Kv4.3 expression and gating
S2 and S3 acidic and S4 innermost basic residues

Matthew R. Skerritt and Donald L. Campbell*

Department of Physiology and Biophysics; University at Buffalo, SUNY; Buffalo, NY USA

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Introduction

Kv4 (Shal-type) channel α subunits, in association with various regulatory β subunits, underlie generation of rapidly recovering transient (inactivating) outward potassium currents in various excitable cell types. These currents include I subfast in cardiac myocytes3-5 and specific "Ia," phenotypes in neurons.4-6 The importance of I subfast in regulating working cardiac myocyte function, including action potential repolarization and excitation-contraction coupling, is well recognized.1,2 In the nervous system, evidence exists for the importance of Kv4-mediated Ia in regulation of somal and dendritic action potentials,10 LTP-mediated memory and learning effects,8,9 and pain perception and associative activation, inactivation, and recovery in Shaker/Kv1.4,12-21 phosphorylation18,37 and changes in [K+]o do so through alteration of CSI characteristics. Mechanisms underlying Kv4 CSI may thus provide a basis for regulation of several functions mediated by the nervous and cardiovascular systems.

With regard to mechanisms underlying voltage sensing, mutagenic charge neutralization and gating current studies in Shaker have demonstrated primary involvement of the four outermost arginine residues ("R1-R4") in transmembrane segment S4 of the voltage sensing domain (VSD [segments S1-S4]).18,21,38,39 However, charge neutralization (by glutamine [Q]) of either of the two innermost S4 basic residues (lysine K374, R377) results in nonfunctional channels that fail to generate either ionic or gating currents.40-42 In addition to basic S4 residues, negatively charged acidic residues (aspartate [D], glutamate [E]) are present in Shaker S2 (E283, E293) and S3 (D316) (refer to Fig. 1). Evidence exists for (i) contribution of S2 E293 to gating current59 and (ii) combined involvement of S2 E293 and S3 D316 in forming state-dependent "electrostatic networks" with the two innermost basic residues in S4 (K374 in the closed state, R377 in the open state).42 Disruption of the latter "electrostatic network" underlies apparently "lethal" effects of S4 mutants K374Q and R377Q.41,42

*Correspondence to: Donald L. Campbell; Email: dc25@buffalo.edu
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either alanine [A] or glutamine [Q]) strongly suggest (but do not prove) S4 R residues are responsible for regulation of not only Kv4.3 activation and deactivation (as predicted by the Shaker model), but also CSI and recovery (CSI is not included in the Shaker model). We thus proposed R290 may lie closer to the septum in the closed state than its S4 “R2” counterpart in Shaker.6 The illustrated alignment of S3 is also speculative.

Figure 1. Two dimensional schematic of transmembrane domains S1-S4 of the Kv4.3 putative voltage-sensing domain (VSD). Assumed extracellular and intracellular linker domains not illustrated. Negatively charged (acidic) residues (S2:D230, E240; S3:D263) and positively charged (basic) S4 arginine (R) and lysine (K) residues are indicated in boxes (as well as S4 V287 and the putative hydrophobic septum between extracellular and intracellular VSD crevices). S1, S2 and S4 sequences have been putatively aligned according to a minimal closed-state model of Shaker.6 This model proposes isoleucine (I) residues in S1 I198, S2 I236 and S4 I241) have thus been putatively assigned to the septum. As Kv4.3 lacks the corresponding Shaker S4 “R1” residue (replaced by V287), we have previously proposed R290 may lie closer to the septum in the closed state than its S4 “R2” counterpart in Shaker.6

Overall, effects of V287R suggest absence of “R1” can only partially account for differences in gating characteristics between Shaker and Kv4.3.31 In particular, V287R still displays significant CSI (see Discussion for further details).

Our prior studies suggest S4 R residues may play more complicated roles in regulating voltage-sensitive gating transitions in Kv4.3 than their counterparts in Shaker. Furthermore, in contrast to “lethal” effects of innermost S4 R377Q in Shaker, the similar Kv4.3 mutant R302A gives rise to measurable ionic currents (although they are reduced in amplitude compared to WT Kv4.3).29 This suggests there may be subtle yet functionally important differences in the structures, registers, and/or interactions of S4 with other α subunit domains among the two Kv channel types. One possibility would be that effects of acidic residues in S2 and S3 may differ. Kv4.3 has comparable S2 and S3 acidic residues (S2 D230, E240; S3 D263; Fig. 1) to those present in Shaker (S2 E283, E293:S3 D316). Individual neutralization of each of these acidic residues in Shaker has been reported to express functional channels.39,42 However, to the best of our knowledge, no results on involvement of (i) S2 and S3 acidic residues and (ii) S4 basic K299 in regulating Kv4 expression and/or gating have been reported.

Results

Sequences of the putative transmembrane segments S1-S4 of the Kv4.3 voltage-sensing domain (VSD) are illustrated in Figure 1. As no crystal structure data presently exists for any Kv4 channel, a conventional two-dimensional schematic is presented, with S2, S3 and S4 putatively aligned as suggested by a proposed minimal closed-state model of Shaker.35

Each individual S2, S3 and S4 mutant to be described introduced two perturbations: (i) single charge deletion (electrostatic perturbation); and (ii) side chain volume perturbation (resulting in possible “structural/conformational” effects). Either one and/or both of these perturbations may have been predominant and capable of altering Kv4.3 gating properties (further details given in Materials and Methods; see also Discussion). In the absence of Kv4.3 state-dependent structural data, to provide an initial physical framework for possible structural effects we have used the data discussed and summarized by Zamyatnin on apparent molar volumes (estimated in aqueous solution) of individual amino acids. Changes in local volume produced by a given mutation were estimated by taking the difference in reported apparent molar volumes of the two residues involved without any further adjustments. Hence, individual S2 and S3 D→Q and E→Q mutations eliminated one negative charge while partially maintaining localized polar...
properties, with D→Q reducing estimated local (assumed side chain) volume by -20.1 cm$^3$/mole and E→Q by -8 cm$^3$/mole. Corresponding S4 K299A, K299Q and R302Q mutants each eliminated one positive charge while maintaining localized polar properties, with K299A reducing side chain volume by -66.9 cm$^3$/mole, K299Q by -14.6 cm$^3$/mole, and R302Q by -33.4 cm$^3$/mole.

