Molecular determinants of external barium block in *Shaker* potassium channels

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Abstract Mutations in the outer pore region of *Shaker* K^+ channels (T^{449} and D^{447}) can influence external Ba^{2+} block. Substitution of T^{449} by A, V or Y differentially reduced Ba^{2+} block primarily by decreasing the blocking rate. Substitution of D^{447} by N resulted in a non-conducting channel with apparently normal gating currents. External Ba^{2+} can speed the OFF gating current of a different non-conducting mutant, W434F; this effect was markedly attenuated by the D447N substitution. These results suggest that D^{447} contributes to an external Ba^{2+} binding site while T^{449} imposes a barrier to the access of that site.

Key words: Barium; Potassium; Gating current; Unitary current; Pore

1. Introduction

Barium ions block a wide variety of functionally and structurally distinct K^+ selective ion channels [1–10]. In many cases Ba^{2+} can block these channels from either side of the membrane suggesting that Ba^{2+} can interact with a structural feature common to many K^+ selective pores [4,11,12]. In fact, it has been proposed that Ba^{2+} blocks these channels by binding at a K^+ site within the permeation pathway [1–4]. Consistent with this idea, K^+ can act as a competitive inhibitor of Ba^{2+} block [3,4,11,13]. When added to the internal side of the squid axon delayed rectifier, Ba^{2+} blocks at a site that can only be accessed when the channel pore is open, deep within the membrane field [2,3]. Molecular biological approaches have given some insight into the structural domains influencing internal block of voltage-gated K^+ channels. For the *Shaker* K^+ channel, both the S4–S5 loop and the S6 region contribute to internal Ba^{2+} sensitivity [14,15]. In addition, internal Ba^{2+} sensitivity of a cloned delayed rectifier can be modified by a point mutation in the putative pore forming region [12].

Our previous work suggests that externally applied Ba^{2+} can interact with at least two distinct and sequential binding sites on the *Shaker* K^+ channel, resulting in a fast and a slow component of ionic current block [13]. The present work identifies two amino acid positions at the outer pore that appear to influence binding at the site associated with the slow component of block. One position (T^{449}) influences the access of Ba^{2+} to the slow component binding site and can therefore explain the very slow blocking rate of this component. A second position (D^{447}) appears to contribute to the binding site associated with the slow component of block as evidenced by measurements of gating current.

2. Materials and methods

2.1. Molecular biology and oocyte preparation

The non-inactivating deletion *Shaker* H4 (Δ6-46) [16], also called ShH4-IR, was the parental clone from which all mutants were made. RNA was transcribed in vitro with T7 RNA polymerase using unmethylated CAP analogue (Pharmacia, Piscataway, NJ) according to published methods [17]. RNA (2.5–50 ng) was injected into collagenase treated *Xenopus laevis* oocytes (stage V–VI) in 50 nl of water. Oocytes were maintained at 18°C in amphibian saline supplemented with gentamicin (50 μg/ml). Voltage clamp recordings were performed at room temperature 2–5 days following RNA injection.

2.2. Electrophysiology

Macroscopic ionic and gating currents were recorded using the cut-open oocyte vaseline gap (COVG) technique [17]. The external solution of the oocyte was exchanged by a manual perfusion system requiring ~5 s for complete solution exchange. Bath solutions were made by mixing stock isotonic solutions (240 mOsm) of the main cation buffered with 10 mM *N*-[2-hydroxyethyl]piperazine-*N'*-[2-ethanesulfonic acid] (HEPES) at pH 7.0; methanesulfonate (MES) was used as the main anion. The external solution in control experiments was 115 mM sodium methanesulfonate (Na-MES), 2 mM calcium methanesulfonate [Ca-$(MES)_2$], 2 mM potassium methanesulfonate (K-MES), 0.1 mM EGTA and 10 mM HEPES. In experiments to measure Ba^{2+} block of ionic current, isotonic barium methanesulfonate [Ba-$(MES)_2$] replaced Na-MES in the following solution: 117 mM Na-MES, 2 mM K-MES, and 10 mM HEPES. Ba^{2+} solutions for measurement of gating currents had a constant divalent concentration of 2 mM with Ba-$(MES)_2$ replacing Ca-$(MES)_2$, e.g. the solution containing 0.1 mM Ba^{2+} was as follows: 0.1 mM Ba-$(MES)_2$, 1.9 mM Ca-$(MES)_2$, 2 mM K-MES, 117 mM Na-MES. The solution in contact with the intracellular compartment was 120 mM potassium glutamate buffered with 10 mM HEPES. The intracellular microelectrode was filled with 2.7 M Na-MES, 10 mM NaCl, 10 mM EGTA, and 10 mM HEPES.

