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Determinants of Voltage-Gated Potassium Channel Surface Expression and Localization in Mammalian Neurons

Hiroaki Misonou and James S. Trimmer

Department of Pharmacology, School of Medicine, University of California, Davis, CA, USA

Neurons strictly regulate expression of a wide variety of voltage-dependent ion channels in their surface membranes to achieve precise yet dynamic control of intrinsic membrane excitability. Neurons also exhibit extreme morphological complexity that underlies diverse aspects of their function. Most ion channels are preferentially targeted to either the axonal or somatodendritic compartments, where they become further localized to discrete membrane subdomains. This restricted accumulation of ion channels enables local control of membrane signaling events in specific microdomains of a given compartment. Voltage-dependent K^+ (Kv) channels act as potent modulators of diverse excitatory events such as action potentials, excitatory synaptic potentials, and Ca^{2+} influx. Kv channels exhibit diverse patterns of cellular expression, and distinct subtype-specific localization, in mammalian central neurons. Here we review the mechanisms regulating the abundance and distribution of Kv channels in mammalian neurons and discuss how dynamic regulation of these events impacts neuronal signaling.

Keywords electrical excitability, Kv channels, trafficking, Kvβ subunits, KChIPs, clustering

INTRODUCTION

The brain is a unique tissue in that changes in function arise not from changes in cellular composition but from the plasticity of a fixed population of its neuronal constituents. Much recent effort has focused on mechanisms of synaptic plasticity, whereby short- and long-term changes in synaptic strength thought to be associated with learning and memory are achieved. However, dynamic regulation of the intrinsic electrical excitability of neurons also confers tremendous plasticity to neuronal function (Daoudal & Debanne, 2003). Neurons use diverse mechanisms to precisely control electrical excitability. In doing so, they effectively control the relationship between synaptic input to dendrites and neurotransmitter release from axon terminals. Neurons can fail to effectively regulate an acceptable range of intrinsic excitability and fall into pathophysiological conditions such as epilepsy (Chang & Lowenstein, 2003). However, a tremendous normal physiological range of intrinsic excitability is observed in mammalian neurons. Moreover, dynamic changes in excitability are observed in neurons in response to developmental, hormonal, and synaptic cues (Levitan, 1999).

The intrinsic electrical excitability of neurons is conferred by the repertoire of voltage-gated ion channels active in the neuronal membrane (Llinas, 1988). These membrane proteins act as selective channels for the flux of specific ions into or out of cells (Hille, 2001). Neurons express a wide variety of ion channels with specific selectivity to Na^+, Ca^{2+}, Cl^-, and K^+. These channels also differ in the rate of ion flux, the level and kinetics of their activation and inactivation at different membrane potentials, and their sensitivity to modulation (Hille, 2001). Altering the density or functional characteristics of these neuronal ion channels could profoundly affect neuronal signaling (Daoudal & Debanne, 2003).

Voltage-dependent K^+ (Kv) channels are especially diverse components of the channel repertoire that determine a neuron's intrinsic electrical excitability (Pongs, 1999). Neurons express a wide variety of Kv channels that can contribute to diverse aspects of neuronal signaling, depending on the functional characteristics, abundance, and distribution of the channel subtypes (Song, 2002). In simple terms, the biophysical properties of Kv channels determine at which membrane potentials they will be available for opening, whether and how quickly they open in response to particular changes in membrane potential, and if they do open how long they remain open and at what rate do

Editor: Irwin B. Levitan
Address correspondence to James S. Trimmer, Department of Pharmacology, School of Medicine, University of California, One Shields Avenue, Davis, CA 95616, USA. E-mail: jtrimmer@ucdavis.edu

they flux K^+ across the membrane (MacKinnon, 2003). Kv channels as a group vary much more extensively in these biophysical properties than other voltage-gated channels (Jan & Jan, 1997). While these inherent differences in biophysical properties are clearly encoded within the primary structure of the particular channel subtype, they can also be modified through posttranslational events, including covalent modifications (usually phosphorylation) and noncovalent protein–protein interactions (Jonas & Kaczmarek, 1996; Yi *et al.*, 2001). The biophysical characteristics can also be dramatically modified pharmacologically, a fact that serves as the basis for a diverse array of promising therapeutics (Wickenden, 2002). However, it remains that specific modulators for many Kv channel subtypes have not yet been identified.

The contribution of a particular Kv channel to governing neuronal excitability also depends on its abundance in the neuronal membrane. Membrane protein biosynthetic mechanisms dictate that translation occurs on rough endoplasmic reticulum (ER)-bound ribosomes, and channel proteins, like other integral membrane proteins, are initially inserted into the ER membrane. Exit from the ER is highly regulated, both by ER quality control machinery that detects the folding state of proteins and by trafficking control machinery that recognizes specific trafficking determinants on certain membrane proteins, and determines rate of incorporation into ER-derived transport vesicles (Ellgaard & Helenius, 2003; Trombetta & Parodi, 2003). As we discuss in detail below, Kv channels are notable for the dramatic differences in trafficking exhibited by highly related subtypes. This controls both the abundance and subunit composition of Kv channels found in the plasma membrane, where they influence neuronal excitability.

That neurons exhibit such complex morphology results in the fact that subcellular localization of Kv channels can dramatically impact specific and localized neuronal functions. For example, certain Kv channels are found primarily on axon terminals, where they play a prominent role in regulating the electrical events controlling neurotransmitter release (Meir *et al.*, 1999). Other Kv channels are found on distal portions of dendrites, where they impact signaling events at the subset of synapses arising from neural connections localized to these regions (Johnston *et al.*, 2003). As Kv channel synthesis occurs in the rough ER located in the neuronal somata, specific mechanisms must exist to first sort these channels into axon- or dendrite-destined transport vesicles, and then insert and/or retain the delivered channels at these highly restricted and precise locations. While much has been learned about the general mechanisms used to establish the two major polarized (somatodendritic and axonal) domains of neurons (Horton & Ehlers, 2003), the molecular machinery and determinants for the more specialized localizations exhibited by Kv channels (e.g., perisynaptic, juxtaparanodal, axon initial segments, distal dendrites) are as yet undefined.

This review focuses on recent insights into the distinct subcellular localization of major Kv channels and how the surface expression and specific localization of Kv channels are regulated in neurons.

MOLECULAR PROPERTIES OF Kv CHANNELS

Kv Channel Subunit Composition

Kv channels are composed of a tetrameric array of principal pore-forming and voltage-sensing α subunits. Each integral membrane subunit contains six transmembrane helices and a membrane-inserted P-loop that forms the bulk of the K^+ conduction pathway (Figure 1). Homotetrameric complexes of four identical α subunits can generally form functional voltage-gated K^+-selective Kv channels. However, in many cases these homomeric channels reach the plasma membrane in very low numbers due to trafficking constraints. As discussed below, heteromeric assembly of different Kv α subunits can yield channel complexes with not only diverse functional characteristics but also distinct trafficking efficiencies, the basis for which is discussed in detail below. Native Kv channel complexes can also contain auxiliary subunits, which in themselves cannot form functional channels but which can dramatically alter diverse aspects of Kv channel function. A growing body of evidence suggests that these auxiliary subunits can play prominent roles in regulating the exit of diverse Kv channels from the ER, as well as aspects of post-Golgi trafficking to specific subcellular domains. As discussed below, these auxiliary subunits can either be cytoplasmic peripheral membrane proteins (Kvβs, KChIPs) or integral transmembrane proteins (DPPX, MiRPs).

Kv Channel α Subunits

The K^+ channel α subunit gene family is diverse, yet some sense can be made of both evolutionary and functional relationships from the systematic nomenclature that was originally proposed by Chandy (Chandy, 1991) and is now widely accepted (Gutman *et al.*, 2003). This nomenclature is based on the chemical symbol for the principal physiologically permeant ion (K for K^+), followed by the abbreviation of the ligand, which, in the case of this review, is always voltage (v). The prototypical Kv channel α subunit polypeptides have been further divided into four families (Kv1-Kv4), based on sequence homology and on their sequence similarity to single-gene orthologues in *Drosophila* (name given in italics): Kv1 (*Shaker*), Kv2 (*Shab*), Kv3 (*Shaw*), and Kv4 (*Shal*). The human genes encoding these α subunits are named KCN*, with the four gene families assigned the letters A–D (*i.e.*, Kv1 = KCNA,

