1521-0111/12/8204-658–667$25.00
MOLECULAR PHARMACOLOGY
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Mol Pharmacol 82:658–667, 2012

Vol. 82, No. 4
78923/3794048

Block of Human Ca_V3 Channels by the Diuretic Amiloride

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Received March 24, 2012; accepted July 5, 2012

ABSTRACT

Previous studies in native T-type currents have suggested the existence of distinct isoforms with dissimilar pharmacology. Amiloride was the first organic blocker to selectively block the native T-type calcium channel, but the potency and mechanism of block of this drug on the three recombinant T-type calcium channels ($Ca_V3.1$, $Ca_V3.2$, and $Ca_V3.3$) have not been systematically determined. The aim of the present study was to investigate whether there is differential block of Ca_V3 channels by amiloride, to establish the mechanism of block, and to obtain insights into the amiloride putative binding sites in Ca_V3 channels. By performing whole-cell patch-clamp recordings of human embryonic kidney 293 cells stably expressing human Ca_V3 channels, we found that amiloride blocked the human Ca_V3 channels in a concentration-response manner; the IC_{50} for $Ca_V3.2$ channels (62 μM) was 13-fold lower than that for $Ca_V3.1$ and $Ca_V3.3$. Block is voltage-independent (except for $Ca_V3.3$ channels) and targets mainly closed-state channels, although a small use-dependent component was observed in $Ca_V3.1$ channels. In addition, amiloride block of $Ca_V3.2$ channels is mainly due to an extracellular effect, whereas in $Ca_V3.1$ and $Ca_V3.3$ channels, the amiloride inhibition is equally effective from both sides of the membrane. The results demonstrate that amiloride blocks human Ca_V3 channels differentially through a mechanism involving mainly the closed state of the channel and suggest a negative allosteric interaction with at least two putative binding sites with different affinities. The preferential block of $Ca_V3.2$ channels labels amiloride as the only organic blocker to be selective for any T-type channel.

Introduction

Voltage-gated calcium (Ca_V) channels are crucial mediators of a wide range of physiological functions, including neuronal communication, muscle contraction, hormone secretion, enzyme regulation, and gene transcription (Catterall, 2011). Therefore, these channels are key pharmacological targets in the treatment of disorders such as hypertension, epilepsy, and pain. There are two major classes of Ca_V channels: low-voltage-activated (LVA) and high-voltage-activated (HVA) (Ertel et al., 2000). For the last 25 years, the study of Ca_V channels that carried HVA currents has been aided by the existence of specific blockers for each of the five channels of this family: L, N-P/Q, and R types. Clinically relevant calcium channel blockers, which target HVA channels, are widely used in the treatment of hypertension and include dihydropyridines, benzothiazepines, and phenylalkylamines (Haller, 2008). Conversely, the systematic study of LVA currents, transported exclusively by T-type channels, has been hindered by the lack of a specific blocker and a delay in the cloning of their molecular substrate, which slowed down research on key aspects of their tissue distribution and pathophysiological roles. With the cloning of three human α1 subunits ($Ca_V3.1$, $Ca_V3.2$, and $Ca_V3.3$) that generate LVA or T-type currents (Cribbs et al., 1998; Perez-Reyes et al., 1998; Gomora et al., 2002), the search for selective blockers that discriminate among these channels has become more imperative in this century. Mibefradil, once described as the first organic blocker effective at submicromolar concentrations for native T-type channels (Clozel et al., 1997), inhibits the three recombinant Ca_V3 channels with practically the same potency (Martin et al., 2000).

Amiloride is a potassium-sparing diuretic, first approved for use in 1967 and used in the management of hypertension and congestive heart failure (Bull and Laragh, 1968; Thomas and Thomson, 1983), by targeting the epithelial sodium channel within the distal tubule of the kidney (Garty and Benos, 1988). However, amiloride has been used as a pharmacological tool to distinguish native T-type channels from HVA channels, (Tang et al., 1988; Hirano et al., 1989; Tytgat

This work was supported by the Consejo Nacional de Ciencia y Tecnología [Grant J50250Q] and the Instituto de Ciencia y Tecnología del Distrito Federal [Grant PICDS08−28] (to M.R.).

Parts of this work were previously presented: Rivera M, López-Charcas O, and Gomora JC (2011) Amiloride docking to T-type calcium channels. *Proceedings of the 55th Annual Meeting of the Biophysical Society*; 2011 Mar 5–9; Baltimore, MD. The Biophysical Society, Rockville, MD.

Article, publication date, and citation information can be found at http://molpharm.aspetjournals.org.

http://dx.doi.org/10.1124/mol.112.078923.

ABBREVIATIONS: Ca_V, voltage-gated calcium; LVA, low-voltage-activated; HVA, high-voltage-activated; HP, holding potential; HEK, human embryonic kidney; AMI, amiloride.

et al., 1990); although significant differences in the amiloride concentration that cause 50% of T-type current inhibition (IC_{50}) have been reported (Tang et al., 1988; Herrington and Lingle, 1992). In addition, it has been shown that the IC_{50} for amiloride blocking of mouse $Ca_V3.1$ channels is approximately 25-fold higher than the observed in human $Ca_V3.2$ channels (Williams et al., 1999; Lacinová et al., 2000). The sensitivity of the third member of the T-type channel subfamily, $Ca_V3.3$ channels, to amiloride has not been reported yet. Here, we have studied systematically the amiloride block of the three human recombinant Ca_V3 channels stably expressed in HEK-293 cells. The goals were to determine the amiloride sensitivities of the three Ca_V3 channels and to investigate the mechanism of action through which the diuretic exerts its blocking effects on these channels.

Materials and Methods

Expression of Human Recombinant Ca_V3 Channels and Electrophysiology. HEK-293 cells stably transfected with human cDNAs encoding $Ca_V3.1$ (AF190860), $Ca_V3.2$ (AF051946), or $Ca_V3.3$ channels (AF393329), were grown as described previously (Díaz et al., 2005; Balderas et al., 2012). Ca_V3 channel activity was recorded at room temperature (20–23°C), with the whole-cell patch-clamp technique (Hamill et al., 1981; Marty and Neher, 1995) using an Axopatch 200B amplifier, a Digidata 1320 A/D converter, and pCLAMP software (Molecular Devices, Sunnyvale, CA). Currents were digitized at 10 to 20 kHz, after 5 kHz analog filtering. Whole-cell series resistance and cell capacitance were estimated from optimal cancellation of the capacitive transients with the built-in circuitry of the amplifier and was compensated electrically by 60 to 70%. Unless otherwise stated, the holding potential (HP) was −100 mV. Cells were bathed in a solution containing 5 mM $CaCl_2$, 160 mM tetraethylammonium chloride, and 10 mM HEPES, pH 7.4. The internal (pipette) solution contained 135 mM CsCl, 10 mM EGTA, 4 mM Mg-ATP, 0.3 mM Tris-GTP, and 10 mM HEPES, pH 7.3. Amiloride [3,5-diamino-6-chloro-*N*-(diaminomethylidene)pyrazine-2-carboxamide hydrochloride; Sigma-Aldrich, St. Louis, MO] was dissolved in water (2× stocks) and then in external or internal 2× solution to reach the final 1× concentration. Under our recording conditions, pH 7.4, amiloride (a weak base) is 95% ionized with a positive charge.

