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Dominant negative chimeras provide evidence for homo and heteromultimeric assembly of inward rectifier K^+ channel proteins via their N-terminal end

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Abstract Chimeras have been constructed using three different fragments (N-terminal, central and C-terminal) of IRK3, a constitutive inward rectifier K^+ channel subunit, and GIRK2, a G-protein activated inward rectifier K^+ channel subunit and have been coinjected into *Xenopus* oocytes together with IRK3 or IRK1 (another constitutive inward rectifier) cRNA. Both IRK1 and IRK3 expression was inhibited by coinjection with chimeras containing a N-terminal fragment of IRK3 suggesting that subunits of K^+ channels in the IRK family form a functional multimeric assembly where the N-terminal end has an important role. In situ hybridization shows that IRK1 and IRK3 are coexpressed in the same areas of the brain and probably in the same cells. Taken together both the localization and the oocyte expression results suggest that not only homomultimeric IRK1 or homomultimeric IRK3 assemblies take place but that heteromultimeric IRK1/IRK3 assemblies are also formed.

Key words: Inward rectifier K^+ channel; Heteromultimer; Colocalization; *Xenopus* oocyte

1. Introduction

The first cloned K^+ channel corresponds to the *Shaker* Drosophila gene and encodes a protein with six transmembrane segments. Numerous genes encoding for the same type of channel proteins have now been identified in the mammalian system. When expressed in *Xenopus* oocytes, this family of K^+ channel clones gives rise to functional voltage dependent (Kv) outward rectifier K^+ channels (see for review [1,2]). The second family of cloned K^+ channels corresponds to proteins with only two transmembrane domains and represents the family of inward rectifier (Kir) K^+ channels which appears to be widely expressed in excitable as well as in non-excitable cells (see for review [3]). These channels are characterised by the absence of outward currents (inward rectification) and by an activation threshold potential close to the K^+ equilibrium potential. There are five known subfamilies of inward rectifier K^+ channels, the Kir1.0 (ROMK1, ROMK2,...), Kir2.0 (IRK1, IRK2, IRK3,...) Kir3.0 (GIRK1, GIRK2, ...) and the Kir4.0 and Kir5.0 subfamilies (see for review [3]). The Kir3.0 subfamily requires activation by G proteins.

It has been previously demonstrated that outward rectifier K^+ channels are multimeric membrane proteins [4–6]. G-protein activated inward rectifiers also necessitate multimeric assembly for function and unlike outward rectifiers they require assembly of different types of subunits [7–10].

The purpose of this paper is to show that inward rectifiers that do not necessitate G-protein activation and that constitute an important family of brain inward rectifiers also require multimeric assembly for function. The strategy used to demonstrate that point has been to create dominant negative chimeras of Kir channels that inhibit both IRK3 and IRK1 expression.

2. Materials and methods

2.1. RNA isolation and chimeric constructions

Cloning of IRK3 and GIRK2 channels cDNA and synthesis of cRNA have been previously described [11].

To construct the chimeras, the IRK3 sequence was mutated at position 147 and 1056 (the A of the initiating codon taken as base (1) to introduce *Mun*I and *Nhe*I restriction sites, respectively. Mutagenesis promoted the following changes in the amino acids sequence: T491, C50G and Q353A. The GIRK2 sequence was mutated at position 255 to introduce a *Mun*I restriction site. Leucine in position 86 was changed into isoleucine. The *Nhe*I site is naturally found in the GIRK2 sequence. Site directed mutagenesis was performed using oligonucleotides primers according to the manufacturer's protocol (Promega). The introduction of these restriction sites divided the two channels into three parts named $G_2G_2G_2$ for GIRK2 and $K_3K_3K_3$ for IRK3. The chimeric constructs were carried out by exchanges of the different parts of each channel. Before cRNA synthesis, each chimera was sequenced on both strands using the dye terminator method on an automatic sequencer (Applied Biosystem model 373A).

2.2. mRNA expression in Xenopus oocyte

Preparation of oocytes, mRNA injection and electrophysiological measurements were previously described [12]. The quantity of cRNA injected per oocyte were 1.0 ng, 0.5 ng and 5.0 ng for IRK1, IRK3 and the chimeric proteins, respectively. A K^+-rich solution containing 98 mM KCl, 1.8 mM $CaCl_2$, 2 mM $MgCl_2$ and 5 mM HEPES at pH 7.4 with KOH was used.

