ABSTRACT

Mechanisms underlying Kv4 channel inactivation and recovery are presently unclear, although there is general consensus that the basic characteristics of these processes are not consistent with Shaker (Kv1) N- and P/C-type mechanisms. Kv4 channels also differ from Shaker in that they can undergo significant inactivation from pre-activated closed-states (closed-state inactivation, CSI), and that inactivation and recovery kinetics can be regulated by intracellular KChIP2 isoforms. To gain insight into the mechanisms regulating Kv4.3 CSI and recovery, we have analyzed the effects of increasing [K+]o from 2 mM to 98 mM in the absence and in the presence of KChIP2b, the major KChIP2 isoform expressed in the mammalian ventricle. In the absence of KChIP2b, high [K+]o promoted Kv4.3 inactivated closed-states and significantly slowed the kinetics of recovery from both macroscopic and closed-state inactivation. Coexpression of KChIP2b in 2 mM [K+]o promoted non-inactivated closed-states and accelerated the kinetics of recovery from both macroscopic and CSI. In high [K+]o, KChIP2b eliminated or significantly reduced the slowing effects on recovery. Attenuation of CSI by the S4 charge-deletion mutant R302A, which produced significant stabilization of non-inactivated closed-states, effectively eliminated the opposing effects of high [K+]o and KChIP2b on macroscopic recovery kinetics, confirming that these results were due to alterations of CSI. Elevated [K+]o therefore slows Kv4.3 recovery by stabilizing inactivated closed-states, while KChIP2b accelerates recovery by destabilizing inactivated closed-states. Our results challenge underlying assumptions of presently popular Kv4 gating models and suggest that Kv4.3 possesses novel allosteric mechanisms, which are absent in Shaker, for coupling interactions between intracellular KChIP2b binding motifs and extracellular K+‐sensitive regulatory sites.

INTRODUCTION

Voltage-sensitive Kv4 (Shal-type) potassium channels are expressed highly in both the nervous and cardiovascular systems. These channels generate rapidly activating and inactivating currents designated “I_A” in neurons and “I_to,fast” in cardiac myocytes.1-4 Because of their very close kinetic overlap with voltage-sensitive L-type calcium channels, I_A/I_to,fast current phenotypes have been implicated in the regulation of a wide range of basic neuronal and cardiac functions, including axonal action potential propagation,2,3 somatodendritic interactions and long term potentiation,2,3,5,6 pain perception,7 and phase I repolarization and excitation-contraction coupling in cardiac myocytes.1,4 Alterations of I_A/I_to,fast characteristics have in turn been implicated in several pathological conditions of the nervous and cardiovascular systems, including epilepsy, Alzheimer’s disease and cardiac arrhythmias and hypertrophy.1,4 As a result, extensive experimental effort has been devoted to determining the basic molecular and biophysical mechanisms underlying Kv4 voltage-dependent gating transitions, in particular inactivation and recovery.1,2

Among Kv channels, the mechanisms underlying inactivation and recovery have been most thoroughly analyzed in Shaker (Kv1) channels. Kv1.4 inactivates and recovers by well characterized N-type (intracellular N-terminal inactivation domain) and P/C-type (external conduction pore vestibule closure) mechanisms.8-11 Evidence indicates N- and P/C-type inactivation are allosterically coupled, and recovery is regulated by the P/C-type process.1,12 Due to these two mechanisms, increases in [K+]o slow macroscopic inactivation kinetics and accelerate recovery kinetics, via either “knock-off” or “foot-in-the-door” mechanisms.1,8-10
Despite the universal acceptance of N- and P/C-type mechanisms for explaining inactivation and recovery characteristics in Kv4.1,4,8-11 initial studies on Kv4 channels produced results that did not conform to the basic predictions of the Shaker model.1,2 Two sets of experimental observations were particularly noteworthy: i) For Kv4.1, 4.2 and 4.3, increases in \([K^+]_o\), accelerated inactivation kinetics and slowed recovery kinetics, effects opposite to those predicted for Shaker;1,2,13 and ii) For Kv4.2, deletion of the intracellular N-terminal domain associated with the Shaker inactivation “ball” or “peptide”8-11 failed to significantly alter recovery kinetics.14 Most investigators have therefore concluded that Kv4 channels do not inactivate or recover by “conventional” Shaker-like N- and/or P/C-type mechanisms.1,2 Nonetheless, the mechanisms underlying Kv4 channel inactivation and recovery, and the anomalous effects of \([K^+]_o\) on these two processes, are still unclear.

In Shaker, rapid N-type inactivation appears to be obligatorily coupled to activation. As a result, the kinetics of rapid macroscopic inactivation do not display inherent voltage-dependence, and there is essentially no closed-state inactivation (CSI).10,11 In contrast, studies on ferret right ventricular myocytes originally demonstrated that native I\(_{to,fast}\) could undergo significant inactivation from non-conducting closed-states at subthreshold hyperpolarized potentials.15,16 Subsequent studies on cloned Kv4 channels have verified the existence of CSI.1,5,17-19 Our laboratory has also recently presented evidence that Kv4.3 CSI may possess inherent voltage-dependence.19 Therefore, Kv4 channels possess a prominent CSI mechanism that is both absent in Shaker and displays novel gating characteristics distinct from N- and P/C-type mechanisms. However, the potential involvement of CSI in the anomalous effects of \([K^+]_o\) on Kv4 channel gating kinetics has not been determined, and the relative importance of CSI in overall Kv4 channel gating is controversial.1,2,14,17-20

