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Molecular Properties of Voltage-Gated K^+ Channels

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Subfamilies of voltage-activated K^+ channels (Kv1–4) contribute to controlling neuron excitability and the underlying functional parameters. Genes encoding the multiple α subunits from each of these protein groups have been cloned, expressed and the resultant distinct K^+ currents characterized. The predicted amino acid sequences showed that each α subunit contains six putative membrane-spanning α-helical segments (S1–6), with one (S4) being deemed responsible for the channels' voltage sensing. Additionally, there is an H5 region, of incompletely defined structure, that traverses the membrane and forms the ion pore; residues therein responsible for K^+ selectivity have been identified. Susceptibility of certain K^+ currents produced by the Shaker-related subfamily (Kv1) to inhibition by α-dendrotoxin has allowed purification of authentic K^+ channels from mammalian brain. These are large ($M_r \sim 400$ kD), octomeric sialoglycoproteins composed of α and β subunits in a stoichiometry of $(\alpha)_4(\beta)_4$, with subtypes being created by combinations of subunit isoforms. Subsequent cloning of the genes for β_1, β_2 and β_3 subunits revealed novel sequences for these hydrophilic proteins that are postulated to be associated with the α subunits on the inner side of the membrane. Co-expression of β_1 and Kv1.4 subunits demonstrated that this auxiliary β protein accelerates the inactivation of the K^+ current, a striking effect mediated by an N-terminal moiety. Models are presented that indicate the functional domains pinpointed in the channel proteins.

KEY WORDS: Dendrotoxins; K^+ channels; K^+ current activation; K^+ current inactivation; K^+ filter/pore; Shaker; α and β-subunit.

I. INTRODUCTION

Voltage-gated K^+ channels are the oldest and most diverse group of cation channels. In the nervous system, they serve a variety of important functions, particularly the control of cell excitability, contributing to the maintenance of resting membrane potential, membrane repolarization, and the regulation of action potential duration and frequency (Hille, 1992). This variety of functions necessitates that K^+ channels be highly diverse in their biophysical properties. How such K^+ currents are integrated into neuronal function is influenced by channel availability at the resting membrane potential, activation threshold, rates of inactivation plus recovery, and channel density. Hence, assessment of the functional role(s) of a given K^+ channel can be aided by deciphering its biophysical properties. Also, this is facilitated by inhibition of a particular current with selective blockers, α-dendrotoxin (α-DTX) and other neurotoxins (Table I) having proved especially useful (Dolly *et al.*, 1984, 1994a). At least four types of voltage-activated K^+ currents have, thus, been distinguished electrophysiologically in mammalian neurons (for an excellent review, see Halliwell, 1990).

Delayed rectifier (I_k): This current is activated after some delay (hence, its name) by depolarization (threshold more positive than −20 mV) and inactivates very slowly (>10 secs). It is blocked by tetraethylammonium (TEA) but is relatively insensitive to 4-aminopyridine (4-AP). Its main function is to repolarize the membrane and, thereby, limit the duration of action potentials.

Fast, transient (I_A): A current that activates quickly (few msecs) upon depolarization (threshold

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Table I. Nomenclature of Subfamilies and Properties of Voltage-Dependent K^+ Channels Produced by Expression of Mammalian Cloned α Subunits in *Xenopus* Oocytes

Subfamily	Activation threshold (mV)	Activation rate[a] (ms)	Inactivation rate[b] (ms)	Pharmacology[c] TEA (mM)	4-AP (mM)	α-DTX (nM)	CTX (nM)	MCDP (nM)	References
Shaker-related									
$K_v1.1$	−40	16	17%*	0.6	1	12	22	45	1
$K_v1.2$	−40	6	40%*	129	0.8	4	6	175	1
$K_v1.3$	−40	14	86%*	50	1.5	>600	1	>1000	1
$K_v1.4$	−55	3	109	>100	12.5	>200	>40	>2000	1
$K_v1.5$	−40	n.r.	slow	>40	0.5	>200	>200	>200	2
$K_v1.6$	−40	26	72%*	4	0.3	9	20	n.r.	2
Shab-related									
$K_v2.1$	−15	20–100	non	10	0.5	n.r.	>1000	n.r.	3
$K_v2.2$	−20	50–100	non	7.9	n.r.	n.r.	n.r.	n.r.	4
Shaw-related									
$K_v3.1$	−5	10–100	13%#	0.1	0.6	n.r.	n.r.	n.r.	5
$K_v3.2$	−10	80	very slow	85% (1 mM)	85% (1 mM)	n.r.	n.r.	n.r.	6
$K_v3.4$	−10	3.4(50mV)	15.2(50mV)	0.3	0.5	>100	n.r.	n.r.	7
Shal-related									
$K_v4.1$	−45	10.1(20mV)	τ_1:22.6(0.34) τ_2:86.4(0.37) τ_3:368(0.29)	>10 (2mM)	18%	n.r.	n.r.	n.r.	8
$K_v4.2$									

[a] Activation rates are measured at 0 mV (unless stated otherwise) and refer to the time to reach 90% of maximal current amplitude.

[b] Inactivation rate is measured at 0 mV unless specified; in cases where there is at least two time constants to the decay, the values in parentheses give the relative contributions from each component.

[c] Values are IC_{50}s except in some cases where % block of the K^+ current by a given concentration is indicated.

* denotes percent inactivation after a 3.2 test pulse, and # denotes percent inactivation after 1 s. n.r. means not reported. Details of K^+ channel nomenclature are given by Gutman and Chandy (1993). Data were taken from: 1, Stuhmer *et al.* (1989); 2, Swanson *et al.* (1990); 3, Frech *et al.* (1989); 4, Hwang *et al.* (1992); 5, Yokohama *et al.* (1989), 6, McCormack *et al.* (1990); 7, Schröter *et al.* (1991); 8, Pak *et al.* (1991).

−50 mV) and inactivates in less than ~100 msec. As its inactivation is complete at membrane potentials more positive than −55 mV, a neuron with a resting potential around this value can only be affected by I_A following a hyperpolarization to remove inactivation prior to depolarization. Functionally, both firing patterns and threshold for firing are affected by the A current in neurons with a negative resting potential. This was clearly demonstrated by blocking I_A in hippocampal pyramidal neurons with α-DTX (see below) or 4-AP (Halliwell *et al.*, 1986; Dolly *et al.*, 1986).

Fast-activating, slowly-inactivating (I_D): This K^+ current, described by Storm (1988), activates rapidly (within msec) but inactivates slowly (over ~1 sec). It differs from the delayed rectifier in being TEA insensitive, highly susceptible to 4-AP, and activated at membrane potentials negative to −60 mV (a threshold ~20 mV more negative than that of I_A). I_D delays (hence, its name) firing of action potentials in a manner that allows temporal integration of depolarizing inputs to the neuron.

Inward (or anomalous) rectifiers: These mediate inward rather than the normal outward K^+ currents.

The potential for diversity of these K^+ channels in mammalian cells has been highlighted over the last half decade by the cloning of a multiplicity of genes

for their α subunits. This progress was afforded by identification of the single locus coding for the A-type K^+ channel α subunit, using the *Drosophila* Shaker mutant that has a defect in this gene [reviewed by Pongs (1992a) and Jan and Jan (1992)]. Its subsequent cloning provided an effective means for probing homologous nucleotide sequences in mammalian cells, resulting in the cloning of four subfamilies (Kv 1, 2, 3, and 4) of α subunit genes called Shaker-, Shab-, Shaw-, and Shal-related after the original genes cloned from *Drosophila* (Table I). As there are multiple members in each subfamily, Kv XY is used to identify each (for accepted nomenclature, see Gutman and Chandy, 1993). Although expression of each of the latter in heterologous systems yielded distinct voltage-activated K^+ currents, their biophysical and pharmacological properties do not normally match those of the native K^+ channels, with a few exceptions. Also, there could be fewer actual K^+ channel subtypes than the large number of α subunits identified to date because, at least in the case of Kv 1 subfamily, several α and newly-discovered β subunits appear to be combined in multimeric complexes (to be detailed later). Hence, it is imperative to establish the oligomeric and subunit properties of these authentic proteins from neurons so that insights into structure and function can be gleaned from their exact reconstruction, by recombinant means, followed by detailed biophysical analysis of the K^+ currents. For this reason, a fundamental goal of our research has been to biochemically characterize the naturally-occurring channel proteins from mammalian brain, both in the membrane-bound and purified states (reviewed by Dolly *et al.*, 1994a, b). α-DTX, an effective blocker of members of this K^+ channel family or its homologue toxin I (Tables I and II), served as very useful probes both for localizing the channels in CNS, monitoring channel activity, and acting as affinity-chromatography ligands for purification purposes. This overview will focus on structural aspects of α-DTX-sensitive K^+ channels because they are the only authentic K^+ channel proteins that have been purified and their molecular properties deciphered, at the oligomeric and subunit levels. In this regard, they serve as prototypes; it is expected that information gained from studies with such members of the Shaker-related subfamily of K^+ channels will aid future progress with the others (Shab-, Shaw-, and Shal-related); as the native structures of these latter channels have yet to be determined, they are not covered in this chapter but have been reviewed by Salkoff *et al.* (1992).

II. α-DTX-SUSCEPTIBLE K^+ CURRENTS IN MAMMALIAN BRAIN—PROTOTYPES K^+ CHANNELS

1. α-DTX and Homologous K^+ Channel Blockers: Structure and Activity

Several neurotoxins have been isolated from the venoms of the green (*Dendroaspis angusticeps*) and black (*Dendroaspis polyepis*) mamba snakes (reviewed in Dolly, 1992a; Dolly *et al.*, 1994a). They are single-chain polypeptides of $M_r \sim 7$ kDa, which show significant sequence homology to bovine pancreatic trypsin inhibitor (BPTI) and other Kunitz type protease inhibitors. However, the most potent neurotoxins (α-DTX; toxin I) exhibit negligible protease inhibitor activity while, conversely, the weakly or nontoxic homologues (e.g., toxin E) are able to inhibit some proteolytic activity, albeit with considerably less potency than BPTI. An additional significant homology also exists between the mamba toxins and the smaller B chain of β-bungarotoxin (β-BuTX). Unlike the former, this protein from the banded krait *Bungarus multicinctus* occurs disulfide-linked to a phospholipase A_2 chain of $M_r \sim 14$ kDa. The structure of several α-DTX homologues has recently been deduced either by X-ray diffraction of protein crystals (α-DTX; Skarzynski, 1992) or by solution NMR spectroscopy (toxins K and I; Berndt *et al.*, 1993; Lancelin *et al.*, 1994). As expected, the overall carbon backbone structure is similar for all these toxin homologues: an N-terminal region either with little structure (α-DTX) or containing a short 3.10 helix (toxins I and K), a loop responsible (in BPTI) for its antiprotease activity, a twisted double-stranded antiparallel β sheet, and, at the C-terminus, a short α-helix. Much interest has focused upon the β turn between the two β strands, since this element contains a region conserved between the toxins which contains two (toxin K, δ-DTX) or three (α-DTX, toxin I) basic lysine residues and may be responsible for their K^+ channel blocking activity (reviewed by Harvey, 1993). However, a recent report (Danse *et al.*, 1994) in which the three lysines were substituted in α-DTX appears to contradict this notion. Nevertheless, knowledge of the high-resolution structure of these molecules should facilitate identification of the amino acids responsible for their toxicity and, importantly, their subtle and selective blockade of K^+ channels (see below). These neurotoxins facilitate neurotransmitter release at a number of peripheral (Harvey

Table II. Blockade by α-DTX and Other Toxins of Voltage-Activated K^+ Currents in Neurons and Those Produced by Expression of Kv α Genes[a]

A. Authenic K^+ currents

Source	*Rate of inactivation*[b]	α-DTX IC_{50} (nM)	*References*
Rat nodose ganglion A cells	slow (secs)	2.1	Stansfeld *et al.* (1987)
Neonatal rat dorsal root ganglion	0	2.0	Stansfeld and Feltz (1988)
Rat hypothalamic magnocellular neurosecretory cell	200 msecs	4.0	Bourque (1988)
Rat node of Ranvier	0 over 100 msecs	8.0	Corrette *et al.* (1991)
Frog node of Ranvier	slow (secs)	11.0	Bräu *et al.* (1990)
Rat hippocampal CA1 pyramidal cells	50–100 msecs	~50.0	Dolly *et al.* (1984) Halliwell *et al.* (1986)
Rat nodose ganglion C cells	18–24 msecs	>800	Stansfeld *et al.* (1987)

B. Expressed in transfected mammalian cells

	α-DTX	Charybdotoxin	Noxiustoxin	Kaliotoxin
Mouse Kv 1.1	20	>1000	>25	41
Rat Kv 1.2	17	14	2	>1000
Mouse Kv 1.3	250	2.6	1	0.65
Human Kv 1.5	>1000	>100	>25	>1000
Mouse Kv 3.1	>1000	>1000	>25	>1000

C. Expressed in *Xenopus* oocytes

	δ-DTX	Toxin K	Toxin I	α-DTX	MCDP
Human Kv1.1	0.003	0.047	0.5	1.7	103
Rat Kv1.1	1	2.5	4	5	45
Rat Kv1.2		2100			

[a] Data in B and C taken from Grissmer *et al.* (1994) and Owen (1995), respectively.
[b] Time taken for the current to decay completely after a voltage step to + 40 mV.

and Karlsson, 1980; Petersen *et al.*, 1986; Rowan and Harvey, 1988; Hu *et al.*, 1991) and central (Dolly *et al.*, 1986; Tibbs *et al.*, 1989; reviewed in Dreyer, 1990; Dolly, 1992b; Dolly *et al.*, 1994a) synapses. This effect may be explained by the toxins' blockade of certain voltage-sensitive K^+ currents in these nerves.

