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# Supplementary Information for

## TRPV1 pore turret dictates distinct DkTx and capsaicin gating

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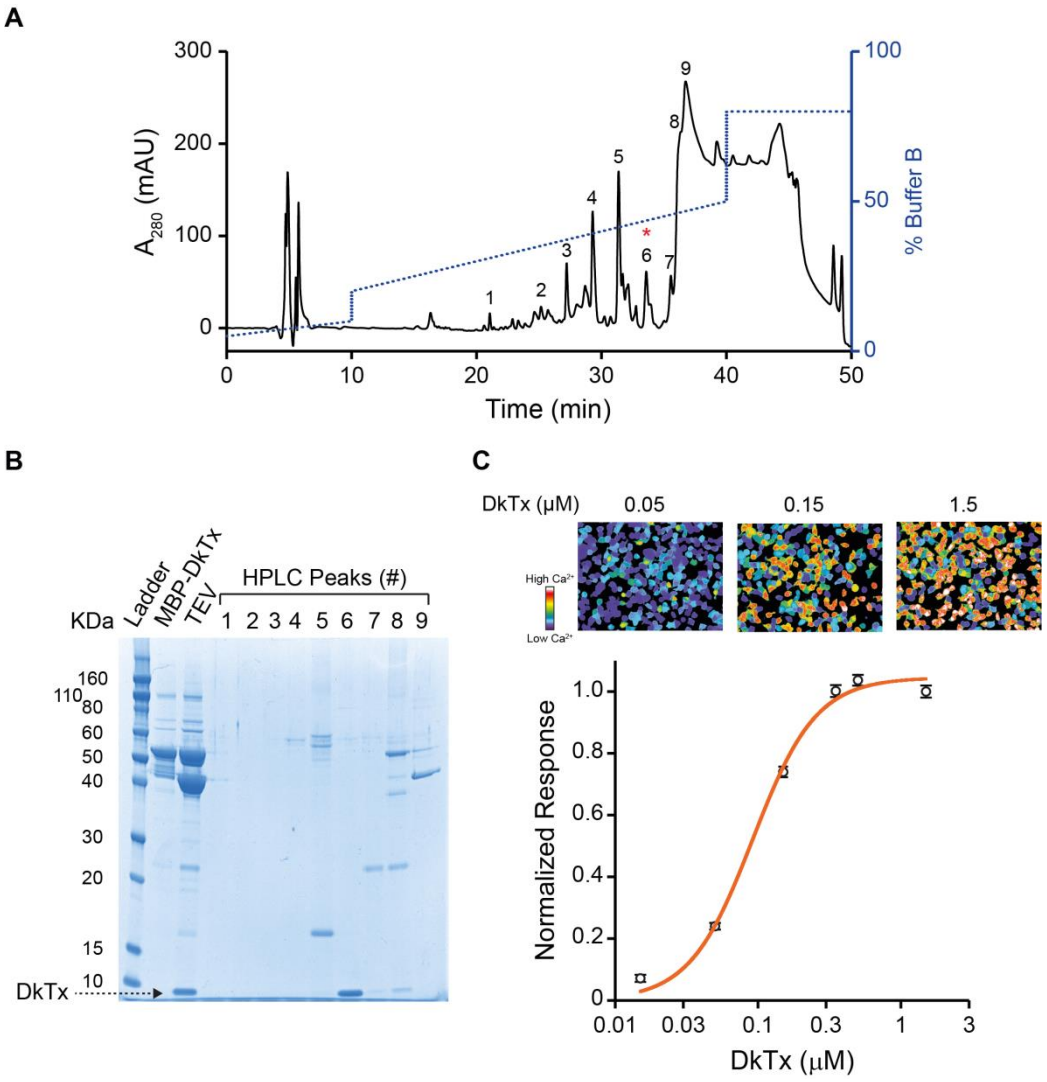