Inactivation and recovery kinetics of Kv4 channels can be significantly modulated by a class of intracellular ancillary \(\beta\) subunits termed K Channel Interacting Proteins (KChIPs).1,2,21,22 of which KChIP2b is the predominant isoform expressed in the mammalian ventricle.1,17,23,24 Most conventional KChIP isoforms regulate Kv4 channels by both slowing macroscopic inactivation kinetics and accelerating macroscopic recovery kinetics to rates similar to those of native I\(_{A}^{\infty}/I_{\text{to,fast}}^{\infty}\)1,2 CSI is believed to play a central role in these physiologically important regulatory effects. However, at present there are two mutually exclusive models for how KChIPs may accelerate Kv4 channel recovery. Beck et al.20 originally reported that KChIP1 accelerated the kinetics of development of CSI (developed at ~50 mV), and proposed that KChIPs promote inactivated closed-states. In contrast, our laboratory reported that KChIP2b slowed the kinetics of development of CSI (also at ~50 mV), and therefore proposed that KChIP2 isoforms promote non-inactivated closed-states.17 Both of these studies, and the proposed models resulting from them, were limited in that neither analyzed the effects of the respective KChIP isoforms on the kinetics of recovery from CSI. As a result, the effects that KChIP isoforms exert on Kv4 channel CSI, and how these effects result in acceleration of recovery kinetics, are still unresolved.

To begin to address these functionally important issues, we have quantitatively analyzed the effects of increasing \([K^+]_o\) from 2 to 98 mM on the basic steady-state and kinetic characteristics of Kv4.3 expressed in the absence and in the presence of KChIP2b. We demonstrate that elevated \([K^+]_o\) and coexpression of KChIP2b exert opposing regulatory effects on channel transitions governing deactivation and recovery. Our results suggest that the inactivation and recovery characteristics of Kv4.3 are distinct from, and more complicated than, those of Shaker. In particular, we demonstrate that, in contrast to Kv4.1, increases in \([K^+]_o\) stabilize Kv4.3 inactivated closed-states, and as a result slow macroscopic recovery kinetics. Coexpression of KChIP2b attenuates the effects of high \([K^+]_o\) on Kv4.3 closed-state inactivation, indicating the presence of novel mechanisms for allosterically coupling the regulatory effects of intracellular KChIP2b to extracellularly accessible K\(^{\text{r}}\)-sensitive regulatory sites. These results in turn provide strong evidence in support of the Kv4.3/KChIP2b regulatory model (previously proposed by our laboratory17).

**METHODS**

**Molecular biology.** Kv4.3 and KChIP2b were cloned from ferret heart (Kv4.3: long form, GenBank AF454388; KChIP2b, GenBank AF454387) as described previously17,23,24 and maintained in the pBluescript KS(+) vector. Site directed mutagenesis was performed using the Quick Change II Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA, USA) and primers designed to positively charged residues (R302, Invitrogen, Carlsbad, CA, USA) in the S4 transmembrane segment exactly as previously described in ref. 19. Specificity of the mutation was confirmed by sequencing of the entire clone (Roswell Park Cancer Institute DNA Sequencing Core Facility, Buffalo, NY, USA).

**In vitro transcription and oocyte preparation.** Kv4.3 wild-type and mutant clone plasmids were linearized with the restriction endonuclease XhoI (Promega, Madison WI, USA). cRNA was synthesized by the mMessage mMachine T7 Ultra Kit (Ambion, Austin, TX, USA). cRNA quantity and quality was evaluated by spectroscopy and agarose gel electrophoresis.

All animal protocols were conducted according to the NIH-approved guidelines of the Institutional Animal Care and Use Committee, University at Buffalo, SUNY. Oocytes were obtained from mature female Xenopus laevis anaesthetized by soaking in 1.0 g L\(^{-1}\) ethyl-3-aminobenzoate methanesulphonate salt and defeciliated as previously described.17,19,23,24 Twelve to 24 hours after isolation, oocytes were injected with 4–9 ng cRNA (Nanoject II; Drummond Scientific, Broomall, PA, USA). For coinjection measurements, the Kv4.3:KChIP2b cRNA ratio was always 1:117,19 (see below, Potential Limitations of Analysis). Injected oocytes were then incubated for 2–4 days at 18°C.

**Electrophysiology.** Two-microelectrode voltage clamp recordings (GeneClamp 500B, Axon Instruments, Union City, CA, USA) were performed on injected oocytes as described previously.27 Voltage clamp recordings were conducted at the maximal gain of the amplifier (10,000X) and clamp rise time stability settings of 60–120 μs. Currents were acquired (filtered at 1 kHz, digitized at 5 kHz) with a Digidata 1320A 16-bit acquisition system run under pCLAMP 9 software control (Axon Instruments). Control recordings (22 ± 2°C) were first conducted in ND96 solution (in mM: 96 NaCl, 2 KCl, 1 MgSO\(_4\), 1.8 CaCl\(_2\), 5 HEPES, pH = 7.40). For subsequent analysis of the effects of increasing \([K^+]_o\), (5, 10, 20, 50 and 98 mM) the same solution was employed except both [NaCl] and [KCl] were varied while maintaining the sum of [NaCl] + [KCl] constant at 98 mM. All chemicals for making electrode and recording solutions were obtained from Sigma-Aldrich (St. Louis, MO, USA).
Protocols and analysis. Quantitative analysis was conducted using pCLAMP 9 and Origin 7.5 (Origin Lab, Northampton, MA, USA). Analysis of approximate steady-state gating relationships (“$a^4$”, “$i$”) and kinetic properties (activation, deactivation, inactivation, recovery from inactivation, closed-state inactivation [CSI] and recovery from CSI; see below, Potential Limitations of Analysis) were conducted using standard voltage clamp protocols as described previously in detail.\textsuperscript{17,19} with any modifications given in the corresponding “Results” section or figure captions.