The first K^+ channel identified as a target for α-DTX and toxin I was that giving rise to a fast-activating, rapidly-inactivating "A" current of rat hippocampal neurons (Dolly *et al.*, 1984; Halliwell *et al.*, 1986). However, its IC_{50} (~50 nM) for block of that current is somewhat higher than would be expected from its dissociation constant ($K_D \sim 0.5$ nM) in rat brain synaptosomal membranes (Black *et al.*, 1986), suggesting that this current may not be the toxin's preferred target. In fact, the transient K^+ currents of guinea-pig dorsal root ganglion cells (Penner *et al.*, 1986), motor nerve terminal (Dreyer and Penner, 1987; Harvey, 1993), rat nodose C cells (Stansfeld *et al.*, 1987), and superior cervical ganglion cells (Stansfeld *et al.*, 1986) are all unaffected by α-DTX. However, a rapidly-inactivating voltage-sensitive K^+ current in magnosecretory neurons of the rat hypothalamus is very sensitive to α-DTX (Bourque, 1988), but this is Ca^{2+}-dependent and quite distinct from the neuronal A current. On the other hand, α-DTX and toxin I block slowly-inactivating K^+ currents from a number of preparations with relatively high potency. At the frog node of Ranvier, toxin I preferentially inhibits a slowly-inactivating component of the K^+ current (I_{KFI}) with IC_{50} of 0.4 nM (Benoit and Dubois, 1986); α-DTX is about 25-fold less potent (Brau *et al.*, 1990). A similar current is blocked by α-DTX at the rat node (Corrette *et al.*, 1991). Slowly-inactivating K^+ currents in dorsal root ganglion cells (Penner *et al.*, 1986; Stansfeld and Feltz, 1988) and rat nodose A cells (Stansfeld *et al.*, 1987) are also inhibited by α-DTX ($IC_{50} \sim 2$ nM). Thus, α-DTX blocks preferentially a family of neuronal K^+ currents with slow inactivation rates but relatively fast activation kinetics. Although it is preferable to study the action of α-DTX (and other toxins) on neuronal K^+ currents, it is not possible to identify the molecular

components comprising these authentic K^+ channels. However, expression in *Xenopus* oocytes and vertebrate cells of mammalian cloned K^+ channel subunits has allowed the toxins' specificity to be further investigated (see Tables I and II). Only the Shaker-related (Kv1.x) subfamily has been extensively investigated. In both expression systems, α-DTX preferentially blocks Kv1.2, 1.1, and 1.6; mast cell degranulating peptide (MCDP) also blocks the former two in oocytes. The pattern of blockade by charybdotoxin (CTX) is less clear, possibly due to variability of the toxin preparations used; in *Xenopus* oocytes, it inhibits Kv1.1, 1.2, 1.3, and 1.6 currents but fails to block Kv1.1 expressed in mammalian cells. In the latter system, noxiustoxin has the same rank order of potency as CTX, with Kv1.3 currents being most susceptible. On the other hand, while Kv1.3 K^+ current is also the primary target of kaliotoxin, the latter is unable to affect K^+ channels formed with Kv1.2 subunits. The potency of the α-DTX homologues, toxin K and δ-DTX, depends on the species from which the Kv1.x subunit is cloned. Thus, human Kv1.1 expressed in mammalian cells is exquisitely sensitive to δ-DTX (IC_{50} = 3 pM) while the rat form is considerably less susceptible (IC_{50} = 1 nM). A similar though less exaggerated pattern is also seen with toxin K. Although none of these expressed channels are identical to the native, neuronal targets for α-DTX and the other toxins, these studies may enable the identification of some of their components. In fact, good correlations can be made between these electrophysiological data and the deduced subunit composition(s) of purified α-DTX-sensitive K^+ channels (see below).

2. Identification of K^+ Channels Using α-DTX

Of all the toxins known to block K^+ currents in neurons (see above), α-DTX and the closely related toxin I have been most useful in probing the structures of the channel proteins. A fully-active radioiodinated form of α-DTX (^{125}I-α-DTX) binds to an apparently homogeneous set of sites in cerebrocortical synaptosomes from rat (Black *et al.*, 1986), bovine (Parcej and Dolly, 1989), and guinea pig (Muniz *et al.*, 1990a) with an affinity of 0.1–0.5 nM. In contrast, two classes of ^{125}I-α-DTX binding site are observed (Black and Dolly, 1986) in chick brain nerve endings, only one of which is blocked by β-BuTX. Similarly, complex interactions between α-DTX or toxin I and a number of other toxins including MCDP (Bidard *et al.*, 1987; Schmidt *et al.*, 1988; Stansfeld *et al.*, 1987), δ-DTX (Muniz *et al.*, 1990a), CTX (Harvey *et al.*, 1989), and β- and γ-DTX (Sorensen and Blaustein, 1989) have also been observed, indicating that α-DTX binds to several subtypes within a family of K^+ channels. This notion was supported by the differential distribution of binding sites for α-DTX, δ-DTX, β BuTX, and MCDP in sections of whole rat brain (Pelchen-Matthews and Dolly, 1988, 1989; Awan and Dolly, 1991; Mourre *et al.*, 1988).

3. Labelling of Polypeptides Constituting the Toxin-Sensitive K^+ Channels

The first identification of a component of native K^+ channels was achieved by covalent cross-linking of ^{125}I-α-DTX to its binding protein in rat brain synaptosomes, with subsequent separation by SDS-PAGE (Mehraban *et al.*, 1984). This labelled a component of about 65,000 M_r, after allowance for the size contribution of one bound toxin molecule. Later, using β-BuTX (Schmidt and Betz, 1989) and MCDP (Rehm *et al.*, 1988), bands of similar size were observed in rat brain synaptosomes, as well as a much smaller one (M_r 28,000) which was labelled only by β-BuTX. In chick brain, two proteins of M_r 75,000 and 69,000 were crosslinked to ^{125}I-α-DTX (Black and Dolly, 1986); labelling of both could be prevented by unlabelled α-DTX or β-BuTX. Conversely, radiolabelled β-BuTX (^{125}I-β-BuTX) itself labelled only a single band of 95,000 in this system (Rehm and Betz, 1983). Similarly two components (M_r = 74,000 and 97,000) were also identified in guinea-pig brain synaptosomes using ^{125}I-δ-DTX as the probe; although labelling of both bands could be inhibited by α-DTX, the latter only appeared to bind to the smaller component (Muniz *et al.*, 1990a). These studies represent the initial biochemical characterization of putative K^+ channel subunits which appear to correspond to the α subunits subsequently identified after purification (see Section II 4). It is possible that the differences in molecular masses of the components labelled by the various toxins in these experiments, even when membranes from the same species were employed, could be due to use of different cross-linking agents or variabilities in the gel systems employed. Certainly, it was subsequently shown that the molecular masses of purified K^+ channel α subunits calculated from SDS-PAGE increased with the percent acrylamide used (Scott *et al.*, 1990). More interesting is the possibility that several different subunits are labelled—

this is particularly plausible where one toxin (e.g., δ-DTX in guinea pig brain synaptosomes or α-DTX in chick brain membranes) labels two bands (though labelling of a single channel component by two toxin molecules cannot be excluded). This notion is consistent with the complex binding interactions observed between the toxins, and with the ensuing discovery of a number of mammalian K^+ channel α subunits which may assemble together to form many oligomeric subtypes (see later).

4. Purification of K^+ Channels Sensitive to α-DTX: Oligomeric Properties

After solubilization with mild detergents which indicated the intrinsic membrane-spanning nature of the proteins, the selective, high-affinity binding of α-DTX and toxin I was exploited by two groups for the purification of K^+ channel proteins. In one approach, essentially pure channels were obtained from bovine brain by a single affinity chromatography step on immobilized toxin I (Fig. 1A; Parcej and Dolly, 1989; Parcej *et al.*, 1992). Although the toxin affinity procedure alone employed in the second approach yielded a lower level of purity, additional anion-exchange and lectin affinity chromatography together with sucrose density gradient centrifugation steps allowed the purification of K^+ channels from rat brain (Rehm and Lazdunski, 1988). In both cases two major subunits were observed: a broad diffusely-staining α subunit of approximately 78,000 M_r, which corresponds to the bands observed by toxin cross-linking, and a sharp band of M_r 39,000 that was called β. The α, but not β, subunit was N-glycosylated as shown by the reduction in M_r upon treatment with neuraminadase and PNGase F (Fig. 1B). The molecular mass of the α subunit can also be reduced further by incubation by alkaline phosphatase (Fig. 1B), indicating the presence of phosphorylation. Both subunits also acted as substrates for exogenous protein kinase A (Rehm *et al.*, 1989; Scott *et al.*, 1994a). An additional 56,000 M_r band was also observed in the bovine preparation after phosphorylation; this may be another α subtype not visible after protein staining. In addition, endogenous

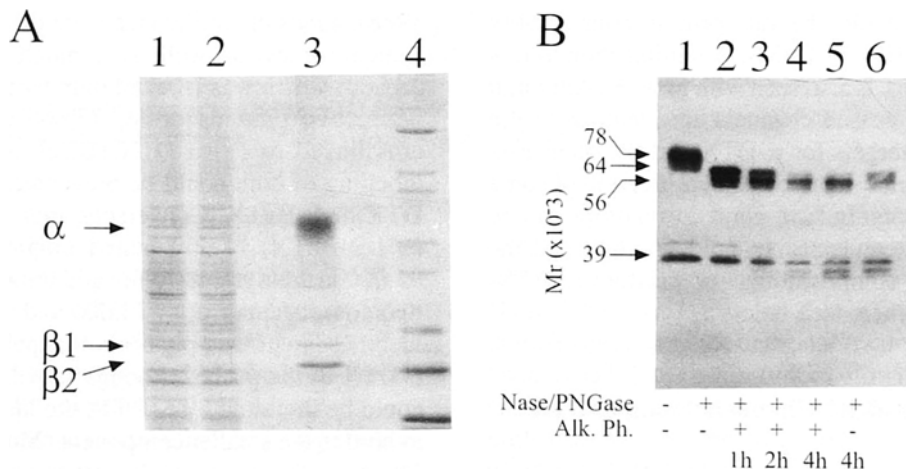

Fig. 1. Purification of α-DTX sensitive voltage-dependent K^+ channels from bovine brain: subunit structure and covalent modification. A. Silver stain of a 10% SDS-PAGE gel showing stages of K^+ channel protein purification (Parcej and Dolly, 1989; Parcej *et al.*, 1992). Lanes 1, crude detergent extract of bovine synaptic plasma membranes; 2, breakthrough of the toxin I affinity column; 3, DTT eluate of the affinity column. Note the presence of major α and β_2 subunits plus the faint (possibly) β_1 band just above the β_2 subunit; 4, molecular weight markers. B. Purified K^+ channels were digested with glycosidases and/or alkaline phosphatase to show the presence of covalently-linked sugar chains and phosphate on the α subunit. After electrophoresis and electroblotting, the channel constituents were detected using antibodies reactive with α or β subunits. Detection was by an enhanced chemiluminescence system. Lanes 1: purified K^+ channels; 2: K^+ channels incubated for 1 h at 37°C with neuramidase and peptidase-N-glycosidase F to remove sialic acid and N-linked core sugars, respectively (Scott *et al.*, 1990); 3–5: deglycosylated K^+ channels treated subsequently for 1, 2, or 4 h at 37°C with alkaline phosphatase; 6: deglycosylated K^+ channels incubated for 4 h at 37°C in the absence of alkaline phosphatase. Note the shift of the upper α band observed in deglycosylated K^+ channels to a lower M_r in the presence of alkaline phosphatase.

kinase activity co-purified with the K^+ channels, but its properties were slightly different in the rat and bovine preparations. In the former, only α subunits could be labelled and the phosphorylation reaction was supported by Mn^{2+}. In the latter, however, both α and β subunits (but not the M_r 56,000 band) could be phosphorylated but all the kinase activity was lost when Mn^{2+} was used in place of Mg^{2+}. The identity of the co-purifying kinase(s) is not yet known but it is possible that phosphorylation may be important in regulating K^+ channel function.

