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Differential Asparagine-Linked Glycosylation of Voltage-Gated K^+ Channels in Mammalian Brain and in Transfected Cells

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Abstract. Glycosylation of ion channel proteins dramatically impacts channel function. Here we characterize the asparagine (N)-linked glycosylation of voltage-gated K^+ channel α subunits in rat brain and transfected cells. We find that in brain Kv1.1, Kv1.2 and Kv1.4, which have a single consensus glycosylation site in the first extracellular interhelical domain, are N-glycosylated with sialic acid-rich oligosaccharide chains. Kv2.1, which has a consensus site in the second extracellular interhelical domain, is not N-glycosylated. This pattern of glycosylation is consistent between brain and transfected cells, providing compelling support for recent models relating oligosaccharide addition to the location of sites on polytopic membrane proteins. The extent of processing of N-linked chains on Kv1.1 and Kv1.2 but not Kv1.4 channels expressed in transfected cells differs from that seen for native brain channels, reflecting the different efficiencies of transport of K^+ channel polypeptides from the endoplasmic reticulum to the Golgi apparatus. These data show that addition of sialic acid-rich N-linked oligosaccharide chains differs among highly related K^+ channel α subunits, and given the established role of sialic acid in modulating channel function, provide evidence for differential glycosylation contributing to diversity of K^+ channel function in mammalian brain.

Key words: Ion channel — Central nervous system — Immunoblot — Pulse-chase — Biosynthesis — Neuronal excitability

Introduction

Voltage-gated ion channels are multisubunit membrane proteins that confer electrical excitability to neurons, cardiac cells and muscle fibers (Hille, 1992). In each case, the multispan (polytopic) integral membrane α subunit contains the functionality typical of the specific channel type, including selective ion conductance, voltage-dependent gating and sensitivity to external drugs and toxins (Catterall, 1988). Peripheral and integral membrane auxiliary subunits can also contribute to the expression efficiency, localization and function of the channel complex (Trimmer, 1998). Voltage-gated K^+ channels are composed of four α-subunit polypeptides, each ranging in size from 350–850 amino acids and containing a highly conserved core region consisting of six transmembrane α helices, interhelical segments that provide the extracellular portions of the channel, and highly divergent cytoplasmic amino and carboxyl termini (Chandy and Gutman, 1995). During the course of their biosynthesis, K^+ channel α subunits assemble into tetramers, in some cases associate with cytoplasmic β-subunits, and transit through the endomembrane system in the process of reaching the plasma membrane (Shi et al., 1996).

Structure-function studies have identified a number of determinants of K^+ channel function that reside within the core polypeptide. These include regions involved in the mechanisms of voltage-dependent activation, K^+-selective conductance, and inactivation (Chandy & Gutman, 1995). Much less is known about how specific post-translational modifications of these core polypeptides can affect the abundance, distribution and function of K^+ channels. A variety of co- or post-translational events can occur to the immature α-subunit polypeptide, including the cotranslational covalent linkage of aspara-

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Abbreviations: DTX, α dendrotoxin; PNGase F, peptide N-glycosidase F; SDS, sodium dodecyl sulfate; RBM, rat brain membrane fraction

gine (N)-linked oligosaccharides, and the post-translational addition of phosphate, sulfate, and lipid moieties. Post-translational processing of N-linked chains, and addition of O-linked chains can also occur. Most K^+ channel α subunits contain a single consensus N-linked glycosylation site (Hubbard & Ivatt, 1981) within their primary amino acid sequence (Chandy & Gutman, 1995). To better understand the functional contribution of N-linked glycosylation on K^+ channel α subunits, a systematic analysis of the extent and nature of this modification is necessary. In the following study, N-glycosylation of a number of K^+ channel proteins is characterized in both rat brain and transfected mammalian COS-1 cells, using sensitivity to digestion with specific glycosidases, and to pharmacological inhibition of N-linked oligosaccharide addition. The rat brain Kv1.1, Kv1.2, and Kv1.4 α subunits, which associate with one another in hetero-oligomeric channel complexes (Sheng et al., 1993; Wang et al., 1993; Rhodes et al., 1997) and have a consensus N-linked site in the extracellular loop between the first and second transmembrane segments are found to be modified with sialic acid-rich N-linked oligosaccharide chains. The Kv2.1 α subunit, which has a consensus N-linked site in the extracellular loop between the third and fourth transmembrane segments, is not N-glycosylated. In transfected cells, the extent of processing of the N-linked chains on recombinant Kv1.1, and Kv1.2 differs from that in brain, probably reflecting the previously observed differences in the efficiency of transit of these K^+ channel α subunits through the endomembrane system. The observed differences in glycosylation reported here may have important functional differences as the nature and extent of N-linked glycosylation of extracellular domains of voltage-gated ion channels can have dramatic effects on channel function (Thornhill et al., 1996; Bennett et al., 1997).

Materials and Methods

Materials

Dulbecco's modified Eagle's medium (DMEM), methionine-free DMEM and phosphate-free DMEM were from Gibco-BRL (Bethesda, MD). Bovine calf serum was from Hyclone Laboratories (Logan, UT). Neuraminidase (from *Vibrio cholerae*) was from Boehringer Mannheim (Indianapolis, IN). PNGase F was kindly provided by Dr. R. Haltiwanger (SUNY at Stony Brook, NY). COS-1 cells were purchased from the Microbiology Department Tissue Culture Facilty (SUNY at Stony Brook, NY). The enhanced chemiluminescence (ECL) reagents were from Amersham (Arlington Heights, IL). [^{35}S]Methionine (Expre^{35}S^{35}S) was from DuPont-NEN (Boston, MA). Pansorbin was from Calbiochem (La Jolla, CA). Prestained molecular weight standards and Non-Enzymatic Cell Dissociation Solution were from Sigma (St. Louis, MO). All other reagents were from Sigma or Boehringer Mannheim.

Transient Transfection of COS-1 Cells

Cells were transfected with mammalian expression vectors for various K^+ channel subunits (Nakahira et al., 1996) by the calcium phosphate precipitation method (Shi et al., 1994). Cells were seeded at 10% confluence and grown at 37°C in DMEM containing 10% calf serum. The calcium phosphate-DNA mixture was added within 24 hr of seeding, when cells were approximately twice the original plating density, and left for 18–24 hr. The transfection media was then removed, and fresh media was added and then incubated at 37°C continued for the desired time.