Statistical significance was determined using either paired t-tests (e.g., results obtained from the same oocyte and same expression conditions but under different potassium concentrations [K$^+$]) or one-way ANOVA (e.g., comparison of results among different oocytes and different expression conditions). For all cases, significance was determined at $p < 0.01$, and in the Results section all references to “significantly different” correspond to appropriately calculated “p values” of less than 0.01. In figures, all data points are mean ± SEM values obtained from the indicated number (n) of oocytes.

Potential limitations of analysis. Activation and deactivation kinetics. Although the use of two microelectrode voltage clamp is widely used for kinetic analysis of voltage-sensitive ion channels expressed in Xenopus oocytes, the technique does have quantitative limitations, particularly in regard to analysis of rapid gating transitions associated with activation and deactivation. Specifically, in our experience attainment of new steady-state voltage clamp conditions (dV/dt = 0) in response to a voltage-clamp step pulse typically occurs with time constants ($\tau$ - $R_{\text{series}} \times C_m$) on the order of ~2–4 ms.\textsuperscript{17,19}

These relatively slow voltage-clamp “rise times” in turn generate large and long lasting passive capacitive transients ($I_{\text{cap}} = C_m \times dV/dt$), the overlap of which can significantly obscure meaningful analysis of rapid voltage-sensitive channel gating transitions. Furthermore, during such capacitive transients it is important to recognize that the membrane potential is not clamped (dV/dt = 0), but is approaching its final steady state value with a characteristic time constant $\tau$. The degree of error introduced during this “charging period” in turn depends upon the relative rates and voltage-dependencies of the specific gating transitions under analysis—for rapid processes (activation, deactivation [time constants on the order of ms]) errors can be very significant, while for slower processes (inactivation, recovery [time constants > 10–1,000 ms]) errors are negligible. Furthermore, although it is common practice to subtract out these capacitive transients (using linear scaling methods), such subtraction techniques do not circumvent the inherent limitation of voltage-clamp rise-times, and extrapolation of voltage-dependent kinetics back to actual step pulse time $t = 0$ ms can result in artifacts regarding activation and deactivation kinetics.\textsuperscript{15}

To account for this inherent limitation, for analysis of Kv4.3 ± KChIP2b activation kinetics we employed the “90% rise-time criteria” utilized in our previous studies.\textsuperscript{15,17,19} For each oocyte, a passive capacitive transient was generated in response to a 10–20 mV sub-threshold depolarizing step pulse (typically applied from HP = -100 mV to -90 or -80 mV), and the time required for decline of $I_{\text{cap}}$ to 90% of its peak value (t$_{90\%}$) was determined. This sub-threshold $I_{\text{cap}}$ was then linearly scaled and subtracted from net (passive + active) currents recorded at more depolarized potentials. Fits to activation kinetics from such subtracted currents were then only begun at t$_{90\%}$, i.e., the time at which the membrane potential was estimated to be within 10% of its final steady-state value. For the oocytes used to generate the $\tau_{\text{act}}$ - $V_{\text{m}}$ curves illustrated in Figure 3, the mean t$_{90\%}$ values were 3.33 ± 0.29 ms (n = 32 oocytes). We therefore make no quantitative conclusions regarding either activation kinetics or degree of sigmoidicity prior to this mean t$_{90\%}$ value. At each potential fits were also restricted to only the early rising phases of the currents (i.e., before significant inactivation was evident), and no attempts were made to correct for potential overlapping effects of inactivation. It has been previously demonstrated that this analytical approach gives valid measurements of Kv4.3 activation kinetics that are not significantly influenced by overlapping inactivation kinetics, since Kv4.3 activation kinetics are approximately an order of magnitude faster than inactivation kinetics.\textsuperscript{17,18,19,25} For deactivation kinetics, fits were begun either at t$_{90\%}$ or t = 3–5 ms after initiation of a step pulse. Therefore, we also make no quantitative conclusions regarding deactivation transitions prior to these initial fit times. Although these methods resulted in deactivation kinetics that were well-described as single exponential processes (Fig. 3),\textsuperscript{17,19,25} and hence the derived time constants to the fitted currents flowing after this time are accurate, it is possible that more rapid deactivation transitions occurring prior to these initial fitting times may exist but could not be resolved.\textsuperscript{15,17,19}

Ratio of Kv4.3:KChIP2b cRNA injected. Neither in this, nor previous studies,\textsuperscript{17,19} did we vary the ratios of Kv4.3:KChIP2b cRNA injected. While this is an important question, which is deserving of future careful study, in the present analysis we were concerned with both measuring macroscopic currents that were large enough to allow accurate fits, while attempting to maximize voltage clamp efficiency. Simultaneously meeting these two concerns resulted in practical experimental comprises. In this regard, we have consistently observed that peak Kv4.3 current amplitudes of greater than 10 μA (at +50 mV) showed obvious signs of voltage clamp failure, thus preventing their use in any meaningful kinetic analysis. As per our previous analyses,\textsuperscript{17,19} we therefore opted to utilize a 1:1 ratio of Kv4.3:KChIP2b cRNA injected at concentrations (4–9 ng) that gave peak current amplitudes of typically 1–5 μA at +50 mV. The data presented therefore does not allow us to address the question of whether expression of KChIP2b protein was sufficient to saturate binding to Kv4.3 protein. As a result, this is another potential limitation to our analysis.

