kv2.1, it is found at high levels in the hippocampus and neocortex (Trimmer, 1991; Hugnot et al., 1996; Castellano et al., 1997). Hence, the selective coexpression of Kv2.3 and Kv2.1 in individual neurons could be a mechanism involved in the fine regulation of their intrinsic electrophysiological properties.

Because K^+ channel modulation by regulatory α-subunits is a novel concept of broad functional interest, the present work was undertaken to identify the molecular determinant for the effect of Kv2.3 on Kv2.1. We show that the modulatory action of Kv2.3 depends on a domain of 59 amino acids located at the N terminus that participates in the normal gating of Kv2.1 channels. The sequence of this fragment of Kv2.3 is highly divergent with respect to the same region in Kv2.1 and Kv2.2 channels.

A preliminary account of these data has appeared in abstract form (Chiara et al., 1998).

MATERIALS AND METHODS

Plasmid constructions

All cDNAs encoding wild-type or chimeric α-subunits were cloned into the p513 eukaryotic expression vector (a derivative of pSG5; Stratagene, La Jolla, CA) using standard cloning techniques (Sambrook et al., 1989). The construction of the various Kv2.1/Kv2.3 chimeras was as follows.

Chimera Ch1. Appropriate primers were used to amplify the 176 C-terminal amino acids of Kv2.3 and to create a silent *Bam*HI site in the

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Drs. Chiara and Monje contributed equally to this work.

Correspondence should be addressed to Dr. José López-Barneo, Departamento de Fisiología Médica y Biofísica, Facultad de Medicina, Avenida Sánchez Pizjuan, 4 E-41009 Sevilla, Spain.

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sequence encoding the Kv2.3-S4 segment. The PCR-amplified product was digested with *Bam*HI and *Kpn*I, and the isolated DNA fragment was used to replace the *Bam*HI-*Kpn*I segment of Kv2.1 cDNA, which encodes the 549 C-terminal amino acids of this channel. In this construct, the 121 N-terminal amino acids of Kv2.1 were deleted by digestion with *Eco*RI and *Cla*I and replaced by an *Eco*RI-*Cla*I fragment of the Kv2.3 cDNA (containing amino acids 1–146).

Chimera Ch2. We sequentially fused in frame the sequences encoding the first 314 amino acids of Kv2.3, followed by amino acids 293–415 of Kv2.1 and the 65 C-terminal amino acids of Kv2.3. The coding sequences were amplified by PCR from the p513-Kv2.1 or p513-Kv2.3 plasmids using the appropriate primers that introduced silent mutations to create *Bgl*II and *Eco*RI sites on the sequences encoding the Kv2.1/Kv2.3 fusions, i.e., the beginning of the S4 and the end of the S6 segments, respectively.

Chimera Ch3. The p513-Ch3 plasmid was obtained using specific primers containing *Eco*RI (5′ primer) and *Bgl*II (3′ primer) to synthesize by PCR the sequence encoding the first 314 amino acids of Kv2.3. The amplified fragment was digested with *Eco*RI and *Bgl*II and cloned into equally digested p513 plasmid. This construct was then linearized with *Bgl*II and ligated to a *Bgl*II fragment containing the sequence encoding the 560 C-terminal amino acids of Kv2.1. Subsequently, an *Eco*RI-*Cla*I fragment encoding the first 146 amino acids of Kv2.3 was replaced by the equivalent fragment of Kv2.1, which encodes its first 121 amino acids.

Chimera Ch4. A silent point mutation, which generates an *Eco*RI site, was introduced into the nucleotide 666 of the Kv2.1 cDNA by using the Altered Sites II *in vitro* Mutagenesis Systems (Promega, Madison, WI) according to the manufacturer's instructions. This construct, named p513-Kv2.1/RI, was digested with *Eco*RI and *Afl*II to delete the amino acids 217–803 of Kv2.1. The resulting 5.7 Kb fragment was gel purified and ligated to an *Eco*RI/*Afl*II PCR fragment containing amino acids 244–314 of Kv2.3, followed by amino acids 293–803 of Kv2.1. The PCR-amplified product was synthesized using the Ch3 cDNA and primers that carry *Eco*RI and *AflII* sites.

Chimera Ch5. This construct was made by replacing the *Cla*I-*Eco*RI fragment of the p513-Kv2.1/RI plasmid, which encodes amino acids 122–214 of Kv2.1, with a *Cla*I-*Eco*RI PCR fragment containing the sequence encoding amino acids 147–241 of Kv2.3.

Chimera Ch6. The p513-Ch6 plasmid was made by replacing the *Cla*I-*Eco*RI fragment of Ch5 cDNA with a *Cla*I-*Eco*RI PCR fragment containing the Kv2.1 coding sequence from amino acid 122 to 178, followed by an *Eco*RI-*Eco*RI PCR fragment containing amino acids 208–241 of Kv2.3.

Chimera Ch7. The p513-Ch7 construct was made as the p513-Ch6 plasmid, except that the *Cla*I-*Eco*RI and the *Eco*RI-*Eco*RI PCR fragments contain the Kv2.3 coding sequence from amino acid 147 to 206 and the Kv2.1 coding sequence from amino acid 179 to 214, respectively.

Chimera Ch8. To make this construct, we synthesized by PCR the sequence encoding amino acids 122–179 of Kv2.1 using primers containing *Cla*I (5′ primer) and *Eco*RI (3′ primer) sites. The amplified fragment was digested with *Cla*I and *Eco*RI and cloned into equally digested pBluescript SK $^{+/-}$ plasmid. This construct was then digested with *Eco*RI and *Sma*I and ligated to an *Eco*RI/*Sma*I-digested PCR fragment containing the 295 C-terminal amino acids of Kv2.3. Then, the Kv2.1-Kv2.3 fused coding sequences were obtained from this construct by digestion with *Cla*I and *Not*I and used to replace the *Cla*I-*Not*I fragment (357 last amino acids of Kv2.3) in the p513-Kv2.3 plasmid.

The sequences of the chimeric cDNAs were verified by restriction enzyme analysis and DNA sequencing

In vitro *transcription and translation*

In vitro translations were performed using 0.5 μg of the indicated plasmids in 12.5 μl of TNT-coupled transcription–translation reaction (Promega) as per the manufacturer's instructions. [^{35}S]Methionine-labeled proteins were resolved in a 9% SDS-polyacrylamide gel and visualized by autoradiography. All the chimeras studied (Ch1–Ch8) were transcribed *in vitro* into proteins of the predicted molecular weight.