Single channel recordings were performed in the cell attached patch clamp configuration. A depolarizing bath solution was used containing 117 mM K-MES, 2 mM $MgCl_2$, 0.1 mM EGTA, and 10 mM HEPES. The pipette solution contained 117 mM Na-MES, 2 mM KCl and 10 mM HEPES for control measurements or 115 mM Na-MES, 2 mM KCl, 2 mM Ba-$(MES)_2$, 10 mM HEPES for Ba^{2+} measurements. Data were digitized at 100 μs/point after low pass filtering at 2 kHz. Single channel records were corrected off line for linear leak and capacity currents using customized software. Open and closed transitions were detected by the half amplitude threshold criterion using the TRANSIT software (A.M.J. VanDongen, Duke University, Chapel Hill, NC).

2.3. Data analysis

Ba^{2+} block of ionic currents was measured from the outward K^+ current at the end of sequential 8 ms depolarizing commands to 30 mV repeated once every 5 s; the holding potential was −90 mV and Ba^{2+} was washed out between each application. The slow decaying phase was fitted to a single exponential term to derive the initial and steady state levels of the slow component of block; the fast blocking component was taken as the difference between the current amplitude in the absence of Ba^{2+} and the initial value of the slow component [13]. Ba^{2+} inhibition data for the individual components were fitted to $K = 100 \cdot [Ba]^n/([Ba]^n + K^n)$ where K is the apparent dissociation constant and n is the Hill coefficient. The slow blocking rate as a function of Ba^{2+} concentration was fitted to a two site binding scheme as

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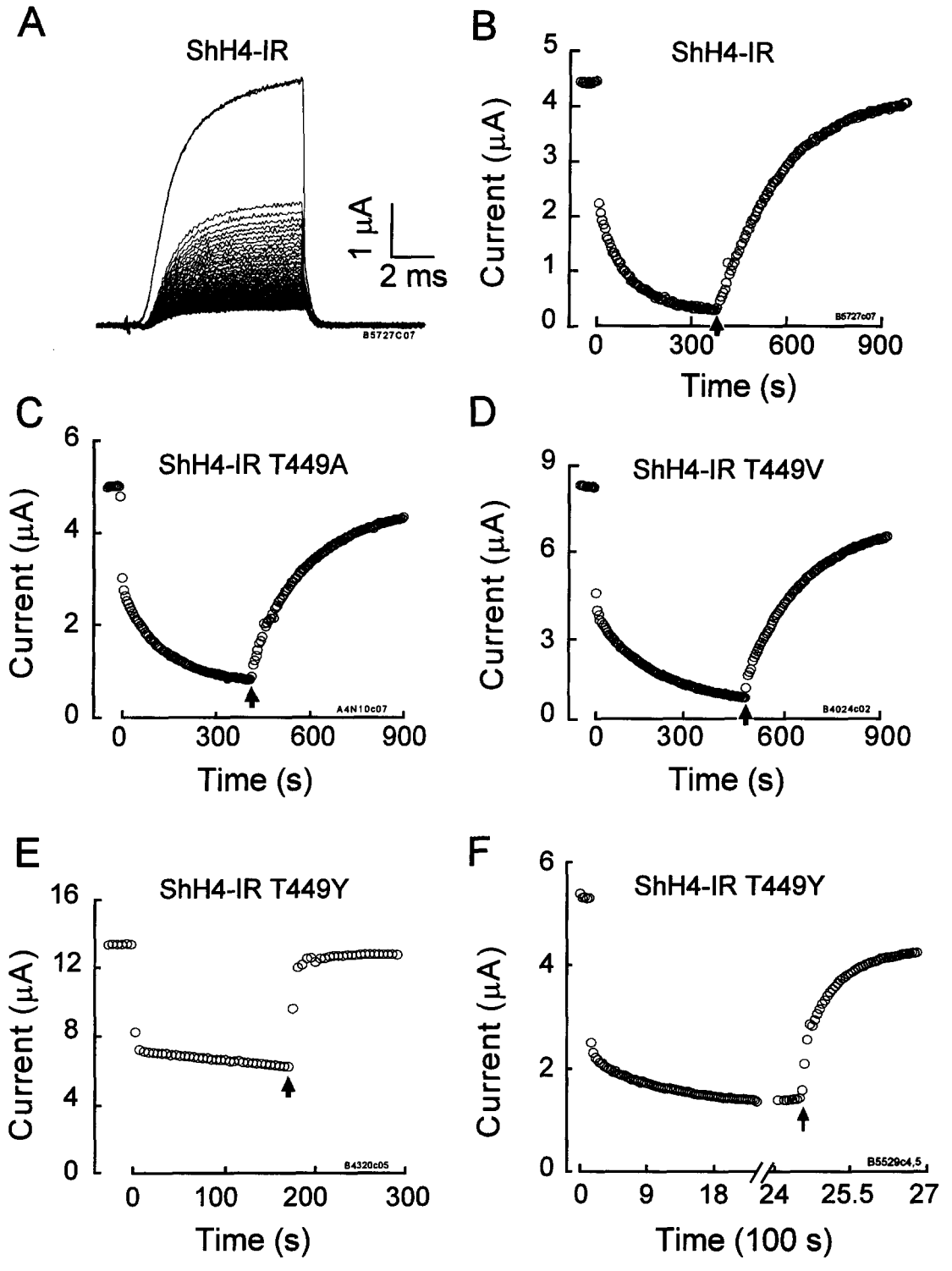

