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Short Communication

Removal of the outermost arginine in IVS4 segment of the $Ca_V3.1$ channel affects amplitude but not voltage dependence of gating current

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Abstract. Positively charged amino acids in S4 segments of voltage-dependent $Ca_V3.1$ channel form putative voltage sensor. Previously we have shown that exchange of uppermost positively charged arginine in IVS4 segment for cysteine (mutation R1717C) affected deactivation and inactivation, but not activation of macroscopic current. Now we compared gating currents from both channels. Maximal amplitude of charge movement in R1717C channel decreased but voltage-dependent characteristics of charge movement were not significantly altered. We concluded that mutation of R1717C affects the coupling between S4 activation and pore opening, but not the S4 activation itself.

Key words: T-type calcium channel — Charge movement — $Ca_V3.1$ channel — S4 segment — Channel gating

Voltage-dependent calcium channels including T-type calcium channels are composed of four homological domains each containing six transmembrane segments S1–S6 (Lacinova 2005). S4 segments containing 5–6 basic amino acids form putative voltage-sensing part of the channel. According to currently accepted model (Bezanilla 2008) membrane depolarization causes an outward movement of S4. This movement represents an initial step in channel activation followed by a conformational change of pore region formed by segments S5–S6 and short pore loop between them (Swartz 2008). This conformational change results in opening ion conducting pathway manifested by an activation of macroscopic inward current. Inflow of calcium ions is terminated by transition of open channels into inactivated and/or closed conformation. Before the channels could be reactivated, S4 segments must return to their initial positions. Because S4 segments contain 5–6 positively charged amino acids, their ON- and OFF-movement can be monitored in absence of inward currents as so-called gating current or charge movement (Lacinova et al. 2002; Talavera and Nilius 2006).

Previously we have investigated role of S4 segments in activation, inactivation and deactivation of the $Ca_V3.1$ channel using channel mutants in which uppermost basic arginines were one by one replaced by neutral cysteines (Kurejova et al. 2007). These mutations have affected basic characteristics of macroscopic inward current, i.e., process of opening, closing and inactivation of the channel pore, in a complex way. Because these mutations did manipulate directly the putative voltage sensor and its net charge, alterations of charge movement measured from mutated channel can provide additional information about underlying mechanism. Therefore we extended this analysis by comparing gating currents originating from $Ca_V3.1$ channel (Klugbauer et al. 1999) and mutant R1717C channel (Kurejova et al. 2007) in which uppermost arginine R1717 in IVS4 segment was replaced by cysteine.

Both channels were permanently transfected in HEK 293 cells. Ion and gating currents were measured by HEKA-10 patch clamp amplifier (HEKA Electronic, Lambrecht, Germany) in whole cell configuration. The extracellular solution contained (in mM): CsCl 105, HEPES 10, glucose 10, TEA-Cl 40, $CaCl_2$ 2, $MgCl_2$ 1, pH 7.4 (CsOH). The