RESULTS

We have used the term “macroscopic” to denote any Kv4.3 kinetic transition that could proceed once channels had reached open-state(s). In contrast, “closed-state” denotes kinetic transitions that could proceed without any previously measurable open-state activity during a conditioning pre-pulse. Our use of the term macroscopic thus does not necessarily exclude kinetic transitions that may have also been capable of occurring from non-conducting closed-states.\textsuperscript{1,2,14-20}

Effects of [K$^+$]$_{\text{o}}$ on Kv4.3 (± KChIP2b) macroscopic gating characteristics. Peak current-voltage (I-V) Relationships. For general orientation, representative recordings of currents generated by Kv4.3 first in 2 mM and then in 98 mM [K$^+$]$_{\text{o}}$ are illustrated in Figure 1A. The mean peak current-voltage (I-V) relationships for Kv4.3 and Kv4.3 + KChIP2b recorded in 2 and 98 mM [K$^+$]$_{\text{o}}$ are illustrated in Figure 1B. Consistent with previous results, coexpression of KChIP2b resulted in increased peak current amplitudes.\textsuperscript{1,17,23,24} As expected,
for both expression conditions 98 mM [K+]o both depolarized the reversal potential and altered the peak I-V relationships. However, for both expression conditions 98 mM [K+]o also shifted the apparent threshold for activation in the hyperpolarized direction by -10 mV.

Steady-state gating relationships \( \sigma^4 \) and \( \iota \). Consistent with our previous analysis,17 in 2 mM [K+]o coexpression of KChIP2b produced no significant effects on the steady-state activation relationship \( \sigma^4 \) (Fig. 2A), but did produce a depolarizing shift in the steady-state inactivation relationship \( \iota \) (mean \( \Delta V_{1/2} = +8.4 \) mV, \( n = 13 \); Fig. 2B). For both expression conditions overlays of the mean \( \sigma^4 \) and \( \iota \) relationships in 2 mM [K+]o indicated that a high degree of steady-state inactivation developed at hyperpolarized potentials where minimal activation developed (Fig. 2C and D). Hence, the depolarizing shift in \( \iota \) (Fig. 2B) indicated that KChIP2b stabilized non-inactivated closed-states.17

For both expression conditions, increasing [K+]o to 98 mM produced hyperpolarizing shifts in \( \sigma^4 \) and \( \iota \) (Fig. 2C and D). High [K+]o therefore stabilized both Kv4.3 open-state(s) (in agreement with Wang et al.25) and inactivated closed-states. In the presence of KChIP2b, the mean shift in \( \iota \) (mean \( \Delta V_{1/2} = +8.1 \) mV; \( n = 13 \)) was similar to that of Kv4.3 (mean \( \Delta V_{1/2} = +8.3 \) mV; \( n = 13 \)); however, the mean shift in \( \sigma^4 \) (mean \( \Delta V_{1/2} = -9.3 \) mV; \( n = 16 \)) was 2-fold greater than that of Kv4.3 (mean \( \Delta V_{1/2} = -4.5 \) mV; \( n = 11 \)).

Activation kinetics. Both our laboratory17,19 and Wang et al.25 have previously demonstrated that Kv4.3 voltage-dependent activation kinetics are sigmoidal and can be well described using a Hodgkin-Huxley-like independent subunit \( \sigma^4 \) formulation.26 A sigmoidal \( \sigma^4 \) formulation for activation kinetics was therefore applied in these measurements, the mean results of which are summarized in Figure 3. For Kv4.3 expressed alone, increasing [K+]o from 2 to 98 mM accelerated the rate of activation at -40 to -0 mV (in agreement with Wang et al.18), while its effects were minimal at more depolarized potentials. In the presence of KChIP2b, 98 mM [K+]o produced minimal effects on activation kinetics over the entire voltage range analyzed.

The largest effects of 98 mM [K+]o on activation kinetics occurred over the range of -40 to 0 mV, which corresponded to the same potential range over which the major shifts in \( \sigma^4 \) occurred (Fig. 2C and D). Alterations in inherent activation kinetics produced by 98 mM [K+]o could thus not be separated unambiguously from “simple” effects due to shifts in the voltage-dependence of \( \sigma^4 \). Nonetheless, since at +50 mV the value of \( \sigma^4 \) was virtually 1.0 for both expression conditions (Fig. 2C and D), additional alterations in inherent activation kinetics produced by 98 mM [K+]o appeared to be minimal.

Deactivation kinetics. Under all expression and recording conditions Kv4.3 deactivation kinetics could be well described as single exponential processes.17-19 In contrast to its relatively minimal effects on activation kinetics, at all potentials analyzed (-50 to -120 mV) 98 mM [K+]o slowed deactivation of Kv4.3 expressed alone (Fig. 3A and inset). These results indicated that 98 mM [K+]o stabilized the Kv4.3 open-state(s). Because the value of \( \sigma^4 \) in both 2 and 98 mM [K+]o was zero at -50 mV (Fig. 2C), these slowing
effects could be attributed to genuine alterations in inherent Kv4.3 deactivation kinetics. In contrast, when KChIP2b was coexpressed deactivation kinetics in 98 mM $[K^+]_o$ were either not altered (-120 to -80 mV) or only slightly slowed (-70 to -50 mV) (Fig. 3B).