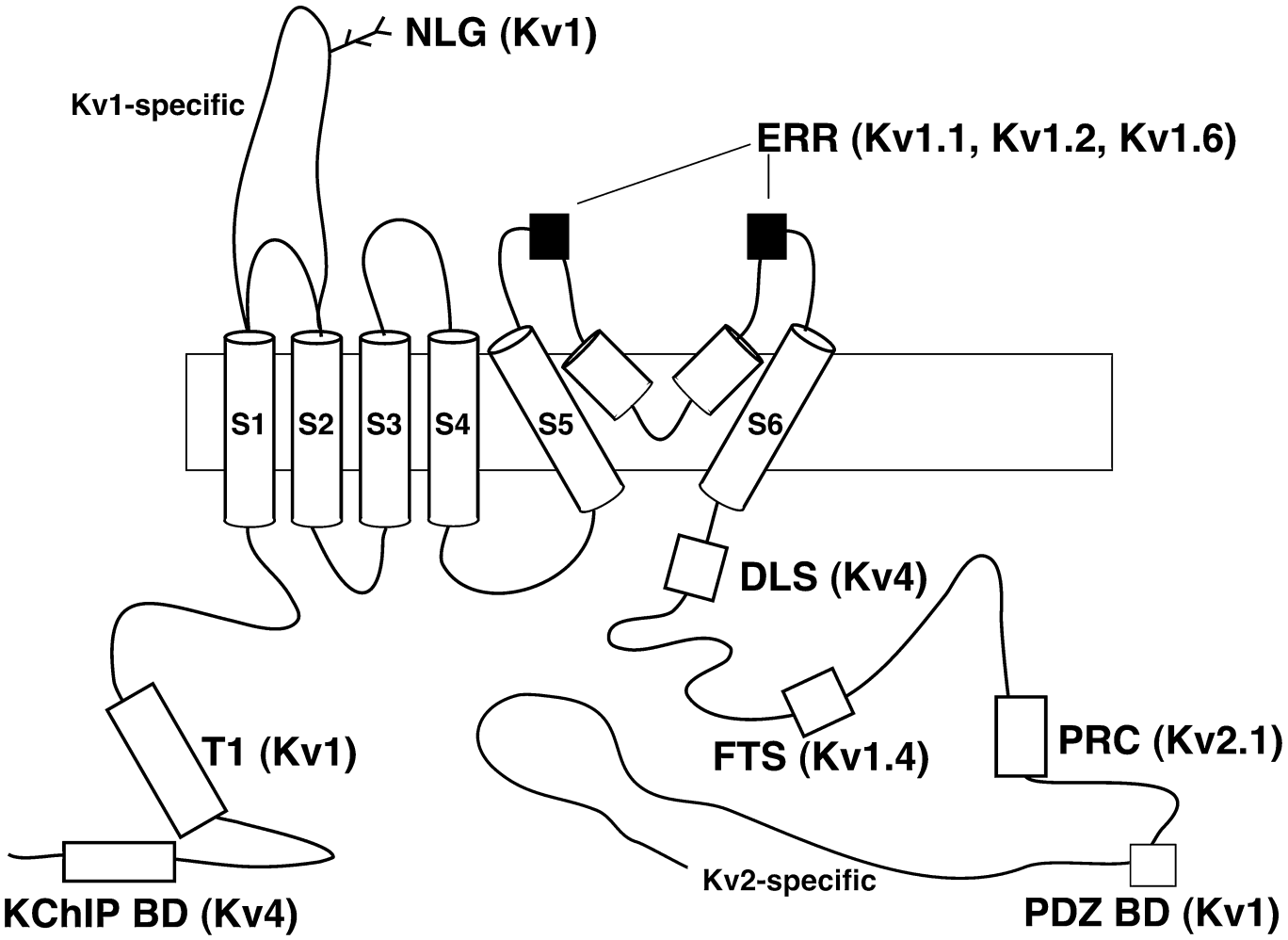

FIG. 1. Cartoon summary of predicted topology and trafficking signals on Kv α subunits. Cartoon shows the predicted transmembrane topology of Kv α subunits and sites defined as important determinants of intracellular trafficking and polarized expression. No attempt has been made to incorporate other known structural details in this cartoon. KChIP BD, KChIP binding domain important for regulating intracellular trafficking of Kv4 α subunits; T1, tetramerization domain critical for Kvβ-mediated polarized trafficking of Kv1 α subunits; NLG, N-linked glycosylation site of Kv1 α subunits used in monitoring ER to Golgi trafficking; ERR, ER retention motif found in Kv1.1, Kv1.2 and Kv1.6; DLS, dendritic localization signal in Kv4 α subunits; FTS, forward-trafficking signal in Kv1.4; PRC, proximal dendritic clustering signal in Kv2.1; PDZ BD, PDZ-binding domain in Kv1 α subunits.

Kv2 = KCNB, Kv3 = KCNC, and Kv4 = KCND). The specific gene number is then derived from the Kv nomenclature, such that Kv1.1 = KCNA1, Kv1.4 = KCNA4, Kv2.1 = KCNB1, Kv4.2 = KCND2, etc.

The biochemical approach that was so successful in the cloning of many other channels and receptors did not yield amounts of Kv channel α subunits amenable for protein sequencing. This is due to the diversity of Kv channel α subunit genes and their potential for oligomerization, and the complex repertoire of Kv channels expressed by any given excitable cell. The breakthrough for Kv channel molecular characterization came from concerted genetic and molecular analyses of K^+ channel mutants in the fruit fly *Drosophila melanogaster* (Jan & Jan, 1997). These efforts resulted in the isolation of cDNAs encoding the Kv channel α subunit encoded at the *Shaker* gene locus (Kamb *et al.*, 1987; Papazian *et al.*, 1987). It was immediately clear from the deduced *Shaker* amino acid sequence that this Kv channel α subunit strongly resembled one of the four internally repeated homologous pseudosubunit domains of a voltage-dependent Na^+ or Ca^{2+} channel. This led to the proposal, later substantiated by direct experimentation (MacKinnon, 1991), that Kv channels are comprised of functional tetramers of individual α subunits. In *Drosophila*, Salkoff and colleagues showed that in addition to *Shaker*, three other Kv channel α subunit genes

were present, named *Shab*, *Shal*, and *Shaw* (Salkoff *et al.*, 1992).

cDNAs encoding multiple members of each of the corresponding mammalian gene families (*Shab* = Kv2 or KCNB, *Shaw* = Kv3 or KCNC, and *Shal* = Kv4 or KCND) have now been isolated and expressed. Using the Shaker cDNAs as probes, Tempel and coworkers isolated the first mammalian Kv channel cDNA, Kv1.1 (Tempel *et al.*, 1988). In rapid succession, cDNAs encoding other Kv1 (*i.e.*, *Shaker*-related) family members (Kv1.2–Kv1.7, the products of the KCNA1–7 genes) were isolated (reviewed in Chandy & Gutman, 1995). These different mammalian Kv1 family members had distinct functional properties when expressed alone (*i.e.*, as homotetramers) in heterologous cells (Stuhmer *et al.*, 1989). Different Kv1 family members could also coassemble into channels with mixed subunit composition, and such heterotetrameric channels exhibited functional properties intermediate between those of channels formed from homotetramers of the constituent subunits (Hopkins *et al.*, 1994; Isacoff *et al.*, 1990; Ruppersberg *et al.*, 1990).

Of the Kv1 α subunits expressed at appreciable levels in the brain, Kv1.1–Kv1.5, but not Kv1.6, α subunits each contain a single consensus N-linked glycosylation (NLG) site in the extracellular linker regions between transmembrane segments S1-S2 (Figure 1). In native brain Kv1.1, Kv1.2, and Kv1.4 α subunits, this site carries oligosaccharide chains bearing sialic acid, conferring a strong negative charge to the extracellular glycan chain (Manganas & Trimmer, 2000; Sheng *et al.*, 1993; Shi & Trimmer, 1999; Thornhill *et al.*, 1996). Moreover, the spatial segregation of oligosaccharide-processing enzymes allows the biosynthetic trafficking of Kv1 α subunits to be tracked by straightforward biochemical analysis of the glycan chain (Trimmer, 1998a). Kv1 α subunits are also extensively modified by phosphorylation, which can affect both function and expression. The extensive literature on this subject of the functional modulation of Kv channels is outside the realm of this review, but the diverse role of phosphorylation in regulating Kv channel trafficking will be discussed below.

The cDNA encoding the rat brain Kv2.1 α subunit was isolated by expression cloning in *Xenopus* oocytes (Frech *et al.*, 1989). The manner of cloning Kv2.1 is noteworthy, as it reflects the high level expression of Kv2.1 in mammalian brain. In mammalian brain, Kv2.1 α subunits appear to exist predominantly as homotetrameric channels, although some evidence suggests that in some cases auxiliary subunits may also be associated with Kv2.1 channels (McCrossan *et al.*, 2003; Trimmer, 1991). Although Kv2.1 α subunits carry a single consensus N-linked glycosylation site on the S3-S4 linker domain, native brain Kv2.1 channels and recombinant Kv2.1 channels expressed in heterologous systems are not N-glycosylated (Shi & Trimmer, 1999). This is consistent with systematic analyses of other polytopic membrane proteins that show a marked preference for glycosylation site usage in the first extracellular loop (as in Kv1 α subunits) over sites in other segments (Landolt-Marticorena & Reithmeier, 1994). Kv2.1 is also extensively modified by phosphorylation (Murakoshi *et al.*, 1997; Tiran *et al.*, 2003). Low stringency hybridization screening of mammalian circumvallate papilla cDNA libraries led to the later isolation of the highly related Kv2.2 (Hwang *et al.*, 1992). Kv2.1 and Kv2.2 apparently do not form heteromultimeric channels with one another in mammalian brain, as they exhibit contrasting patterns of subcellular distribution in coexpressing cells (Hwang *et al.*, 1993b). However, some of these initial data should be interpreted with some caution due to extensive revisions of the original Kv2.2 sequence, upon which antibodies used in some of these studies were based.

Low stringency hybridization screening of mammalian brain cDNA libraries led to the isolation of cDNAs encoding Kv3 α subunits (Luneau *et al.*, 1991; McCormack *et al.*, 1991; Rudy *et al.*, 1991; Yokoyama *et al.*, 1989). Among Kv α subunits Kv3 mRNAs are somewhat unique in that they are subjected to extensive alternative splicing to generate subunits that differ at their cytoplasmic carboxyl termini (Luneau *et al.*, 1991). Kv3 α subunits contain two N-linked glycosylation sites in the S1-S2 linker. A comprehensive biochemical analysis of the oligosaccharide chains on these sites has not been performed. The full nature and extent of the association and colocalization of Kv3 channels in mammalian neurons is not yet known, in part due to the additional complexity conferred to such analyses by the extensive alternative splicing.

Kv4 α subunit cDNAs were also originally isolated using low stringency hybridization (Baldwin *et al.*, 1991; Roberds & Tamkun, 1991). Kv4.2 and Kv4.3 are expressed at high levels in mammalian brain, whereas Kv4.1 expression is much lower. Kv4 α subunits do not have glycosylation sites in their sequence, but they are extensively modified by phosphorylation (Adams *et al.*, 2000; Anderson *et al.*, 2000; Schrader *et al.*, 2002). The extent of Kv4 hetero-oligomerization is as yet unknown, but certain neurons coexpress Kv4.2 and Kv4.3, and as their the subcellular localization precisely overlaps heteromeric Kv4.2/Kv4.3 channels may exist.