Functional expression of ion channels and electrophysiological measurements

Functional expression of the various K^+ channel α-subunits was done using Chinese hamster ovary (CHO) cells grown in McCoy's 5A culture medium (BioWhittaker, Walkersville, MD) supplemented with L-glutamine and antibiotic solutions. CHO cells were transiently transfected with 1–6 μg of the K^+ channel cDNAs by electroporation using a Gene Pulser apparatus (Bio-Rad, Hercules, CA). In all experiments, 2 μg of the green fluorescent protein cDNA was cotransfected with the α-subunit cDNAs to identify by fluorescence the cells that had been efficiently transfected. K^+ currents were recorded 24–48 hr after electroporation using the whole-cell configuration of the patch-clamp technique as adapted to our laboratory (Hamill et al., 1981; Castellano and López-Barneo, 1991). We used low-resistance electrodes (1–3 MΩ), capacity compensation, and subtraction of linear leakage and capacity currents. Series resistance compensation (up to 50%) was systematically used. The holding potential was −80 mV in all the experiments. Inactivation and closing rates were estimated by fitting the time courses of the currents with a single exponential function. Activation kinetics were estimated by the time interval elapsed between 20 and 80% of maximal current amplitude (20–80% rise time). Inhibition by external Zn^{2+} was calculated from the current amplitude measured at the end of 200 msec pulses. Kv2.1 channels are relatively resistant to inhibition by external Zn^{2+} in the millimolar range (De Biasi et al., 1993a; Castellano et al., 1997), but sensitivity to Zn^{2+} blockade increases two to three times in heteromeric Kv2.3+Kv2.1 channels (Castellano et al., 1997). In preliminary experiments, we tested the potency of various concentrations of external Zn^{2+} (0.1–5 mM) to inhibit the macroscopic K^+ currents mediated by either homomeric Kv2.1 or heteromeric Kv2.3+Kv2.1 channels. In the present work, we used routinely 1 mM Zn^{2+} because, in these conditions, reduction of current amplitude was fairly reversible, thus allowing a quantitative comparison of Zn^{2+} blockade of the various channels studied. Recovery of current amplitude after Zn^{2+} washout was slow and in many cases incomplete when higher concentrations of the cation were used. Standard composition of the external solutions were (in mM): 140 NaCl, 2.7 KCl, 2.5 $CaCl_2$, 4 $MgCl_2$, and 10 HEPES, pH 7.4. In some experiments, 1 mM $ZnCl_2$ was added to this solution. To study deactivation kinetics, 70 mM NaCl was replaced by 70 mM KCl. Pipette solution was (in mM): 80 KCl, 30 K-glutamate, 20 K-fluoride, 4 ATP-Mg, 10 EGTA, and 10 HEPES, pH 7.2. In the tables and text, average values of the kinetic parameters of the potassium currents are given by mean ± SD and in parentheses the number of observations. Statistical analysis of Zn^{2+} blockade was done using the nonparametric Mann–Whitney *U* test.

RESULTS

Kv2.3 interacts selectively with Kv2.1 channels

Kv2.3 (or Kv8.1) produce profound functional changes of Kv2.1 or Kv2.2 channels expressed in CHO (Castellano et al., 1996, 1997) and COSm6 (Salinas et al., 1997a) cells, respectively. In contrast, Kv2.3 does not modify the K^+ current kinetics of Rbk1 (Kv1.1) or *Shaker* B channels (Castellano et al., 1997). To further evaluate the specificity of the interaction of Kv2.3 with other Kv α-subunits, we extended our previous study to various channels representative of four genetic families of voltage-dependent K^+ channels. Figure 1 shows typical records of potassium currents elicited by short- and long-lasting depolarizing pulses applied to voltage-clamped cells transfected with the indicated K^+ channel α-subunits. The current traces are scaled to the same peak amplitude to facilitate the comparison of the activation (Fig. 1, *left*) and inactivation (*right*) kinetics in the various experimental conditions. Note that coexpression of Kv2.3 with Kv1.2 (*A*), Kv3.3 (*C*), and Kv4.2 (*D*) resulted in currents without appreciable alterations compared with those obtained when the channels were expressed alone. Deactivation time courses of these three channel types were also unaltered by coexpression with the regulatory α-subunit (data not shown). As described previously (Castellano et al., 1997), coexpression of Kv2.3 with Kv2.1 leads to marked deceleration of activation and inactivation (Fig. 1*B*), as well as channel closing (see Fig. 3). These results indicate that, as suggested in our previous work (Castellano et al., 1997), Kv2.3 interacts selectively with channels of the Kv2 family.

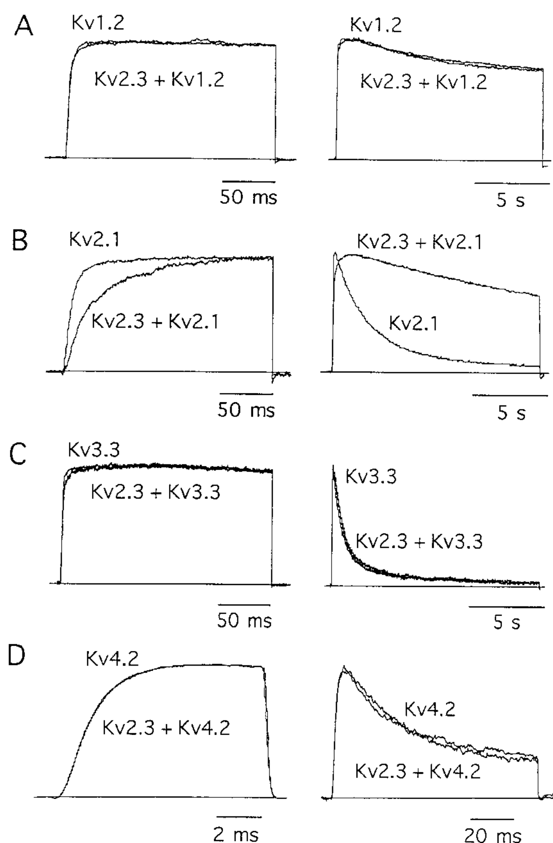

Figure 1. Effect of Kv2.3 on Kv1.2 (*A*), Kv2.1 (*B*), Kv3.3 (*C*), and Kv4.2 (*D*) channels. In each case, normalized K^+ current traces of cells transfected with a K^+ channel α-subunit alone (Kv1.2, Kv2.1, Kv3.3, and Kv4.2) or with a 50% mixture of each subunit and Kv2.3 are shown superimposed. In all cases, the short- and long-lasting depolarizing pulses were applied to +20 mV from a holding potential of −80 mV.