2.3. In situ hybridization

All experiments were performed by using standard procedures [13]. Brain sections were hybridized with RNA probes complementary to IRK1 and IRK3 genes. RNA probes were generated by in vitro transcription using [^{33}P]α-UTP (3000 Ci/mmol, ICN Radiochemicals). Antisense probes were generated with T3- and T7-RNA polymerases (Boehringer) from linearized fragments containing respectively a 61 bp *Bgl*II fragment of IRK1 cDNA in the 3'-coding sequence and a 115 bp *Bgl*I fragment of IRK3 cDNA in the 5'-untranslated sequence. Sections (12 μm) were hybridized overnight at 65°C, treated and probed as previously described [13] and exposed to Amersham β-max Hyperfilm for 5 days at 4°C. Selected slides were dipped in Amersham LM1 photographic emulsion and exposed for 2 weeks at 4°C and then developped in Kodak D-19 for 4 min. All slides were counterstained with hematoxylin/eosin. For control experiments, adjacent sections were hybridized with sense riboprobes or digested with RNAse before hybridization.

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2.4. Statistical analysis

The variability of the results was expressed as the standard error of the mean (S.E.M.) with N indicating the number of tested batches and n indicating the number of oocytes contributing to the mean.

3. Results

3.1. Comparative localizations of IRK1 and IRK3 transcripts

IRK1 transcripts are expressed in many tissues and are at a high level in brain, heart and skeletal muscle [14]. IRK3 transcripts are essentially present in the brain [11].

Fig. 1a shows characteristic autoradiographs illustrating the overall distribution profile of mRNAs encoding IRK1 and IRK3 genes in sagittal sections of rat brain. In situ hybridization revealed that these transcripts display overlapping expression patterns. IRK1 and IRK3 mRNAs are widely expressed throughout the brain and found in the majority of the neuronal perikarya but were not detected in glial cells. Higher expression levels appeared in the neo- and allocortical regions, hippocampus and cerebellum. The level of IRK1 and IRK3 mRNA expression was moderate in other regions of the brain. The labeling of the cortical areas was intense and uniformly distributed in the cortical layers II to VI. In the hippocampus, the major hybridization signal of both genes were concentrated over the cell bodies of the hilar cells, the granule cells of the dentate gyrus and the pyramidal cells in CA1 through CA3, with a level of expression higher in dentate granule cells. Very strong labeling was observed in all lobules of the cerebellum. IRK1 and IRK3 mRNAs were detected in granule cells and Purkinje cells. Basket and/or stellate cells in the molecular layer showed high levels of hybridization. No labeling was seen in the deep cerebellar nuclei. The potential significance of heteromultimeric assembly of IRK1 and IRK3 in the brain was confirmed by microscopic analysis of emulsion-dipped sections. Coexpression of both transcripts in the same neuron type was observed in all strongly labeled brain structures. Fig. 1b gives an example of the high degree of colocalization of IRK1 and IRK3 in the dentate granule cells and the hilar cells, reflecting the highly overlapping distribution of IRK1 and IRK3 transcripts within the rat hippocampus. These results suggest the possibility that IRK1 and IRK3 may form functional heteromultimeric inward K^+ channels in neurons in many brain structures as previously demonstrated for the G-protein activated inward rectifiers GIRK1 and GIRK2 [10].

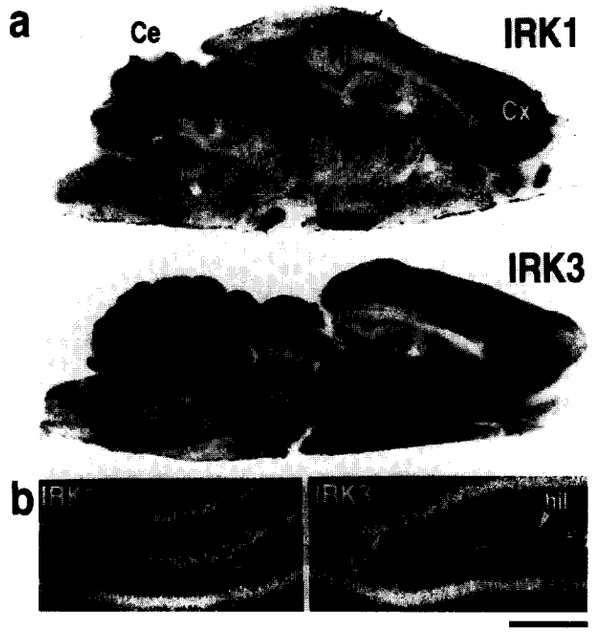

Fig. 1. (a) X-ray film autoradiograpghs illustrating expression patterns of IRK1 and IRK3 channel mRNAs in sagittal rat brain sections following in situ hybridization with specific IRK1 and IRK3 RNA probes. Abbreviations: Cpu, caudate putamen; Cx, cortex cerebral; Ce, cerebellum, Th, thalamus; Hi, hippocampus. (b) Dark field photomicrographs of emulsion autoradiograms illustrating coexpression of IRK1 and IRK3 transcripts in dentate granule cells (gr) and hillar cells (hil) of the hippocampus. Scale bar: 400 μm.

