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K^+ Channel Subunit Isoforms with Divergent Carboxy-Terminal Sequences Carry Distinct Membrane Targeting Signals

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Abstract. Kv3 K^+ channel genes encode multiple products by alternative splicing of 3′ ends resulting in the expression of K^+ channel proteins that differ *only* in their C-termini. This divergence does not affect the electrophysiological properties of the channels expressed by these proteins. A similar alternative splicing with unknown function is seen in K^+ channel genes of other families. We have investigated the possibility that the alternative splicing serves to generate channel subunits with different membrane targeting signals by examining the sorting behavior of three alternatively-spliced Kv3.2 isoforms when expressed in polarized MDCK cells. Two Kv3.2 proteins, Kv3.2b and Kv3.2c were expressed predominantly in the apical membrane, while Kv3.2a was localized mainly to the basolateral side (thought to be equivalent to the axonal and somatodendritic compartments in neurons, respectively). The Kv3.2 mRNA transcripts used in these studies are *identical* except for their 3′ sequence, encoding the extreme C-terminal domain of the protein and the 3′UTR of the mRNA. However, the proteins achieve the same localizations in MDCK cells when expressed from constructs containing or lacking the 3′UTR, indicating that the differential localization is due to targeting signals present in the C′ terminal domain of the protein. These results suggest that the alternative splicing of Kv3 genes is involved in channel localization. Since the precise localization of any given ion channel on the neuronal surface has significant functional implications, the results shown here suggest an important function for the alternative splicing of 3′ ends seen in many K^+ channel genes.

Key words: Potassium channels — Membrane targeting — Protein sorting — Alternative splicing — Targeting signals — Kv3

Introduction

Potassium channels constitute a very diverse group of ion channels and are composed of variable combinations of subunits encoded in large multigene families (Hille, 1992; Rudy, 1988; Jan & Jan, 1990; Perney & Kaczmarek, 1991; Rudy, Kentros & Vega-Saenz de Miera, 1991; Salkoff et al., 1992; Pongs, 1992; Vega-Saenz de Miera et al., 1994; Gutman & Chandy, 1995; Doupnik, Davidson & Lester, 1995; Robertson, Warmke & Ganetzky, 1996). This diversity contributes to the ability of specific neurons to respond uniquely to a given input. One of these groups of genes, the Kv family, encodes subunits of tetrameric voltage-gated K^+ channels, and is divided into several subfamilies based on sequence similarities and hence probable evolutionary relationships (Jan & Jan, 1990; Perney & Kaczmarek, 1991; Rudy et al., 1991*a;* Salkoff et al., 1992; Pongs, 1992). Subunits of the same subfamily, but not from different subfamilies, can form heteromeric channels, suggesting that each group of genes encodes a distinct system of channels (Christie et al., 1990; Isacoff, Jan & Jan, 1990; Ruppersberg et al., 1990; McCormack, K. et al., 1990; Covarrubias, Wei & Salkoff, 1991; Vega-Saenz de Miera et al., 1994). One of these subfamilies known as Kv3 consists of four genes (Kv3.1, Kv3.2, Kv3.3 and Kv3.4; Vega-Saenz de Miera et al., 1994), but each Kv3 gene encodes multiple products by alternative splicing of 3′ ends re-

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sulting in the expression of K^+ channel proteins that differ only in short C-terminal sequences (Luneau et al., 1991*a,b;* Vega-Saenz de Miera et al., 1994). There is a total of 12 Kv3 transcripts known which are expressed in several neuronal populations in the CNS (Perney et al., 1992; Rudy et al., 1992; Vega-Saenz de Miera et al., 1994; Weiser et al., 1994, 1995; Moreno et al., 1995). Voltage-gated K^+ channels containing Kv3 subunits arising from different genes display different electrophysiological properties when expressed in heterologous expression systems (Vega-Saenz de Miera et al., 1994). However, alternatively spliced products from the same gene express identical currents, and therefore the functional role for the alternative splicing of these genes is not known (Vega-Saenz de Miera et al., 1994). A similar alternative splicing of 3′ ends of unknown function is seen in other Kv genes and in K^+ channel genes of other families (Lau et al., 1994; Kamb, Tseng-Crank & Tanouye, 1988; Schwartz et al., 1988; Pongs et al., 1988; Attali et al., 1993; Zhu et al., 1995; Wei et al., 1996).

One possibility that has not been previously explored experimentally is that the alternative splicing serves to generate channel subunits with different membrane targeting signals. Since neurons are assymetric cells consisting of structural domains with specific functions, the role of any given K^+ channel will depend on its precise location on the neuronal surface (Kandel, Schwartz & Jesell, 1991; Llinas, 1988; Hille, 1992). Protein localization in different membrane domains depends on various specialized sorting mechanisms, as well as on selective exclusion and retention mechanisms such as stabilization by components of the submembrane cytoskeleton, or interactions with matrix proteins (Kelly & Grote, 1993; Nelson 1992; Wollner & Nelson 1992; Craig & Banker, 1994; Mostov et al., 1992; Rodriguez-Boulan & Powell, 1992; de Hoop & Dotti, 1993).

It has been postulated that neurons and epithelial cells utilize similar mechanisms to target membrane proteins (Dotti & Simons, 1990; Nelson, 1992; Craig & Banker, 1994; Ahn et al., 1996; Rodriguez-Boulan & Powell, 1992; de Hoop & Dotti, 1993; Kelly & Grote, 1993). In both cases sorting of plasma membrane proteins takes place at the trans-Golgi network (TGN). Here proteins are thought to be separated into distinct transport vesicles destined for either the basolateral or the apical membrane in the case of epithelial cells and the somatodendritic or axonal compartments, respectively, in the case of neurons. To explore the possibility that the alternative-splicing of Kv3 genes plays a role in channel localization we have investigated the targeting behaviour of three alternatively-spliced products (Kv3.2a, Kv3.2b and Kv3.2c) of the Kv3.2 gene after transfection into Madin-Darby Canine Kidney (MDCK) cells. MDCK cells form polarized monolayers with epithelial properties in culture (Cereijido et al., 1978), and are a classical cell model for the study of the targeting of membrane proteins of both neuronal and epithelial origin (Dotti & Simons, 1990; Rodriguez-Boulan & Powell, 1992; Nelson, 1992; Mostov et al., 1992; de Hoop & Dotti, 1993; Craig & Banker, 1994; Ahn et al., 1996).

