A Functional Spliced-Variant of β2 Subunit of Kv1 Channels in C6 Glioma Cells and Reactive Astrocytes from Rat Lesioned Cerebellum†

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ABSTRACT: Voltage-gated K^+ channels (Kv1) are important in glia, being required for cell proliferation. Herein, reactive astrocytes from a rat cerebellar lesion were shown to contain Kv1.1, -1.2, -1.3, -1.4, and -1.6 α plus β1.1 subunits, as well as an unusual β2.1 constituent; the latter was also found in a glioblastoma C6 cell line, together with Kv1.1, -1.3, and -1.6 and β1.1 subunits. Reverse transcriptase−polymerase chain reaction revealed a novel product of the β2 gene in C6 cells and reactive astrocytes, but not in cultured astrocytes or rat normal brain. Its cloning identified a variant, Kvβ2.1A, alternatively spliced between I24 and Y39. Despite this 14 residue deletion, Kvβ2.1A assembled cotranslationally with Kv1.1 or -1.2 and, when coexpressed with Kv1.4 in oocytes, increased the inactivation rate of this K^+ current. Whereas the full-length β2.1 gave a large increase in the amplitude of the Kv1.1 current in oocytes, the effect of β2.1A varied from a modest elevation of the current to a slight suppression in some cases. In summary, this is the first report of the existence of an alternatively spliced product of the Kvβ2.1 gene in C6 cells and reactive astrocytes, and supports the involvement of its core region (residues 39 onward) in assembly with α subunits while excluding a contribution of the adjacent 14 residues to accelerating the inactivation of Kv1.4.

Electrophysiological recordings of K^+ currents in astrocytes have detected inward rectifiers, A-type, and delayed rectifier channels (*1*−*3*). These cells also possess Na^+ and Ca^{2+} channels; yet, they are nonexcitable due to the ratio of Na^+/K^+ conductances being the inverse of that in neurons (*4*). The inwardly rectifying K^+ channels in glial cells are implicated in the regulation of extracellular K^+ levels (*5*−*7*), while voltage-sensitive (Kv1)[1] channels are known to be important in cell proliferation (*8, 9*). For example, blockade of K^+ currents with 4-aminopyridine or tetraethylammonium (TEA) inhibits proliferation of glia (*10, 11*) and melanoma cells (*12*). Whereas neurons become mitotically inactive upon maturation, astrocytes remain quiescent but they respond to injury by reentering the proliferating cycle to form glial scars. A shift in the pattern of ionic currents expressed accompanies this transition from glia to a 'glioma-like' state; mature nonreactive astrocytes in hippocampal slices and in culture (*6, 13*) predominantly express inwardly rectifying channels, whereas mitotically active astrocytes (i.e., during development, following mechanical injury or in astrocytomas) largely exhibit outward K^+ currents (*11, 14*). Despite the physiological importance of Kv1 channels expressed in proliferating astrocytes, their molecular identities have yet to be established.

Progress in addressing this important question should be aided by the data available on the *Shaker*-related Kv1 subfamily of K^+ channels in neurons. Kv1 channels from mammalian brain (reviewed by ref *15*) are known to be large sialoglycoproteins ($M_r \sim 400$K) consisting of transmembrane, channel-forming α subunits ($M_r \sim 78$K) and auxiliary β proteins ($M_r \sim 39$K), usually associated in $(\alpha)_4(\beta)_4$ stoichiometry (*16*). Seven major α subunit genes (Kv1.1−1.7) have been cloned and when heterologously expressed generate K^+ currents with distinct properties (*15*). A common N-terminal region termed NAB (or T1) allows coassembly only between members of this subfamily, yielding homo- or heterotetrameric combinations of K^+ channel α subunits (*17, 18*). Multiple genes encoding the smaller accessory β subunits of K^+ channels include three subfamilies: Kvβ1, which has three alternatively spliced isoforms (1.1, 1.2, and 1.3) (*19*−*23*); Kvβ2.1 (*20, 24*); and Kvβ3.1 (*25*). These β subunits associate specifically, through a highly conserved C-terminal region, with the NAB domain of Kv1 α subunits (*18, 26, 27*). Kvβ1 members and β3 accelerate the inactivation of K^+ currents produced by certain Kv1 α subunits with variable potency; this important function is mediated by an N-terminal region (*20, 25, 28, 29*). However, Kvβ2.1, which lacks an N-type inactivation domain, also increases the rate of inactivation of Kv1.4 by an unknown mechanism (*30*−*32*). Additionally, β2.1 is known to speed up the activation of both Kv1.4 (*32*) and -1.5 (*31*), to associate with Kv1 α subunits early in their synthesis and promote folding which, in turn, increases their cell surface expression (*33*−*35*). Ion

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[1] Abbreviations: Kv, voltage-sensitive K^+ channels; TEA, tetraethylammonium; RT-PCR, reverse transcriptase−polymerase chain reaction; PMSF, phenylmethylsulfonyl fluoride; pA, polyadenylation; M_r, relative molecular weight; IgG, immunoglobulin class G; bp, base pair(s); SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; $V_{1/2}$, half-maximal activation voltage.

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channel gating and molecular chaperoning may not be the only roles of Kvβ subunits, as suggested by Gulbis et al. (*36*) based on the recently solved crystal structure of the core region of Kvβ2.1. This revealed that the β2.1 subunit exists as a tetramer, consistent with the $(\alpha)_4(\beta)_4$ oligomer found in neurones (*16*). Moreover, it possesses an oxido-reductase active site with bound NADP(H) coenzyme; the authors propose that the enzyme may interact directly or indirectly with the voltage sensor of α subunit, providing a link between the redox state of the cell and its membrane potential, an intriguing postulation that has to be demonstrated experimentally. Thus, the functional properties of β subunits warrant further investigation.