The oligomeric composition of the purified K^+ channels was deduced by measurement of their molecular mass by sucrose density-gradient centrifugation and gel-filtration (Parcej *et al.*, 1992). Preparation of gradients in both ^1H_2O and ^2H_2O allowed the contribution of bound detergent to be accounted for. The vast majority of the channels contained both α and β subunits and had a molecular mass of approximately 400,000. However, a minor species with a size of a 250,000 was also observed but this possesses only α subunits. The mass of the sparse peak could be accounted for by four α subunits, while the difference between the masses of the two populations, if contributed by β subunits only, would suggest that the larger, more abundant form contains four α and four β subunits.

The $(\alpha)_4(\beta)_4$ stoichiometry observed for the naturally-occurring channels is consistent with indirect studies of K^+ channels expressed in *Xenopus* oocytes after injection of cRNAs encoding α subunits *only* of either *Drosophila* Shaker or mammalian Kv1.1 (Shaker-related) proteins. These recombinant products are equivalent to the α subunits of the purified channel (see below). MacKinnon (1991) utilized cRNAs of Shaker variants which produce distinct K^+ channels when expressed in oocytes, i.e., either sensitive (wild-type) or insensitive (mutant) to CTX. By mixing the two types of cRNAs in various ratios and observing the amount of CTX-sensitive and insensitive K^+ current produced, it was possible to calculate that the channels assembled as either tetramers (α_4) or nonomers (α_9). Since the K^+ channel α subunits appear to be equivalent to one of four repeating domains of Na^+ and Ca^{2+} channel α subunits, and given the observation that α subunit cRNAs linked together in pairs also form active K^+ channels when injected in oocytes (Isacoff *et al.*, 1990), it was suggested that the K^+ channels are tetrameric. Liman *et al.* (1992) constructed tandem linked Kv1.1 (and mutant) cDNAs comprising two, three, four or five K^+ channels α subunits and found that the highest expression level was obtained with the four α subunit construct, reaffirming that the channels form as tetramers. Recently, the oligomeric structure of both active and expressed K^+ channels has been examined by electron microscopy. Shaker α subunits were expressed in insect SF9 cells after transfection with the baculovirus vector. After purification, images of these K^+ channels (Li *et al.*, 1994) showed a distinct 4-fold symmetry, as expected of an "end-on" view of a tetrameric structure. Using this technique, images of the authenic K^+ channels purified from brain are being obtained in this laboratory so that the topography of α and β subunits can be established.

5. Identification of the α Subunits Present in Neuronal Purified K^+ Channel Oligomers

When the α subunit was subjected to solid-phase microsequencing (Scott *et al.*, 1990), its N-terminal amino acid sequence was found to be almost identical to Kv1.2 (Stuhmer *et al.*, 1989; McKinnon, 1989). Interestingly, this clone, when expressed gives rise to K^+ currents which are highly sensitive to α-DTX (Tables I and II B), confirming that the α-DTX binding component is a K^+ channel protein. Subsequent cloning (Reid *et al.*, 1992) of the bovine α-DTX K^+ channel α subunit confirmed its identity as part of the Shaker-like mammalian K^+ channel family. A large number of K^+ channel α subunits have been cloned from mammalian brain, and other excitable tissues, initially using sequences of K^+ channel cDNAs cloned from the *Drosophila* Shaker locus. From the translated amino acid sequences of the *Drosophila* and mammalian K^+ channel α subunits, a simple model has been proposed (Fig. 2A). The protein contains six putative transmembrane domains (S1–S6)—possibly α-helical—together with the H5 domain which is believed to be part of the K^+ conducting pore (see later). Both N- and C-termini are likely to be intracellular since the proteins lack a typical N-terminal leader sequence and cross the membrane an even number of times. Also identifiable are a number of possible phosphorylation sites and an N-glycosylation motif-consistent with data obtained with pure, native K^+ channels (Section II 4). However, *Drosophila* and mammals appear to use quite different mechanisms for generating K^+ channel variants. In the former, a single, large Shaker gene complex generates a number of K^+ channel transcripts by alternative splicing (Fig. 2B). The S1–S5 and H5 regions are encoded by a block of exons to yield an invariant core domain. Various 5′

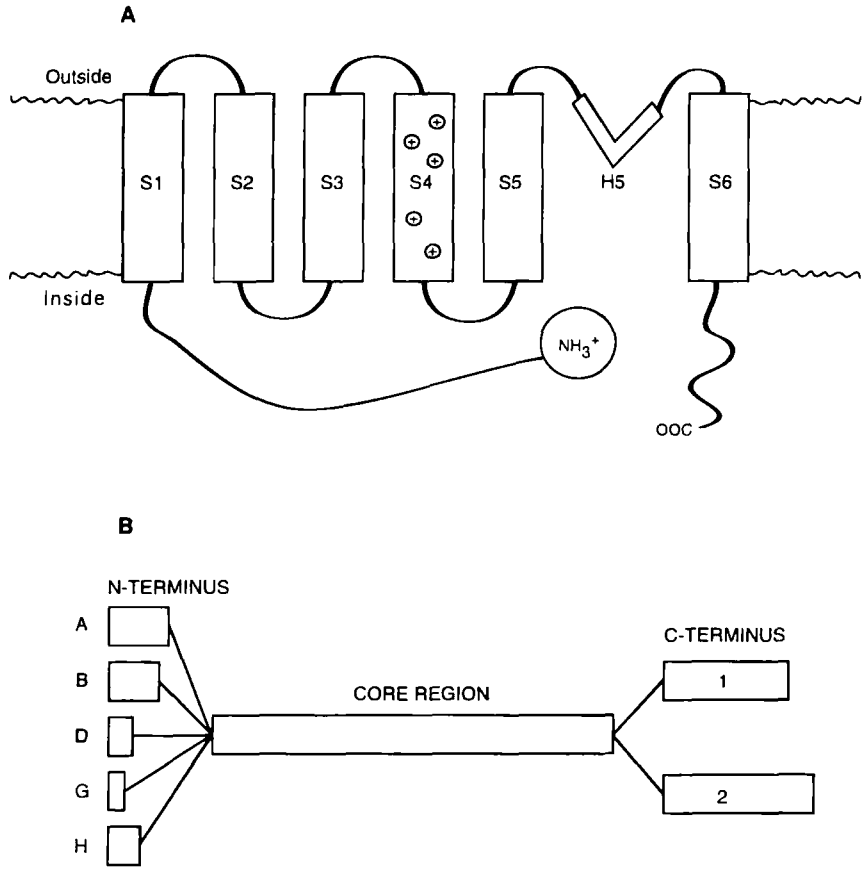

Fig. 2. Diagrammatic representation of gross structural features common to all Kv channels' α subunits and the dissimilar terminal sequences in the alternatively-spliced variants of Shaker. A. Both the N- and C- termini are thought to be located on the cytoplasmic side of the membrane. Six putative, hydrophobic, membrane-spanning segments (S1–6) are present, with S4 containing a conserved, positively-charged, repeat motif (Arg/Lys XX) that comprises the voltage sensor (see text). It is believed that H5 forms a hairpin loop in the membrane, and contains residues comprising the ion pore. The N-terminal region of rapidly-activating A-type K^+ channels (Shaker B, Kv 1.4 and 3.4), but not others, has a domain (containing hydrophobic and positively charged amino acids) that mediates N-type fast inactivation (detailed later). B. Illustrates the core region of the single-gene locus of Shaker that encodes, in every variant, all the membrane-traversing segments (shown in A) except S6; it is flanked by five alternative 5′ ends and two 3′ ends (the latter encodes S6, as well as the C-termini). Alternative splicing in Shaker, using all combinations of core region and termini, yields several channel variants; in contrast, diversity of the mammalian α subunits arises from multiple genes. Redrawn from Pongs (1992a).

and 3′ ends are then used to produce different N and C terminal (including S6) domains. Upon translation, the mRNAs produced yield K^+ channel variants which differ in kinetic properties. In contrast, mammals possess numerous intronless genes with highly homologous, though not identical, transmembrane domains; diversity is generated by the latter and variations in the 5′ and 3′ ends of these different genes. In particular, Kv 1.4 and 3.4 contain extended N-termini encompassing an inactivation "ball" domain (Fig. 2A; see later).

Western blotting with α subtype specific polyclonal antibodies, raised against the divergent C-termini of the Shaker-like mammalian proteins (Scott *et al.*, 1994a, Fig. 3A), revealed the presence of several members of the Kv 1 subfamily—demonstrating that the purified α-DTX-sensitive K^+ channel preparation contained a number of these α subunit variants. This

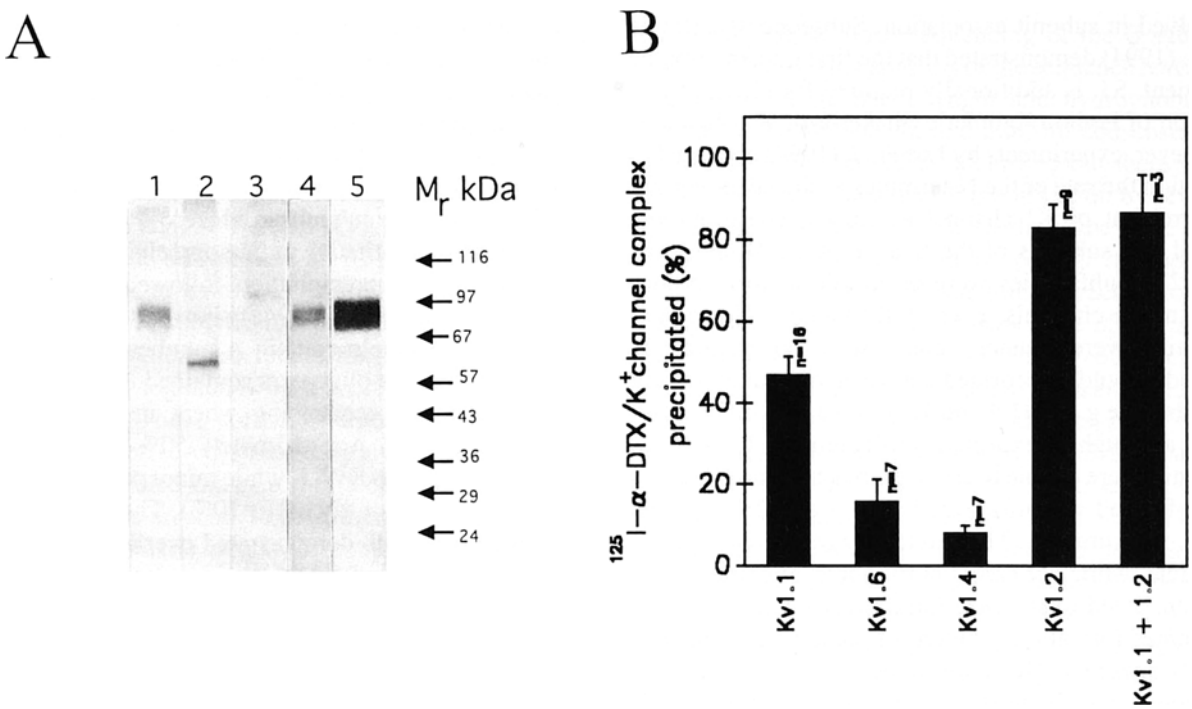

Fig. 3. Immunoprecipitation and Western blotting of α-DTX sensitive K^+ channels with α-subunit specific antibodies: demonstration of oligomeric subtypes. A. K^+ channels purified by toxin affinity chromatography (Parcej and Dolly, 1989) were separated by SDS-PAGE, electroblotted onto PVDF membrane, probed with α-subunit specific antibodies, and visualized after addition of anti-species antibody coupled to alkaline phosphatase. Lanes 1: anti-Kv 1.1 antibody; 2: anti-Kv 1.6; 3: anti-Kv 1.4; 4: anti-Kv 1.2 (rabbit polyclonal); 5: anti-Kv 1.2 (mouse monoclonal antibody). The positions of molecular mass markers are also shown. B. α-DTX sensitive K^+ channels were labelled in crude detergent extracts by incubation with ^{125}I-labelled α-DTX. Anti-α subunit antibodies were added, followed by anti-IgG-sepharose. After centrifugation and washing to remove unbound ^{125}I-α-DTX, pellets were γ-counted to assess the amount of K^+ channel-toxin complex precipitated. Data are expressed relative to the ^{125}I-α-DTX binding in crude extract. From Scott *et al.* (1994b).

suggests a structural basis for the multiple α-DTX sensitive K^+ channel subtypes observed from binding and cross-linking studies (Section II 3), though the presence of homo- and/or hetero-oligomeric K^+ channels had to be established.