Peak current values and exponential fits of current recordings were obtained by using the Clampfit application of pCLAMP software. Concentration-response relationships for amiloride block were fit with the following Hill equation: $Y = 1/(1 + 10^{[(\log IC^{50} - X) \cdot h]})$, where X is the logarithm of concentration, Y is the fraction of current remaining after addition of the drug, IC_{50} is the concentration required for 50% block of current, and h is the Hill coefficient. For this analysis, current in control external solution was normalized to 100%, and we assumed complete block of current with sufficient drug concentration. The voltage dependence of current activation was estimated using a modified Boltzmann function to fit normalized current-voltage (I-V) relationships data: $I = I_{max}(V_m - V_{rev})/(1 + \exp[(V_{1/2} - V_m)/k])$, where I is current, V_m is the test potential, V_{rev} is the apparent reversal potential, $V_{1/2}$ is the midpoint of activation, and k is the slope factor. Steady-state inactivation relationships (or availability curves) were obtained by fitting averaged data to a standard Boltzmann function: $I = I_{max}/(1 + \exp[(V_m - V_{1/2})/k])$, where I_{max} is the maximal current recorded at −30 mV, $V_{1/2}$ is the midpoint of steady-state inactivation, and k is the slope.

All quantitative results are given as the mean ± S.E.M. Differences in means were tested with an unpaired two-tailed Student's t test and were accepted as significant if $P < 0.05$.

Results

Concentration-Response Block of Ca_V3 Channels by Amiloride. To determine an IC_{50} for each Ca_V3 channel, we tested the effect of increasing concentrations of amiloride on the peak current evoked by step depolarizations to −30 mV from a HP of −100 mV applied every 10 s. An example of such experiments is illustrated in Fig. 1, A and B, for $Ca_V3.2$ channels. Steady-state block was observed after each amiloride concentration and after exposure to 3 mM concen-

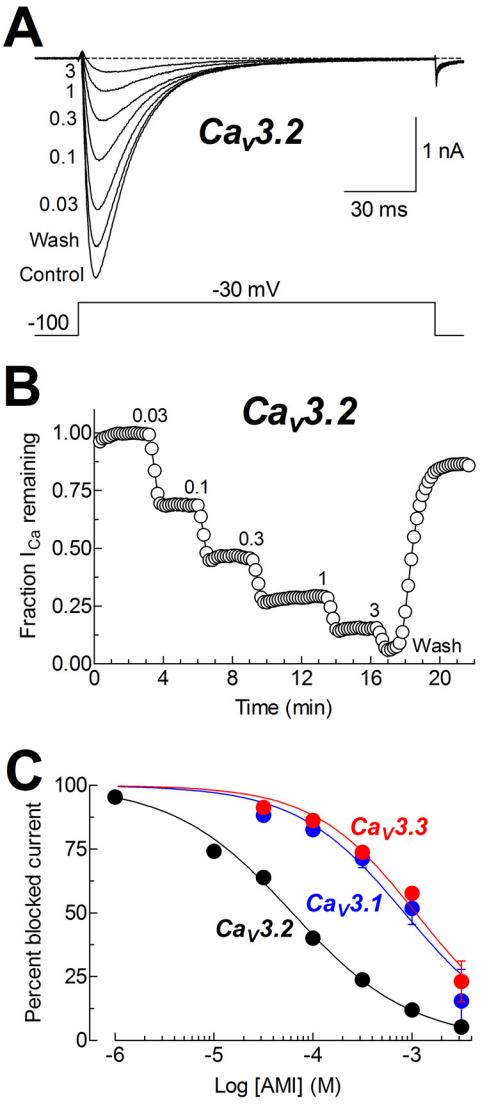

Fig. 1. Concentration-dependent block of human Ca_V3 channels by amiloride. A, representative records showing steady-state block by various concentrations (in millimolar concentrations) of amiloride on $Ca_V3.2$ currents. Whole-cell patch-clamp recordings were made from human $Ca_V3.2$ channel stably expressed in a HEK-293 cell. Currents were evoked by voltage steps to −30 mV from a holding potential of −100 mV applied every 10 s. The dotted line represents zero current. B, time course of $Ca_V3.2$ currents block by amiloride. Peak currents were normalized to control amplitude (before drug exposure), defined as the fraction of I_{Ca} remaining. Data from the same cell are shown in A. C, concentration-response relationships for the effect of amiloride on Ca_V3 channels. Percent blocked current was calculated from peak current measurements from step voltages to −30 mV in the presence of several amiloride concentrations (n = 5–31 cells). Data for each channel were fitted using a Hill equation (smooth lines) with IC_{50} values and Hill slope (h) parameters as follows: $Ca_V3.1$, 840 ± 127 μM and −0.80 ± 0.11; $Ca_V3.2$, 62 ± 3 μM and −0.73 ± 0.03; and $Ca_V3.3$, 1091 ± 117 μM and −0.84 ± 0.09.

TABLE 1
Concentration-dependent block of Ca_V3 channels by amiloride

	IC_{50}	Hill Coefficient
	μM	
$Ca_V3.1$	840 ± 127	−0.80 ± 0.11
$Ca_V3.2$	62 ± 3	−0.73 ± 0.03[a]
$Ca_V3.3$	1091 ± 117	−0.84 ± 0.09

[a] Significantly <1.

trations of the drug, a small fraction of remaining current (approximately 10%) was observed. Block was almost 100% reversible in most of the cells. We did not use higher concentrations of amiloride to keep water as the only solvent in the recording solutions. By performing this type of experiments, concentration-response curves were obtained for the block of human Ca_V3 channels by amiloride (Fig. 1C). The data clearly indicate that $Ca_V3.2$ channels are more sensitive to the amiloride block than $Ca_V3.1$ and $Ca_V3.3$ channels. This was measured by fitting Hill equations to the experimental data (smooth lines, Fig. 1C). Amiloride blocked half the $Ca_V3.2$ current with a concentration of 62 μM; in contrast, the IC_{50} values for $Ca_V3.1$ and $Ca_V3.3$ currents were at least 13-fold higher (Table 1). In addition, the Hill coefficient for the $Ca_V3.2$ curve was approximately 0.7, significantly less than 1, suggesting the possibility of more than one binding site for amiloride in the protein of $Ca_V3.2$ channels. Thus, amiloride blocks $Ca_V3.2$ channels preferentially among the T-type calcium channel subfamily.

Amiloride Block Is Voltage-Dependent Only for $Ca_V3.3$ Channels. We next investigated the voltage dependence of amiloride block of Ca_V3 channels. Representative families of current recordings obtained from a HEK-293 cell expressing $Ca_V3.2$ channels in the absence and the presence of 100 μM amiloride are shown in Fig. 2A. Under control recording conditions, the maximum peak current was reached at −30 mV. Amiloride significantly inhibited $Ca_V3.2$ currents, without modifying the voltage for the maximum peak current. Current amplitudes were normalized to the C_m value of each cell, averaged, and plotted as a function of the test potential to generate *I-V* relationships for each experimental condition (Fig. 2B). Then, to facilitate comparison among experimental conditions, normalized *I-V* relationships were constructed for each Ca_V3 channel. A modified Boltzmann function fitted to the data indicates that amiloride block did not modify the voltage dependence of activation of $Ca_V3.1$ and $Ca_V3.2$ channels (Fig. 2, C and D); on the contrary, the current activation of $Ca_V3.3$ channels was shifted 7.5 mV toward more positive potentials (Fig. 2E; Table 2), which was reversed upon washout (Recovery, Fig. 2E). The voltage dependence block of $Ca_V3.3$ channels by amiloride was also observed in the percentage block of the peak current as a function of the test potential (inset, Fig. 2). Amiloride block was stronger at negative potentials and decreased monotonically at more positive potentials. These results indicate that amiloride exerts voltage-dependent effects only on $Ca_V3.3$ channels. This could be explained by assuming that the binding site of amiloride in $Ca_V3.3$ channels is localized within the pore and partway across the electric field of the membrane, such that stronger depolarizations are needed to open these channels. It is worth mentioning that outward currents carried by the $Ca_V3.3$ channels were poorly blocked by amiloride (data not shown), which also suggests that amiloride physically plugs the pore and that outward currents partially unblock the channels.