Fig. 1. The slow component of Ba^{2+} block is influenced by mutations at position 449. A: Ionic currents evoked from an oocyte expressing ShH4-IR by 8 ms depolarizations to 30 mV before (upper traces) and after the addition of 20 mM Ba^{2+}, pulses were given every 5 s. B–E: Symbols show the current evoked at the end of successive 8 ms depolarizations to 30 mV; pulses were repeated once every 5 s. In each case, 20 mM Ba^{2+} was added to the external solution at time zero and the arrow indicates the time of wash. B: ShH4-IR, C: T449A, D: T449V, and E: T449Y. F: Long exposure of T449Y to Ba^{2+}. The depolarizing commands were given once every 30 s during the application of Ba^{2+}; during the wash-out, the membrane was depolarized to 30 mV once every 5 s (note the change in scale along the time axis).

previously described [13],

$$Ba^{2+} + \text{Site1}-\text{Site2} \underset{k_{off(1)}}{\overset{k_{on(1)}}{\rightleftharpoons}} Ba^{2+} : \text{Site1}-\text{Site2} \underset{k_{off(2)}}{\overset{k_{on(2)}}{\rightleftharpoons}} \text{Site1}-\text{Site2} : Ba^{2+}$$

where Site 1 and Site 2 correspond to the fast and slow components of macroscopic current block, respectively. The blocking rate ($1/\tau_{block}$) as a function of Ba^{2+} concentration was fitted to $1/\tau_{block} = k_{on}(2)\cdot([Ba]/$