The aim of this study was to identify and characterize the α and β subunits of Kv1 channels in proliferating glia. For this purpose, a well-established C6 glioma cell line (*37*), derived from a rat brain tumor, provided a defined source of reactive astrocytes; this was used in conjunction with an in vivo model of gliosis in brain in which kainic acid served as a lesioning agent (*38*). Administration of this excitatory neurotoxin into adult rat cerebellum results in neuronal degeneration leading to induction of gliosis, where reactive astrocytes are the major contributor, and up-regulation of various proteins associated with astrogliosis, e.g., glial fibrillary acidic protein (GFAP), vimentin, and nestin (*39*, *40*). Herein, Kv1.1 and several other α subunits were identified with specific antibodies in C6 cells and proliferating astrocytes, along with Kvβ1.1 and a smaller form of Kvβ2.1. cDNA cloning from C6 cells and gliotic tissue led to the discovery of an alternatively spliced variant, Kvβ2.1A, which was shown to assemble with Kv1.1, -1.2, or -1.4 and influence the properties of these channels.

EXPERIMENTAL PROCEDURES

Analytical Procedures. C6 cells were grown as detailed elsewhere (*37*) and homogenized in 2.5 mM phosphate buffer (pH 7.2)/15 mM NaCl containing protease inhibitors (2.5 mM benzamidine, 20 μg/mL bacitracin, 0.5 mM PMSF, and 25 μg/mL trypsin inhibitor). Type I astrocyte-enriched cultures, using rat normal cerebellum as a source, were prepared as detailed elsewhere (*38*). Reactive astrocytes were obtained from rat cerebellum in which gliosis had been induced for 3−10 days after injection of kainic acid (*38*). The carefully dissected scar tissue, C6 cells, and bovine cortex or rat brain membranes were extracted in SDS−PAGE sample buffer and subjected to Western blotting, using affinity-purified antibodies specific for Kv1 α or β subunits (*41*, *42*), and horseradish peroxidase-conjugated antispecies secondary antibodies (1:1000−1:2500), with visualization by the ECL system (Amersham).

Cloning of Kvβ2.1A from C6 Cells and Gliotic Tissue: Construction of Various Plasmids. Total RNA samples (isolated from C6 cells, lesioned cerebellum, cultured astrocytes, and rat brain) and oligo(dT) primers (Promega) were utilized to generate single-stranded cDNAs which were later amplified by PCR, using β1.1 or -2.1 specific primers. These reactions were performed for 1 min at 95 °C, 1 min at 54 °C (β1.1) or 56 °C (β2.1), and 5 min at 72 °C for 40 cycles using the primer pair: β2.1 sense 5′-CCAGCCGGCCG-GAATCAACCACGGGG-3′ and antisense 5′-TCAGTC-GACTTAGGATCTATAGTCCTTTTTG-3′ or β1.1 sense 5′-CCGGGG ATCCAATGCAATGCAAG TTCCATAGCC-3′ and antisense 5′-GCAAAAAGGACTATAGATCCTAA-GGGTCGAC-3′. All PCR products were sequenced. The samples generated from C6 cells and lesioned tissue represented a mixture of Kvβ2.1 and -2.1A; these were ligated into pGEMT vector for sequencing. For in vitro translation, each of the two fragments from C6 cells was subcloned into pCITE-2b(+) vector (Novagen). To achieve functional expression in oocytes, the cDNAs were amplified using sense 5′-GAAAAGACACGCTGATCTAGATAA-3′ and antisense 5′-TCAGTCGACTTAGGATCTATAGTCCTTTTTG-3′ primers to introduce appropriate restriction sites for subcloning into pβUTpA vector, which contains untranslated regions of *Xenopus* β-globin gene and an extended polyA (268 nucleotides) sequence. The constructs made were confirmed by restriction mapping and dideoxy DNA sequencing.

Subcloning of Kv1.1 and -1.2 into pCITE-2b(+) Vector for in Vitro Translation. Rat brain Kv1.1 and -1.2 coding sequences were PCR-amplified from pAKS plasmids, using the following sense and antisense primers: 1.1, 5′-TGCG-GATCCCATACGTAGCAGG-3′ and 5′-CTGGTG CT-TCTCGTAGAACATCGGTCAG-3′ (minus the stop codon); 1.2, 5′-CAACCATGGCAGTGGCTACCGGAGACCC-3′ and 5′-CAACTCGAGATCAGTTAACATTTTGGTAA-3′ (excluding the stop codon). PCRs were performed for 40 cycles at 95 °C for 1 min and at 56 °C (Kv1.1) or 58 °C (Kv1.2) for 1 min followed by 10 min at 72 °C, and the products were ligated into pCITE-2b(+) after digestion with the requisite enzymes. The final constructs had a 6-His tag inserted at the 3′ end, followed by a stop codon; both strands of each cDNA were sequenced as above.

cRNA Synthesis and Protein Expression. cDNA constructs were linearized with appropriate enzymes and used as templates to generate capped or uncapped cRNA for expression in oocytes or in vitro translation, respectively. Capped cRNAs were made using the SP6 (pAKSKv1.4) or T3 (pβUTpA constructs) RNA polymerase Cap-Scribe system (Boehringer Mannheim), whereas uncapped cRNAs were generated from the pCITE-2b(+) constructs using T7 RNA polymerase (RiboMAX, Promega). The uncapped cRNAs were translated using nuclease-treated rabbit reticulocyte lysate (Promega) in the presence of [^{35}S]methionine with or without (in the case of Kvβ subunits) canine microsomal membranes (1.5 μL, Promega) in 25 μL with 500 ng for single subunits or 250 ng each of two different cRNAs. Microsomal membranes greatly enhanced translation of Kv1.1 cRNA but had a more modest effect on Kv1.2. Translates (1−2 μL) were solubilized in sample buffer and subjected to SDS−PAGE; the gels were dried under vacuum and exposed to X-ray film for 24−48 h at 80 °C prior to development.

Purification of His-Tagged α and $\alpha-\beta$ Subunit Complexes. Either Kv1.1 and -1.2 cRNAs were cotranslated with that for Kvβ2.1A from C6 cells, or their cRNAs were translated separately and mixed in pairs (α/β) following treatment with RNase A at 37 °C for 30 min. The samples (12.5 μL) were solubilized in Thesit extraction buffer at 4 °C for 2 h (*41*) and centrifuged at 12000*g* for 30 min, and the supernatant was diluted into binding buffer before purification on 200 μL of Ni^{2+}-NTA agarose beads (Qiagen); after elution by 500 mM imidazole buffer, the samples were subjected to SDS−PAGE/autoradiography.