We present evidence showing that the proteins explored in this study are expressed in different membrane domains of MDCK cells and we localize the differential targeting determinants to the C-terminal domain of the proteins. These results lend support to the hypothesis that the alternative splicing of Kv3 genes is involved in channel localization, and raise the possibility that the alternative splicing of 3′ ends of other K^+ channel genes has a similar role. The results shown here have been presented in Abstract form (Ponce et al., 1995).

Materials and Methods

Construction of Recombinant Vectors

For transfection, the cDNAs were subcloned into PCVN (a kind gift of Dr. Rich Chris, NYUMC), a mammalian expression vector that features an SV40 promoter, an ampicilin resistant gene for selection in bacteria, and a neomycin resistant gene for selection in mammalian cells (Moreno et al., 1995). The Kv3.2a-PCVN construct was described in Moreno et al., (1995). The Kv3.2b cDNA (a kind gift of Dr. Rick Swanson, Merck Laboratories) cloned in pGEMa and the Kv3.2c cDNA in pBluescript were cut with SfiI-NotI and XbaI-XhoI, respectively, gel purified, blunt-ended with Klenow and ligated to EcoRV digested, and dephosphorylated PCVN. The orientation of the cDNA inserts was assessed by restriction mapping. Selected colonies were grown and plasmid DNA purified with Qiagen resin (Qiagen, Germany), following suppliers protocols.

Cell Culture

MDCK cells were obtained from Dr. Enrique Rodriguez-Boulan (Cornell University Medical Center). They were cultured in Dulbbeco's Modified Eagle Medium (GIBCO) supplemented with 10% Fetal Bovine Serum (GIBCO) in the presence of Penicillin and Streptomycin and maintained at 37°C in 5% CO2 in 100 mm dishes (Falcon). For passage the monolayers were rinsed twice with PBS without Ca^{2+}, and incubated with 0.05% trypsin-EDTA (GIBCO) for 10 min. Cells were further dissociated mechanically and diluted 1 to 10 in new 100 mm dishes. The media was changed every 3 days and cells passaged every 5 days.

Expression of Kv3 Proteins in MDCK Cells

To prepare stably transfected cell lines, the cells were trypsinized one day prior to transfection, plated at 60% confluence in 100 mm dishes, and transfected with recombinant pCVN plasmids (Moreno et al., 1995) utilizing DOTAP (Boeringher-Manheim) according to the manufacturer's protocols, in the presence of geneticin (1 gr/lt, GIBCO) to select transfected colonies. The selected colonies grown in Geneticin, were tested for expression of the foreign genes by Western blot analysis of solubilized membrane proteins as well as by electrophysiological

and immunohistochemical analysis. For immunohistochemical study, the transfected cells were plated at confluence on sterile 12 mm circular glass coverslips (Fisher) and grown in Dulbeco's modified Eagle Medium (GIBCO) supplemented with 10% fetal bovine serum in the presence of Penicillin, Streptomycin and Geneticin, and grown for four to five days on 24 well plates to allow maturation of the monolayer.

Preparation of Membrane Extracts

Membrane fractions were prepared from 3-to-5-day-old monolayers grown on 150 mm petri dishes. The monolayers were rinsed twice with ice-cold PBS with 1 mM Ca^{2+} (Ca-PBS), scraped and dounce homogenized in an ice-cold lysis solution containing 25 mM potassium phosphate buffer (pH 7.4), 2 mM EDTA and a cocktail or protease inhibitors (1 μM pepstatin A, 0.2 mM PMSF and 1 mM iodoacetamide). Membranes were pelleted at 35,000 × g for 1 hr, resuspended in the same solution at 2–3 mg/ml, and stored at −70°C. Before use the membranes were thawed and solubilized for 1 hr in a 2% Triton-X100 solution containing 50 mM potassium phosphate buffer pH 7.4, 50 mM KCl, 2 mM EDTA and the cocktail of protease inhibitors. The suspension was spun at 35,000 × *g* to remove nonsolubilized material, and the top 2/3 of the supernatant used for further procedures.

Immunoprecipitation

Before immunoprecipitation, 300 μl of solubilized membranes (~400 μg protein) were precleared for 30 min at 4°C with protein A-sepharose beads (Pharmacia). After removing the beads, the extracts were incubated for 4 hr at 4°C with Kv3.2 antibodies at a 1:30 dilution. At the end of the incubation period fresh protein A-sepharose beads were added and the suspension incubated for 2–3 hours at 4°C. The complexed beads were collected and washed 2 times in 1% Triton-X100 in 50 (mM): Tris, 150 NaCl, 1 EDTA, 1 EGTA, pH 7.4. Proteins were then extracted with a sample buffer (10% (vol) glycerol, 5% (vol) β-mercaptoethanol; 60 mM Tris-HCl pH 6.8; 0.001% (weight) bromphenol blue and 3% SDS), heated for 3 minutes at 80°C and electrophoresed in a 8% SDS polyacrylamide gel (Harlow & Lane, 1988). For immunoblotting, the electrophoresed proteins were dry transferred onto a Nitrocellulose membrane (Bio Rad). The membrane was blocked with 5% dry milk, 0.1% Tween 20 in PBS for 1 hr, followed by an incubation with Kv3.2 antibodies at a 1:300 dilution in the same solution. The membranes were rinsed 3× for 15 min each with PBS containing 1% Tween 20 and incubated with peroxidase-linked antirabbit secondary antibody (Amersham) at a 1:200 dilution. Bound antibodies were detected using chemilluminescence with an ECL detection kit (Amersham).