To determine whether amiloride block has any effects on the Ca_V3 current kinetics, we analyzed the current traces obtained from the *I-V* protocols (Fig. 2A) by fitting the whole current trace with two exponentials, one corresponding to

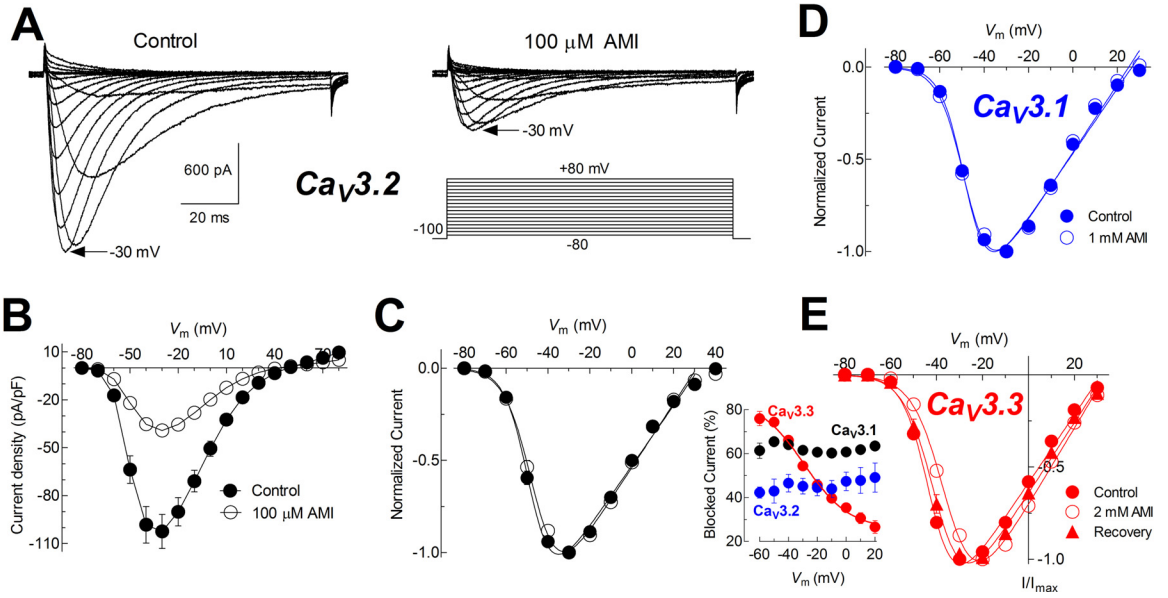

Fig. 2. Effects of amiloride on the voltage dependence of activation of Ca_V3 channels. A, family of $Ca_V3.2$ current recordings obtained before (Control) and after exposure to amiloride (100 μM AMI) conditions, in response to the illustrated voltage protocol. B, Current-voltage relationships of $Ca_V3.2$ channels obtained under the indicated experimental conditions. Peak current amplitudes were normalized to the C_m value of each cell. Data points are averages of 11 cells. C, normalized *I-V* curves for the same cells are shown in B. D and E, Normalized *I-V* curves for $Ca_V3.1$ and $Ca_V3.3$ channels, respectively. Because only $Ca_V3.3$ current showed a shift in the voltage dependence of activation, the respective washout (Recovery) condition is shown. Solid lines in C, D, and E show the fits to the data obtained using a modified Boltzmann function that takes into account changes in driving force (see *Materials and Methods*). The corresponding parameters are shown in Table 2. Inset, percentage block of current as a function of test potential from the same cells shown in C to E. Only $Ca_V3.3$ currents display a voltage-dependent block by amiloride.

TABLE 2

Voltage and kinetic parameters of recombinant Ca_V3 channels in the absence and the presence of amiloride

Data represent mean ± S.E.M. V_{50}, k, and V_{rev} are given in millivolts and were obtained from *I-V* relationship fits with the modified Boltzmann functions. τ_{act} and τ_{inact} are shown in ms, and were obtained from two exponential fits of current recordings at −30 mV. τ_h (in milliseconds) was obtained from single exponential fits to the recovery from inactivation data at −100 and −70 mV. Recovery data are given as the maximum fraction of current recovered after the longest time interval between both pulses to −30 mV. The number of investigated cells was 8 for $Ca_V3.1$, 11 for $Ca_V3.2$, and 6 for $Ca_V3.3$ channels, except for the recovery data, for which the numbers of cells were five, four, and six for −100 mV and five, three, and three for −70 mV, respectively.

	$Ca_V3.1$		$Ca_V3.2$		$Ca_V3.3$	
	Control	AMI	Control	AMI	Control	AMI
V_{50}	−48.0 ± 0.6	−47.7 ± 0.7	−48.3 ± 0.8	−46.5 ± 0.9	−42.3 ± 0.6	−34.8 ± 0.7*
k	5.2 ± 0.1	5.7 ± 0.1	5.4 ± 0.1	6.2 ± 0.1	5.6 ± 0.2	6.8 ± 0.2
V_{rev}	26.7 ± 0.9	25.3 ± 0.9	32.2 ± 0.7	30.9 ± 0.7	31.6 ± 0.4	33.9 ± 0.4
τ_{act}	2.9 ± 0.2	4.1 ± 0.2*	5.2 ± 0.4	5.1 ± 0.3	21.3 ± 1.6	29.9 ± 1.9*
τ_{inact}	18.5 ± 0.9	21.5 ± 1.2	15.5 ± 0.8	22.1 ± 1.2*	44.9 ± 1.2	73.3 ± 2.1*
τ_h at −100 mV	135 ± 4	430 ± 28*	344 ± 13	370 ± 18	441 ± 24	661 ± 58*
Rec at −100 mV	0.99 ± 0.01	0.91 ± 0.02*	0.97 ± 0.01	0.94 ± 0.01*	1.1 ± 0.02	0.89 ± 0.04*
τ_h at −70 mV	287 ± 20	549 ± 151*	800 ± 26	687 ± 153	1148 ± 77	1535 ± 226*
Rec at −70 mV	0.15 ± 0.02	0.13 ± 0.02	0.13 ± 0.02	0.12 ± 0.02	0.17 ± 0.02	0.32 ± 0.04*

Rec, recovery.
* Significantly different from control ($P < 0.05$).

activation and the other to inactivation. The summarized results for $Ca_V3.2$ channels indicate that amiloride has no effects on the kinetics of current activation (Fig. 3A) and only a very modest, although significant (Table 2), effect on current inactivation (Fig. 3B). Similar effects were observed on the current kinetics of $Ca_V3.1$ channels (Table 2). More drastic effects of amiloride were observed in $Ca_V3.3$ current kinetics of activation (Fig. 3C; Table 2), which were slowed down significantly between −40 and +20 mV. In the same range of voltages, inactivation was also slowed down by the diuretic (Fig. 3D). The shift in the current kinetics to more depolarized potentials agrees with the amiloride voltage-dependent effect on the $Ca_V3.3$ current activation (Fig. 2E), rather than a separate effect on the opening kinetics of the channel.