Electrophysiological Recordings from Oocytes. For expression in oocytes, the coding sequences of Kv1.1, β2.1A, and β2.1 were PCR-amplified and the products ligated into the pβUTpA vector; the constructs were confirmed by sequencing. Capped cRNAs were made for the aforementioned constructs, and for pAKS Kv1.4, as outlined earlier; the concentration of the stocks was adjusted to 500 ng/μL. Oocytes from *Xenopus laevis* were prepared and injected with cRNA in a total volume of 13.8 nL in all cases in Ca^{2+}-free Barth's solution, as reported before (*32*). A constant amount (0.6 ng) of α subunit cRNA was injected into each oocyte, with or without a fixed quantity (6.3 ng) of cRNA for β2.1 or -2.1A; this represents α:β molar ratios of 1:19 (for Kv1.4) and 1:16 (for Kv1.1), to maximize the probability of coassembly as $(\alpha)_4(\beta)_4$ multimers. Two-microelectrode voltage−clamp recordings were made at 22−24 °C, as described before but using the solution specified in the figure legends (*32*). Steady-state activation curves were obtained by converting peak outward current into conductance and plotting it as a function of the command potential, using the equation: $G = I/(V - E_k)$, where V is the command voltage and E_k is the reversal potential. Data were then fitted with a Boltzmann function in the form:

$$I = (I_{max}/\{1 + \exp[(-V - V_{1/2})/K]\})^4$$

where I is the current, I_{max} is the maximum whole cell current, V is the command potential, $V_{1/2}$ is the voltage of half-maximal activation per subunit, and k is the slope factor. Activation time constants (time to peak) were estimated using a single-exponential function [$I(t) = a_0 + a_1 \exp(-t/\tau_1)$] fitted to the rising phase of the current (*31*). Inactivation time constants for Kv1.4 were determined using a single-exponential function fitted to the decline of the outward current from peak to steady state. Statistical probability was determined with a Mann−Whitney U-test and considered significant if $P < 0.05$. All data presented represent the mean value ± SEM, where n = number of oocytes.

RESULTS

Immunoidentification of Kv Channel α and β Subunits in C6 Cells and Reactive Astrocytes: Demonstration Therein of an Unusual β 2.1 Subunit That Is Absent from Rat Normal Brain. Immunoblotting of C6 cells and tissue from gliosis in cerebellum of rats revealed the presence of several Kv1 subfamily members (Figure 1), using antibodies specific for individual α and β subunits whose characterization has been reported (*41, 42*). Anti-Kv1.1 and -1.6 IgGs labeled bands with respective $M_r \sim 84$K and 58K in C6 cells and kainate-lesioned cerebellar tissue, approximating the apparent sizes of those found in synaptic membranes from bovine brain. A strong signal for Kv1.2 ($M_r \sim 80$K) was obtained with reactive astrocytes and brain, but not the C6 cell line (Figure 1). Anti-Kv1.3 antibody recognized a doublet ($M_r \sim 68$K and 70K) or a broad band ($M_r \sim 70$K) in C6 cells and astrocytes, respectively, corresponding in size to that of the smaller component ($M_r \sim 70$K) in brain synaptic membranes (Figure 1); the second larger protein found in the latter ($M_r \sim 82$K) was virtually undetectable in the other two tissues. It appears that posttranslational modification in the various membrane samples underlies these multiple forms of Kv1.3.

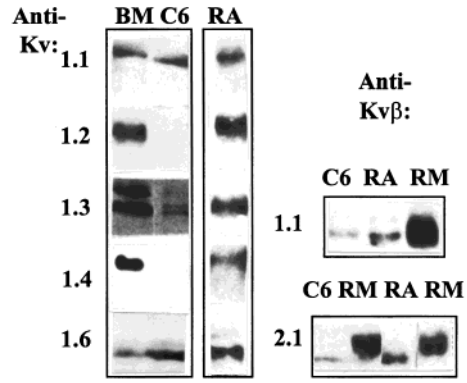

FIGURE 1: Immunoblots showing Kv1 $\alpha\beta$ and α subunits present in C6 cells, reactive astrocytes, and brain membranes. SDS−PAGE (8 or 10% gels for α and β subunits) was performed with glioma cells (C6; 20 μg), bovine (BM; 10 μg) or rat synaptic membranes (RM; 10 μg), and reactive astrocytes (RA; 10 μg) taken from gliosis induced in the cerebellum of 6 rats with kainic acid for 3 days. Following electrophoretic transfer of the protein bands onto PVDF membranes, they were incubated with rabbit polyclonal IgG (1−3 μg/mL) specific for individual α subunits (Kv1.1, -1.3, -1.4, and -1.6) or monoclonal antibody 5 (diluted 1:500-fold) reactive exclusively with Kv1.2. β subunits were detected by incubation with guinea pig IgG specific for β1.1 (to be described elsewhere) or 2.1 (antisera 1:500 and 1:1000, respectively). After washing, the various bound antibodies were complexed to anti-species peroxidase-conjugated IgG and detected using the ECL system (see Experimental Procedures for details). Molecular weights of the various subunits were calculated using the mobilities observed for standard protein markers.

Also, Kv1.4 was visualized in the lesioned tissue and brain membranes ($M_r \sim 95$K) but not in C6 cells.

IgG produced in guinea pigs against a peptide corresponding to a unique N-terminal sequence of Kvβ2.1 detected a single band (Figure 1) in both C6 cells and glial cells from the lesioned cerebellum of rats. Importantly, its size ($M_r \sim 37$K) is smaller than that of the β2.1 ($M_r \sim 39$K) subunit found in rat brain membranes, though all these tissues contained a Kvβ1.1 band of the same mobility ($M_r \sim 42$K) that was revealed with IgG specific for the distinct N-terminus of β1.1. A notable absence of the unusual smaller β2.1 from whole brain, together with its contrasting presence in the lesioned tissue and C6 cells (Figure 1), highlights that it is a characteristic of proliferating astrocytes and, thus, worthy of further characterization.