Immunohistochemical Analysis in MDCK Cells

The preparation and characterization of polyclonal antibodies to Kv3.2 raised in rabbits, has been previously described (Moreno et al., 1995). These antibodies have been shown to be highly specific and not to cross-react with other Kv3 proteins (Moreno et al., 1995 and Weiser et al., 1995). MDCK cell monolayers in coverslips were rinsed twice with Ca-PBS, fixed with 4% PFA in Ca-PBS for 20 min, permeabilized with PBST (0.2% Triton X-100 in Ca-PBS) for 15 min, and incubated for 1 hr in a blocking solution (PGBA) containing 10% Fetal Bovine Serum, 1% BSA, 0.2% normal goat serum, 0.2% Triton X-100 in PBS) and 40 μl/ml Avidin (Vector), rinsed 3 × 15 min with PBST, and then incubated with the primary antibodies (1:300 for Kv3.2) in PGBA plus 40 μl/ml of Biotin (Vector) in a humidified chamber for 2–3 hr at 37°C, or overnight at room temperature. To enhance the signal, the samples were then incubated with biotinylated antirabbit IgG (Boehringer-Manheim) diluted 1:200 in PGBA for 1 hr, rinsed 3 × 15 min with PBS and incubated with either streptavidin-fluorescein or streptavidin-Texas Red (Vector) diluted 1:200 in PBS. Samples were rinsed 3 × 15 min with PBS and mounted on slides with Prolong (Molecular Probes). For the double staining experiments the following monoclonal antibodies, raised in mouse, were used: anti- Na-K ATPase (at a 1:100 dilution), anti-gp 114 glycoprotein (at a 1:10 dilution) or anti-gp 135 glycoprotein (at a 1:50 dilution). Both primary antibodies (one against a channel protein and one against an internal MDCK marker) were added at the same time and then the samples stained serially with species-specific biotinylated antirabbit IgG (Boehringer-Manheim), followed by incubation with streptavidin-fluorescein, and finally with Texas Red conjugated anti mouse secondary antibodies (Boehringer-Manheim). Images were obtained by Laser Scanning Confocal Microscopy (LSCM) in a Sarastro 2000 system (Molecular Dinamycs) equipped with a Silicon Graphics (SGI) Indy computer with Imagespace software for acquisition and analysis of data. The data was stored in magneto-optical disks. For graphic analysis and preparation of figures the files were transferred to a Macintosh computer with graphics software (Adobe Photoshop).

Electrophysiological Analysis

Whole cell currents were obtained at room temperature by the whole-cell configuration of the patch-clamp technique (Hamill et al., 1981) with an Axopatch 1A amplifier (Axon Instruments, Foster City, CA). The patch pipettes contained a solution of (mM): 130 KCl, 10 EGTA, 1 $MgCl_2$ and 10 Hepes (pH adjusted to 7.4 with KOH). The extracellular solution contained 135 NaCl, 3.5 KCl, 1.5 $CaCl_2$, 1.0 $MgCl_2$, 5.0 Glucose and 10 Hepes (pH adjusted to 7.4 with NaOH). Seal resistance was typically >10 GΩ. Recordings were obtained with partial series resistance compensation (50%–70%) and most of the cell capacitance cancelled. The currents were low-pass filtered at 2 kHz using an 8 pole Bessel filter (Frequency Devices) and digitized at 2.5 kHz. Subtraction of leak and remaining capacitance was obtained using a P/4 protocol. To generate voltage clamp protocols, and for data acquisition and analysis we used the PCLAMP software (Axon Instruments).

Expression of Kv3 Transcripts in MDCK Cells and Brain Tissue

First strand cDNA was synthesized with reverse transcriptase (BRL) from total RNA isolated from mature MDCK cell monolayers or brain and used for 35 cycles of PCR as described in Vega-Saenz de Miera and Lin (1990) using the following degenerate oligonucleotide primers:

Sense:
5′-C TCG AAT TCI TT(C/T) TG(C/T) (C/T)T(N) (G/A)A(A/G) AC(N) CA-3′
Antisense:
5′-CTC GAA TTC GGA (G/A)TA (G/A)TA CAT (N)(C/G)C(G/A)AA(G/A)TT-3′

These primers amplify the sequence flanking from the beginning of the S1-S2 linker to the end of the S6 membrane spanning domain of all four mammalian Kv3 genes and the fly Shaw homologue. In mammals the expected product of the PCR reaction ranges from 742 bp for Kv3.3 to 715 bp for Kv3.2.