[^{35}S]Methionine Pulse Chase Metabolic Labeling

Labeling was performed essentially as described (Shi et al., 1994). Cells grown on 60-mm dishes were pre-incubated in methionine-free DMEM ("starved") for 15 min at 37°C, followed by incubation in methionine-free DMEM containing 300 μCi/ml of [^{35}S]methionine at 37°C for desired times. Cells were washed twice in PBS and incubated in serum-containing DMEM supplemented with an additional 5 mM L-methionine for various periods of time before being lysed and subject to immunoprecipitation.

Immunoblots and Immunoprecipitation

All antibodies used in these studies have been described previously (Trimmer, 1991; Rhodes et al., 1995; Bekele-Arcuri et al., 1996; Nakahira et al., 1996; Shi et al., 1996). To harvest cells and prepare detergent lysates, cells were first washed twice in ice-cold PBS and then lysed for 5 min on ice in 1 ml of an ice-cold lysis buffer solution containing TBS (150 mM NaCl, 10 mM Tris, pH = 8.0), 1% Triton X-100, 1 mM iodoacetamide, and a protease inhibitor mixture (2 μg/ml aprotinin, 1 μg/ml leupeptin, 2 μg/ml antipain, 10 μg/ml benzamidine, and 0.2 mM phenylmethylsulfonyl fluoride). The lysate was spun in the microcentrifuge for 2 min to pellet nuclei and debris, and the resulting supernatant saved for analysis. Protein assays were performed by the bicinchoninic acid method (BCA; Pierce) using bovine serum albumin as the standard, and samples were normalized for protein concentration by dilution in lysis buffer.

For immunoblots, 25 μg of protein was added to reducing SDS sample buffer, boiled, and fractionated on 9% polyacrylamide-SDS gels. Lauryl sulfate (Sigma) was the SDS source used for all SDS-PAGE, to accentuate differences between glycosylated and deglycosylated forms of α subunits (Shi et al., 1994). After electrophoretic transfer to nitrocellulose paper, the resulting blots were blocked in TBS containing 4% low fat milk (Blotto), incubated in affinity purified antibodies diluted 1:20–1:100 in Blotto for 1 hr, and washed three times in Blotto for 30 min total. Blots were then incubated in horseradish peroxidase-conjugated secondary antibody (1:1000 dilution in Blotto) for 1 hr and then washed in TBS three times for 30 min total. The blots were then incubated in substrate for ECL for 1 min and exposed to preflashed (to OD_{545} = 0.15) Fuji RX film. If reprobing an immunoblot was desired, the blot would be stripped with stripping buffer (100 mM 2-mercaptoethanol, 2% SDS, 62.5 mM Tris-HCl pH = 6.7) at 50°C for 30 min before probing with another primary antibody.

To harvest cells and prepare detergent lysates for immunoprecipitation, cells were washed twice in ice-cold PBS and then lysed in 1 ml of an ice-cold lysis buffer solution (*see above*) containing 1 mg/ml bovine serum albumin. The lysate was spun in the microcentrifuge for 2 min to pellet nuclei and debris, and for [^{35}S]labeled samples, the resulting supernatant was used directly for immunoprecipitation. Typically, 100 μl of detergent lysate was diluted to 1 ml in ice-cold lysis

buffer. Affinity-purified antibodies was added (2.5–5 μl; 1:200–1:400 final dilution), and the lysates incubated on a rotator at 4°C for 16 hr. The antibody-antigen complex was immobilized by absorption onto 50 μl of fixed *Staphylococcus* aureus cells (Pansorbin, Calbiochem, La Jolla). After a 1 hr incubation at 4°C, the precipitated pellet was washed three times in ice-cold lysis buffer. The protein was eluted from the pellet by boiling in reducing sample buffer. The samples were analyzed on either 6%, 7.5% or 9% polyacrylamide-SDS gels. Gels containing [^{35}]labeled samples were prepared for fluorography by a 1 hr incubation in Autofluor (National Diagnostics, Manville, NJ). Fluorography on these gels was performed for the indicated times at −70°C on preflashed Kodak XAR-5 film using a Lightening Plus intensifying screen (DuPont-NEN).

Glycosidase Digestion

Products of immunoprecipitation reactions were washed twice in incubation buffer (PNGase F:75 mM Tris-HCL pH = 8.0, 10 mM EDTA; Endo H: 50 mM sodium citrate; and neuraminidase (from *Vibrio cholerae*): 10 mM sodium phosphate pH = 7.0), resuspended in 10 μl of incubation buffer with 10% SDS. Samples were heated at 100°C for 5 min. After cooling, incubation buffer (with a protease inhibitor mixture (2 μg/ml aprotinin, 1 μg/ml leupeptin, 2 μg/ml antipain, 10 μg/ml benzamidine, and 0.2 mM phenylmethylsulfonyl fluoride)) was added to bring the final volume to 100 μl. PNGase F (final concentration of 8 U/ml), Endo H (0.1 U/ml) or neuraminidase (0.25 U/ml) was added to the incubation buffer. After overnight incubation of both aliquots at 37°C, an equal volume of 2× sample buffer was added and digestion products were analyzed by SDS gels/fluorography. If rat brain membrane was used, SDS (Bio-Rad, Hercules, CA) was added to 200 μg of protein containing incubation buffer at a final concentration of 1%. The sample was then heated at 100°C for 5 min before treatment with the appropriate glycosidase.

Tunicamycin Treatment

Transfected cells (36–48 hr post-transfection) were pre-incubated in methionine-free DMEM in the presence of 10 μg/ml tunicamycin for 30 min. Then, cells were labeled with 300 μCi/ml of [^{35}S]methionine for 1–2 hr. Tunicamycin (10 μg/ml) was present in the medium for the entire labeling period before cells were lysed and subjected to immunoprecipitation.