PCR Detection of a Novel Form of Kvβ2.1: Cloning Identifies a Variant, β2.1A, Only in C6 Cells and Reactive Astrocytes. PCR was performed with Kvβ2.1-specific primers and oligo(dT)-primed cDNA prepared using dissected scar tissue from kainate-lesioned rat cerebellum, C6 glioma cells, rat brain, and cultured astrocytes. PCR products from C6 and reactive astrocytes gave a band of slightly faster mobility than the single component (~1100 bp) obtained with brain and cultured astrocytes (Figure 2A). The later component amplified from rat brain and cultured astrocytes gave a DNA sequence which is identical to that published for β2.1 (*20*). The DNA sequences obtained for the 3′ end (up to 700 bp) and the first 50 bp of the PCR product from C6 cells and those induced by gliosis were identical to those of β2.1, but their intervening region could not be resolved. Insight was gained into this anomaly by restriction mapping where three fragments were obtained for C6 cells and gliotic tissue (681, 423, and 381 bp) compared to the two expected fragments

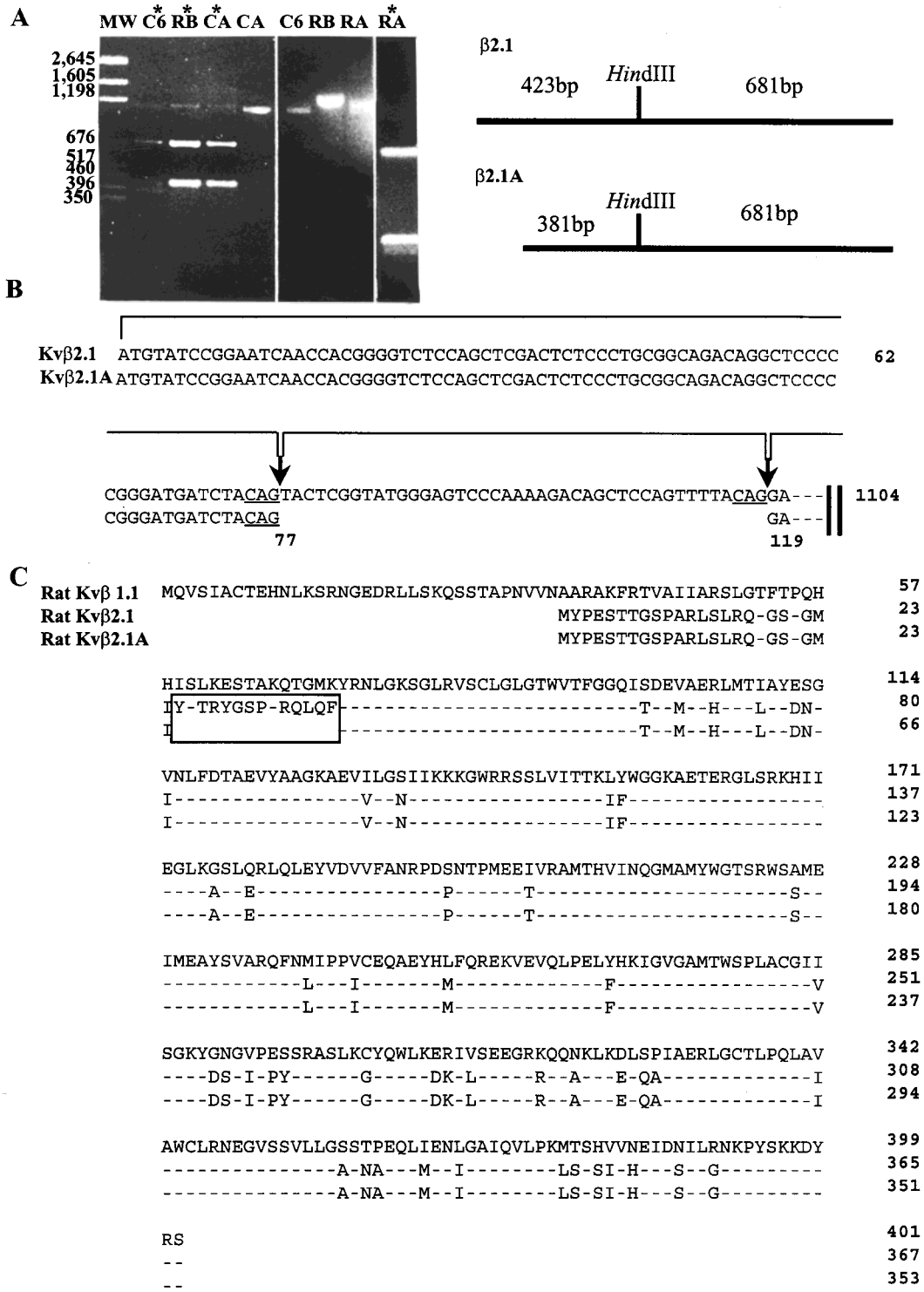

FIGURE 2: Analysis of PCR products from rat brain, C6 cells, and reactive and cultured astrocyte cDNAs: alignments of the sequences for β2.1 and β2.1A. (A) Each PCR product was incubated with and without *Hin*dIII prior to electrophoresis or loaded directly onto a 2% agarose gel made in TAE buffer and run for 2 h unless otherwise stated. Different gels have been aligned to give relative sizes. pGEM DNA markers (MW); digested (*) PCR fragments from C6 cells (C6), rat brain (RB), and cultured astrocytes (CA); undigested PCR products from CA, C6, RB, and RA, respectively; PCR-digested products from RA (this gel was run for 3 h). A schematic diagram showing the fragments expected after restriction digestion; C6 cells and the lesioned cerebellar tissue contained β2.1A as well as β2.1, whereas rat brain and cultured astrocytes had the latter only. (B) Comparison of the sequences of Kvβ2.1 and Kvβ2.1A cDNA, cloned from C6 and reactive astrocytes, surrounding the proposed splice junction. Part of the consensus splice site sequence is indicated by underlining, with arrows marking the end of exons. The boldface vertical lines indicate that the two sequences are identical up to the 3′ end. (C) Comparison of amino acid sequences of rat brain cDNAs encoding Kvβ1.1 and Kvβ2.1 with the new variant β2.1A. Identical amino acids are indicated by dashes, and the residues removed by splicing are boxed.

