The TRPV2 channel mediates Ca2+ influx and the Δ9-THC-dependent decrease in osmotic fragility in red blood cells

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The TRPV2 channel mediates Ca\(^{2+}\) influx and the Δ9-THC-dependent decrease in osmotic fragility in red blood cells

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**Running head:** TRPV2 channel in mouse and human RBCs

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**Author contributions**
ABelkacemi, CFT, RT, DF, MM, and ABeck performed experiments, ABelkacemi, CFT, DF, ABeck and VF analyzed data. MRM provided reagents. ABelkacemi, ABeck and VF conceived and supervised the study. CFT, ABeck, CW, MRM edited the manuscript. ABelkacemi and VF wrote the manuscript.

**Conflict of Interest Disclosures**
The authors declare no competing interests.
Water and ionic homeostasis of red blood cells (RBCs) is regulated by various active and passive transport mechanisms in the RBC membrane, including channels like aquaporins, the mechanically activated non-selective cation channel Piezo1 and the Ca\(^{2+}\)-activated potassium channel KCa3.1. The human genome contains 27 TRP channel-coding genes. The only TRP channel protein that has been detected in circulating mouse RBCs is TRPC6, which might be associated with basal Ca\(^{2+}\) leakage and stress stimulated Ca\(^{2+}\)-entry. TRPC2 and TRPC3 are expressed by murine erythroid precursors, splenic erythroblasts, and erythropoietin stimulates in these cells an increase in intracellular calcium concentration via TRPC2 and TRPC3. In this study we identified the TRP vanilloid (TRPV) 2 channel protein in mouse and human RBCs by specific antibodies and mass spectrometry. TRPV2-dependent currents and Ca\(^{2+}\)-entry were activated by the TRPV2 agonists cannabidiol (CBD) and \(\Delta 9\)-tetrahydrocannabinol (\(\Delta 9\)-THC) resulting in a left-shift of the hypotonicity-dependent haemolysis curve. This effect was reversed in the presence of the KCa3.1 inhibitor TRAM-34, whereas the knockout of Trpv2 right-shifted the haemolysis curve to higher tonicities.

We separated mouse RBCs from other blood cells by centrifugation and analysed protein lysates by nanoflow liquid chromatography tandem mass spectrometry (nano-LC-MS/MS). Among the identified proteins was TRPV2 (Figure S1A). To enrich the TRPV2 protein we generated an antibody which recognized the TRPV2 protein in RBCs from wild-type (WT) animals but not in RBCs from Trpv2 gene-deficient (KO) mice (Figure 1A). As an additional control, we used anti-TRPC6 antibody and identified TRPC6 in RBCs (Figure 1B). Total eluates of anti-mTRPV2 affinity purifications from RBCs membranes of wild-type mice were analysed by nano-LC-MS/MS that retrieved peptides covering 54% of the accessible TRPV2 primary sequence (Figure S1B).

To obtain a more comprehensive protein profile, we lysed wild-type and Trpv2-KO RBCs, extracted the proteins, and measured the resulting tryptic peptides by nano-LC-MS/MS. A
total of 1450 proteins were identified (Figure S1E), with TRPV2 present in all WT samples. Eighty-seven of the identified proteins were detected exclusively or with more than 2-fold increase in WT RBCs, while 13 proteins were detected with more than 2-fold increase in Trpv2-KO RBCs (Figure 1C, Figure S1F) by semiquantitative exponentially modified protein abundance index (emPAI) analysis. Next, we evaluated the frequency of the identified proteins by spectral counting and normalized the data to band 3 (Figure 1D). TRPV2 ranked at position 560, about 0.4% of band 3, 50% and 84% less than ferroportin and Piezo1, respectively. In addition to TRPV2 and Piezo1, other channels such as aquaporin1 and transmembrane channel like 8 (Figure S1D) were identified. The KCa3.1 protein, on the other hand, seemed to be much less abundant, as we could identify only one KCa3.1 peptide in our experiments, which was below the threshold for unambiguous protein identification.