Results

N-linked Glycosylation of Native K^+ Channel α Subunits in Rat Brain Membranes

Although all cloned mammalian voltage-gated K^+ channel α-subunits are expressed in the brain (Chandy & Gutman, 1995), the biochemical characterization of these K^+ channel proteins has been primarily performed in heterologous expression systems due to the low abundance and heterogeneity of K^+ channels in native tissues. The recent availability of a panel of subtype-specific antibodies generated against K^+ channel α subunits allows for the identification, isolation and characterization of these K^+ channels from rat brain tissues. Here we have characterized the N-linked glycosylation of the

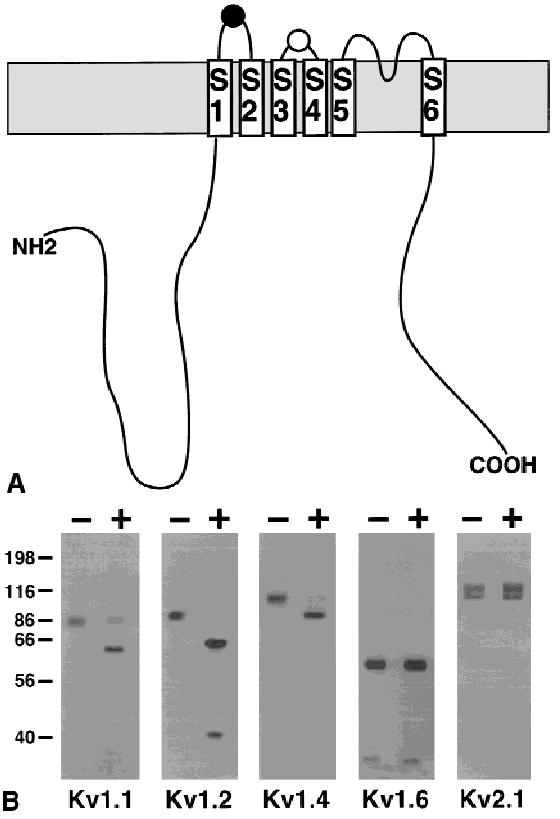

Fig. 1. N-linked glycosylation of rat brain K^+ channel α subunits. (*A*) Schematic diagram of the membrane topology of voltage-gated K^+ channel α-subunit polypeptides. Symbols refer to extracellular consensus N-linked glycosylation sites of (●) Kv1.1, Kv1.2, Kv1.4, and Kv1.5; and (○) Kv2.1. (*B*) PNGase F treatment of K^+ channel α-subunits. RBM (25 μg) were incubated in incubation buffer (75 mM Tris-HCL pH = 8.0, 10 mM EDTA) in the presence (+) or absence (−) of PNGase F (8 U/ml) at 37°C overnight. Digested samples were fractionated 9% SDS-PAGE and transferred to nitrocellulose. The same blot was then probed sequentially with each of the indicated affinity-purified rabbit polyclonal antibodies specific for different K^+ channel α subunit after stripping with stripping buffer (100 mM 2-mercaptoethanol, 2% SDS, 62.5 mM Tris-HCl pH = 6.7) at 50°C for 30 min. The numbers to the left of the figure denote mobility of prestained molecular weight standards in kDa.

Kv1.1, Kv1.2, Kv1.4, Kv1.5, Kv1.6 and Kv2.1 K^+ channel α subunit polypeptides. A schematic diagram of the topology of voltage-gated K^+ channel α subunit polypeptides is shown in Fig. 1*A*, such a topology is predicted by hydropathy analysis (Durell & Guy, 1992) and has been confirmed by insertional epitope mapping (Shih & Goldin, 1997). Each of the Kv1 family members with the exception of Kv1.6 has a single N-linked glycosylation site on the extracellular loop between the first and second transmembrane domains (Fig. 1*A*). Kv1.6 lacks consensus N-linked sites in predicted extracellular domains, while the single consensus N-linked site for Kv2.1 is on the extracellular loop between the third and fourth transmembrane domains (Fig. 1*A*).

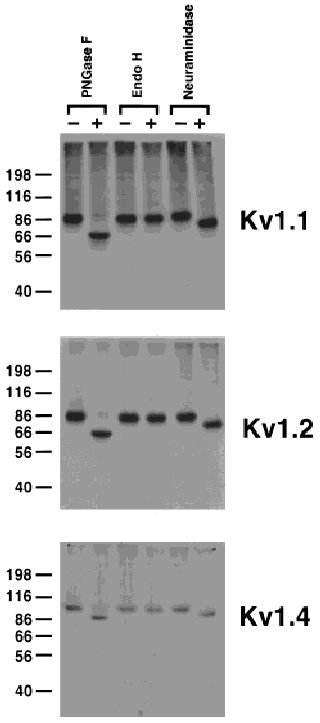

Fig. 2. Endoglycosidase digestion of K^+ channel α subunits in rat brain. RBM (50 μg) were digested in the presence (+) or absence (−) of PNGase F (8 U/ml), Endo H (0.1 U/ml), or Neuraminidase (from *Vibrio cholerae*, 0.25 U/ml) overnight at 37°C. Samples were then fractionated on a 7.5% SDS gel and probed sequentially with anti-Kv1.1, anti Kv1.2, and anti-Kv1.4 antibodies as indicated. Each primary antibody was stripped with stripping buffer at 50°C for 30 min before reprobing with another primary antibody. The numbers to the left of each blot denote mobility of prestained molecular weight standards in kDa.

To directly answer the question whether a given K^+ channel α subunit is N-glycosylated, crude rat brain membrane extracts were digested with PNGase F, an endoglycosidase which removes N-linked sugar moieties from the asparagine residue. The reaction product was fractionated on 9% SDS-PAGE followed by immunoblotting with K^+ channel α-subunit-specific antibodies. Upon PNGase F digestion, the mobility of the Kv1.1 polypeptide shifts from 84 kDa to 60 kDa (Fig. 1*B*). A similar shift in the apparent M_r of ≈ 20 kDa was observed upon PNGase F treatment of the Kv1.2 (86 kDa to 63 kDa) and Kv1.4 (110 kDa to 88 kDa) α-subunits (Fig. 1*B*). The characteristic mobility shift of Kv1.1, Kv1.2 and Kv1.4 by PNGase F digestion indicates that these K^+ channels are N-linked glycoproteins in rat brain. However, PNGase F digestion did not cause any changes in the apparent M_r of Kv1.6 or Kv2.1 (Fig. 1), suggesting a lack of N-linked oligosaccharides on these channel polypeptides. Immunoblot analyses of Kv1.5 in these brain preparations were not performed due to a lack of consistent immunoblotting results with anti-Kv1.5 antibodies. Because the same nitrocellulose filter was used sequentially in the different immunoblotting experiments, the different digestion patterns most likely represent real differences in PNGase F sensitivity of these K^+ channels. It should be noted that the apparent M_r of some of these K^+ channel polypeptides, even after deglycosylation, are larger than that calculated from their deduced amino acid sequences (Kv1.1: 56.4 kDa; Kv1.2: 56.8 kDa; Kv1.4: 73.4 kDa; Kv1.6: 45.6 kDa), suggesting either deviations in M_r determination using SDS-PAGE, or the presence of other types of post-translational modification, such as phosphorylation, sulfation, or lipidation, that could contribute to increased mass or anomalous mobility.