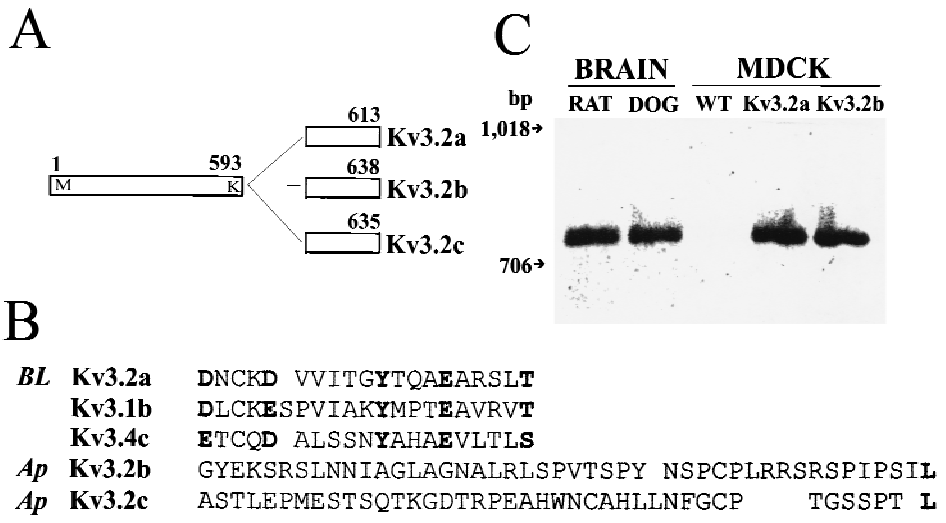

Fig. 1. (*A*) Diagram of Kv3.2 K^+ channel subunit isoforms. Kv3.2 proteins have identical sequence from the starting methionine to lysine 593 and then diverge into different short C-terminal sequences. (*B*) Comparison of the C-terminal domains of five Kv3 proteins including the three whose targeting behavior was investigated in this study. Note the conserved tyrosine and negatively charged residues in Kv3.2a (basolateral), Kv3.1b and Kv3.4c. The two proteins that are expressed apically terminate in a hydrophobic amino acid and have a serine or a threonine 1 or 2 residues from the C-terminal residue. Kv3.2a, Kv3.1b and Kv3.4c terminate in threonine or serine. (*C*) MDCK cells do not express Kv3 mRNA transcripts. Agarose gel of amplified products of PCR reactions with degenerate primers to four known Kv3 genes using as template first strand cDNA synthesized from RNA obtained from rat and dog brain (first and second lanes, respectively), untransfected MDCK cells (WT, third lane), and Kv3.2a and Kv3.2b transfected MDCK cells (fourth and fifth lanes, respectively).

Results

MDCK Cells do not Express Kv3 mRNA Transcripts

The Kv3.2 gene generates three to four alternatively spliced proteins that differ only in short C-terminal sequences (Rudy et al., 1992; Luneau et al., 1991*b;* Vega-Saenz de Miera et al., 1994; *see* Fig. 1*A* and *B*). Most neurons containing Kv3 proteins express more than one isoform (Rudy et al., 1992) and antibodies that distinguish among alternatively spliced products are not available. Therefore, to investigate whether different Kv3.2 isoforms have distinct targeting determinants, it is necessary to analyze the subcellular localization of individual Kv3.2 subunits in a model system. Such model system should not express native Kv3 subunits in order to avoid the possibility that these subunits redirect the heterologous protein by forming heteromultimeric complexes as seen with $GABA_A$ receptors (Perez-Velazquez & Angelides, 1993).

We chose for this study Madin-Darby Canine Kidney (MDCK) cells, a cell line forming polarized, epithelial-like, monolayers in culture (Cereijido et al., 1978; Contreras et al., 1989). MDCK cells can be transfected and have been used extensively as a model system to study the targeting of heterologous membrane proteins of both epithelial and neuronal origin (Rodriguez-Boulan & Powell, 1992; Ahn et al., 1996; Craig & Banker, 1994).

We first utilized RT-PCR to explore whether MDCK cells express any Kv3 products, and thus whether they are suitable to investigate the presence of targeting signals in Kv3.2 proteins. RNA was extracted from MDCK cells grown under conditions that result in the formation of polarized epithelial monolayers. First strand cDNA synthesized with reverse transcriptase was used for a PCR reaction utilizing degenerate primers capable of amplifying the constant region (prior to the alternatively spliced 3′ ends) of all known Kv3 cDNAs. The primers were designed based on the sequence of the four known Kv3 genes in mammals and the *Drosophila* Shaw gene, the Kv3 homologue in flies, and have proven useful to amplify transcripts of this subfamily in several vertebrate and invertebrate species (Vega-Saenz de Miera et al., 1994).

No amplification product was detected when cDNA derived from wild type MDCK cells was used (Fig. 1*C*). However, reactions with cDNA derived from Kv3.2a or Kv3.2b transfected MDCK cells produce an amplification band of similar size to that obtained with cDNA derived from rat or dog brain.

Immunoblot Analysis of Kv3.2 Proteins Expressed in MDCK Cells

Kv3.2 proteins were immunoprecipitated from solubilized membranes obtained from transfected MDCK cells and analyzed in immunoblots. A prominent band, simi-

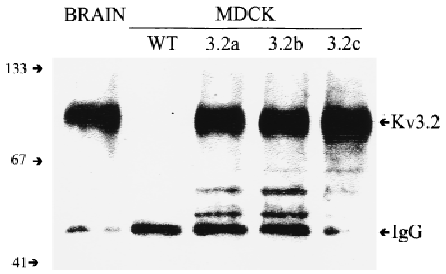

Fig. 2. Immunoblot analysis of transfected Kv3.2 proteins expressed in MDCK cells. Immunoblots with Kv3.2 antibodies of immunoprecipitation reactions with Kv3.2 antibodies of detergent extracts of membranes from rat brain (BRAIN), untransfected MDCK cells (WT), and Kv3.2a, Kv3.2b and Kv3.2c transfected MDCK cells. A band, similar in size to the band obtained from brain membranes is seen in membrane extracts from transfected MDCK cells.

lar in size to the band obtained with rat brain membranes (BRAIN) was detected in extracts from membranes from transfected MDCK cells (Kv3.2a, Kv3.2b and Kv3.2c), but not from membranes from untransfected MDCK cells (WT) (Fig. 2). The size of this band (~80 kDA) is larger than the size of the core polypeptides (65–70 kDa), indicating that as in brain (Moreno et al., 1995), the Kv3.2 proteins undergo postranslational modification, probably glycosylation, in MDCK cells. Minor bands of smaller molecular weight were also seen in transfected MDCK cells but not in brain. These bands may represent unprocessed Kv3.2 proteins or proteolytic products.

Kv3.2 Proteins Expressed in MDCK Cells Reach the Membrane and Form Functional Channels

To determine whether the Kv3.2 proteins expressed in transfected MDCK cells reach the membrane and are capable of forming functional channels, we compared the electrophysiological properties of transfected and wild-type MDCK cells utilizing the whole cell configuration of the patch-clamp technique (Hamill et al., 1981). MDCK cells have intrinsic voltage-dependent K^+ currents that are prominent at voltages more positive than +40 mV (Ponce & Cereijido, 1991), therefore, we only used step depolarizations to voltages more negative than this value. Transfected cells had outward currents which were much larger than wild type cells in the voltage range used (Fig. 3). The extra current seen in transfected cells starts activating when the membrane is depolarized between −20 and −10 mV, and has delayed-rectifier-type kinetics as for Kv3.2 currents expressed in *Xenopus* oocytes or Chinese Hamster Ovary (CHO) cells (McCormack, Vega-Saenz de Miera & Rudy, 1990; Vega-Saenz de Miera et al., 1994; Moreno et al., 1995). The currents are also similar in pharmacological properties (*data not shown*).