Auxiliary Subunits of Kv Channels

Kv channels also contain auxiliary subunits, which in themselves cannot form functional channels but which can modify the function of channels formed from Kv α subunits. The best characterized of these are the cytoplasmic Kvβ subunits associated with Kv1 family members (Pongs *et al.*, 1999). The bulk of Kv1 channel complexes in mammalian brain have associated Kvβ subunits (Rhodes *et al.*,

1996) such that native channels are $\alpha_4\beta_4$ octomers (Orlova *et al.*, 2003). Four Kvβ subunit genes exist in the human genome, and alternative splicing can generate a number of functionally distinct isoforms (Pongs *et al.*, 1999). Inclusion of the Kvβ1.1 subunit in Kv channel complexes containing Kv1.1 α subunits dramatically alters the channel gating properties, converting the channels from sustained, or delayed-rectifier type, to rapidly inactivating, or A-type (Rettig *et al.*, 1994). Moreover, the specific α and β subunit composition of native complexes can dramatically impact both the expression level, localization, and function of Kv1 channels in mammalian neurons (Trimmer, 1998b).

Accessory subunits for Kv4 channels have also been identified recently and are encoded by two distinct sets of proteins. One set is a family of calcium-binding proteins, called KChIPs, that are members of the neuronal calcium sensor gene family (An *et al.*, 2000). At least four KChIP genes have been reported to exist in mammals (An *et al.*, 2000; Holmqvist *et al.*, 2002), and multiple, alternatively spliced isoforms of each KChIP gene product have been reported. With the exception of the KChIP4a splice variant, in heterologous expression systems all KChIP isoforms increase the surface density (Shibata *et al.*, 2003) and slow the inactivation gating and speed the kinetics of recovery from inactivation (An *et al.*, 2000) of Kv4 channels. The importance of KChIPs to Kv4 channel function is exemplified by knockout of KChIP2 expression in heart, which eliminates all of the Kv4-based I_{to} current (Kuo *et al.*, 2001). Importantly, Kv4 α subunits and KChIPs exhibit extensive association and colocalization in mammalian brain, supporting a *bona fide* role for KChIPs in modulating native Kv4 channels (An *et al.*, 2000; Trimmer & Rhodes, 2004). More recently, Rudy and colleagues (Nadal *et al.*, 2003) reported the identification of a dipeptidyl-peptidase–like protein (DPPX) as an accessory subunit for Kv4 channels. Coexpression of DPPX, KChIPs, and Kv4 α subunits gives rise to A-type currents whose biophysical properties very closely match the properties of native somatodendritic A-type currents.

Another family of candidate auxiliary subunits for Kv channels is the MiRPs, or mink-related peptides, members of the KCNE gene family. These single-pass type I transmembrane proteins exhibit promiscuous association with many Kv family members in heterologous expression systems (Abbott *et al.*, 2001). Moreover, mutations in MiRPs are associated with cardiac and skeletal muscle pathophysiology via effects on Kv channels (Abbott & Goldstein, 2002). The wide array of MiRPs and their nonselective interaction with diverse members of the Kv family, including many within and outside of the prototypical Kv1–Kv4 subset, in heterologous cells raises many questions as to the representation of MiRPs in native Kv channel complexes (McCrossan *et al.*, 2003).

In addition to these stereotypical auxiliary subunits, there exist in the genome a number of "electrically silent" α subunit-like polypeptides (Drewe *et al.*, 1992). In heterologous expression systems, these can coassemble with and functionally modify *bona fide* Kv α subunits (Patel *et al.*, 1997). However, while there is some information available as to the expression pattern of these genes in mammalian tissues (Drewe *et al.*, 1992; Salinas *et al.*, 1997), very little is known of their expression at the protein level or their contribution to native mammalian brain Kv channels.

GENERAL MECHANISMS FOR ION CHANNEL TRAFFICKING IN NEURONS

Control of Surface Expression Levels of Ion Channels

The relative contribution of individual ion channel subtypes to neuronal excitability is primarily determined by their functional characteristics, and their abundance and distribution in the neuronal membrane. The functional expression of ion channels in the plasma membrane of neurons is regulated by a hierarchical regulatory system operating at multiple levels within the cell. Initially, the expression levels of ion channel genes are highly regulated, with specific promoter elements acting in concert with transcriptional machinery to achieve precise temporal and spatial patterns of gene expression (Mandel & McKinnon, 1993). This mechanism can affect both the abundance and subunit composition of surface channels via mass action. While regulation of ion channel mRNA levels via effects on mRNA stability have been observed (Yanagita *et al.*, 2003), the more common posttranscriptional effects occur *via* alternative splicing that can yield both dramatic changes in channel function and channels with altered trafficking characteristics (see below). RNA editing of transcripts encoding at least three different channel types (amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid [AMPA] receptors, serotonin receptors and Kv channels) is also used as a posttranscriptional mechanism to generate channels with distinct functional characteristics (Seeburg & Hartner, 2003). Evidence for dynamic regulation of ion channel translation has not been provided.

An emerging theme in neuronal cell biology is the major role of posttranslational mechanisms in regulating the surface expression and subunit composition of many ion channel proteins (Bredt & Nicoll, 2003). Like other cell surface proteins, the steady-state expression level of ion channels in the neuronal membrane is determined by the delicate balance between biosynthetic delivery, and endocytic recycling and degradation. The rough ER is the initial site where channels are synthesized, inserted into the lipid bilayer, folded, and assembled. Successful completion of these events with resultant ER export is the

rate-limiting step for posttranslational regulation of ion channel surface expression. A number of compelling studies have revealed that the ER functions as the quality control center for sensing the proper folding and assembly of channel complexes (reviewed in Deutsch, 2003), as it does for other membrane proteins (Ellgaard & Helenius, 2003; Pelham, 1989; Trombetta & Parodi, 2003). In addition to this general role for the ER quality control system in regulating export, there exist signal-based mechanisms to regulate further ER export of ion channels and other membrane proteins containing anterograde export signals, and ER retention/retrieval signals (reviewed in Ma & Jan, 2002).

Transport of newly synthesized membrane proteins is accomplished by COPII-coated vesicles that package protein in the ER and transport them to the Golgi complex. A long-held view was that in the absence of specific ER retention information a properly folded membrane protein would progress by default to the Golgi complex (Pfeffer & Rothman, 1987). An emerging theme is that efficient transport from the ER is not a default process, and that specific signals exist to facilitate interaction of the cargo membrane protein with components of the COPII complex, and as such enhance ER export (Barlowe, 2003a). These forward or anterograde trafficking signals are diverse in nature and have been found on a number of proteins, including ion channels (Barlowe, 2003b). Several general forward trafficking signals have been identified, including diacidic and dihydrophobic motifs (Barlowe, 2003b). Inwardly rectifying Kir channels contain diacidic motifs (Ma & Jan, 2002; Ma *et al.*, 2001). A unique forward-trafficking motif present in the Kv1.4 α subunit (Li *et al.*, 2000) that was the first such motif identified in an ion channel protein, will be discussed in detail below.

ER retention/retrieval signals have been long recognized as providing potent mechanisms for regulating membrane protein trafficking (Munro & Pelham, 1987; reviewed in Lippincott-Schwartz *et al.*, 2000). ER retention signals presumably mediate interaction with resident ER proteins that lead to retention in the ER, while retrieval signals lead to incorporation into retrograde Golgi to ER transport vesicles (Lippincott-Schwartz *et al.*, 2000). Among the more interesting examples of ion channels whose expression is regulated by ER retention signals are ATP-sensitive K^+ channels (K_{ATP}) and N-methyl-D-asparate (NMDA) receptors. K_{ATP} channels are formed from four inwardly rectifying K^+ channel α subunits (Kir6.1 or 6.2) and four sulphonylurea-binding β subunits. Both α and β subunits contain a cytoplasmic ER retention RXR motif (RKR in Kir6), which was originally identified in single-pass type II membrane proteins (Teasdale & Jackson, 1996). This motif can retain α subunits in the ER in the absence of coassembled β subunits, and *vice-versa*. However, upon assembly of an octomeric channel complex with the correct α/β subunit stoichiometry, the RKR retention signals on both α and β subunits are masked, and K_{ATP} channel complexes exit the ER and are transported to the cell surface (Zerangue *et al.*, 1999). ATP-binding to the Kir6 subunit can substantially alter intra- and/or intermolecular subunit interactions (Tsuboi *et al.*, 2004) raising the possibility that K_{ATP} channel assembly and trafficking can be dynamically regulated by cellular metabolism.

NMDA receptor surface expression is regulated by a similar retention signal. NMDA receptor subunit type 1 (NR1) is an essential subunit of NMDA receptors. The cytoplasmic carboxyl-terminal region of NR1 undergoes variable mRNA splicing to generate eight different NR1 subtypes (NR1a-h) (Ehlers *et al.*, 1995). Sequence comparisons with Kir6 revealed that one of the possible carboxyl-terminal cassettes (NR1a) contains an RXR consensus ER retention motif that controls trafficking of NR1 and of NR1/NR2 complexes (Scott *et al.*, 2001). Interestingly, alternative splicing of NR1 is regulated by neuronal activity, which *via* these trafficking mechanisms can lead to activity-dependent effects on NMDA receptor surface expression (Mu *et al.*, 2003). Kainate receptors also contain retention signals that control expression (Ren *et al.*, 2003). These are but a few examples of a growing list of neuronal ion channels for which regulation of ER export plays a primary role in determining cell surface expression levels. As we detail below, similar mechanisms acting through distinct trafficking signals that suggest interaction with a separate subset of the ER trafficking machinery are used to regulate Kv channel expression.