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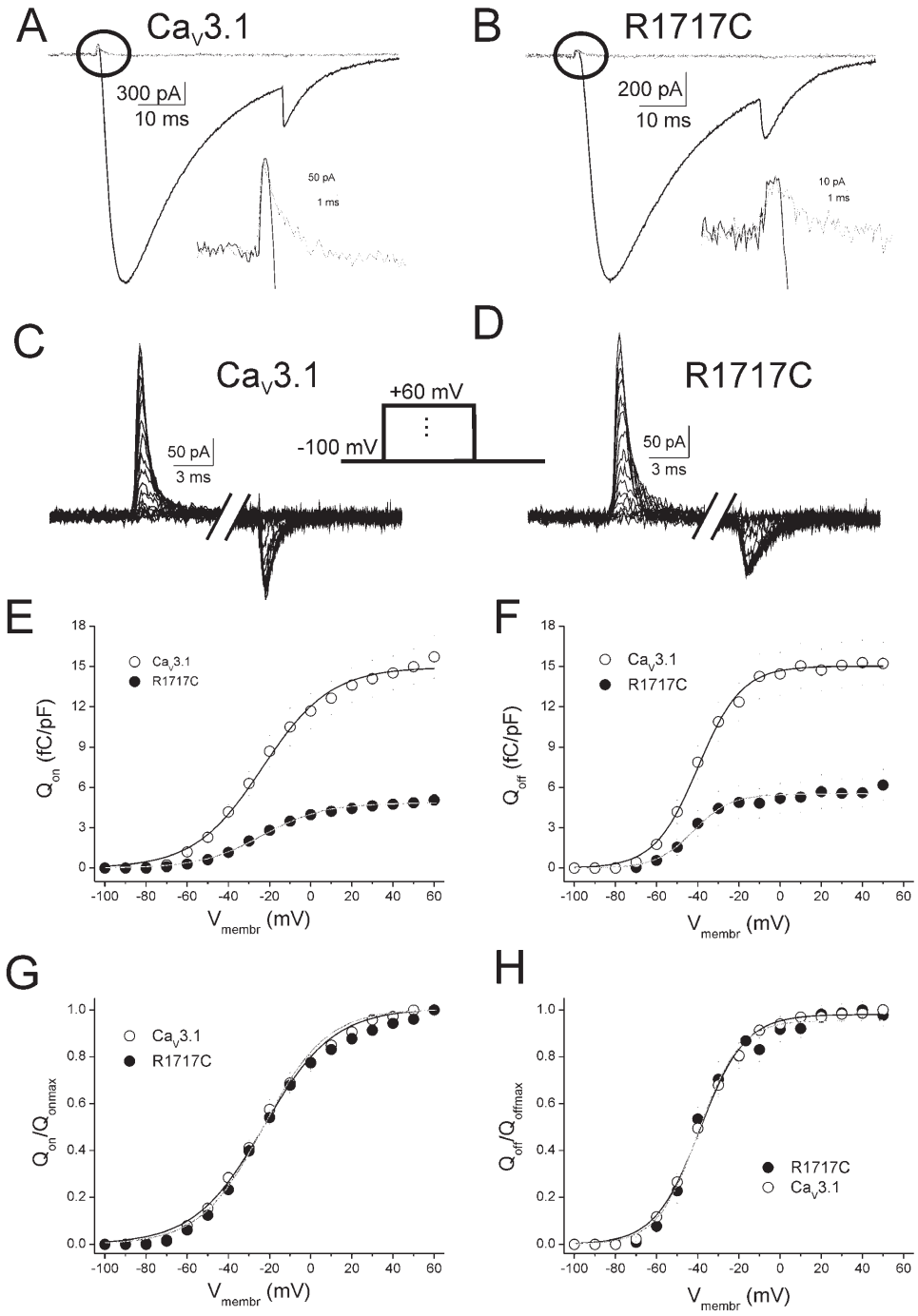

Figure 1. Effect of removal of uppermost arginine R1717 in IVS4 segment on voltage dependence of gating currents. Traces of inward currents activated by a pulse to –30 mV (maximum of an I-V relationship) under control conditions (solid black lines) and in equilibrium with 30 μM of Er^{3+} (dashed grey lines) measured from the $Ca_V3.1$ (**A**) and R1717C (**B**) channels. Initial phase of current activation (marked by circles) is enlarged in insets. Exemplar gating currents measured from cells expressing $Ca_V3.1$ channels (**C**) and R1717C channels (**D**) activated by a series of 40 ms long depolarizing pulses from a holding potential (HP) of –100 mV to voltages between –100 and +60 mV. Voltage protocol is shown between panels. **E**. Averaged Q_{on} values evaluated by integrating the area under the gating current traces at the beginning of each depolarizing pulse. Solid lines are fits of experimental data by the Boltzmann equation. **F**. Averaged Q_{off} values evaluated by integrating the area under the gating current traces at the end of each depolarizing pulse. Solid lines are fits of experimental data by the Boltzmann equation. To facilitate comparison of voltage dependencies Q-V relations from panels E and F were normalized in respect to their maximal values in panels **G** and **H**.

intracellular solution contained (in mM): CH_3SO_3Cs 130, EGTA 10, $MgCl_2$ 5, TEA-Cl 10, Na-ATP 5, and HEPES 10, pH 7.4 (CsOH). For measurement of gating current inward currents were blocked by 30 μM Er^{3+}. 10 mM stock solution of $ErCl_3$ was prepared daily in deionized water and diluted in the bath solution to a desired concentration prior to the experiment. When measuring gating currents three identical pulses were averaged for each trace. Linear component of leak current and capacity transients were subtracted using the P/8 procedure. Averaged data are presented as mean ± S.E.M.