In summary, the effects of increasing $[K^+]_o$ from 2 to 98 mM on the kinetics of Kv4.3 activation and deactivation were consistent with elevated $[K^+]_o$ stabilizing the open-state(s). These effects were in turn eliminated or attenuated by coexpression of KChIP2b. Elevated $[K^+]_o$ and KChIP2b thus exerted opposing effects on Kv4.3 activation and deactivation.

Macroscopic inactivation kinetics. For all expression and recording conditions the kinetics of macroscopic inactivation at +50 mV (a potential where the value of “i" was zero [Fig. 2C and D]) could be well fit as a double exponential process. In 2 mM $[K^+]_o$ and then 98 mM (hollow symbols) $[K^+]_o$ for (A) Kv4.3 (squares) and (B) Kv4.3 + KChIP2b (circles). Mean data points based upon the following number of paired measurements: $r_{act}$, Kv4.3: n = 11; Kv4.3 + KChIP2b: n = 12; $r_{deact}$, Kv4.3: n = 23; Kv4.3 + KChIP2b: n = 18. Inset (A): Representative recordings of deactivating tails currents recorded at -100 mV first in 2 mM and then in 98 mM $[K^+]_o$. Peak current amplitudes normalized. Calibration bar: 20 ms.

Figure 3. Activation and deactivation kinetics. (A and B) Overall voltage-dependence of mean $r_{fast}$ and $r_{deact}$ values recorded in 2 mM (solid symbols) and then 98 mM (hollow symbols) $[K^+]_o$ for (A) Kv4.3 (squares) and (B) Kv4.3 + KChIP2b (circles). Mean data points based upon the following number of paired measurements: $r_{act}$, Kv4.3: n = 11; Kv4.3 + KChIP2b: n = 12; $r_{deact}$, Kv4.3: n = 23; Kv4.3 + KChIP2b: n = 18. Inset (A): Representative recordings of deactivating tails currents recorded at -100 mV first in 2 mM and then in 98 mM $[K^+]_o$. Peak current amplitudes normalized. Calibration bar: 20 ms.

In 2 mM $[K^+]_o$ coexpression of KChIP2b produced an overall net slowing of Kv4.3 macroscopic inactivation (Fig. 4A). Quantitatively, this effect was manifested as a slowing (-1.4 fold) of the fast time constant ($r_{fast}$) of inactivation, an acceleration (-1.9 fold) of the slow time constant ($r_{slow}$), and a decrease (-1.4 fold) in the initial relative amplitude of the fast component of inactivation ($A_{fast}$) (Fig. 4D).

For both expression conditions, subsequently increasing $[K^+]_o$ to 98 mM produced similar effects: $r_{fast}$ was slowed (-1.4 fold) and $A_{fast}$ was increased, the latter effect being particularly pronounced in the presence of KChIP2b (Fig. 4D). In addition, when KChIP2b was coexpressed, in many cases macroscopic inactivation in 98 mM $[K^+]_o$ could be well-approximated as a single exponential process (Fig. 4C), indicating a major alteration in the relative contributions of the two inactivation components (i.e., the kinetics of both processes were apparently similar and hence difficult to resolve).

Wang et al. previously demonstrated that the regulatory effects of altering extracellular K+ on Kv4.3 activation and deactivation kinetics were a function of $[K^+]_o$. To demonstrate this was also the case in our study, we determined the overall $[K^+]_o$-response relationships on Kv4.3 ± KChIP2b macroscopic inactivation characteristics at +50 mV (Fig. 5). Although these measurements indicated that effects on inactivation were a function of $[K^+]_o$, there were clear differences in the $[K^+]_o$-response relationships among the two expression conditions. For Kv4.3 alone, $r_{fast}$, $r_{slow}$, and $A_{fast}$ all displayed a conventional monotonic dependence upon increasing $[K^+]_o$, while in the presence of KChIP2b all three of these parameters displayed a marked non-monotonic $[K^+]_o$ dependence. The latter effect was prominent for the fast inactivation parameters over the range of -5 to -10 mM $[K^+]_o$ (Fig. 5D). At higher $[K^+]_o$ the values of these parameters either approached or were very similar to those for Kv4.3 expressed alone.

Macroscopic recovery kinetics. For all expression and recording conditions the kinetics of recovery from macroscopic inactivation (developed during 1 s pulses to +50 mV; recovery measured at HP = -100, -85 and -70 mV) could be described as single exponential
Effects of $[K^+]_o$ on Kv4.3 (± KChIP2b) closed-state inactivation gating characteristics. The overlap of the “a” and “i” relationships in 2 mM $[K^+]_o$ (Fig. 2) indicated that over the normal range of resting membrane potentials (-90 to -50 mV) Kv4.3 would undergo significant closed-state inactivation, while the degree of CSI for Kv4.3 + KChIP2b would be markedly reduced. CSI may therefore have been playing an important role in the complex interactive effects of $[K^+]_o$ and KChIP2b that we observed on Kv4.3 macroscopic gating characteristics (Figs. 2–7). We therefore next analyzed the effects of increasing $[K^+]_o$ on Kv4.3 (± KChIP2b) CSI and recovery characteristics.
Kv4.3 Closed State Inactivation

Kinetics of development of CSI. In the previous studies of both Beck et al.20 and Patel et al.17 the kinetics of development of CSI were measured at -50 mV. Based upon the overlap of our measured “a” and “i” relationships (Fig. 2), there would have been minimal activation at this potential, and CSI would have been the dominant kinetic process under all expression and recording conditions. We therefore analyzed kinetics of development of CSI at -50 mV in the present study.

