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Jürgen Kupper · Mark R. Bowlby
Shimon Marom · Irwin B. Levitan

Intracellular and extracellular amino acids that influence C-type inactivation and its modulation in a voltage-dependent potassium channel

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Abstract The rate of C-type inactivation of the cloned voltage-gated potassium channel, Kv1.3, measured in membrane patches from *Xenopus* oocytes, increases when the patch is detached from the cell; the structural basis for this on-cell/off-cell change was examined. First, four serine and threonine residues, that are putative sites for phosphorylation by protein kinases A and C, were mutated to alanines. Mutating any one of these residues, or two or three of them simultaneously, does not eliminate the change in C-type inactivation. However, the basal rate of C-type inactivation in the cell-attached patch is markedly slower in the triple phosphorylation site mutant. Second, a homologous potassium channel, Kv 1.6, does not exhibit the on-cell/off-cell change. When an extracellular histidine at position 401 of Kv1.3 is replaced with tyrosine, the residue at the equivalent position (430) in Kv1.6, the resulting Kv1.3 H401Y mutant channel does not undergo the on-cell/off-cell change. The results indicate that several potentially phosphorylatable intracellular amino acids influence the basal rate of C-type inactivation, but are not essential for the on-cell/off-cell change in inactivation kinetics. In contrast, an extracellular amino acid is critical for this on-cell/off-cell change.

Key words C-Type inactivation · Phosphorylation · Voltage-gated potassium channel · Channel modulation · Potassium channel pore · Patch clamp

J. Kupper[1] · M. R. Bowlby · S. Marom[2] · I. B. Levitan (✉)
Biochemistry Department and Center for Complex Systems, Brandeis Universisty, Waltham MA 02254, USA

Present addresses:
[1]Laboratoire de Neurobiologie, Ecole Normale Supérieure, 46 Rue D'Ulm, F-75005 Paris, France
[2]Rappaport Institute of Medical Sciences, Faculty of Medicine, Technion, PO Box 9697, Haifa 31096, Israel

Introduction

Neurons and other excitable cells exhibit a striking diversity and plasticity of physiological properties. One fundamental mechanism underlying plastic changes in neuronal electrical activity is the modulation of membrane ion channels. Ion channel modulation can be mediated by mechanisms such as binding of extracellular or intracellular ligands and ions, direct interaction with guanyl nucleotide binding proteins, and actions of second messenger systems that generally act via protein phosphorylation [24]. Among the ion channels the activity of which is influenced by phosphorylation and dephosphorylation are voltage-gated potassium channels [12, 18, 22, 32].

Many voltage-gated ion channels not only activate in response to membrane depolarization, but also inactivate during the depolarizing pulse. The rapid inactivation exhibited by the *Shaker* family of potassium channels results from blockade of the channel pore by a sequence of about 20 amino acids at the amino terminal of the channel protein [9, 20, 38]; this rapid inactivation is slowed by intracellular tetraethylammonium (TEA) and has been termed N-type inactivation [8]. Many voltage-gated potassium channels, including *Shaker*, exhibit a much slower inactivation process during a prolonged depolarizing pulse [3, 15, 25, 29]; this slower inactivation is interfered with by extracellular TEA [3, 8, 15] and has been termed C-type inactivation. Recent site-directed mutagenesis of *Shaker* channels has demonstrated that the time course of C-type inactivation can vary over a wide range [25].

We have investigated the mechanism and modulation of C-type inactivation in the cloned voltage-gated potassium channel, Kv1.3 (nomenclature according to [7]). When Kv1.3 channels are expressed in *Xenopus* oocytes, macroscopic currents can be recorded in both cell-attached and detached membrane patches. We showed previously [29] that the inactivation of Kv1.3 changes dramatically when the patch is detached from

the cell, suggesting that one (or more) kinetic time constants is (are) modulated by some cytoplasmic factor. In the present study we show that the time constant for the transition of Kv1.3 into C-type inactivation during a prolonged pulse changes in the detached as compared to the cell-attached patch recording mode. Furthermore we make use of chimeric channels and site-directed channel mutants to identify specific amino acid residues that influence Kv1.3 channel basal inactivation rate, and the change in inactivation when the patch is detached from the cell.

Materials and methods

Construction of chimeric and mutant channels

Standard methods for plasmid deoxyribonucleic acid (DNA) preparation and deoxyribonucleic acid DNA sequencing were used [34]. The parent clones Kv1.3, Kv1.6 and *Shaker*, the Kv1.3/Kv1.6 and *Shaker*/Kv1.3 chimeras, and the single amino acid mutants were propagated in *E. coli* DH-1, in the modified plasmid vector pGemA [29]. Chimeric channels were constructed by cutting the parent plasmids with appropriate restriction enzymes, gel purifying the fragments, and ligating fragments together as described previously [29].

Pore domain channel mutants were constructed using two sequential polymerase chain reactions (PCR), with the linearized plasmid containing the channel gene serving as template. Three oligonucleotides, each 18–30 bases in length, were synthesized. Two of them – the outside primers – were complementary to sequences on opposite sides of the putative channel pore domain (see Fig. 5), and the third was a mutagenic primer with a 1– to 2– base mismatch binding within the pore domain. The first PCR reaction used the mutagenic oligonucleotide and the upstream primer. The second PCR reaction used the amplified product of the first reaction and the downstream oligonucleotide as primers. In this way a stretch of mutant DNA flanked by the two restriction sites, *Acc*I and *BamH*I, was obtained. Both the PCR product and the parent channel were double digested with those two restriction enzymes and the mutant DNA was ligated into the parent channel backbone. The resulting plasmid DNA was amplified and sequenced across the entire pore domain to confirm the mutation and to check for errors that might have occurred during the PCR reaction.

Phosphorylation site mutants were constructed using the same approach. The outside primers for the PCR reactions defined a region of the channel cDNA, within which were two unique restriction sites flanking the putative phosphorylation site. The two PCR reactions and ligation into the parent Kv1.3 channel were as described above.