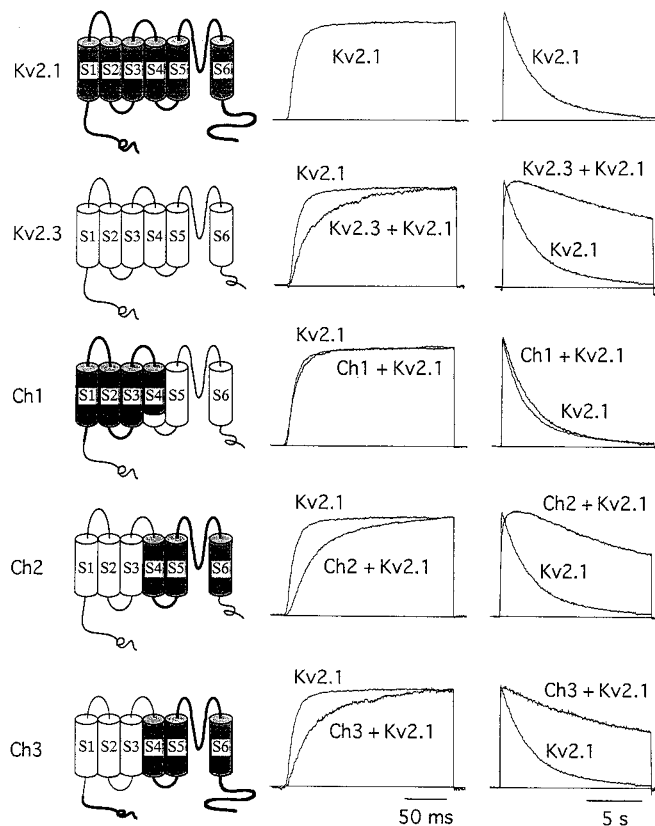

Figure 2. Comparison of the time course of K^+ currents recorded from cells transfected with Kv2.1 or cotransfected with Kv2.1 and Kv2.3, Ch1, Ch2, or Ch3. The proposed transmembrane topology of Kv2.1, Kv2.3, and the chimeric α-subunits are represented by schemes close to each set of traces. The protein sequences are represented by *thick lines* and *filled cylinders* (Kv2.1) or *thin lines* and *open cylinders* (Kv2.3). Activation and inactivation time courses of the currents are shown by traces in the *left* and *right columns* superimposed in all cases on the same scaled Kv2.1 records to facilitate comparison. In all the experiments, depolarizing pulses were applied to +20 mV from a holding potential of −80 mV.

Identification of the structural domain responsible for the modulatory action of Kv2.3

To identify the molecular determinant of Kv2.3 modulating Kv2.1 channel gating, we constructed chimeric proteins by swapping different regions between Kv2.3 and Kv2.1 α-subunits. Each of these chimeric proteins was coexpressed with Kv2.1 in CHO cells to study the resulting macroscopic K^+ currents. Representative current traces recorded from cells transfected with Kv2.1 alone or cotransfected with Kv2.1 plus either Kv2.3 or a chimera are shown in Figure 2. Currents recorded from cotransfected cells are superimposed on scaled Kv2.1 current records to facilitate comparison. We first created chimeras Ch1 and Ch2 by replacing either of the two halves of the Kv2.3 core region (S1–S6) by the corresponding fragments of Kv2.1. Coexpression of Ch1 plus Kv2.1 gave rise to a potassium current with time course similar to the currents produced by Kv2.1 alone, whereas coexpression of Ch2 and Kv2.1 resulted in currents with clear deceleration of activation and inactivation kinetics. These modulatory effects on Kv2.1 channels were retained in chimera Ch3, which differs from Ch2 in that its N terminus (121 amino acids) and C terminus (560 amino acids) are from Kv2.1. Ch2 and Ch3, but not Ch1, also induced a clear slowing of closing time course as studied by measuring tail currents in cells exposed to high external K^+ (Fig. 3). Average values of the activation and inactivation parameters (rise time and time constant, respectively) of cells expressing Kv2.1 or various types of heteromeric K^+ channels are summarized in Table 1. Mean values of closing kinetics are given in Figure 3 legend. The effects of Ch2 and Ch3 on Kv2.1 appeared to be qualitatively similar to those produced by wild-type Kv2.3 (Figs. 2, 3), thus suggesting that the modulatory role of Kv2.3 depended on a regulatory element located in the region spanning from amino acid at position 147 in the N terminus (adjacent to S1) to the beginning of the S4 segment of the Kv2.3 protein.

External Zn^{2+} is known to block more strongly Kv2.3+Kv2.1 heteromers than Kv2.1 homomers (Castellano et al., 1997) (Fig. 4) and, although with quantitative differences, high sensitivity to Zn^{2+} was maintained in the channels resulting from the coexpression of Kv2.1 and those chimeras conserving any of the extracellular domains of Kv2.3 (Table 1). The mechanism of Kv2.1 channel blockade by external Zn^{2+} is unknown, and its characterization was not an objective of the present work; however, sensitivity to external Zn^{2+} was used as a tool to check whether chimeric α-subunits were effectively forming oligomers with Kv2.1. It is obvious that this Zn^{2+} block assay was particularly important in the study of chimeras that did not alter the kinetics of Kv2.1 to demonstrate that they were able to coassemble with the Kv2.1 α-subunit. Figure 4 shows that, regardless of the kinetic of the currents, external Zn^{2+} blocked heteromeric channels formed by chimeras Ch1, Ch2, or Ch3 plus Kv2.1 with