For both expression conditions, in 2 mM [K+]o, the development of CSI at -50 mV was well described as a single exponential process (Fig. 8A and B). Interestingly, in this series of measurements the mean kinetics of CSI was significantly faster in the presence of KChIP2b (Kv4.3: τCSI = 2071 ± 150 ms, n = 8; Kv4.3 + KChIP2b: τCSI = 1289 ± 470 ms, n = 5). Nonetheless, the mean percent magnitude of CSI was significantly attenuated by KChIP2b (Kv4.3: 32.6 ± 4.2% inactivation; Kv4.3 + KChIP2b: 4.4 ± 1.7%; Fig. 8). Upon increasing [K+]o to 98 mM τCSI values for both expression conditions were accelerated, with the relative degree of acceleration being greater for Kv4.3 (-2.2X) than Kv4.3 + KChIP2b (-1.4X). However, the mean τCSI values in 98 mM [K+]o were not significantly different among the two expression conditions (Kv4.3: τCSI = 942 ± 97 ms, n = 8; Kv4.3 + KChIP2b: τCSI = 901 ± 160 ms, n = 5; Fig. 9B). Finally, although the mean percent magnitude of CSI was increased for both expression conditions, it was still significantly less in the presence of KChIP2b (Kv4.3: 82.2 ± 2.8%; Kv4.3 + KChIP2b: 55.8 ± 8.3%).

Consistent with our initial results on the effects of 98 mM [K+]o on the mean inactivation “i” relationships (Fig. 2), the relative magnitude of CSI for both expression conditions monotonically increased as [K+]o increased (Fig. 9A). At all values of [K+]o the degree of CSI was always significantly less in the presence of KChIP2b. Similarly, the mean τCSI, -50 mV values also displayed a monotonic dependence upon [K+]o and were progressively accelerated to final saturating values (Fig. 9B). For Kv4.3, saturation was reached at -20 mM [K+]o, while in the presence of KChIP2b saturation was reached at ~5–10 mM [K+]o. At sub-saturating concentrations, KChIP2b reduced the relative degree of acceleration of τCSI. Overall, the net effect of elevated [K+]o resulted in mean τCSI values for the two expression conditions that were not significantly different at 20 mM and above.

DISCUSSION

Our [K+]o-response relationships indicate that Kv4.3 gating transitions are regulated by changes in [K+]o (see also Wang et al.25). However, in the presence of KChIP2b these relationships are much more complicated and display a marked non-monotonic behavior. The latter may likely indicate the existence of multiple, physically distinct binding sites and/or anomalous mole fraction effects (among two or more adjacent binding sites).27 Considering that Kv4.3 has at least two major inactivation mechanisms, such non-monotonic behavior may not be surprising. It is thus interesting to note that all non-monotonic [K+]o effects were observed while using protocols involving pulses to depolarized potentials where both closed- and
open-state inactivation processes could occur. This suggests that these "anomalous" [K+o] effects may have been due to the presence of the open-state inactivation mechanism.\textsuperscript{17-19}

Our results also challenge the proposal of Eghbali et al.\textsuperscript{28} that the effects of [K+o] on Kv4.3 inactivation and recovery kinetics are due to an external K+-sensitive regulatory site that is normally saturated in 2 mM [K+o]. Our extracellular potassium-response relationships clearly indicate that this is not the case. Rather, our results demonstrate that significant alterations in Kv4.3 inactivation and recovery kinetics can be produced by changes in [K+o], corresponding to the normal physiological to pathological ranges (2–10 mM), and that these alterations can be efficiently opposed by the presence of KChIP2b. Although the pathophysiological significance of these effects is unclear, they may be important under specific circumstances. For example, under hypokalemic conditions the presence of KChIP2b may help to minimize the effects of decreased [K+o] on Kv4.3 gating kinetics.

Although we have primarily focused on the effects of [K+o] on Kv4.3 inactivation and recovery, the effects of 98 mM [K+o] on activation and deactivation kinetics (Fig. 4A) we observed were in very good agreement with those (previously reported by Wang et al.\textsuperscript{25}). In particular, there was a noticeable discontinuity (at -50 mV) in the voltage-dependence of \( \tau_{\text{act}} \) and \( \tau_{\text{deact}} \) an effect which was markedly increased by 98 mM [K+o] (Fig. 3A; see also Fig. 5A in Wang et al.\textsuperscript{25}). Our results demonstrate that this \( \tau_{\text{act}} - \tau_{\text{deact}} \) discontinuity was effectively eliminated by KChIP2b, which also markedly reduced the discontinuity produced by 98 mM [K+o]. Wang et al.\textsuperscript{25} have proposed that this discontinuity arises from a voltage-insensitive transition between the final closed- and open-states (which have significantly different forward and backward rate constants). This in turn implies that the discontinuity produced by 98 mM [K+o] would be due to slowed deactivation. Our results on the effects of KChIP2b in 2 and 98 mM [K+o] (Fig. 4B) are also consistent with such a proposal.\textsuperscript{25}

While our results on [K+o] on Kv4.3 activation and deactivation kinetics were in agreement with previous studies, the effects of [K+o] on Kv4.3 macroscopic gating characteristics were clearly inconsistent with basic predictions of the "conventional" Shaker model.\textsuperscript{1,2,8-12} However, even more novel were our findings that these effects were highly dependent upon expression conditions (whether KChIP2b was absent or present) and much more prominent on certain Kv4.3 gating transitions than others. In particular, the most striking result was that 98 mM [K+o] and KChIP2b exerted opposing regulatory effects on Kv4.3 deactivation and recovery from both macroscopic and closed-state inactivation (CSI).