Channel expression and electrical recording

After plasmid linearization with *Not*I, T7 ribonucleic acid (RNA) polymerase was used for transcription as described previously [29]. Defolliculated *Xenopus* oocytes were injected with approximately 40 nl cRNA (≈1 mg/ml) and incubated for up to 7 days at 18°C in ND-96 medium. Macroscopic current measurements were carried out at room temperature (20–22°C) in the cell-attached and detached patch recording mode using conventional methods. Because of the variability of C-type inactivation from one batch of oocytes to another, we measured inactivation of chimeric and mutant channels only in batches of oocytes in which inactivation of wild-type Kv1.3 channels was also measured. Patch electrodes were pulled from soft precision glass (Garner Glass, Claremont, Calif., USA), fire polished to 1–2 MΩ resistance and coated with a thin film of bees wax. Unless otherwise mentioned, solutions are as follows (concentrations in mM): (1) bath solution, which is equivalent to the cytoplasmic side in the inside-out recording mode – 100 KCl, 1 ethylenebis(oxonitrilo)tetraacetate (EGTA), 10 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), pH 7.5; (2) pipette solution – 30 KCl, 70 NaCl, 2 $CaCl_2$, 10 HEPES, pH 7.5. Macroscopic currents were recorded with a List EPC-7 or an Axopatch 200A amplifier. The currents were filtered at 1–2 kHz using an eight-pole Bessel filter (Frequency Devices 902LPF) and sampled at frequencies greater than 2 kHz. InClamp V1.5 software, kindly provided by Dr. Peter Reinhart of Duke University, or DAPclamp software, was used for pulse generation and data acquisition. There were no observable endogenous currents in uninjected oocytes under our recording conditions. We used patches from injected oocytes with peak currents ranging from 250 pA to 1.5 nA; patches with larger currents were discarded to avoid complications due to potassium accumulation in the electrode. Leak current for all experiments shown here was less than 0.5% of the peak current within the pulse, and accordingly we did not carry out leak subtraction. Selected current traces were exported to SigmaPlot V5.0 (Jandel Scientific) or ORIGIN V2.8 (MicroCal Software) and fitted to extract individual kinetic parameters.

Results

Modulation of C-type inactivation of Kv1.3

mRNA transcribed from the cloned Kv1.3 potassium channel cDNA produces a potassium selective, delayed-rectifier-like current when expressed in *Xenopus* oocytes [11, 17, 19, 36, 37]; the expression level is so high that macroscopic currents can be measured routinely in cell-attached and detached membrane patches [29]. We have shown previously that, when the patch is detached from the cell, there is an increase in the degree of cumulative inactivation of the Kv1.3 current in response to a series of short depolarizing pulses [29]. Furthermore, the cumulative inactivated state is identical to the C-type inactivated state into which channels enter during a prolonged depolarization [28, 29]. The degree of C-type inactivation also increases when the patch is detached from the cell (Fig. 1A and [29]). Shown in Fig. 1A is the Kv1.3 current in response to a prolonged depolarizing pulse, in the cell-attached patch mode and 5 min after detaching the patch from the cell. The peak current values have been normalized to illustrate better the change in C-type inactivation kinetics. The time course of the change in inactivation rate is shown in Fig. 1B, C. Note that the change is not instantaneous, as might be expected if it were due simply to an alteration in the intracellular ionic environment or pH [10, 16]. Rather the increase in inactivation rate occurs over a period of several minutes, consistent with the involvement of some enzymatic process. Furthermore the peak current amplitude does not decrease (Fig. 1C), indicating that there is no rundown of channel activity. In fact the peak current amplitude often increases slightly (5–10%), immediately after patch excision (see Fig. 1C). This effect may involve a change in ionic environment [10, 16]; its time course is very different from the change in inactivation kinetics. The time

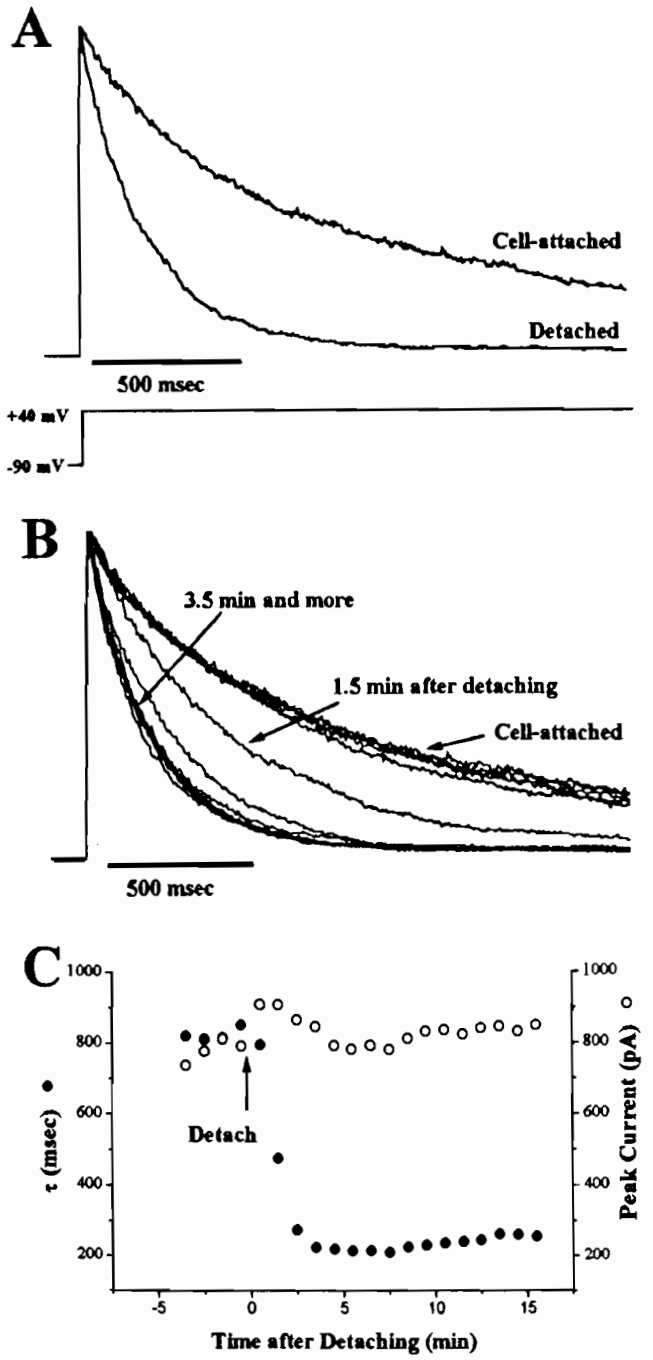


Fig. 1A–C Modulation of C-type inactivation of Kv1.3 channels. Kv1.3 channels were expressed in *Xenopus* oocytes and macroscopic currents were recorded in membrane patches. Depolarizing pulses (5 s duration) were given from a holding potential of −90 mV to a pulse potential of +40 mV (only 2 seconds are shown for clarity). Current traces are normalized to the peak value in this and all subsequent figures. **A** Current traces for the same patch in the cell-attached recording mode and 5 min after detaching the patch from the cell in the inside-out configuration. The experiment shown here is representative of results with >100 patches from >50 oocytes. **B** Traces at different times before and after detaching the patch from the cell to show the time course of the change in C-type inactivation. **C** The inactivation time constant τ (●), obtained by fitting a single exponential to the inactivating portion of the current trace, and the peak current amplitude (○), are plotted as a function of time before and after detaching the patch

course of the change in inactivation kinetics varies considerably from one batch of oocytes to another, but is less variable within a batch. No consistent differences in time course were observed between wild-type channels, and those mutant channels (see below) that exhibit the on-cell/off-cell change. For the sake of convenience we use the term "modulation" here to describe this increase in C-type inactivation rate when the patch is detached from the cell.