The relatively large mobility shift resulting from PNGase F digestion of the Kv1.1 and Kv1.2, and Kv1.4 polypeptides implies their sugar moieties had been processed into the complex type structures, perhaps including multiple sialic acid residues. Indeed, neuraminidase (sialidase) digestion of rat brain membranes resulted in shifts in the M_r of Kv1.1, Kv1.2, and Kv1.4 to positions intermediate between the intact polypeptides and those seen after PNGase F treatment, resulting from selective removal of sialic acid from the ends of the oligosaccharide chains (Fig. 2). Consistent with their neuraminidase sensitivity was the observed resistance of these same K^+ channels to digestion with Endo H (Fig. 2). Under the same experimental conditions, effective Endo H digestion was shown for several K^+ channel polypeptides in transfected COS-1 cells (*see below*). Therefore, the lack of an observed shift in the M_r of rat brain Kv1.1, Kv1.2, and Kv1.4 upon Endo H digestion is consistent with the presence of complex or hybrid type oligosaccharide chains. Treatment with PNGase F plus neuraminidase did not yield any additional shifts in mobility compared to treatment with PNGase F alone, indicating that all of the detectable sialic acid was on N-linked structures (*data not shown*).

Interestingly, in addition to the major M_r = 86 kDa pool of Kv1.2 in brain, on much darker exposures a trace amount of a minor lower M_r pool, migrating around 66 kDa, was also observed (Fig. 3). Upon treatment with Endo H, an additional Kv1.2 population appeared at M_r = 63 kDa, this appears to be a digestion product of the 66 kDa band (Fig. 3, "Kv1.2" inset). Combined with the study of Kv1.2 in transfected COS-1 cell (*see below*), the 66 kDa form most likely represents a biosynthetic intermediate of brain Kv1.2 with an unprocessed high mannose oligosaccharide chain, while the 63 kDa form is the endo H-deglycosylated core polypeptide of Kv1.2. The appearance of deglycosylated Kv1.2 upon the treatment with Endo H serves as an excellent internal control for the Endo H activity. However, consistent with the results above (Fig. 2), the 86 kDa Kv1.2 polypeptide pool in rat brain is resistant to Endo H digestion (Fig. 3).

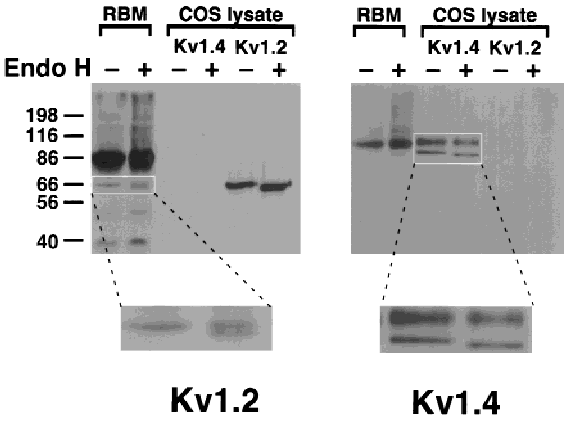

Fig. 3. Endo H digestion of K^+ channel α subunits in rat brain membrane and in transfected COS-1 cells. RBM (50 μg) and COS-1 cells lysates expressing Kv1.2, or Kv1.4 polypeptide were digested in the presence (+) or absence (−) of Endo H (0.1 U/ml) overnight at 37°C. Samples were then fractionated on a 7.5% SDS gel and probed with anti-Kv1.2 antibody (left panels). The same blot was then stripped and reprobed with anti-Kv1.4 antibody (right panels). On the lower left panel, the region of the gel corresponding to the brain Kv1.2 polypeptide pool with components migrating at 66 kDa (−Endo H), and 63 kDa (+Endo H) has been enlarged. On the lower right panel, the region of the gel corresponding to the Kv1.4 pool in Kv1.4-transfected COS-1 cells migrating at 110 kDa, 90 kDa (−Endo H), and 88 kDa (+Endo H) has been enlarged.

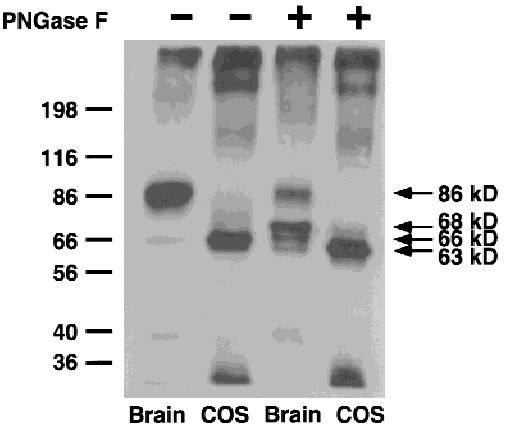

Fig. 4. Comparison of Kv1.2 expressed in rat brain and in transfected COS-1 cells. RBM (50 μg, lanes labeled "brain") and COS-1 cell lysates expressing recombinant Kv1.2 polypeptide (lanes labeled "COS") were digested in the presence (+) or absence (−) of PNGase F (8 U/ml) overnight at 37°C. Samples were then fractionated on a 7.5% SDS gel and probed with anti-Kv1.2 antibody. Arrows denote mobility of Kv1.2 pools. Numbers on left indicate mobility of prestained molecular weight markers.

N-Linked Glycosylation of Recombinant K^+ Channels in Transfected COS-1 Cells

A monkey fibroblast cell line, COS-1, was employed as a mammalian expression system to study K^+ channel biosynthesis by virtue of its null and homogeneous cellular background and robust expression of introduced K^+ channel polypeptides (Shi et al., 1994; Nakahira et al., 1996).

Kv1.2

When Kv1.2 expressed in COS-1 cells was compared with that from rat brain, a difference in the apparent M_r is observed (Fig. 3, 4). Kv1.2 from both sources was sensitive to PNGase F digestion indicating N-glycosylation occurred in each case (Fig. 2, 4). Higher resolution SDS-PAGE analyses revealed heterogeneity in the deglycosylated Kv1.2 polypeptides from rat brain: Three populations of Kv1.2 can be found, with M_r = 63 kDa, M_r = 66 kDa and M_r = 68 kDa (Fig. 4). The first two forms comigrate with the deglycosylated and the glycosylated forms from COS-1 cells, respectively. Multiple bands given by the PNGase F digestion is unexpected, because only a single consensus site is predicted in the cloned Kv1.2 amino acid sequence (Shi et al., 1996). It is not clear if they represent subsets of Kv1.2 polypeptides with additional post-translational modifications, for example, phosphorylation or lipidation.