Non-Shaker-like effects of elevated [K+o] on Kv4 channel gating transitions have been noted in previous studies.\textsuperscript{1,2} although the basis for such effects on inactivation and recovery have remained unclear. Our results now provide a functional interpretation. Due to the lack of CSI in Shaker, elevated [K+o] exerts its regulatory effects on this particular Kv channel by interactions with the N- and/or P/C-type mechanisms. These interactions occur within specific regions of the K+-selective conduction pore. In contrast, by stabilizing inactivated closed-states, elevated [K+o] slows Kv4.3 recovery.\textsuperscript{17,19} In the absence of additional mutagenic data, the effects of [K+o] on Kv4.3 recovery kinetics which we have observed should therefore not be automatically assumed to be due sites located exclusively within the K+-selective conduction pore. For example, the effects we have observed may arise from direct interactions of [K+o] with domains located within S4 and/or other transmembrane domains which S4 residues interact with.\textsuperscript{19,28} Such interactions could in turn arise from the presence of protein crevices allowing extracellular K+ access to transmembrane domains conventionally assumed to be inaccessible to extracellular ions, a mechanism similar to that proposed in Shaker

Figure 10. Kinetics of recovery from CSI. CSI was developed during a during a 2 s P1 pulse to -50 mV (not illustrated). Recovery from CSI was quantified (HP = -85 mV) by fitting peak recovering waveforms generated by P2 pulses to +50 mV following variable durations back to HP = -85 mV first in 2 mM and then in 98 mM [K+o]. Representative recovering P2 current waveforms in 2 mM and 98 mM [K+o], for (A) Kv4.3 and (B) Kv4.3 + KChIP2b. Recovery time constants as indicated. Kv4.3 data empirically fit with an “a2” sigmoidal formulation, Kv4.3 + KChIP2b data fit with conventional single exponential functions. (C) Comparison of mean \( \tau_{\text{csi}, -85 \text{mV}} \) values in 2 and 98 mM [K+o]. Mean data based upon: Kv4.3, 2 mM [K+o]: n = 4, 98 mM [K+o]: n = 4; Kv4.3 + KChIP2b, 2 mM [K+o]: n = 5, 98 mM [K+o]: n = 5.

Figure 11. S4 charge deletion mutant R302A. Effects of 2 mM and 98 mM [K+o] on the kinetics of recovery (HP = -85 mV) from macroscopic inactivation developed at +50 mV. Mean data based upon: Kv4.3 R302A, n = 3; Kv4.3 R302A + KChIP2b, n = 5.
Figure 12. Preliminary Kv4.3 gating model. C₄→C₀, non-inactivated closed-states; IC₄→IC₀, inactivated closed-states; O, open-state; and IO, inactivated open-state. Forward (a) and backward (b) rate constants between specific states as indicated. In contrast to previous Kv4 channel gating models, the preliminary model proposes that i) the inactivated open-state (IO) is absorbing; and ii) IO can directly communicate with the closed-inactivated state (IC₀). Individual α and β values are not only functions of voltage, but also [K⁺], and KChIP2b expression levels. Increases in [K⁺] primarily promote stabilization of inactivated closed-states (IC₄→IC₀), thus slowing recovery from both macroscopic and CSI. Coexpression of KChIP2b promotes stabilization of non-inactivated closed-states (C₄→C₀), thus accelerating recovery from both macroscopic and CSI.

studies to account for apparent focusing of the transmembrane electrical field to a highly localized region of S₄.

Almost all Kv4 channel studies agree on the presence of CSI, although its overall importance in Kv4 channel gating transitions, and in particular macroscopic recovery, has recently been questioned. With regard to Kv4.3, elevated [K⁺]o increased both the percent magnitude and rate of development of CSI and slowed its kinetics of recovery. KChIP2b efficiently opposed or significantly attenuated all of these effects. These opposing effects were in turn consistent with general predictions based on the steady-state inactivation "i" relationships obtained for the two expression conditions. Although there was variability among oocytes in some of the kinetic parameters measured (e.g., the degree of CSI developed at -50 mV), we nonetheless consistently observed the same overall general trends: 98 mM [K⁺]o slowed Kv4.3 deactivation, promoted CSI and slowed recovery from both macroscopic and CSI, effects which were eliminated or significantly reduced by coexpression of KChIP2b. We therefore conclude that elevated [K⁺]o slows Kv4.3 recovery by promoting inactivated closed-states, while KChIP2b accelerates recovery by promoting non-inactivated closed-states. This conclusion is further supported by our confirmation of the predicted differential effects of 98 mM [K⁺]o, and KChIP2b on macroscopic recovery characteristics of the S4 positive charge deletion mutant R302A. Hence, the mechanisms governing Kv4.3 recovery appear to be quite distinct from those of Shaker.