Detaching the patch increases the rate of entry into the inactivated state

We have shown previously that transition of Kv1.3 into inactivation occurs from the open state (or from silent states closely associated with the open state) [28, 29]. According to this scheme, the change in the rate at which the current decays during the prolonged depolarization, when the patch is detached from the cell, must be due to a change in the time constant for either entry into, or recovery from, the C-type inactivated state. In order to distinguish between these possibilities, we used an analysis similar to that of DeCoursey [9]. Kv1.3 current was measured during depolarizations long enough (7 s) for the current to reach steady-state during the pulse. At steady-state, the rates of entry into and recovery from inactivation are equal, and thus the rate constant (β) for recovery from inactivation can be determined from the ratio (R) of the steady-state current to the peak current, and the measured time constant of current decay (τ), by the equation:

$$\beta = R/\tau \quad (1)$$

The rate constant (α) for entry into inactivation is obtained from the equation:

$$\alpha = (1/\tau) - \beta \quad (2)$$

From rate constants α and β, the time constants $1/\alpha$ and $1/\beta$ for entry into and recovery from inactivation, respectively, are easily determined. It has been found previously [21, 28] that these parameters do not vary as a function of voltage, and in fact C-type inactivation of Kv1.3 is a state-dependent rather than a voltage-dependent process in both cell-attached and detached patches [28]. As shown in Table 1, the time constant for recovery from inactivation does not change when the patch is excised; in contrast, the time constant for entry into inactivation is several-fold faster in the detached patch, sufficient to account for the modulation of C-type inactivation observed.

A role for phosphorylation?

Because many ion channels, including Kv1.3 [2, 32], can be modulated by protein phosphorylation, we tested the possibility that phosphorylation or dephosphorylation mediates the change in C-type inactivation upon detaching the patch from the cell. Kv1.3 contains

Table 1 Time constants for entry into and recovery from inactivation for the cloned voltage-gated potassium channel Kv1.3. The Kv1.3 current was measured during 7 s depolarizations from −90 mV to +40 mV, in cell-attached and detached membrane patches from *Xenopus* oocytes. A single exponential time constant (τ) was fitted to the inactivating portion of the current, and the ratio (R) of the steady-state current to the peak current was measured. The time constants for entry into and recovery from inactivation were then calculated as described in the text (see also reference [9]). Data are means ± SEM for nine patches. The τ values and the time constants for entry into inactivation were significantly different in cell-attached and detached patches ($P < 0.05$)

Recording mode	Parameter			
	τ (ms)	R	Time constants (ms)	
			Entry	Recovery
Cell-attached patch	940 ± 85	0.15 ± 0.02	1100 ± 106	7130 ± 1036
Detached patch	415 ± 46	0.06 ± 0.01	440 ± 53	8040 ± 1269