(681 and 423 bp) observed for the PCR product for rat brain and cultured astrocytes (Figure 2A). This fragmentation pattern, and the identical 3′ sequence observed (~700 bp), could be explained by the product(s) from the C6 cells and the proliferating glia containing a mixture of full-length β2.1 (producing 423 + 681 bp) and a 5′-deleted form (generating

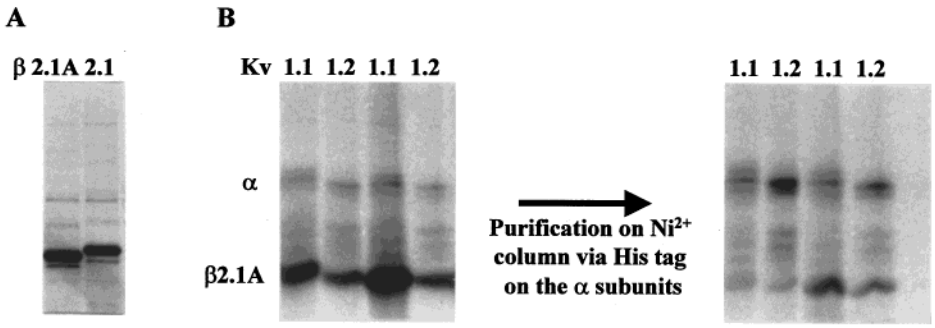

FIGURE 3: Autoradiographs showing in vitro translation of α and β subunits: their cotranslation, posttranslational mixing, and chromatographic isolation. In vitro translations were performed using reticulocyte lysate in the presence of [^{35}S]methionine; after subjection of the reaction mixtures to SDS−PAGE, autoradiograms were prepared as outlined under Experimental Procedures. (A) β subunits translated individually: β2.1A and β2.1. (B) cRNAs encoding the α and β 2.1A subunits were cotranslated or these proteins mixed following translation. cRNA for β2.1A was cotranslated with Kv1.1 or -1.2 [left panel in (B), lanes 3 and 4]; additionally, these respective pairs of subunits were mixed following treatment with RNase [left panel in (B), lanes 1 and 2]. Aliquots of these reaction mixtures were subjected to chromatography on Ni^{2+}-NTA agarose, prior to electrophoreses [right panel in (B)]; the last lane shows in vitro translated β2.1A alone, subjected to the latter purification protocol. As the very small volumes used by necessity for the reactions caused variations in the translation levels, only the relative intensities of the α and β subunit bands should be noted.

381 + 681 bp) (Figure 2A). These cDNAs from reactive astrocytes or C6 cells were ligated into a pGEMT vector and the clones sequenced; the data from these two samples revealed full-length β2.1 and a shorter form, termed β2.1A, which had an identical sequence in both cases and was indistinguishable from β2.1 at the 5′ and 3′ ends but with a 42 bp deletion between 77 and 119 bp (Figure 2B). This 42 bp segment encodes a 14 amino acid sequence, YSTRYGSPKRQLQF, with a calculated molecular weight of ∼2K. Such a deletion would account for the difference in M_r observed between the β2.1 subunit found in rat normal brain and its shorter, β2.1A, variant expressed in C6 cells and proliferating astrocytes. Finally, the cDNA obtained for the full-length β2.1 from C6 cells and reactive astrocytes (see Figure 2) gave a sequence identical to that published (*24*), but, curiously, its mRNA is not translated as shown by the absence of the normal sized β2.1 subunit from these cells (cf. Figure 1). DNA sequencing of RT-PCR products using Kvβ1.1-specific primers confirms that the cDNA sequences amplified from C6 cells and rat brain tissues were identical to the published Kvβ1.1 sequence (*20*), but no product was generated when cultured astrocytes were used (data not shown).

β2.1A Is Generated by Alternative Splicing. The nucleotide sequence CAG is present just before and at the end of the sequence deleted from β2.1 (Figure 2B) and resembles the splice sequence CAGGTA (*43*) predicted for the Kvβ1 gene (*22*, *30*) where the CAG marks the end of an exon. Based on these data and by analogy with findings for the closely related β1 gene, it is reasonable to postulate that the β2 gene may be composed of exons 1−77 and 78−119 and possibly several in the remaining sequence (120−1104 bp), with β2.1A being generated by alternative splicing which excludes the second one (Figure 2B). In the case of the β1 gene, a splice sequence occurs at Y73 which corresponds in the aligned sequence (Figure 2C) to the Y39 demonstrated here for β2; notably, the whole of the core region following this Y residue is conserved (70−80% identity) among all the β subunits, including Kvβ2.1A. Thus, alternative splicing of the β2 gene further extends the scope for increasing K^+ channel diversity, at least in reactive astrocytes.