According to the proteomic profiling, Piezo1 and aquaporin1 proteins were present in equal amounts in murine RBCs from Trpv2-KO and WT animals. In contrast, several proteins that affect ion and fluid homeostasis were significantly less abundant in Trpv2-KO RBCs, including the STE20-like- and the WNK1-serine/threonine protein kinases SLK (in humans also dubbed SPAK) and WNK1 (Figure 1C). Both kinases regulate the Na⁺-K⁺-Cl⁻ symporter NKCC1 present in the erythrocyte membrane, resulting in the flux of NaCl and KCl into the cell with subsequent rehydration. This mechanism would be attenuated in Trpv2-KO RBCs with decreased WNK1, similar to renal cells that are also equipped with NKCC1 and WNK1 and in which WNK1 is inhibited under hypotonic conditions. Similarly, the significantly reduced amount of the casein kinase II (CKII)-alpha subunit (CSK21) in Trpv2-KO RBCs (Figure 1C) could be part of mechanisms that compensate for the absence of TRPV2, as pharmacological inhibition of CKII-alpha causes shrinkage of RBCs.

Haematological parameters from blood of Trpv2-KO and WT animals (Figure 1E), were not significantly different. However, when RBCs were exposed to hypotonic solutions, keeping
extracellular [Ca\(^{2+}\)] at 76 µM, haemolysis of Trpv2-KO RBCs occurred at higher tonicity (Figure 1F). The relative tonicity at half maximal lysis (C\(_{50}\)) (Figure 1G) was 49.19±0.62 (WT, n=5) and 53.7±0.68 (Trpv2-KO, n=5; P<0.0001).

To isolate TRPV2 currents from murine RBCs we applied the non-specific TRPV2 agonist 2-APB. Inward and outward currents with the outwardly rectifying current-voltage (IV) relationship typical of TRPV2 currents were recorded by whole cell patch-clamping (Figure 2A, B) or by a miniaturized patch system (Figure 2C, D). A fraction of these 2-APB induced currents was blocked by ruthenium red (RR). Similar but much larger currents were recorded from COS-7 cells, which over-express the murine Trpv2 cDNA (Figure S2E). Upon application of 2-APB, cytoplasmic [Ca\(^{2+}\)] increased in WT RBCs (Figure S2A). The Ca\(^{2+}\) increase was blocked in the presence of RR but could also be induced in Trpv2-KO RBCs (Figure S2B-D). 2-APB blocks TRPC6 and KCa3.1 present in RBCs and acts on additional targets.\(^9\) Thereby it may affect the RBC membrane potential and Ca\(^{2+}\)-signalling pathways independent of TRPV2 during monitoring cytoplasmic Ca\(^{2+}\). As shown in COS-7 cells (Figure S2E-I and S3A), which do not endogenously express TRPC6 and KCa3.1, the 2-APB-induced increase in cytosolic Ca\(^{2+}\) and plasma membrane currents required the presence of over-expressed mouse or human TRPV2. We therefore applied the more specific TRPV2 agonist Δ9-THC, which elicited Ca\(^{2+}\) influx in WT RBCs, which was significantly reduced in Trpv2-KO RBCs (Figure 2E, F) indicating that part of the Ca\(^{2+}\) increase was mediated by TRPV2. The antagonists of the G protein-coupled cannabinoid receptors type1 (CB1) and type2 (CB2), AM251 (AM) and JTE907 (JTE), had no effect on the Δ9-THC-elicited Ca\(^{2+}\)-response in WT RBCs (Figure 2G, H) demonstrating that TRPV2 mediates a significant fraction of THC-elicited Ca\(^{2+}\)-influx and that the action of THC on TRPV2 is direct, and not mediated by CB1 or CB2 receptors.
The primary sequences of human and mouse TRPV2 are 80.4 percent identical, but the antibody against mTRPV2 does not recognize the hTRPV2 protein. We therefore generated an antibody, which recognized the hTRPV2 protein by Western blot (Figure 3A). Next, total eluates of anti-hTRPV2 affinity purifications from RBCs membranes were analysed by nano-LC-MS/MS that retrieved peptides covering 54.8% of the hTRPV2 primary sequence (Figure S1G). In similar experiments but with an antibody for hTRPC6, TRPC6 was not detectable in human RBCs by Western blot nor by nano-LC-MS/MS.