6. Evidence for Hetero-oligomeric K^+ Channels in Brain

Co-injection of cRNAs encoding different α subunits (Isacoff *et al.*, 1990; Ruppersberg *et al.*, 1990; Christie *et al.*, 1990) lead to the generation of K^+ currents with properties intermediate between those produced by each α subunit cRNA when injected alone, indicating the formation of hetero-oligomeric K^+ channel tetramers. However, hetero-oligomeric channels were not formed (Covarrubias *et al.*, 1991) when Shaker α subunit cRNAs were co-injected with those encoding α subunits of different K^+ channel subfamilies. The primary determinants for the specificity of assembly of K^+ channel α subunits appear to lie in the cytosolic N-terminal region. Li *et al.* (1992) and Shen *et al.* (1993) identified a stretch of amino acids (residues 83–196 in Shaker B and 66–194 in Kv 1.1) that were conserved between Shaker subfamily members and appeared essentially for the formation of homo-oligomeric channels. Mutation of small groups of amino acids within the larger region was also sufficient to disrupt oligomer formation, suggesting that the structural integrity of the whole N terminal domain was required for efficient assembly (Hopkins *et al.*, 1994a; Shen and Pfaffinger, 1995). However, some assembly was observed between Kv 1.1 subunits that contained the N-terminal domain and those that did not (Shen *et al.*, 1993); probably it was sufficient for each tetramer to contain a single copy of the latter. This indicated that other parts of the protein were

involved in subunit association. Subsequently, Babila *et al.* (1994) demonstrated that the first transmembrane segment, S1, is additionally required for efficient formation of homo-oligomeric Shaker-type K^+ channels. However, experiments by Lee *et al.* (1994) appeared to question the role of the N-terminus as the all-important determinant of K^+ channel assembly. These authors found that subunits of the Shaker (Kv 1.4) and Shab (Kv 2.1) subfamilies were each able to form homo-multimeric channels, even if their entire N-terminal domains were deleted; conversely, no intrafamily hybrids could be formed between truncated family members (e.g., Kv 1.4 and 1.5). Most strikingly, however, although as expected full-length Kv 1.4 and 2.1 subunits were unable to co-assemble, hetero-oligomers were formed when truncated Kv 1.4 and 2.1 subunits were co-expressed. These data suggested that the N-terminal, while not essential for homo-oligomeric formation, acted to stabilize intrafamily interactions and prevented formation of hetero-oligomers between non-family subunits. This latter assertion agrees well with studies in which the N-terminus of either Shaker B (Li *et al.*, 1992) or Kv 1.1 (Shen and Pfaffinger, 1995) was transplanted into Kv 2.1 or 3.1, respectively. In both cases, the recipient subunit inherited Shaker-like specificity of assembly. The possible role of the C terminus in K^+ channel assembly has also been addressed recently (Hopkins *et al.*, 1994a). Kv 1.1 subunits with deletions of amino acids 480–495 were able to form homo-multimers or co-assemble into hetero-multimeric structures with full-length Kv 1.3 subunit—suggesting that these residues have no role in subunit association. However, Kv 1.1 subunits lacking residues 424–495 were unable to form homo-multimeric structures but could associate with Kv 1.3 subunits. Thus, although the N-terminal region appears to regulate the subfamily specificity of K^+ channel subunit association, it is not the only region required for stable assembly of multimeric structures. This is not unexpected since the four subunits in the tetrameric K^+ channel would be expected to contact each other in a large number of regions, particularly the hydrophobic core surrounding the ion translocating pore.

Evidence for the formation of such hetero-oligomers in the brain was sought using antibodies specific for each of the α subunits (Scott *et al.*, 1994a). As alluded to in Section II 5, a number of α-subunit variants are detected in the purified preparation of α-DTX-sensitive K^+ channels (Fig. 3A). The presence of Kv1.1, 1.2 (also detected by direct N-terminal sequencing), and 1.6 was not surprising since α-DTX-sensitive K^+ currents are obtained when their cRNA is injected into *Xenopus* oocytes (Tables I and IIB,C). Importantly, however, Kv1.4, which gave rise to α-DTX-insensitive K^+ currents when expressed, was also observed. This could only have been adsorbed and eluted from the toxin I affinity column if it was in association with α-DTX-sensitive α subunits in a hetero-oligomer. Further analysis by quantitative immunoprecipitation (Fig. 3B) and by immunoprecipitation followed with Western blotting (Scott *et al.*, 1994a) showed that the pure α-DTX K^+ channels contain a number of oligomeric subtypes. All the oligomers contained Kv1.2, confirming N-terminal sequencing where it gave the most abundant signal. Approximately 50% of these oligomers also possess Kv1.1, while minor populations have Kv1.6 (~20%) or Kv1.4 (~10%). These data appear to agree well with demonstrated overlapping distributions of Kv1.2 and 1.1 in the juxta-paranodal regions of nodes of Ranvier in mouse brain stem and in the terminal fields of the basket cells in mouse cerebellum (Wang *et al.*, 1993) and of Kv1.2 and 1.4 in the middle third of the dentate gyrus, molecular layer, and stratum lacunosum moleculare of rat hippocampus (Sheng *et al.*, 1993) which may indicate hetero-multimeric complexes. However, it is informative to compare the labelling in these brain regions observed with anti-Kv antibodies (Wang *et al.*, 1993, 1994; McNamara *et al.*, 1993; Sheng *et al.*, 1993, 1994) with that obtained using ^{125}I-α-DTX (Pelchen-Matthews and Dolly, 1989) and δ-DTX (Awan and Dolly, 1991). In cerebellum, the area corresponding to the basket cell termini is labelled strongly with both the antibodies as well as by ^{125}I-α-DTX. Additionally, Kv1.1 and 1.2 subunits and α-DTX binding sites are visible, albeit less noticeably, in both the granule and molecular layers. On the other hand, high-affinity δ-DTX acceptors, while also present in molecular and granule layers, appear at this low resolution to be absent from the basket cell terminal regions (Awan and Dolly, 1991). Similarly in hippocampus, Kv1.1, 1.2, and 1.4 are all present in the middle third of the dentate gyrus and possibly in the stratum lacunosum moleculare (Sheng *et al.*, 1993, 1994; Wang *et al.*, 1994) while ^{125}I-α-DTX binding is visible in both these areas but the latter lacks high-affinity sites for ^{125}I-δ-DTX. Perhaps, δ-DTX only binds efficiently to channels consisting solely, or largely, of Kv1.1 subunits. α-DTX, on the other hand, may be able to bind Kv1.2 and/or Kv1.1 subunits, even when they are associated in hetero-oligomeric complexes with nontoxin-binding α subunits. The existence of Kv1.1/1.2 hetero-multimers in mouse

whole brain was shown by immunoprecipitation of solubilized membrane proteins, followed by Western blotting (Wang *et al.*, 1993). Anti-Kv1.1 antibodies precipitated 30% of Kv1.2 protein together with >90% Kv1.1; likewise, Kv1.2-specific antisera were able to sediment 30% of Kv1.1 K^+ channel subunit along with >90% Kv1.2. Thus, 30% of all Kv1.1-containing K^+ channel oligomers also possess Kv1.2 subunit and vice versa. Likewise, in extract of whole rat brain membranes, antibodies specific for either Kv1.1 or Kv1.2 were able to immunoprecipitate the majority of the cognate antigen together with a fraction of the other Kv α subunits. These collective studies indicate the occurrence of hetero-oligomers containing pairs of Kv α subunits, but also indicate that a complex mixture of K^+ channels is present. To date, there is no evidence for hetero-oligomeric structures containing three (or four) different α subtypes—although their existence is not unlikely.

7. β Subunits: Integral Modulators of α-DTX-Sensitive K^+ Channel Function

When α-DTX-sensitive K^+ channels are purified from brain (Section II. 4), the Kv α subunit is, in about 90 out of 100 cases, associated with a smaller ($M_r \sim$ 39,000) β subunit. The strength of interaction between α and β subunits appears to be very strong since it persists from solubilization (Black *et al.*, 1988) through various purification steps including anion-exchange and toxin- and lectin-affinity chromatography (Rehm and Lazdunski, 1988; Parcej and Dolly, 1989). In the latter case, when channels are adsorbed via the sugars present on the α subunit and the resin washed with a high salt concentration, β subunits are still found to be associated. The β protein is, therefore, an integral part of the K^+ channel complex which has an $(\alpha)_4(\beta)_4$ stoichiometry (Parcej *et al.*, 1992). In order to elucidate the possible functional role(s) of the β subunit, it was electrophoretically isolated and proteolytically digested. After purification, several peptides were sequenced (Scott *et al.*, 1994b) and used to design oligonucleotide primers. These were used in the polymerase chain reaction to probe a bovine brain cDNA library and amplify a cDNA which allowed the isolation of a full-length clone (Scott *et al.*, 1994b). The predicted mass of the encoded protein (now named β_2, 40 983 Da) was close to the relative molecular mass of the β subunit of the purified channel (39,000). In addition, the predicted sequence included those obtained by direct microsequencing of the β subunit peptides. Hydropathy analysis of the sequence revealed no domains of sufficient length and hydrophobicity to span the membrane. In addition, no consensus N-glycosylation sites could be identified but there were a number of potential sites for modification by several different protein kinases. These data, the lack of a typical leader sequence, and its overall hydrophilic nature, suggest that the β subunit interacts strongly with α subunits on the cytoplasmic side of the membrane (detailed later). Although many other cation channels contain auxiliary proteins (Isom *et al.*, 1994), in addition to the pore-forming subunits, none show any significant sequence homology to this K^+ channel β subunit. However, it has some gross structural similarities to Ca^{2+} channel β subunits (Isom *et al.*, 1992). Both appear to be cytoplasmic and are predicted to have a similar secondary structure, using several simple algorithms. Using the Kv β_2 subunit as a probe, additional β subunit clones (Fig. 4A) were isolated first from rat brain (Kv β_1; Rettig *et al.*, 1994) and, more recently, from ferret and human heart (Kv β_3; Morales *et al.*, 1995; Majumder *et al.*, 1995). Although both Kv β_1 and β_3 subunits possess an extended N-terminus compared to Kv β_2, there is no similarity between the first 72 residues of Kv β_1, 79 of β_3, and 38 of β_2. However, 85% identity is observed for the last 329 amino acids of each subunit. Kv β_1 and β_3 subunits also vary in their tissue distribution. Kv β_1 mRNA is detectable in brain tissue but not heart, skeletal muscle, or kidney (Rettig *et al.*, 1994); Kv β_3 is prominent in aorta and left ventricle but is also observed in brain, skeletal muscle, and kidney (Morales *et al.*, 1995).