**Fig. S1.** Purification of bioactive recombinant DkTx. (*A*) Reversed-phase (C18) chromatogram of recombinant MBP-DkTx fusion protein after TEV protease cleavage. Red asterisk denotes the retention of the active recombinant DkTx. (*B*) SDS-PAGE analysis of DkTx purification. Lane 1, molecular weight markers. Lane 2, MBP-DkTx extracted from bacteria. Lane 3, TEV protease cleavage of proteins shown in lane 2. Lanes 4-12, HPLC fractions indicated in *A*. (*C*) *Top*: representative pseudo-colored images of cells after application of DkTx in the indicated concentrations. Scale bar indicates levels of intracellular calcium. *Bottom*: dose-response analysis for recombinant DkTx ($EC_{50}$ = 0.11 ± 0.01 μM, $n_H$ = 1.5 ± 0.1) in TREx HEK293T cells stably expressing rTRPV1 as determined by live-cell calcium imaging. Values are normalized to highest DkTx concentration (1.5 μM) - evoked responses. Each point represents an average of 50–150 cells.

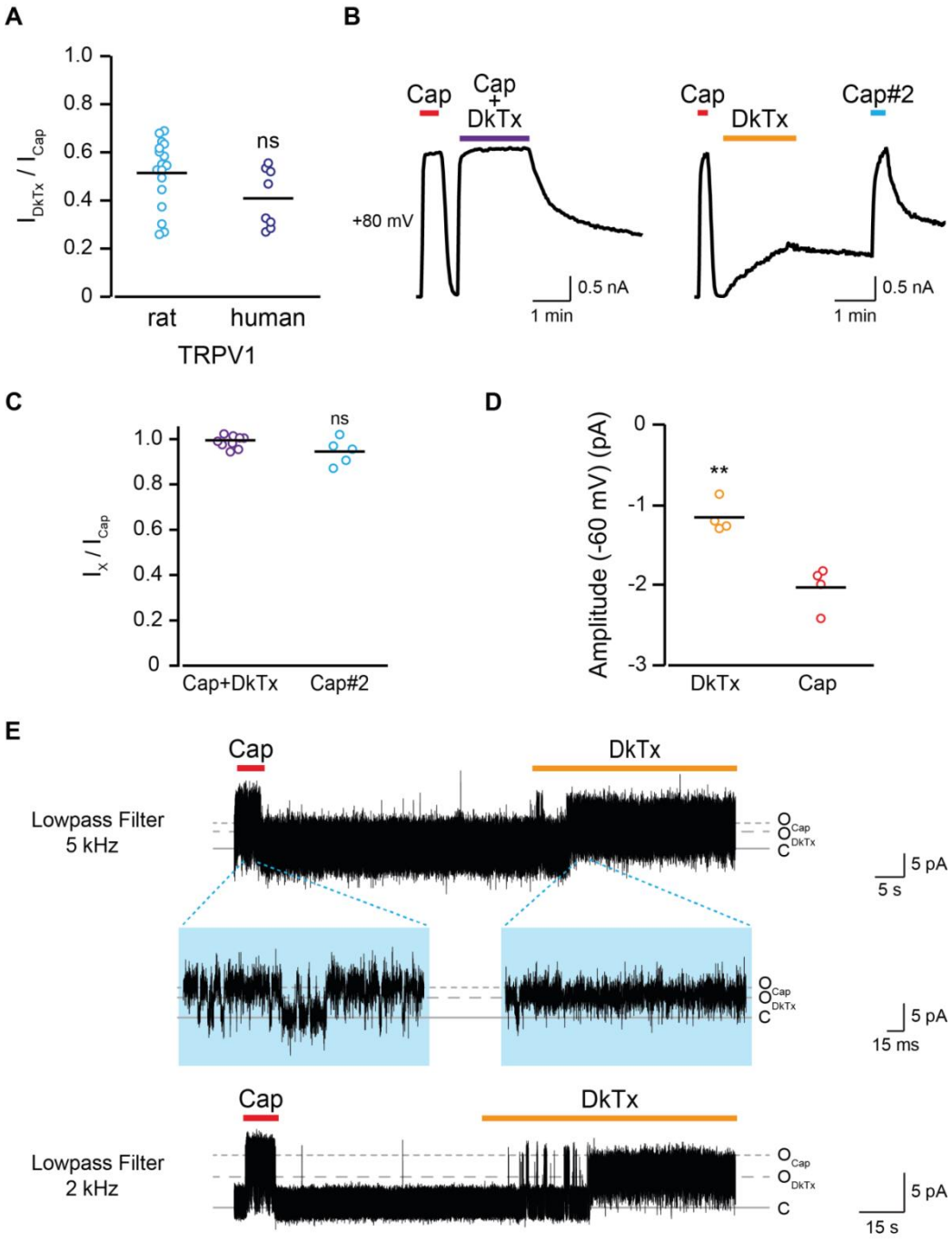


**Fig. S2.** DkTx evokes a moderate TRPV1 activation in various tested conditions. (*A*) Mean/scatter-dot plot representing the amplitude of DkTx-evoked (2 µM) whole cell currents in TREx HEK293T cells transiently expressing rTRPV1 ('rat'; cyan circles) or hTRPV1 ('human'; dark blue circles) ($V_m$= +80 mV) normalized to the current amplitude of the saturating capsaicin response (1 µM) (n = 8-17). Statistical significance is indicated as ns, not statistically significant (Unpaired student's *t*-test). (*B*) *Left*: representative whole-cell current traces from TREx HEK293T cells stably expressing rTRPV1 upon application of capsaicin (1 µM; red bar) followed by co-application of DkTx (1 µM) and capsaicin (1 µM)

(purple bar) (n = 9). Vm = +80 