Immunoprecipitation of Kv1.2 from [^{35}S]methionine pulse-chase labeled COS-1 cell lysates followed by Endo H treatment showed the co-translational N-glycosylation of Kv1.2 (Fig. 5). To confirm this, tunicamycin, which prevents the formation and subsequent attachment of the N-linked oligosaccharide chain, was used to treat [^{35}S]methionine labeled COS-1 cells expressing Kv1.2. Tunicamycin treatment causes the disappearance of the major 66 kDa form of Kv1.2, with a subsequent increase in the amount of the unglycosylated 63 kDa form (Fig. 7). Unlike its brain counterpart, the N-glycosylated Kv1.2 is not further processed into the complex, Endo H resistant form, such that even at long (e.g., 24 hr) chase periods Kv1.2 retains its sensitivity to endo H digestion (Fig. 5). The difference in oligosaccharide chain processing is likely the cause of the observed difference in M_r of Kv1.2 expressed in COS-1 cell and in rat brain. In summary, Kv1.2 expressed in COS-1 cells is N-glycosylated, but, unlike Kv1.2 in brain, the majority of Kv1.2 in COS-1 cells does not have its oligosaccharide structure processed into the complex type and remains Endo H sensitive. Also, additional post-translational modifications exist in brain Kv1.2 which do not occur in COS-1 cells.

Kv1.1

[^{35}S]Methionine pulse-chase experiments (Fig. 6*A* and *B*) showed three bands at M_r = 60 kDa, M_r = 63 kDa, and M_r = 65 kDa (minor) at the earliest time point (30

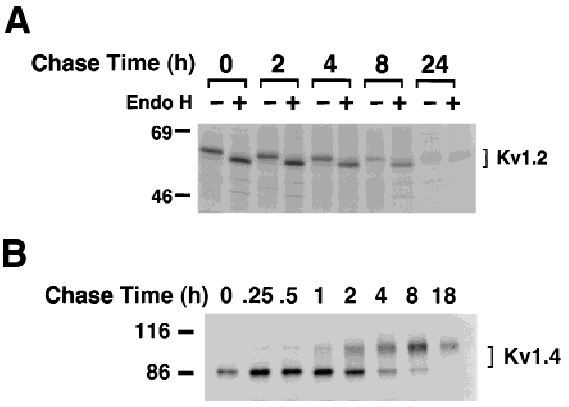

Fig. 5. Pulse chase analysis of Kv1.2 and Kv1.4 expressed in COS-1 cells. (*A*) Endo H treatment of metabolically labeled Kv1.2 in COS-1 cells. COS-1 cells co-transfected with Kv1.2 and Kvβ2 cDNAs (Shi et al., 1996) were labeled with [^{35}S]methionine for 30 min, then incubated at 37°C with unlabeled methionine for the indicated times ("chase"). Cells were lysed and subjected to immunoprecipitation with anti-Kv1.2 antibody, followed by incubation in the presence (+) or absence (−) of Endo H (0.1 U/ml), fractionation by SDS-PAGE (7.5%) and fluorography. Numbers on left indicate mobility of [^{14}C]-labeled molecular weight markers in kDa. The mobility of Kv1.2 polypeptides is as indicatd. (*B*) Biosynthetic maturation of Kv1.4 in COS-1 cells. Kv1.4 transfected COS-1 cells were labeled with [^{35}S]methionine for 15 min, then subjected to chase with unlabeled methionine for the indicated times at 37°C. Cells were lysed and subjected to immunoprecipitation with anti-Kv1.4 antibody, followed by SDS-PAGE (7.5%) and fluorography. Numbers on left indicate mobility of prestained molecular weight markers in kDa, arrows denote the two populations of recombinant Kv1.4 in COS-1 cells.

min pulse, 0 min chase). Upon PNGase F or Endo H digestion, a major population of Kv1.1 migrated at M_r = 60 kDa, indicating it is the unglycosylated core polypeptide (Fig. 6*A* and *B*). Kv1.1 remains Endo H sensitive throughout the chase period (Fig. 6*B*). Thus, in contrast to Kv1.1 in rat brain, the oligosaccharide structure of Kv1.1 expressed in COS-1 cells was not processed into the complex type. In addition to the 60 kDa form, a minor population can be observed at M_r = 62 kDa when treated with either endoglycosidase (Fig. 6*A* and *B*). It is likely that this newly formed 62 kDa population represents deglycosylated 65 kDa form. In the presence of 10 μg/ml of tunicamycin, two major protein populations with M_r of 60 and 62 kDa were immunoprecipitated by anti-Kv1.1 antibody (Fig. 7); these bands are identical in M_r to those observed upon glycoside digestion (Fig. 6*A*). The fact that his 2 kDa addition to the core (60 kDa) and N-glycosylated (63 kDa) Kv1.1 polypeptides is insensitive to PNGase F digestion suggests that the 2 kDa shift probably resulted from other forms of post-translational modification. It should be noted that similar results were obtained from the study of Kv1.1 biosynthesis in transfected mouse fibroblast L cells (Deal, Lovinger & Tamkun, 1994).

Kv1.4

When cell lysates from COS-1 cells expressing Kv1.4 were analyzed by immunoblotting, two immunoreactive bands of M_r = 110 kDa and M_r = 90 kDa, were detected (Fig. 3). The upper (M_r = 110 kDa) Kv1.4 band in COS-1 cells not only comigrated on SDS gels with the brain form, but also shared a similar resistance to Endo H digestion (Fig. 3). However, the lower Kv1.4 band (M_r = 90 kDa) changed mobility (to 88 kDa) when digested with Endo H (Fig. 3). Consistent with this observation, tunicamycin treatment of COS-1 cells labeled for a short period (1 hr) with [^{35}S]methionine showed that both the 110 kDa and 90 kDa Kv1.4 bands were replaced by the 88 kDa form in the presence of tunicamycin (Fig. 7). The 1 hr labeling period used in this experiment results in the majority of Kv1.4 still appearing as the 88 kDa precursor form, in that [^{35}S]methionine pulse-chase experiments showed that the co-translationally glycosylated precursor (M_r = 90 kDa) was further processed into the fully processed, mature form (M_r = 110 kDa) with a $t_{1/2} \approx 3$ hr (Fig. 5*B*). In summary, the majority of the Kv1.4 polypeptide pool in COS-1 cells has N-linked sugar chains that are processed into Endo H resistant structures, while a minor pool remains Endo H sensitive.