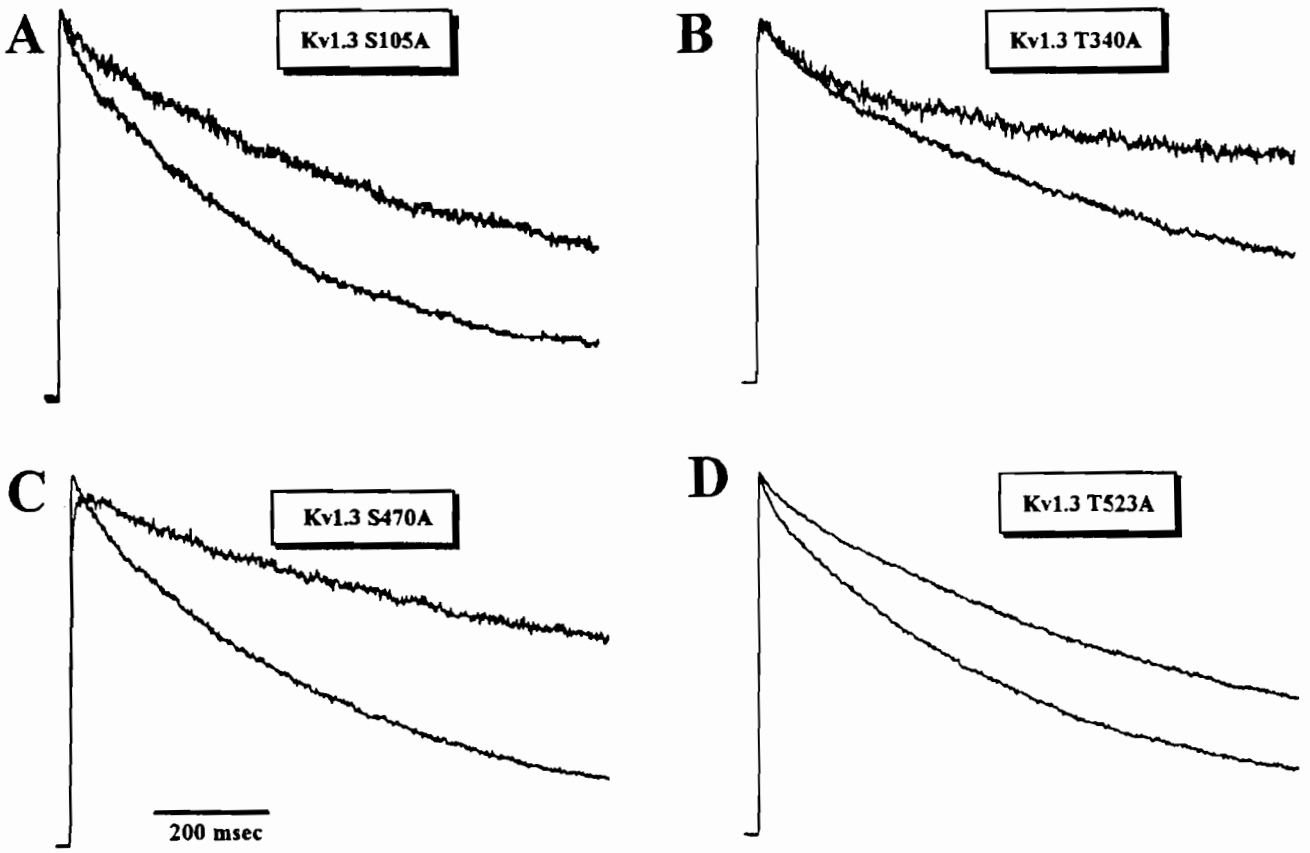


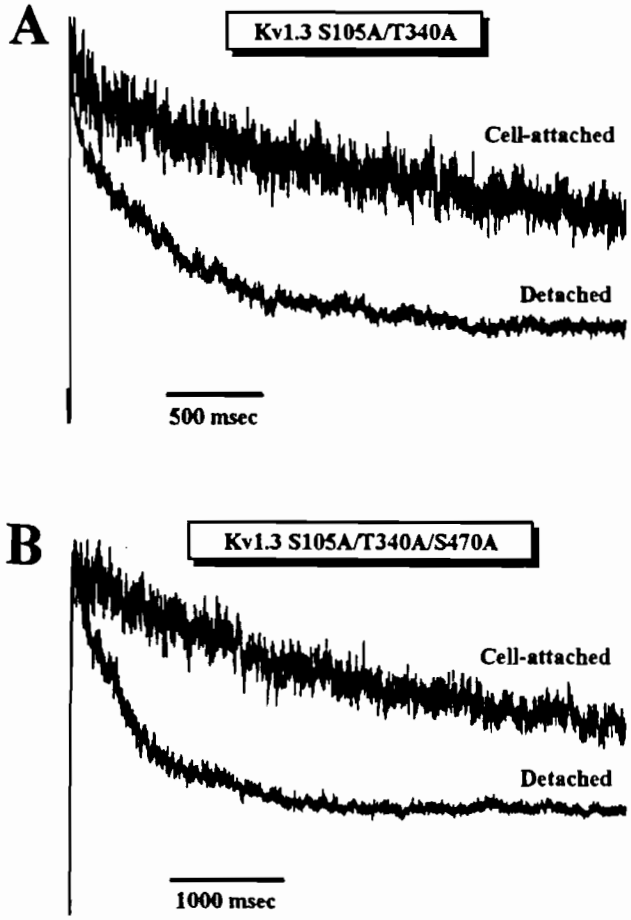
Fig. 2A–D Mutation of single phosphorylation sites does not eliminate the on-cell/off-cell change in C-type inactivation. Individual serines (S) or threonines (T) within phosphorylation site consensus sequences were mutated to alanines (A) as described in the text. Mutant channels were expressed in *Xenopus* oocytes and C-type inactivation was measured in the cell-attached and detached patch recording modes. In each panel the more rapidly inactivating current trace is from the detached patch recording mode. **A–D** Illustrations of the four mutants for which patch recordings could be made. A fifth mutant (S344A) was not expressed sufficiently to permit macroscopic current measurements to be made in the patch

a number of serine and threonine residues that sit within consensus sequences for phosphorylation by protein kinases A and C [23]. We used site-directed mutagenesis to convert several of these serines (S) and threonines (T) to alanines (A), and examined C-type inactivation and its modulation in the mutant channels. As shown in Fig. 2 and Table 2, modulation of C-type inactivation upon detaching the patch from the cell was observed in all four of the individual phosphorylation site mutants tested (S105A; T340A; S470A; and T523A where S, T and A have been defined and the number is the site of substitution). The time course of the modulation in these mutants (not shown) was similar to that in wild-type Kv1.3 channels (Fig. 1C). Furthermore the basal rate of C-type inactivation was similar in the wild-type channel and the individual phosphorylation site mutants (Table 2). A fifth phosphorylation site mutant, S344A, was not expressed sufficiently for macroscopic currents to be easured in membrane patches. However, from two-electrode

Table 2. Inactivation time constants for phosphorylation site mutants of Kv1.3. Channel constructs were expressed individually in *Xenopus* oocytes and macroscopic currents were recorded in response to depolarizations to +40 mV from a holding potential of −90 mV, in the cell-attached and detached patch recording modes. Time constant (τ) values, estimated from exponential fits to the inactivating portion of the current, are shown as means ± SEM. A fast initial component was observed in some cases so that two exponentials were necessary to fit the time course of current decay (data not shown). The ratio of the slow inactivation time constant of the detached patches to that of the cell-attached patches is shown in the *last column*. A paired *t*-test indicated that for all channel constructs the time constants for on-cell and off-cell were significantly different ($P < 0.05$). (*S105A* Conversion of serine at position 105 to alanine, *T340A* conversion of threonine at position 340 to alanine, *S470A* conversion of serine at position 470 to alanine, *T523A* conversion of threonine at position 523 to alanine, *S105A/T340A* double mutation, *S105A/T340A/S470A* triple-mutation)

Channel construct		Parameter		
		Inactivation time constants (τ, ms)		$\tau_{detached}/\tau_{attached}$
		Cell-attached	Detached	
Wild-type	(n = 8)	803 ± 185	404 ± 72	0.50
S105A	(n = 5)	1200 ± 295	517 ± 58	0.43
T340A	(n = 5)	1230 ± 29	726 ± 69	0.59
S470A	(n = 5)	658 ± 78	425 ± 17	0.65
T523A	(n = 5)	900 ± 174	610 ± 94	0.68
S105A/T340A	(n = 5)	1410 ± 140	606 ± 56	0.43
S105A/T340A/S470A	(n = 5)	3181 ± 387	601 ± 61	0.19

Fig. 3A, B C-type inactivation in double and triple phosphorylation site mutants. C-type inactivation was measured in cell-attached and detached patches, for Kv1.3 channels containing double (S105A/T340A - *panel* **A**) or triple (S105A/T340A/S470A - *panel* **B**) phosphorylation site mutations. Note the different time scales in **A** and **B**



voltage-clamp experiments (not shown), it appears that the basal rate of C-type inactivation of Kv1.3 S344A is also similar to that of the wild-type channel.

Since no particular one of the individual phosphorylation sites tested appears to be necessary for the modulation of C-type inactivation, we investigated the effects of changing two or three of them simultaneously. Both a double mutant (S105A/T340A; Fig. 3A) and a triple mutant (S105A/T340A/S470A; Fig. 3B) still exhibit the modulation of C-type inactivation when the patch is detached from the cell (Table 2). Interestingly, the triple mutant exhibits a much slower basal inactivation rate in the cell-attached patch than the wild-type channel or any of the individual phosphorylation site mutants (Table 2). Since the rate of C-type inactivation in the detached patch is similar for all the channel constructs, the extent of the change in going from the cell-attached to the detached patch mode is in fact greatest in the triple phosphorylation site mutant (see the ratios of time constants in Table 2).