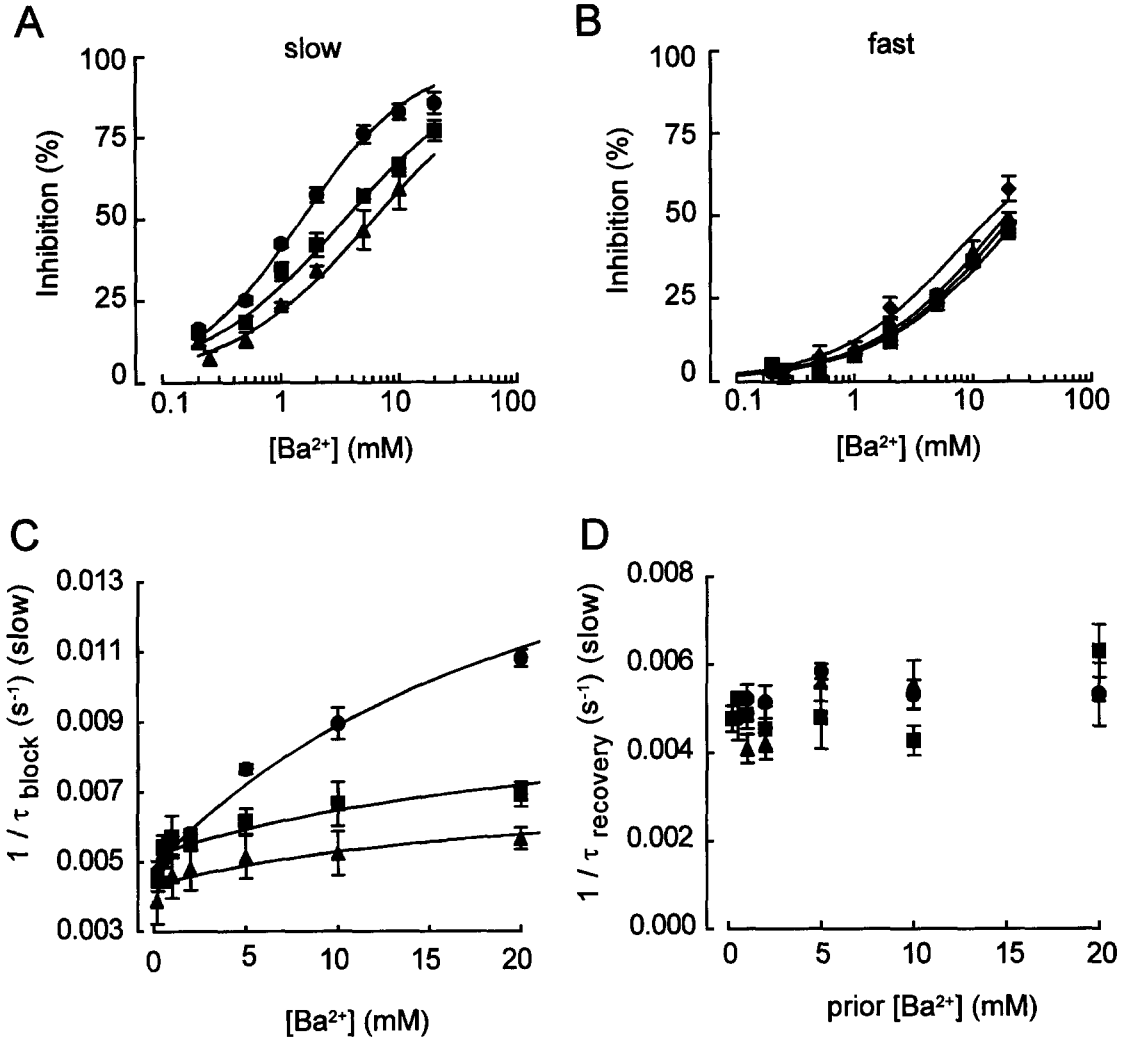

Fig. 2. Dose-response relationships for the slow and fast component of block. A: Dose response of the slow component of block for ShH4-IR (filled circles), T449A (filled squares) and T449V (filled triangles). The continuous curves show the fits to a single binding isotherm. The apparent dissociation constants for the slow components (K_S) were as follows (mean ± S.D.): for ShH4-IR, 1.5 ± 0.4 mM (n = 5); for T449A, 3.5 ± 1.2 mM (n = 8); for T449V, 6.1 ± 3.0 mM (n = 5). K_S could not be determined for T449Y. B: Dose response of the fast component of block for ShH4-IR (filled circles), T449A (filled squares), T449V (filled triangles), and T449Y (open circles). The apparent dissociation constants for the fast components (K_F) were as follows (mean ± S.D.): for ShH4-IR, 23.3 ± 3.6 mM (n = 5); for T449A, 24.2 ± 4.3 mM (n = 6); for T449V, 20.6 ± 6.0 mM (n = 6); for T449Y, 11.7 ± 6.4 (n = 5). C: Blocking rate ($1/\tau_{block}$) of the slow component as a function of Ba^{2+} concentration (for legend, see A). Continuous curves are the fit of the blocking rate to a sequential two site blocking model (see section 2). D: Recovery rate ($1/\tau_{recovery}$) upon removal of Ba^{2+} (for legend, see A). $1/\tau_{recovery}$ obtained from all the experiments were as follows (mean ± S.D.): for ShH4-IR, 0.005 ± 0.001 s^{-1} (n = 28); for T449A, 0.005 ± 0.001 s^{-1} (n = 25); for T449V, 0.005 ± 0.001 s^{-1} (n = 18).