Kv1.5

In Kv1.5-transfected COS-1 cells, bands of M_r = 72 kDa and M_r = 68 kDa were present at the earliest time point of [^{35}S]methionine pulse-chase experiments (30 min pulse, 0 min chase, Fig. 6*A* and *B*). As indicated by their Endo H sensitivity, the higher band represents the high mannose type N-glycosylated polypeptide; while the lower band represents the unglycosylated core polypeptide (Fig. 6*A* and *B*). Tunicamycin treatment of Kv1.5-expressing cells is consistent with this interpretation, as the 72 kDa upper band disappeared in the presence of 10 μg/ml tunicamycin; while the lower 68 kDa band remained intact (Fig. 7). Like Kv1.1 and Kv1.2, Kv1.5 remains Endo H sensitive throughout the entire chase period (Fig. 6*A*).

Kv1.6

Kv1.6 lacks consensus N-glycosylation sites in its primary structure. As expected, in transfected COS-1 cells, [^{35}S]methionine pulse-chase experiments showed that recombinant rat Kv1.6 is expressed as a single M_r = 61 kDa band with no evidence of post-translational addition at chase periods up to 4 hr (*data not shown*). In contrast with other α-subunits from Kv1 family, Kv1.6 did not show any apparent mobility shift in cells treated with tunicamycin (Fig. 7).

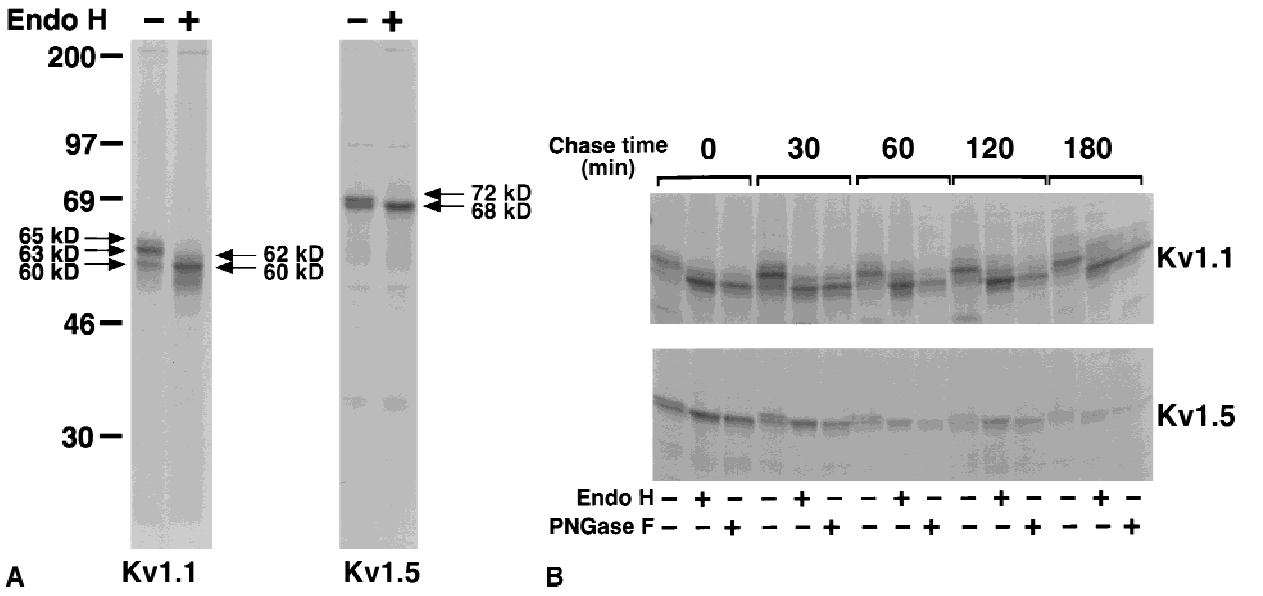

Fig. 6. Endoglycosidase treatment of metabolically labeled Kv1.1 and Kv1.5 in COS-1 cells. (*A*) The earliest time point (30 min pulse, 0 min chase) of [^{35}S]methionine pulse chase labeled Kv1.1 or Kv1.5 was digested in the presence (+) or absence (−) of Endo H (0.1 U/ml). Mobilities of different Kv1.1 and Kv1.5 pools are denoted by arrows. (*B*) COS-1 cells transfected with Kv1.1 or Kv1.5 cDNA were labeled with [^{35}S]methionine for 30 min, then subjected to chase at 37°C with unlabeled methionine for the indicated times. Cells were lysed and subjected to immunoprecipitation with anti-Kv1.1 or anti-Kv1.5 antibody, followed by incubation in the presence (+) or absence (−) of Endo H (0.1 U/ml) or PNGase F (8 U/ml) as indicated, fractionation by SDS-PAGE (7.5%) and fluorography. Numbers on left refer to mobility of [^{14}C]-labeled molecular weight standards in kDa.

Kv2.1

Previously, we have shown that as in rat brain (Fig. 1*B*) recombinant rat Kv2.1 expressed in COS-1 cells is not N-glycosylated, with the only detectable post-translational modification alkaline phosphatase-sensitive phosphorylation (Shi et al., 1994), which affects voltage-dependent activation of the channel (Murakoshi et al., 1997).

Discussion

Voltage-gated K^+ channel α subunits for the most part contain a single consensus N-linked glycosylation site within their primary amino acid sequence (Chandy & Gutman, 1995). Glycosidase treatment of the DTX-binding fraction isolated from bovine or rat brain demonstrated that the higher M_r, toxin-binding subunit was sensitive to enzymatic deglycosylation in vivo (Rehm, 1989; Scott et al., 1990). It is now known that this pool is quite heterogeneous at the molecular level and contains at a minimum the Kv1.1, Kv1.2, Kv1.4 and Kv1.6 α subunits (Scott et al., 1994). Mutagenesis studies of the *Drosophila* Shaker (Santacruz-Toloza et al., 1994) and mammalian Kv1.1 (Deal et al., 1994) K^+ channel polypeptide expressed in transfected cells, and of *Aplysia* Kv1.1 expressed in an in vitro translation system (Shen et al., 1993) showed that N-glycosylation occurred at the consensus sites in the S1–S2 loop.

