KCR1, a Membrane Protein That Facilitates Functional Expression of Non-inactivating $K^+$ Currents Associated with Rat EAG Voltage-dependent $K^+$ Channels*

(Received for publication, June 1, 1998, and in revised form, June 26, 1998)

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Cerebellar granule neurons possess a non-inactivating K$^+$ current, which controls resting membrane potentials and modulates the firing rate by means of muscarinic agonists. kcr1 was cloned from the cerebellar cDNA library by suppression cloning. KCR1 is a novel protein with 12 putative transmembrane domains and enhances the functional expression of the cerebellar non-inactivating K$^+$ current in Xenopus oocytes. KCR1 also accelerates the activation of rat EAG K$^+$ channels expressed in Xenopus oocytes or in COS-7 cells. Far-Western blotting revealed that KCR1 and EAG proteins interacted with each other by means of their C-terminal regions. These results suggest that KCR1 is the regulatory component of non-inactivating K$^+$ channels.

K$^+$ channels are essential components of the plasma membranes of both excitable and non-excitable cells. Three major classes have been described for voltage-gated K$^+$ currents in mammalian neurons: A-type currents with rapid inactivation, delayed rectifier currents with slow inactivation, and non-inactivating outward currents (1, 2). The first two classes play an important role in shaping action potentials during repolarization and in determining the interval of an action potential. The non-inactivating outward currents can modulate firing frequency upon receptor stimulation by neurotransmitters or produce resting membrane potentials.

Typical K$^+$ currents that show no inactivation upon depolarization are M currents (3, 4), S currents (5), the standing outward current, $I_{KSO}$, recently reported in cerebellar granule cells (6), and resembling currents in myoblasts (7). These currents have a common feature in that they begin to be activated from deep membrane potentials and are inhibited by neurotransmitters such as muscarinic agonists (4, 6), bradykinin (3), and serotonin (5). Except for the many important items of physiological relevance identified for such currents, molecular bases for the responsible channels are poorly understood. The cloned K$^+$ channels that exhibit non-inactivating currents are the aKv5.1 (8) and ether $\alpha$-go-go (EAG)$^1$ K$^+$ channels (9, 10).

Recently, it has been reported that the expression of Drosophila EAG K$^+$ channels in oocytes results in a slow relaxation current resembling M currents (11). However, whether a rat homologue of EAG (r-EAG) contributes to the M channel has been the subject of much debate (12, 13).

To characterize low-threshold non-inactivating K$^+$ currents, we measured K$^+$ currents in Xenopus oocytes injected with the cerebellar poly(A)$^+$ RNA, in accordance with the report by Hoger et al. (14). We realized that low-threshold non-inactivating K$^+$ currents ($I_{Kni}$), which are similar to $I_{KSO}$, are expressed in oocytes injected with cerebellar poly(A)$^+$ RNA. To clarify the molecular information for $I_{Kni}$, we isolated a cDNA clone required for the functional channel protein for $I_{Kni}$ by suppression cloning, which in turn is based on the capacity of antisense single strand DNA to block the expression of $I_{Kni}$ in oocytes. This strategy enabled us to obtain a clone that encodes either an integral component or an important regulatory polypeptide (15). We could obtain one clone which specifically suppresses $I_{Kni}$ and the full-length cDNA termed kcr1. We describe here the functional characterization of the encoded protein designated KCR1 in oocytes and COS-7 cells and the biochemical interaction with r-EAG K$^+$ channels.

**EXPERIMENTAL PROCEDURES**

cDNA Cloning—A pool of fractionated rat cerebellar poly(A)$^+$ RNA (6–12 kb) was used to construct a randomly primed cDNA library in λZAPII (Stratagene). Excised ssDNA pools were used for the simplified version of suppression cloning with exogenously added ribonuclease H as described elsewhere (15). Briefly, heat-denatured ssDNA and poly(A)$^+$ RNA were mixed and incubated at 37 °C for 1 h in a solution containing 50 mM sodium acetate (pH 7.8), 2 mM MgCl$_2$, 3 mM dithiothreitol, and 1.1 units of ribonuclease H (Takara Shuzo), 2.6 units of ribonuclease inhibitor. The treated samples were directly injected into Xenopus oocytes. When Kv1.2 or Kv3.1 cDNA (2.6 µg/µl) was treated with antisense Kv1.2 ssDNA (1.6 µg/µl), the expression of Kv1.2 current was suppressed to 20.4 ± 26% ($n = 13$) and expression of Kv3.1 current to 99 ± 17% ($n = 5$). In each suppression experiment, expression of $I_{Kni}$ was evaluated by using $I_{KAN}$ as an internal control.