(I) S2 D230Q. (a) Activation and deactivation. D230Q gave rise to large currents in response to depolarizing voltage clamp steps (Fig. 2A, inset). However, compared to WT K,4.3 (which displays an “activation threshold” of -40 mV$^{24,28}$), the D230Q peak current-voltage (I-V) relationship was significantly depolarized (Fig. 2A, main panel), with an “activation threshold” of +10 mV. This suggested D230Q produced significant stabilization of K,4.3 closed states.

To quantify this effect, the steady-state activation “a” relationship was determined (saturating tail-current protocol; mean data points [n = 11] fit as a single Boltzmann relationship raised to the fourth power: $1/(1 + \exp[-(V - V_{1/2})/k])^4$). Compared to WT K,4.3, D230Q significantly depolarized “a” (mean shift of $D\Delta V_{1/2} = +52.7$ mV), an effect which corresponded to a relative change in the voltage independent free energy of activation (per subunit) of $D\Delta G_{vi} = 3.67$ RT. However, in contrast to its large depolarizing effect on $V_{1/2}$, D230Q produced only minimal effect
exponential function with a slightly decreased voltage-sensitivity and associated decrease in kinetic estimate of \( q_{\text{act}} \) (D230Q, 1.47 e\(_o\); WT, 1.56 e\(_o\)). At the most depolarized potentials, D230Q \( \tau_{\text{act}} \) values were very similar to those of WT K\(_{V4.3}\). Due to the depolarized shift in \( \alpha_4 \) (Fig. 2B), D230Q deactivation kinetics (single exponential fits\(^{24,28-31,47}\)) could be analyzed over the entire hyperpolarized range of potentials (0 to -120 mV). Compared to WT (measured at -40 mV and more hyperpolarized), D230Q significantly accelerated deactivation. Due to our previous studies\(^{24,28-31}\), activation kinetics of the early rising phases of D230Q currents were directly fit to a Hodgkin-Huxley-like independent sigmoidal \( \alpha \) exponential relationship (Fig. 2C, inset). The D230Q \( \tau_{\text{act}} - V_{m} \) curve could be described as a single exponential function with a slightly decreased voltage-sensitivity and associated decrease in kinetic estimate of \( q_{\text{act}} \) (D230Q, 1.47 e\(_o\); WT, 1.56 e\(_o\)).

Table 1. Summarized mean effects of K\(_{V4.3}\) VSD charge mutants on steady-state \( \alpha \) and isochronal (1 sec) \( \gamma \) relationship\(^{29-31}\)

| V\(_{1/2}\) (mV) | k (mV) | q (e\(_o\)) | \( G_{\text{vi}} \) (RT) | \( G_{\text{vi}} \) (RT) | Predominant effect(s) |
|----------------|--------|------------|----------------|----------------|-----------------------|
| **(A) \( \alpha \)** | | | | | |
| WT | -36 | 14.5 | 1.75 | 2.48 | - |
| R290A | -69 | 19.1 | 1.33 | 3.61 | -1.13 | E + S |
| R290Q | -78 | 19.2 | 1.32 | 4.06 | -1.58 | E + S |
| R291A | -36 | 17.8 | 1.43 | 2.02 | 0.46 | E |
| R293Q | -40 | 15.8 | 1.61 | 2.53 | -0.05 | E |
| R296A | -7 | 14.7 | 1.73 | 0.48 | 2.00 | S |
| R296Q | -5 | 16.3 | 1.56 | 0.31 | 2.17 | E + S |
| K299A | Expressed, ND | | | | S* |
| K299Q | Expressed, ND | | | | S* |
| R302A | Expressed, ND | | | | S* |
| R302Q | Expressed, ND | | | | S* |
| D230Q | +16.7 | 14.0 | 1.82 | -1.19 | 3.67 | S |
| E240Q | Not expressed | | | | |
| D263Q | Not expressed | | | | |
| V287R | +1.4 | 11.6 | 2.19 | -0.12 | 2.60 | E + S |
| **(B) \( \gamma \)** | | | | | |
| WT | -60.1 | 6.2 | 4.10 | 9.69 | - |
| R290A | -106.7 | 13.1 | 1.94 | 8.15 | 1.54 | E + S |
| R290Q | -118.7 | 8.4 | 3.03 | 14.13 | -4.44 | E + S |
| R293A | -80.7 | 11.7 | 2.17 | 6.90 | 2.79 | E + S |
| R293Q | -76.6 | 7.4 | 3.44 | 10.33 | -0.66 | E + S |
| R296A | -86.9 | 14.5 | 1.75 | 5.99 | 3.70 | E + S |
| R296Q | -61.5 | 7.7 | 3.30 | 7.99 | 1.70 | E |
| K299A | -61.8 | 5.3 | 4.80 | 11.66 | -1.97 | E |
| K299Q | -38.6 | 7.9 | 3.23 | 4.90 | 4.79 | E + S |
| R302A | -38.7 | 7.5 | 3.40 | 5.16 | 4.53 | E + S |
| R302Q | -34.1 | 7.9 | 3.71 | 4.70 | 4.99 | E + S |
| D230Q | -0.2 | 7.3 | 3.48 | 0.03 | 9.66 | E + S |
| E240Q | Not expressed | | | | |
| D263Q | Not expressed | | | | |
| V287R | -51.5 | 7.3 | 3.50 | 7.05 | 2.64 | E + S |

Mean values as follows: \( V_{1/2} \) (half-maximal potential) and k (slope factor) values from fits to \( \alpha \) (fourth order Boltzmann relationships) and \( \gamma \) (single Boltzmann relationships); q, minimal effective gating charges for either activation or CSI (units of e\(_o\) [1.60 x 10\(^{-19}\) C] calculated assuming \( h = 1.0 \); \( \Delta G_{\alpha} = \Delta G_{\text{vi}} \) \( W_{\alpha} + \Delta G_{\text{vi,mean}} \) relative change in voltage independent free energy, units of RT [=0.59 kcal at 22°C]). Estimated values derived from the following equations (see Material and Methods): (i) q = (RT/k); and (ii) \( G_{\text{vi}} = -(V_{1/2}/k) RT \) (tabular values were calculated from the single, aggregate best fits to the associated \( \alpha \) and \( \gamma \) curves [smooth curves as summarized in Fig. 7], and thus have no associated ±SEM values). For initial purposes of categorization, we functionally defined a “predominant effect” by the following criteria: (i) electrostatic (“E”), a greater than ±5% change in associated mean q value compared to mean WT q value; and (ii) structural (“S”), a greater than ±5 mV shift in mean value of \( V_{1/2} \) compared to mean WT \( V_{1/2} \) value. Note that this categorization is not an absolute dichotomy (see text for further details). In column “Predominant effect(s)” an * indicates only partially determined effects.
Nonetheless, the voltage-sensitivity of the corresponding $\tau_{\text{deact}}$ - $V_m$ curves were similar (Fig. 2D), giving similar kinetic estimates of $q_{\text{deact}}$ (D230Q, 1.08 e; WT, 1.04 e$^{30}$).