The cannabinoid TRPV2 agonists CBD and Δ9-THC elicited Ca^{2+}-influx in human RBCs (Figure 3B-D). Inward and outward currents with the outward rectifying IV relation were obtained by patch clamp recordings from human RBCs after application of Δ9-THC (Figure 3E-G). Although the currents obtained from human RBCs are small in amplitude, their IVs match the TRPV2 current signature obtained from COS-7 over-expressing human TRPV2 cDNA upon Δ9-THC or CBD application (Figure S3B, C).

Assessment by confocal microscopy revealed that 95.3±2.4% of the human RBCs had a biconcave disc-shaped form. Adding CBD or Δ9-THC shifted the morphology from these biconcave discocytes to concave RBCs, the stomatocytes, which in the presence of CBD and Δ9-THC make up 92.7±1.3% (CBD) and 66.3±17.1% (THC) of the total RBCs, the remaining being discocytes and more spherical-shaped spherocytes (Figure 3H, I). The TRPV2 agonist-induced shape change of the RBCs was maintained in the presence of the CB1 and CB2 antagonists (Figure S3D, E), indicating that the major fraction of the cannabinoids’ effect on RBC morphology is mediated by TRPV2. After addition of Δ9-THC, human RBCs showed reduced osmotic fragility, as shown by the left-shifted haemolysis curve as response to the hypotonicity challenge, no matter whether cannabinoid receptor antagonists were absent or present (Figure 3J, K). Similarly, but to a lesser extent, Δ9-THC shifts the C_{50} value after treating wild-type murine RBCs to lower tonicities (C_{50}, in the absence, 49.05±1.53, and in
the presence of Δ9-THC, 46.08±1.55). This effect was reversed by pre-treatment with the KCa3.1 antagonist TRAM-34, both in the presence of 76 µM (Figure S3F, G) or 2 mM extracellular Ca²⁺ (Figure 3L, M). The data indicate that TRPV2, like Piezo1 and, maybe TRPC6, enables an influx of cations including Ca²⁺. The increase of intracellular Ca²⁺ by TRPV2 activates KCa3.1 which allows K⁺-efflux, resulting in the shift of the haemolysis curve. This shift does not occur in the presence of the KCa3.1 antagonist TRAM-34 (Figure 3L-N).

Stabilization of the RBC membrane against hypotonic haemolysis by Δ9-THC and CBD has been described¹⁰ and it has been shown that in the presence of Δ9-THC at concentrations of > 15 µM almost all RBCs assume a stomatocyte-like concave shape.¹¹ Some of those results were attributed to the interactions between the hydrophobic, naturally occurring cannabinoids and the membrane lipids of the RBCs. However, membrane partitioning experiments, electron spin resonance spectrometry and experiments with artificial liposomes of different compositions which were tested for the release of trapped markers in the presence of Δ9-THC, suggested additional mechanisms.¹²-¹⁴

The data described in our study point to TRPV2 as a specific molecular target for Δ9-THC and CBD in RBCs. Activation of the TRPV2 channel by the compounds present in the Cannabis sativa plant makes RBCs more resistant to lysis in response to hypotonic solutions. Whether our data explain why hemp medicines have been used in folk medicine to treat malaria since ancient times¹⁵ needs to be shown by further studies.
References