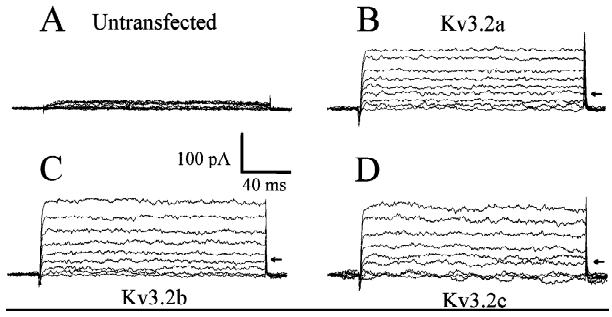

Fig. 3. Kv3.2 proteins expressed in MDCK cells reach the membrane and form functional channels. Representative examples of ion currents obtained from MDCK cells in monolayers using whole cell patch clamp. (*A*) series of currents obtained from an untransfected cell during depolarizations from −60 to +40 mV, in 10 mV increments, from a holding potential of −90 mV. (*B*), (*C*) and (*D*) currents during the same depolarizing series as in (*A*) from cells transfected with Kv3.2a, Kv3.2b and Kv3.2c, respectively. Arrows point to the currents recorded at −10 mV. Whole cell currents were obtained at room temperature as described in Moreno et al., 1995.

Membrane Sorting of Kv3.2 Isoforms in MDCK Cells

MDCK cells permanently transfected with cDNAs encoding three different Kv3.2 isoforms (Kv3.2a, Kv3.2b, and Kv3.2c) were grown in cultures forming polarized monolayers and processed for immunofluorescence utilizing the Kv3.2 antibodies.

When observed in a plane parallel to the monolayer (*en face*), the immunofluorescent signals from cells expressing the Kv3.2a isoform show the pattern (Fig. 4*A*) characteristic of basolateral membrane protein localization in epithelial cells (e.g., Pietrini et al., 1994), which results from the staining of the lateral membrane surrounding each cell. In contrast, monolayers containing cells transfected with Kv3.2b or Kv3.2c (Fig. 4*B* and *C*, respectively) show the punctate pattern on the apical surface of the monolayer characteristic of apical labeling (Hanzel et al., 1991). Vertical cross sectional projections of the images were constructed from stepwise scans perpendicular to the surface of the monolayer. The results of this analysis verify the basolateral expression of Kv3.2a proteins (Fig. 4*D*) and the apical expression of Kv3.2b and Kv3.2c (Fig. 4*E* and *F*, respectively). These results were confirmed in at least three different colonies for each Kv3.2 isoform. Quantification of the fluorescent signals shows that >90% of the Kv3.2a is in the basolateral membrane and >80% of Kv3.2b and Kv3.2c in the apical domain (Fig. 5).

To confirm the conclusions reached from the analysis of the patterns of immunostaining we utilized double labeling of monolayers of Kv3.2 transfected MDCK cells with the Kv3.2 antibodies (raised in rabbit) and monoclonal antibodies raised in mouse against native MDCK

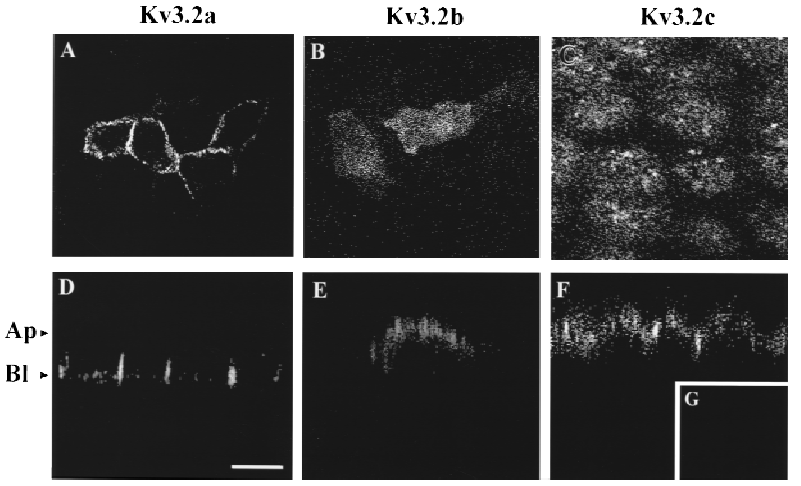

Fig. 4. Membrane sorting of Kv3.2 isoforms in MDCK cells. LSCM images of monolayers of MDCK cells stably-transfected with Kv3.2a (*A* and *D*), Kv3.2b (*B* and *E*) and Kv3.2c (*C* and *F*) cDNAs, after immunostaining with Kv3.2 antibodies. (*A, B,* and *C*) *en face* images; (*D, E,* and *F*) vertical cross sectional projections of the monolayers. (*G*) Background staining in a monolayer of untransfected cells. Note the staining of the lateral domain in Kv3.2a-transfected MDCK cell monolayers in the images obtained *en face*, while those transfected with either Kv3.2b or Kv3.2c show a punctate pattern on the apical surface. The vertical cross sectional projections of the monolayers verify the basolateral expression of Kv3.2a proteins (*D*) and the apical expression of Kv3.2b (*E*) and Kv3.2c (*F*). Scale bar: 15 μm.