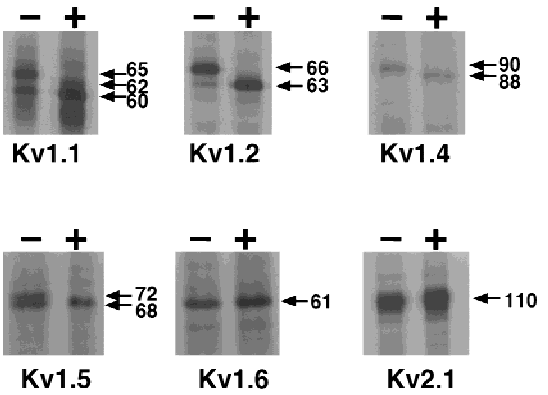

Fig. 7. Tunicamycin treatment of [^{35}S]methionine labeled K^+ channel α subunits in transfected COS-1 cells. COS-1 cells transfected with the indicated K^+ channel α-subunit cDNAs were labeled with 300 μCi/ml[^{35}S]methionine in the presence (+) or absence (−) of 10 μg/ml tunicamycin for 1 hr. Then cells were lysed and subjected to immunoprecipitation with the appropriate α-subunit-specific antibody followed by SDS-PAGE (7.5%) and fluorography. Arrows on right denote the mobility of K^+ channel α-subunits.

As predicted from their amino acid sequences, N-glycosylation of the Kv1.1, Kv1.2 and Kv1.4 α subunits occurs on both native channels in rat brain and recom-

binant polypeptides expressed in transfected cells. These polypeptides each have a single glycosylation site in the extracellular loop between the first two transmembrane segments. Given the considerable overall divergence in both the sequence and the length of this S1–S2 loop between different Kv1 family members (Chandy & Gutman, 1995), the fact that the glycosylation site itself is conserved in each of the Kv1 family members except Kv1.6 is quite striking, and implies that N-glycosylation at this site may have functional importance for these channels. The fact that in brain, Kv1.1, Kv1.2 and Kv1.4 are heavily sialylated, and co-assemble as components of hetero-oligomeric K^+ channel complexes is intriguing, given that the extent of sialylation can have dramatic effects on channel function (Thornhill et al., 1996; Bennett et al., 1997).

It should be noted that the presence of the consensus N-linked glycosylation site in all of the Kv1 family members except Kv1.6 (and the absence of this site in Kv1.6), is conserved in the mouse, rat and human orthologues (Chandy & Gutman, 1995). The mammalian Kv2.1 α-subunit differs from these Kv1 family members in that it contains a consensus N-glycosylation site in the S3–S4 extracellular loop. The lack of N-glycosylation on Kv2.1 could result from the inaccessibility of this N-glycosylation site, located near the S3 transmembrane segment, to the enzyme oligosaccharyl transferase. The sequence and position of this N-glycosylation site are both absolutely conserved in Kv2.2, another member of mammalian *Shab* subfamily. A comprehensive survey of other mammalian polytopic membrane proteins revealed that in most cases only glycosylation sites in the first extracellular loop are used (Landolt-Marticorena & Reuthmeier, 1994); our data on glycosylation of Kv1 family members and lack of glycosylation of Kv2.1 provide additional data on a group of highly related polytopic polypeptides to support this model.

The role of N-glycosylation in K^+ channel function *in situ* is as yet unknown. Studies of recombinant Kv1.1 expressed in Lec mutant CHO cells have shown that sialylation of the N-linked chain of Kv1.1 affects the voltage-dependence of channel activation in a manner consistent with effects of the negatively charged sialic acid group on the surface charge of the channel (Thornhill et al., 1996). Similar results have also been obtained for voltage-gated Na^+ channels (Bennett et al., 1997), showing that the nature and extent of glycosylation can dramatically affect channel function.

It has also been suggested that correct glycosylation of channel proteins is required for efficient cell surface expression in neurons (Gilly, Lucero & Horrigan, 1990; Zona, Eusebi & Miledi, 1990). Tunicamycin treatment of COS-1 cells expressing either Kv1.2 or Kv1.4 did not have any effects on surface expression efficiency (G. Shi & J.S. Trimmer, *unpublished data*), implying that N-glycosylation of these α subunits is not absolutely essential for subunit assembly and surface expression. Previous studies using glycosylation site mutants of *Shaker* (Santacruz-Toloza et al., 1994) and mammalian Kv1.1 (Deal et al., 1994) K^+ channel α subunits also provided evidence that functional channels are formed in the absence of the normal N-glycosylation. While the presence of N-linked oligosaccharides on native K^+ channels in neurons and recombinant channels expressed in transfected COS-1 cells is qualitatively consistent, the processing of these N-linked chains differs substantially between the different cellular backgrounds for Kv1.1 and Kv1.2, but not for Kv1.4. The major population of Kv1.1 and Kv1.2 polypeptides expressed in COS-1 cells remains in the simple, high mannose (or hybrid) structures as manifested by their Endo H sensitivity, while the native channels in brain are in higher M_r, Endo H-resistant, and sialidase-sensitive forms. This is probably a reflection of the lack of efficient surface expression of Kv1.1 and Kv1.2 in COS-1 cells, as the bulk of expressed channel protein appears to be trapped in the endoplasmic reticulum (Bekele-Arcuri et al., 1996; Shi et al., 1996) and thus never reaches the Golgi apparatus where the major steps in the processing of N-linked oligosaccharides, including sialylation, occur. By contrast, Kv1.4 appears to be efficiently expressed on the surface of COS-1 (Bekele-Arcuri et al., 1996) and also contains efficiently processed N-linked oligosaccharide chains. The basis for the dramatic differences in oligosaccharide chain processing and surface expression efficiency between these distinct but highly related members of the Kv1 family when expressed in transfected cells are not known, but presumably involve subunit-specific differences in the efficacy of folding of the channel polypeptide within the endoplasmic reticulum. This apparent correlation between the efficiency of channel surface expression and the extent of oligosaccharide chain processing will allow for the identification and selective isolation of surface K^+ channels based on the structure of their N-linked oligosaccharide chains, and may lead to identification of factors other than auxiliary subunits (Shi et al., 1996) that are important in controlling the abundance of functional ion channels in mammalian cells.