[Ba] + K_1)) + $k_{off}(2)$, where K_1 is the dissociation constant of Site 1 and the ratio ([Ba]/[Ba] + K_1) is the probability that Site 1 is occupied by Ba^{2+}. Recovery from Ba^{2+} block was assayed by an 8 ms depolarizing command to 30 mV repeated once every 5 s following the washout of Ba^{2+}; the recovery phase was fitted to a single exponential term to obtain the time constant of recovery ($\tau_{recovery}$). Gating currents were measured by a 20 ms pulse to 30 mV given once every 5 s. Subtraction pulses (P/4) were given from a subtraction holding potential (SHP) of 20 mV.

3. Results

3.1. Mutations at T^{449} influence Ba^{2+} block

Previous studies demonstrated that an aromatic group at the position 449 in *Shaker*, or its equivalent, can play a critical role in block of ionic current by extracellular tetraethylammonium (TEA) [18–20]. We therefore mutated this position (T^{449}) in the non-inactivating deletion of *Shaker* H4 (ShH4 Δ6-46, also called ShH4-IR) [16] to determine its influence on external Ba^{2+} sensitivity. The naturally occurring threonine (T) at position 449 (T^{449}) was substituted by alanine (A), valine (V), or tyrosine (Y).

We previously reported that external Ba^{2+} can reduce *Shaker* K^+ current evoked by a brief depolarization without changing the kinetics of the remaining current [13]. We also found that the time course of Ba^{2+} block had a fast and a slow component and proposed that these two components resulted from separate and sequential binding sites. Fig. 1 illustrates the time course of Ba^{2+} block, and the recovery from block, for the wild type and the pore mutants at position T^{449}. While the mutations had little influence on the fast component of block, they slowed to different degrees the slower phase of block. T449A and T449V had relatively modest ef-

