

The role of LRRC8 in the hypotonic stress response of human keratinocytes

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Cultured human keratinocytes (HaCaT) were kindly provided by BRAIN Biotech AG



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Summary

The human skin is constantly exposed to various stress factors such as temperature changes, mechanical stress, different humidity levels, air pollution or radiation. These factors can have a tremendous impact on the skin and can contribute to barrier disruption and inflammation, dry and fragile skin as well as premature ageing¹. Recent advances in different research areas point to an important role of LRRC8 volume regulated anion channels (VRACs) in a plethora of different physiological processes². The function of LRRC8 has been characterized in human keratinocytes and in the native human epidermis³ and the LRRC8 ion channel has been proposed to be a novel molecular target to modulate keratinocyte differentiation in a recent patent⁴.

LRRC8A (also named SWELL1) has been identified as the first essential component of VRACs in various cell types^{5,6}. LRRC8A is composed of four transmembrane domains and a C-terminal domain containing up to 17 leucine-rich repeats^{7,8}. Together with four additional LRRC8 family members (LRRC8B-E) it assembles into hetero-hexameric complexes^{5,6}. Interestingly, the LRRC8 subunit composition differs between cell types and influences VRAC properties such as inactivation kinetics, voltage-dependence and selectivity of the transported osmolyte⁹⁻¹³. The generation of LRRC8A^{-/-} knockout HaCaT keratinocytes has provided evidence for the essential function of LRRC8A in hypotonic stress response of human keratinocytes³.

In this Application Note we show electrophysiological data from WT and LRRC8A^{-/-} knockout HaCaT keratinocytes which corroborate the essential function of LRRC8A in keratinocytes.

Results

LRRC8A expressed in wild type HaCaT keratinocytes was activated by hypotonic solution and blocked by DIDS on the Patchliner. In contrast, no activation by hypotonic solution was observed in the LRRC8A^{-/-} knockout HaCaT keratinocytes (Figure 1).

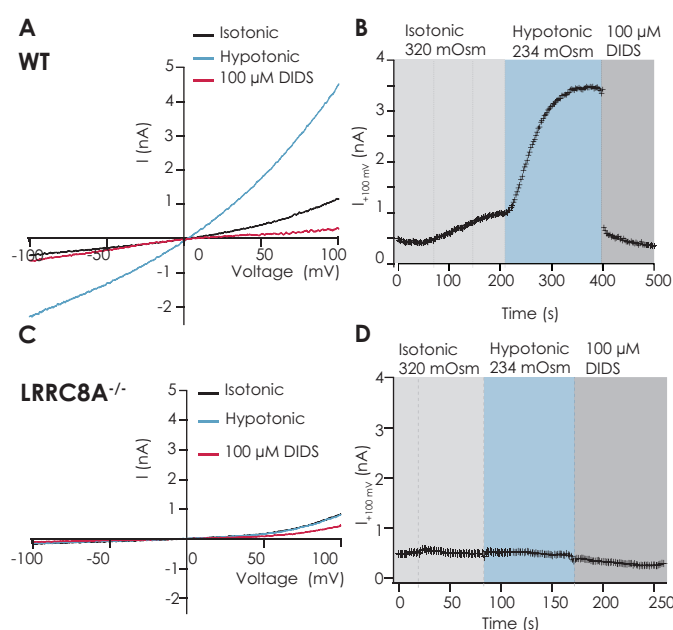


Figure 1: Typical recordings from WT and LRRC8A^{-/-} KO HaCaT keratinocytes. **A** LRRC8 current was recorded using a voltage ramp protocol from -100 mV to +100 mV over 200 ms. Hypotonic solution had an osmolarity of 234 mOsm, isotonic solution had an osmolarity of 320 mOsm. **B** Timecourse of the experiment shown in A, after addition of hypotonic solution, there was a large increase in current at both +100 mV and -100 mV (not shown). The LRRC8 current was blocked by DIDS. **C** There was no activation of LRRC8 current in the knockout keratinocytes recorded on the Patchliner using a voltage ramp protocol. **D** Corresponding timecourse of the experiment, there was no activation of LRRC8 current and no block of the current by DIDS.