In our previous analysis of macroscopic inward current (Kurejova et al. 2007) we have shown that replacement of uppermost arginine in the domain IV affected current deactivation and inactivation stabilizing open state of conducting pore and decreasing voltage sensitivity of both processes. However, it did not affect current activation and recovery from inactivation. Phenomena, which have been described in Kurejova et al. (2007) were related to activity of channels conducting pore. In present work we looked at a preceding step, i.e., at a movement of S4 segments. As demonstrated in the Fig. 1A and B, 30 μM Er^{3+} completely inhibited inward current without affecting amplitude of gating current. Upon inhibition of inward calcium current by Er^{3+} we recorded families of gating currents from the wild type $Ca_V3.1$ channel (Fig. 1C) and from the mutant R1717C channel (Fig. 1D). Total charge transferred during each pulse was evaluated by integrating the area below gating current trace at the beginning (Q_{on}) and after the end of each depolarizing pulse (Q_{off}). Density of inward current amplitude measured in a peak of I-V relation I_{max} decreased 2.5-fold (Table 1). Previous analysis of macroscopic inward current (Kurejova et al. 2007) did not allow distinguish whether this effect was due to decreased number of channels targeted into the surface membrane or due to altered opening probability of the channel. Maximal amplitude of charge movement Q_{max} normalized in respect to cell capacity was three-fold lower in mutant channel (Fig. 1E, F and Table 1). Ratio I_{max}/Q_{max} was significantly increased by R1717C mutation (Table 1).

Maximal inward current is proportional to the number of channels available for opening, unitary current amplitude and opening probability of a single channel. Maximal charge movement is proportional to the number of available channels and total charge transferred during activation of a single channel. Value of I_{max}/Q_{max} is considered to be a measure of opening probability (Agler et al. 2005; Baumgart et al. 2008). Such approximation is valid if we can consider unitary current amplitude and total charge *per* channel to be constant. Because replacement of charged arginine by neutral cysteine did decrease total charge in R1717C channel, this decrease accounts at least for part of observed I_{max}/Q_{max} increase. Increased opening probability of the R1717C channel may contribute another part, nevertheless, from our data we cannot make equivocal conclusion.

Voltage dependence of ON and OFF charge was not altered in R1717C channel (Fig. 1G, H and Table 1). Lack of alteration of Q_{on}-V relation corresponds to lack of such shift in I-V relations of both channels (Kurejova et al. 2007). Nevertheless, previously we have shown that voltage dependence of inactivation of macroscopic current was significantly shifted to more negative membrane potentials and it's slope factor significantly increased in R1717C channel (Kurejova et al. 2007), while parameters of OFF-gating were not altered (Fig. 1H and Table 1). We can speculate that return movement of S4 segments is related to channel closing rather than to channel inactivation. Our previous finding that prolonged depolarization does not immobilize Q_{off} (Lacinova et al. 2002) supports this suggestion. Therefore we analyzed in more details parameters of OFF-gating.

We activated gating current by 40 ms long depolarizing pulse to +30 mV, i.e., to a potential at which charge movement is already saturated. Repolarization potential varied between –100 mV and –20 mV. Representative families of gating current recorded under such conditions are shown in Fig. 2A and B. Total charge transferred during repolarization is drawn as a function of repolarizing potential in Fig. 2C. Kurejova and coauthors (2007) have shown tail current amplitude sharply decreases with increasing repolarization potential (see also Fig. 2E). In contrast, total OFF-charge was constant between –100 and –80 mV and decreased at membrane potential