Mechanisms for Polarized Expression and Clustering of Ion Channels in Neurons

Neurons are extremely polarized cells separated into two structurally and functionally distinct domains; axonal and somatodendritic. Most cell surface ion channels are preferentially targeted to either the axonal or somatodendritic membrane (Craig & Banker, 1994), where they become further localized to discrete membrane domains, the most familiar of which is the excitatory chemical synapse (Grant, 2003). The sequestration of ion channels in high-density clusters at synapses is necessary for effective synaptic signaling and also allows for the independent structural and functional plasticity of individual synapses (Bredt & Nicoll, 2003). A growing body of evidence suggests that the localization of many other types of ion channels at specific yet diverse extrasynaptic subcellular sites is fundamental to proper neuronal function (Trimmer & Rhodes, 2004). An especially prominent example is the highly localized expression of transient Kv channels on pyramidal cell dendrites (Johnston *et al.*, 2003), hyperpolarization-activated and cyclic-nucleotide–gated cation channels at the very distal tips of pyramidal cell

apical dendrites (Lorincz *et al.*, 2002), and voltage-gated sodium channels at the node of Ranvier of myelinated axons (Rasband & Trimmer, 2001).

There are several distinct molecular events that provide mechanisms for achieving such precision in polarized expression and local clustering of membrane proteins. These consist of (1) polarized delivery of membrane protein-containing transport vesicles to the dendrites or axon, (2) insertion of these proteins into dendritic or axonal membranes *via* targeted exocytosis, (3) specific retrieval of un- or mistargeted proteins from the dendritic or axonal membranes via endocytosis, and (4) selective retention or concentration at the specific site *via* protein–protein interactions. It should be noted that there is growing evidence for polarized transport of mRNA to, and local protein synthesis in, neuronal processes (Steward & Schuman, 2003). Kv channel mRNA has not been detected on processes in these published reports. It can also be argued on quantitative terms that this mechanism should be a minor component of the total level of membrane protein biosynthesis needed to maintain steady-state levels of receptors and ion channels in the extensive dendritic and axonal membranes of neurons, as the amount of rough ER and Golgi components in neuronal processes is quite small and highly localized to specific subcellular sites (*e.g.*, to the base of dendritic spines). Moreover, for most ion channels transfection of cDNAs that lack the 5′- and 3′-untranslated regions thought to direct mRNA trafficking to processes yields proper subcellular localization (*e.g.*, Garrido *et al.*, 2001; Lim *et al.*, 2000), suggesting that the observed patterns of subcellular localization can be obtained solely through posttranslational mechanisms. Thus the classical soma-localized biosynthetic processes followed by packaging of membrane proteins into distinct vesicles and delivery to the axon or dendrites *via* specific adaptor and motor proteins appears to provide the bulk of the ion channel population (Horton & Ehlers, 2003).

Recent studies have provided examples of a number of scenarios leading to polarized membrane protein localization on neuronal processes. It is likely that adaptor complexes coordinate the formation of specific vesicle populations at the trans-face of the trans-Golgi network and then bind to specific motor proteins such as kinesins (Schnapp, 2003). For example, AMPA-type glutamate receptor subunits are delivered to the dendrites by a complex of kinesin-heavy chains and GRIP1 (Setou *et al.*, 2002). In contrast, the adhesion molecule NgCAM/L1 is transported to both dendrites and axons but exhibits selective insertion only into axonal membranes (Burack *et al.*, 2000). For such specific insertion the "classic" SNARE hypothesis is still attractive, where specific combinations of vesicular SNAREs and target SNAREs (v- and t-SNARE) restrict the fusion of transport vesicles with the plasma membrane to specific sites (Malhotra & Emr, 2002). The vesicular SNARE protein VAMP-2 is localized to axon terminals via a third mechanism, where it is delivered and inserted into both somatodendritic and axonal membranes (Sampo *et al.*, 2003). However, selective retrieval from the dendritic membrane generates axonal localization of this protein, although the molecular mechanisms for the domain-specific retrieval remain unclear.

Once membrane proteins are inserted into specific subcellular plasma membrane sites by vesicular fusion, specific mechanisms must exist to maintain the achieved localization of these proteins against the entropic forces of lateral diffusion. Proteins that interact with and anchor ion channels at specific sites have been identified through a combination of protein biochemistry and molecular interaction assays (Sheng & Wyszynski, 1997). A well-known example is the PSD-95/discs large/ZO-1 domain (PDZ)-containing scaffolding protein PSD-95, which interacts with plasma membrane NMDA receptor subunits and a number of other proteins in the postsynaptic density, and creates a macromolecular signaling complex (Grant, 2003).

Compared to synaptic glutamate receptors, relatively little is known about the dynamic processes and macromolecular machinery underlying localization of Kv channels. As discussed below, recent studies have begun to dissect the various mechanism that regulate trafficking and localization of distinct members of the diverse family of mammalian Kv channels.

MECHANISMS REGULATING EXPRESSION AND LOCALIZATION OF Kv1 CHANNELS

Cellular and Subcellular Localization of Kv1 α Subunits

The distribution of Kv and other ion channels in mammalian brain was recently reviewed (Trimmer & Rhodes, 2004), and it is outside the scope of this article to discuss in detail the localization of each and every Kv channel in every brain region. However, in each of the following sections we present a brief description of the general aspects of localization of each Kv channel family as background to our critical discussion of the underlying targeting mechanisms.

Mammalian genomes contain a total of 7 Kv1 (KCNA) α subunit genes (Gutman *et al.*, 2003), of which six (Kv1.1–Kv1.6) are expressed in mammalian brain. When expressed in heterologous expression systems, Kv1 α subunits comprise either transient (Kv1.4) or sustained (Kv1.1–Kv1.3, Kv1.5, and Kv1.6) K^+ currents. Since these Kv1 α subunits can form heterotetrameric channels with one another and can complex with auxiliary Kvβ subunits, combinatorial assembly into $\alpha_4\beta_4$ octomeric complexes can theoretically generate an impressive array of structure and function.

The three most abundant Kv1 α subunits expressed in mammalian brain are Kv1.1, Kv1.2, and Kv1.4. Each of these is found predominantly localized to axons and nerve terminals (Trimmer & Rhodes, 2004). These α subunits seem to form heterotetrameric channel complexes, as Kv1.1, Kv1.2, and Kv1.4 exhibit virtually identical patterns of localization and biochemical association as shown by copurification (Rhodes *et al.*, 1997). Immuno-electron microscopic studies have demonstrated that Kv1.1, Kv1.2, and Kv1.4 are concentrated along the axons and in the axonal membrane immediately preceding or within axon terminals (Cooper *et al.*, 1998; Sheng *et al.*, 1992; Wang *et al.*, 1994). Activity of Kv1 channels at these sites can play a critical role in regulating the extent of nerve terminal depolarization and thereby regulate neurotransmitter release. The Kv1 subunits Kv1.1 and Kv1.6 are also found colocalized in many interneurons (Rhodes *et al.*, 1997).

Kvβ2 subunits appear to be components of many, if not all, Kv1-containing channel complexes in mammalian brain, as immunoreactivity for Kvβ2 is present in each and every location where immunoreactivity for Kv1-family α subunits is found (Rhodes *et al.*, 1996, 1997). Moreover, immunopurification of Kvβ2-containing complexes depletes mammalian brain preparations of all detectable α subunit immunoreactivity (Trimmer & Rhodes, 2000). The Kvβ1 subunit, which exerts dramatic effects on the inactivation kinetics of Kv1 channels, appears to be included more selectively (Rhodes *et al.*, 1997).

Mechanisms Regulating Surface Expression of Kv1 α Subunits

The overall steady-state cell surface expression levels and subunit composition of Kv1 α subunits appear to be regulated by a hierarchical system of regulatory steps (Table 1). The primary determinant for regulating trafficking appears to be an ER retention (ERR) signal consisting of residues in the turret region at the external face of the channel pore domain (Figure 1). A cytoplasmic C-terminal forward-trafficking signal (FTS) also acts to regulate intracellular trafficking of Kv1.4 channels, as does interaction of N-terminal T1 sequences with cytoplasmic Kvβ subunits (Figure 1). Studies of these determinants for Kv1 channel surface expression have been primarily conducted in heterologous expression systems, although critical experiments have been reproduced in cultured neurons.

When expressed in a wide variety of heterologous expression systems (the COS-1, HEK293, CHO, MDCK cell lines), and in cultured hippocampal neurons, Kv1.4 α subunits, presumably in homotetrameric Kv1.4 channels, are efficiently expressed on the cell surface (Bekele-Arcuri *et al.*, 1996; Li *et al.*, 2000; Manganas & Trimmer, 2000; Manganas *et al.*, 2001b; Shi & Trimmer, 1999; Tiffany *et al.*, 2000; Zhu *et al.*, 2003b). This conclusion comes from a wide variety of technical approaches. Immunohistochemical approaches include immunofluorescence staining and conventional or confocal imaging of plasma membrane channels in permeabilized cells, and staining of cell surface Kv1.4 in intact cells with ectodomain-directed anti-Kv1.4 antibodies. Diverse biochemical analyses, including characterization of N-linked oligosaccharide chains (whose spatially segregated processing allows for determination of subcellular localization), proteinase K digestion of cell surface Kv1.4, and biotinylation analyses using membrane-impermeant reagents corroborate the staining results. Taken together, these results are consistent with the fact that a large proportion of the steady-state Kv1.4 cellular pool (between 80–90%) is present on the cell surface. This is due to the lack of the critical amino acids in the turret region that mediate ER retention (Manganas *et al.*, 2001b), combined with the presence of a unique ER export (FTS) signal (VKESL) in the cytoplasmic carboxyl-terminal region (Li *et al.*, 2000). This cytoplasmic FTS (Figure 1) appears to be recessive to the turret domain ERR signal (which would be luminal in

TABLE 1
Summary of determinants of the abundance and distribution of Kv channels

Kv subfamily	Localization	Trafficking determinants	Polarity determinants
Kv1	Axonal	T1 (N-terminus, via Kvβ subunit interaction) ERR (P-loop) FTS (C-terminal) PDZ BD (C-terminus, via MAGUK interaction)	T1 (N-terminus, via Kvβ subunit interaction)
Kv2	Somatodendritic		PRC (C-terminus)
Kv3	Mixed		C-terminus (*via* splicing)
Kv4	Somatodendritic	KChIP BD (N-terminus, via KChIP interaction)	DLS (C-terminal)

the ER), as Kv1.4 chimeras with active ERR signals from Kv1.1 (see below) but that are otherwise composed of Kv1.4 are efficiently retained in the ER. However, simple deletion of this VKESL ER export/FTS signal decreases the level of Kv1.4 surface expression (Li *et al.*, 2000; Zhu *et al.*, 2003b). Kvβ subunits do not dramatically affect the trafficking of wild-type Kv1.4 α subunits. However, this may be simply due to the fact that the inherent trafficking properties of these proteins appear to be near the maximum efficiency possible.