mV. *Right*: representative whole-cell current trace from TREx HEK293T cells stably expressing rTRPV1 upon application of capsaicin (1 µM; red bar), DkTx (1 µM; orange bar) followed by a second application of capsaicin ('Cap#2'; 1 µM; cyan bar) (n = 5). Vm = +80 mV. (*C*) Mean/scatter-dot plot representing the amplitude of whole-cell currents in TREx HEK293T cells stably expressing rTRPV1 (Vm= +80 mV) of DkTx and capsaicin co-application ('Cap + DkTx'; both at 1 µM; purple circles) or capsaicin (1 µM) applied following activation by DkTx (cyan circles), normalized to the current amplitude of the initial capsaicin response (1 µM) (n = 5-9). ns, not statistically significant (Paired student's t-test). (*D*) Mean/scatter-dot plot representing the amplitude of single-channel currents at the holding potential of -60mV evoked by DkTx (1 µM) and capsaicin (1 µM) (n = 4). Statistical significance is indicated as $^{**}p \leq 0.01$ (Paired student's t-test). (*E*) *Top:* representative current recordings low-pass filtered at 5 kHz from an excised outside-out membrane patch of TREx HEK293T cells stably expressing rTRPV1 (treated as described in Fig. 1). Representative recording is shown after exposing the patch to capsaicin (1µM; red bar) and DkTx (1 µM; orange bar) ($V_m$ = +60 mV) (n = 3). Blue background insets show higher magnification of indicated 0.2 s of capsaicin or DkTx -evoked responses. *Bottom:* representative current recordings low-pass filtered at 2 kHz from an excised outside-out membrane patch of TREx HEK293T cells stably expressing rTRPV1 (treated as described in Fig. 1). Representative recording is shown after exposing the patch to capsaicin (1 µM; red bar) and DkTx (1 µM; orange bar) ($V_m$= +60 mV) (n = 6).