Table 1. Comparison of basic properties of charge movement measured from wild type $Ca_V3.1$ channel and mutant R1717C channel

	$Ca_V3.1$	R1717C
I_{max}/C (pA/pF)	106 ± 7 (16)	42 ± 3*** (16)
Q_{onmax}/C (fC/pF)	15.6 ± 1.3 (16)	5.0 ± 0.4*** (16)
I_{max}/Q_{onmax} (pA/fC)	7.3 ± 0.5 (16)	9.1 ± 0.4* (17)
Q_{on} $V_{0.5}$ (mV)	–20.2 ± 2.7 (16)	–21.7 ± 1.8 (17)
Q_{on} dV (mV)	17.1 ± 1.3 (16)	15.3 ± 0.9 (17)
Q_{off} $V_{0.5}$ (mV)	–38.6 ± 1.7 (16)	–37.7 ± 3.5 (6)
Q_{off} dV (mV)	10.4 ± 0.8 (16)	10.5 ± 1.6 (6)

I_{max}, averaged amplitude of inward current activated by depolarization to –30 mV, i.e., to maximum of a current-voltage relationship; C, capacitance of individual cells; Q_{onmax}, averaged maximal ON-charge; $V_{0.5}$, dV, half-maximal activation voltages and slope factors of voltage dependencies of activation of ON- (Q_{on}) and OFF- (Q_{off}) charge movements. These parameters were obtained by fitting the data in the Fig. 1E and F by Boltzmann equation. Significance of the difference between $Ca_V3.1$ and R1717C channels was tested by unpaired Student's *t*-test. *** $p < 0.001$; * $p < 0.05$. Number of cells tested is given in brackets.

higher as –80 mV only. Dependence of OFF-charge on repolarization potential was virtually identical for $Ca_V3.1$ and R1717C channels (Fig. 2C). Kinetics of Q_{off} was analyzed for repolarizing potentials up to –50 mV by fitting of OFF-gating current traces by monoexponential function. At higher membrane potentials gating current amplitude was too small for reliable fitting. As shown in the Fig. 2D, time constants of OFF-gating were virtually