By each of the above immunohistochemical and biochemical criteria, the trafficking phenotype of Kv1.1 differs dramatically from that of Kv1.4 (Bekele-Arcuri *et al.*, 1996; Deal *et al.*, 1994; Manganas & Trimmer, 2000; Manganas *et al.*, 2001b; Shi & Trimmer, 1999; Tiffany *et al.*, 2000; Zhu *et al.*, 2003b). Moreover, [^{125}I]-α dendrotoxin (DTX)-binding assays reveal that only a small fraction ($\approx$5%) of the total toxin binding in Kv1.1-expressing cells is to cell surface channels (Manganas *et al.*, 2001a). The large (18-fold) increases in toxin binding revealed upon cell disruption also indicate that ER-retained Kv1.1 may be correctly folded and assembled. However, these data should be interpreted with caution as the precise relationship between Kv1.1 folding and α DTX binding activity have not been performed, although a clearly misfolded Kv1.1 α subunit mutant (ΔC79) that is present in certain episodic ataxia type I patients does not bind [^{125}I]-α DTX (Manganas *et al.*, 2001a).

The Kv1.1 α subunit has in its external turret domain (Figure 1) the critical amino acids that define a potent ERR signal (Manganas *et al.*, 2001b). The detailed chimera study presented in this article revealed that any Kv1.4 α subunit containing the Kv1.1 pore region (P-loop), including the turret domain, was ER-retained. Conversely, any Kv1.1 α subunit containing the Kv1.4 P-loop was efficiently exported from the ER. An alignment of the Kv1.1 and Kv1.4 P-loop sequences revealed three key positions, all in the turret domain that differed between Kv1.1 and Kv1.4. Mutation of those in Kv1.1 to those in Kv1.4 yield functional Kv1 channels that have Kv1.4-like trafficking patterns, and vice-versa, by each of the immunohistochemical and biochemical criteria detailed above, and by electrophysiological analyses of functional channel levels in transfected cells (Manganas *et al.*, 2001b). This Kv1.1 ERR signal was also shown to function in cultured hippocampal neurons (Manganas *et al.*, 2001b). As discussed above, analyses of a large number of Kv1.1/Kv1.4 chimeras and mutants reveal that this luminal ERR motif is dominant over the cytoplasmic C-terminal forward trafficking signal (Manganas *et al.*, 2001b). Subsequent studies yielded similar results (Zhu *et al.*, 2003b). This luminal ERR motif is also dominant over any robust effects of Kvβ subunit coexpression on Kv1 α subunit trafficking. Although small but measurable increases of Kv1.1 surface expression have been observed in cells coexpressing Kvβ2 subunits (Shi *et al.*, 1996) the effects are small compared to auxiliary subunit effects on other Kv channels.

The intriguing aspect of the identity of the three P-loop residues that control Kv1 family trafficking is that they are the same as those that determine high-affinity binding to α DTX, which in Kv1.1 are A352, E353, and Y449 (Hurst *et al.*, 1991; Imredy & MacKinnon, 2000; Tytgat *et al.*, 1995). Moreover, each of the Kv1 family members that bind α DTX (Kv1.1, Kv1.2, and Kv1.6) exhibit a strong degree of ER retention relative to the Kv1 family members (Kv1.3, Kv1.4, and Kv1.5) that lack the critical binding residues and as such do not bind α DTX (Bekele-Arcuri *et al.*, 1996; Manganas & Trimmer, 2000; Manganas *et al.*, 2001b; Shi & Trimmer, 1999; Tiffany *et al.*, 2000). This allows for speculation that the ER retention of Kv1.1, and of Kv1.2 and Kv1.6, may be mediated by a resident ER retention receptor protein that binds to the P-loop of Kv1.1 in a fashion similar to the binding of α DTX. This mechanism is attractive in that it may block the pore of ER-retained Kv1 channels, preventing any inadvertent effects of active ER-localized channels on cell physiology. This hypothetical ER-receptor for Kv1.1 and other α DTX-sensitive Kv1 α subunits remains to be identified.

Analyses of the trafficking characteristics of heteromeric channels composed of Kv1 α subunit led to insights that trafficking may play a role in determining not only surface expression levels of Kv1 homotetramers but also the subunit composition of surface Kv1 heterotetramers. These studies revealed that in cells coexpressing Kv1.1 and Kv1.4, three Kv1.4 α subunits are necessary to promote surface expression of Kv1.1 α subunit-containing heterotetramers (Manganas & Trimmer, 2000). Other Kv1.1/Kv1.4 heteromeric channels are retained intracellularly. Subsequent studies yielded similar results (Zhu *et al.*, 2003a). This suggests that the Kv1 trafficking determinants are not only responsible for the lack of Kv1.1 homotetramers in the brain (Coleman *et al.*, 1999; Shamotienko *et al.*, 1997) but can also shape subunit composition. This result further underscores the dominance of the Kv1.1 P-loop luminal ER retention signal over the cytoplasmic ER export signal in Kv1.4.

In contrast to the extreme differences in trafficking between Kv1.1 and Kv1.4 homotetrameric channels, Kv1.2 homotetrameric channels display intermediate trafficking characteristics (Bekele-Arcuri *et al.*, 1996; Campomanes *et al.*, 2002; Manganas & Trimmer, 2000; Manganas *et al.*, 2001b; Shi *et al.*, 1996; Shi & Trimmer, 1999; Tiffany *et al.*, 2000). This suggests that Kv1.2 α subunits have an active although less-potent ER retention signal than that found on Kv1.1 (and Kv1.6) α subunits. This conclusion is supported by data on Kv1.2 α subunits in heteromeric channels, whose trafficking characteristics

can be dominated by heteromeric assembly with either Kv1.1 or Kv1.4 (Manganas & Trimmer, 2000), by addition of the Kv1.4 C-terminal forward trafficking determinant (Li *et al.*, 2000), or by Kvβ subunit coexpression (Campomanes *et al.*, 2002; Shi *et al.*, 1996).

The native Kv1.1, Kv1.2, and Kv1.4 α subunits are highly phosphorylated in brain, as shown by shifts in SDS gel electrophoretic mobility upon alkaline phosphatase treatment (H. Misonou & J. Trimmer, unpublished data). This raises the question as to whether phosphorylation can dynamically regulate Kv1 channel trafficking. Large increases in Kv1.1 current density have been observed in transfected cells upon stimulation of specific kinases (*e.g.*, Winklhofer *et al.*, 2003), although effects on biosynthetic trafficking, as opposed to endocytosis, unmasking of silent surface channels, or changes in the biophysical properties of active surface channels, have not been directly assessed. However, these results raise the possibility that surface expression of neuronal Kv1 channels could be dynamically regulated via phosphorylation.

Control of Polarized Localization and Clustering of Kv1 α Subunits

In native mammalian central neurons, Kv1 α subunits are preferentially localized to axons and nerve terminals (Trimmer & Rhodes, 2004), where in some cells they are present in high-density clusters at precise sites (Cooper *et al.*, 1998). The precise determinants for the polarized expression and local clustering of Kv1 channels are not well characterized. One of the problems has been recapitulating the heteromeric channels found in native neurons in model cell systems (*e.g.*, polarized epithelial cells), compounded by the inefficient intracellular trafficking inherent to many of the Kv1 α subunits. Moreover, for reasons that are not well understood, efficiently expressed Kv1 α subunits, such as Kv1.4, when expressed in such model systems do not "behave." As described in detail below for Kv2.1, in most cases the apical membrane of polarized epithelial cells is analogous to the axonal membrane of neurons and the basolateral to the somatodendritic (Dotti & Simons, 1990). However, when expressed in polarized Madin-Darby Canine Kidney (MDCK) epithelial cells, axonal Kv1.4 is found in the basolateral membrane (Le Maout *et al.*, 1996), negating any opportunity to characterize axonal targeting determinants in these cells. Some studies of polarized trafficking of Kv1 α subunits have been performed in transfected neurons. When expressed in organotypic slice cultures from neocortex, Kv1.4 does achieve an axonal localization (Arnold & Clapham, 1999), although the contribution of endogenous Kv1 α and Kvβ subunits to this targeting cannot be directly ascertained.