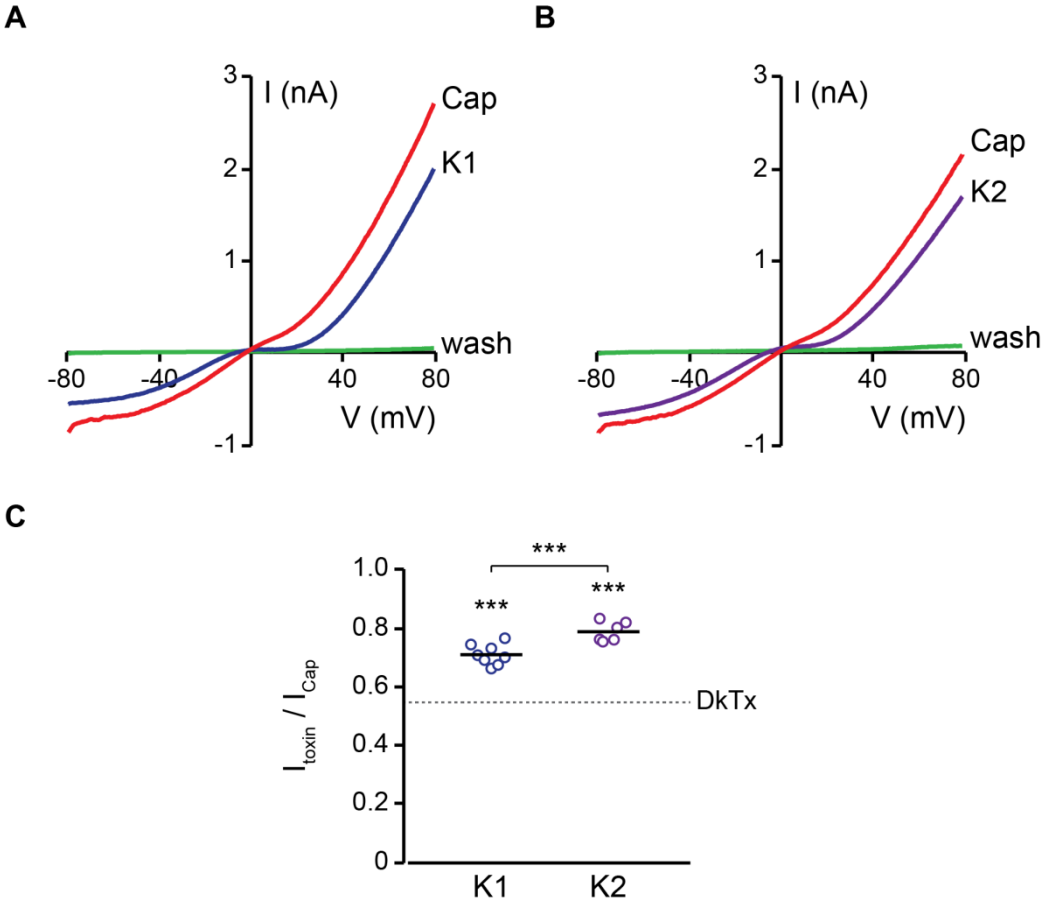


**Fig. S3.** The single knots, K1 and K2, evoke higher current amplitude in whole-cell recordings in respect to the full-length DkTx. (*A*) Representative current-voltage relationship traces recorded using the whole-cell configuration of the patch-clamp technique (in 1 $s^{-1}$ voltage-ramps between -80 and +80 mV) from TREx HEK293T cells stably expressing rTRPV1. Cells were exposed to capsaicin ('Cap'; 1 µM; red line) followed by application of K1 ('K1'; 300 µM; blue line) (n = 8). (*B*) Representative current-voltage relationship traces from TREx HEK293T cells stably expressing rTRPV1 (treated as in A). Cells were exposed to capsaicin ('Cap'; 1 µM; red line) followed by application of K2 ('K2'; 50 µM; purple line) (n = 6). (*C*) Mean/scatter-dot plot representing the ratio between the indicated toxin and capsaicin-evoked whole-cell current amplitudes at +80 mV (n = 6-8). The dashed line indicates the average ratio of DkTx and capsaicin-evoked peak amplitudes at +80 mV. Statistical significance between normalized responses of K1, K2, and wild-type DkTx are indicated as ***$p \leq 0.001$ (ANOVA followed by a multiple comparison test).