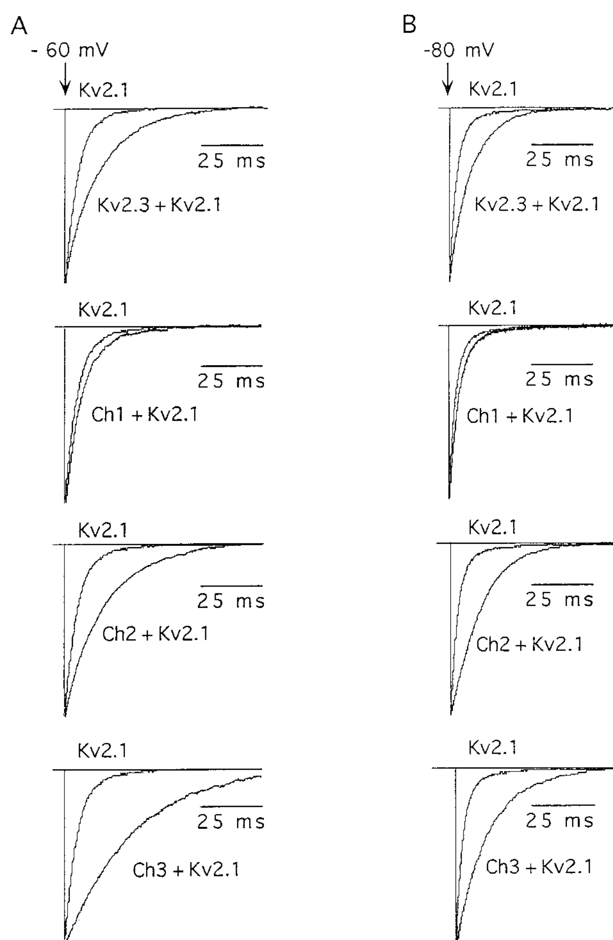

Figure 3. Comparison of the closing time course of Kv2.1 channels and the heteromeric channels resulting from the coexpression of Kv2.1 with Kv2.3 or chimeras Ch1, Ch2, and Ch3. Inward K^+ tail currents were recorded at the instant of repolarization (*vertical arrow*) to either −60 (*A*) or −80 (*B*) mV after depolarizing pulses to +20 mV. Tail currents are superimposed on the same scaled Kv2.1 currents to facilitate comparison. The external solution contained 70 mM K^+. Closing time constants (in milliseconds) were, at −60 mV, as follows: Kv2.1, 5.3 ± 1.1 (6); Kv2.3+Kv2.1, 22.6 ± 4 (6); Ch1+Kv2.1, 5.2 ± 1.1(4); Ch2+Kv2.1, 23 ± 3 (6); and Ch3+Kv2.1, 21.4 ± 6.1(6). Closing time constants (in milliseconds) were, at −80 mV, as follows: Kv2.1, 3.5 ± 1 (6); Kv2.3+Kv2.1, 11.1 ± 1.3 (6); Ch1+Kv2.1, 3.6 ± 1.1(4); Ch2+Kv2.1, 12.3 ± 1.1(6); and Ch3+Kv2.1, 11.6 ± 2.7(6). Values are given by mean ± SD, and the number of experiments is given in *parentheses*.

almost perfect reversibility and significantly higher potency than that observed for homomeric Kv2.1 channels (Table 1). Hence, these observations suggested that, like the parental Kv2.3 protein, the chimeric α-subunits were able to form heteromeric channels with Kv2.1. These heteromers retained the high sensitivity to blockade by external Zn^{2+} independently of their kinetic parameters, which appeared modified only in those channels containing the regulatory domain of Kv2.3.

To define more precisely the location of the Kv2.3 regulatory domain, we constructed several derivatives of Ch3 (Ch4–Ch6), conserving different parts of Kv2.3. Although with variable effects on Kv2.1, these chimeras were also able to form heteromeric channels with Kv2.1 as evidenced by the high sensitivity of the currents to external Zn^{2+} (Fig. 5, Table 1). For the sake of simplicity, in studying the effects of chimeras derived from Ch3, we focused our analysis on activation and inactivation time courses, although similar qualitative effects were observed on channel closing. Ch5, but not Ch4, induced modifications in the kinetic parameters of the K^+ current similar to those elicited by Kv2.3 (Fig. 5, Table 1). Ch5+Kv2.1 heteromers appeared to have a particularly slow activation time course, but this was not studied in detail. The modulatory effect of Ch5 was abolished by replacing the fragment of the N terminus close to S1 (Fig. 5, *asterisks*) by the equivalent segment of Kv2.1. Coexpression of this new chimera (Ch6) with Kv2.1 resulted in currents with fast activation and inactivation time courses (indistinguishable from Kv2.1 currents) but high sensitivity to external Zn^{2+} (Fig. 5, Table 1). Therefore, the data suggested that the N-terminal region adjacent to S1 contained the regulatory structure of Kv2.3. Further evidence supporting this idea was obtained by studying the effects of chimera Ch7, which only differs from Kv2.1 in that the N-terminus fragment proximal to S1 belongs to Kv2.3 (Fig. 5). Ch7+Kv2.1 currents exhibited the deceleration of activation and inactivation characteristic of Kv2.3+Kv2.1 heteromers (Fig. 5). As expected, because Ch7 does not contain any of the extracellular domains of Kv2.3, external Zn^{2+} had a small inhibitory effect on Ch7+Kv2.1 channels, similar to that of the cation on Kv2.1 currents (compare Fig. 4, *top records*, with Fig. 5, *right column*; Table 1). A definitive test in favor of the regulatory role of the N-terminal fragment was obtained by studying chimera Ch8, which is almost identical to Kv2.3, with the sole modification that the N-terminal fragment adjacent to S1 is replaced by the equivalent sequence of Kv2.1. In contrast to wild-type Kv2.3, coexpression of Ch8 with Kv2.1 gave rise to K^+ currents similar to the Kv2.1 currents. Ch8+Kv2.1 heteromers were, however, highly sensitive to external Zn^{2+} (Fig. 5). Comparison of the results obtained with Ch7 and Ch8 indicated that the same fragment of Kv2.3 that conferred regulatory function to Kv2.1 was necessary to avoid the loss of function in Kv2.3 (Fig. 5). Therefore, a stretch of 59 amino acids adjacent to S1 (*Ch5*, *Ch7*, *Ch8*, *asterisks*) constitutes an N-terminus regulatory domain (NRD) responsible for the modulatory action of Kv2.3 on Kv2.1 channels.

Kinetic properties of chimeric (Kv2.3/Kv2.1) homomers

The fact that the coexpression of Kv2.3, or the chimeras that had the NRD of Kv2.3, with Kv2.1 resulted in heteromeric channels with altered kinetics led us to postulate that the same changes should be present in homomeric channels formed by the various chimeric proteins. Among all the chimeras studied, only Ch3–Ch7 were capable of forming functional channels by themselves. Representative current traces obtained from cells transfected with cDNAs of these chimeras are shown in Figure 6*A*. As in previous figures, each set of currents obtained with short- and long-lasting depolarizing pulses are shown superimposed on scaled Kv2.1 currents to facilitate comparison. Average values of the activation and inactivation parameters are given in Table 2. As expected, only the chimeras containing the NRD of Kv2.3 (Ch3, Ch5, and Ch7) formed channels exhibiting the deceleration of kinetics characteristic of Kv2.3+Kv2.1 heteromers. Currents resulting from the expression of Ch4 and Ch6 were practically similar to those mediated by Kv2.1. All the chimeric currents were blocked by external Zn^{2+} with excellent reversibility but differential sensitivity, depending on the presence of extracellular domains of Kv2.3 (Fig. 6*B*). The potency of Zn^{2+} to block the chimeric currents was qualitatively similar to the effect of the cation on Kv2.1+chimera heteromeric channels. In general, homomeric channels made of chimeric α-subunits exhibited more

Table 1. Comparison of the kinetic parameters of Kv2.1 α-subunit expressed alone or coexpressed with either Kv2.3 or chimeric α-subunits