The possible influence of the β subunits upon K^+ channel function was investigated by co-injection of rat β and α subunit cRNA into oocytes. None of the Kv β subunits produced K^+ currents when injected alone. However, Kv β_1 had dramatic effects on Kv α subunit channels (Rettig *et al.*, 1994). Inactivation of both the delayed rectifier Kv1.1 α subunit and the rapidly-inactivating Kv1.4 was markedly increased by Kv β_1 (Fig. 5). Aldrich (1994) has speculated that the absence of β_1 subunit may explain why none of the α subunits expressed in oocytes give K^+ channels inactivating as quickly as their native counterparts. Despite the N-terminus of Kv β_3 being distinct from that of β_1, it also proved able to increase the rate of inactivation of a full-length Kv1.4 channel (Morales *et al.*, 1995; Majumder *et al.*, 1995). In these studies, inactivation of the K^+ current carried by Kv1.4 could be resolved

A

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rKvβ1          MQVSIACTEHNLKSRNGEDRLLSKQSSTAPNVVNAARAKFRTVAIIARSLGTF  53
rKvβ2                                                 MYPESTTGSPARLSLRQTG  19
bKvβ2                                                 MYPESTTGSPARLSLRQTG  19
fKvβ3     MHLYKPACADIPSPKLGLPKSSESALKCRRHLAVTKPPPQAACWPARPSGAAERKFLEKF  60
hKvβ3     MHLYKPACADIPSPKLGLPKSSESALKCRWHLAVTKTQPQAACKPVRPSGAAEQKYVEKF  60

rKvβ1     TPQHHISLKESTAKQTGMKYRNLGKSGLRVSCLGLGTWVTFGGQISDEVAERLMTIAYES 113
rKvβ2     SPGMIYSTRYGSPKRQLQF:::::::::::::::::::::::::T::M::H:::L::DN  79
bKvβ2     SPGMIYSTRYGSPKRQLQF:::::::::::::::::::::::::T::E::Q:::L::DN  79
fKvβ3     LRVHGISLQETTRAETGMA:::::::::::::::::::::::::S::V::R:::I::ES 120
hKvβ3     LRVHGISLQETTRAETGMA:::::::::::::::::::::::::S::V::R:::I::ES 120
                      *
rKvβ1     GVNLFDTAEVYAAGKAEVILGSIIKKKGWRRSSLVITTKLYWGGKAETERGLSRKHIIEG 173
rKvβ2     :I:::::::::::::::V::N:::::::::::::::::IF:::::::::::::::::::: 139
bKvβ2     :I:::::::::::::::V::N:::::::::::::::::IF:::::::::::::::::::: 139
fKvβ3     :V:::::::::::::::I::S:::::::::::::::::LY:::::::::::::::::::: 180
hKvβ3     :V:::::::::::::::I::S:::::::::::::::::LY:::::::::::::::::::: 180

rKvβ1     LKGSLQRLQLEYVDVVFANRPDSNTPMEEIVRAMTHVINQGMAMYWGTSRWSAMEIMEAY 233
rKvβ2     ::A::E:::::::::::::::P::::::T::::::::::::::::::::S::::::: 199
bKvβ2     ::A::E:::::::::::::::P::::::T::::::::::::::::::::S::::::: 199
fKvβ3     ::G::Q:::::::::::::::S::::::I::::::::::::::::::::A::::::: 180
hKvβ3     ::G::Q:::::::::::::::S::::::I::::::::::::::::::::A::::::: 180

rKvβ1     SVARQFNMIPPVCEQAEYHLFQREKVEVQLPELYHKIGVGAMTWSPLACGIISGKYGNGV 293
rKvβ2     :::::::L:::I::::::::M:::::::::::::F:::::::::::::::V::::DS:I 259
bKvβ2     :::::::L:::I::::::::M:::::::::::::F:::::::::::::::V::::DS:I 159
fKvβ3     :::::::M:::V::::::::L:::::::::::::Y:::::::::::::::I::::GN:V 300
hKvβ3     :::::::M:::V::::::::L:::::::::::::Y:::::::::::::::I::::GN:V 300

rKvβ1     PESSRASLKCYQWLKERIVSEEGRKQQNKLKDLSPIAERLGCTLPQLAVAWCLRNEGVSS 293
rKvβ2     :PY::::::G:::::DK:L:::::R::A:::E:QA:::::::::::::I:::::::::: 259
bKvβ2     :PY::::::G:::::DK:L:::::R::A:::E:QA:::::::::::::I:::::::::: 259
FKvβ3     :ES::::::C:::::ER:V:::::K::N:::D:SP:::::::::::::V:::::::::: 300
hKvβ3     :ES::::::C:::::ER:V:::::K::N:::D:SP:::::::::::::V:::::::::: 300

rKvβ1     VLLGSSTPEQLIENLGAIQVLPKMTSHVVNEIDNILRNKPYSKKDYRS              401
rKvβ2     ::::A:NAE::M::I::::::::LS:SIVH:::S::G:::::::::               367
bKvβ2     ::::A:SAD::M::I::::::::LS:SIIH:::S::G:::::::::               367
FKvβ3     ::::S:TPE::I::L::::::::MT:HVVN:::N::R:::::::::               408
hKvβ3     ::::S:TPE::I::L::::::::MT:HVVN:::N::R:::::::::               408
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Fig. 4. K^+ channel β subunits. A. Primary sequence alignment of the 5 Kv β subunits cloned to date. rKv β_1, β_1 subunit cloned from rat brain (Genbank accession number X70662; Rettig *et al.*, 1994); rKv β_2 and bKv β_2, β_2 subunit cloned from rat (X76724; Rettig *et al.*, 1994) and bovine cortex (X70661; Scott *et al.*, 1994b), respectively; fKv β_3 and hKv β_3, β_3 subunit from ferret (U17966; Morales *et al.*, 1995) and human atrium (U16953; Majumder *et al.*, 1995). Amino acids conserved in all sequences are displayed as ":". Conserved cysteine residues in the inactivation domains of Kv β_1 (residue 7) and Kv β_3 (residues 8 and 28) are underlined. The putative catalytic tyrosine, conserved in oxidoreductases, is marked with an asterisk (residue 90 of Kv β_2). B. Model of the β subunits based on the known structure of a homologous oxidoreductase enzyme. The crystal structure of aldose reductase (Wilson *et al.*, 1992; pdb code 1IADS) was used to model homologous domains in the K^+ channel β subunit (residues 37–366 of Kv B_2), using ProMod (Peitsch and Jongeneel, 1993) at the Glaxo Institute for Molecular Biology, SA. Molscript (Kraulis, 1991) was used to generate the schematic output.

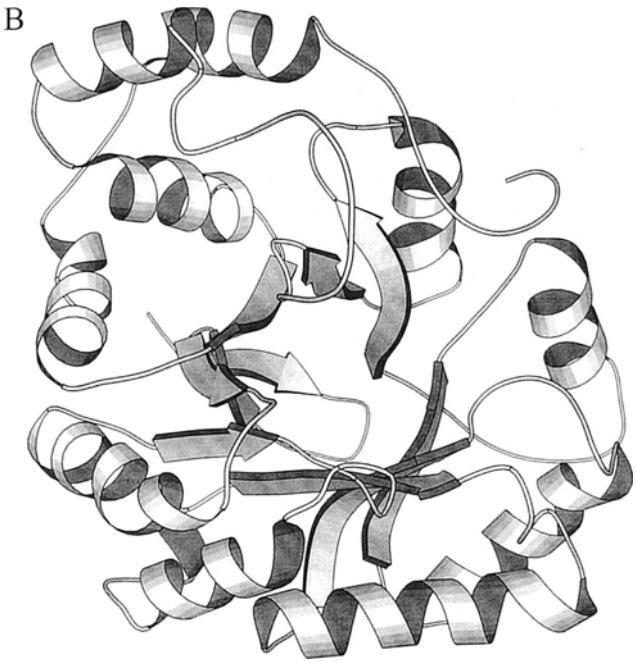


Fig. 4. Continued.

into two components, both of which were accelerated by Kv β_3. Likewise, the noninactivating Kv1.5 current (Majumder *et al.*, 1995) and a truncated Shaker B channel (Morales *et al.*, 1995) also inactivated faster in the presence of Kv β_3. However, Kv1.1 and 1.2 were not affected by Kv β_3, either because α and β subunits could not assemble or specific components (i.e., ball and receptor) are unable to associate. Thus, association of α subunits in different tissues with various β subunits offers the potential for the generation of diverse K^+ channels, on top of that generated by multiple α subunits assembled into homo- or hetero-oligomeric combinations.

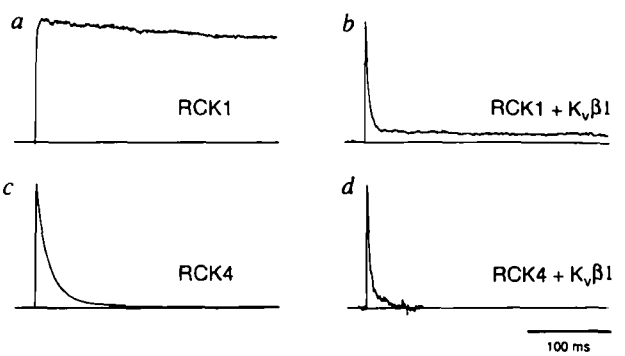


Fig. 5. Co-expression of Kv β_1 with Kv 1.1 or 1.4 in oocytes accelerates the channels' inactivation rates. *Xenopus* oocytes were injected with cRNA (12.5 ng) for rat cortex Kv 1.1 (RCK1) or 1.4 (RCK4) alone or with that for β_1. Patch-clamp recordings were carried out, 1–7 days later, in the cell-attached configuration; outward K^+ currents were evoked by depolarizing pulses to +50 mV from a holding potential of −100 mV. Taken from Rettig *et al.* (1994).

Although Kv β_1 and β_3 have clear effects on the K^+ channels with which they are associated, the function of β_2 is not known. No readily detectable effects were observed on the activation or inactivation properties of any of the Kv α subunits tested (Rettig *et al.*, 1994). This is particularly surprising since Kv β_2 is the major β subunit associated with purified α-DTX sensitive K^+ channels (Rehm and Lazdunski, 1988; Parcej and Dolly, 1989; Scott *et al.*, 1994a). In fact, a band which maybe to Kv β_1 is barely visible in these preparations (see Fig. 1A). However, a possible novel role for Kv β subunits was proposed recently by McCormack and McCormack (1994) who identified some (15–30%) sequence homologies between the K^+ channel Kv β subunits and a superfamily of oxidoreductase enzymes. In particular, there is a conservation of residues which define secondary structure elements of the $(\alpha/\beta)_8$ barrel structures of some oxidoreductases (Rondeau *et al.*, 1992; Wilson *et al.*, 1992). The structural similarities between the enzymes and Kv β subunits suggest that they may share a common 3-dimensional fold and could be used to model the subunit structure (Fig. 4B). Additionally, a possible catalytic Y residue and some of the residues believed to contact the NAD(P)H co-factor are also present in the Kv β subunits, indicating that they may be functional enzymes. This raises the intriguing notion that the K^+ channel Kv β subunits might provide a link between K^+ channel activity and the redox state of the cell, perhaps influenced by osmolarity, oxidoreductive metabolism, or even the metabolism of free radicals. β subunits, then, may be capable of influencing K^+ channel activity in several subtle ways.

III. PINPOINTING FUNCTIONAL DOMAINS IN α AND β SUBUNITS

Much of the early data on structure/function relations of K^+ channels has been reviewed by Brown (1993), Pongs (1992a, b), Jan and Jan (1992), and Durell and Guy (1992).