Application Note

In 11/11 WT keratinocytes, an increase in current at -100 mV from -0.2 ± 0.02 nA to -1.2 ± 0.1 nA, an increase of over 400%, and at 100 mV from 0.7 ± 0.1 nA to 2.8 ± 0.3 nA, an increase of 360%, was observed (Figure 2, left). The current at -100 mV and +100 mV was blocked by DIDS in 11/11 cells tested. In LRRC8A^{-/-} keratinocytes, 7/8 cells did not respond to hypotonic solution (<100% increase in current) and a moderate decrease in current was observed upon addition of DIDS (Figure 2, right), although this was not statistically significant.

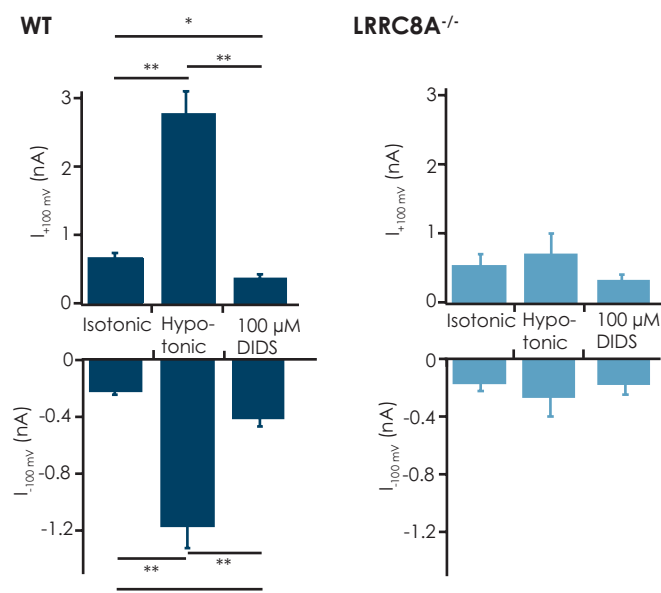


Figure 2: Effect of hypotonic solution on WT and KO keratinocytes on the Patchliner. Average current size in isotonic, hypotonic and in DIDS for WT (left) and LRRC8A^{-/-} (right) keratinocytes. **P < 0.05; **P<0.001, paired Student's *t* test.

LRRC8 was also measured using a voltage step protocol on the Patchliner (Figure 3). LRRC8 expressed in HEK and HCT116 cells has been shown to display prominent inactivation at positive potentials⁶. On the Patchliner, LRRC8 expressed in HaCaT keratinocytes was activated using hypotonic solution and also displayed inactivation at positive potentials above 100 mV (Figure 3A). In contrast, LRRC8A^{-/-} cells did not show any activation at any of the voltages tested in 5/5 cells (Figure 3B, right).

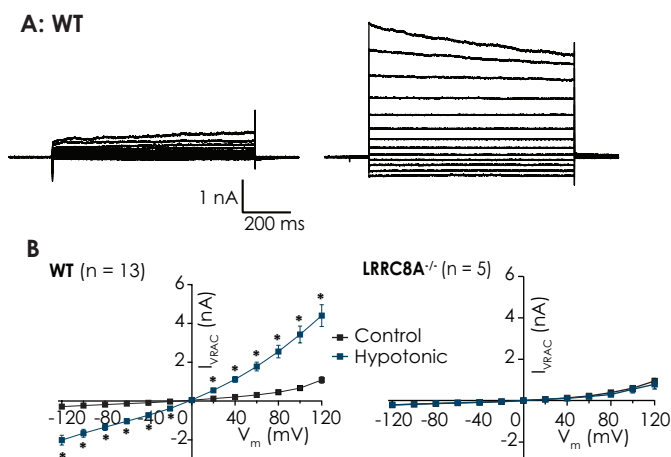


Figure 3: Effect of hypotonic solution on WT and LRRC8A^{-/-} HaCaT keratinocytes using a voltage step protocol on the Patchliner. A Traces from a representative cell in isotonic (left) and hypotonic (right) conditions. B Current-voltage-plot for an average of 13 WT cells (left) and 5 LRRC8A^{-/-} cells (right).