Clones containing the entire coding sequence of kcr1 were obtained by screening a second rat brain library. r-eag cDNA clones were obtained by screening the same library using reverse transcriptase-polymerase chain reaction sequencing primers. The sequences of the oligonucleotides for PCR were 5′-CGGGATCCCTTCGCGGTC-TGGCAGC (forward) and 5′-CGCAGCTTTGAAATGACCTCA-GCCTG (reverse). The nucleotide sequence of one clone (pBS- SKS/eag3a) carrying nucleotides 47–3164 of the r-eag gene was identical to the published sequence except for nucleotide 1334, where T was replaced by C without changing the amino acid sequence (numerical designations correspond to the published sequence (16)).

Expression Plasmids—The coding sequence of kcr1 was amplified by PCR with following primers containing additional restriction sites of ether $\alpha$-go-go; ssDNA, single-stranded DNA; GST, glutathione S-transferase; MBP, maltose-binding protein; kb, kilobase(s).
Electrophysiological Measurements—Xenopus laevis oocytes were isolated and different RNAs were injected as described previously (20). In the rescue experiment, ssDNA containing the antisense strand of the open reading frame was used to deplete corresponding mRNAs species from cerebellar poly(A)+ RNA by using the suppression cloning protocol. cRNA (0.5–5 ng/oocyte) was injected. Oocytes were then pelleted and treated with 2 μg/ml of Boehringer Mannheim) or 5 mM NaOH as described elsewhere (19, 19).

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14%. Calibration against the retardation coefficient determined from Ferguson plots (27), in other words, a comparison of the slopes of KCR1 and molecular weight standards in the plot, yielded an apparent molecular weight of 55,500 ± 6,100 which was close to the predicted molecular weight from the deduced amino acid sequence (M, 55,571). When pancreatic microsomes were included, translated proteins predominated in the membrane pellet, indicating that the KCR1 is a membrane protein. In the presence of microsomes, the mobility of the product was also shifted to 42 kDa. This shift of 2 kDa was resistant to endoglycosylase H treatment (19). The β-elimination in a mild alkaline condition (18) could partially remove this shift to 40 kDa (Fig. 2B).

Suppression of I_{K(ni)} by kcr1 Antisense and Restoration by kcr1 cRNA in Oocytes—The function of KCR1 in ion channel activity was examined. Xenopus oocytes injected with kcr1 cDNA exhibited small outward currents of 41.3 ± 30.2 nA (n = 40) at +40 mV, which were not significantly different from the endogenous currents in control oocytes (32.4 ± 18.5 nA, n = 49; Fig. 3A, left panel). The inability of KCR1 to permeate K⁺ is not surprising, because the amino acid sequence lacks the conserved pore sequence.

We next tested whether KCR1 was necessary for the formation of the functional channels producing I_{K(ni)} in oocytes injected with cerebellar poly(A)⁺ RNA. Depletion of corresponding mRNA from cerebellar poly(A)⁺ RNA with the antisense ssDNA of kcr1 cDNA inhibited expression of I_{K(ni)} (49 ± 22%,
n = 13; p < 0.001). This inhibition was restored to 88 ± 41% (n = 6) by re-injecting kcr1 cRNA (Fig. 3A, top). The suppression and rescue effects of KCR1 were restricted to I_{K(ni)} since identical effects were not observed for I_{K(A)} (Fig. 3A, bottom) or voltage-dependent sodium currents in 4–9 oocytes (data not shown). The wave shape of the rescued I_{K(ni)} was indistinguishable from that of the original I_{K(ni)} (Fig. 3A), as was K⁺ selectivity (Fig. 3B) and the activation curve (Fig. 3C). Taken altogether, these results show KCR1 is a regulatory protein of the non-inactivating K⁺ channel.