Coassembly of Kv1 α Subunits and β2.1A. To assess if β2.1A could associate with Kv1α subunits during or after translation, their cDNAs were subcloned into a high-level expression vector, pCITE-2b(+), with the introduction of a 6-His tag at the C-terminus of the α subunits only. Kv1.1 and -1.2 were selected for study because of their presence in proliferating astrocytes. Individually, β2.1A and -2.1 could be readily translated in the reticulocyte lysate system; notably, the β2.1A protein showed a slightly smaller size (M_r ∼ 37K) than the full-length version (Figure 3A), confirmatory of the results for the tissues seen in Figure 1. β2.1A and Kv1.1 or -1.2 were translated separately and the pairs mixed to allow their interaction, or the cRNAs for Kv1.1/β2.1A or Kv1.2/β2.1A were combined prior to translation. SDS−PAGE of aliquots of each showed that a larger amount of β2.1A was produced than either of the α subunits, under both sets of conditions (Figure 3B); this is to be expected because the hydrophilic nature of the β protein would allow it to be more easily translated than the membrane-spanning α subunits. In each case, any α/β complexes formed were purified via the 6-His sequence in the α subunits on Ni^{2+}-charged resin. When Kvβ2.1A was cotranslated with either Kv1.1 or Kv1.2, proportionately more β subunit was detected in the copurified α/β complexes compared to that obtained from posttranslational mixing of either with the β2.1A protein (Figure 3B), indicating that α and β subunits associate more readily when they are translated together. If β2.1A was translated alone and subjected to the same chromatographic procedure, no protein was detected (Figure 3B), demonstrating the lack of nonspecific absorption to the column and, thereby, validating the α/β interaction observed. When similar experiments were performed using Kvβ2.1, posttranslational assembly was also observed using Kv1.1 or -1.2 (data not shown). These collective findings support the deduction that the deleted 14 amino acids preceding the core region of the β are not involved in their assembly with α subunits.

Functional Coexpression of β2.1A and α Subunits: Alterations of Kv1.4 and -1.1 K^+ Currents. To ascertain if β2.1A could modify the properties of Kv1.1 or -1.4 channels that were detected immunologically in reactive astrocytes, their cRNAs were coexpressed in *Xenopus* oocytes. Coding sequences of Kv1.1, β2.1, or 2.1A were subcloned into the pβUT2pA vector, which contained 5′- and 3′-untranslated regions of the β-globin gene from *Xenopus*; this has the

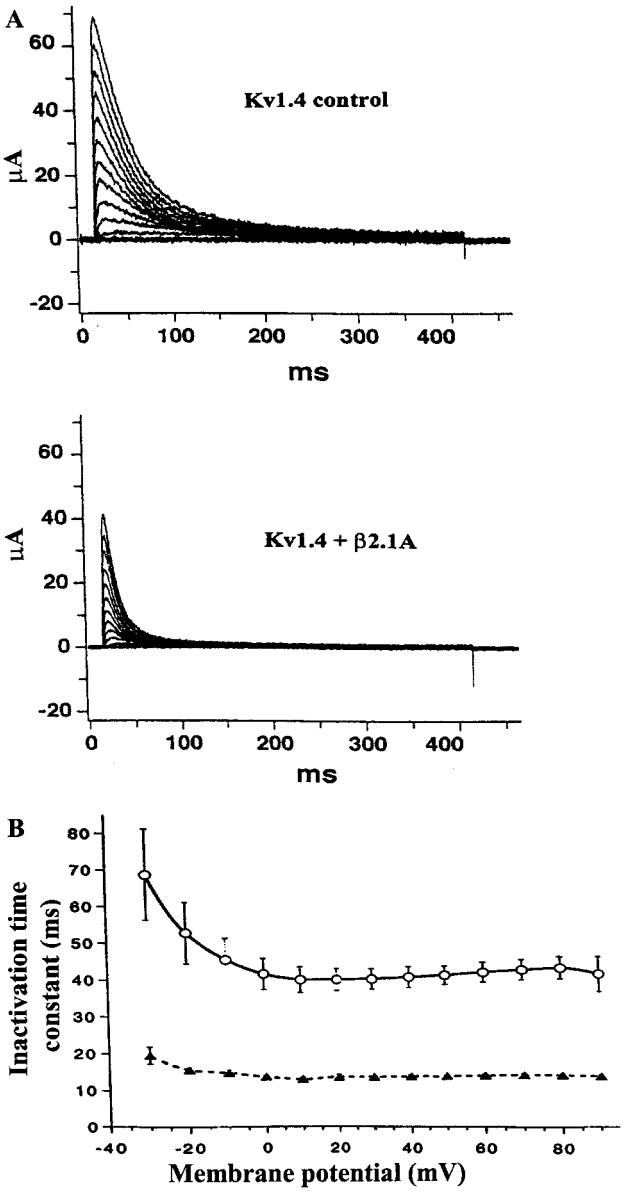

FIGURE 4: Inactivation time constant of the Kv1.4 current in oocytes is modified by coexpression with β2.1A. Recordings were made in ND96 solution (mM: NaCl, 96; KCl, 1; $MgCl_2$, 1; HEPES, 5; $CaCl_2$, 1.8; adjusted to pH 7.2 with NaOH). Depolarizing voltage pulses of 200 ms were applied with a two-electrode voltage clamp in 10 mV increments from a holding potential of −90 mV to a maximum command potential of +100 mV, with leak and capacity subtraction on-line. (A) Currents from oocytes injected with cRNA for Kv1.4 alone, and with that for β2.1A. (B) Inactivation time constants were estimated by single-exponential fits for Kv1.4 (○) and with β2.1A (▲). Data are shown for 5 oocytes ± SEM; in some cases, the symbols encompass the error bars.

advantage of giving a substantially increased level of K^+ channel expression (*44*). Capped cRNA was in vitro transcribed from these constructs and pAKSKv1.4 using T3 and SP6 RNA polymerase, respectively. Each α subunit cRNA alone yielded voltage-activated K^+ currents within 36 h of microinjection, with Kv1.4 typically producing a much larger outward current than Kv1.1. Expression of Kv1.4 gave a K^+ current which exhibited rapid activation, and fast inactivation to steady state (Figure 4A) due to its N-terminal inactivation domain (*20, 32, 45*). Notably, when coexpressed with β2.1A, a significant acceleration of inactivation of Kv1.4 current was seen (Figure 4A), the inactivation time constant being changed at all potentials (Figure 4B). Values for the inactivation time constant at +60 mV were Kv1.4 = 42.2 ± 5 ms and Kv1.4 + β2.1A = 15.5 ± 0.9 ms (n = 5−6) and are the same as those seen with full-length β2.1 (15.3 ± 1.1 ms) (*32*). This result demonstrates that the altered subunit both coassembles with Kv1.4 and functionally modifies the channel kinetics to a similar extent as β2.1.