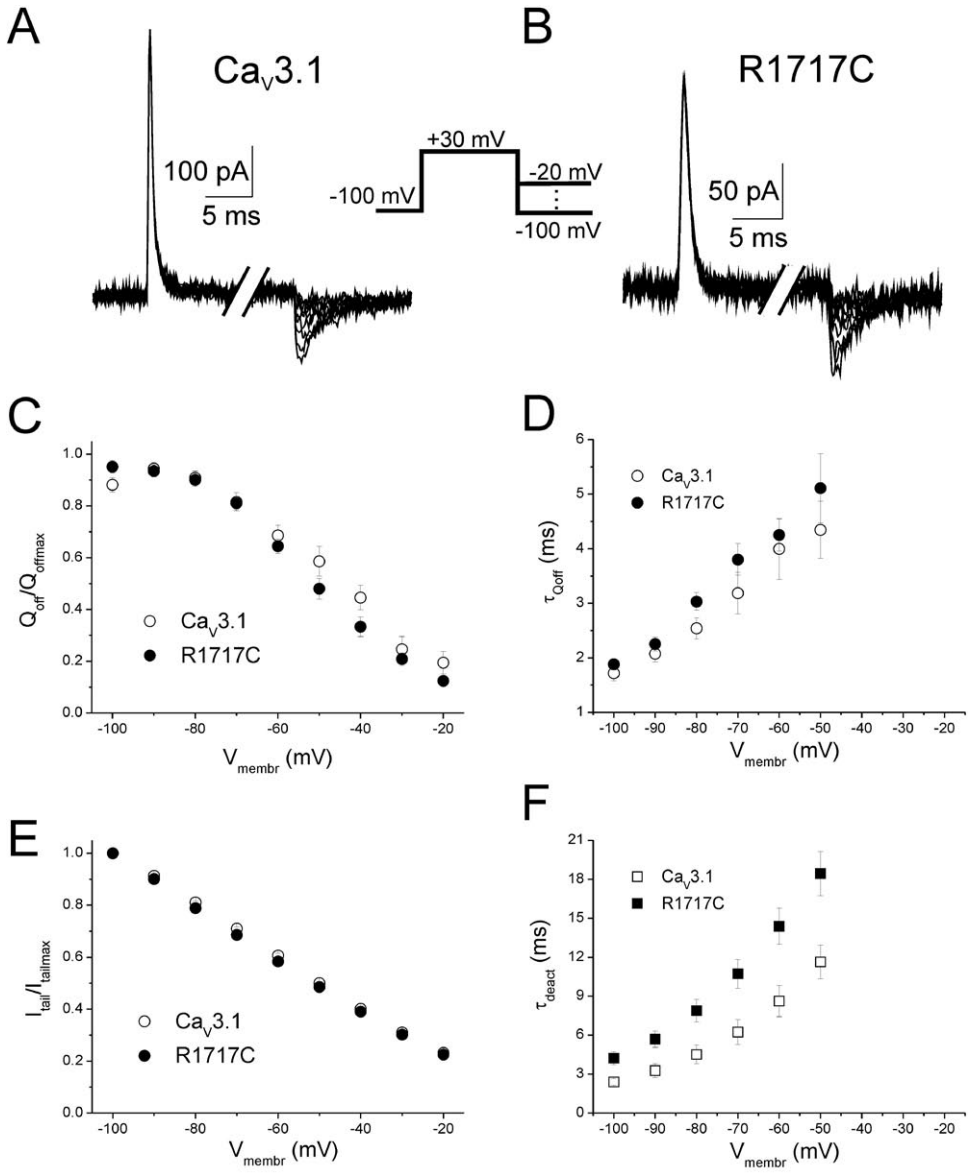

Figure 2. Effect of increasing membrane potential on the amplitude and kinetics of OFF-charge. Recordings of gating currents measured from cells expressing $Ca_V3.1$ channels (**A**) and R1717C channels (**B**) by series of 40 ms long depolarizing pulses from a HP of –100 mV to the membrane potential of +30 mV followed by repolarization to membrane voltages increasing from –100 mV to –20 mV. Voltage protocol is shown between panels. Total OFF-charge was evaluated by integrating the area under the gating current traces at the end of each depolarizing pulse. Dependence of total OFF-charge on repolarization voltage normalized in respect to maximum is shown in the panel **C**. Panel **D** shows voltage dependencies of time constants of OFF-gating. Time constants were evaluated by fitting time course of Q_{off} traces by single exponential. 10 cells were averaged for both channels. Normalized amplitudes of tail currents measured in the absence of Er^{3+} at the same repolarizing voltages are shown in the panel **E**. Voltage dependencies of deactivation time constants of tail currents measured without inward current block are shown for comparison in the panel **F**. For analysis of tail currents same voltage protocol was used as in experiments shown in panels A–D except for the length of depolarizing pulse, which was 10 ms.

identical for $Ca_V3.1$ and R1717C channels and increased with increasing membrane potential. Such voltage dependence sharply contrasts with voltage dependencies of deactivation time constants (see Kurejova et al. 2007 and Fig. 2F), which differed significantly ($p < 0.001$) at each investigated repolarization potential. Further, dependence of deactivation time constant on membrane potential was clearly exponential (Fig. 2F) while voltage dependence of Q_{off} time constant (Fig. 2D) was nearly linear in analyzed interval of membrane potentials. These differences allow us to suggest that closing of conducting pore and OFF-charge movement of the $Ca_V3.1$ channel are independent processes.

Altogether, analysis of gating currents from the $Ca_V3.1$ and R1717C channels supplied following additional information about mutated channel properties: i) decreased current density in R1717C channel is caused mostly by decreased number of channels targeted into surface cell membrane while minor increase in opening probability of the mutated channel cannot be either confirmed or excluded; ii) characteristics of Q_{off} differ from characteristics of both inactivation and deactivation of macroscopic inward current suggesting that closing/inactivation of channel pore and returning movement of S4 segments are independent processes; iii) R1717C mutation affected coupling between voltage sensor activation and pore opening rather than activation of voltage sensor itself.

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