Figure Legend

Figure 1. TRPV2 protein in mouse RBCs. (A, B) Western blot from wild-type, Trpv2 KO (A) and Trpc6 KO (B) RBC proteins, using antibodies against mouse TRPV2, TRPC6 and β-actin. (C) Semi-quantitative analysis of differentially expressed proteins identified by mass spectrometry in wild-type and Trpv2 KO RBCs lysates. Up- and down regulated proteins were identified based on at least 2-fold changes with a P-value <0.05, calculated by unpaired two-tailed Student’s t test. The heatmap shows the Z-scores of the emPAI values from independent mass spectrometry measurements from five independent wild-type and four independent Trpv2 KO samples. (D) Relative abundance of the TRPV2 protein compared to the 1450 proteins identified in mouse erythrocyte membrane fractions. Rank represents the order of the identified protein obtained by spectral counting with the P-value calculated by unpaired Student’s t test. NA, not applicable. (E) Haematological parameters of the blood from wild-type and Trpv2 KO. RBC, red blood cell; HGB, haemoglobin; HCT, haematocrit; MCV, mean corpuscular volume; MCH, mean corpuscular haemoglobin; MCHC, mean corpuscular haemoglobin concentration; RDW, red cell distribution width with P-value calculated by unpaired two-tailed Student’s t-test. (F) Haemolysis (%) of RBCs collected from wild-type (black) and Trpv2 KO mice (red) in buffer A (149 mM NaCl, 2 mM CaCl2, 4 mM KCl, 2 mM HEPES, pH 7.4), diluted 26-fold in buffer B (0-149 mM NaCl, 2 mM HEPES, pH 7.4) as indicated; extracellular [Ca2+] was kept at ~76 µM. (G) Tonicity at which 50% lysis occurred (C50), calculated by sigmoidal fitting from experiments in F. Single values and mean ± SEM from 5 independent experiments performed in triplicates are shown with P-value calculated by unpaired two-tailed Student’s t-test.