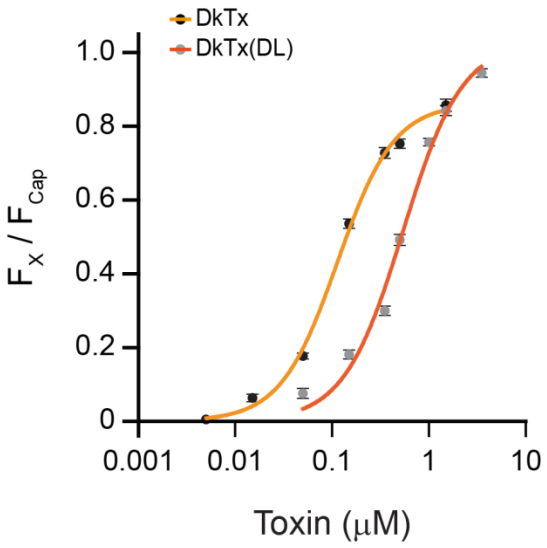


**Fig. S4.** DkTx(DL) is less potent than wild-type toxin. Dose-response analysis for DkTx(DL) ($EC_{50}$ = 0.54 ± 0.08 μM, $n_H$ = 1.4 ± 0.2) and DkTx ($EC_{50}$ = 0.11 ± 0.01 μM, $n_H$ = 1.5 ± 0.1) in TREx HEK293T cells stably expressing rTRPV1 as determined by calcium imaging. Values are normalized to saturating capsaicin (1 μM) evoked responses. Each point represents an average of 50–150 cells.

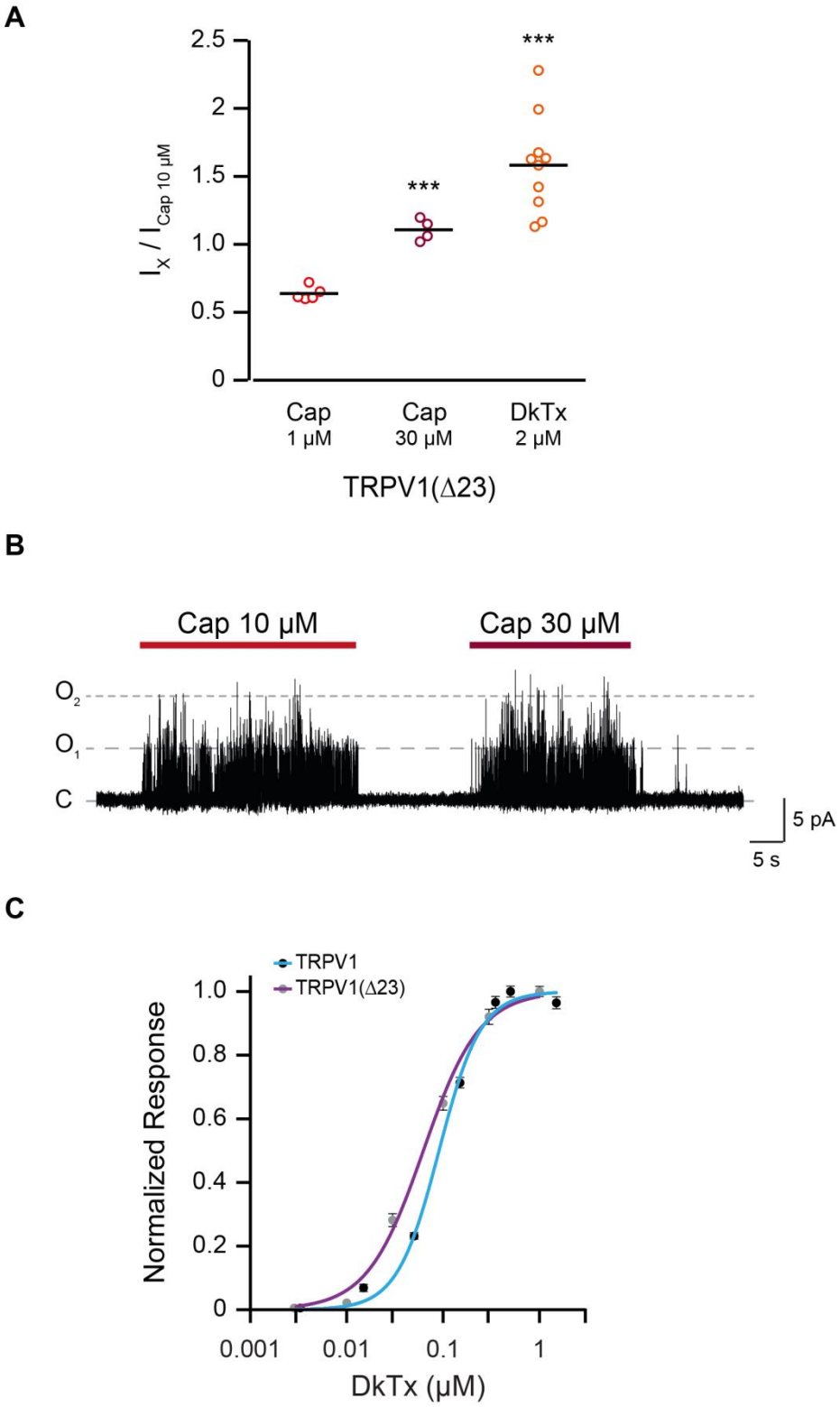
A
2.5
2
1.5
1.0
0.5
0
$I_X$ / $I_{Cap\ 10\ \mu M}$
***
***
Cap
1 μM
Cap
30 μM
DkTx
2 μM
TRPV1(Δ23)
B
Cap 10 μM
Cap 30 μM
$O_2$
$O_1$
C
5 pA
5 s
C
TRPV1
TRPV1(Δ23)
Normalized Response
1.0
0.8
0.6
0.4
0.2
0
0.001
0.01
0.1
1
DkTx (μM)