In summary, effects of D230Q on K$_{+}$,4.3 activation and deactivation characteristics were consistent with stabilization of closed states. While there were minor alterations that could be attributed to electrostatic perturbations, effects of D230Q could primarily be attributed to structural perturbations$^{29-31}$ (further details in Table 1 caption, Discussion, and Materials and Methods).

(b) Development of macroscopic and closed state inactivation (CSI). Similar to its general effects on “a” (Fig. 2B), D230Q significantly depolarized the mean isochronal (1 s) inactivation “i” relationship by $\Delta V_{1/2}$ = +59.9 mV ($n = 14$) (Fig. 3A). This large depolarizing shift corresponded to an estimated relative change in voltage-independent free energy of $\Delta G$$_{\text{i,rel}}$ = 9.93 RT. D230Q also slightly reduced “i” voltage-sensitivity (D230Q, k = 7.32 mV; WT, k = 6.2 mV$^{30}$), corresponding to a small reduction (~15%) in estimated minimal closed state inactivation (CSI) charge (D230Q, $q_{\text{CSI}}$ = 3.47 e; WT, $q_{\text{CSI}}$ = 4.10 e$^{30}$). Thus, although both “a” and “i” were depolarized by D230Q, there was still very little overlap between the two relationships (Fig. 3A, inset), indicating existence of prominent CSI. D230Q therefore significantly stabilized non-inactivated closed states.

Macroscopic inactivation kinetics (+20 to +50 mV) could be well fit as a double exponential process (Fig. 3B) (at +15 mV only a single exponential component could be reliably obtained). The $\tau_{\text{fast}}$ - $V_m$ curve (Fig. 3C) could be well described as a single exponential function whose voltage-dependency and estimated $q_{\text{inact,fast}}$ was very similar (~3% difference) to that of WT K$_{+}$,4.3 (D230Q, $q_{\text{inact,fast}}$ = 2.43 e; WT, $q_{\text{inact,fast}}$ = 2.36 e). The mean relative amplitude of the fast component of inactivation at +50 mV was $A_{\text{fast}}$ = 0.86 ± 0.01, a value comparable (but somewhat
In contrast, D230Q failed (n = 9) to show any measurable variability compared to the \( \tau \) values that were similar to those of WT K\(_V\)4.3 at more depolarized potentials. This was found to be the case, with significant CSI developing exponentially with a mean time constant of \( \tau_{\text{CSI,+10 mV}} = 536 \pm 81 \text{ ms} \) (n = 4) (Fig. 3D, main panel).

(c) Recovery from macroscopic and closed state inactivation (CSI). The kinetics of recovery (HP = -100 mV) from both macroscopic inactivation (developed at +50 mV) and CSI were significantly altered by D230Q. Compared to WT (which displayed a mean \( \tau_{\text{rec,macro}} = 206 \text{ ms} \)), D230Q greatly accelerated macroscopic recovery (single exponential fits) by over an order of magnitude (\( \tau_{\text{rec,macro}} = 14.0 \pm 0.6 \text{ ms}; n = 10 \)) (Fig. 4A). With regard to CSI, after a 2 sec pulse to -50 mV WT K\(_V\)4.3 displayed sigmoidal kinetics of recovery from CSI (empirically fit as an “a\(^2\)” formulation [HP = -100 mV] with a mean \( \tau_{\text{rec,CSI}} = 171 \text{ ms} \)). For comparative D230Q measurements, recovery from CSI developed during a 2 second pulse to either 0 or +10 mV was determined. At these potentials there was significant development of CSI but no or minimal open state activity (Fig. 3B). Compared to WT, D230Q recovery from CSI (HP = -100 mV) was greatly accelerated and well-described as a single exponential process with a mean time constant of \( \tau_{\text{rec,CSI}} = 23.0 \pm 3.2 \text{ ms} \) (n = 9). These significant acceleratory effects on both macroscopic and CSI recovery (summarized in Fig. 4B) could not be attributed to a change in the value of “i” (at HP = -100 mV, “i” = 1.0 for both conditions; Fig. 3A). In this regard, while it is theoretically possible \( \tau_{\text{rec}} \) could increase with further hyperpolarization even though the value of “i” is virtually constant at 1.0, our previous studies\(^{24,28}\) indicate K\(_V\)4.3 \( \tau_{\text{rec}} \) displays signs of saturation once the value of “i” = 1.0 has been reached, i.e., \( \tau_{\text{rec}} \) displays no to minimal changes with further hyperpolarization. Nonetheless, more comprehensive measurements of the overall voltage-sensitivity of recovery of D230Q compared to WT K\(_V\)4.3 (i.e., the corresponding \( \tau_{\text{rec}} - V_m \) curves measured over a sufficiently wide hyperpolarized potential range\(^{29}\)) should give more definitive support to this conclusion. Such comprehensive \( \tau_{\text{rec}} - V_m \) measurements were not conducted in the present study.