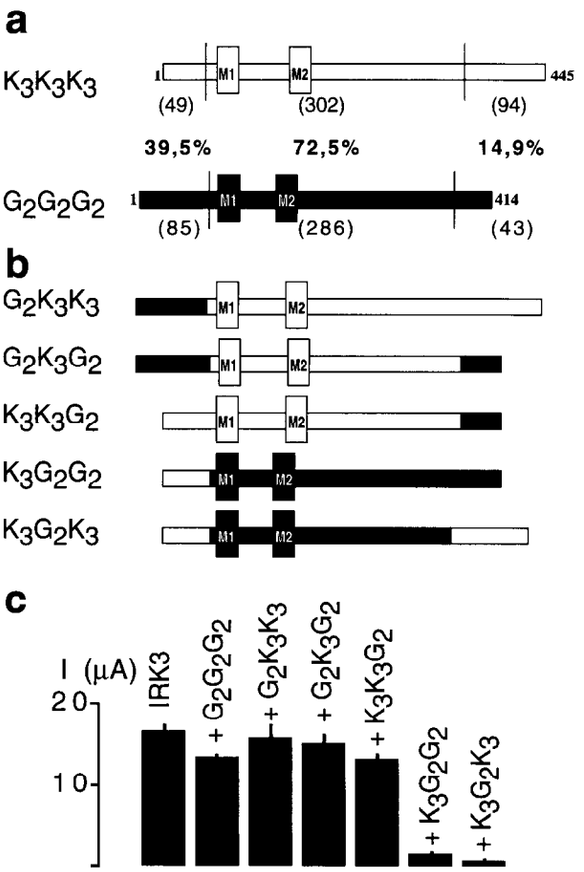

Fig. 2. (a) Schematic representation of the mutated channels GIRK2 ($G_2G_2G_2$) and IRK3 ($K_3K_3K_3$). The rectangles represent the two transmembrane domains M1 and M2 flanking the pore forming region (P). The vertical bars indicate the place where mutagenesis was done to create restriction sites. The numbers in brackets indicates each fragment size in amino acids. Proteic sequences comparisons were carried out between GIRK2 and IRK3 for the N-terminal, the central and the C-terminal fragments. Percentages of similarities are indicated in bold. (b) The name of each chimeric construction reflects the origin of the N-terminal, the central and the C-terminal fragment. Filled portions symbolize GIRK2 parts whereas emptied portions indicate an IRK3 origin. (c) Bar graphs showing the effects of the coinjection of various chimeric proteins on IRK3 peak currents recorded at −130 mV. Vertical bars indicate the S.E.M. with $n = 10$ and $N = 2$. The IRK3 current inhibition by $K_3G_2K_3$ is dose-dependent and 50% of inhibition was obtained with a $[K_3G_2K_3]/[IRK3]$ cRNA ratio of 2.5 (not shown).