State-Dependent Block of $Ca_V3.1$ Channels by Amiloride. Binding to inactivated states is a distinctive property that confers selectivity to many drugs. To investigate whether Ca_V3 channels sensitivity to amiloride is state-dependent, we determined the effect of the drug on the steady-state inactivation curve of the three T-type channels. This was studied using inactivating prepulses to several V_m values of the following duration: 10 s for $Ca_V3.1$ and $Ca_V3.2$ and 15 s for $Ca_V3.3$ channels; then the fraction of not-inactivated channels was estimated by a test pulse to −30 mV. Figure 4A illustrates families of $Ca_V3.1$ currents recorded at −30 mV after 10-s prepulses to the illustrated voltages, in the absence (Control) and the presence of amiloride (2 mM AMI). Currents were normalized to that evoked after the prepulse to −110 mV, averaged across each experimental condition and plotted versus the prepulse potential. Only in the case of $Ca_V3.1$ was amiloride capable of shifting the inactivation curve to more negative potentials (Fig. 4B). The $V_{1/2}$ shift was small (3.5 mV) but significant (from −75.5 ± 0.4 mV in Control to −79.0 ± 0.7 mV with 2 mM AMI; $n = 7$; $P < 0.05$) and was not reversible even after several minutes of washing out of the drug. The steady-state inactivation curves of $Ca_V3.2$ (Fig. 4C) and $Ca_V3.3$ (Fig. 4D) channels were not modified by the presence of the diuretic.

An additional strategy for measuring amiloride binding to inactivated states of the channel was to measure the block by using different holding potentials. We performed experi-

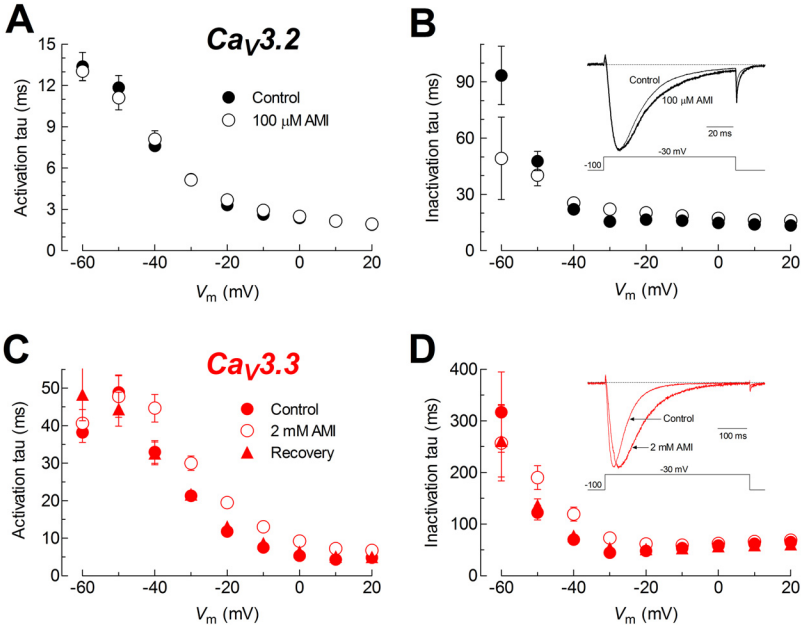

Fig. 3. Amiloride slows down the activation and inactivation kinetics of $Ca_V3.3$ currents. Voltage-dependence of time constants (τ) of activation (A and C) and inactivation (B and D) for $Ca_V3.2$ and $Ca_V3.3$ channels, respectively, before and after the indicated concentrations of amiloride. Currents like those illustrated in Fig. 1A were fitted with two exponentials, one for the activation and the other for the inactivation of the current, and the respective constants were plotted versus membrane potential. Same cells as those in Fig. 2, C and E, are shown. Insets, amiloride effects on current kinetics of normalized traces of $Ca_V3.2$ (B) and $Ca_V3.3$ (D) channels.

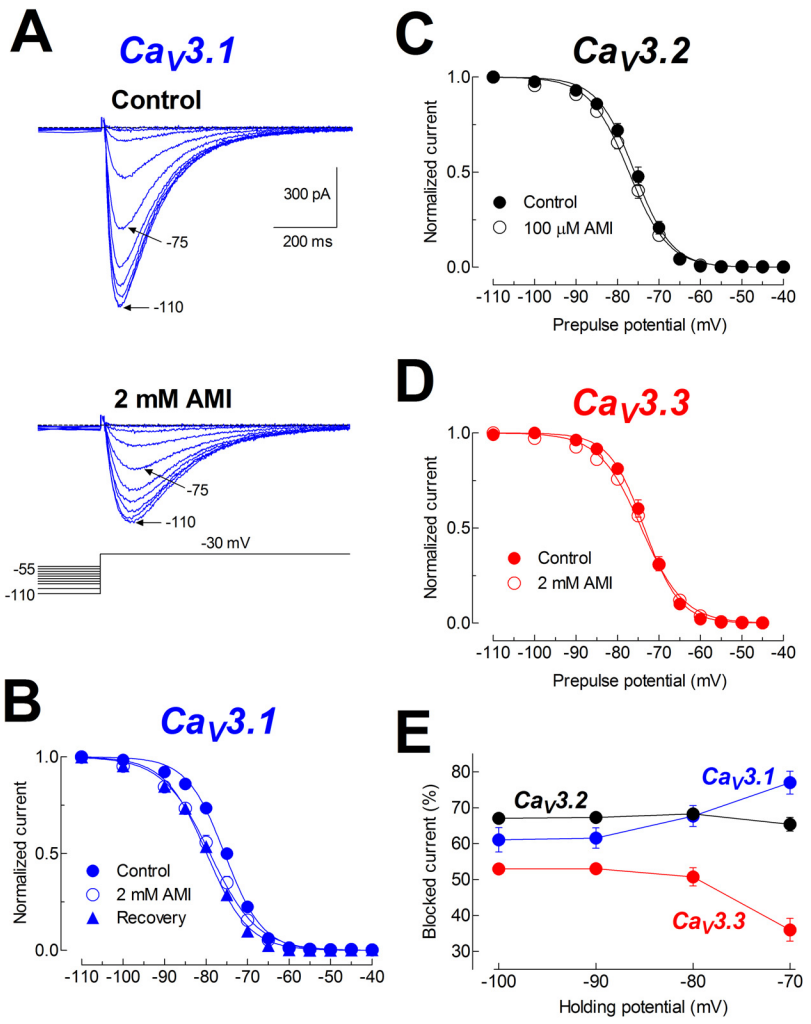

Fig. 4. Amiloride effects on Ca_V3 channels steady-state inactivation. A, families of $Ca_V3.1$ currents at −30 mV after 10-s prepulses to potentials between −110 and −55 mV, before and during exposure to 2 mM amiloride. For the purpose of clear presentation, only the last 200 ms of the prepulses are shown. B, C, and D, steady-state inactivation curves for the three Ca_V3 channels. Currents at −30 mV like those shown in A were normalized to the value at −110 mV at each experimental condition and plotted as a function of the prepulse potential. Data are from seven to eight cells. Smooth lines are fits to Boltzmann functions. Only the $Ca_V3.1$ channel curve was significantly shifted to more hyperpolarized voltages (Control: $V_{1/2}$ = −75.5 ± 0.4 mV, k = 4.5 ± 0.1 mV; 2 mM AMI: $V_{1/2}$ = −79.0 ± 0.7 mV, k = 5.5 ± 0.1 mV; Recovery: $V_{1/2}$ = −79.8 ± 0.9 mV, k = 4.8 ± 0.1 mV; $P < 0.05$). E, opposite effects of amiloride on the block of $Ca_V3.1$ and $Ca_V3.3$ channels at different holding potentials. Data points are the percentage of blocked current at −30 mV with use of the indicated holding potentials by the presence of 2 mM AMI ($Ca_V3.1$ and $Ca_V3.3$ channels) or 100 μM AMI ($Ca_V3.2$ channels). Data are from 5 to 16 cells for each observation.