(II) S2 E240Q and S3 D263Q. In contrast to D230Q, both individual mutants E240Q (S2) and D263Q (S3) (Fig. 1) failed to give rise to measurable ionic currents during depolarizing pulses up to +50 mV. This lack of macroscopic current was observed at both control HP = -100 mV and with 1 second hyperpolarized prepulses applied “down” to -150 mV.\(^{29,30}\) Deletion of negative charge and/or introduction of structural perturbations at either of these more intracellular S2 and S3 positions thus prevented functional expression of K\(_V\)4.3 ionic currents. These observations are different from those observed in Shaker, where comparable mutants S2 E293Q and S3 D316 express functional channels.\(^{42}\)

In Shaker, Papazian et al.\(^{42}\) have demonstrated the “lethal” S4 single mutant K374Q can be “rescued” by second site mutations S2 E293Q and S3 D316N, i.e., neutralization of both positive and negative charges in the putative inner VSD “electrostatic network” is capable of overcoming defects produced by deletion of only one innermost S4 positive charge. Following the general rationale of this Shaker “double charge deletion” strategy,\(^{42}\) to determine if we could rescue (at least partially) E240Q and/or D263Q negative charge deletion mutants, we attempted to analyze effects of the four comparable K\(_V\)4.3 double charge deletion mutants E240Q-K299Q, E240Q-R302Q, D263Q-
K299Q and D263Q-R302Q. However, similar to the single site mutants E240Q and D263Q, none of these double charge deletion mutants generated measurable K,V,4.3 macroscopic currents (again, using HP = -100 mV and prepulses applied “down” to -150 mV29,30). Thus, “rescuing” effects of innermost VSD double charge deletion mutants displayed by Shaker42 appear not to be present in K,V,4.3.

(III) S4 K299 and R302. In Shaker, S4 innermost positive charge deletion mutants K374Q and R377Q fail to express functional channels.40-42 However, in K,V,4.3 we have previously demonstrated the innermost S4 charge deletion mutant R302A gives rise to reduced yet measurable currents which display both depolarized activation properties (as judged by the peak I-V relationship) and significantly accelerated recovery kinetics.29 To expand upon these prior R302A results,29 as well as to determine effects of neutralizing K,V,4.3 K299 (Fig. 1), we determined effects of the S4 individual mutants R302Q, K299A and K299Q.

R302Q, K299A and K299Q each gave rise to measurable ionic currents at depolarized potentials (Fig. 5A). Compared to WT K,V,4.3, which displays an apparent macroscopic “activation threshold” of ~-40 mV,24,28 all three mutants began to generate measurable currents at more depolarized potentials (K299A at ~-20 to -10 mV, K299Q and R302Q at ~-10 to 0 mV). However, the mean peak currents at +50 mV were all smaller (~10X) than those typical for WT K,V,4.3 and the outermost S4 R mutants29,30 (K299A, Ipeak,+50 mV = 330 ± 65 nA [n = 17]; K299Q, Ipeak,+50 mV = 241 ± 40 nA [n = 13]; R302Q, Ipeak,+50 mV = 279 ± 53 nA [n = 13]).

The depolarized “activation threshold” potentials of R302Q, K299A, and K299Q suggested all three mutants stabilized K,V,4.3 closed states. Unfortunately, reduced current amplitudes of all three mutants prevented accurate determination of their associated “a” activation relationships by the saturating tail current protocol.29-31 It similarly proved infeasible to measure accurately voltage-dependence of deactivation kinetics. Nonetheless, we were able to obtain limited quantitative measurements on some functional CSI and recovery characteristics.

R302Q produced a mean (n = 6) depolarizing shift in “i” (DV,1/2 = +26 mV) and a decrease in slope factor (k = 7.93 mV) (Fig. 5B). These effects corresponded to ΔΔG,*/c = 4.99 RT and a reduction (~22%) in d∞a (K299Q, 3.21; c; WT, 4.10 e,30). Overall, these
effects were comparable to those of R302A\textsuperscript{29} (Table 1, Fig. 7, and Discussion). Similarly, K299Q also depolarized “i” (mean [n = 5] $\Delta V_{1/2} = +21.5$ mV) and decreased slope factor [k = 7.88 mV; corresponding to $\Delta G_{i}^{C} = 4.79$ RT and $-21\%$ reduction in $q_{\text{CSI}}$]. However, in contrast to K299Q, K299A produced essentially no effects on $V_{1/2}$ of “i”, although it did increase mean slope factor (k = 5.3 [n = 8]), corresponding to an $-17\%$ increase in $q_{\text{CSI}}$ (see Table 1). Consistent with the mean “i” relationships, at -50 mV K299A displayed significant development of CSI (single exponential) with a mean (n = 7) time constant of $\tau_{\text{CSI,50 mV}} = 230.1 \pm 14.1$ ms (and associated value of relative inactivation of “i” = 0.153 $\pm$ 0.015) (Fig. 5C, upper inset), while both K299Q (Fig. 5C, lower inset) and R302Q each displayed no to minimal development of CSI at -50 mV.

Figure 6. (A and B) Macroscopic recovery kinetics (HP = -100 mV) for (A) R302Q (solid squares, n = 8) and R302A (smooth curve with no data points [data from 29]) and (B) K299A (hollow squares, n = 9) and K299Q (solid squares, n = 8). In both panels mean WT recovery illustrated as smooth curve with no data points (data from 29). Similar to R302A,\textsuperscript{29} all three mutants significantly accelerated macroscopic recovery and produced a pronounced overshoot in the amplitude of the recovering P2 current (measurements conducted up to 70 ms). Mean data points fit as single exponential functions with the following parameters: K299A, $\tau_{\text{rec}} = 72.5$ ms, overshoot 70 ms = 1.60; K299Q, $\tau_{\text{rec}} = 37.8$ ms, overshoot 70 ms = 1.98; and R302Q, $\tau_{\text{rec}} = 19.9$ ms, overshoot 70 ms = 1.49. Mean times for the relative P2 current to fully recover ($P2/P1 = 1.0$) and the associated extrapolated effective recovery time constant ($t_{0.632}$) were as follows: K299A, $t_{1.0} = 30$ ms, $t_{0.632} = 15$ ms; K299Q, $t_{1.0} = 26$ ms, $t_{0.632} = 14$ ms, and R302Q, $t_{1.0} = 24$ ms, $t_{0.632} = 13$ ms. Insets: Representative recordings of P2 overshoot at 70 ms of (A) R302Q (calibration bars: 150 nA, 300 ms) and (B) K299Q (calibration bars: 100 nA, 300 ms). Dashed lines indicate the relative value of the control P1 current. (C) K299A, representative recordings of recovery (HP = -100 mV) from CSI developed at -50 mV (protocol as per 29,30). Peak currents fit with a single exponential function with $\tau_{\text{rec,CSI}} = 58$ ms. Unlike recovery from macroscopic inactivation developed at +50 mV (A and B), recovery from K299A CSI showed no obvious overshoot in the recovering P2 current amplitude (dashed line corresponds to the relative value of the control P1 current [not illustrated]). Calibration bars: 50 nA, 50 ms. (D) Comparison of mean $\tau_{\text{rec,CSI}}$ values of K299A (n = 6) and WT (data from 29). Note the logarithmic time scale.