C-type inactivation of Kv1.6

To determine the specificity of the change in the rate of C-type inactivation upon detaching the patch from the cell, the gating of the homologous Kv1.6 channel [37] was examined under identical conditions. In contrast to Kv1.3, C-type inactivation of Kv1.6 during a prolonged depolarization is not modulated upon going from the cell-attached to the detached patch recording mode (Fig. 4; compare with Kv1.3 in Fig. 1A). Fitting the current decay during C-type inactivation of Kv1.6 frequently requires two exponential time constants, with a fast component ranging from 140 ms to 600 ms and a slow component ranging from 3,400 ms to

Table 3. Inactivation time constants for chimeric Kv channels and site-directed channel mutants. Experimental conditions were identical to those indicated in Table 2. The *Shaker* channel had its rapid inactivation removed by deletion of a portion of the N-terminus (see text). (*N6P6C3* Replacement of the Kv1.6 C-terminus with a Kv1.3 C-terminus, *N3P3C6* replacement of the Kv1.3 C-terminus with a Kv1.6 C-terminus, *N6P3C6* replacement of the Kv1.6 P-domain with that of Kv1.3, *Shaker-P3* replacement of the P-domain of the *Shaker* channel with that of Kv1.3, *Kv1.3H401Y* replacement of the histidine in position 401 with tyrosine, *Kv1.6Y430H* replacement of the tyrosine in position 430 of Kv1.6 with histidine, *Kv1.3 H401W* replacement of the histidine in position 401 of Kv1.3 with tryptophan)

Channel Construct		Parameter: Inactivation Time Constants (τ, ms)		$\tau_{detached}/\tau_{attached}$
		Cell-attached	Detached	
Wild-type				
Kv1.3	(n = 8)	803 ± 185	404 ± 72	0.50*
Kv1.6	(n = 7)	8660 ± 1678	8590 ± 1667	0.99
Shaker	(n = 7)	3210 ± 352	3320 ± 287	1.03
Chimeras				
N6P6C3	(n = 5)	2610 ± 550	2580 ± 671	0.99
N3P3C6	(n = 5)	1130 ± 124	580 ± 59	0.51*
N6P3C6	(n = 5)	821 ± 19	521 ± 84	0.63*
Shaker-P3	(n = 5)	1450 ± 255	790 ± 78	0.54*
Mutants				
Kv1.3 H401Y	(n = 7)	7830 ± 1220	7650 ± 1152	0.98
Kv1.6 Y430H	(n = 7)	151 ± 37	93 ± 26	0.62*
Kv1.3 H401W	(n = 5)	162 ± 36	92 ± 25	0.57*

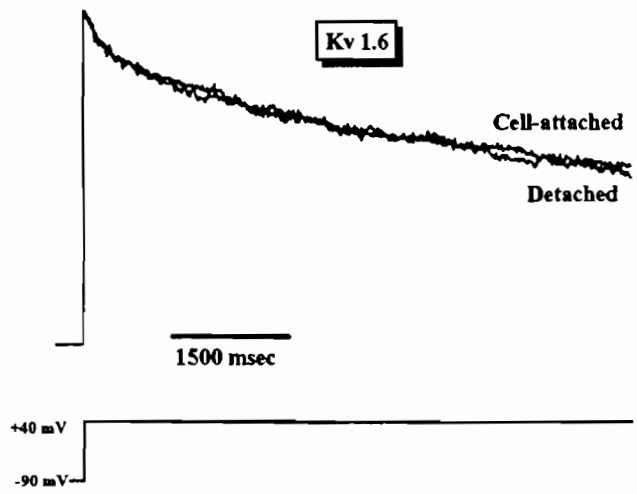
*Time constants on and off cell were significantly different ($P < 0.05$)

11,200 ms in different patches. Averages of the slower time constant for seven patches in both the cell-attached and detached patch modes are shown in Table 3. A paired t-test demonstrates that neither the slow time constant nor the faster one differs significantly between the cell-attached and detached patches, again in contrast to Kv1.3 (Table 3).

Chimeric channels

Because of the extensive sequence identity between Kv1.3 and Kv1.6, the finding that only Kv1.3 and not Kv1.6 C-type inactivation is modulated provided a convenient way to screen for channel structural features that participate in the modulation of inactivation kinetics. Accordingly we constructed Kv1.3/Kv1.6 chimeric channels, taking advantage of several unique restriction sites engineered into equivalent positions in Kv1.3 and Kv1.6 [29]. The nomenclature for the chimeric constructs is based on the location of the unique restriction sites and is displayed in Fig. 5. The N-domain contains the amino terminus and includes the putative transmembrane helices one to five. Two of the unique restriction sites define a stretch of 46 amino acids that encompasses the putative pore region together with some flanking regions and is referred to as the P-domain. The C-domain comprises transmembrane helix six and the C-terminus. Thus a wild-type Kv1.3 channel is referred to as N3P3C3. In initial experiments we exchanged the C-domains of Kv1.3 and Kv1.6 because this is the region where they are most diverse. Replacing the Kv1.6 C-terminus with a Kv1.3 C-terminus produces a chimeric channel, N6P6C3, that behaves like a wild-type Kv1.6 channel in that it does not undergo modulation of C-type inactivation (Fig. 6A; see also [29]). Furthermore the Kv1.3 C-terminus is not necessary for modulation, since a chimeric channel consisting predominantly of a Kv1.3 channel with a Kv1.6 C-terminus, N3P3C6, is modulated in the same way as wild-type Kv1.3 (Fig. 6B). To test the contribution of another region of the channel protein, the P-domain of Kv1.6 was replaced with that of Kv1.3. This construct, referred to as N6P3C6, exhibits modulation of inactivation identical to that of wild-type Kv1.3 (Fig. 6C). The time constants for C-type

Fig. 4 C-type inactivation of Kv1.6 is not modulated. Same as Fig. 1A, except that measurements were made from oocytes expressing Kv1.6 channels. This experiment is representative of >30 patches from >20 oocytes