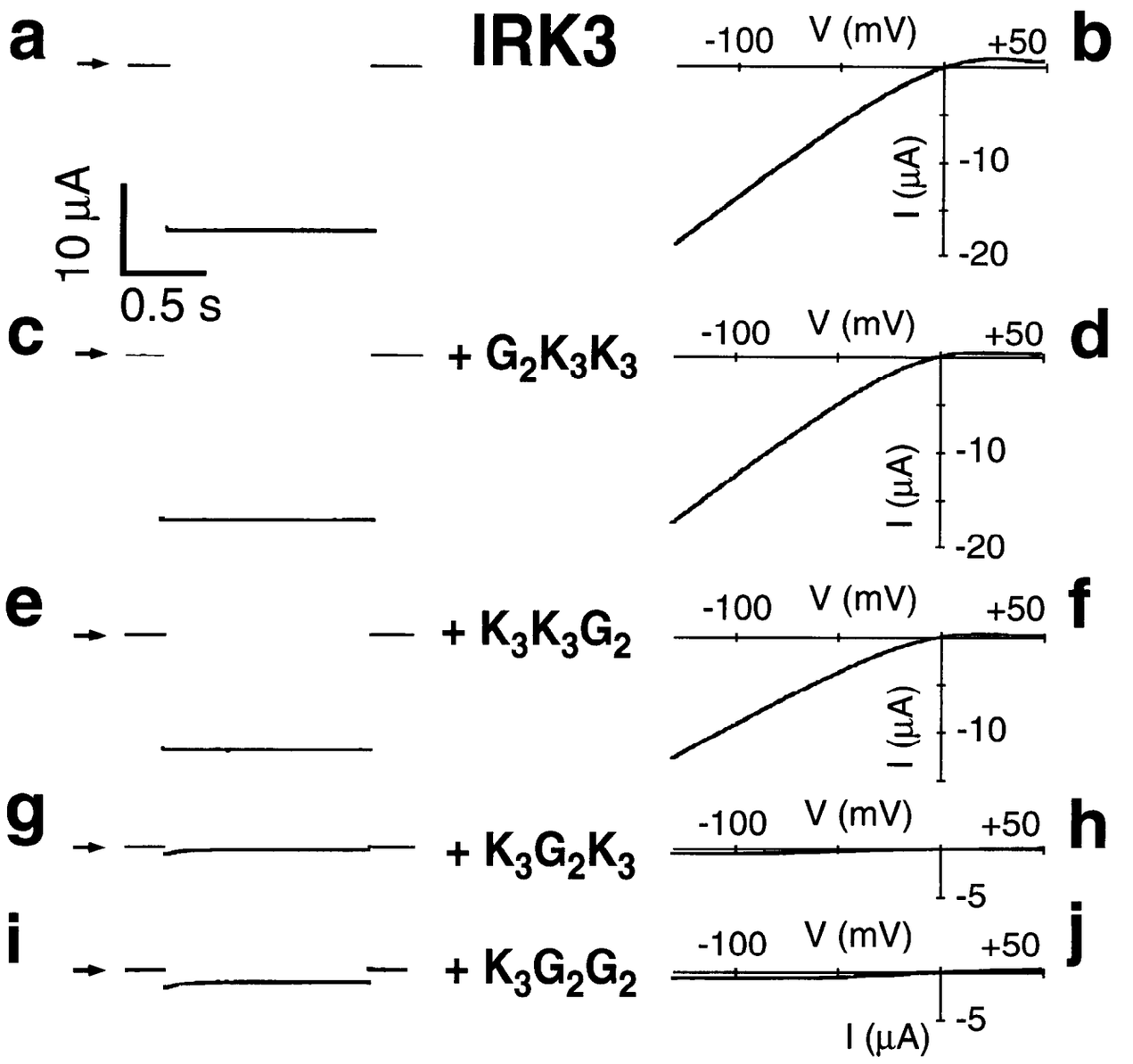

Fig. 3. (a,c,e,g,i) Examples of the currents recorded at −130 mV during 1.4 s pulses with IRK3 alone or coinjected with chimeric proteins cRNA. Scaling for all pulses is indicated in a. (b,d,f,h,j) Examples of the *I–V* relationships recorded with voltage ramps, 0.9 s in duration, on the same combinations as in a,c,e,g,i. Voltage ramps ranged from +50 mV to −130 mV. In all cases the holding potential was 0 mV (K^+ equilibrium potentia

3.2. Construction and functional effects of chimeras

Proteic sequences comparisons between GIRK2 and IRK3 proteins have shown a 55% total amino acids similarity. Taking advantage of the fact that the most important differences are located in the N and C terminal fragments with 39.5% and 14.9% amino acids similarity, respectively, which flank a more conserved 'core' fragment with 72.5% of similarities, our strategy was to construct chimeras between IRK3 and N-terminal, C-terminal and core fragments belonging to GIRK2 and to co-express them with either IRK3 or IRK1 in *Xenopus* oocytes.

Fig. 2a shows the N-terminal, C-terminal and core fragments of IRK3 and GIRK2 channel proteins used for the construction of chimeras presented in Fig. 2b. All constructed chimeras were functionally inactive, none of them had K^+ channel activity (2 batches of oocytes, not shown).