**Fig. S5.** While TRPV1Δ23 is less sensitive to capsaicin, DkTx potency is preserved in this mutated channel. (*A*) Mean/scatter-dot plot representing the ratio between the whole-cell current amplitudes of 1 µM capsaicin ('Cap 1 µM'; red circles), 30 µM capsaicin ('Cap 30 µM'; dark red circles), 2 µM DkTx (orange circles) and 10 µM capsaicin in TREx HEK293T cells transiently expressing TRPV1Δ23 ($V_m$= +80 mV) (n = 4-10). High capsaicin concentration and DkTx normalized responses were compared to the 1 µM capsaicin normalized response. Statistical significance is indicated as ***$p \leq 0.001$ (ANOVA followed by a multiple comparison test). (*B*) Representative current recording from an excised outside-out membrane patch of TREx HEK293T cells transiently expressing TRPV1Δ23 (treated as described in Fig. 1). A representative recording is shown after exposing the patch to 10 µM capsaicin (red bar) and 30 µM capsaicin (dark red bar) ($V_m$= +60 mV) (n = 4). (*C*) Dose-response analysis for DkTx in TREx HEK293T cells transiently expressing rTRPV1 (TRPV1: $EC_{50}$ = 90 ± 5 nM, $n_H$ = 2 ± 0.2) or TRPV1Δ23 (TRPV1Δ23: $EC_{50}$ = 60 ± 4 nM, $n_H$ = 1.5 ± 0.1) as determined by live-cell calcium imaging. Values are normalized to the maximal DkTx-evoked response in each construct. Each point represents an average of 50–100 cells.