Recent studies have provided intriguing data that cytoplasmic Kvβ subunits may be involved in polarized trafficking of Kv1 α subunits (Table 1). While Kv1.2 α subunits exogenously expressed in cultured hippocampal neurons exhibit somatodendritic localization, cotransfection of Kv1.2 with the Kvβ2 subunit yields a pronounced axonal localization of Kv1.2 (Campomanes *et al.*, 2002). Consistent with these studies, subsequent analyses of Kv1.2 deletion mutants and chimeric channels revealed that the amino terminal T1 domain (Figure 1), which comprises the Kvβ-subunit–binding site (Gulbis *et al.*, 2000; Sewing *et al.*, 1996; Yu *et al.*, 1996), is essential for axonal expression of Kv1.2 channels (Gu *et al.*, 2003). Specific mutations to disrupt Kvβ2 binding also disrupted axonal expression of Kv1 α subunits in transfected neurons, further suggesting that endogenous Kvβ subunits were contributing to the axonal Kv1 localization (Gu *et al.*, 2003). Remarkably, the wild-type Kv1.2 T1 domain, but not mutant isoforms with altered Kvβ binding, was able to direct the axonal localization of single-pass transmembrane reporter proteins (Gu *et al.*, 2003). Thus, the cytoplasmic Kvβ subunits may affect not only early biosynthetic processing events and ER export (Shi *et al.*, 1996), but also axonal localization (Campomanes *et al.*, 2002; Gu *et al.*, 2003), of Kv1 channels. It should be noted that the proposed oxidoreductase activity of Kvβ subunits is apparently not involved in any of these trafficking effects, as mutation of critical catalytic site residues have no effect on either the ER export or axonal trafficking properties (Campomanes *et al.*, 2002). The important role of Kvβ2 is underscored by the finding that deletion of the human Kvβ2 gene in chromosome 1p36 deletion syndrome is closely linked to epilepsy, which is present in a subset of these patients (Heilstedt *et al.*, 2001). This finding suggests that in humans haploinsufficiency of Kvβ2 may be sufficient to induce neuronal hyperexcitability, perhaps via effects on Kv1 channel trafficking. Interestingly, Kvβ2 null mice also exhibit occasional seizures and cold swim-induced tremors similar to that observed in Kv1.1-null mice (McCormack *et al.*, 2002). However, dramatic changes in Kv1 α subunit localization were not observed in these mice, presumably due to compensatory action of other Kvβ subunits (Campomanes *et al.*, 2002). These results together suggest that Kv1 channel trafficking is crucial to regulate intrinsic neuronal excitability.

Membrane-associated guanylate kinases (MAGUKs) such as PSD-95 and SAP97 function as scaffolding molecules to promote coclustering of membrane receptors and ion channels (Sheng & Sala, 2001). Mutational and structural analyses showed that PSD-95 and other MAGUKs bind to the PDZ-binding motif (S/TxV) in the carboxyl-termini (Figure 1) of all Kv1 α subunits (Doyle *et al.*, 1996; Kim *et al.*, 1995). Studies in COS-1 cells showed that PSD-95 coexpression did not affect steady-state levels of Kv1 surface expression (Tiffany *et al.*, 2000). However, PSD-95:Kv1 clustering could be used as a reliable readout

for surface expression and/or ER export (see Campomanes *et al.*, 2002; Manganas *et al.*, 2001a), as the ability of PSD-95 to cluster Kv1 channels correlated precisely with surface expression efficiency (Tiffany *et al.*, 2000). Similar results were obtained in another mammalian cell line (HEK293 cells), where PSD-95 clustering also did not increase steady-state surface expression levels of Kv1.4 (Jugloff *et al.*, 2000) although Kv1.4 internalization was suppressed by PSD-95–mediated clustering. In contrast to the lack of effects of PSD-95 on Kv1 channel surface expression efficiency, in heterologous cells SAP-97 acts as a potent inhibitor of Kv1 channel surface expression by biosynthetic clustering of Kv1 channels in the ER, leading to intracellular retention (Tiffany *et al.*, 2000). The role of MAGUKs in Kv1 channel trafficking and clustering in neurons is unclear. In most mammalian central neurons, MAGUKs are found at synapses, generally in the postsynaptic density. Kv1 channels in many of the same cells are localized to axons, suggesting that Kv1:MAGUK interaction does not occur at high levels in native cells. There are a few examples where Kv1 channels and MAGUKs, in this case PSD-95, colocalize in neurons, for example in cerebellar basket cells terminals (Kim *et al.*, 1995; Laube *et al.*, 1996) and at juxtaparanodal regions of nodes of Ranvier (Rasband & Trimmer, 2001). However, even at these sites deletion of PSD-95 does not affect Kv1 channel clustering (Rasband *et al.*, 2002), raising questions as to the role of MAGUKs in clustering native Kv1 channels in mammalian neurons.

MECHANISMS REGULATING EXPRESSION AND LOCALIZATION OF Kv2 CHANNELS

Cellular and Subcellular Localization of Kv2.1 and Kv2.2

Kv2 (KCNB) family members form delayed rectifier K^+ channels. Unlike Kv1 α subunits, Kv2.1 and Kv2.2 do not form heteromeric channels and show discrete localization in brain and in neurons (Hwang *et al.*, 1993b; Lim *et al.*, 2000). Kv2 α subunits are prominently expressed in mammalian brain, where they are localized in the somatodendritic domain of neurons. Kv2.1 was the first member of this family and is unique in that it was identified and isolated by expression cloning (Frech *et al.*, 1989). Thus it was not surprising when immunostaining revealed that Kv2.1 was highly expressed and has an extensive distribution throughout the mammalian brain (Du *et al.*, 1998; Hwang *et al.*, 1993b; Rhodes *et al.*, 1997; Trimmer, 1991). However, in spite of this broad neuronal expression, within individual neurons the staining for Kv2.1 is highly restricted and is present on only the somatic and proximal dendritic membrane and is absent from axons and nerve terminals. Immuno-electron microscopy has unambiguously confirmed the somatodendritic localization of Kv2.1 (Du *et al.*, 1998). The striking subcellular distribution is accentuated by the fact that within these domains Kv2.1 is present in large clusters (Hwang *et al.*, 1993b; Lim *et al.*, 2000; Maletic-Savatic *et al.*, 1995; Scannevin *et al.*, 1996; Trimmer, 1991), which are present on the cell surface membrane immediately facing astrocytic processes, and over subsurface cisterns underlying the plasma-membrane–facing astrocytes (Du *et al.*, 1998). The physiological requirement for the highly clustered, discrete localization of Kv2.1 to these specialized membrane domains is not known.

In spite of its widespread cellular distribution, certain cells stand out for having especially prominent Kv2.1 expression. In the cortex, pyramidal cells in layers II/III and layer V are especially striking for their high levels of Kv2.1 expression. Kv2.1 is also present in high levels throughout the hippocampus, although the levels in dentate granule cells and CA1 pyramidal cells exceed those found in CA3 and CA2 pyramidal cells in both rat and mouse. However, it should be stressed that Kv2.1 is found on both principal cells and interneurons throughout the hippocampus (Du *et al.*, 1998; Martina *et al.*, 1998). Among interneurons, Kv2.1 is found in the majority of cortical and hippocampal parvalbumin, calbindin, and somatostatin-containing inhibitory interneurons (Du *et al.*, 1998).

Kv2.2 is expressed in many of the same cells that express Kv2.1 (Hwang *et al.*, 1993b; Hwang *et al.*, 1992). However, unlike other Kv channels (Kv1, Kv3, and Kv4 family members), the two members of the mammalian Kv2 family apparently do not readily form heteromultimers in native neurons, as the subcellular localizations of Kv2.1 and Kv2.2 expressed in the same cells are distinct (Hwang *et al.*, 1993a). Kv2.2 is uniformly present on dendrites and along the entire length of the dendrite. The clustered, proximal dendritic localization of Kv2.1 is not observed for Kv2.2 (Hwang *et al.*, 1993b; Lim *et al.*, 2000). Kv2.2 is present at high levels in olfactory bulb neurons and in cortical pyramidal neurons.

Mechanisms Regulating Surface Expression of Kv2 α Subunits

Kv2.1 channels are likely to be expressed on the cell surface *via* default trafficking pathway. Kv2.1 α subunits expressed in heterologous cells exhibit prominent plasma-membrane–localized staining with little or no intracellular accumulation (Bekele-Arcuri *et al.*, 1996; Shi *et al.*, 1994). No obvious retention or forward-trafficking signals are present in the Kv2.1 sequence, and none of numerous mutants of Kv2.1 has ever shown loss of surface expression (Bentley *et al.*, 1999; Lim *et al.*, 2000; Scannevin *et al.*, 1996). The regulation of Kv2.2 surface expression has not been characterized.

Control of Polarized Expression and Clustering of Kv2 α Subunits

A number of studies have focused on defining the determinants of the dramatic polarized and clustered localization of Kv2.1. Kv2.1 is expressed only in the soma and proximal dendrites in pyramidal neurons of cortex and hippocampus and forms unique clusters with 1$\sim$2 μm diameter (Scannevin *et al.*, 1996; Trimmer, 1991). Initial studies of the polarized and clustered localization of Kv2.1 were performed in polarized epithelial MDCK cells, as Kv2.1 is neither polarized nor clustered in COS-1 cells (Shi *et al.*, 1994). In MDCK cells, Kv2.1 localizes in the basolateral membrane (Scannevin *et al.*, 1996), consistent with the analogous membrane hypothesis, whereby the apical and basolateral membranes of epithelial cells correspond to the axonal and somatodendritic membranes, respectively, of neurons (Dotti & Simons, 1990). Interestingly, Kv2.1 is also present in clusters in MDCK cells (Scannevin *et al.*, 1996). Expression of truncation mutants that lacked relatively large portions of the cytoplasmic Kv2.1 carboxyl terminus revealed that an $\approx$130 amino acid segment approximately midway in the 440 amino acid carboxyl terminus of Kv2.1 was necessary for both polarized expression and clustering in MDCK cells (Scannevin *et al.*, 1996).