WT and LRRC8A^{-/-} HaCaT keratinocytes were also used on the high throughput automated patch clamp device, the [SyncroPatch 384](#). Figure 4 shows the hypotonic activated currents measured using a voltage ramp protocol and partial reversal following washout in isotonic solution.

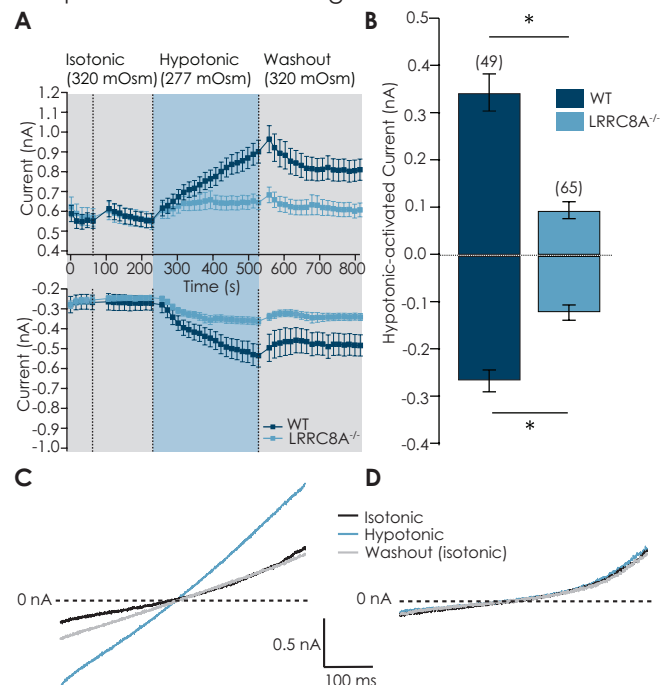


Figure 4: Hypotonic-activated current in WT and LRRC8A^{-/-} HaCaT keratinocytes on the SyncroPatch 384. A Current amplitude at 100 mV (top) and -100 mV (bottom) from WT (dark blue) and LRRC8A^{-/-} (light blue) HaCaT keratinocytes in isotonic (grey region), hypotonic (light blue region) and following washout (isotonic; grey region) is shown. B Average hypotonic-activated current amplitude at +100 mV and -100 mV for WT (dark blue) and LRRC8A^{-/-} (light blue) cells is shown as a bar graph, number of cells shown in brackets. The hypotonic-activated current was significantly larger in WT vs. LRRC8A^{-/-} cells (P<0.05, unpaired Student's *t* test).

Application Note

LRRC8 was also measured using a voltage step protocol on the SyncroPatch 384 (Figure 5). As shown on the Patchliner above, LRRC8 expressed in HaCaT keratinocytes was activated using hypotonic solution and also displayed inactivation at positive potentials above 100 mV (Figure 5B). In contrast, LRRC8A^{-/-} cells showed little activation upon addition of hypotonic solution (Figure 5C). The hypotonic-activated current was significantly larger in WT vs. LRRC8A^{-/-} cells for an average of 112 and 91 cells, respectively ($P < 0.05$, Student's *t* test; Figure 5D).