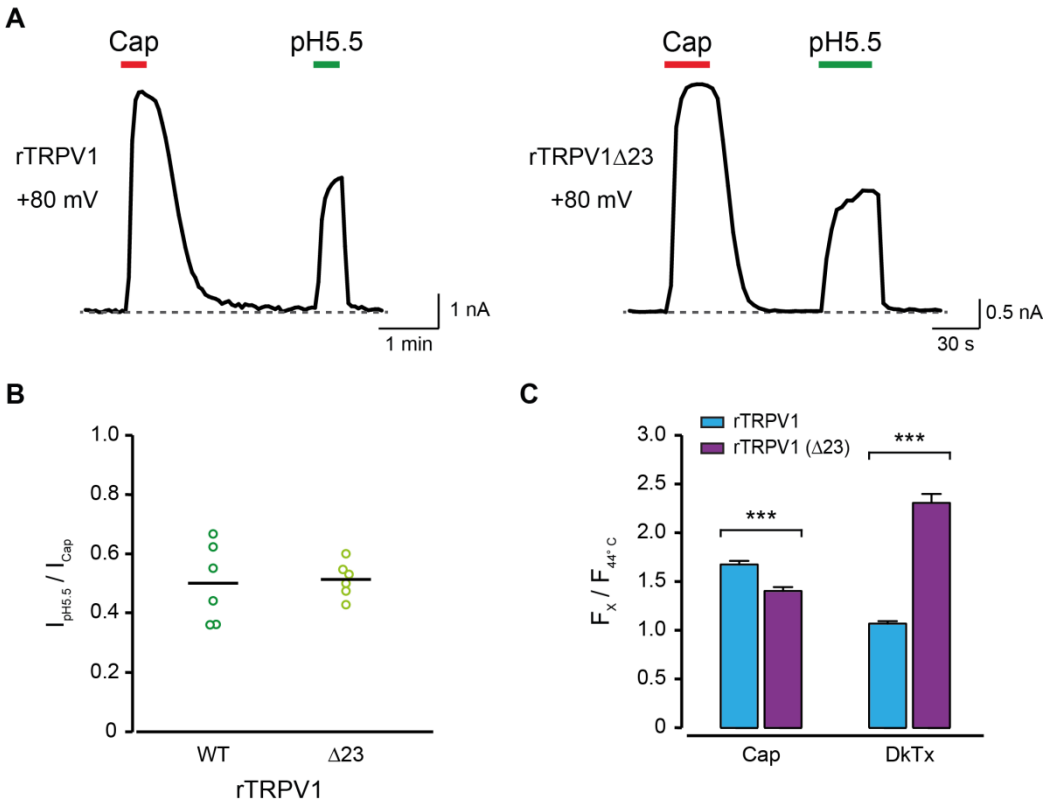


**Fig. S6.** pH and heat -evoked responses are reduced in TRPV1Δ23. (*A*) *Left*: representative whole-cell current trace from CHO cells transiently expressing rTRPV1 upon application of capsaicin (1 µM; red bar) and pH 5.5 (green bar) (n = 6). Vm = +80 mV. *Right*: representative whole-cell current trace from CHO cells transiently expressing rTRPV1Δ23 upon application of capsaicin (1 µM; red bar), and pH 5.5 (green bar) (n = 6). Vm = +80 mV. (*B*) Mean/scatter-dot plot representing the ratio between pH 5.5 and capsaicin-evoked whole-cell current amplitudes in CHO cells transiently expressing rTRPV1 ('wt'; dark green circles) or TRPV1Δ23 ('Δ23'; light green circles) ($V_m$= +80 mV) (n = 6). Statistical significance is indicated as ns, not statistically significant (Unpaired student's *t*-test). Note that the ratio between pH 5.5 and capsaicin responses in TRPV1Δ23 is comparable to wild-type. As the capsaicin-evoked response is reduced in the mutated channel, thus the pH 5.5 response is also reduced proportionally in TRPV1Δ23. (*C*) Bar graph represents the average (± SEM) ratio of the calcium response evoked by capsaicin (1 µM) or DkTx (1 µM) relative to heat (44°C) in TREx HEK293T cells transiently expressing either TRPV1 (cyan bars) or TRPV1Δ23 (purple bars) as determined by live-cell calcium imaging. Each bar represents an average of 50–150 cells. Statistical significance is indicated as ***$p \leq 0.001$ (ANOVA followed by a multiple comparison test).