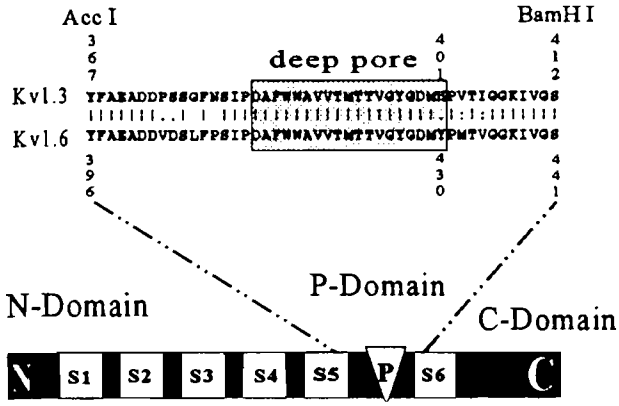


Fig. 5 Schematic diagram of the putative transmembrane topology and amino acid sequence homology of Kv1.3 and Kv1.6 channels. The presumed transmembrane regions S1 to S6 are *boxed* and the deep pore region (P) is symbolized by a *triangle*. The unique restriction sites *Acc*I and *Bam*HI, which were used for constructing chimeric channels, determine the borders of the N-, P- and C-domains. The deduced amino acid sequences (one-letter notation) of the P-domains of Kv1.3 and Kv1.6 are illustrated, and the equivalent positions 401 for Kv1.3 and 430 for Kv1.6 are noted

inactivation of these chimeric channels, in both the cell-attached and detached patch modes, are given in Table 3.

It was also of interest to ask whether the P-domain of Kv1.3 would confer modulability on other voltage-gated potassium channels. Accordingly we examined C-type inactivation in a *Shaker* channel from which N-type inactivation had been removed by deletion of amino acids 6 to 46 [20]. It was found previously [8, 21, 25] that this *Shaker* construct undergoes C-type inactivation during a prolonged depolarizing pulse. As shown in Table 3, this construct does not exhibit modulation of inactivation when the patch is detached from the cell. Replacing the P-domain of *Shaker* with that of Kv1.3 produces a chimeric channel (*Shaker*-P3) that does exhibit modulation of inactivation (Table 3). Thus the P-domain of Kv1.3 is sufficient to confer modubility of C-type inactivation on several different kinds of voltage-gated potassium channels.

Mutant channels

Due to the high degree of sequence identity between Kv1.3 and Kv1.6 in the P-domain, N6P3C6 differs from wild-type Kv1.6 by only seven amino acids on the shoulders of the putative pore region (Fig. 5). Thus one or more of those seven amino acids is sufficient to render Kv1.6 channels susceptible to modulation of inactivation (Fig. 6). We focused our attention on one particular amino acid residue that must be accessible from the extracellular milieu, because it has been shown to interact with external TEA in voltage-dependent potassium channels [3, 26]. It has been reported previously that replacing the histidine (H) in this position (i.e. 401) of Kv1.3, with the tyrosine that is in the

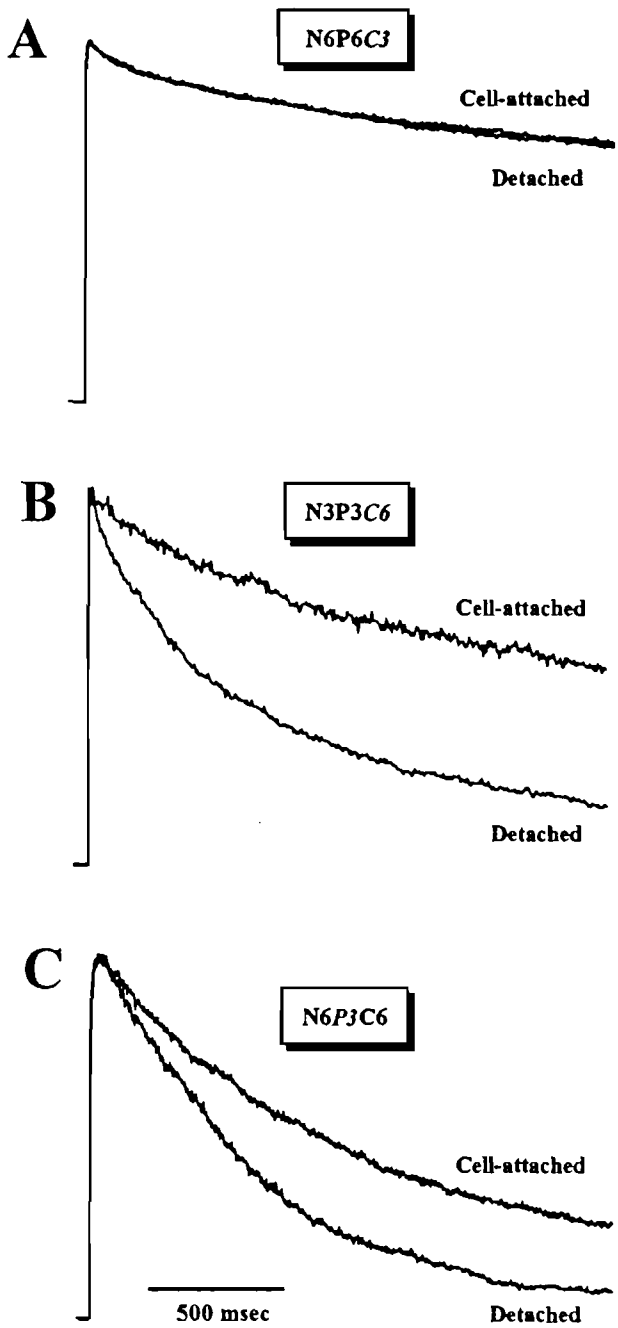


Fig. 6A–C Modulation of C-type inactivation in Kv1.3/Kv1.6 chimeric channels. Shown are normalized currents in patches from *Xenopus* oocytes expressing one of three chimeric channels. Currents were evoked by depolarizations from a holding potential of −90 mV to a pulse potential of +40 mV in the cell-attached configuration and >7 min after detaching the patch from the cell. The three chimeric channels shown are: a Kv1.6 channel in which the C-domain has been replaced with that of Kv1.3 (*N6P6C3*, **A**); a Kv1.3 channel in which the C-domain has been replaced with that of Kv1.6 (*N3P3C6*, **B**); and a Kv1.6 channel in which the P-domain has been replaced with that of Kv1.3 (*N6P3C6*, **C**). The two chimeras that contain the P-domain of the Kv1.3 channel exhibit modulation of C-type inactivation when the patch is detached from the cell

equivalent position (i.e. 430) of Kv1.6, slows inactivation [3]. Furthermore, in *Shaker* potassium channels,