	Rise time (msec)			τ Inactivation (sec)	% of inhibition of current amplitude (1 mM Zn^{2+})		
	0 mV	+20 mV	+40 mV	+20 mV	0 mV	+20 mV	+40 mV
Kv2.1	22 ± 6 (10)	13 ± 3 (10)	11 ± 2 (10)	3 ± 1 (11)	21 ± 4 (7)	16 ± 4 (8)	13 ± 8 (9)
Kv2.1+Kv2.3	51 ± 9 (14)	21 ± 3 (10)	19 ± 3 (22)	12 ± 3 (26)	40 ± 5 (9)*	34 ± 4 (24)*	29 ± 4 (28)*
Kv2.1+Ch1	21 ± 6 (8)	13 ± 5 (8)	10 ± 3 (8)	5 ± 1 (10)	39 ± 7 (5)#	24 ± 4 (4)§	20 ± 5 (4)
Kv2.1+Ch2	86 ± 6 (8)	43 ± 8 (8)	24 ± 4 (8)	13 ± 3 (11)	66 ± 4 (4)&	45 ± 1 (4)#	33 ± 4 (4)#
Kv2.1+Ch3	100 ± 13 (9)	58 ± 12 (9)	34 ± 9 (9)	19 ± 1 (8)	50 ± 8 (5)#	43 ± 6 (5)#	38 ± 6 (5)*
Kv2.1+Ch4	21 ± 5 (13)	12 ± 4 (13)	10 ± 3 (13)	4 ± 1 (7)	56 ± 8 (5)#	41 ± 9 (5)#	30 ± 6 (5)#
Kv2.1+Ch5	97 ± 8 (10)	78 ± 15 (11)	49 ± 14 (11)	14 ± 3 (9)	66 ± 9 (9)*	55 ± 8 (9)*	42 ± 6 (9)*
Kv2.1+Ch6	22 ± 4 (19)	12 ± 3 (15)	10 ± 2 (15)	2 ± 1 (10)	42 ± 7 (5)#	32 ± 6 (5)#	26 ± 5 (5)&
Kv2.1+Ch7	106 ± 9 (10)	42 ± 15 (6)	14 ± 3 (7)	21 ± 10 (6)	15 ± 5 (4)	13 ± 3 (4)	11 ± 5 (4)
Kv2.1+Ch8	24 ± 4 (8)	15 ± 3 (8)	13 ± 2 (8)	3 ± 1 (6)	37 ± 3 (7)*	27 ± 3 (7)*	21 ± 4 (7)

Values are given by mean ± SD, and the number of observations is given in parentheses. Inhibition by Zn^{2+} of each channel construct is compared with the potency of Zn^{2+} to block Kv2.1 channels at the corresponding membrane potential. Statistical significance (Mann–Whitney *U* test) is indicated by the following symbols: *$p<0.001$; #$p<0.005$; &$p<0.01$; and §$p<0.05$.

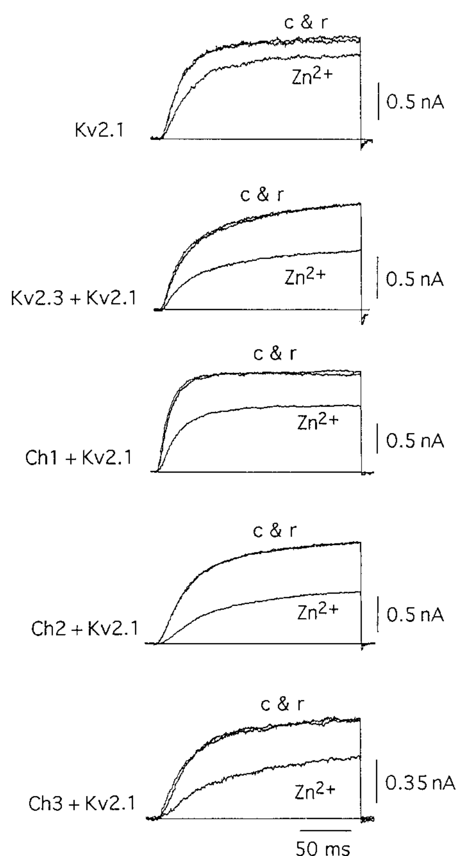

Figure 4. Blockade by external Zn^{2+} of K^+ currents recorded from cells transfected with Kv2.1 or cotransfected with Kv2.1 and Kv2.3, Ch1, Ch2, or Ch3. All records illustrate the reversible (*c & r*, control and recovery) reduction of the various types of currents by application of 1 mM Zn^{2+} to the external solution. In all experiments, depolarizing pulses were applied to +20 mV from a holding potential of −80 mV.

pronounced kinetic modifications and sensitivity to external Zn^{2+} than heteromeric channels resulting from the coexpression of the corresponding chimera and Kv2.1 (compare Tables 1, 2). However, these differences were not too large, suggesting that the presence of one or two mutated subunits in heteromeric channels is enough to produce almost full regulatory effect. This is consistent with previous observations in other heteromeric channels in which a single subunit can impose new functional properties (Monyer et al., 1992; Waldmann et al., 1995). Thus, these data indicate that the NRD is of critical importance for the gating of Kv2.1 channels.

The aligned amino acid sequences of the N-terminal regions of Kv2.3 and the two known functional channels of the Kv2 family (Kv2.1 and Kv2.2) are shown in Figure 7. The NRD of Kv2.3 spans from residue 148 (a few amino acids after the end of the B box) to near the beginning of the first transmembrane segment S1 (residue 206). Given that the last few amino acids of the NRD region are conserved in the three channel types, it is most likely that its regulatory role depends on the fragment between amino acids 148 and 196, whose sequence in Kv2.3 is highly divergent compared with the equivalent regions in Kv2.1 and Kv2.2 channels. The NRD amino acid sequence is almost identical in Kv2.1 and Kv2.2 channels, but the percentage of identity in this region of the channels with Kv2.3 falls to <20%. Interestingly, other fragments of the N terminus, such as the B box, involved in protein tetramerization have an amino acid sequence much more conserved among the three channel types (Castellano et al., 1997).

DISCUSSION

The major finding in this paper is the identification of the structural domain determining the regulatory effects of Kv2.3 on Kv2.1 channels. This domain is within a fragment of 59 amino acids located at the N terminus adjacent to the first transmembrane segment. Our results strongly indicate that this N-terminal region has a critical role in gating of Kv2.1 channels.