Very similar to our previous observations on R302A,\textsuperscript{29} R302Q produced a marked acceleration in macroscopic recovery kinetics (HP = -100 mV) and a pronounced overshoot in the amplitude of the recovering P2 current (Fig. 6A). Up to 70 ms (the maximal recovery interval analyzed in these series of measurements) the mean (n = 8) recovering P2 current waveform could be fit as a single exponential function with $\tau_{\text{rec}} = 19.9$ ms and a relative overshoot value (at 70 ms) = 1.49. The mean time for the P2 current to fully recover (i.e., $P2/P1 = 1.0$) was $t_{1.0} = 24$ ms, from which an effective time constant for full recovery ($t_{0.632}$) could be extrapolated. Similar to R302Q, both K299A and K299Q (Fig. 6B) each also significantly accelerated macroscopic recovery and produced a pronounced overshoot in P2 current amplitude at 70 ms (K299A [n = 9]: $\tau_{\text{rec}} = 72.5$ ms, overshoot 70 ms = 1.60 [t_{1.0} = 30 ms, t_{0.632} = 15 ms]; K299Q [n = 8]; $\tau_{\text{rec}} = 37.8$ ms, overshoot 70 ms = 1.98 [t_{1.0} = 26 ms, t_{0.632} = 14 ms]).

Since, similar to WT K$_{V_{4.3}}$, K299A displayed significant development of CSI at -50 mV, we also determined the kinetics
KV4.3 S2 and S3 acidic residues. Within the framework of the 2 state gating model employed (see Methods and Materials), effects of S2 D230Q on activation and deactivation could be attributed primarily to structural perturbations which resulted in stabilization of closed states and marked acceleration of deactivation. We conclude S2 D230 is not significantly involved in conferring voltage-sensitivity to KV4.3, a conclusion in good general agreement with the prior finding that the corresponding S2 residue in Shaker (E283) does not contribute to single subunit gating charge.

D230Q also stabilized KV4.3 non-inactivated closed states and significantly accelerated macroscopic recovery. In turn, parallel effects of D230Q on deactivation and recovery were consistent with our prior proposal that KV4.3 recovery is coupled to deactivation. Thus, while lack of electrostatic perturbation of D230Q on activation and deactivation is similar to the corresponding Shaker mutant S2 E283, the acceleratory effects D230Q on CSI and recovery are clearly “non-Shaker-like.”

It should also be noted that Shaker S2 possesses a corresponding R residue (R297), to the best of our knowledge this residue appears not to be primarily involved in regulating Shaker voltage sensitivity. Considering significant differences in gating characteristics between Shaker and KV4.3 channels and in particular differences in effects of neutralizing innermost S4 R residues and S2/S3 acidic residues, at present we cannot rule out a role for S2 R244 in regulating KV4.3 gating and/or expression.

Discussion

In combination with our prior studies of S4 charge deletion mutants R290A/Q, R293A/Q and R296A/Q, the results of the present study represent the first complete survey of potential roles of individual basic (S4) and acidic (S2, S3) residues in the KV4.3 putative voltage-sensing domain (VSD). A caveat nonetheless is in order: S2 also possesses an innermost arginine (R244; Fig. 1), potential effects of which we have yet to determine. While Shaker S2 possesses a corresponding R residue (R297), to the best of our knowledge this residue appears not to be primarily involved in regulating Shaker voltage sensitivity. Considering significant differences in gating characteristics between Shaker and KV4.3 channels and in particular differences in effects of neutralizing innermost S4 R residues and S2/S3 acidic residues, at present we cannot rule out a role for S2 R244 in regulating KV4.3 gating and/or expression.
mutants significantly depolarized “a4” (Table 1 and Fig. 7), effects attributable to structural perturbations. Both mutants also effectively increased net positive charge in the putative outer region of the VSD; nonetheless, V287R exerted significant electrostatic perturbations on “a4” (corresponding to an ~25% increase in estimated minimal \( q_{act} \)), while D230Q produced virtually no electrostatic perturbation. These comparative effects again argue S2 D230 is not primarily involved in bestowing voltage sensitivity to K4.3. Both mutants also stabilized non-inactivated closed states; however, depolarizing shifts in “a” and “i” were not parallel among the mutants, as reflected in differences (ΔV) between mean \( V_{1/2} \) values of “a” and “i” (D230Q, ΔV = +52.9 mV; V287R, ΔV = +16.9 mV) [Table 1, Fig. 7, and further discussion to follow]. Finally, D230Q and V287R each accelerated macroscopic recovery, effects which again correlated with accelerated deactivation kinetics.3,28,31

In contrast to D230Q, neither S2 E240Q nor S3 D263Q gave rise to measurable ionic currents. These effects are different from those reported for corresponding mutants in Shaker (S2 E293Q and S3 D316N, both of which express channels generating macroscopic currents44). Also, in contrast to Shaker,42 we were unable to “rescue” these effects using various combinations of innermost VSD double charge deletion mutants. Involvement of acidic residues E240 and D263 in K4.3 gating therefore presently remains uncharacterized. The combined use of membrane protein expression assays and gating current analysis may yield further insights into this question.

**K4.3 S4 innermost basic residues.** In Shaker, while neutralization of the outermost S4 R residues (“R4”) gives rise to measurable ionic currents, neutralization (by Q) of either innermost K374 or R377 fails to result in expression of functional channels (neither ionic nor gating currents are observed41,42). This “lethality” is believed to result from disruption of structurally important “electrostatic networks” with acidic residues S2 E293 and S3 D316.42 In contrast, individual neutralization (by either Q or) of the comparable K4.3 S4 residues (K299, R302) produces functional channels, although their peak ionic current amplitudes are reduced compared to WT K4.3. This reduced current amplitude may be due, at least in part, to the apparent depolarizing shift in voltage-dependence of activation (as presently assessed by shifts in peak I-V relationships and apparent “activation thresholds”; Figs. 5A and 7F). However, our present results do not allow us to exclude the additional possibility that K299A/Q and/or R302A/Q mutants may also be reducing efficacy of “trafficking” or expression of subunit proteins.