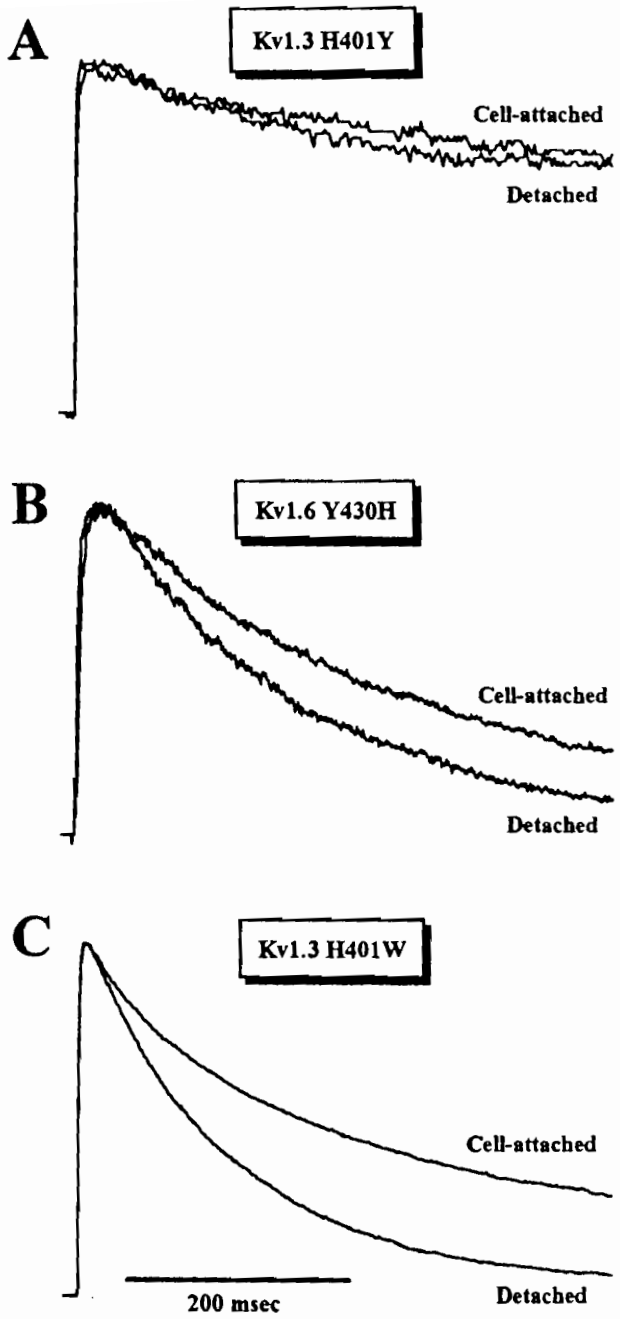


Fig. 7A–C Site-directed channel mutants. Shown are normalized currents in patches from *Xenopus* oocytes expressing either **A** a Kv1.3 channel in which the histidine at position 401 was replaced by a tyrosine (*Kv1.3 H401Y*), **B** a Kv1.6 channel in which the tyrosine at position 430 was replaced by a histidine (*Kv1.6 Y430H*); or **C** a Kv1.3 channel in which the histidine at position 401 was replaced by a tryptophan (*Kv1.3 H401W*). C-type inactivation was examined in the cell-attached patch and >7 min after detaching the patch from the cell

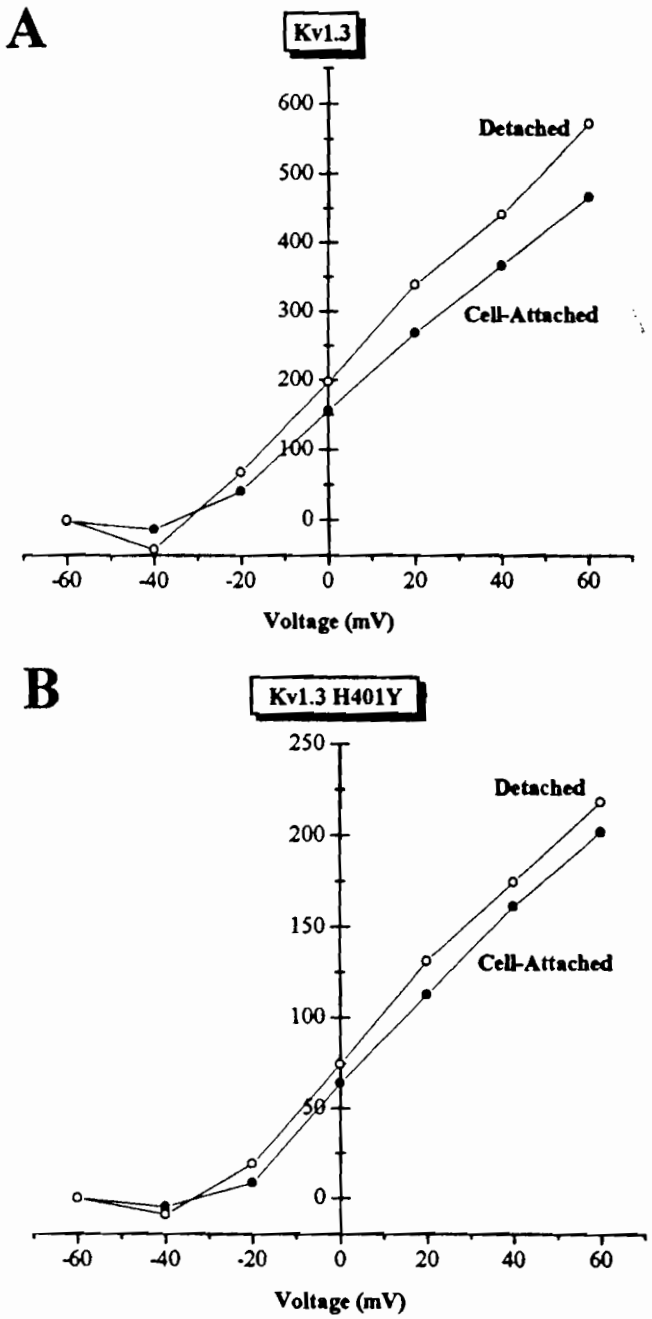


Fig. 8A, B Voltage dependence of activation is the same in wild-type and mutant channels. Voltage dependence of channel activation was measured by stepping from a holding potential of −90 mV to various test potentials for 500 ms, in the cell-attached (●) or detached (○) patch recording modes. Peak current amplitudes are plotted for wild-type Kv1.3 **A** and Kv1.3 H401Y mutant **B** channels