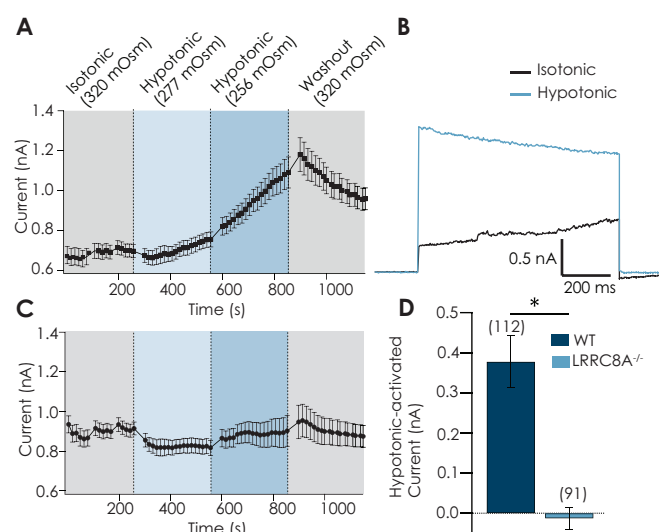


Figure 5: Hypotonic-activated current in WT and LRRC8A^{-/-} HaCaT keratinocytes on the SyncroPatch 384 using a voltage step protocol. **A** Current amplitude elicited using a voltage step to +100 mV in isotonic (grey) and hypotonic (blue) solution of WT HaCaT keratinocytes. **B** Example traces from a WT cell in isotonic (black) and hypotonic (blue) solution. **C** Current amplitude elicited using a voltage step to 100 mV in isotonic (grey) and hypotonic (blue) solution of LRRC8A^{-/-} HaCaT keratinocytes. **D** Average hypotonic-activated current amplitude at +100 mV for WT (dark blue) and LRRC8A^{-/-} (light blue) keratinocytes is shown as a bar graph, number of cells shown in brackets. The hypotonic-activated current was significantly larger in WT vs. LRRC8A^{-/-} cells ($P < 0.05$, unpaired Student's *t* test).

The hypotonic-activated current from WT HaCaT keratinocytes was partially blocked by the chloride channel blocker, NPPB (Figure 6) but not LRRC8A^{-/-} keratinocytes (Figure 6). In both WT and LRRC8A^{-/-} keratinocytes a moderate, but statistically significant, current was activated at different voltages on the SyncroPatch 384 upon addition of hypotonic solution (Figure 7). This current was more prominent in WT keratinocytes.

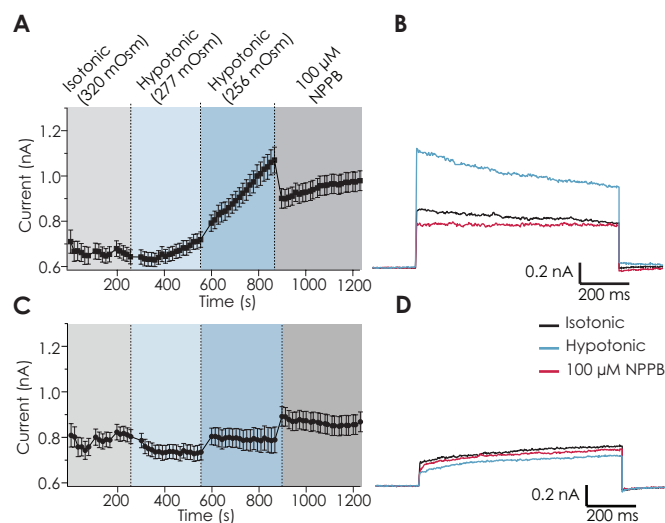


Figure 6: Hypotonic-activated current in WT and LRRC8A^{-/-} HaCaT keratinocytes on the SyncroPatch 384 and block by NPPB. **A** Average current amplitude of 108 cells elicited using a voltage step to +100 mV in isotonic (grey) and hypotonic (blue; 256 mOsm) solution of WT HaCaT keratinocytes was blocked by NPPB (dark grey). **B** Example traces from a WT cell in isotonic (black) and hypotonic (blue) solution and block by NPPB (red). **C** Average current amplitude of 103 cells elicited using a voltage step to +100 mV in isotonic (grey) and hypotonic (blue; 256 mOsm) solution of LRRC8A^{-/-} HaCaT keratinocytes. **D** Example traces from a LRRC8A^{-/-} cell in isotonic (black), hypotonic (blue) solution and NPPB (red).

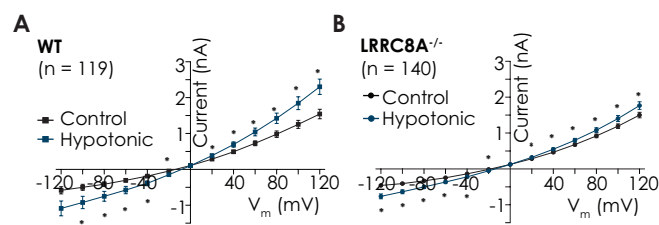


Figure 7: Effect of hypotonic solution on WT and LRRC8A^{-/-} HaCaT keratinocytes at different voltages. **A** Current voltage-plot for an average of 119 WT cells. **B** Current voltage-plot for an average of 140 LRRC8A^{-/-} cells.