Since the N-terminal end of outward rectifier K^+ channels of the *Shaker* family have previously been implicated in their multimeric formation [15,16], we first studied the effects of coinjection of two chimeras sharing an IRK3 N-terminal extremity and a GIRK2 central core on IRK3 currents. IRK3 currents were strongly inhibited by both $K_3G_2G_2$ and $K_3G_2K_3$ (Fig. 2c) suggesting that these chimeras are able to interact with IRK3. As a control, the effects of $K_3G_2K_3$ on other types of K^+ channels such as the outward rectifier Kv1.5 channel [17] were also studied. $K_3G_2K_3$ was without effect on the outward rectifier K^+ channel activity ($n = 10$, $N = 2$, not shown). Other chimeras

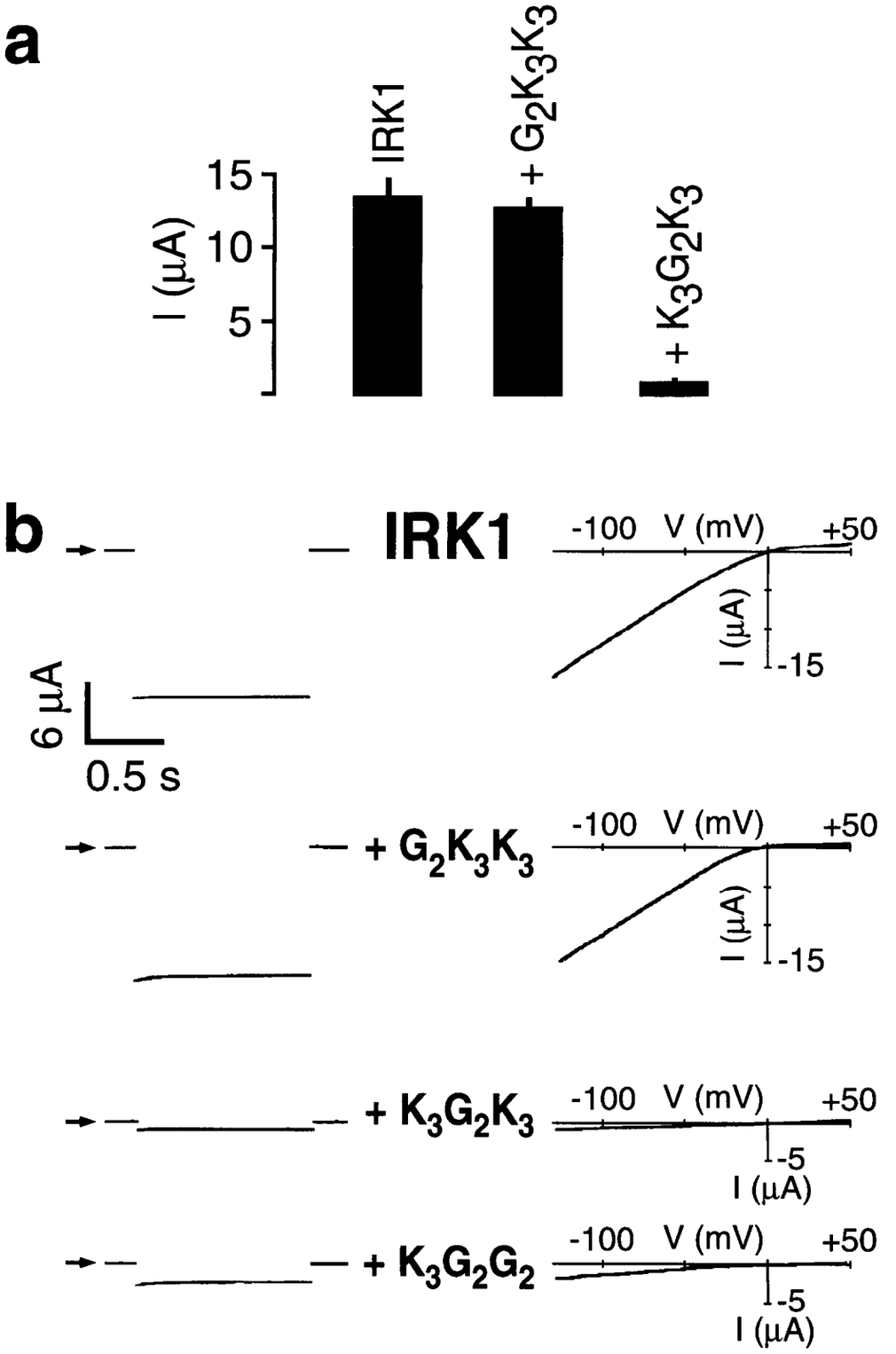

Fig. 4. (a) Bar graphs showing the effects of the coinjection of various chimeric proteins on IRK1 peak currents recorded at −130 mV. Vertical bars indicate the S.E.M. with $n = 10$ and $N = 2$. (b) left panel: examples of the currents recorded at −130 mV during 1.4 s pulses with IRK1 alone or coinjected with chimeric proteins cRNAs. (b) right panel: examples of the *I–V* relationships recorded with voltage ramps, 0.9 s in duration. Voltage ramps were ranging from +50 mV to −130 mV. In all cases the holding potential was 0 mV (K^+ equilibrium potential).