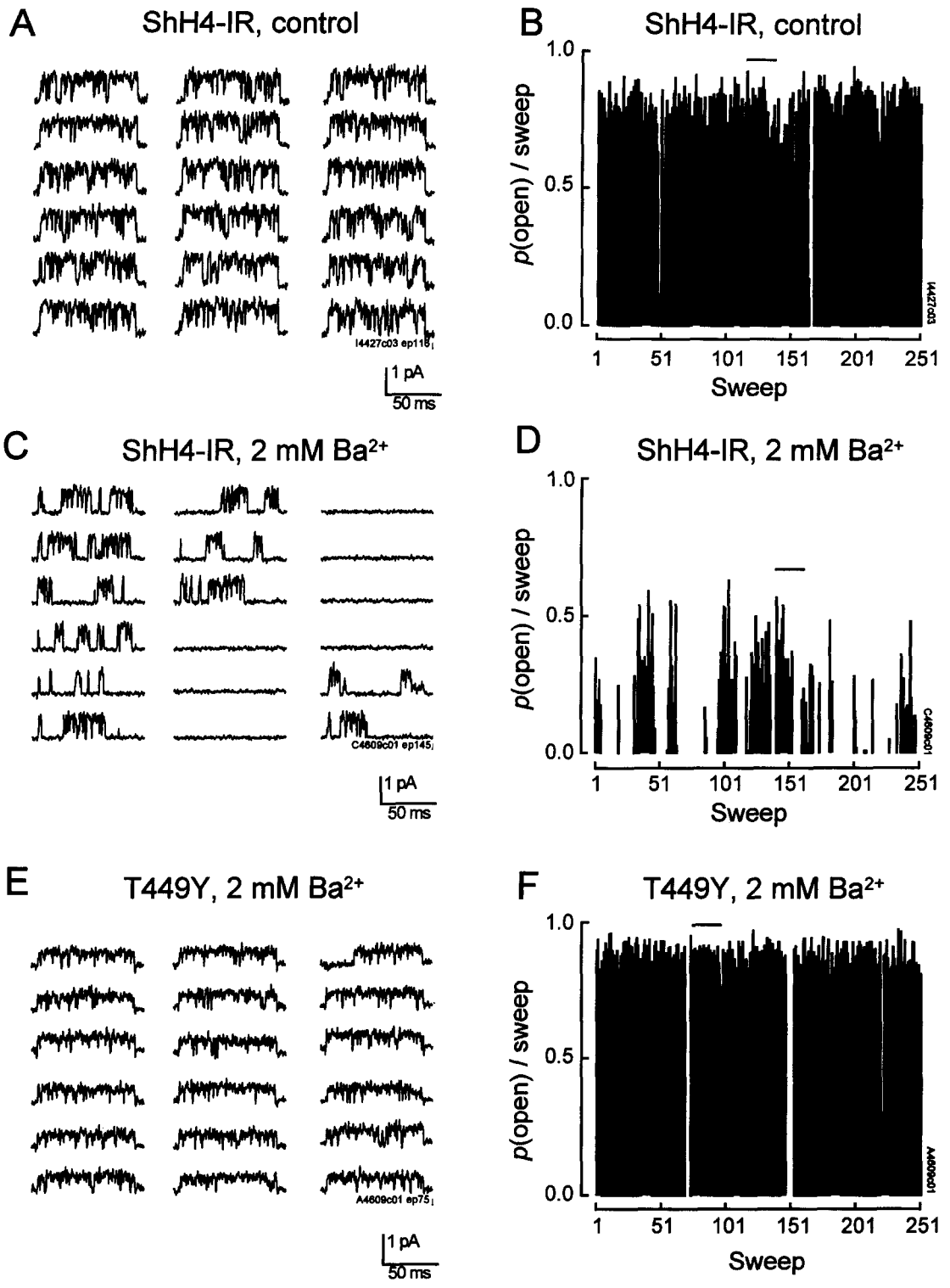

Fig. 3. T449Y reduces the frequency of discrete blocking events. A, C, E: Consecutive sweeps from single channel patches containing (A) ShH4-IR in control conditions, (C) ShH4-IR in 2 mM Ba^{2+} and (E) T449Y in 2 mM Ba^{2+}. The membrane was held at −90 mV and stepped to 30 mV for 100 ms repeated once per second. B, D, F: Plots of open probability per sweep as a function of sweep number for (B) ShH4-IR in control conditions, (D) ShH4-IR in 2 mM Ba^{2+} and (F) T449Y in 2 mM Ba^{2+}. The over bars indicate the region from which the individual sweeps shown in the left panels were taken.

fects (Fig. 1C,D). In contrast, T449Y decreased the blocking rate to the point where exposure times to Ba^{2+} of 30–45 min were required to visualize the slow component of block and subsequent recovery upon wash-out (Fig. 1E,F).

The mutations T449A and T449V decreased the steady state level of block, as estimated by a single exponential fit (see section 2), by about 2- and 4-fold, respectively (Fig. 2A). The steady state level of block for T449Y could not be reliably estimated due to the extremely slow kinetics and concomitant 'run-down' of ionic current. None of these mutations