Regardless of underlying mechanism(s), our K299A/Q and R302A/Q results are different from those reported for comparable S4 mutants in Shaker.41,42,43 In combination, these two sets of independent results on VSD acidic and basic residues appear to be functionally opposite (Shaker: individual S2 E299Q and S3 D316N mutants are functional,42 while S4 innermost K374Q and R377Q are “lethal”,40-42 K4.3: individual S2 E240Q and S3 D263Q are apparently “lethal” [at least with regard to macroscopic current generation], while S4 K299A/Q and R302A/Q are functional). These comparative results suggest functionally important differences in structures, registers, and/or interactions of S4 with S2, S3 and/or other α subunit domains among the two Kα channel types. Since our results indicate deletion of just one S2 (E240) or S3 (D263) acidic residue leads to non-functional K4.3 channels (effects which are apparently not “rescued” by innermost VSD inter-subunit double charge deletion mutants42), while K299A/Q and R302A/Q generate reduced but functional channels, it is reasonable to propose that interactive effects of the S4-S2/S3 “electrostatic networks” existing in Shaker45-49 are not directly applicable to K4.3.

The most striking effects of K299A/Q and R302A/Q were significant acceleration of macroscopic recovery kinetics and production of a marked overshoot in recovering P2 current amplitude (Fig. 6A and B). WT K4.3,24,28,29 outermost S4 R residue mutants (R290A/Q, R293A/Q, R296A/Q),29,30 V287R31 and D230Q (Fig. 4) all displayed altered recovery kinetics with no pronounced P2 overshoot. Hence, a recovering P2 current overshoot appears to be specifically associated with charge deletion mutants at S4 positions K299 and R302. While our present K299A/Q and R302A/Q measurements did not address the kinetics of “return” to the “baseline” value of the P2 current, the fact that the P1 current was virtually constant during application of the double pulse recovery protocol (applied at a frequency of 0.125 Hz) indicates return to “baseline” P1 amplitude (at HP = -100 mV) was complete within 8 seconds. Nonetheless, the basis of the P2 overshoot is presently unclear.

Potentially important roles for membrane lipids in modulating ion channel function have recently become recognized. With regard to Kα channels, molecular dynamic modeling studies50 (conducted up to 50 ns) of the isolated VSD of Kα AP (modeled in either zwitterionic or anionic lipid bilayers) have suggested significant interactions between S4 R residues and lipid phosphate groups. This modeling study50 further suggests these interactions lead to significant local deformation of the bilayer, resulting in production of a focused transmembrane electrical field, a prediction in good general agreement with previous biophysical and electrophysiological studies (reviewed in refs. 19-21). Experimental studies of isolated Kα AP channels incorporated into lipid bilayers of various compositions51 have provided further evidence in very good support of the general predictions of the molecular dynamic studies.50 These experimental bilayer studies51 indicate negatively charged lipid phosphodiester groups interact with and help to energetically stabilize positively charged S4 R residues, thus allowing the VSD to properly function. With regard to K4.3 channels, the roles of phospholipids in channel modulation are presently unknown, and thus can only be speculated upon. Altered accessibility of phospholipids to individual charged residues in the VSD may thus be an important factor contributing to differences between Shaker and K4.3 in effects of neutralizing S2, S3 and S4 innermost charged residues. It will be very interesting once results on effects of phospholipids on K4.3 channel function (and associated VSD charge mutants) become available.

**Implications for functional roles of K4.3 VSD basic and acidic residues.** Due to obligatory coupling of N-type inactivation to channel opening,13-17,40 Shaker S4 mutants which alter voltage-dependence of activation similarly alter voltage-
dependence of inactivation. However, as summarized in Table 1 and Figure 7, in Kv4.3 while R290A and R290Q produced hyperpolarizing shifts in both “a” and “i” (effects consistent with at least partial coupling of CSI to activation) (Fig. 7A), effects of R293 and R296 mutant pairs were unique: R293A and R293Q produced no to minimal shift in “a” yet hyperpolarized “i” (Fig. 7B), R296A depolarized “a” yet hyperpolarized “i” (Fig. 7C), and R296Q depolarized “a” yet did not significantly shift “i” (Fig. 7C). While S4 V287R (Fig. 7D) and S2 D230Q (Fig. 7E) both depolarized “a” and “i”, V287R produced a greater shift between “a” and “i” than D230Q. Finally, while we could not accurately construct “a” curves for K299A/Q and R302A/Q, all of these innermost S4 mutants depolarized the potential for macroscopic current activation, and, with the notable exception of K299A, depolarized “i” (Fig. 7F). These non-paralleling effects argue for Kv4.3 inactivation either possessing inherent voltage-sensitivity and/or a significantly different mechanism of coupling inactivation to activation from that existing in Shaker.13-17

It is tempting to interpret a change in effective gating charge produced by a single charge mutant as a simple additive effect due to reduced charge moving across the entire transmembrane electrical field. However, previous gating current studies in Shaker have clearly indicated individual VSD charge mutants can produce non-additive effects.18-19,38,39 For example, both Aggarwal and MacKinnon39 and Seoh et al.39 have reported individual VSD mutants that alter Shaker single alpha subunit gating charge by up to as much as 7 echarge (alteration of 4 echarge would be the additive prediction). These seemingly paradoxical non-additive effects are proposed18,19,38,39 to be due to charge neutralization mutants producing not only a direct electrostatic perturbation but also secondary indirect alterations in either charge distribution and/or the electrical field existing across neighboring charges, as well as additional unknown physical effects. We have lumped these complicated indirect perturbations into the general category “structural” (using as initial indices measured ΔV1/2 values and associated derived ΔΔG◦v values [Table 1], and referencing estimated changes in residue molar volumes46 as a plausible physical basis). Such complicated non-additive effects are almost certainly present in our measurements and analysis summarized in Table 1, e.g., they may account for the seemingly paradoxical effect charge deletion mutant K299A increased apparent effective qCSI. Hence, while we feel our initial categorization of predominant effects of Kv4.3 VSD charge mutants into “electrostatic” and “structural” is both empirically and conceptually useful, it is important to recognize this categorization is not an absolute dichotomy.