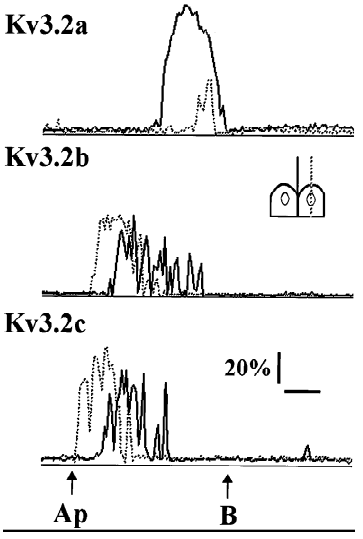

Fig. 5. The majority of Kv3.2a proteins are expressed basolaterally while the majority of Kv3.2b and Kv3.2c proteins are expressed apically. The intensity of fluorescent signals was quantified in the center of a cell (broken lines) and in the interface between two cells (contiguous lines) by scanning the image from the apical (Ap) to the basal (*B*) surface. Normalized fluorescence intensity is shown on the vertical axis and distance on the horizontal axis. This type of analysis with these and other representative experiments shows that >90% of Kv3.2a is in the basolateral membrane and >80% of Kv3.2b and >85% of Kv3.2c is in the apical domain. Horizontal scale: 6 μm.

membrane proteins with known basolateral and apical distributions. Two different fluorescent markers (fluorescein or FITC and Texas red or TR) were used to distinguish between Kv3.2 proteins (FITC, green) and the internal marker (TR, red).

In monolayers of Kv3.2a transfected cells, both the antibodies against the Na-K ATPAse, a basolateral marker (Nelson 1992; Hanzel et al., 1991) (Fig. 6*B* and *C*), and the antibodies to Kv3.2 proteins (Fig. 6*A* and *C*) stain mainly the lateral membrane. The staining of the cell in the center has similar intensities with both antibodies and appears yellow when the FITC and the TR fluorescence are superimposed (Fig. 6*C*). In monolayers of cells transfected with Kv3.2c (Fig. 6*D–I*) or Kv3.2b (*not shown*) the antibodies to the Na-K-ATPase stain the lateral membrane while the Kv3.2 antibodies stain the apical membrane (Fig. 6*D–F*); in monolayers double stained with the Kv3.2 antibodies and the antibodies to the apical gp114 (Fig. 6*G–I*) or gp 135 (*data not shown*) glycoproteins (Ojakian, Schwimmer, & Herz, 1990; Balcarova-Stander et al., 1984), the apical domain is immunostained with both types of antibodies.

Counterstaining of cell nuclei with propidium iodide (Molecular Probes), which produces a red fluorescence, has been recently introduced as a means to facilitate distinction between apical and basolateral localization of proteins detected by indirect green immunofluorescence in epithelial cells (Zurzolo & Rodriguez-Boulan, 1993). Utilizing this method we further confirmed that Kv3.2a is expressed mainly, if not exclusively, in the basolateral membrane and Kv3.2b and Kv3.2c in the apical compartment. The results for Kv3.2b are illustrated in Fig. 7 (upper panels).

Localization of Targeting Signals

Kv3.2 proteins are alternatively spliced products and differ *only* in a C-terminal domain of 20–45 residues (Fig. 1*A* and *B*). The mRNA transcripts however, differ also in the sequence following the stop codon (the 3′ untranslated sequence or 3′ UTR). To test whether the differential targeting described above is due to differences in the variable C-terminal domains of the protein or to differences in the 3′ UTRS's, we compared the localization of Kv3.2a and Kv3.2b proteins expressed in MDCK cells from constructs containing the 3′ UTR, with the location of the proteins when expressed from mutant cDNAs in which the 3′ UTR has been deleted. The Kv3.2b protein is expressed apically (Fig. 7), and the Kv3.2a protein

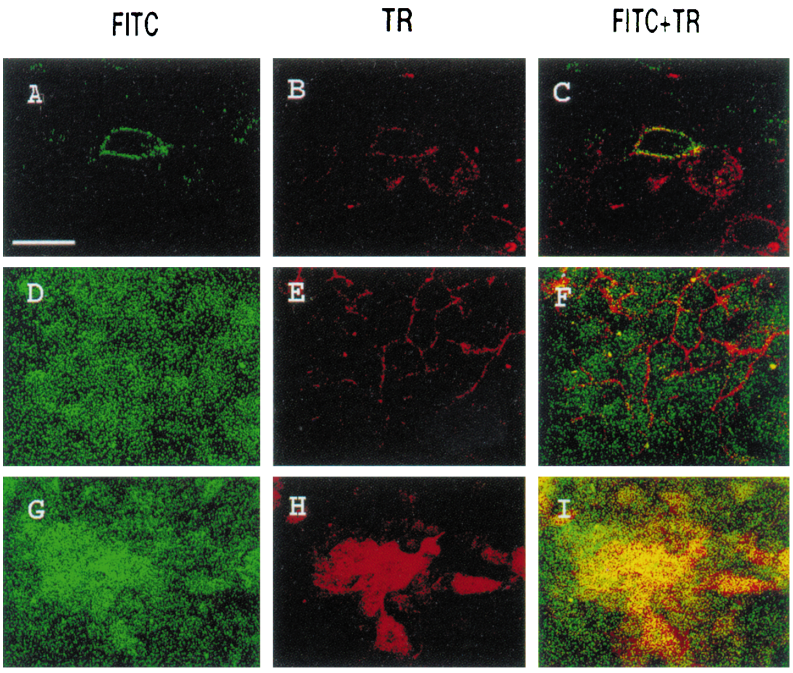

Fig. 6. Kv3.2a colocalizes with the Na-K-ATPase, a basolaterally distributed protein, and Kv3.2c colocalizes with an apical but not with a basolateral protein. (*A–C*) Monolayers of cells transfected with Kv3.2a were incubated with the antibody against Kv3.2 proteins, along with an antibody against the α subunit of Na-K-ATPase, utilizing fluorescein (FITC) to recognize Kv3.2 proteins and Texas Red (TR) to recognize the Na-K-ATPase. Both the antibody against the Na-K-ATPase (*B*) and the Kv3.2 antibodies (*A*) stain the lateral membrane. The cell in the center is strongly stained with both antibodies and appears yellow when the fluorescence of the two fluorochromes is superimposed (*C*). (*D–I*) Monolayers of cells transfected with Kv3.2c were incubated with the antibody against Kv3.2 proteins along with an antibody against the gp 114 glycoprotein (*G–I*) or an antibody against the α subunit of Na-K-ATPase (*D—F*). In the latter experiment, the antibody against the Na-K-ATPase stains the lateral membrane (*E* and *F*) while the Kv3.2 antibodies stain the apical membrane (*D* and *F*). In monolayers incubated with the antibodies to the gp114 glycoprotein and Kv3.2c (*G–I*), the apical domain is immunostained with both antibodies. Scale bar 16 μm.