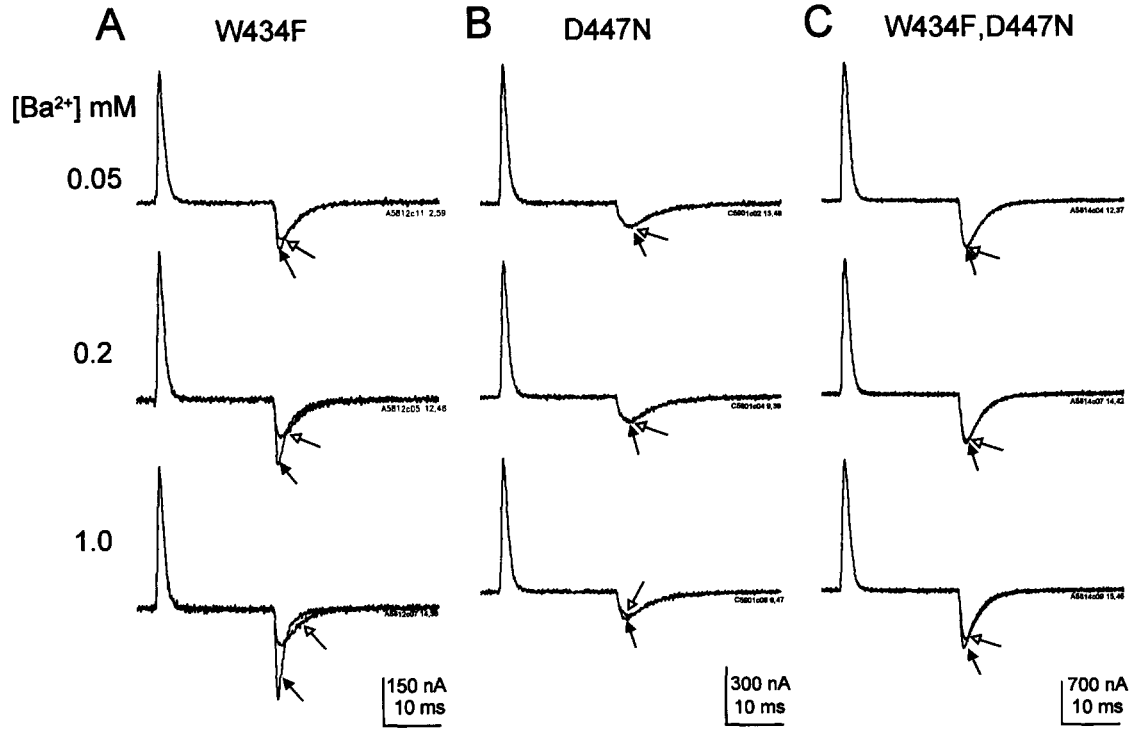

Fig. 4. D447N reduces the ability of Ba^{2+} to speed the return of gating charge. Gating currents from three non-conducting mutants; A: ShH4-IR (W434F), B: ShH4-IR (D447N) and C: ShH4-IR (W434F,D447N). Each panel shows a control trace (open arrow) and a trace recorded in the indicated concentration of external Ba^{2+} (filled arrow). In some cases, the currents recorded in Ba^{2+} superimpose the control records. Gating currents were evoked by a depolarizing command to 30 mV from a holding potential of −90 mV.

significantly altered the dose dependence of the fast component of block (Fig. 2B). Fig. 2C,D shows that the primary cause for the decreased Ba^{2+} sensitivity of T449A and T449V was a reduction in blocking rate ($1/\tau_{block}$) while the unblocking rate ($1/\tau_{recovery}$) was practically unaltered. The unblocking rate for T449Y ($\tau_{recovery}$ = 96.3 ± 4.9 s, n = 6; mean ± S.D.) was faster than the wild type but this difference was small compared to the reduction in blocking rate (see Fig. 1F). These results suggest that the mutations at T^{449} slowed the arrival of Ba^{2+} to the slow blocking site but had relatively little effect on the length of time Ba^{2+} occupied that site. In other words, it appears that the residue at position 449 can influence the access of Ba^{2+} to a binding site deeper in the pore.

3.2. Single channel analysis of T449Y

Divalent cations are known to influence the gating properties of voltage sensitive K^+ channels (e.g. [21–25]). For example, external Zn^{2+} substantially slows the activation time course of squid axon [21] and *Shaker* K^+ currents [23]. We therefore investigated the effect of Ba^{2+} on single T449Y channels to determine if the decreased slow component reflected changes in gating properties. At the single channel level, the slow component of external Ba^{2+} block of ShH4-IR results from discrete blocking events, often observed as sweeps which lack channel opening [13]. Consistent with the idea that T449Y decreases the access of Ba^{2+} to the binding site associated with the slow component, this mutation markedly reduced the frequency of these long blocked times (Fig. 3). In contrast, the T449Y mutation had no effect on the single channel conductance when measured in the absence of Ba^{2+}; the chord between −40 mV and +60 mV was 8.6 ± 0.8 pS for ShH4-IR (± S.D., n = 6) and 9.3 ± 0.4 pS for ShH4-IR T449Y (± S.D., n = 4).

3.3. Neutralization of D^{447} and the effect of Ba^{2+} on gating currents

Relative to T^{449}, an aspartate (D^{447}) resides two positions deeper in the pore and is predicted to face the aqueous lumen [26]. Neutralization of this highly conserved D has previously been reported to eliminate ionic current [27,28]. However, when this position is substituted by asparagine (D447N), the resulting proteins are still expressed and undergo voltage-dependent conformational changes as evidenced by the measurement of gating current (Seoh and Papazian, personal communication) (for review on gating currents, see [29]).

External Ba^{2+} can increase the speed of gating charge return in the non-conducting mutant ShH4-IR (W434F) [30] but only at potentials positive to about −55 mV. Since this is near the threshold for channel activity, this effect can be explained by a destabilization of the open state by Ba^{2+} [Hurst et al. (1996) Biophys. Soc. Abst.]. Fig. 4A shows that following a depolarization to 30 mV, Ba^{2+} markedly increased the rate of OFF gating current (Q_{off}) in the non-conducting W434F mutant. In contrast, Ba^{2+} had relatively little effect on the gating currents of the D447N mutant following the same depolarization (Fig. 4B). To insure that the effect of Ba^{2+} on Q_{off} was not a result of the W434F mutation, D447N was introduced into the background of ShH4-IR (W434F). This double mutant, ShH4-IR (D447N,W434F), was also insensitive to Ba^{2+} (Fig. 4C). Therefore, the most likely explanation for these results is that the D447N mutation disrupts the site necessary for Ba^{2+} to speed Q_{off}.