While Kv4.3 gating currents have yet to be measured, and acknowledging the above interpretive caveat, our combined macroscopic current results (Table 1 and Fig. 7) are consistent with (but do not prove) S4 outmost residues R290, R293 and R296 (equivalent to Shaker “R2–R4”) are primarily involved in bestowing voltage-sensitivity to Kv4.3 activation and deactivation. Each of these corresponding S4 R⇒A/Q mutant pairs29,30 reduced estimated minimal effective qCSI, following a general pattern of maximal reduction at R290 (R290A/Q) to minimal reduction at R296 (R296A). These results suggest R290 and R293 may traverse a significant fraction of the transmembrane electrical field during activation gating. In particular, R290 appears to be a prominent residue in regulating voltage sensitivity of Kv4.3 activation. Overall, these effects are consistent with the general predictions of the Shaker S4 R activation model.18-21

S4 outermost R mutants also altered characteristics of Kv4.3 CSI and recovery, effects outside of the predictive realm of the Shaker gating model.13-17 In particular, with the exception of R296Q,40 each of the Kv4.3 S4 outermost R mutants significantly stabilized inactivated closed states. While there were quantitative differences between individual mutant pairs, all pairs reduced apparent effective qCSI, effects consistent with S4 imparting voltage sensitivity to CSI. These results argue that in the WT Kv4.3 channel at normal resting membrane potentials S4 R290, R293 and R296 are also importantly involved in stabilizing non-inactivated closed states. However, the apparent correlation between relative S4 position and mutant effects on qCSI was less clear than that obtained for qact. We suggest two possibilities: (i) While development of CSI is reasonably described as a single exponential process, for WT Kv4.3 we have consistently observed recovery from CSI is sigmoidal. Such sigmoidicity suggests the presence of multiple inactivated closed states. Since we hypothesize CSI results from S4-mediated voltage sensitive transitions occurring prior to final channel opening, individual S4 outermost mutant R mutants may produce distinct and/or prominent effects on different multiple CSI gating transitions, e.g., R290 and R296 may be involved in regulating different sequential steps in the CSI gating process. These possible effects could be overlooked or obscured by our present two state gating analysis. Due to such limitations, and present lack of relevant structural data on Kv4 channels (either crystallographic or state-dependent inter-subunit interactions of individual residues), we choose to refrain from further speculation on potential structural correlates underlying these effects; and (ii) For consistency and ease of comparison, all of our qCSI estimates are based upon isochronal (1 second) “i” measurements (a protocol which arose from experimental necessities originating in our initial Kv4.3 S4 mutagenic study29). Hence, all qCSI estimates are potentially dependent upon this 1 second isochronal condition (reviewed in ref. 52), a consideration which should be kept in mind when evaluating the data summarized in Table 1. Future studies on effects of different durations of P1 pulses on the “i” curve should be able to address this issue, at least for some of the Kv4.3 S4 mutants analyzed. Nonetheless, even if such studies reveal increases in slope factor and/or more hyperpolarized shifts in “i”, the corresponding parameters for “a” should not change. The general implications of non-parallel (i.e., “non-Shaker-like”) trends between shifts in “a” and “i” produced by individual Kv4.3 VSD mutants summarized in Figure 7 will thus still be valid, and some non-paralleling shifts in “a” and “i” produced by specific Kv4.3 VSD charge mutants may be even greater than indicated.

In contrast to corresponding S4 mutants in Shaker,41,42 K299A/Q and R302A/Q gave rise to reduced yet functional Kv4.3 channels. While our present data did not allow us to estimate electrostatic perturbations produced by K299A/Q and R302A/Q, both sets of mutants (i) depolarized the potential at
which macroscopic currents could be first detected (an effect consistent with stabilization of K<sub>4,3</sub> closed states produced by introduced structural perturbations<sup>29,31</sup>), and (ii) significantly accelerated macroscopic recovery kinetics. While many questions remain regarding effects of these two innermost S4 basic mutants, our present results argue that K299 and R302 may be important residues involved in regulation of K<sub>4,3</sub> recovery via “reverse” (deactivating) S4 transitions<sup>3,24,28-31</sup>.

Finally, variation in K<sub>4,3</sub> VSD residue side chain properties may selectively alter interactions within unique aqueous, lipid and/or protein microenvironments encountered during S4-mediated “forward” versus “reverse” gating transitions<sup>31,29-31</sup>. These S4 mutagenic effects<sup>29-31</sup> are predominant on “reverse” K<sub>4,3</sub> gating transitions (deactivation, recovery). Our results on differential effects of K299A/Q on the inactivation relationship “i” (Figs. 5 and 7) versus similar effects on macroscopic recovery (Fig. 6) further support these proposals<sup>29-31</sup>. Our results raise the possibility VSD targeted drugs and/or molecular stratagems may act as a substrate for therapeutic agents aimed at alleviating cellular hyperexcitability associated with pathologies such as cardiac arrhythmia and epilepsy<sup>53-55</sup>.

**Materials and Methods**

Mutagenesis, in vitro transcription and oocyte preparation. K<sub>4,3</sub> (long form, GenBank AF454388) was originally cloned from ferret heart<sup>34</sup> and maintained in pBluescript KS(+) vector. S2, S3 and S4 mutants (single site mutants: S2 D230Q, E240Q; S3 D263Q; S4 K299A/Q, R302Q; double site mutants: E240Q-K299Q, E240Q-R302Q, D263Q-K299Q, D263Q-R302Q) were constructed using the Quick Change II Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA). Mutant specificity was confirmed by sequencing (DNA Sequencing Core Facility, Roswell Park Cancer Institute, Buffalo, NY). K<sub>4,3</sub> WT and mutant clone plasmids were linearized with the restriction endonuclease Xhol (New England Biolabs, Ipswich, MA). cRNA was synthesized by the mMessage mMachine T7 Ultra Kit (Ambion, Austin, TX), and evaluated by spectroscopy and agarose gel electrophoresis.

All animal protocols were conducted in accordance with NIH-approved guidelines of the Institutional Animal Care and Use Committee, University at Buffalo, SUNY. Oocytes were obtained from mature female *Xenopus laevis* (euthanized by soaking in a lethal concentration of 6.0 g/L ethyl-3-aminobenzoate methanesulfonate salt), enzymatically defolliculated (collagenase), injected (12–24 hrs after isolation) with 4–9 ng cRNA (Nanoject II; Drummond Scientific, Broomall, PA), and subsequently incubated (18°C) for 2–4 days<sup>29-31</sup>.