ments similar to those in Fig. 1A (i.e., determining the steady-state block by amiloride at −30 mV, but applying the step depolarizations from four different HPs: −100, −90, −80, and −70 mV). Figure 4E shows the percentage block of current at each HP for the three Ca_V3 channels. No significant differences were observed in $Ca_V3.2$ channels block regardless of the HP. A small but significant increase in the percentage block (26 ± 3%, n = 11 cells) with use of a HP of −70 mV was observed for $Ca_V3.1$ channels. This agrees with the shift in the voltage dependence of inactivation observed for this channel in Fig. 4B, suggesting that amiloride block of $Ca_V3.1$ channels also includes binding to the inactivated state. On the contrary, an unexpected result was found in the case of $Ca_V3.3$ channels: the percentage block was significantly reduced (32 ± 3%, n = 16 cells) when step depolarizations where made from −70 mV. These opposite effects on $Ca_V3.1$ and $Ca_V3.3$ channels indicate that amiloride blocks the inactivated state of the former more potently, whereas in the latter the binding is actually weaker, resulting in a smaller fraction of blocked channels at −70 mV compared with that observed at −100 mV.

State-Dependent Enhancement of $Ca_V3.3$ Channels by Amiloride. Because of the results presented in Fig. 4, we next sought the effects of amiloride on the recovery from inactivation of Ca_V3 channels at two different potentials, −100 and −70 mV. Recovery from the inactivated state was evaluated with a classic two-pulse voltage protocol from a HP of −100 mV (Fig. 5A). Currents were inactivated by step depolarizations to −30 mV, followed by increasing periods of time at −100 mV (or −70 mV), and finally the recovered current was evoked by a second pulse to −30 mV. Representative currents illustrating the recovery of $Ca_V3.3$ channels at −100 mV in the absence and the presence of 2 mM amiloride are shown in Fig. 5A. Relationships of normalized current amplitudes versus time were plotted for the three Ca_V3 channels (Fig. 5, B–D). Under amiloride conditions, the time constants of recovery (τ_h) for $Ca_V3.1$ and $Ca_V3.3$ were significantly slowed down; however, the time course of recovery for the $Ca_V3.2$ channels was practically unaffected (Table 2). In addition, recovery was clearly incomplete (by ~20%) for $Ca_V3.3$ channels in the presence of amiloride (Fig. 5B). On the other hand, recovery from inactivation at −70 mV produced striking results for $Ca_V3.3$ channels. Figure 5E show examples of current recordings at −30 mV before and after a HEK-293 cell expressing $Ca_V3.3$ channels was kept at −70 mV during 3 s. Without amiloride in the bath, the fraction of current recovered after such time was approximately 20% of the control; of interest, when amiloride was bathing the same cell, the fraction recovered was close to 40% of the control current. This behavior was observed in the whole interval of times explored, as shown in Fig. 5F. The percentage of current recovered after 3 s at −70 mV was increased from 17 ±

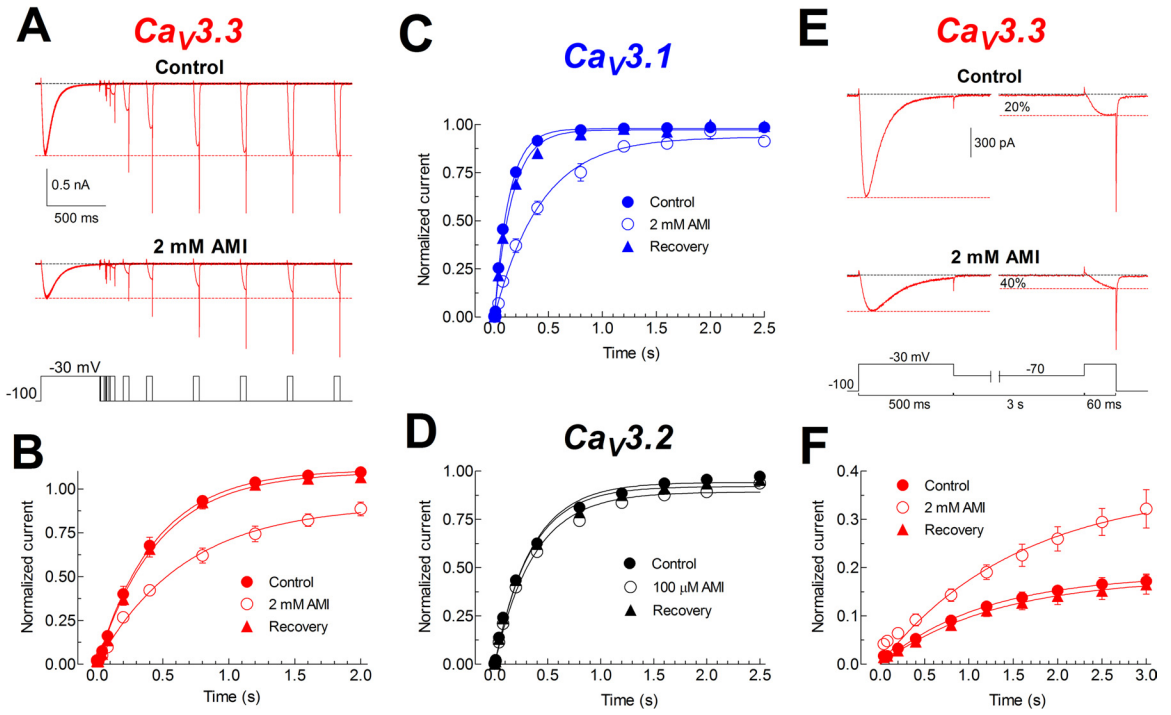

Fig. 5. Effects of amiloride on the recovery from inactivation of Ca_V3 channels. A, recovery from inactivation at −100 mV of $Ca_V3.3$ channels in the absence and the presence of 2 mM AMI. The two-pulse protocol used is shown at the bottom. Ca^{2+} currents were inactivated by a 500 ms pulse to −30 mV; then the membrane potential was stepped to −100 mV for periods ranging from 1 to 2000 ms, and at that time a 50-ms activating voltage step to −30 mV was applied. Tail currents generated by repolarizing to −100 mV are off scale. Red dotted lines indicate the 100% recovery level. B, time course of recovery from inactivation at −100 mV for $Ca_V3.3$ channels under the indicated conditions. The values are the peak current during the 50-ms pulse, normalized to the peak current in the 500-ms pulse. Smooth curves are fits to the data using a one-phase exponential association equation. τ values and number of investigated cells are given in Table 2. Similar experiments were performed for determining the corresponding time course of recovery from inactivation at −100 mV for $Ca_V3.1$ (C) and $Ca_V3.2$ (D) channels. E, recovery from inactivation at −70 mV of $Ca_V3.3$ channels is facilitated by amiloride. Illustrative recordings of $Ca_V3.3$ currents at −30 mV showing the recovery from inactivation after 3 s at −70 mV, in the absence and the presence of amiloride. Currents were evoked in response to a similar protocol as that in A, with the exception that the potential between the test pulses was −70 mV. The percentages indicate the proportion of current recovered after the 3 s at −70 mV in each experimental condition. F, time course of recovery from inactivation at −70 mV for $Ca_V3.3$ channels under the indicated conditions. Experimental data were fitted by single exponential for each condition. τ values and number of investigated cells are given in Table 2.