These findings were later extended by studies in primary cultures of hippocampal neurons (Lim *et al.*, 2000). Expression of epitope-tagged wild-type Kv2.1 and the two aforementioned mutants in hippocampal neurons yielded results consistent with those in MDCK cells. Wild-type Kv2.1 was found in large clusters on the soma and proximal dendrites of cultured neurons, and the truncation mutants delineated the same $\approx$130 segment as being critical to this localization. Kv2.2 exhibited a uniform localization on axons and dendrites, and Kv2.1/Kv2.2 chimeras revealed that the disparate carboxyl-terminal regions of the channels controlled these subtype-specific localizations (Lim *et al.*, 2000). Further deletion analysis of Kv2.1 revealed a $\approx$25 amino acid segment (the PRC signal; Table 1) in the cytoplasmic carboxyl-terminus (Figure 1) that was necessary for polarized and clustered localization. That this region was sufficient for dendritic localization and clustering was shown by generation of chimeric Kv1.5 α subunit having this segment appended to the carboxyl terminus. However, this targeting signal does not function when appended to single-pass type I transmembrane proteins, suggesting that structural background of Kv channels, perhaps the tetrameric quaternary structure, is required for proper targeting signal function (Lim *et al.*, 2000). That the amino acid sequence of this targeting signal is fairly well conserved in Kv2.2 also suggests determinants of the signal function outside of the primary structure (Lim *et al.*, 2000). An alanine scan through this segment revealed that three of the four critical amino acids are serine residues, raising the possibility that changes in phosphorylation state may dynamically regulate Kv2.1 localization.

Some candidates for Kv2.1-interacting proteins have been proposed, such as syntaxin-1 (Leung *et al.*, 2003) and MiRP-2 (McCrossan *et al.*, 2003). However, these proteins show poor overlap of localization with the very obvious Kv2.1 clusters in neurons. As Kv2.1 is also expressed outside of the brain, it may be that it is there that these interactions are physiologically relevant. Electron microscopic analysis has shown that plasma membrane Kv2.1 clusters on the somata and proximal dendrites of pyramidal neurons lie over subsurface cisternae (Du *et al.*, 1998), which are intracellular ER-derived membranes rich in inositol triphosphate and ryanodine receptors. These specialized sites where intracellular Ca^{2+} stores come into close apposition with the plasma membrane represent a specialized neuronal signaling domain that may also contain elevated levels of voltage-dependent Ca^{2+} channels (Westenbroek *et al.*, 1990). A functional relationship between Kv2.1 and dendritic $[Ca^{2+}]i$ transients were revealed by antisense knockdown of Kv2.1 in rat hippocampal neurons (Du *et al.*, 2000). Moreover, ryanodine receptor intracellular Ca^{2+} release channels colocalize with Kv2.1 clusters in cultured hippocampal neurons (Antonucci *et al.*, 2001). These findings suggest that the clustered localization of Kv2.1 could affect dendritic Ca^{2+} signaling at these sites.

MECHANISMS REGULATING EXPRESSION AND LOCALIZATION OF Kv3 CHANNELS

Cellular and Subcellular Localization of Kv3 α Subunits

Kv3 (KCNC) family members consist of four genes in human and rodents, and have unique functional characteristics, including fast activation at voltages positive to -10 mV and very fast deactivation rates. Kv3 currents can have either sustained (Kv3.1, Kv3.2) or transient (Kv3.3, Kv3.4) characteristics and can form hetero-oligomeric channels with intermediate gating characteristics (Rudy *et al.*, 1999). These properties are thought to facilitate sustained high-frequency firing, and Kv3 α subunits are highly expressed in fast-spiking neurons, such as neocortical and hippocampal interneurons, as well as in midbrain auditory neurons (Rudy & McBain, 2001). Each of four Kv3 α subunit genes generates multiple products *via* alternative splicing, which differ at their cytoplasmic carboxyl termini (Luneau *et al.*, 1991). This complicates studies of localization of these subunits because one needs to distinguish among reports on the basis of the reagents used (*i.e.*, their specificity for sequences conserved

among the alternative forms or unique to individual variants).

Initial *in situ* hybridization analyses revealed that unlike many other Kv subunits, Kv3.1 and Kv3.2 transcripts are expressed in only a small subset of cells in the cerebral cortex and hippocampus (Perney *et al.*, 1992; Weiser *et al.*, 1995). Interestingly, the *in situ* hybridization patterns of Kv3.1 and Kv3.2 were distinct, suggesting a strict cellular specificity to expression of these highly related Kv channel α subunits (Weiser *et al.*, 1995). Immunolocalization studies were performed using antibodies raised against the major splice variant of Kv3.1, termed Kv3.1b, which has a longer carboxyl terminus than the less abundant Kv3.1a variant. These studies revealed that Kv3.1b was highly expressed in interneurons and that expression was very low or undetectable in principal cells, such as neocortical and hippocampal pyramidal cells and dentate granule cells (Sekirnjak *et al.*, 1997; Weiser *et al.*, 1994). Kv3.1b is also robustly expressed in fast-spiking cells in the cochlear nucleus (Perney & Kaczmarek, 1997). Double-labeling experiments revealed that the subset of cortical cells labeled with anti-Kv3.1b antibodies corresponded to GABAergic interneurons (distinguished by their fast-spiking properties), which express the calcium-binding protein parvalbumin (Sekirnjak *et al.*, 1997). Interestingly, Kv3.2 α subunits were found in non-fast-spiking, somatostatin- and calbindin-containing interneurons (Chow *et al.*, 1999). Thus, the expression patterns of Kv3.1 and Kv3.2 can distinguish different populations of interneurons, raising the possibility that interneuron firing patterns rely to some extent on the subtype of Kv3 channels expressed (Kawaguchi & Kondo, 2002).

Kv3.3 α subunits are also widely expressed in brain at the mRNA level (Rudy *et al.*, 1999). Both Purkinje cells in cerebellar cortex and deep cerebellar nuclei contain high levels of Kv3.3b message (Goldman-Wohl *et al.*, 1994). Most brainstem auditory neurons also express Kv3.3 mRNA (Li *et al.*, 2001), where it may coassemble with Kv3.1 in a subset of cells. Immunofluorescence staining reveals prominent staining for Kv3.3 in Purkinje-cell somata and dendrites where a Kv3 channel complex of Kv3.3 and Kv3.4 may play a role in shaping large depolarizing events (Martina *et al.*, 2003). Unlike Kv3.1 and Kv3.2, in neocortex and hippocampus Kv3.4 is present in principal cells (Rettig *et al.*, 1992; Weiser *et al.*, 1994). Moreover, Kv3.4 appears to be localized to axons and nerve terminals of these cells, such that in a number of brain regions Kv3.4 is found colocalized with Kv1 family members. Combined *in situ* hybridization and immunohistochemistry showed that Kv3.4 protein is found in many regions rich in nerve terminals (perforant path and mossy fibers in hippocampus, basket cell terminals in cerebellum (Laube *et al.*, 1996; Rettig *et al.*, 1992; Veh *et al.*, 1995). Immuno-electron microscopy on different Kv channels in basket cell terminals revealed Kv1.1 and Kv1.2 are present in septate-like junctions formed between basket cell terminals and Purkinje-cell axons, whereas Kv3.4 is found in nonjunctional regions of the terminals (Laube *et al.*, 1996). These findings highlight the extent to which different highly related ion channel subunits can be precisely localized in neuronal membrane domains.

Mechanisms Regulating Surface Expression of Kv3 α Subunits

Little is known about regulation of the surface expression of Kv3 α subunits. However, Kv3.2 α subunits are efficiently expressed on the cell surface in transfected MDCK cells (Ponce *et al.*, 1997). However, a systematic analysis of the intracellular trafficking determinants on Kv3 α subunits has not been presented.

Control of Polarized Expression and Clustering of Kv3 α Subunits

The extensive alternative splicing of Kv3 mRNAs in the carboxyl termini suggests a possible additional mechanism for differential targeting. In fact, studies on the exogenous expression of the three different Kv3.2 splice variants in polarized MDCK cells revealed that alternative splicing led to differences in subcellular localization (Table 1). The Kv3.2a splice variant was localized to the basolateral membrane, whereas the Kv3.2b and Kv3.2c isoforms were expressed predominantly in the apical membrane (Ponce *et al.*, 1997). The epithelial cell:neuron analogous membrane hypothesis (Dotti & Simons, 1990) predicts that in neurons Kv3.2a would be localized to the somatodendritic domain and Kv3.2b and Kv3.2c to the axon. This has not yet been directly tested, although Kv3.2-specific antibodies directed against a constant domain label both axons and dendrites of cortical GABAergic interneurons (Chow *et al.*, 1999). However, a recent study using alternative-splice, variant–specific anti-Kv3.1 antibodies revealed differences in the polarized expression of Kv3.1 variants in mammalian neurons (Ozaita *et al.*, 2002). The carboxyl-terminus of Kv3.1b bears some similarity to that of Kv3.2a, which directs basolateral localization in MDCK cells (Ponce *et al.*, 1997). The subcellular localization of Kv3.1b in mammalian central neurons is predominantly somatodendritic, although some axonal staining is also observed (Ozaita *et al.*, 2002). In contrast, Kv3.1a was prominently expressed in the axons of some of the same neuronal populations, and there was little or no Kv3.1a protein expression in somatodendritic membrane (Ozaita *et al.*, 2002). Thus, alternative splicing of Kv3.1 transcripts can generate functionally similar variants of the same channel with altered subcellular distributions.

MinK-related peptide 2 (MiRP2) has been shown to bind to Kv3.1 α subunits in heterologous expression systems, in skeletal muscle, and in brain (McCrossan *et al.*, 2003). While MiRP2 coexpression modifies Kv3.1 channel function, a role of MiRP2 in the trafficking and localization of Kv3 channels is not known.