1. Voltage-Sensor: Voltage-Dependent Activation

Extensive electrophysiological measurements (reviewed by Jan and Jan, 1992) have shown that

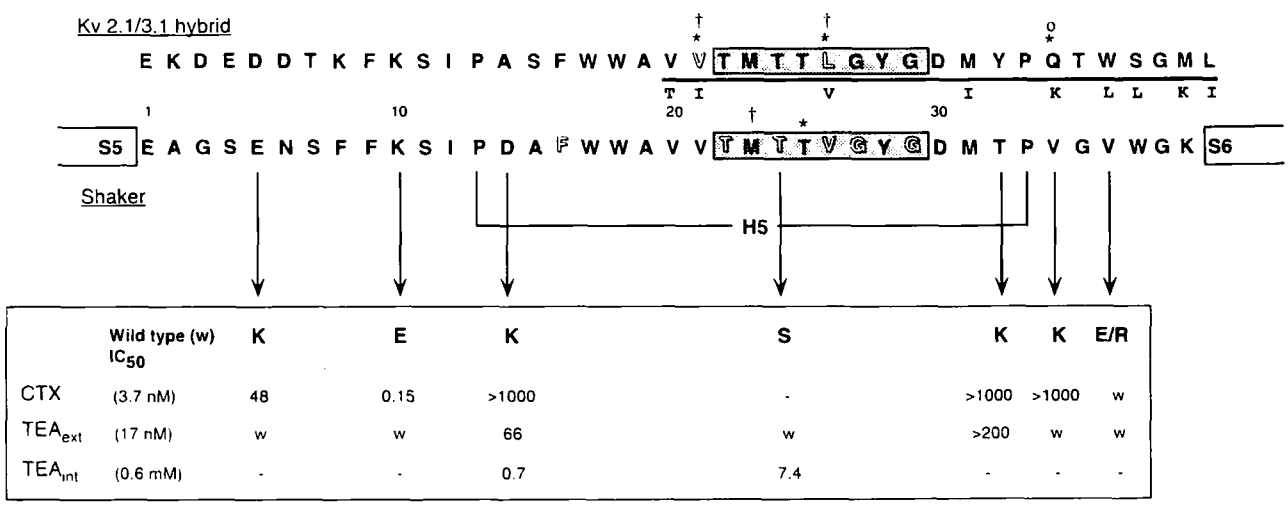
voltage-gated K^+ (and, indeed, Na^+ and Ca^{2+}) channels possess intrinsic voltage sensors, enabling them to respond to depolarization and undergo voltage-driven conformational changes to a state from which opening of their pore can occur in a voltage-independent step (Koren *et al.*, 1990; Zagotta and Aldrich, 1990). The voltage sensor was predicted to carry charges or dipoles and to reside in the hydrophobic membrane interior, which has a low dielectric constant and where the electrical potential difference across the membrane is greatest. Accordingly, the S4 membrane-spanning segment of the α subunits contain regularly-spaced positive residues (Fig. 2) interspersed with two hydrophobic (usually) amino acids. There can be 4–7 repeats of this motif (Arg/Lys-X-X) in each α subunit of all Kv sub-families (cf. Table I). In a widely supported hypothesis (reviewed by Hille, 1992), it is assumed that movements of these voltage-sensor "particles" in the membrane following depolarization are reflected in outward movement of 4–8 positive charges, recorded as gating currents using Kv1.1 and Shaker channels (Koren *et al.*, 1990; Zagotta and Aldrich, 1990). These small outward currents are associated with conformational changes of the protein that culminate in channel opening; they precede the latter and can be measured separately from the subsequent and very much larger flow of K^+ through the activated channel pore. Direct evidence for the S4 acting as a voltage sensor has been obtained by mutagenesis of various residues in this region, followed by expression of the channels and electrophysiological measurement of the voltage-dependent K^+ conductance activation curves; also, gating charges were estimated indirectly from the slopes of the latter. Neutralization of positive amino acids in S4 of *Drosophila* Shaker (reviewed by Jan and Jan, 1992) α subunits decreased the observed gating charges and shifted the voltage-activation plots along the voltage axis, consistent with these conserved charged residues being involved in voltage sensing and gating. A similar pattern of results was seen with rat cortex Kv1.1 (Tytgat *et al.*, 1993), but most of such mutants of positive residues in S4 were nonfunctional unless tetrameric cDNAs were used (three wild-type and one mutant in tandem). Neutralization of R 2 or 4 gave leftward shifts, reflecting increased stability of the open-channel conformation; in contrast, similar mutations of R 3, 5, or 6 yielded rightward shifts. Clearly, complex mechanisms underlie these changes, which differed in their magnitudes, making interpretation of the data difficult; this can be complicated further if certain types of mutations alter cooperativity known to occur in voltage-dependent gating of the four subunits (Hurst *et al.*, 1992). Further to the major contribution of positively charged amino acids to the activation process, hydrophobic residues in S4 are also important. For example, mutating one hydrophobic residue (usually L) in S4 of Shaker to another (e.g., A) resulted in large shifts of the voltage-dependence of channel activation; notably, similar mutations of conserved leucines in other regions did not produce such changes (Lopez *et al.*, 1991), emphasizing the uniqueness of the S4 segment in mediating voltage activation. Collectively, these experimental findings lend strong support to S4 segments acting as voltage sensors, with the positive residues therein contributing to gating charges and others also participating in the cooperative interactions between various parts of the channel that occur during the voltage-dependent transitions of the closed channel into an open state. Importantly, certain mutations in the voltage sensor (R377K and H378Q) of Shaker that shift (in the positive direction) the voltage dependency of activation attenuated inhibition of the channel by internally-applied TEA (Choi *et al.*, 1993). The decreased levels of blockade observed for these mutants at lower voltages was explained by assuming that block can only occur when the channel gate is open. In other words, this provides evidence for the coupling of voltage-dependent channel opening and TEA block. 'Sliding helix' or 'helical screw' models have been proposed for the movement of S4 across the membrane (Catterall, 1993, 1994; Durell and Guy, 1992).

In the α subunit of many, but not all voltage-gated K^+ channels, a leucine-containing heptad is repeated (up to five times) in the ends of S4 and 5 plus adjoining regions and has been termed the leucine zipper motif (detailed in Pongs, 1992a). Importantly, substitution of these leucines by valine residues altered the voltage dependence of the resultant expressed K^+ conductances (McCormack *et al.*, 1991), in a manner resembling those of the above-noted S4 mutations. As the equilibria of voltage-dependent steps of gating were changed in these mutated K^+ channels (Schoppa *et al.*, 1992), it has been deduced that movement of charged residues is a prelude to depolarization-induced activation and the ensuing voltage-independent transitions, involving hydrophobic groups (Pongs, 1992a). This interpretation accords with the demonstration, using rat cortex Kv1.1 channel, that the transition from the last closed intermediate to the open state is voltage-independent.

2. Ion Pore: K^+ Permeation and Selectivity

Activated K^+ channels allow K^+ to permeate across the membrane at a high rate of 10^6/sec/ion pore, several orders of magnitude faster than for Na^+. Such amazing selectivity involves, among other things, several binding sites for K^+ within its pore (diameter of narrowest point ~ 3 Å) through which the ions pass in single file (Hille, 1992). Site-directed mutagenesis of α subunits followed by expression of the resultant K^+ currents in oocytes, and monitoring of alterations in their blockade by various agents, identified the H5 region as comprising the ion pore (Fig. 6); it is also called the "P" region. This is a highly conserved, short stretch of uncharged and largely hydrophobic amino acids between S5 and 6 (Fig. 2); note that for clarity, the residues between these two segments are numbered 1–39, starting with E^{418} in Shaker (Fig. 6) to allow comparisons to be made among α subunits from different sources or subfamilies. The outer and inner limits of the channel lining in the membrane were defined by the location of residues established to be essential in Shaker K^+ channel for blockade of its conductance by TEA, acting at the extra- or intra-cellular sites (Fig. 6). Mutating D^{14} or T^{32} to K diminished sensitivity to externally-applied TEA (MacKinnon and Yellen, 1990; Yellen *et al.*, 1991); furthermore, either of these two changes abolished inhibition by CTX but the more distal E^5 and V^{34} also contributed to blockade by the larger toxin *only* (MacKinnon and Miller, 1989; MacKinnon *et al.*, 1990). Notably, α-DTX—another blocker of certain K^+ channels (Tables I and II)—also binds near the outer mouth of the channel; although its site seems to overlap with that for CTX, it is not identical (Stocker *et al.*, 1991). Mutation of A^{352}, E^{353}, and Y^{378} (singly or together) in Kv1.1 (equivalent to positions 5, 6, and 32 in Shaker, Fig. 6), to residues found at corresponding positions in a K^+ channel showing little α-DTX sensitivity (Kv1.3; Table I, IIB), diminished inhibition by α-DTX or toxin I (Hurst *et al.*, 1991; Hopkins *et al.*, 1994b). On the other hand, Kv1.3 became susceptible to α-DTX when E or Y were introduced singly or together at the positions equivalent to where each occurs in Kv1.1 (Hurst *et al.*, 1991).

Confirmation that the presence of T, or even better, a Y at position 32, is essential for TEA blocking from the outside was gained by investigating rat cortex Kv1.4 (Stocker *et al.*, 1991); consistently, it only assumes sensitivity when its K (at the equivalent posi-



	Wild type (w) IC_{50}	K	E	K	S	K	K	E/R
CTX	(3.7 nM)	48	0.15	>1000	-	>1000	>1000	w
TEA_{ext}	(17 nM)	w	w	66	w	>200	w	w
TEA_{int}	(0.6 mM)	-	-	0.7	7.4	-	-	-

Fig. 6. Schematic of the amino acid sequences between S5 and S6 in Shaker (lower) and Kv 2.1/3.1 hybrid (upper) K^+ channels showing identified functional residues in H5 and adjoining regions. Amino acids are shown in single letter code and numbered starting with E^{418} in Shaker. Arrows illustrate the residue changes introduced by site-directed mutagenesis. The IC_{50} values listed are the concentrations of blockers required to give 50% inhibition of wild type or mutant K^+ currents expressed in oocytes. CTX and TEA_{ext} were applied externally and TEA_{int} was administered internally. The bar indicates the residues transplanted from Kv 3.1 into Kv 2.1 to yield the hybrid shown: those in smaller lettering represent the amino acids present originally in the host Kv 2.1. Shaded area represents the signature sequence (TXXTXGYG) conserved in all K^+ channel types. Also shown are other residues identified by mutagenesis to affect K^+ selectivity (light gray), channel open times (*), or contribute to binding of internal (†) or external (○) TEA, respectively. Adopted from Pongs (1992b); data taken from MacKinnon and Yellen (1990); Yellen *et al.* (1991); Yool and Schwarz (1991); MacKinnon and Miller (1989); MacKinnon *et al.* (1990); Hartmann *et al.* (1991); Kirsch *et al.* (1992a, b); Choi *et al.* (1993); Heginbotham *et al.* (1994).

tion) is substituted by Y. Likewise, Kv1.1 has a Y at this position and is very susceptible to TEA (Table I). In fact, location of a Y there correlates with very high susceptibility to externally-administered TEA and affinity for the latter was found to increase with the number of α subunits containing this residue, in specially-constructed tetramers of Kv1.1 (Hurst *et al.*, 1992). Compelling evidence that H5 spanned the membrane and provided both the external and internal binding sites for TEA was obtained by analysis of a chimeric K^+ channel (Hartmann *et al.*, 1991), constructed by substituting a large part (residues 20 onwards) of H5 from rat Kv3.1 for the equivalent part of rat Kv2.1 (Fig. 6). The hybrid channel exhibited the distinct characteristics of Kv3.1 (single-channel conductance, sensitivity to external and internal TEA), establishing that the residues transferred were responsible for the ion permeability properties. Furthermore, changing T^{24} to S in Shaker decreased blockade by TEA applied inside the oocyte membrane (Fig. 6; Yellen *et al.*, 1991); there was a more dramatic reduction in the effectiveness of internal TEA when M^{23} was changed to I (Choi *et al.*, 1993). Notably, the mutation $T^{24} \rightarrow S$ also altered the ion selectivity, NH_4^+ and Rb^+ permeabilities being increased without change in the exclusion of other cations; a similar change resulted from mutating F^{16} to a less hydrophobic residue (Yool and Schwarz, 1991). Using the above-noted Kv2.1/3.1 hybrid channel, substituting L with V at the position equivalent to 26 in Shaker (Fig. 6) was shown to increase Rb^+ permeability and decrease that of K^+ (Kirsch *et al.*, 1992a, b). In fact, simultaneously altering this and another nonpolar residue (V^{21}; Fig. 6) with which it may interact proved most effective in switching the relative K^+:Rb^+ permeabilities, plus improving blockade by internal TEA, while mutating $Q \rightarrow K$ at equivalent position 34 decreased the efficacy of external TEA (Fig. 6) in inhibiting the K^+ current (Kirsch *et al.*, 1992a, b). These changes, singly or together, also affected channel open times (Fig. 6). The close proximity of this special pair of residues deep in the pore to the functionally important Thr^{24} indicate that these contribute to the cation binding site essential for ion selectivity. Most importantly, alignment of the cloned sequences from all the major K^+ channel types (voltage-sensitive, Ca^{2+} activated and inward rectifiers) revealed a homologous stretch of eight residues (TXXTXGYG) in the pore region (Fig. 6), called the signature sequence (Heginbotham *et al.*, 1994). Mutagenesis studies on Shaker demonstrated that four of these residues (T^{22}, V^{26}, G^{27}, and G^{29} but not T^{24}, in contrast to the above-noted results) contribute to the channel's selectivity for K^+. Such an essential involvement of the signature sequence in this process accords with the proposal of a conserved conduction pathway that is common to all K^+ channels. Also, the latter investigation showed that mutant channels without a hydroxyl-containing residue at position 24 or 25 retain ion selectivity, as did that in which Y^{28} was changed to a nonaromatic amino acid, V, questioning the importance of such groups in this facet of channel activity. However, replacing T^{25} with S dramatically slowed the inactivation rate of the K^+ channel (Yool and Schwarz, 1991). Considering these and other experiments not reviewed here, it is clear that the H5 constitutes a major part of the K^+ channel pore.