Functional Interaction of KCR1 with Cloned a Subunits of K⁺ Channels in Oocytes—Since we assumed that KCR1 could be a part of channel complexes producing I_{K(ni)}, we examined several α subunits of K⁺ channels, which have been reported to be expressed in cerebellum (16, 28–30) as a potential target of functional interaction with KCR1. Co-expression of KCR1 with the rKv1.2, rKv2.1, or mKv3.1a channel (28, 31, 32) in Xenopus oocytes did not have any appreciable effect on the functional expression and activation kinetics of these channels (each co-expression in 7–9 cells; data not shown). Tutsense, or cerebellar α subunits could rescue I_{K(ni)} when co-expressed with KCR1. Thus, the expression of KCR1 caused a 10 mV hyperpolarizing shift (p < 0.01) in the voltage dependence of the occupancy by the faster component, whereas the steady state activation remained unchanged (Fig. 4C). The half-activation voltages (V_{1/2}) were +2.5 ± 1.7 mV (n = 10) for control oocytes and +2.4 ± 1.2 mV (n = 11) with KCR1. This implies that the KCR1 protein accelerates the rate of transition in r-EAG channels toward the open state.

Fast Activation Kinetics of r-EAG Accelerated by KCR1 in COS-7 Cells—The above data were confirmed in a mammalian
cell expression system with much smaller membrane capacitance. In COS-7 cells, both r-EAG and r-EAG + KCR1 currents showed two activating components with fast (12.9 ± 1.8 ms (n = 12) and 10.3 ± 1.8 ms (n = 13)) and slow (146 ± 15 ms and 125 ± 13 ms) time constants, when the currents were evoked by stepped depolarization from a holding potential of −80 mV to +40 mV for 500 ms. Again, a marked difference was that the co-expressed currents showed rapid activation resulting from the reduction of the slow component.

Since the slow component of the r-EAG current has been reported to be affected by holding potentials and extracellular Mg²⁺ (21), we measured the voltage and Mg²⁺ dependence of the slow component in COS-7 cells. The relative amplitudes of the slow component seemed to decrease as the prepulses were being depolarized (Fig. 5, A–C), with the shift from the midpoint voltage of −65.0 ± 1.2 mV (n = 11) for r-EAG currents to that of −75.5 ± 1.4 mV (n = 6) for r-EAG + KCR1 currents (p < 0.001). Thus, the expression of KCR1 caused a 10 mV hyperpolarizing shift in the voltage dependence of the slow kinetics in the presence of Mg²⁺, with the slope factor unchanged: 8.2 ± 0.7 mV for r-EAG currents and 9.1 ± 0.3 mV for r-EAG + KCR1 currents. This hyperpolarizing shift was eliminated in the absence of Mg²⁺. The midpoint voltage in this case was −137.4 ± 2.6 mV (n = 8) for r-EAG currents and −139.5 ± 4.2 mV (n = 5) for r-EAG + KCR1 currents. The modifying effect of KCR1 on the activation kinetics of the r-EAG channels was further evidenced by an estimate of the number of Mg²⁺-dependent slow gating particles (Fig. 5D) (21). The power needed for fitting r-EAG currents at a prepulse of −160 mV was 3.5 ± 0.2 (n = 11) for r-EAG currents in the presence of 2 mM Mg²⁺, indicating nearly four gating particles were in the Mg²⁺-dependent slow activating mode. However, the corresponding value was 2.5 ± 0.1 (n = 5) for r-EAG + KCR1 currents, which suggests that at least one particle of the r-EAG channels was released to the Mg²⁺-independent fast activating mode by the expression of KCR1.

Molecular Association between KCR1 and r-EAG—The molecular association between KCR1 and r-EAG was examined by in vitro binding of recombinant fusion proteins. The GST fusion protein containing the S6-S7 loop and the C-terminal tail of KCR1 carrying 17 amino acids were tested for binding to a MBP fusion protein containing the C-terminal tail of the r-EAG protein and to the MBP alone. Binding of a GST-KCR1 tail fusion protein with a MBP-r-EAG fusion protein could be detected (Fig. 6A, lane 1), but not with a GST fusion protein containing the S6-S7 loop (data not shown). Progressive deletion analysis of the C-terminal tail of r-EAG channels revealed that the 50 amino acids (788–837th; see Fig. 1 by Ludwig et al. (16)) are sufficient for interaction (Fig. 6, A–C). However, the fusion proteins lacking amino acid residues
837–962 showed weaker intensity than those containing that region.