Figure 2. TRPV2 function in mouse RBCs. (A-D) In- and outward currents at -80 and +80 mV shown as mean ± SEM, recorded from mouse RBCs using the patch pipette (A) or a miniaturized patch clamp system (port-a-patch) (C) plotted versus time (number of cells in brackets). TRPV2 currents were activated by the application of 2-APB (black line) in the absence and presence of 10 µM ruthenium red (RR, blue line) with the corresponding current-voltage relationships (IVs) at the peak net currents (I_{max net}), shown as mean ± SEM in (B) and (D). Patch pipette resistances were 10 - 15 MΩ when filled with standard internal solution (in mM): 120 Cs-glutamate, 8 NaCl, 1 MgCl2, 10 HEPES, 10 1,2-bis(2-aminophenoxy)ethane-N,N,N’,N’-tetraacetic acid tetracesium salt (Cs-BAPTA), 3.1 CaCl2 (100 nM free Ca2+, calculated with WebMaxC), pH7.2 with CsOH. Standard external solution contained (in mM): 140 NaCl, 2 MgCl2, 1 CaCl2, 10 HEPES, 10 glucose, pH7.2 with NaOH. For experiments with the miniaturized patch system, the intracellular solution contained (in mM): 60 Cs-methansulfonate, 8 NaCl, 1 MgCl2, 3.1 CaCl2, 60 CsF, 10 HEPES, 10 BAPTA (100 nM free Ca2+, calculated with WebMaxC), 10 glucose, pH7.2 with CsOH and the extracellular solution contained (in mM): 140 NaCl, 2 MgCl2, 1.35 CaCl2, 10 HEPES, 10 glucose, pH7.2 with NaOH. (E, G) Mean Fluo-4 fluorescence (F/F0) traces showing changes in the cytosolic [Ca2+] of RBCs isolated from wild-type (black) and Trpv2 KO mice (red) in the absence (E) and presence (G, blue) of the CB1/CB2-receptor antagonists AM251 and JTE907 (100 nM each), challenged by the application of 30 µM Δ9-tetrahydrocannabinol (Δ9-THC, line). Ca2+-imaging measurements were performed in the presence of a Tyrode’s solution (in mM): 135 NaCl, 5.4 KCl, 1 MgCl2, 10 HEPES, 10 Glucose, and 1.8 CaCl2, pH7.35; RBCs were loaded with 5 µM Fluo-4 and the fluorescence was excited at 488 nm every 3 seconds with the emitted fluorescence detected at >515 nm. (F, H) Summary of peak amplitudes from E and G shown as mean ± SEM with P-values calculated by the unpaired two-tailed Student’s t test (ns, not significant). Numbers of measured cells (x) within (y) independent experiments are indicated in brackets and bars.
**Figure 3. TRPV2 protein and function in human RBCs.** (A) Western blot of protein lysates of COS-7 cells transfected with human TRPV2 cDNA, the cDNA of GFP as control, and human RBCs using anti-human TRPV2. (B, C) Representative traces of cytosolic Ca\(^{2+}\) changes, detected as Fluo-4 fluorescence (F/F\(_0\)), in human RBCs challenged by the application of 100 µM cannabidiol (CBD, B) or 30 µM Δ9-tetrahydrocannabinol (Δ9-THC, C). (D) Summary of the peak amplitudes in B and C as mean±SEM (78 and 82 cells measured in 3 independent experiments each). (E) In- and outward currents at -80 and +80 mV in the absence and presence of Δ9-THC (black line) recorded from human RBCs and plotted versus time. The corresponding current-voltage relationships (IVs) of the basic current (I\(_{\text{min}}\)) and the peak net current in Δ9-THC (I\(_{\text{max net}}\)) are depicted in F and G. Data are shown as mean ± SEM (number of cells indicated in brackets). (H) Confocal microscopic images of human RBCs non-treated (control, left) or treated with 100 µM cannabidiol (CBD, middle) or 30 µM Δ9-THC (right). (I) Bar graphs showing the percentage of discocytes (black), stomatocytes (red) and spherocytes (blue) as mean ± SEM, from three independent healthy donors, treated as in H, with P-values calculated by one-way ANOVA, followed by Bonferroni’s multiple comparison. The classification was done with 3-D stacks of confocal images. (J) Haemolysis (%) of human RBCs treated with the vehicle (control, black), 30 µM Δ9-THC (grey open circle) or 30 µM Δ9-THC in the presence of 100 nM CB1- and CB2-receptor antagonists AM251 and JTE907 (Δ9-THC + AM + JTE, blue) plotted versus the extracellular NaCl concentration (mM) and respective tonicity (%), with extracellular [Ca\(^{2+}\)] kept at 76 µM as described in Figure 2F. (K) Tonicity at which 50% lysis occurred (C\(_{50}\)) calculated by sigmoidal fitting of the individual experiments in J. (L) Haemolysis (%) of human RBCs in buffer A (149 mM NaCl, 2 mM CaCl\(_2\), 4 mM KCl, 2 mM HEPES, pH7.4), treated with vehicle (control, black), 30 µM Δ9-THC (grey open circle), 2 µM TRAM-34 (green) or 30 µM Δ9-THC plus TRAM-34 (red) for 30 min, after 26-fold dilution in buffer B (0-149 mM NaCl, 2 mM CaCl\(_2\), 4 mM KCl, 2 mM HEPES, pH 7.4); extracellular [Ca\(^{2+}\)] was kept at 2 mM. (M) Tonicity at which 50% lysis occurred (C\(_{50}\)) calculated by sigmoidal fitting of the individual experiments in L. Data in K and M are shown as means ± SEM from 2 independent experiments performed in triplicates with P-value calculated by one-way ANOVA, followed by Bonferroni’s multiple comparison. (N) Working model for how TRPV2, activated by Δ9-THC, modulates the TRAM-34 sensitive KCa3.1 activity in a human RBC. Note that TRPC6 was not detectable in human RBCs.
The TRPV2 channel mediates Ca\(^{2+}\) influx and the Δ9-THC-dependent decrease in osmotic fragility in red blood cells