Detailed modelling studies (Guy and Conti, 1990) predicted that H5 forms a β-hairpin that partially traverses the membrane and forms the ion selectivity region of the pore. Thus, a model of the pore was proposed (Hartmann *et al.*, 1991; Yellen *et al.*, 1991; Yool and Schwarz, 1991; Durell and Guy, 1992) in which a stretch of 19 amino acids—flanked by proline residues—spans the membrane as a β-hairpin composed of two antiparallel β strands. The latter was postulated, rather than a helical structure, because in an extended conformation of a β strand there would be adequate residues to transverse the membrane. As the channel is known to be tetrameric with 4-fold symmetry (see above), the pore would be formed by an 8-stranded β-barrel. However, direct evidence for a β-hairpin is lacking (see later). Further insight into the structure of the ion permeation pathway was gleaned from individually mutating residues in the pore of Kv2.1 to cysteines; alterations of the K^+ currents expressed in oocytes was monitored after extra- or intracellular application of membrane-impermeable methanethiosulfonates that covalently label the thiols introduced (Pascual *et al.*, 1995). The several residues modified appeared to span the pore as a nonperiodic structure. Another major deduction from the investigation is that D^{378} and V^{374} (equivalent to positions 26 and 30 in Fig. 6) are accessible to the reagent from the external and internal mouths of the channel, respectively (Fig. 7). Hence, the authors proposed that impermeability to the reagent is created by three intervening residues (G^{377}, Y^{376}, and G^{375}) that must form the narrowest span of the channel, as shown in the revised model (Fig. 7). The extent of accessibilities observed for such a large reagent, from either side of the membrane, was deemed to be incompatible with the tight packing of the β-hairpin model, and associated filter-

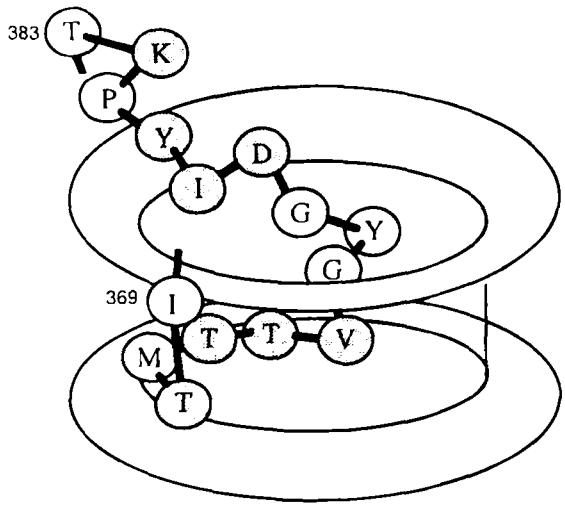


Fig. 7. Proposed membrane topology of the pore of K^+ channels. The 15-amino acid segment shown is the C-terminal half of the pore region from Kv 2.1; this can be aligned with residues 21–35 in Fig. 6. Shaded residues represent those found to be accessible to thiol-labelling methanethiosulfonates when applied extra- (upper) or intra-cellularly (lower) to oocytes expressing Kv 2.1 in which each residue was mutated singly to cysteine. The triplet GYG (clear circles) was not labelled by the reagent from either side of the membrane and is, thus, regarded as forming the narrowest part of the pore; other residues not tested or apparently inaccessible are shown as clear circles. The polypeptide chain is shown surrounded by a reference cylinder that opens into wider mouths. Apparently, a turn occurs in the area of residues 374–370, predicting that residues in the N-terminal half of the pore region upstream of I 369 exit at the external surface. Taken from Pascual *et al.* (1995).

ing in the outer and inner mouth of the channel. Instead, it is envisioned that most of the pore consists of wide vestibules on either side of a restriction formed by amino acid side-chains clustered into a nonperiodic structure (Pascual *et al.*, 1995). A similar series of experiments on cysteine mutations of residues in the pore region of Shaker was performed by Lü and Miller (1995) except that accessible amino acids were identified from the inhibition of the resultant K^+ current by thiol-reactive Ag^+; the latter has the advantage of being similar in size to K^+ but can only be applied externally because of reactivity with other cysteines. The profile of residues (13–22 in Fig. 6) found to be exposed to the lumen of the channel pore is not consistent with the β-barrel model but could be reconciled with α-helical periodicity. Also, there is an increasing amount of evidence for the proposal that a large portion of the pore loop resides near the external mouth of the channel, with only a segment extending into the membrane to form the ion selectivity filter (Heginbotham *et al.*, 1994); notably, in the revised model of Durell and Guy (1992), the ion-selective region is a β-barrel that spans only the outer half of the membrane. Additionally, the intracellular loop between S4 and S5 and the intracellular end of S6 segment appear to line the inner mouth of the pore because mutations in these regions of Shaker alter ion selectivity and conductance (Slesinger *et al.*, 1993; Lopez *et al.*, 1994). Most of these salient features are depicted in an updated hypothetical model (Fig. 8) showing the N-terminal part of H5 as a helix and the gating pore region as a folded structure, with the S4/5 loop and S6 tail lining the inner mouth of the pore.

3. Inactivation

This involves one or more nonconducting states of a channel that are distinct from the closed conformation. Although the inactivation process of K^+ channels has little intrinsic voltage dependency, it is coupled to activation (Zagotta and Aldrich, 1990). Following a refractory period, channels recover from inactivation by undergoing conformational changes that entail going through an open state before reaching the resting closed state (for a review see Pongs, 1992a). There are two types of inactivation, termed N (fast, over milliseconds) and C (rate can vary from 10 msec to seconds), that occur via different mechanisms, although these appear to be coupled (Hoshi *et al.*, 1991). External TEA competes with C-type inactivation and, thus, this process is assumed to involve conformational changes that result in closure of the extracellular entrance to the channel. Also the nature of one residue (V or A at position 463 in Shaker) in the extracellular end of S6 was found to influence its kinetics and this actually explains the different inactivation time courses of two variants (Hoshi *et al.*, 1991). On the other hand, C-type inactivation was unaffected by the cytoplasmic C-terminal region. Much more detailed information is available on the molecular basis of the other inactivation process.

α *Subunit-Mediated Inactivation.* Fast inactivation of Shaker can be removed by the action of trypsin applied on the cytoplasmic side of the membrane, implicating intracellularly-exposed protein domain(s) (Hoshi *et al.*, 1990). The voltage independence of this process is also consistent with channel domains not spanning the membrane being involved. Accordingly, dissimilar rates of inactivation observed for spliced variants of Shaker K^+ channel have been attributed to the variable N- and C-termini contributing to N- and

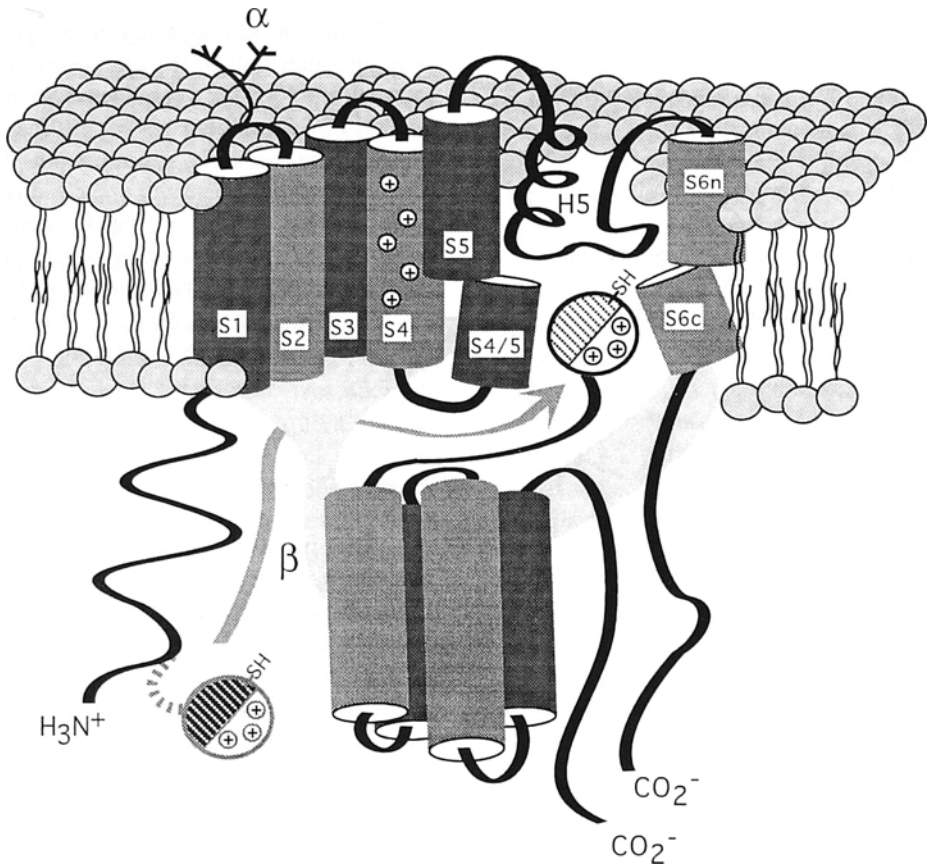


Fig. 8. A hypothetical model of K^+ channel α/β subunits highlighting the structural domains concerned with ion permeation and inactivation. In addition to six membrane-spanning, putative α helical segments (S1–6; S4 and 6 are shown in two sections as modelled by Durell and Guy, 1992), α subunits contain the conserved pore-forming H5 region. A positively-charged, repeated motif in S4 serves as a voltage sensor. The 19 amino acids (14–32 in Fig. 6) comprising H5, delimited by a proline residue at each end, forms a loop into the membrane. Its N-terminal half is depicted as α-helical while the C-terminal portion, which is largely responsible for ion selectivity and permeability, forms the nonperiodic structure detailed in Fig. 7 and shown here in an arbitrary fashion. The latter contains an identified triplet of amino acids that is ascribed as being the most restricted part of the pore. The inner mouth of the pore is lined by the S4/5 loop and residues from the cytoplasmic end of S6. Only the channels displaying A-type rapid inactivation (e.g., Kv 1.4 and 3.4) have an N-terminal inactivation domain (a ball similar to that detailed below); when the channel is activated, it diffuses and binds to a receptor that becomes exposed on the cytoplasmic mouth of the channel (arrow) and, thereby, blocks ion flux. The β subunits, whose structures were originally predicted to include 4 putative helical regions (but see the modelled structure in Fig. 4B), are placed on the cytoplasmic side of the membrane because of their hydrophilic nature and functional effects intracellularly, where they are associated tightly with the channel through unidentified contacts (light gray). β_1 (and β_3), but not β_2, possess an N-terminal inactivation domain (ball) containing hydrophobic and positive residues, together with a C(SH) responsible for sensitivity of its effect to oxidation. Considering its resemblances in gross structural and functional characteristics to the N-type inactivation α ball of Shaker B, it is reasonable to predict that it occludes the pore by binding to a site on the cytoplasmic mouth of the pore, as documented for the α inactivation particle (see text). As sequences of the α and β balls are distinct, different shading is used. For simplicity, only one α and β subunit is illustrated from the octomeric complex $[(\alpha)_4\ (\beta)_4]$ found in authentic K^+ channels purified from brain (Parcej *et al.*, 1992). Note that the structures are not drawn to scale Y, represents N-glycosylation. This tentative model is based largely on data for β subunits from Scott *et al.* (1994b) and Rettig *et al.* (1994) together with sources of information cited in the text and Figs. 6 and 7 for the α subunits.