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References

Bekele-Arcuri, Z., Matos, M.F., Manganas, L., Strassle, B.W., Monaghan, M.M., Rhodes, K.J., Trimmer, J.S. 1996. Generation and characterization of subtype-specific monoclonal antibodies to K^+

channel α- and β-subunit polypeptides. *Neuropharmacology* **35:**851–865

Bennett, E., Urcan, M.S., Tinkle, S.S., Koszowski, A.G., Levinson, S.R. 1997. Contribution of sialic acid to the voltage dependence of sodium channel gating. A possible electrostatic mechanism. *J. Gen. Physiol.* **109:**327–343

Catterall, W.A. 1988. Structure and function of voltage-sensitive ion channels. *Science* **242:**50–61

Chandy, K.G., Gutman, G.A. 1995. Voltage-gated K^+ channel genes. *In:* Ligand and Voltage-Gated Ion Channels. R.A. North, editor. pp. 1–71. CRC Press, Boca Raton, FL

Deal, K.K., Lovinger, D.M., Tamkun, M.M. 1994. The brain Kv1.1 potassium channel: in vitro and in vivo studies on subunit assembly and post-translational processing. *J. Neurosci.* **14:**1666–1676

Durell, S.R., Guy H.R. 1992. Atomic scale structure and functional models of voltage-gated potassium channels. *Biophys. J.* **62:**238–247

Gilly, W.F., Lucero, M.T., Horrigan, F.T. 1990. Control of the spatial distribution of sodium channels in giant fiber lobe neurons of the squid. *Neuron* **5:**663–674

Hille B. 1992. Ionic Channels of Excitable Membranes, 2nd Ed., Sinauer, Sunderland, MA

Hubbard, S.C., Ivatt, R.J. 1981. Synthesis and processing of asparagine-linked oligosaccharides. *Annu. Rev. Biochem.* **50:**555–583

Landolt-Marticorena, C., Reuthmeier, R.A. 1994. Asparagine-linked oligosaccharides are localized to single extracytosolic segments in multi-span membrane glycoproteins. *Biochem. J.* **302:**253–260

Murakoshi, H., Shi, G., Scannevin, R.H., Trimmer, J.S. 1997. Phosphorylation of the Kv2.1 K^+ channel alters voltage-dependent activation. *Mol. Pharm.* **52:**821–828

Nakahira, K., Shi, G., Rhodes, K.J., Trimmer, J.S. 1996. Selective interaction of voltage-gated K^+ channel β-subunits with α-subunits. *J. Biol. Chem.* **271:**7084–7089

Rehm, H. 1989. Enzymatic deglycosylation of the dendrotoxin-binding protein. *FEBS Lett.* **247:**28–30

Rhodes, K.J., Keilbaugh, S.A., Barrezueta, N.X., Lopez, K.L., Trimmer, J.S. 1995. Association and co-localization of K^+ channel α- and β-subunit polypeptides in rat brain. *J. Neurosci.* **15:**5360–5377

Rhodes, K.J., Strassle, B.W., Monaghan, M.M., Bekele-Arcuri, Z., Matos, M.F., Trimmer, J.S. 1997. Association anbd colocalization of Kvβ1 and Kvβ2 with Kv1 α-subunits in mammalian brain K^+ channel complexes. *J. Neurosci.* **17:**8246–8258

Santacruz-Toloza, L., Huang, Y., John, S.A., Papazian, D.M. 1994. Glycosylation of shaker potassium channel protein in insect cell culture and in *Xenopus* oocytes. *Biochemistry* **33:**5607–5613

Scott, V.E., Muniz, Z.M., Sewing, S., Lichtinghagen, R., Parcej, D.N., Pongs, O., Dolly, J.O. 1994. Antibodies specific for distinct Kv subunits unveil a hetero-oligomeric basis for subtypes of alpha-dendrotoxin-sensitive K^+ channels in bovine brain. *Biochem* **33:**1617–1623

Scott, V.E., Parcej, D.J., Keen, J.N., Findlay, J.B., Dolly, J.O. 1990. Alpha-dendrotoxin acceptor from bovine brain is a K^+ channel protein. Evidence from the N-terminal sequence of its larger subunit. *J. Biol. Chem.* **265:**20094–20097

Shen, N.V., Chen, X., Boyer, M.M., Pfaffinger, P.J. 1993. Deletion analysis of K^+ channel assembly. *Neuron* **11:**67–76

Sheng, M., Liao, Y.J., Jan, Y.N., Jan, L.Y. 1993. Presynaptic A-current based on heteromultimeric K^+ channels detected in vivo. *Nature* **365:**72–75

Shi, G., Kleinklaus, A.K., Marrion, N.V., Trimmer, J.S. 1994. Properties of Kv2.1 K^+ channels expressed in transfected mammalian cells. *J. Biol. Chem.* **269:**23204–23211

Shi, G., Nakahira, K., Hammond, S., Schechter, L.E., Rhodes, K.J., Trimmer, J.S. 1996. Kvβ2 β-subunits promote K^+ channel surface expression through effects early in biosynthesis. *Neuron* **16:**843–852

Shih, T.M., Goldin, A.L. 1997. Topology of the Shaker potassium channel probed with hydrophilic epitope insertions. *J. Cell Biol.* **136:**1037–1045

Thornhill, W.B., Wu, M.B., Jiang, X., Wu, X., Morgan, P.T., Margiota, J.F. 1996. Expression of Kv1.1 delayed rectifier potassium channels in Lec mutant Chinese hamster ovary cell lines reveals a role for sialidation in channel function. *J. Biol. Chem.* **271:**19093–19098

Trimmer, J.S. 1991. Immunological identification and characterization of a delayed rectifier K^+ channel polypeptide in rat brain. *Proc. Nat. Acad. Sci. USA* **88:**10764–10768

Trimmer, J.S. 1998. Regulation of ion channel expression by cytoplasmic subunits. *Curr. Opin. Neurobiol.* **8:**370–374

Wang, H., Kunkel, D.D., Martin, T.M., Schwartzkroin, P.A., Tempel, B.L. 1993. Heteromultimeric K^+ channels in terminal and juxtaparanodal regions of neurons. *Nature* **36:**75–79

Zona, C., Eusebi, F., Miledi R. 1990. Glycosylation is requierd for maintenance of functional voltage-activated channels in growing neocortical neurons of the rat. *Proc. R. Soc. Lond. B. Biol. Sci.* **239:**119–127