MECHANISMS REGULATING EXPRESSION AND LOCALIZATION OF Kv4 CHANNELS

Cellular and Subcellular Localization of Kv4 α Subunits

The Kv4 (KCND) α subunits Kv4.1, Kv4.2, and Kv4.3 form transient or A-type Kv channels. Experimental knockdown of Kv4 α subunit expression in mammalian neurons results in suppression of A-type Kv channels in superior cervical ganglion neurons (Malin & Nerbonne, 2000). In hippocampal CA1 pyramidal neurons, dendritic transient A-type Kv4 channels, which are expressed in high densities in the distal dendrites, exert profound control over dendritic excitability by limiting dendritic depolarization (Hoffman *et al.*, 1997). Kv4.1 is expressed at very low levels in mammalian brain (Serodio & Rudy, 1998), and what expression that can be detected in neurons does not correlate with A-type current density (Hattori *et al.*, 2003). In contrast, Kv4.2 and Kv4.3 are expressed at relatively high levels, and the expression of these subunits correlates well with neuronal A-type current density in a number of neuronal types (Hattori *et al.*, 2003; Shibata *et al.*, 1999; Song, 2002; Tkatch *et al.*, 2000). *In situ* hybridization analyses show that the expression of Kv4.2 and Kv4.3 is widespread throughout the brain, and whereas in many brain regions the cellular expression of these two Kv4 genes is reciprocal or complementary, there are also cells in which Kv4.2 and Kv4.3 are coexpressed (Serodio & Rudy, 1998). Immunoreactivity for Kv4 α subunits is concentrated primarily in the dendrites of central neurons. Kv4.2 is expressed at high levels in many principal cells, whereas Kv4.3 is found in a subset of principal cells and in many interneurons (Trimmer & Rhodes, 2004). Although a detailed report of the expression and localization of the Kv4 auxiliary subunits KChIP and DPPX has not yet been published, some images of KChIP and DPPX localization have been published within the scope of broader papers (An *et al.*, 2000; Nadal *et al.*, 2003). These studies have revealed that immunoreactivity for KChIP2 is concentrated in dendritic membranes where their distribution corresponds closely with that described for Kv4.2. In contrast, the distribution of KChIP1 closely matches the distribution of Kv4.3, particularly in the somatodendritic domain of neocortical, hippocampal, and striatal interneurons.

Mechanisms Regulating Surface Expression of Kv4 α Subunits

KChIP and DPPX auxiliary subunits dramatically regulate Kv4 channel intracellular trafficking and surface expression. The KChIP family consists of four genes (KChIP1–4; An *et al.*, 2000; Holmqvist *et al.*, 2002; Morohashi *et al.*, 2002; Shibata *et al.*, 2003), and these genes yield at least 16 products through alternative splicing of KChIP mRNA. Most of these gene products are then posttranslationally modified by covalent acylation (O'Callaghan & Burgoyne, 2003; Takimoto *et al.*, 2002). Detailed biochemical analyses showed four KChIPs associate with each Kv4 tetramer (Kim *et al.*, 2004). KChIPs are Ca^{2+}-binding proteins containing four EF hand domains and are structurally related to frequenin/neuronal Ca^{2+} sensor-1. As KChIP3 was also identified as a transcription factor (Carrion *et al.*, 1999), and KChIP3 and KChIP4 as binding proteins for presenilins, the Alzheimer's disease–causative gene products (Buxbaum *et al.*, 1998; Morohashi *et al.*, 2002), KChIPs may have multiple cellular roles. When expressed alone in heterologous cells, Kv4.2 is not efficiently expressed on the cell surface (An *et al.*, 2000; Bahring *et al.*, 2001; Shibata *et al.*, 2003; Takimoto *et al.*, 2002). Moreover, Kv4.2 is misfolded, hypophosphorylated, and relatively unstable when expressed in the absence of KChIPs (Shibata *et al.*, 2003).

KChIP coexpression dramatically increases the density of surface Kv4.2 channels and alters their inactivation kinetics and the rate of recovery from inactivation, and also enhances Kv4.2 protein folding, phosphorylation, and stability (Table 1; An *et al.*, 2000; Bahring *et al.*, 2001; Shibata *et al.*, 2003; Takimoto *et al.*, 2002). ER retention of Kv4 channels expressed in the absence of KChIPs appears to be the primary mechanism preventing surface expression (Shibata *et al.*, 2003). Interestingly, large deletions of the amino-terminus of Kv4.2, where KChIPs bind, allow surface expression of Kv4.2 in the absence of KChIPs (Bahring *et al.*, 2001). This led to a model, recently supported by detailed structural studies (Scannevin *et al.*, 2004; Zhou *et al.*, 2004), that the amino-terminus of Kv4.2 contains an ER retention signal or folding determinant that is masked by KChIP-binding to allow export of Kv4.2/KChIP complexes from ER (Figure 1). While the amino-terminus of Kv4.2 contains a classic dibasic ER retention signal (RXR motif, RKR in Kv4.2), mutation of this motif does not rescue ER export (Shibata *et al.*, 2003). The cytoplasmic amino-terminal region of Kv4.2 has a stretch of hydrophobic amino acid residues upstream of the RKR motif, and deletion of this hydrophobic region rescued Kv4.2 export from the ER. These findings suggest that in the absence of KChIPs, this hydrophobic segment mediates aggregation of Kv4.2 α subunits, leading to activation of the quality control

machinery of ER which results in retention and degradation. The KChIP structure has a hydrophobic binding groove that accommodates and as such masks this hydrophobic segment (Scannevin *et al.*, 2004; Zhou *et al.*, 2004), allowing for appropriate tetramerization and ER export. This model is consistent with the critical role for KChIPs in regulating expression of Kv4 channels.

Recently, DPPX was identified as another binding partner of Kv4 α subunits after immunoaffinity purification of native Kv4 channel complexes from rat brain (Nadal *et al.*, 2003). DPPX is structurally related to the dipeptidyl aminopeptidase family and the cell adhesion molecule CD26, and has a very small cytoplasmic domain (33 or 89 amino acid, depending on alternative splicing) and a large extracellular domain that contains an amino peptidase-like domain and an extracellular matrix-binding domain (Nadal *et al.*, 2003). DPPX has dramatic effects on the physiological properties of Kv4.2 that are distinct from those induced by KChIPs. However, surface expression of Kv4.2 in heterologous expression systems is also increased by DPPX coexpression (Nadal *et al.*, 2003). The mechanism whereby the transmembrane DPPX polypeptide mimics the effects of the cytoplasmic KChIPs on Kv4 expression is not known. These findings together indicate that the trafficking and surface expression of Kv4 channels can be regulated by diverse protein–protein interactions.

Control of Polarized Expression and Clustering of Kv4 α Subunits

As described above, Kv4 channels exhibit strict polarized somatodendritic localization in neurons (Trimmer & Rhodes, 2004). The determinants of dendritic targeting of Kv4 α subunits have been recently investigated in organotypic cortical slice cultures (Rivera *et al.*, 2003). Analyses of these chimeras revealed a critical 16-amino acid dileucine-containing dendritic localization signal (DLS; Table 1) in the cytoplasmic carboxyl-terminal region of Kv4.2 (Figure 1) that is conserved in all Kv4 family members, from nematodes to mammals (Rivera *et al.*, 2003). Unlike the determinant for polarized localization and clustering of dendritic Kv2.1 described above, the Kv4 targeting signal also targets type 1 membrane protein to dendrites (Rivera *et al.*, 2003). These results have to be interpreted in the light of the neuronal expression background used for these studies, which contain endogenous KChIPs, DPPX, and other potential Kv4-interacting partners (see below). Thus, it will be important to determine whether this trafficking signal directs polarized Kv4 targeting in an expression background lacking these proteins (*e.g.*, MDCK cells).

Besides KChIPs and DPPX, three other proteins have been shown to interact with Kv4 α subunits to increase surface expression. The MAGUK PSD-95 can bind to the carboxyl terminus of Kv4.2 to enhance the surface expression level and clustering of Kv4.2 in HEK293 cells (Wong *et al.*, 2002). It is interesting that in cultured hippocampal neurons, the obvious clusters of Kv4.2 and PSD-95 do not colocalize but rather appear interdigitated along the dendrites (see online supplemental data for Shibata *et al.*, 2003). Kvβ subunits also bind to the carboxyl terminus of Kv4 channels and influence expression levels in HEK293 cells (Wang *et al.*, 2003; Yang *et al.*, 2001) but not COS-1 cells (Shibata *et al.*, 2003). Filamin, an actin-binding protein, interacts with the carboxyl terminus of Kv4.3 α subunits (Petrecca *et al.*, 2000). Expression of Kv4.3 in filamin+ and filamin− variants of a human malignant melanoma cell line (M2) revealed filamin-dependent increases in Kv4.3 currents, although the mechanism of the enhanced expression was not characterized.

FUTURE DIRECTIONS

The abundance and distribution of Kv channels in the neuronal membrane is regulated by a diversity of molecular determinants. A major regulatory step regulating the abundance of surface Kv channels is biosynthetic exit from the ER. Diverse mechanisms appear to operate on this key regulatory step, including specific retention signals, forward-trafficking signals, and folding determinants. Such a highly regulated trafficking of Kv channels is consistent with the critical role of Kv channels in neuronal function and the dramatic impact that even small changes in the expression level of these channels have on neuronal excitability. While much has been learned about how these determinants affect Kv channel trafficking and expression in heterologous cells, with the exception of a small number of key confirmatory experiments relatively little has been accomplished in studying these events in real neurons. While there are a number of technical issues that restrict the practical feasibility of such approaches, it is clear that detailed studies of these processes in neurons are needed to confirm and extend those already performed in heterologous systems.

The localization of Kv channels to specific subcellular domains can influence local signaling events, and the localization of different Kv channels in neurons is both highly restricted and incredibly diverse. Many insights have been gained into the determinants of the gross aspects of polarized localization (axonal versus somatodendritic) of Kv channels. However, the signals that restrict specific channels to very small subdomains within these larger compartments, and the molecular machinery mediating the localization, are not well understood.

A critical question remains as to the level of dynamic regulation of the abundance and distribution of Kv channels in neurons. There exist hints that the trafficking and

localization of Kv channels are dynamically influenced by phosphorylation state, but the extent of nature of the contribution of these and other signaling events to post-translational regulation of Kv channel expression is not fully appreciated.

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