C-type inactivation, respectively (detailed by Pongs, 1992a). In an elegant and extensive series of mutations (including deletions, truncations, and insertions), the group of Aldrich pinpointed the N-type inactivation particle to the N-terminus of Shaker channel, expressed in oocytes (Hoshi *et al.*, 1990). It consists of two functional domains: mutations in the first 19 amino acids causes slowing or complete removal of the inactivation while deletions in the second region (residues 20–83) speed up the process and insertions retard it. Such intriguing findings provided direct evidence for the "ball and chain" model (Figs. 2 and 8). Substitution of positive residues in the ball slowed inactivation whereas switching Leu^7 to a hydrophilic amino acid prevented the process. Covalent attachment of the ball peptide is not essential because internal application of a 20-residue synthetic peptide, corresponding to the N-terminal sequence *only* of variants exhibiting fast inactivation, proved effective in restoring this property to a noninactivating mutant (Zagotta *et al.*, 1990). Thus, it was proposed that inactivation occurs by movement of the ball, containing positively charged plus hydrophobic residues and tethered to a chain, into the inner mouth of the open channel followed by binding to a receptor site revealed when the channel opens, thereby occluding the pore. This explains how the activation and inactivation processes could be coupled. The two identified mammalian A-type channels (Kv 1.4 and 3.4; Fig. 2) displaying fast inactivation also possess an N-terminal ball (Ruppersberg *et al.*, 1991a, b), as illustrated for Kv 1.4 (Fig. 9); deletion of the first 110 residues abolishes the rapidly-inactivating component. Notably, Kv 1.4 (Fig. 9a, b) and Kv 3.4 (Ruppersberg *et al.*, 1991b) contain a cysteine near the N-terminus that renders the effects of their inactivation balls susceptible to oxidation. It is noteworthy that peptides corresponding to the sequence of these inactivation domains (Ruppersberg *et al.*, 1991a, b) function with different K^+ channels. For example, Shaker ball peptide has been found to speed up inactivation of rat Kv 1.1 and 2.1 (Zagotta *et al.*, 1990; Isacoff *et al.*, 1991) and the corresponding sequence from human Kv 3.4 selectively causes mouse Kv 1.1 K^+ current in CHO cells to decay more quickly (Stephens and Robertson, 1995) without altering other parameters (e.g., voltage activation); the observed susceptibility of this action to oxidation was attributed to C at position 6 in the peptide. Moreover, the Shaker peptide accelerates inactivation of Ca^{2+}-activated (Foster *et al.*, 1992) but not ATP-dependent K^+ channels in skeletal muscle (Beirão *et al.*, 1994). Such a degree of promiscuity raises the question of the nature of the receptor(s) for the N-terminal domains. The binding site for the ball is known to include residues in the intracellular loop between S4 and S5 because their mutation reduces fast inactivation (Isacoff *et al.*, 1991). When an A for L mutation, which stabilizes fast inactivation of Shaker K^+ channel, was introduced into this region of Kv 2.1, it enhanced the rapid inactivation induced by the Shaker ball peptide (Isacoff *et al.*, 1991). Thus, with the exception of this crucial substitution, the fast inactivation domain receptor (consisting of the equivalent four residues in Shaker) must be conserved in Kv 2.1—normally, a slowly-inactivating K^+ channel. This is also the case for other K^+ channels; moreover, the spacing of the five residues in S4/5 loop shown to be concerned with fast inactivation is consistent with them being on the same face of an α-helix (Fig. 8). It had been assumed that such a receptor for the inactivation particle ought to be near the cytoplasmic mouth of the pore because internally-applied TEA mimics N-type inactivation. Moreover, certain mutations in S4/5 loop of Shaker directly reduce single-channel conductance, demonstrating that such residues lie in the permeation pathway (Isacoff *et al.*, 1991). Proximity of this receptor to the permeation path is also revealed by the observation that fast inactivation is attenuated by K^+ within the pore; hence, inactivation cannot occur while the pore is occupied by permeant ions. Collectively, all these findings provide strong evidence that the ball directly blocks K^+ flux though the pore. Also, inactivation is known to immobilize the gating charge movement that initiates channel activation; consistently, when the inactivation particle is deleted from Shaker K^+ channel, such movement of the gating charge is not prevented during depolarization (Bezanilla *et al.*, 1991). Thus, dissociation of the inactivation particle from the receptor must precede the channel going via the open state to the resting closed conformation.

In view of some mutations in one face of the helix in S4/5 loop altering channel conductance, voltage dependence of activation, and rates of inactivation and recovery, and considering the modelling data of Durell and Guy (1992), it has been suggested that S4/5 helix may act as the channel gate. In this scenario (see Pongs, 1993), movement of the S4 voltage sensor in response to depolarization leads to a voltage-independent conformational change in which the S4/5 gate moves and, thereby, allows the channel to open. Subsequent binding of the N-terminal inactivation ball to the abovementioned receptor would lock the gate open and, at the same time, occlude the inner side of the pore,

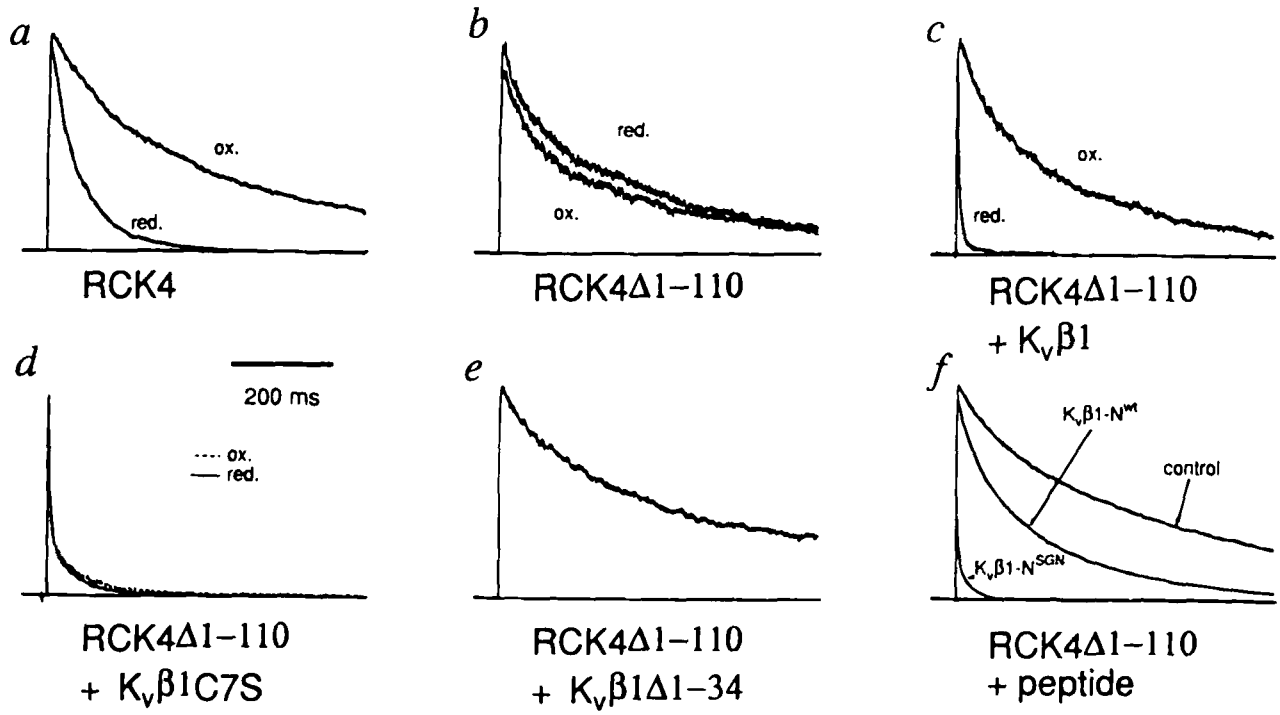


Fig. 9. Inactivation mode of Kv 1.4 channels is determined by the N-terminal moiety of Kv β_1 subunit. Kv 1.4 (RCK4) or its N-terminally-truncated form (Δ1–110) were expressed in *Xenopus* oocytes alone, or together with Kv β_1 subunit, its mutated ($C^7 \rightarrow S$) or N-terminus deleted (Δ1–34) forms by injecting each cRNA (12.5 ng), as indicated. Some days (1–7) later, outward membrane K^+ currents were recorded in the inside-out patch-clamp configuration following depolarizing pulses to +50 mV from a holding potential of −100 mV. Oxidizing (ox; 0.1% H_2O_2) or reducing (red; 5 mM reduced glutathione) bath solutions were used where specified. In *f*, currents were recorded before (control) and after addition of 100 μM peptide corresponding to the first 24 residues of wild-type (Kv β_1-N^{wt}) or mutated (Kv β_1-N^{SGN}, GI7S, E18G, D19N) β_1 subunit. For details, see Rettig *et al.* (1994).

thereby inactivating the channel. After eventual dissociation of the inactivation particle from the receptor, the resultant open state of the channel could become closed again (i.e., reach the resting state) by the gate going back in place.

N-terminal Inactivation Domain of β_1 *Subunit.* To pinpoint the region of rat brain β_1 subunit that mediates the acceleration of K^+ channel inactivation outlined above (Fig. 5), N-terminal truncated Kv 1.4 was used in co-expression studies (Rettig *et al.*, 1994) because it is bereft of its normal fast inactivation (Fig. 9). Oocytes injected with mRNA for Kv β_1 together with that for Kv 1.4 Δ1–110, yielded a very much faster inactivating K^+ current, resembling the time course of wild-type Kv 1.4 (Fig. 9c). When an oxidizing reagent was added to the inside out patches, the rate of inactivation was dramatically retarded. As β_1 influenced the inactivation in a similar way to the N-terminal ball of Kv 1.4, their sequences were checked for the presence of homologous domains. An alignment of the amino acid sequences of the two fast-inactivating K^+ channels (Kv 1.4 and 3.4) with β_1 revealed a serine-cysteine motif near the N-terminus together with a cluster of positively charged residues further upstream (Rettig *et al.*, 1994). This raised the exciting possibility of the N-terminus domain of β_1 mediating the effect on K^+ channel inactivation, with C at position 7 being responsible for the observed sensitivity to oxidation. Indeed, after mutation of $C^7 \rightarrow S$ in β_1 its ability to increase the rate of channel inactivation was preserved but susceptibility to oxidation disappeared (Fig. 9d). Furthermore, deletion of the first 34 residues from β_1 abolished its effect on inactivation (Fig. 9), thereby identifying a N-terminal inactivation ball on β_1 subunit. As in the case of α subunit ball, covalent attachment of the β_1 inactivation domain is not a prerequisite because application of a synthetic peptide, corresponding to the first 24 residues, to inside-out patches of oocyte membrane containing Kv 1.4 Δ1–110 also accelerated K^+ current inactivation, an effect accentuated when a more positively charged analogue was employed (Fig. 9f). It was concluded from the clear-cut results of these

various experiments that Kv β_1 contains an inactivating ball at its N-terminus which occludes the internal mouth of the channel, as depicted schematically in the hypothetical model (Fig. 8). Because of structural resemblances between α and β balls, it is likely that they share the same receptor. In this context, it will be informative to establish experimentally whether naturally-occurring K^+ channels possess both types of inactivation domains in the same oligomer. There are widespread functional implications for the presence in K^+ channel multimers of β_1 subunit (or β_3, which also has a cysteine-containing domain), alone or together with an α N-terminal inactivation ball, because these provide cells with sophisticated means of altering their inactivation time courses. This, in turn, would determine to a large extent the firing patterns and waveform of action potentials. Moreover, such influences of β_1 are important in creating diversity and increasing the scope for adaptation to the roles served by a given K^+ channel subtype at a particular cellular location.

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