**DISCUSSION**

\( I_{\text{koni}} \) One type of low-threshold non-inactivating K⁺ current derived from cerebellum, which was nearly identical to \( I_{\text{koni}} \) was characterized in this study. We isolated the \( \text{kr1} \) cDNA from our rat cerebellar cDNA library by suppression cloning and showed that KCR1 facilitates the functional expression of \( I_{\text{koni}} \). The amino acid sequence of KCR1 did not contain the K⁺ channel pore sequence (23) or the voltage sensor region seen in voltage-gated K⁺ channels. Structural characteristics of the 12 transmembrane regions in KCR1 appeared to be somewhat similar to those of the transporter superfamily. Since no homology was found between KCR1 and known transporters, it appears that KCR1 must be a new mammalian protein that does not belong to the family of the α subunit of K⁺ channels or transporters.

The translation products in vitro showed the 2-kDa shift, when microsomes were present. Since this shift was partially removed by β-elimination (Fig. 2), some ester-linked modification such as phosphorylation, acylation, and O-glycosylation might contribute to the shift (33). However, as O-glycosylation occurs only in the Golgi apparatus, the most likely modification in the presence of microsomes is acylation on serine or threonine residues or phosphorylation on a site exposed when KCR1 translocates into the microsomal membrane. On the contrary, this shift was resistant to endoglycosidase H treatment, showing that the two potential N-glycosylation sites are not glycosylated and suggesting that these sites are located in the cytoplasmic side. On the basis of these interpretations, we propose the membrane topology of KCR1 as shown in Fig. 7.

Since co-expression of cerebellar mRNA with \( \text{kr1} \) increased the outward current in our experiments, KCR1 may function as an up-regulator of the expression of \( I_{\text{koni}} \). Similar regulators in ion channel expression have already been reported: the β subunit (Kvβ2) for Shaker-type K⁺ channels (34), the minK (IsK) for \( K_{\text{LQT1}} \) and HERG voltage-gated K⁺ channels (35–37), and the cysteine string protein for voltage-dependent Ca²⁺ channels (15, 38).

By using r-EAG as a candidate target, we could demonstrate a protein-protein interaction of KCR1. As shown in Figs. 6 and 7, the C-terminal tail of KCR1 peptide of 17 (458–474th) amino acid residues interacted with the C-terminal region of the 788–837th amino acid residues of r-EAG. We do not know whether other regions contribute to the interactions between r-EAG and KCR1. However, the presence of r-EAG fusion proteins containing amino acid residues 837–962 results in stronger binding than that of two fusion proteins lacking that region, indicating that other regions of r-EAG may also contribute to the interaction with KCR1. The interaction of KCR1 and r-EAG at the C-terminal region might be specific, because the 788–837th amino acids of r-EAG are not conserved among other K⁺ channels, and Kv1.2, Kv2.1, or Kv3.1 did in fact not display any functional interaction with KCR1. Furthermore, the shift of the activation kinetics of r-EAG currents caused by the coexpression of KCR1 constitutes functional evidence that molecular interaction of KCR1 with r-EAG channels is taking place. We therefore conclude that the association of KCR1 with r-EAG channels leads to faster activation by reducing the Mgs²⁺-dependent slow component.

The finding that both r-EAG and \( \text{kr1} \) antisense ssDNAs could suppress \( I_{\text{koni}} \) suggests that r-EAG and KCR1 are essential components in the channel complexes producing \( I_{\text{koni}} \). On the basis of these observations, we propose the hypothesis that KCR1 combines with r-EAG in forming the K⁺ channels to produce \( I_{\text{koni}} \) in cerebellar granule neurons. In addition, it would be of interest to examine whether KCR1 can bind to other molecules of EAG in different tissues, because the \( \text{kr1} \) message is also expressed widely in peripheral tissues, where r-EAG mRNA is not expressed.

In conclusion, we have isolated the \( \text{kr1} \) gene, which encodes a novel membrane protein, from the cerebellar cDNA library. This KCR1 membrane protein modulates \( I_{\text{koni}} \), expression and specifically binds to r-EAG at the C-terminal region. Therefore, KCR1 is thought to be a new subunit of voltage-dependent K⁺ channels in rat brain. Further validation is necessary to show that KCR1 and EAG are colocalized in the same single neuron.

**Acknowledgments**—We thank R. H. Joho for the gift of rKv2.1 cDNA clone and E. R. Liman for the gift of pgEMEH.

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