Modulation of Kv2 channels by the regulatory α-subunit Kv2.3

We show here that Kv2.3 exerts a selective action on Kv2.1 channels, leaving unaltered the kinetics of Kv1.2, Kv3.3, and Kv4.2 channels. In previous work, we also demonstrated that Kv2.3 does not modify the function of Kv1.1 and *Shaker* B channels (Castellano et al., 1996, 1997). Hugnot et al. (1996) reported that Kv8.1 cRNA (the hamster clone equivalent to rat Kv2.3) injected in *Xenopus* oocytes blocked completely the expression of *Shab* (Kv2) and *Shaw* (Kv3) channels, suggesting that Kv8.1 was regulating the function of these channels. However, the same authors have shown that, in mammalian cells (COSm6), Kv8.1 only modulates Kv2.2 without affecting Kv3.4 currents (Salinas et al., 1997a). Reduction of current amplitude when

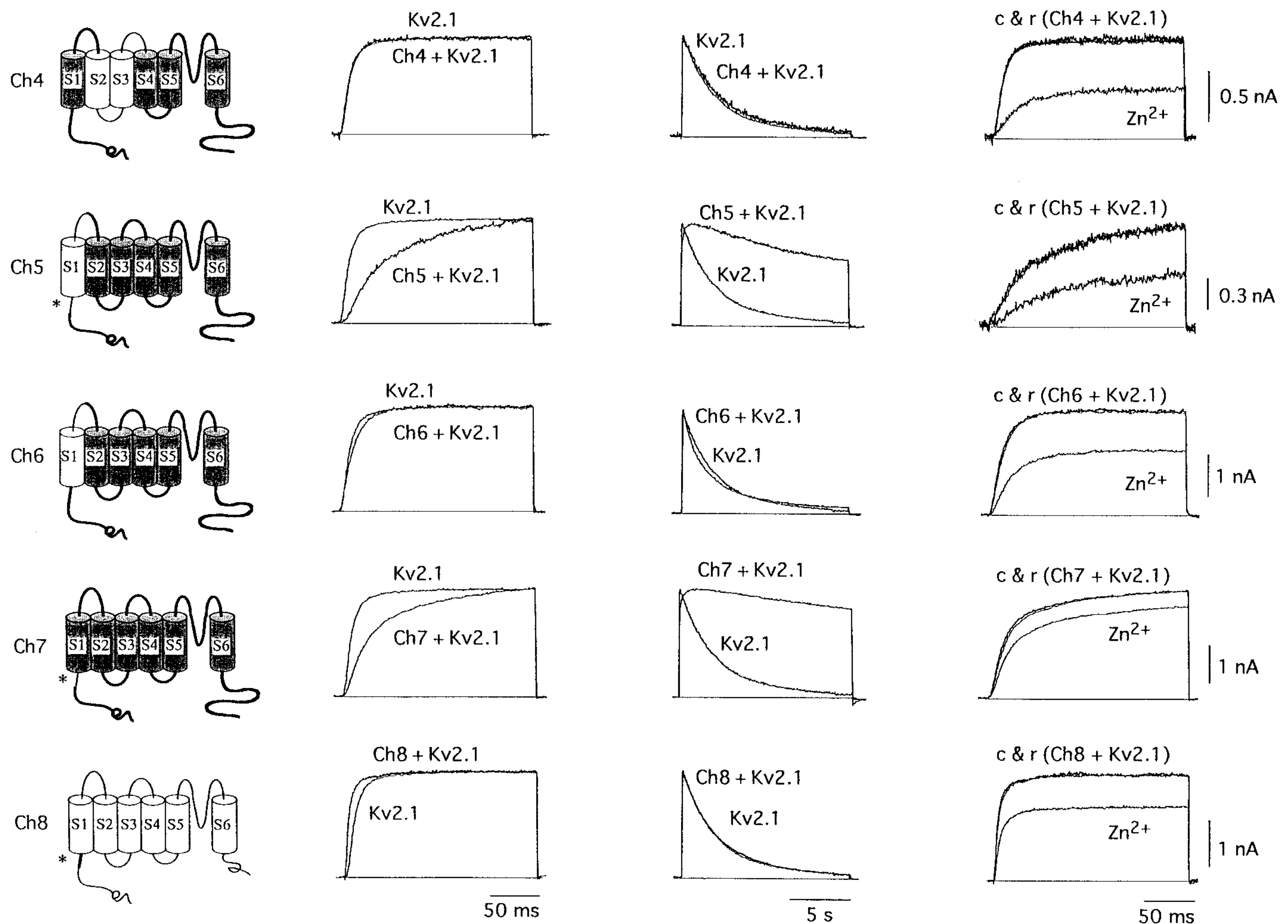

Figure 5. Comparison of the time course of K^+ currents recorded from cells transfected with Kv2.1 or cotransfected with Kv2.1 and Ch4–Ch8. The proposed transmembrane topology of the chimeric α-subunits are represented by schemes close to each set of traces. The protein sequences are represented by *thick lines* and *filled cylinders* (Kv2.1) or *thin lines* and *open cylinders* (Kv2.3). *Asterisks* in *Ch5*, *Ch7*, and *Ch8* indicate the location of the regulatory domain of Kv2.3. Activation and inactivation time courses of the currents are shown by traces in the *left* and *middle columns* superimposed in all cases on the same scaled Kv2.1 records to facilitate comparison. Records in the *right column* illustrate the reversible (*c & r*, control and recovery) reduction of the various types of currents by application of 1 mM Zn^{2+} to the external solution. In all experiments, depolarizing pulses were applied to +20 mV from a holding potential of −80 mV.

Kv2.3 is coexpressed with other K^+ channels does not necessarily indicate the existence of functional interaction between the different α-subunits because similar nonspecific reductions in current amplitude are observed when K^+ channels are coexpressed with other proteins, such as green fluorescent protein or β galactosidase (Castellano et al., 1997; Salinas et al., 1997a). Together, these data suggest that the physiological role of Kv2.3 (or Kv8.1) is to modulate the activity of the functional Kv2 (2.1 and 2.2) channels.

Expression of Kv2.3 is restricted to specific areas of the brain in which there are also high levels of Kv2.1 or Kv2.2 mRNAs (Trimmer, 1991; Drewe et al., 1992; Hwang et al., 1992, 1993; Hugnot et al., 1996; Castellano et al., 1996, 1997). Kv2.1 and Kv2.2 have, in general, a distinct nonoverlapping distribution in mammalian central neurons (Hwang et al., 1992, 1993) and, thus, it is unlikely that these channels form heteromeric complexes (Blaine and Ribera, 1998). However, Kv2.3 could form heteromers with Kv2.1 or Kv2.2 channels to modulate their function. In fact, we have preliminary indications that Kv2.3 and Kv2.1 mRNAs can coexist in the same neuron. The existence of regulatory α-subunits, such as Kv2.3, with a modulatory role on Kv2.1 channels might have special physiological significance because Kv2.1 is abundantly expressed in the mammalian brain (Trimmer, 1991; Drewe et al., 1992). Kv2.1 is a major contributor to the delayed K^+ current in hippocampal neurons (Murakoshi and Trimmer, 1999) and is localized uniquely among brain K^+ channels to large clusters on the soma and on the very proximal portions of dendrites (Trimmer, 1991; Scannevin et al., 1996; Du et al., 1998; Murakoshi and Trimmer, 1999). It is possible that Kv2.1 has a major role in regulating the transmission of electrical signals into and out of the neuronal somata (Murakoshi and Trimmer, 1999); thus, selective coexpression of Kv2.3 and Kv2.1 could confer plasticity to neuronal integration and processing. Another modulatory effect of Kv2.3 on Kv2.1 might result from the increased sensitivity to external Zn^{2+} of Kv2.3+Kv2.1 heteromers because in hippocampal nerve terminals, Zn^{2+} is highly enriched and it can reach concentrations near the millimolar range in the synaptic cleft (Huang, 1997). This type of modula-