A noninactivating K^+ current was observed upon expressing Kv1.1 alone (Figure 5A) because this α subunit lacks an N-terminal inactivation ball (*45*); when coexpressed with β2.1A, the amplitude was boosted but to a lesser extent than that seen with β2.1 (Figure 5A and detailed below). Moreover, the activation time constant was moderately accelerated by either β subunit; with β2.1 this effect was apparent over the entire voltage range whereas in the case of β2.1A a much more modest change occurred, and only then at the most positive potentials (Figure 5B). The half-maximal activation voltage ($V_{1/2}$) obtained for Kv1.1 (Figure 5C) was similar to that reported by others (*25*). Although β2.1A induced a slight leftward shift in the activation curve, this represented a negligible change of −4 mV. Neither β2.1 nor 2.1A subunit caused any significant alteration to k (slope factor); the values observed were as follows: Kv1.1 alone = 5.5 ± 1 mV; +β2.1 = 5.9 ± 1 mV; and +β2.1A = 4.4 ± 0.5 mV. A quantitative study of the effects of β2.1A and its full-length counterpart on K^+ channel expression was performed. A representative trace (Figure 5D) shows that while Kvβ2.1 increased the Kv1.1 current, a more modest change was usually observed with β2.1A; in some experiments, the truncated β subunit reduced the current amplitude. Notably, when the peak currents from several experiments were normalized at each time point for every oocyte (~50 for each from 4 frogs) and plotted as a percentage of the control Kv1.1 current, Kvβ2.1A was found on average to exert little, if any, change.

DISCUSSION

The biochemical properties of the channel proteins responsible for the Kv1 K^+ currents recorded in reactive astrocytes remain poorly defined (*1, 46*). Herein, we report for the first time the sets of K^+ channel subunits expressed in C6 glioma cells and kainate-induced proliferating astrocytes. Compared to brain synaptic membranes or reactive astrocytes, the complement of subunits found in C6 cells is more restricted, containing only three of the isoforms (Kv1.1, -1.3, and -1.6, but not -1.2 and -1.4) found in the other two tissues. The absence of Kv1.4 (which gives a fast-inactivating K^+ current) and a minimal content of β1.1 in C6 cells (see below) are consistent with 90% of their whole-cell voltage-activated K^+ current being classified as a delayed rectifier and attributed to Kv1.1-containing channels (*37*). This current in C6 cells was deemed to be responsible for maintaining the resting membrane potential (*47*). In contrast to C6 cells, the observed presence in reactive astrocytes of Kv1.4, as well as the β1.1 subunit [known to confer fast inactivation on most Kv1 channels (*20, 48*)], indicates that these could be constituents of an oligomer likely to underlie the TEA-insensitive, transient K^+ current reported in proliferating astrocytes (*11*). On the other hand, one or more of the Kv1.1, -1.2, -1.3, and -1.6 subunits also found in the gliotic tissue may comprise different channel subtypes responsible for the noninactivating, delayed rectifier K^+ current that reactive