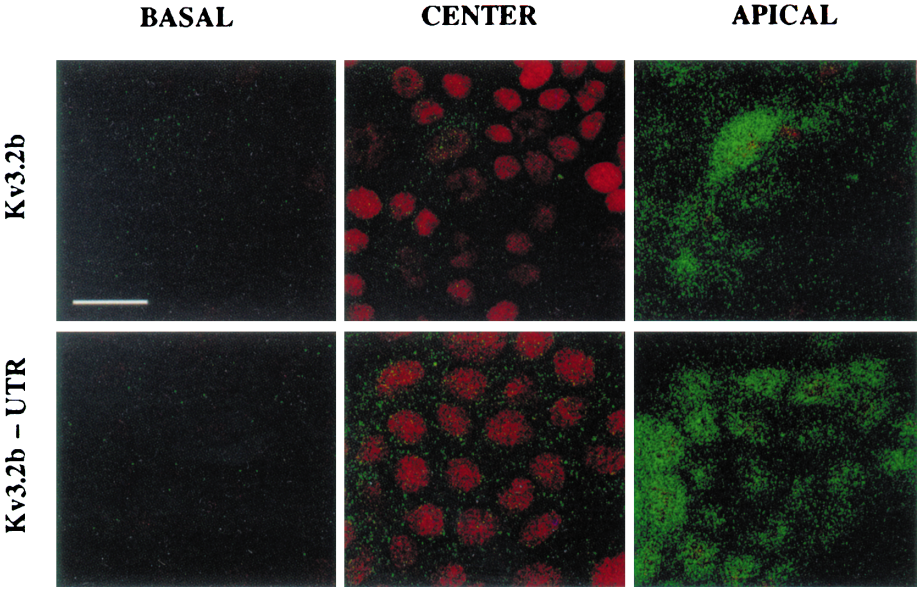

Fig. 7. Deletion of 3′ UTRs does not affect the distribution of Kv3.2 proteins. Localization of Kv3.2b proteins expressed from wild type constructs (Kv3.2b) or from constructs lacking the 3′ UTR (Kv3.2b-UTR) in MDCK cells following immunohistochemical detection of Kv3.2 proteins with FITC (green) and staining (following manufacturers protocols) of cell nuclei with propidium iodide (red). Shown are sections of the monolayers at three focusing levels: (1) Sections taken at the base of the monolayer (Basal), (2) towards the middle of the monolayer) (Center), and (3) at the apical surface of the monolayer (Apical). Scale bar: 16 μm.

basolaterally (*not shown*), whether they are expressed from a construct with or without the 3′ UTR, demonstrating that the differential targeting is due to the differences in the C′ terminal domain of the protein.

Discussion

Functional Role of the Alternative Splicing of C-Terminal Sequences of K^+ Channel Proteins

Alternative splicing generating transcripts with divergent 3′ ends is a common feature of several mammalian genes encoding components of voltage-gated K^+ channels. All four known genes of the Kv3 subfamily encode more than one protein with different C-terminal domains (Luneau et al., 1991*a,b;* Vega-Saenz de Miera et al., 1994). Isoforms with varying C-termini are also known in other Kv subfamilies such as for the products of the Kv2.1 gene (also known as DRK1, Frech et al., 1989), a member of the Kv2 subfamily (Lau et al., 1994), and Kv1.5, a member of the Kv1 subfamily (Attali et al., 1993), as well as for several members of the inward rectifier family (Zhu et al., 1995; Wei et al., 1996). In all these cases,

electrophysiological differences between the isoforms have not been observed.

In the *Drosophila* Shaker gene, there is alternative splicing of both the 5′ and the 3′ ends (Schwartz et al., 1988; Kamb et al., 1988; Pongs et al., 1988). The divergent amino termini produce subunits that express channels with different ''N'' inactivation properties (Iverson et al., 1988; Iverson & Rudy, 1990; Timpe, Jan & Jan, 1988; Zagotta, Hoshi & Aldrich, 1989). Although the isoforms resulting from the alternative splicing at the 3′ end show differences in a process known as ''C inactivation'' (Iverson et al., 1988; Iverson & Rudy, 1990; Timpe et al., 1988; Zagotta et al., 1989; Hoshi, Zagotta & Aldrich, 1991), these are due to a change in a single residue in the sixth membrane spanning domain of the transmembrane portion of the polypeptide and not to differences in the cytoplasmic C-terminal tail (Hoshi et al., 1991). *If this difference is eliminated the divergent C-terminal tails do not affect channel properties.* Moreover, the mechanism of ''C inactivation'' has now been discovered to involve structural changes of the channel's pore which does not involve the C-terminal sequence of the channel subunits (Liu, Jurman & Yellen, 1996).

Thus, although there is extensive alternative splicing generating protein isoforms with different cytoplasmic C-terminal sequences in genes encoding subunits of K^+ channels, the functional consequences of this splicing have remained mysterious. The results of this study suggest that one role for the alternative splicing is to generate isoforms with different membrane localizations. Differential targeting of alternatively spliced products was previously shown for N-CAM, but in this case the isoforms also have different modes of membrane association (GPI-anchored or transmembrane) (Powell et al., 1991). The C-terminal domains of Kv3 proteins also contain different putative sites for phosphorylation (Vega-Saenz de Miera et al., 1994). Therefore, it is possible that targeting of Kv3 subunits to different neuronal regions is associated with channel modulation by different stimuli.