comprising the N-terminal sequence of GIRK2 were also constructed and tested. Co-injections of $G_2K_3K_3$, $G_2K_3G_2$, $K_3K_3G_2$ and GIRK2 (called here $G_2G_2G_2$) cRNAs with IRK3 were essentially without significant effect on IRK3 currents (Fig. 2c). These chimeras were also without effect on the time-course and the inward rectification of IRK3 currents (Fig. 3a–f). Fig. 3g–j shows a very drastic inhibition of IRK3 currents by $K_3G_2K_3$ and $K_3G_2G_2$.

The effects of the coinjection of the most characteristic chimeras on IRK1 currents are shown in Fig. 4. As previously seen for IRK3, co-expression of IRK1 with $G_2K_3K_3$ did not affect IRK1 currents while $K_3G_2K_3$ completely inhibited IRK1 currents (Fig. 4a). Again the time-course and the inward rectification of IRK1 currents remained unchanged when coexpressed with $G_2K_3K_3$, while drastic inhibitions were recorded with $K_3G_2K_3$ or $K_3G_2G_2$ (Fig. 4b).

4. Discussion

It is well known that it takes four P (pore) domains to make a functional voltage dependent Na^+ channel, a voltage dependent Ca^{2+} channel or an outward rectifier K^+ channel [18], (see [19,20] for reviews). It was then expected that inward rectifier subunits which only comprise one P domain also need to form a quaternary structure to be functional. The present work shows that chimeric proteins containing protein parts of both GIRK2 and IRK3 channels can form heteromultimers with the IRK3 protein. Indeed, the most simple interpretation for the inhibition of IRK3 currents by some of the chimeras is that these chimeras associate with the IRK3 subunit to produce heteromultimers which are not functional.

$K_3G_2K_3$ and $K_3G_2G_2$ are the only chimeras that significantly inhibit IRK3 currents. Both chimeras contain the N-terminal end of IRK3 which then appears as a crucial structural element for subunits assembly. The presence of the GIRK2 sequence in the central part of the protein, which contains an important part of the pore P domain, leads to the formation of channels that are apparently blocked in a closed configuration. Interestingly, the N-terminal end of outward rectifiers of the *Shaker* family has also been shown to be essential for the tetrameric assembly of this class of channels [16,19].

Chimeras with the N-terminal ends of GIRK2 such as $G_2K_3G_2$ and $G_2K_3K_3$, did not interfere with IRK3 expression. These chimeras probably do not coassemble with IRK3 subunits despite the presence of a proper pore. The chimera $K_3K_3G_2$ did not inhibit IRK3 expression although it contains both the N-terminus end the P domain of IRK3. The first interpretation of this result is that $K_3K_3G_2$ does not fold properly, another evident possibility is that $K_3K_3G_2$ unlike other chimeras forms a functional multimer. It has recently been shown that heteromultimeric assembly is essential for the functionning of G-protein activated K^+ channels and that homopolymeric assembly of GIRK1, GIRK2 or GIRK4 does not really give rise to K^+ channel activity [7–10]. The situation is different for channels in the IRK family since homomultimers of IRK1 or of IRK3 are functional. However, since these two constitutively active inward rectifiers (IRK1 and IRK3) are colocalized in the same area of the brain (as are GIRK1 and GIRK2 [10]) they can obviously also form a heteromultimeric assembly as indicated by the electrophysiological results.

The particular function of these IRK1/IRK3 heteromultimers versus IRK1 or IRK3 homomultimers, which can easily form in the same brain regions, is not known. One is faced here with the same problem encountered with many of the functional outward rectifier subunits (for example $Kv_{1.1}$, $Kv_{1.2}$, ..., $Kv_{1.6}$) which can also form both functional homomultimers and functional heteromultimers (see for review [21]). This built-in capacity of diversity might be important in terms of subcellular localizations, regulations or/and developmental properties.

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