changing the amino acid that occupies the equivalent position can cause a large change in the rate of C-type inactivation [25]. Thus this Kv1.3 H401Y mutant channel was constructed, and its rate of inactivation in both the cell-attached and the detached patch modes was determined. The results in Fig. 7A and Table 3 confirm the previous finding [3] that this amino acid substitution slows the basal rate of C-type inactivation of the Kv1.3 channel. In addition, our data show that the mutant channel does not exhibit a change in the rate of inactivation when the patch is detached from the cell; in this respect it behaves like Kv1.6 rather than Kv1.3. In order to determine whether a histidine residue

by itself is sufficient to induce modulation of inactivation in Kv1.6, the reverse mutant, Kv1.6 Y430H was constructed. This mutant channel demonstrates C-type inactivation much more rapidly than the parent Kv1.6 channel (shown previously by Busch et al. [3]), and does exhibit modulation of inactivation when the patch is detached from the cell (Fig. 7B and Table 3). However, it is not necessary to have a histidine in this position to observe the on-cell/off-cell modulation; a Kv1.3 channel in which the histidine is mutated to tryptophan (W) also exhibits an increase in the rate of C-type inactivation when the patch is detached from the cell (Kv 1.3 H 401 W, Fig. 7C and Table 3). In each case, the time course of modulation of mutant (not shown) and wild-type (Fig. 1C) channels was similar. Furthermore, other features of channel activity, including voltage dependence (Fig. 8) and kinetics of activation in both cell-attached and detached patches, are similar in wild-type (Fig. 8A) and mutant (Fig. 8B) channels.

Discussion

Many potassium channels exhibit C-type inactivation during a prolonged depolarizing pulse. Although the physiological significance of C-type inactivation has been open to question because of its relatively slow time course, it is now clear that, under some circumstances, it can in fact be a very rapid process [26]. Accordingly, sensitivity to extracellular TEA [3, 8, 15] is a better diagnostic tool of C-type inactivation than is its time course. Furthermore, the cumulative or use-dependent inactivation of potassium current seen often in response to a series of short depolarizations is simply another manifestation of C-type inactivation [1, 5, 9, 28, 29]. Since cumulative inactivation can produce a long-lasting increase in membrane excitability [1, 4, 14, 27, 35], the modulation of C-type/cumulative inactivation can contribute in a major way to long-term changes in neuronal electrical properties.

C-type inactivation influenced by a cytoplasmic factor

Cahalan et al. [5] noted an increase in cumulative inactivation of potassium current in T lymphocytes, as a function of time after rupturing the cell membrane in the whole-cell patch recording mode. Since the potassium current in lymphocytes is carried via Kv1.3 channels [11, 17], this suggests that cumulative inactivation of Kv1.3 is affected by some factor(s) that is lost from the cell during whole-cell recording. Furthermore, the modulation of cumulative inactivation is significantly less when the perforated-patch technique, which allows electrical access to the cell interior while preventing cellular constituents from diffusing into the electrode, is employed in place of conventional whole-cell recording [30].

When Kv1.3 is expressed to high levels in *Xenopus* oocytes and currents are recorded in membrane patches, the rates of cumulative inactivation and C-type inactivation are significantly faster when the patch is detached from the cell [29]. A similar phenomenon has been reported for rat brain sodium channels expressed in *Xenopus* oocytes, which also exhibit a change in inactivation kinetics in detached membrane patches [13]. As we show here, the modulation of C-type inactivation during a prolonged depolarization results from a change in the rate constant for entry into the inactivated state, whereas the rate constant for recovery from inactivation is not affected. The time course of the change in inactivation rate, when the patch is detached from the cell, is consistent with an enzymatic process rather than simply a change in the ionic environment on the intracellular side of the patch. The mechanism of this change in the rate of C-type inactivation in Kv1.3 remains to be investigated in detail, and we emphasize that we have used the term modulation here for convenience and not to imply any particular molecular mechanism for the on-cell/off-cell change.

A role for protein phosphorylation?

Potassium channel inactivation can be influenced by a variety of factors including redox state [33], pH [10], monovalent [25, 31] and divalent [16] cations, agents that disrupt cytoskeleton [19], and protein phosphorylation [12]. In view of the ubiquitous role of protein phosphorylation in modulating the properties of ion channels [24], including Kv1.3 [2, 32], and the fact that Kv1.3 is an excellent substrate for protein kinases ([6]; T. Holmes and I.B. Levitan, unpublished), it is somewhat surprising that the modulation remains intact in the various phosphorylation site mutants we constructed. It is possible that phosphorylation is indeed involved in the on-cell/off-cell change in inactivation rate, but that the relevant phosphorylation site is one that we did not test (for example, the low expression level precluded us from examining the S344A channel mutant in cell-attached and detached patches). The slowing of the basal rate of C-type inactivation in the triple phosphorylation mutant does suggest some role for these serines/threonines in regulating inactivation, but the precise nature of this role remains to be determined.

An extracellular amino acid influences modulation of C-type inactivation

The search for structural correlates of modulation of C-type inactivation in Kv1.3 was facilitated by the finding that the homologous Kv1.6 channel does not exhibit this modulation. Results with Kv1.3/Kv1.6

chimeric channels identify the region around the pore of Kv1.3 as sufficient to confer modulability on Kv1.6. Several amino acid residues, in and downstream from the S5 membrane-spanning domain, have been shown previously to influence the basal rate of C-type inactivation in voltage-gated potassium channels [3, 21, 25]. We show now that an extracellular amino acid residue in this region influences the modulation of C-type inactivation described here. This same residue forms part of the binding site for extracellular TEA [3, 26], and extracellular TEA is known to interfere with C-type inactivation [3, 8, 15]. Furthermore the rate of C-type inactivation in *Shaker* channels can vary over several orders of magnitude depending on the particular amino acid that occupies this position [25]. We have confirmed these earlier findings that this amino acid position is important in determining the basal rate of C-type inactivation. In addition we show that this residue is critical for the modulation of C-type inactivation described here. The degree of modulation is not simply correlated with the basal rate of C-type inactivation, as indicated by the fact that the very slowly inactivating triple phosphorylation site mutant channel is modulated to a far greater extent than more rapidly inactivating channel constructs (Tables 2 and 3). It is particularly interesting that an extracellular amino acid confers modulability on the Kv1.3 channel. Since the modulation involves a change in the environment on the cytoplasmic face of the patch, this implies some interaction between extracellular and intracellular amino acids in regulating channel function. Understanding how such interaction occurs is a major challenge for future structure-function studies of voltage-gated potassium channels.

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