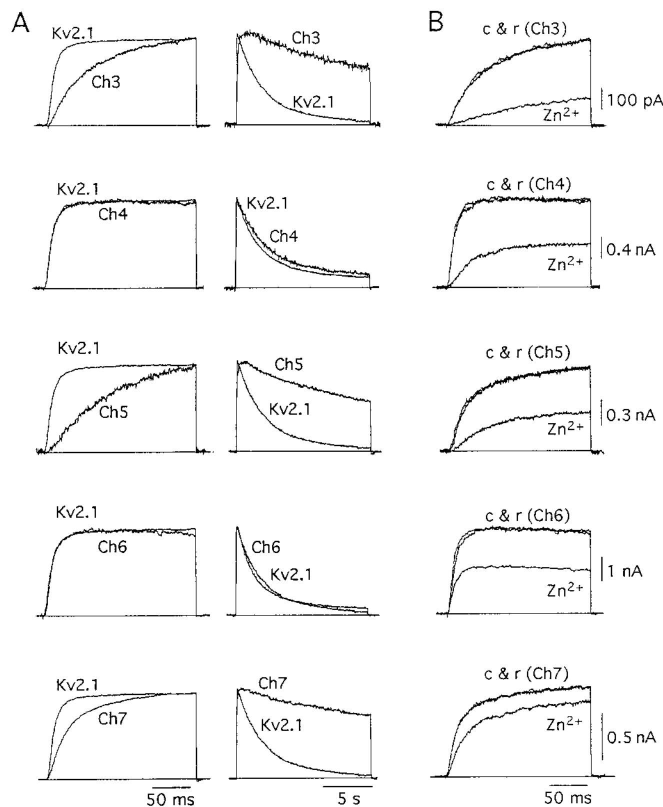

Figure 6. Potassium currents mediated by the Kv2.3/Kv2.1-derived chimeras capable of forming functional homomeric channels. *A*, *Left* and *middle columns*, Activation and inactivation time courses of the various types of potassium currents superimposed in all cases on the same scaled Kv2.1 records to facilitate comparison. *B*, *Right column*, Reversible (*c & r*, control and recovery traces) reduction of the various types of currents by application of 1 mM Zn^{2+} to the external solution. In all experiments, depolarizing pulses were applied to +20 mV from a holding potential of −80 mV.

tion has a precedent in the NMDA receptor inhibition by Zn^{2+} (Westbrook and Mayer, 1987), which depends on the molecular subunit composition of the channels (Chen et al., 1997).

N-terminal regulatory domain of Kv2.3 and gating of Kv2 channels

We have identified a regulatory domain (NRD) in the N terminus of Kv2 channels that determines the functional effects of Kv2.3 on Kv2.1. The sole presence of the NRD of Kv2.3 in Kv2.1 confers to the chimeric protein (Ch7) the ability to modulate native Kv2.1 channels in the same way as Kv2.3. In contrast, replacement of the NRD of Kv2.3 by the same fragment of the Kv2.1 protein results in a chimera (Ch8) with almost complete loss of regulatory function. Salinas et al. (1997a) have reported that the effects of Kv8.1 on Kv2.1 channels are mediated by amino acids in the S6 segment based on the fact that a chimera containing from the N terminal to the pore of Kv8.1 and the S6 segment and carboxyl end of Kv1.3 (Kv8/Kv1) is unable to alter the properties of Kv2.1 currents. However, in these experiments, formation of heteromeric channels by Kv2.1 and the chimera Kv8/Kv1 was not directly tested, so it is possible that the chimera was unable to form heteromers with Kv2.1 channels. Salinas et al. (1997a) have also shown that a mutated Kv8.1 subunit with two amino acid replacements in S6 is less effective than the native Kv8.1 to decelerate inactivation of Kv2.1 channels. Mutations of S6 residues are known to modify inactivation rate (Hoshi et al., 1991); however, these changes cannot fully explain the effect of Kv2.3 on Kv2 channels (deceleration of activation, closing, and inactivation). Our study shows that various chimeras (e.g., Ch1 and Ch8), conserving intact large regions of the native Kv2.3 protein including the S6 segment but lacking the NRD fragment of Kv2.3, are unable to alter the kinetics of Kv2.1 currents. On the contrary, chimeras with the S6 segment of Kv2.1 but containing the NRD of Kv2.3 have full regulatory effects on Kv2 channels. Thus, the inescapable conclusion is that the presence of the NRD sequence in Kv2.3 is the major cause for the modulatory action of the Kv2.3 subunit on the Kv2.1 channel.

Our experiments also indicate that the NRD fragment is of pivotal importance for the gating of Kv2.1 because the deceleration of kinetics imposed by Kv2.3 in heteromeric (Kv2.3+Kv2.1) channels was observed in functional homomers formed by those chimeras having the NRD of Kv2.3 (Ch3, Ch5, and Ch7). The intracellular N-terminal region is known to contain conserved, family-specific, sequences (such as the A and B boxes or T1 domain) that participate in recognition and assembly of voltage-gated potassium channels (Li et al., 1992; Shen and Pfaffinger, 1995; Yu et al., 1996). However, the precise role of the N terminus in channel gating is poorly understood. There are reports indicating that the N terminus determines the voltage-dependent gating behavior of *eag* (Schönherr and Heinemann, 1996; Spector et al., 1996; Terlau et al., 1997) and KAT families of channels (Marten and Hoshi, 1998). For example, deletions in the N terminus of *eag* channels can produce voltage shifts in the activation parameters and marked slowing of closing (Terlau et al., 1997). In addition, deletions in the N and C termini of Kv2.1 channels are also known to result in pronounced modifications of

Table 2. Comparison of the kinetic parameters of Kv2.1 and functional Kv2.1/Kv2.3 chimeric channels