2 to 32 ± 4% (n = 3 cells). Overall, the kinetics of the recovery from inactivation at −70 mV was delayed by amiloride for $Ca_V3.1$ and $Ca_V3.3$, but not for $Ca_V3.2$ channels (Table 2). These results are in agreement with the observation that amiloride binding to partially inactivated $Ca_V3.3$ channels is weaker than that to closed channels (Fig. 4).

Amiloride Blocks the Closed State of Ca_V3 Channels. To investigate the effect on the closed state of the channels, we used a protocol that promotes the interaction of the amiloride with the resting state of the channel. This was achieved by obtaining baseline measurements of peak currents at −30 mV with step depolarizations applied every 10 s, exposing cells to amiloride for 3 min without channel stimulation, and, finally, measuring the current amplitude after resuming stimulation, but in the presence of amiloride. Examples of this type of experiments for the three Ca_V3 channels are displayed in Fig. 6. After exposure to amiloride for 2 to 3 min in the absence of depolarizing steps, peak current of the first pulse after resumption of step depolarizations to −30 mV was practically of the same amplitude as that reached after steady-state block (usually periods of 2–3 min continuously depolarizing to −30 mV) for $Ca_V3.2$ (Fig. 6B) and $Ca_V3.3$ (Fig. 6C) channels. A second nonstimulation period of time (2–3 min) did not relieve any block. This was accomplished only after washing with control solution. In contrast, the block by amiloride induced a discrete use-dependence effect only on $Ca_V3.1$ channels. In this case, when depolarizations were resumed, the peak current was initially inhibited by approximately 47% (Fig. 6A), but the extent of inhibition increased with subsequent test pulses to a maximum of 52.3%. On average, the small, but significant, increase in percentage block was from 49.6 ± 2.5 to 58.9 ± 2.5 ($P < 0.05$, $n = 4$) (Fig. 6D). These results indicate that amiloride strongly targets the closed state of the three Ca_V3 channels and that only $Ca_V3.1$ channels exhibit a small component of use-dependent block by amiloride.

To further investigate the use-dependent block of amiloride on $Ca_V3.1$ channels, we measured the block of calcium currents at −30 mV under different stimulation frequencies. Trains of 35 pulses at −30 mV were applied in control and amiloride conditions at 1 and 3 Hz for $Ca_V3.1$ channels. Figure 6E illustrates traces 1, 2, and 35 of the applied train at 3 Hz in HEK-293 cells expressing $Ca_V3.1$ channels, before (Control) and after exposure to 2 mM amiloride. During drug exposure, cells were depolarized to −30 mV at the usual frequency (0.1 Hz) until steady-state block was reached. The normalized remaining current after the 35th pulse, was clearly smaller with amiloride. In control conditions, there was a 14% decrease in the $Ca_V3.1$ peak current upon the first few pulses with the 1-Hz train and 35% when it was stimulated at 3 Hz (Fig. 6, F and G, blue circles). This was certainly due to the accumulation of inactivated

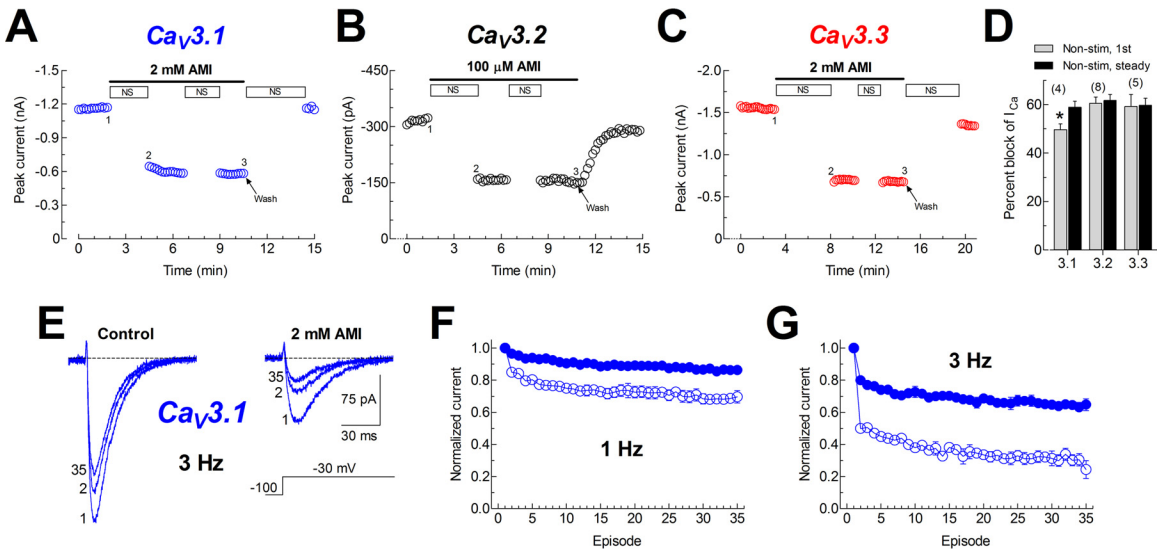

Fig. 6. Strong closed-state block and poor use-dependent block of Ca_V3 channels by amiloride. A–C, amiloride inhibits Ca_V3 currents without channel stimulation. Currents were activated every 10 s by 10-ms voltage steps to −30 mV from a HP of −100 mV. After recording baseline currents (1), cells were superfused with the indicated amiloride concentrations for 3 or 5 min with no stimulation of channels. Depolarizing steps were then resumed (2) in the continued presence of amiloride. A second nonstimulation (NS) period of time was introduced, still in the presence of the drug; then depolarizations were resumed to reach a steady-state block (3). Finally, recovery of blocked current (Wash) was achieved with stimulation of $Ca_V3.2$ channels (B) or without it for $Ca_V3.1$ (A) and $Ca_V3.3$ (C) channels. Note that only $Ca_V3.1$ channel current amplitude decreased after resumption of step depolarizations (from 2 to 3) in the presence of amiloride, which implies a discrete component of use-dependent block by amiloride. D, average percentage block of Ca_V3 currents by amiloride recorded at the first pulse after resumption of step depolarizations (gray columns) and after steady-state block was reached (black columns). ∗, statistical significance with a Student's t test ($P < 0.039$). The number of cells is indicated in parentheses. E–G, use-dependent block of $Ca_V3.1$ channels by amiloride. E, examples of $Ca_V3.1$ currents elicited by test pulses to −30 mV applied at a frequency of 3 Hz in the absence (left traces) and the presence (right traces) of 2 mM amiloride. A train of 35 pulses was applied under each experimental condition; for clarity only the traces 1, 2, and 35 are shown. The peak of each pulse was normalized to the peak of the first pulse for each experimental condition, and the averaged values for $Ca_V3.1$ ($n = 3$ cells) were plotted for 1-Hz trains (F) and 3-Hz trains (G).

channels during the depolarizing train. Amiloride induced an additional reduction in the peak current, which is the combination of inactivated and blocked channels. Here, use-dependent block was calculated by subtracting the fraction of current remaining after the 35th pulse in control conditions from that recorded in the presence of the drug. Thus, the use-dependent block observed for $Ca_V3.1$ channels was 16.7 ± 2.2% at 1 Hz and 40.4 ± 6.6% at 3 Hz ($n = 4$ and 3; Fig. 6, F and G, ○). Effects much more discrete were obtained in similar experiments with $Ca_V3.2$ and $Ca_V3.3$ channels (not shown).