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Supplemental Figure 1. Identification of TRPV2 protein in RBCs and proteome profiling by mass spectrometry (nano-LC-MS/MS). (A) Mouse TRPV2 (SwissProt: Q9WTR1) amino acid sequence coverage (red: identified tryptic peptides) as detected in RBCs lysate with nano-LC MS/MS mass spectrometry covering 13.2% of the protein sequence (black). (B) Mouse TRPV2 amino acid sequence coverage of 54% (red: identified tryptic peptides) as detected after antibody-based enrichment with mouse TRPV2 antibodies from mouse RBCs lysates by nano-LC MS/MS mass spectrometry (n=4). (C) The mouse TRPC6 protein (SwissProt: Q61143) is identified by MS/MS fragmentation covering 33.9% of the protein sequence, (red: identified tryptic peptides). (D) Heatmaps reporting semi-quantitative analysis of transporter and receptor expression. (E) Number of identified proteins in wild-type and TRPV2 KO mice. (F) Heatmaps reporting Log2 fold change in TRPV2 KO compared to wild-type.
receptor and ion channel (water channel) proteins identified by mass spectrometry in wild-type and Trpv2 KO RBCs lysate. Heatmap shows the Z-scores of the Exponentially Modified Protein Abundance Index (emPAI) values from independent mass spectrometrical measurements from five wild-type and four Trpv2 KO samples. Heatmaps were prepared by the “heatmap2” function from the gplots R package (https://cran.r-project.org/web/packages/gplots/). (E) Venn diagram showing the number of MS identified proteins in RBC lysate fractions from wild-type (n=5 experiments) and Trpv2 KO (n=4 experiments) animals. Each experiment was done with the pooled RBCs from 2-3 individual mice. 1450 proteins were identified in both genotypes using the thresholds settings; 2 peptides/protein, protein false discovery rate FDR:0.05, peptide FDR:0.004. (F) The volcano plot of P-value vs. x-fold change (emPAI values, semi quantitative) summarizes the proteins differentially expressed in Trpv2-deficient erythrocytes. Proteins highlighted in green are significantly (unpaired two tailed Student’s t-test, P-value <0.05) upregulated (upper-right) or downregulated (upper-left) in Trpv2 KO compared with wild-type. The TRPV2 protein was exclusively identified in each WT RBCs lysate (n=5) and not detectable in lysates from Trpv2 KO RBCs (n=4). (G) Human TRPV2 amino acid sequence coverage of 54.8% (red, identified tryptic peptides) after antibody-based enrichment with the human TRPV2 antibody (n=8) by nano-LC MS/MS mass spectrometry.
Supplemental Figure 2. 2-APB-activated TRPV2 currents and Ca²⁺ entry in mouse RBCs and COS-7 cells transfected with the murine Trpv2 cDNA. (A-C) Representative traces of cytosolic Ca²⁺ changes, detected as Fluo-4 fluorescence (F/F₀), in RBCs isolated from wild-type (A, B) and Trpv2 KO mice (C) challenged by the application of 500 µM 2-APB in the absence (A, C) and presence of 10 µM ruthenium red (RR, B). Calcium imaging experiments has been performed at 21°C in the presence of a Tyrode’s solution containing (in mM): 135 NaCl, 5.4 KCl, 1 MgCl₂, 10 HEPES, 10 Glucose, and 1.8 CaCl₂, pH7.35. Fluo-4 fluorescence in RBCs was excited at 488 nm every 3 seconds with the emitted fluorescence detected at >515 nm. (D) Bar graphs summarizing the peak amplitudes (ΔF/F₀) from experiments in A-C shown as means ± SEM. (E) In- and outward currents at -80 and 80 mV, respectively, measured from COS-7 cells transiently expressing mouse Trpv2 cDNA (black traces) or GFP cDNA as control (red traces) plotted versus time (left panel). COS-7 cells were challenged by the application of 300 µM 2-APB as indicated by the bar. The corresponding current-voltage relationships (IVs) at the peak net current (Iₘₐₓ net) are depicted in the right panel. Data are shown as