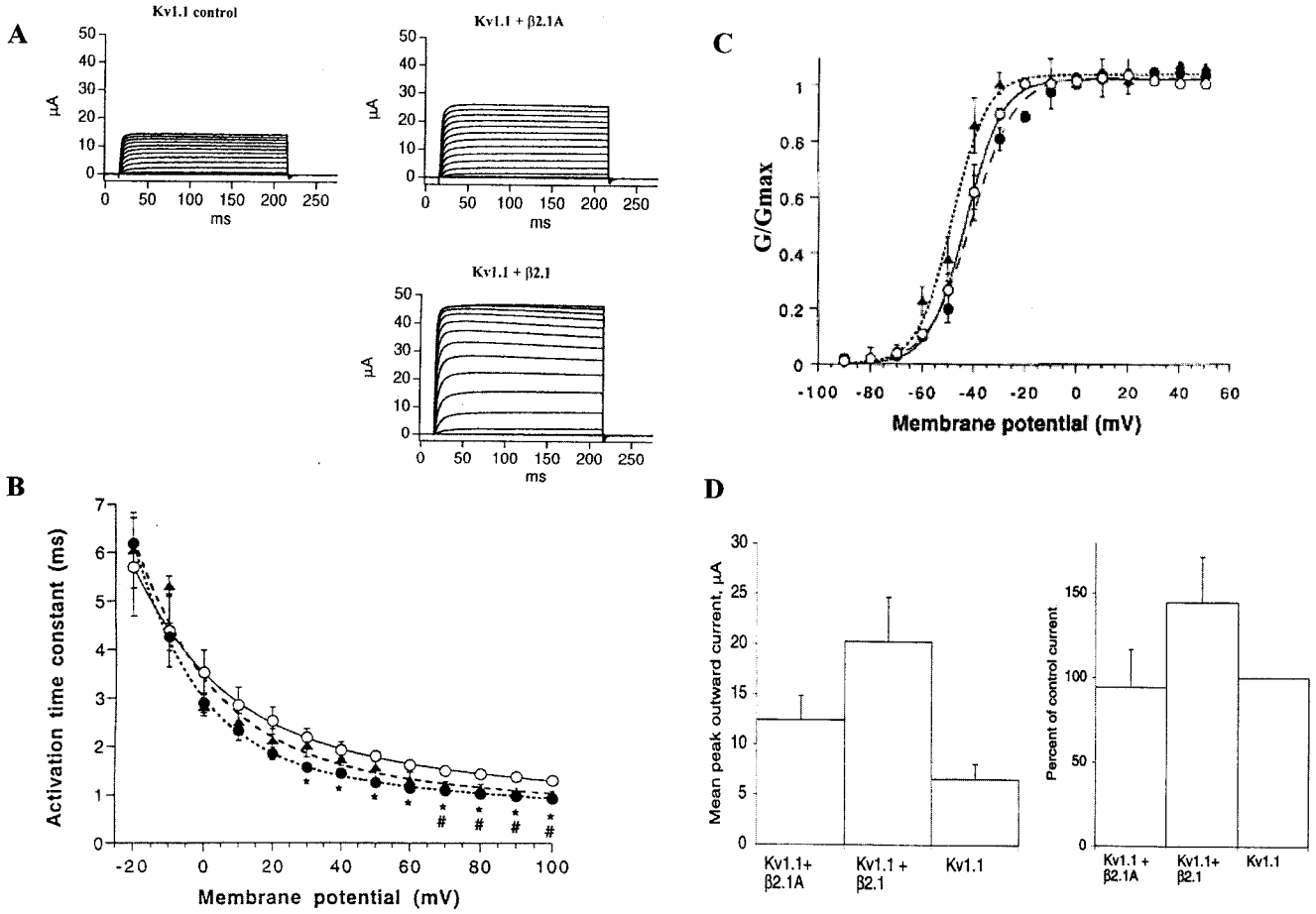

FIGURE 5: Coexpression of β subunits with Kv1.1 alters the current amplitude and activation properties. Recordings were carried out as in Figure 4. (A) Oocytes injected with Kv1.1 cRNA alone and together with β2.1 or β2.1A. (B) Activation time constants were estimated using a single-exponential function fitted to the rising phase of the current (*31*). β2.1 subunit (●) significantly accelerated the activation time constant while β2.1A (▲) had a more modest effect and only at the most positive potentials, relative to that for Kv1.1 alone (○). Significance is indicated (* for β2.1 and # for β2.1A); data are representative of 5 oocytes ± SEM. (C) Voltage-dependency of the steady-state activation was studied using conductance−voltage curves with data normalized to 0 mV. Data were fitted with a Boltzmann function of fourth order (*32*). Coexpression of β2.1(●) or β2.1A (▲) did not significantly alter $V_{1/2}$ activation voltage or the voltage sensitivity of Kv1.1 alone (○), despite β2.1A inducing a slight leftward shift. (D) Peak currents were recorded, as outlined above, in oocytes injected with Kv1.1 cRNA alone, and together with that encoding β2.1 or β2.1A. The left-hand panel shows the mean peak currents (±SEM), at 96 h after injection of cRNA, from a representative set of recordings on 10 oocytes for each combination. The summated data from several experiments are plotted in the other panel. This was calculated by normalizing the peak current amplitude at each time point (48, 72, and 96 h) for every oocyte (150 in total, from 4 frogs) and expressing the values relative to the control (Kv1.1 alone).

astrocytes exhibit (*11*). However, the limited availability of lesioned tissue and the low content of K^+ channels precluded determination of the oligomeric combinations.

Another significant finding is the novel spliced product of the Kvβ2 gene, β2.1A, identified in C6 and reactive astrocytes by immunoblotting, RT-PCR, and DNA sequencing, but which was undetectable in cultured astrocytes or rat whole brain. This variant, cloned from both reactive astrocytes and C6 cells, contains N-terminal (1−24) and C-terminal (39−367) regions identical to those in β2.1, but has a 14 residue deletion. The human Kvβ1 gene, which encodes 3 spliced-variants, is known to consist of 17 exons, with the core region being composed of exons 3−15 (*49*). Thus, the Kvβ2 gene might have a similar genomic organization with the first two exons generating spliced variants having distinctive effects on K^+ channel properties, and a core C-terminal region (39−367) encoded by several common exons, as observed for the β1 gene. However, such variants have escaped detection to date, possibly because their occurrence may be restricted to certain cells or expressed only under unusual physiological conditions, as found herein for β2.1A in reactive astrocytes and rapidly dividing glioma cells.

By examining the interaction of β2.1A with the Kv1 α subunits using biochemical and electrophysiological techniques, we have been able to shed some light on the influence of the 14 residue deletion on the functioning of representative K^+ channels found in both glia and neurons. Using an in vitro translation system, it was shown that β2.1A associates with Kv1.1 and -1.2 predominantly when they are cotranslated, hence probably at an early stage in their biosynthesis as reported for the full-length subunit (*35*). It appears that the core C-terminal region (39−367) of β2.1A is involved in coassembling with α subunits, as reported for the equivalent regions of β1.1 (which is 85% identical to the sequence of β2.1A) (*18*) and β2.1 (*27*); the 14 preceding deleted residues were found not to be essential for such association. Insights into the functional properties of β2.1A were gained by investigating its influence on recombinant K^+ channels, Kv1.1 and -1.4 homomers. When its cRNA

was coinjected with that for Kv1.1, the β2.1A subunit, like β2.1, produced a very slight acceleration of channel activation. Additionally, β2.1A modified the inactivation kinetics of Kv1.4 to an extent virtually identical to that of β2.1 (*30*, *32*), despite lacking residues Y25−F38. Although the precise basis of how these β2 subunits can induce this characteristic change in the absence of an N-terminal inactivating domain (present in β1 and β3) remains to be determined, apparently they promote the effectiveness of the inactivation ball present in the α subunit. In any case, our results establish that the deleted region is not involved. Instead, this function is likely to be mediated by the first 24 residues, as postulated from the compactness of the three-dimensional structure of the core region (*36*); however, a contribution cannot be excluded from amino acids that differ in the core region of the various β subunits (which is ∼80% identical).

A notable consequence of the truncation in β2.1A is that the surface expression of Kv1.1 is elevated to a lesser extent than seen with the full-length subunit. This may arise from a perturbation of the normal α/β or β/β subunit interactions, due to residues 25−38 being deleted; this might compromise the ability of β2.1A to act as a chaperone as effectively as β2.1 (*34*, *35*, *50*). Another speculative interpretation can be made based on the recent suggestion that β subunits might also couple membrane excitability directly to cell chemistry because the crystal structure of β2.1 highlights close resemblances to oxido-reductase enzymes (*36*). Moreover, the postulated positioning of the β subunit tetramer relative to K^+ channel α subunits suggests that they may interact directly or indirectly with the voltage sensor. Although the catalytic site is located in the core region, the N-terminal domain of β subunits may also contribute to the conformational changes in the channel complex underlying such signaling. This intriguing scenario emphasizes the importance of establishing whether the deletion in β2.1A perturbs any facet of this coupling mechanism because the resultant information would help decipher why the truncated subunit occurs only in reactive astrocytes.

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