Intracellular Block by Amiloride Is Rather Weak on $Ca_V3.2$ Channels. Finally, to gain further insights into the interaction site of amiloride with the Ca_V3 channels protein, we decided to measure intracellular block of Ca_V3 channels by adding the drug in the pipette solution instead of bathing the cells with it. Baseline measurements of the current could not be obtained in these experiments, because the drug inhibited the peak current after the second test pulse to −30 mV; therefore, the percentage block was calculated by normalizing the peak current recorded when steady-state block was reached to that of the first test pulse. The results are summarized in Fig. 7. Representative current recordings obtained at −30 mV are shown for each Ca_V3 channel before (Control) and after exposure to the indicated concentrations of amiloride either when it was available from the extra- or intracellular side (Fig. 7A). The fraction of blocked current in $Ca_V3.1$ and $Ca_V3.3$ channels was quite similar regardless of the amiloride location; in contrast, currents carried by $Ca_V3.2$ channels were dramatically less sensitive to amiloride block from the intracellular side. The percentage block of the peak current at −30 mV was 72 ± 2% (calculated with the respective Hill function from Fig. 1C) when amiloride was bathing the cells, and only 14 ± 4% ($n = 8$) when the drug was added to the pipette recording solution (Fig. 7B). These results suggest that amiloride binds with a higher affinity to a site more accessible from the extracellular face of the $Ca_V3.2$ channel protein; conversely. the amiloride block of $Ca_V3.1$ and $Ca_V3.3$ channels is practically the same from both sides.

Discussion

The present study elucidates the preferential inhibition of human $Ca_V3.2$ channels by the diuretic amiloride and the blocking mechanism by which this drug exerts a differential block of human Ca_V3 channels. We also have obtained insights about two different binding regions of amiloride in the protein of $Ca_V3.2$ channels. The preferential inhibition of $Ca_V3.2$ channels by amiloride reported here contributes to explaining previous differences in the amiloride concentration observed to block native T-type currents. The data also suggest that amiloride is the only organic blocker to be selective for any T-type channel.

Block of T-Type Channels by Amiloride. Amiloride blocked the three human Ca_V3 channels in a concentration-dependent manner and exhibited higher potency blocking of $Ca_V3.2$ channels ($IC_{50} = 62$ μM), whereas $Ca_V3.1$ and $Ca_V3.3$ were more than 10-fold less sensitive to the inhibitory effect of the drug (Table 1). The Hill coefficients obtained for the blocking relationship of Ca_V3 channels had values less than 1, suggesting that amiloride might be binding to more than one site in the protein of these channels. Blocking of T-type currents by amiloride was originally reported in mouse N18 cells (Tang et al., 1988). The IC_{50} reported then was 30 μM,

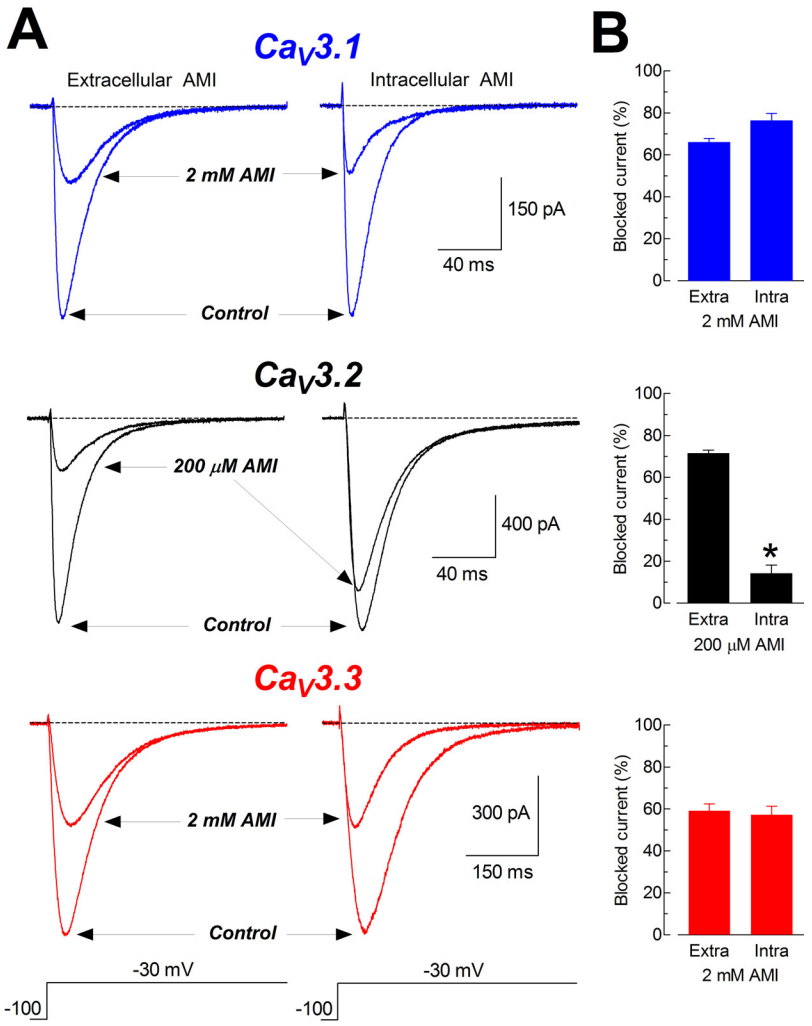

Fig. 7. Comparison between extracellular and intracellular block by amiloride. A, representative traces of calcium currents recorded at −30 mV, showing the steady-state block of amiloride (smaller currents) for the three Ca_V3 channels. Amiloride was added to the bath (Extracellular AMI) or to the recording pipette (Intracellular AMI) at the same concentration for each type of channel. Scales on the left are also for the right traces, with the exception of $Ca_V3.1$ recordings for which the amplitude scale for the left traces is 250 pA. B, percentages of blocked current by extracellular and intracellular amiloride. Columns, means from seven to nine cells; bars, S.E.M. Data for extracellular block was calculated from the concentration-response curves illustrated in Fig. 1C. *, statistical significance with a Student's *t* test ($P < 0.0001$).

and concentrations up to 500 μM had only a discrete inhibitory effect on HVA calcium current. Later, an IC_{50} of 1.55 mM was reported in GH3 cell T-type currents (Herrington and Lingle, 1992). On the basis of our results, we suggest that N18 cells express mainly $Ca_V3.2$ channels (the most sensitive), whereas the T-type current of GH3 cells must be carried mainly by $Ca_V3.1$ channels. In fact, there is already evidence indicating a high expression of $Ca_V3.1$ mRNA in GH3 cells (Mudado et al., 2004). In addition, the observation from Lacinová et al. (2000) showing that 5 mM amiloride only blocked 38% of the mouse $Ca_V3.1$ current implies a species-dependent block for $Ca_V3.1$ channels, whereas the human clone studied here displays higher sensitivity to the diuretic (Table 1). Regarding $Ca_V3.3$ channels, the thalamic nucleus reticularis neuron is one of the very few cell types expressing robust, well defined $Ca_V3.3$ currents (Huguenard and Prince, 1992; Lee et al., 1999), which correlates with the preferential expression of $Ca_V3.3$ mRNA (Talley et al., 1999). Amiloride (500 μM) blocks only 41% of the T-type current in such rat neurons (Huguenard and Prince, 1992). The present work is the first reporting the block of human $Ca_V3.3$ channels by amiloride, and it shows a sensitivity similar to that reported for rat currents (Table 1). In summary, $Ca_V3.2$ channels are the most sensitive T-type channels to amiloride followed by $Ca_V3.1$ and $Ca_V3.3$ channels; in addition, this differential block correlates well with the sensitivity of native T-type currents to amiloride and establishes that this diuretic is the only organic blocker to be selective for any T-type channel.