4. Discussion

4.1. Mutations at position 447 influence the blocking rate of Ba^{2+}

We recently proposed that two distinct and sequential binding sites on the *Shaker* K^+ channel give rise to the fast and slow component of external Ba^{2+} block [13]. The deeper of these two sites, which produces the slow component of block, is preceded by a relatively large energy barrier as evidenced by the very slow blocking kinetics. Substitution of T^{449} by A, V, Y reduced the blocking potency of Ba^{2+} on the slow component to different degrees primarily by slowing the blocking rate; the same mutations had relatively little effect on the unblocking rate. This suggests that the mutations at T^{449} increased the energy barrier that precedes the slow blocking site and thereby decreased the likelihood of Ba^{2+} reaching that site. Consistent with this idea, it has been proposed that T^{449} resides at the outer edge of the narrow pore region and should therefore be well suited to regulate access of ions into the narrow pore region [31].

Another possible explanation for the influence of mutations at T^{449} on Ba^{2+} block could be that Ba^{2+} sensitivity is in some way related to slow or C-type inactivation [32]. Previous studies have demonstrated that the amino acid at position 449 in *Shaker*, or its equivalent, can influence the rate of slow inactivation [33,34]. However, any relationship between inactivation and Ba^{2+} block seems unlikely because the mutations at position 449 differentially affected Ba^{2+} sensitivity and slow inactivation. For example, T449A increases the rate of slow inactivation while T449V and T449Y tend to slow this process [34] yet all of these mutations decrease the blocking potency of external Ba^{2+}.

4.2. D^{447} contributes to the site necessary for Ba^{2+} to facilitate gating charge return

In their original description, Armstrong and Taylor proposed that Ba^{2+} binding stabilized the closed state of squid axon K^+ channels [2]. In addition, they proposed that the very tight interaction of Ba^{2+} implied the existence of negatively charged groups within the channel pore [2]. Following this idea, we removed the negative charge at position 447 (D^{447}) to determine its effect on Ba^{2+} binding. Because neutralization of this highly conserved D eliminates K^+ conduction [27,28], we compared the effects of Ba^{2+} on the gating current of ShH4-IR (D447N) with a different non-conducting mutant, ShH4-IR (W434F). The D447N substitution alone, or when introduced into the background of ShH4-IR (W434F), substantially reduced the ability of Ba^{2+} to speed Q_{off} (Fig. 4). Given the charge on this side chain and its proposed exposure to the aqueous pore [26], the simplest explanation is that D447N disrupts the binding site that allows Ba^{2+} to speed Q_{off}.

The question remaining is whether the binding site at D^{447} is associated with the slow component or the fast component of ionic current block. Since the mutations at position 449 did not alter the fast component of block, the fast blocking site most likely resides external to T^{449}, consistent with the relative voltage-dependence of these two components [13]. The widely accepted topology of this channel places D^{447} internal to T^{449} [35]; therefore, D^{447} probably does not contribute to binding site associated with the fast component of ionic current block. In lack of evidence suggesting a third external Ba^{2+} binding site [13], the simplest interpretation is that the site at D^{447} is therefore associated with the slow component of ionic current block.

In summary, this work identifies two positions in the putative pore forming region of the *Shaker* K^+ channel that appear to influence the binding of external Ba^{2+}. First, mutations at position 449 (T^{449}) decreased the slow component of block primarily by slowing the blocking rate. This suggests that position 449 is an important component to an energy barrier that precedes the narrow pore region. Second, neutralizing a negative charge two positions deeper in the pore (D447N) not only eliminated ionic conduction but also markedly reduced the ability of Ba^{2+} to speed the return of gating charge. This suggests that D^{447} contributes to the binding site associated with the Ba^{2+} facilitation of gating charge return. While it is tempting to speculate that D^{447} also contributes to the binding site associated with the slow component of Ba^{2+} induced ionic current block, this idea is supported only by indirect evidence.

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