**Electrophysiology.** Two-microelectrode voltage clamp recordings (GeneClamp 500B, Axon Instruments, Union City, CA) were conducted (22°C) in control ND96 solution (in mM: 96 NaCl, 2 KCl, 1 MgCl<sub>2</sub>, 1.8 CaCl<sub>2</sub>, 5 HEPES, pH = 7.40). All current recordings were conducted at maximal gain of the amplifier (10,000X), clamp rise time stability settings of 60–120 μs, and filtered at 1 kHz (digitized at 5 kHz; Digidata 1320A system under pCLAMP 9 software control [Axon Instruments]). Quantitative analysis was subsequently conducted offline using pClamp9 and Origin 7.5 (Origin Lab, Northampton, MA, USA).

**General protocols and analysis.** D230Q steady-state activation “α<sub>i</sub>” curves were constructed from direct measurements of saturating P2 tail currents (500 msec P2 pulses to -40 mV) elicited following a series of brief 15 msec depolarizing P1 pulses to progressively depolarized potentials (similar saturating tail current protocols were applied for WT K<sub>4,3</sub> and the other mutants<sup>29-31</sup> summarized in Table 1 and Fig. 7). Assuming independent gating<sup>28</sup> of 4<sub>α</sub> subunits, peak tail current amplitudes were normalized and mean data points best fit to a single Boltzmann relationship raised to the fourth power 1/(1 + exp[-(V - V<sub>1/2</sub>)/k])<sup>4</sup> where k is slope factor (mV), V membrane potential (mV), and V<sub>1/2</sub> potential of half maximal activation (mV). Isochronal inactivation “i” relationships were constructed (HP = -100 mV) by applying a series of 1 second P1 pulses to progressively depolarized potentials, followed by a fixed 1 second P2 pulse to +50 mV (this same general protocol was also applied to WT and other mutants (Table 1 and Fig. 7), although in the case of some S4 outermost R mutants<sup>29,31</sup> application of 1 second hyperpolarizing P1 pulses was required to partially remove significant CSI existing at HP = +100 mV). P2 current amplitudes were normalized, plotted as a function of P1 potential, and mean data points fit to a single Boltzmann relationship 1/(1 + exp[-(V - V<sub>1/2</sub>)/k]), where k is isochronal slope factor (mV), V<sub>mem</sub> membrane potential (mV), and V<sub>1/2</sub> potential of half maximal isochronal inactivation (mV).

For analysis of D230Q activation and deactivation kinetics the 90% voltage-clamp rise time criterion<sup>28-32</sup> was employed. A mean (n = 12) value of τ<sub>90%</sub> = 1.66 ± 0.07 ms was obtained. Fits to activation (general equation of form (1 - exp[-t/τ<sub>rec</sub>])<sup>4</sup>) and deactivation (single exponential) were only begun after τ<sub>rec</sub>, and hence we make no further quantitative claims about more rapid gating transitions that may have occurred prior to τ<sub>rec</sub>. Recovery from macroscopic inactivation was measured using a conventional P1-P2 double pulse protocol<sup>25,28-32</sup>. From HP = -100 mV, a 1 second P1 pulse to +50 mV was applied. Following return to HP = -100 mV for variable periods of time (Δt), an identical P2 was applied. Peak P2 current amplitude was normalized, plotted as a function of Δt, and data points best fit to single exponential functions to determine τ<sub>rec</sub>. Kinetics of development of D230Q CSI were analyzed using a P1 prepulse to +10 mV (applied from HP = -100 mV) of progressively increasing duration, followed by a 1 second P2 pulse to +50 mV (for WT, P1 = -50 mV, as discussed in Results). Analysis of decline of the P2 current as a function of P1 duration (single exponential fits) allowed determination of the τ<sub>rec</sub> of development of CSI at the fixed given potential<sup>29-31</sup>.

Recovery (HP = -100 mV) from CSI (developed during a 2 second P1 prepulse to either 0 or +10 mV for D230Q and -50 mV for WT K<sub>4,3</sub>) was determined by plotting the amplitude of the peak P2 current at +50 mV (1 second pulse) as a function of P1 duration.

Gating charge estimates and potential functional/interpretive implications were determined as follows. When an individual
α subunit undergoes a global gating transition, its total free energy change will be composed of both voltage-dependent and voltage-independent (i.e., structural/conformational) components.\(^{16,56}\) Estimates of minimal effective equivalent gating charges (q) and voltage-independent free energy changes (\(\Delta G^\text{v-independent}\)), the change in nonelectrical energy at 0 mV) were made using a simple two state gating model. From best fits to mean activation “a” and isochronal inactivation “i”, q and \(\Delta G^\text{v-independent}\) values were calculated as follows:\(^{56}\) (i) \(q = (RT/k)\) and (ii) \(\Delta G^\text{v-independent} = -(V_1/2/k)RT\), where k is slope factor, \(V_1/2\) is potential of half maximal activation or inactivation, and R and T have their usual thermodynamic meanings. Under our recording conditions (\(T = 22^\circ C\)), movement of one elementary charge (e\(_q\)) across the entire membrane potential field would correspond to a value of \(k = 25.43\) mV. Within this two state framework: (i) If perturbation in electrostatic effects (changes in q) were prevalent, a change in k with minimal alterations in \(V_1/2\) would be produced; (ii) If structural perturbations were predominant (changes in \(\Delta G^\text{v-independent}\)), a minimal alteration in k but a significant shift in \(V_1/2\) would be produced (reflecting alterations in relative stability of associated closed and open states); and (iii) If both electrostatic and structural perturbations are significant, alterations in both k and \(V_1/2\) may be produced. The limitations of such a two state gating analysis are acknowledged and have been previously discussed.\(^{13,16,18,19,29-31}\)

While the above three predictions are simplistic, in the absence of \(K_V4.3\) gating current analysis (and associated structural data) they nonetheless provide a useful framework for initial categorization of mutant effects (as summarized in Table 1 and Fig. 7). When data permitted, independent kinetic estimates of q associated with a specific gating transition were derived from exponential best fits (e-fold change) of measured \(\tau \sim V_1/2\) curves.\(^{30}\)

Statistical significance (\(p < 0.01\)) was determined by ANOVA (Origin). All data points in figures are mean ± SEM. Unless otherwise indicated: (i) All references to mean WT \(K_V4.3\) data were based upon previous analyses;\(^{24,28-31}\) and (ii) In most figures such prior WT data is illustrated as solid gray curves without data points.

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