Mechanism of Ca_V3 Channel Block by Amiloride. To elucidate the mechanism by which amiloride blocks Ca_V3 channels, we studied in detail the biophysical properties of these channels. Even though $Ca_V3.2$ channels were the most sensitive to amiloride block, there was no evidence of any voltage dependence of the block. The mechanism of block of T-type currents by amiloride was partially studied in guinea pig ventricular myocytes (Tytgat et al., 1990). Because the human $Ca_V3.2$ subunit is expressed in human heart (Cribbs et al., 1998; Williams et al., 1999) and T-type currents of heart cells are highly sensitivity to amiloride (for review, see Perez-Reyes, 2003), it is likely that the current studied by Tytgat et al. (1990) corresponds to that carried mainly by $Ca_V3.2$ channels.

Here, we found that amiloride induces a strong voltage dependence of block in $Ca_V3.3$ channels, characterized by a 7.5-mV shift in the activation curve (Fig. 2). On the contrary, in $Ca_V3.1$ and $Ca_V3.2$ channels, this effect was absent. Of interest, we have shown recently that block of Ca_V3 channels by niflumic acid is also voltage dependent only in $Ca_V3.3$ (Balderas et al., 2012). Sequence alignments of Ca_V3 channel proteins indicates that $Ca_V3.3$ channels share approximately 80% identity with $Ca_V3.1$ and $Ca_V3.2$, but the sequence identity of those two is higher (~ 90%) (Perez-Reyes, 2003); these

differences might be the molecular substrate for the voltage-dependent effects observed exclusively in $Ca_V3.3$ channels. Unfortunately, there are no crystal structures of Ca_V3 channels available; therefore, it will be very relevant to test this hypothesis by using computational approaches to predict three-dimensional structures of these proteins to performing modeling, docking, and site-directed mutagenesis studies.

We also found that amiloride targets mainly the closed state of all three Ca_V3 channels, although a small component of use- and state-dependent block for $Ca_V3.1$ channels was also detected (Figs. 4 and 6). Closed-state block is evidenced by tonic channel inhibition observed after exposing channels to the drug for a period of time long enough to produce steady-state block but without the application of step depolarizations (Fig. 6, A–D); except for $Ca_V3.1$ channels, no further block was obtained when stimulation of the channel was resumed. In addition, stimulation at frequencies higher than 0.1 Hz improved amiloride blocking only in $Ca_V3.1$ channels, whereas the effect was modest on $Ca_V3.2$ and virtually absent in $Ca_V3.3$ channels (Fig. 6, E–G). These observations indicate that amiloride blocking is favored when $Ca_V3.1$ channels are frequently activated (i.e., in the open state conformation). Additional evidence about the preferential binding of amiloride to the closed state was obtained from the recovery from inactivation experiments mainly in $Ca_V3.3$ channels. In comparison with control conditions, the fraction of channels available to be activated after 3 ms at −70 mV increased to almost double in the presence of amiloride (Fig. 5F). However, the fraction of current recovered for $Ca_V3.1$ and $Ca_V3.2$ channels was not modified by amiloride (Table 2). In addition, the percentage of blocked current by the diuretic at −30 mV from a HP of −70 mV was smaller than that at a HP of −100 mV (Fig. 4). Thus, this biophysical property reveals that amiloride prefers binding to the $Ca_V3.3$ channel closed state over the inactivated state. In this regard, $Ca_V3.3$ channels share this property with epithelial sodium channels, for which it has been reported that hyperpolarization of the membrane increases the affinity for amiloride (Palmer, 1984; Warncke and Lindemann, 1985). However, the state-dependent enhancement observed with amiloride for $Ca_V3.3$ channels has not been reported previously for any drug or inorganic blocker in Ca_V channels; the only similar effect described until now is for an A-type potassium current by 4-aminopyridine in mouse neurons (Jackson and Bean, 2007). Nevertheless, we also observed a moderate effect of amiloride on the inactivated state of $Ca_V3.3$ channels, evidenced by a smaller fraction of current recovered at −100 mV (approximately 20% less) in the presence of amiloride (Fig. 5B; Table 2).

A striking component for the mechanism of block of Ca_V3 channels by amiloride was the intracellular effect of the diuretic. Whereas current reduction was very similar in $Ca_V3.1$ and $Ca_V3.3$ channels regardless of the amiloride application (i.e., extracellular or intracellular), for $Ca_V3.2$ channels the block was 6- to 7-fold stronger when amiloride was applied from the extracellular side of the channel (Fig. 7). Besides the novelty of this observation, it also implies that under our experimental conditions amiloride does not escape from the cell by diffusion across the cell membrane. The possibility that amiloride could diffuse out of the cell, be diluted in the bath, and therefore never reach a concentration in the vicinity of the extracellular site to produce appreciable binding and block must be ruled out because if that were the case, the percentage of intracellular block must be equally poor for the tree Ca_V3 channels, rather than just for $Ca_V3.2$ as shown by our results (Fig. 7). Amiloride is an organic base that at physiological pH (pK_a of 8.4) exists as an organic cation (Simchowitz et al., 1987), although several studies have shown that it can act as a permeant weak base (Benos et al., 1983; Briggman et al., 1983). A tertiary or uncharged drug that can leave the cell by diffusion across the cell membrane, amiloride frequently requires at least 10-fold more drug when it is applied through the pipette to produce a block comparable with that induced with the drug in the bath (Bergson et al., 2011). Here, our data indicate that the same amiloride concentration acting from either side of the cell membrane produces comparable blocking effects for $Ca_V3.1$ and $Ca_V3.3$ channels but not for $Ca_V3.2$ channels. Therefore, the reduced intracellular block of $Ca_V3.2$ must be due to the lower affinity of amiloride for the intracellular site rather than a lower concentration of the drug near the channel.

In conclusion, our data suggest that amiloride acts as a pore channel blocker of the Ca_V3 channel proteins. Among those, the diuretic binds with a higher affinity to $Ca_V3.2$ channels (lower IC_{50}). Remarkably, the results also suggest a negative allosteric interaction with at least two putative binding sites with different affinities. In addition, there is an additional intracellular binding site for $Ca_V3.2$ channels with a lower affinity than the extracellular one. To date, amiloride is the only organic blocker to be selective for any T-type channel.

Acknowledgments

We thank Dr. E. Perez-Reyes at University of Virginia for thoughtful comments and discussion of the manuscript and A. M. Escalante-Gonzalbo, F. Perez-Eugenio, and C. E. Diaz-Velásquez at Instituto de Fisiología Celular, Universidad Nacional Autónoma de México, for expert technical assistance.

Authorship Contributions

Participated in research design: Rivera and Gomora.
Conducted experiments: Lopez-Charcas.
Contributed new reagents or analytic tools: Rivera.
Performed data analysis: Lopez-Charcas and Gomora.
Wrote or contributed to the writing of the manuscript: Lopez-Charcas, Rivera, and Gomora.

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