Sorting Signals in Kv3.2 Channel Subunits and the Distribution of Kv3.2 K^+ Channels in CNS Neurons

Neurons are asymmetric cells consisting of several parts such as the soma, the dendrites, the axon, the axon hillock, the presynaptic terminals and the nodes of Ranvier, each responsible for a different aspect of the function of the cell (Kandel et al., 1991). Therefore, the functional implications of any given ion channel will depend on their precise localization on the neuronal surface. The finding that Kv3 subunits are targetted to different membranes in model polarized epithelial cells depending on the sequence of their C-terminus thus suggests an important function for the generation by alternative splicing of isoforms with divergent C-termini. Future experiments will address the relationship between the basolateral and apical targeting of Kv3 K^+ channel subunits in model MDCK cells and their targeting behavior in different neuronal membrane compartments. In other cases it has been found, by comparing the expression of heterologous proteins in epithelial and neuronal cells in culture, that the basolateral compartment of epithelial cells corresponds to the somato-dendritic compartment in neurons, and the apical to the axonal-terminal membrane (Dotti & Simons, 1990; de Hoop & Dotti, 1993; Ahn et al., 1996). It remains to be established whether this correspondence applies in the case of Kv3 proteins.

Utilizing the antibodies that recognize all Kv3.2 isoforms it has been found that native Kv3.2 proteins have different subcellular distributions in distinct neuronal populations of the rat brain (Moreno et al., 1995). For example, they are present in axons and terminals of thalamic relay neurons, but are somatic in a subset of neurons in deep layers of the cerebral cortex, a subset of interneurons in the hippocampus, and neurons in the red nucleus, the globus pallidus, the substantia nigra pars reticulata, the dorsal cochlear nucleus, and deep cerebellar nuclei. Strong axonal staining, but weak somatic labeling, is also seen in the subthalamic nucleus as well as in a group of fibers connecting the subthalamic nucleus with other structures (presumably the globus pallidus). The results presented here suggest that specific combinations of Kv3.2 isoforms expressed in different neuronal populations could be determining whether the Kv3.2 channel complex is somatic or axonal. Antibodies that recognize specific Kv3.2 isoforms will help confirm this hypothesis.

Towards an Understanding of the Nature of the Targeting Signals in Kv3 Subunits

The studies described here provide evidence for differential targeting signals in the cytoplasmic C-terminal domain of Kv3 proteins. Most of what is known about the signals by which membrane proteins are recognized by the targeting machinery comes from studies with heterologous proteins expressed in epithelial cells, and chiefly with the MDCK cell line. Both basolateral and apical targeting determinants have been found in a variety of membrane proteins (Hopkins, 1991; Rodriguez-Boulan & Powell, 1992; Craig & Banker, 1994; Nelson, 1992; Matlin, 1992; Thomas & Roth, 1994; Matter, Hunziker & Mellman, 1992; Matter, Yamamoto & Mellman, 1994).

Intriguing patterns of sequence similarity emerge

when a comparison is made of the C-terminal sequence of Kv3 proteins including the three Kv3.2 isoforms studied in this paper (Fig. 1*B*). For example, the two isoforms that are expressed apically terminate in a leucine and have a serine or a threonine at position −1 or −2., while Kv3.2a, which is expressed basolaterally, terminates in a threonine. Furthermore, all other known (12 total) Kv3 proteins (*see* Fig. 3 in Vega-Saenz de Miera et al., 1994) except perhaps for Kv3.3b (whose C-terminal sequence has been difficult to obtain) terminate either in a bulky hydrophobic residue (leucine, isoleucine or valine) or a residue with an hydroxyl side chain (serine or threonine; tyrosine in one case). In all cases except for one, where the terminal amino acid is hydrophobic, there is a serine or threonine in position −1 to −3. The significance of these apparent relationships remains to be established by studying the targeting behavior of mutant Kv3 proteins. However, the results of the comparison are particularly interesting in the light of recent results showing that the extreme C-terminal sequence of several proteins, including Kv1 K^+ channels and NMDA receptors is responsible for their association with PDZ domain-containing proteins such as PSD-95 (Kornau et al., 1995; Kim et al., 1995; Niethammer, Kim & Sheng, 1996; Songyang et al., 1997). This association might be critical in the clustering and localization of these channels. It is believed that the sequence of the last 3–4 residues of these channel proteins determines their binding to the PDZ domain-containing proteins. A large number of these binding proteins have been discovered, and each appears to have strict requirements in the C-terminal sequence of their binding partners (Songyang et al., 1997). For example, several of them require terminal hydrophobic or aromatic residues and a serine, threonine or tyrosine two residues from the COOH terminus (Songyang et al., 1997). The sequence requirements for binding to these PDZ domains are reminiscent of the terminal sequences we observe in Kv3 proteins that are targeted apically. It is therefore possible that associations with some type of PDZ domain-containing proteins or related protein-protein interactions play a role in the differential targeting of alternatively-spliced Kv3 proteins.

Another feature of note are several similarities between Kv3.2a, shown to be expressed basolaterally and Kv3.1b and Kv3.4c. There is conservation of a tyrosine, a cysteine, and three negatively charged residues and these aminoacids are not present in the two isoforms targeted apically (Fig. 1*B*). This is interesting because basolateral targeting signals that depend on a critical tyrosine residue in the C-terminal tail have been found in a number of membrane proteins (Rodriguez-Boulan & Powell, 1992; Matlin, 1992; Thomas & Roth, 1994; Matter et al., 1994). In the tyrosine-based, basolateral determinant of the low density lipoprotein (LDL), clusters of 1–3 acidic amino acids close to the tyrosine, were also found to be critical for basolateral targeting (Matter et al., 1994). The proteins where tyrosine-based basolateral targeting signals have been discovered have a single transmembrane domain. If future mutagenesis studies provide support for a tyrosine-based basolateral targeting signal in Kv3 proteins, which are believed to have six membrane spanning domains (Miller, 1991; MacKinnon, 1991; Jan & Jan, 1992; Rudy et al., 1991*a;* Pongs, 1992), this will indicate that more complex membrane proteins can also use the tyrosine-based sorting signal.

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