While several mechanistic questions remain, our results verify the general validity of the Kv4.3/KChIP2b gating model (previously proposed by Patel et al.17). Since our results also demonstrate that restricting analysis to only the kinetics of development of CSI at one fixed potential fails to give definitive predictions on the kinetics of recovery from CSI, it will be very interesting to determine if KChIP1 also accelerates Kv4.1 recovery from CSI. It should also be noted that "non-conventional" KChIP2 isoforms (e.g., KChIP2e) have been reported to slow Kv4.3 recovery kinetics. We predict that these isoforms will promote Kv4 channel CSI, i.e., they will mimic the effects of 98 mM [K⁺]o that have observed. If verified, such non-conventional KChIP2 isoforms should prove to be valuable tools for selectively probing mechanisms underlying Kv4 channel CSI.

Barhring et al. previously concluded that recovery from CSI is likely the rate limiting step in Kv4.2 recovery. Both our present and previous results support this proposal. Based upon the absence of measurable reopening currents generated upon membrane hyperpolarization, Barhring et al. have also argued against the presence of a major absorbing open-state inactivation mechanism existing in Kv4.2. However, these previous measurements were conducted in 130 mM [K⁺]o, recording conditions that may have biased results due to promotion of CSI. Furthermore, there are now at least three independent demonstrations of Kv4.2/4.3 reopening currents.17,18,34

Our results thus should not be interpreted as providing evidence against the existence and/or relative importance of Kv4.3 open-state inactivation. Rather, they demonstrate the novel and opposing regulatory effects that [K⁺]o and KChIP2b have on Kv4.3 inactivated closed-states. In this regard, we still support our original hypothesis that KChIP2b exerts its overall regulatory effects by stabilizing Kv4.3 non-inactivated closed-states, an effect that in turn promotes an obligatorily coupled open-state inactivation mechanism that is dominated by CSI in the absence of KChIP2b. Whether this is the mechanism by which other KChIP family isoforms (KChIPs 1, 3 and 4) regulate Kv4 channel inactivation and recovery remains to be determined.

Based upon our present and previous results, as well as those of independent laboratories, a preliminary Kv4.3 gating model is illustrated in Figure 12. In contrast to previous Kv4 channel models, we propose that Kv4.3 open-state inactivation is at least partially absorbing, and the open-inactivated state can directly communicate with closed-inactivated states. The corresponding forward (a) and backward (b) rate constants between both the non-inactivated closed-states (C₄→C₀) and inactivated-closed states (IC₄→IC₀), as well as those between the final closed-state (C₀) and open-state (O), are not only functions of voltage but also [K⁺]o and KChIP2b expression levels, with the latter two exerting opposite effects (increased [K⁺]o promotes inactivated closed-states IC₄→IC₀, while KChIP2b promotes non-inactivated closed-states C₄→C₀). While this model can qualitatively account for our results, we wish to emphasize that a more detailed quantitative testing of it will require future development and analysis.

How can expression of intracellular KChIP2b alter the effects of extracellular [K⁺]o? While we cannot definitively answer this question, our results suggest the presence of novel allosteric coupling mechanisms between extracellularly accessible K⁺-sensitive domains and intracellular domains involved in KChIP2b-mediated regulation. One possibility would be that 98 mM [K⁺]o may be promoting release and/or destabilization of KChIP2b from its intracellular binding domains. However, with regard to Kv4.3, we consider this possibility highly unlikely for the following reasons: i) the slowing of macroscopic recovery by 98 mM [K⁺]o was significantly attenuated by KChIP2b; and ii) recovery (both macroscopic and closed-state) in 98 mM [K⁺]o was significantly faster in the presence of KChIP2b.

Assuming that elevated [K⁺]o does not destabilize Kv4.3/KChIP2b binding, then KChIP2b-mediated allosteric interactions may reduce the steady-state proportion, affinity and/or accessibility of the extracellularly accessible K⁺-sensitive sites. For example, if the intracellular
peripheral columns observed in the low resolution EM-structure of the Kv4.2/KChIP2 complex correspond to the C-termini of Kv4.3 α subunits, then the C-termini may be involved, at least partially, in the novel effects we have observed. With regard to the potential location of the [K+]o-sensitive sites, we have recently demonstrated that individual mutation of each of the first three S4 N-terminal arginines to alanine (R290A, R293A, R296A) produces significant stabilization Kv4.3 inactivated closed-states and slowing of recovery (both from macroscopic and CSI), effects which are very similar to those produced by 98 mM [K+]o. This suggests that 98 mM [K+]o may be effectively competing with sites that S4 R290, R293, and R296 normally interact with in the closed state, e.g., possibly negatively charged glutamate (E) and/or aspartate (D) residues in S2, S3 and/or the extracellular domains associated with S5. Another possibility would be cation-π interactions with various aromatic residues, of which there are several possibilities. Determination of such sites may help to establish, or at least place physical constraints upon, the closed-state configuration of Kv4.3 channels and the “local geography” of putative channel protein crevices that are accessible to extracellular K+ ions in the closed-state(s) conformation.

While the above mechanistic proposals are speculative, they provide plausible initial models for testing and ultimately understanding the structural bases underlying the significant differences in gating characteristics between Kv4.3 and Shaker channels, the latter of which do not display intracellular peripheral columns, are not regulated by KChIP2 isoforms and do not display the regulatory effects of elevated [K+]o that we have observed.

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