means (right panel) or means ± SEM (left panel). Numbers in parenthesis indicate the numbers of measured cells. (F-H) Cytosolic Ca²⁺ changes, detected with Fura-2 (F340/F380), in COS-7 cells (loaded with 5 µM Fura-2) expressing mouse Trpv2 cDNA (F, G) and GFP cDNA as control (H) in the absence (F, H) or presence of 30 µM ruthenium red (G), challenged by the application of 500 µM 2-APB (black bars). (I) Bar graph showing the means ± SEM of the peak amplitudes (ΔF₃₄₀/F₃₈₀) from experiments as in F-H. The P-values are calculated using one-way ANOVA, followed by a Bonferroni’s multiple comparison test. Numbers in the bars indicate the numbers of measured cells. Note: 2-APB blocks TRPC6¹ and KCa3.1² present in RBCs
and acts on additional targets\textsuperscript{3-6}. Thereby it may affect the RBC membrane potential and Ca\textsuperscript{2+}-
signalling pathways independent of TRPV2 during monitoring cytoplasmic Ca\textsuperscript{2+}. As shown in
COS-7 cells (Figure S2E-I and S3A), which do not endogenously express TRPC6 and KCa3.1,
the 2-APB-induced increase in cytosolic Ca\textsuperscript{2+} and plasma membrane currents required the
presence of over-expressed mouse or human TRPV2. (Other targets of 2-APB including
TRPV1, TRPV3, TRPV4, TRPV6, TRPC2, TRPC3, TRPC5, TRPM2, TRPM3, TRPM7.)
Supplemental Figure 3. CBD- and Δ9-THC-mediated currents in COS-7 cells transfected with the human Trpv2 cDNA, and shape and haemolysis of human RBCs. (A, B, C, left panels) In- and outward currents at -80 and 80 mV, respectively, measured from COS-7 cells.
transiently expressing human TRPV2 cDNA (black traces) or GFP cDNA (red traces) as control plotted versus time. Cells were challenged by the application of 1 mM 2-APB (A), 30 µM Δ9-tetrahydrocannabinol (Δ9-THC, B) in the absence (B, top) or presence of either CB1/CB2-receptor antagonists AM251 and JTE907 (100 nM each, B, middle) or 10 µM ruthenium red (B, bottom), 300 µM cannabidiol (CBD, C) in the absence (C, top) or presence of 10 µM ruthenium red (C, bottom). (A, B, C, right panels) Corresponding current-voltage relationships (IVs) at the peak net currents (I_{max net}) from the left panels. Data are shown as means (right panels) and means ± SEM (left panels). Numbers in parenthesis indicate the numbers of measured cells. (D, E) Confocal microscopic images of human RBCs in the presence of the CB1- and CB2-receptor antagonists AM251 and JTE907 (both 100 nM) either non-treated (control, left) or treated with 100 µM CBD (middle) or 30 µM Δ9-THC (right). RBCs were stained with CellMask deep red at a concentration of 5 µg/ml in Tyrode buffer for 30 min and placed into polymeric coverslips. (E) Bar graphs showing the percentage of discocytes (black), stomatocytes (red) and spherocytes (blue) in RBCs as means ± SEM, from three independent healthy donors. P-values were calculated using one-way ANOVA, followed by Bonferroni’s multiple comparison. The classification was done with 3-D stacks of confocal images. (F) Haemolysis (%) of human RBCs in buffer A (149 mM NaCl, 2 mM CaCl_{2}, 4 mM KCl, 2 mM HEPES, pH 7.4), treated with 2 µM TRAM-34 (green) or 30 µM Δ9-THC plus TRAM-34 (red) for 30 min, after 26-fold dilution in buffer B (0-149 mM NaCl, 2 mM HEPES, pH 7.4) as indicated; extracellular [Ca^{2+}] was kept at 76 µM. (G) Tonicity at which 50% lysis occurred (C_{50}), calculated by sigmoidal fitting of the individual experiments in F. Single values are shown and the mean ± SEM from 2 independent experiments performed in triplicates with P-value calculated by unpaired two-tailed Student’s t-test. Note: Cannabinoids found in plants such as cannabidiol (CBD) and Δ9-tetrahydrocannabinol (Δ9-THC) have been described as activators of TRPV2.\textsuperscript{7,9}
Supplemental references