The Patchliner and SyncroPatch 384 are automated patch clamp devices for recording from up to 8 or 384 cells, respectively. Both devices were used to record LRRC8 from keratinocytes in response to hypotonic stress. LRRC8A-E have been identified as volume-regulated anion channels in a number of cell types^{5,6}, which assemble into hetero-hexameric complexes. Cryo-EM structures have also recently confirmed the formation of LRRC8 ion channel pores¹⁴⁻¹⁶. The LRRC8A^{-/-} knockout HaCaT keratinocytes were created to investigate the function of LRRC8 in the hypotonic stress response of human keratinocytes.

Application Note

In previous studies, LRRC8 activity was measured, as well as cell volume changes indirectly by using the fluorescent halide-sensitive YFP and the volume-sensitive calcein-AM dye³. In this study we recorded LRRC8A from WT HaCaT keratinocytes which was activated by hypotonic solution and blocked by the broad-spectrum chloride channel blockers, DIDS or NPPB, on both the Patchliner and SyncroPatch 384. No LRRC8 current was recorded by addition of hypotonic solution of the LRRC8A^{-/-} HaCaT keratinocytes in 7/8 cells tested on the Patchliner, and a much smaller hypotonic-activated current was detected in the LRRC8A^{-/-} cells compared with WT recorded on the SyncroPatch 384. On the Patchliner, the microfluidic design of the chip ensures that complete exchange of the solution occurs resulting in

a lower osmolality of the hypotonic solution compared with experiments on the SyncroPatch 384. This is because the SyncroPatch uses a well-based chip so that addition of a new solution mixes 1:1 with the solution already present in the well. Therefore, we observed a larger effect on current amplitude with a faster time of onset on the Patchliner compared with the SyncroPatch 384.

In conclusion, the Patchliner and SyncroPatch 384 automated patch clamp instruments can be used to record VRAC channels in keratinocytes which, in combination with WT and KO keratinocytes, can be used to elucidate the physiological and pathological role of VRAC channels in various disorders including inflammatory skin diseases, ischemia and even cancer.

References

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Collaboration partner

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Methods

Cells

WT and LRRC8A^{-/-} HaCaT keratinocytes were kindly provided by BRAIN Biotech AG.

Cell culture

Keratinocytes were cultured and harvested according to Nanion's standard cell culture protocols.

Electrophysiology

Whole cell patch clamp recordings were conducted according to Nanion's standard procedures for the [Patchliner](#) and [SyncroPatch 384](#). Isotonic solution with an osmolality of 320 mOsm and hypotonic solution with an osmolality of 234 mOsm were prepared (osmolality was adjusted using mannitol). On the Patchliner, the hypotonic solution was pipetted onto the cells, completely exchanging the external solution resulting in a final osmolality of 234 mOsm. On the SyncroPatch 384, hypotonic solution was added either once, mixing with the isotonic solution present in the well resulting in a final osmolality of 277 mOsm, or twice resulting in a final osmolality of 256 mOsm. A voltage ramp from -100 mV to 100 mV over 200 ms from a holding potential of -50 mV was used on the Patchliner or from -100 mV to 100 mV over 500 ms from a holding potential of -40 mV on the SyncroPatch 384. Current at -100 mV and +100 mV was used for analysis. Current-voltage relationships were constructed using a voltage step protocol from -120 mV for 840 ms increasing in 20 mV steps to 120 mV from a holding potential of -50 mV (Patchliner) or -40 mV (SyncroPatch 384). Current amplitude was measured at the start of the voltage step. For pharmacology experiments using a voltage step protocol, a step from a holding potential of -40 mV to +100 mV was repeated every 15 s. All data are expressed as mean ± SEM.