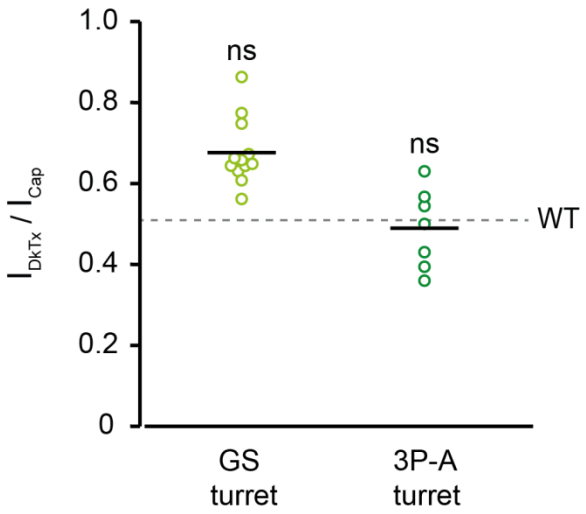


**Fig. S7.** Substitution mutations in the TRPV1 pore turret do not increase DkTx-evoked current amplitude. Mean/scatter-dot plot representing the ratio between the DkTx (2 µM) and capsaicin (1 µM) -evoked whole-cell current amplitudes in TREx HEK293T cells transiently expressing rTRPV1 with an artificial pore turret (‘GS turret'; light green circles) or rTRPV1 containing three point mutations (P608A, P613A, P623A) in its turret (‘3P-A turret'; dark green circles) ($V_m$= +80 mV) (n = 7-12). The dashed line indicates the average ratio of DkTx and capsaicin-evoked peak amplitudes at +80 mV in wild-type rTRPV1. Statistical significance between normalized responses in mutated constructs and wild-type TRPV1 is indicated as ns, not statistically significant (ANOVA followed by a multiple comparison test).

**Table S1. Whole cell and single-channel properties of toxin-evoked rTRPV1 activation**

| Construct | Toxin | Whole cell | | | Single Channel | |
|---|---|---|---|---|---|---|
| | | $I_{Tx(peak)} / I_{cap(peak)}$ | $I_{Tx(wash)} / I_{cap(peak)}$ | $I_{Tx(wash)} / I_{Tx(peak)}$ | $I_{Tx} / I_{cap}$ | Po |
| rTRPV1 | DkTx | 0.56 ± 0.02*** n = 7 | 0.51 ± 0.03*** n = 7 | 0.91 ± 0.03 n = 7 | 0.68 ± 0.04*** n = 6 | 0.99 ± 0.01 n = 6 |
| | K1 | 0.71 ± 0.01***,$$$ n = 8 | NSS n = 8 | NSS n = 8 | 0.75 ± 0.01***, $$ n = 6 | 0.95 ± 0.02 n = 6 |
| | K2 | 0.79 ± 0.01***,$$$ n = 6 | NSS n = 6 | NSS n = 6 | 0.89 ± 0.01***, $$$ n = 7 | 0.88 ± 0.03 n = 7 |
| | K1K1 | ND | ND | ND | 0.58 ± 0.01***, $$$ n = 7 | 0.92 ± 0.05 n = 7 |
| | K2K2 | ND | ND | ND | 0.64 ± 0.01*** n = 5 | 0.93 ± 0.02 n = 5 |
| | DkTx (DL) | 0.80 ± 0.04**,$$$ n = 7 | 0.60 ± 0.06*** n = 7 | 0.73 ± 0.06$$ n = 7 | 0.77 ± 0.03***, $ n = 8 | 0.99 ± 0.01 n = 8 |
| Cryo rTRPV1 | DkTx | 3.25 ± 0.76***, $$$ n=4 | 3.01 ± 0.79***, $$$ n=3 | 0.84 ± 0.01 n=3 | 1.18 ± 0.11$$$ n=4 | 0.99 ± 0.01 n=4 |
| rTRPV1Δ23 | DkTx | 2.67 ± 0.16***, $$$ n=12 | 1.89 ± 0.31***, $$$ n=8 | 0.78 ± 0.05$ n=8 | 1.15 ± 0.07$$$ n=4 | 0.93 ± 0.03 n=4 |

Tx, toxin (at the relevant saturating concentration)

cap, capsaicin (1 μM)

peak, maximal amplitude

wash, steady-state current following 2 minutes of toxin washout

I, current

Po, open probability

ND, not determined

NSS, no steady-state current

*, statistical significance between toxin and maximal capsaicin response (paired *t* test; ** $p < 0.01$ and *** $p < 0.001$)

$, statistical significance between toxin and the relevant DkTx response (unpaired *t* test; $ $p < 0.05$, $$ $p < 0.01$ and $$$ $p < 0.001$)