	Rise time (msec)			τ Inactivation (sec)	% of inhibition of current amplitude (1 mM Zn^{2+})		
	0 mV	+20 mV	+40 mV	+20 mV	0 mV	+20 mV	+40 mV
Kv2.1	22 ± 6 (10)	13 ± 3 (10)	11 ± 2 (10)	3 ± 1 (11)	21 ± 4 (7)	16 ± 4 (8)	13 ± 8 (9)
Ch3	98 ± 15 (5)	67 ± 10 (5)	61 ± 15 (6)	21 ± 3 (4)	88 ± 8 (4)&	82 ± 8 (3)&	59 ± 7 (3)#
Ch4	21 ± 5 (14)	13 ± 3 (14)	10 ± 2 (14)	4 ± 1 (6)	65 ± 4 (8)*	52 ± 7 (8)*	37 ± 4 (8)*
Ch5	110 ± 16 (11)	91 ± 14 (17)	68 ± 13 (17)	18 ± 7 (8)	72 ± 8 (10)*	60 ± 6 (10)*	50 ± 8 (10)*
Ch6	22 ± 5 (16)	14 ± 3 (16)	12 ± 3 (16)	2 ± 0.4 (16)	63 ± 2 (4)&	51 ± 4 (5)#	44 ± 5 (5)*
Ch7	110 ± 9 (7)	62 ± 11 (6)	18 ± 5 (5)	13 ± 3 (6)	14 ± 5 (4)	12 ± 4 (4)	10 ± 3 (4)

Values are given by mean ± SD, and the number of observations is given in parentheses. Inhibition by Zn^{2+} of each channel construct is compared with the potency of Zn^{2+} to block Kv2.1 channels at the corresponding membrane potential. Statistical significance (Mann–Whitney *U* test) is indicated by the following symbols: *$p<0.001$; #$p<0.005$; &$p<0.01$; and §$p<0.05$.

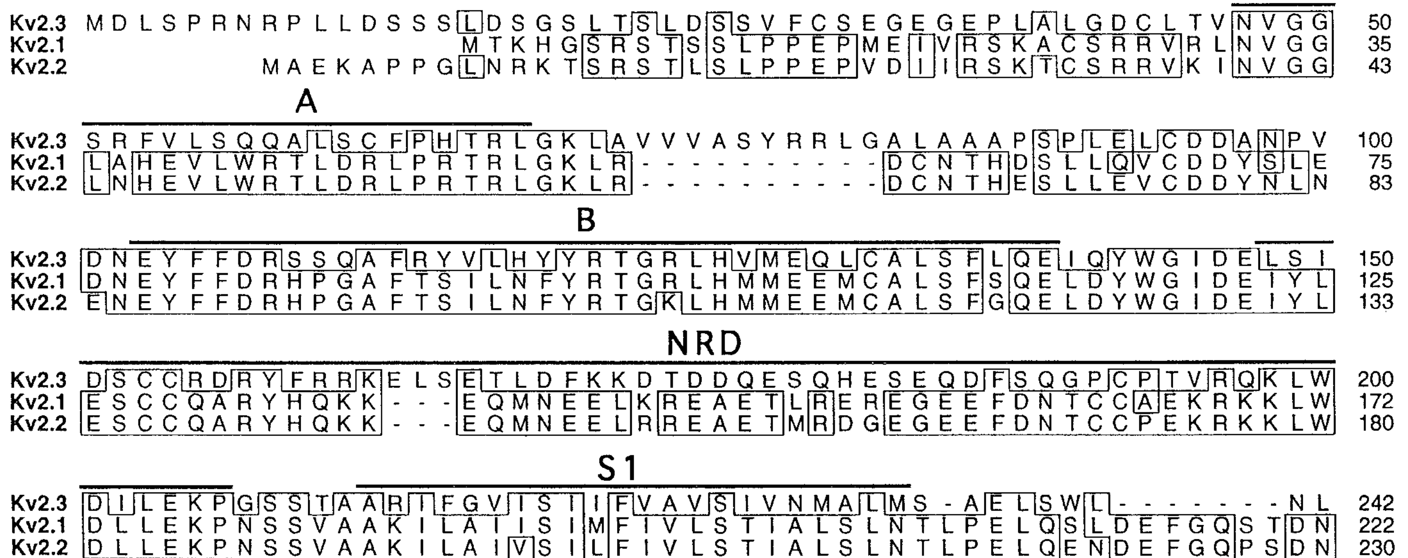

Figure 7. Amino acid sequence alignment of the N terminus of Kv2.3, Kv2.1, and Kv2.2 channels. The position of the A and B boxes, the S1 segment, and the proposed N-terminal regulatory domain (NRD) are indicated. Regions of sequence identity are enclosed in *boxes*.

activation and closing kinetics (VanDongen et al., 1990). Although replacement of cysteine residues in the N terminus of Kv2.1 channels can produce slowing of activation (Pascual et al., 1997), the effects of Kv2.3 on Kv2.1 activation and closing must depend on different residues because the two cysteines present in the NRD fragment of Kv2.1 (C128 and C129) are conserved in Kv2.3 (Fig. 7). Apart from the effects on activation and closing, the NRD of Kv2.3 also induces a marked slowing of inactivation time course. Because inactivation of Kv2.1 channels is much slower than activation, the rate of macroscopic inactivation is not voltage-dependent and is unaffected by moderate deceleration of activation kinetics. Thus, the slowing of inactivation induced by Kv2.3 on Kv2.1 currents is not a manifestation of the coupling between activation and inactivation, but it is most likely a result of primary modification of the inactivation mechanism. This observation is interesting because the mechanism of inactivation in Kv2.1 channels is unknown, and it is believed to be of the C- or P-type rather than of the N-type and, in principle, independent of the N-terminal domain (Choi et al., 1991; Hoshi et al., 1991; De Biasi et al., 1993b; López-Barneo et al., 1993). Participation of N and C termini of Kv2.1 in inactivation was already suggested by VanDongen et al. (1990), who showed that large deletions in the N terminus (first 139 amino acids) produced a slowing of inactivation, which was reverted by additional deletions in the C terminus. However, the NRD region, which determines the regulatory effect of Kv2.3 on Kv2.1 channels, spans from amino acid 148 to 196 and is located closer to S1 than the 139 amino acid fragment deleted by VanDongen et al. (1990).

In conclusion, our results show that Kv2.1 channel activation and closing, as well as inactivation, are regulated by a domain (NRD) in the N terminus between the B box and the S1 segment. Sequence divergence of the NRD fragment of Kv2.3 with respect to the same region in Kv2.1 and Kv2.2 may explain the modulatory role of the regulatory α-subunit on these channels. Elucidation of the mechanisms underlying the interaction of the NRD with the main core of the channel protein and characterization of the processes regulating coexpression of Kv2.3 and Kv2.1 channels in individual central neurons must be the subject of future experimental work.

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