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TRPC channel activation by extracellular thioredoxin

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Abstract

Mammalian homologues of *Drosophila melanogaster* transient receptor potential (TRP) are a large family of multimeric cation channels that act, or putatively act, as sensors of one or more chemical factor^{1,2}. Major research objectives are the identification of endogenous activators and the determination of cellular and tissue functions of these novel channels. Here we show activation of TRPC5 homomultimeric and TRPC5-TRPC1 heteromultimeric channels³⁻⁵ by extracellular reduced thioredoxin acting by breaking a disulphide bridge in the predicted extracellular loop adjacent to the ion-selectivity filter of TRPC5. Thioredoxin is an endogenous redox protein with established intracellular functions, but it is also secreted and its extracellular targets are largely unknown⁶⁻⁹. Particularly high extracellular concentrations of thioredoxin are apparent in rheumatoid arthritis^{8,10-12}, an inflammatory joint disease disabling millions of people worldwide¹³. We show that TRPC5 and TRPC1 are expressed in secretory fibroblast-like synoviocytes from patients with rheumatoid arthritis, endogenous TRPC5-TRPC1 channels of the cells are activated by reduced thioredoxin, and blockade of the channels enhances secretory activity and prevents suppression of secretion by thioredoxin. The data suggest a novel ion channel activation mechanism that couples extracellular thioredoxin to cell function.

Striking activators of TRPC5 are extracellular lanthanide ions^{4,14,15}. Effects of these ions depend on a glutamic acid residue at position 543 in the predicted extracellular loop adjacent to the ion pore (Supplementary Fig. 1-2). This structural feature may, therefore, have functional importance in enabling extracellular factors to activate the channels. Because lanthanides are unlikely physiological activators we were interested in alternatives and developed a hypothesis based on amino acid sequence alignment which showed two cysteine residues near glutamic acid 543 that are conserved in TRPC5, TRPC4 and TRPC1 (Supplementary Fig. 2), a subset of the seven TRPC channels¹⁻⁵. TRPC5 and TRPC4 have similar functional properties⁴ and both form heteromultimers with TRPC1-3, a subunit that has weak targeting to the plasma membrane when expressed in isolation^{3,16}.

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Pairs of cysteine residues may be covalently linked by a disulphide bridge that can be cleaved by reduction. We therefore applied the chemical reducing agent dithiothreitol (DTT) to HEK 293 cells expressing TRPC5^{15,16}. There was channel activation with the characteristic current-voltage relationship (I-V) of TRPC5 and block by 2-APB, an inhibitor of TRPC55 (Fig. 1a, b, d). Current recovered on wash-out of DTT (data not shown). Similarly, the membrane-impermeable disulphide reducing agent TCEP (Fig. 1c, d) activated TRPC5, whereas the thiol reagent MTSET had no effect (Fig. 1d). TRPC5 was inhibited by cadmium ions only after pre-treatment with DTT (Fig. 1e, f), consistent with the metal ion acting by re-engaging cysteines¹⁷. Other TRP channels lacking the cysteine pair in a similar position were unresponsive to DTT (Supplementary Fig. 2-3). The data support the hypothesis that the cysteine pair in TRPC5 normally engages in a disulphide bridge that constrains the channel in a state of limited opening probability, enabling enhanced channel activity when the bridge is broken.

To further test the hypothesis we expressed TRPC5 mutants containing alanine in place of cysteine. Such mutants were constitutively active and not stimulated by reducing agent or lanthanide (Fig. 1g; Supplementary Fig. 4-5). Ionic currents for the single (C553A, C553S or C558A) and double (C553A+C558A) mutants were not significantly different from each other, suggesting the two cysteine residues have a joint role (Fig. 1g). Co-expression of wild-type TRPC1 with the TRPC5 double mutant led to smaller constitutive currents that were not affected by DTT or lanthanide, consistent with TRPC1 suppressing the current amplitude but not conferring a functional effect of reducing agents (Supplementary Fig. 6). Dimers of TRPC5 were not detected under non-reducing conditions, suggesting an intra- rather than inter-subunit disulphide bridge (Supplementary Fig. 7).

Thioredoxin is an important redox protein with established biological roles including in cancer, ischaemic reperfusion injury, inflammation and ageing⁸. It is both an intracellular and secreted protein⁶⁻⁹. It is reduced by the NADPH-dependent flavoprotein thioredoxin reductase and has capability in this form to break disulphide bridges⁸. Extracellular reduced thioredoxin (rTRX) acts like DTT, causing TRPC5 activation (Fig. 2a-b). We therefore hypothesised that rTRX is a novel endogenous extracellular regulator of TRPC5. In taking this idea forward we also considered TRPC1 because many cells endogenously co-expressed TRPC5 and TRPC1, leading to TRPC5-TRPC1 heteromultimers^{3,5,16,18}. Importantly, the TRPC5-TRPC1 channel is also activated by rTRX or DTT (Fig. 2c). Consistent with previous reports^{3,16}, the TRPC5 and TRPC5-TRPC1 channels displayed distinct “fingerprint” current-voltage (I-V) relationships (eg Fig. 3b and Supplementary Fig. 14).

Thioredoxin concentrations up to a mean value of 0.41 $\mu\text{g}\cdot\text{ml}^{-1}$ (maximum 1.2 $\mu\text{g}\cdot\text{ml}^{-1}$) have been detected in serum and synovial fluid from patients with rheumatoid arthritis^{8,10-12}. Furthermore, there is reducing capability of thioredoxin in serum and thioredoxin reductase occurs in human joints and has activity correlated with disease severity^{10,19,20} (see also Supplementary Results and Discussion). We therefore considered whether rTRX activation of TRPC5 is relevant to the cells that secrete synovial fluid, the CD55-positive fibroblast-like synoviocytes (FLS cells). Strikingly, CD55-positive FLS cells (Supplementary Fig. 8) exhibited non-selective cationic current in response to DTT or rTRX (Fig. 2d-g; Supplementary Fig. 9-10). Mean current evoked by rTRX at -80 mV in FLS cells from the knee joint of patients with rheumatoid arthritis was -0.85 ± 0.42 nA ($n=14$). Oxidised TRX (oTRX) had no effect (Fig. 2f). The effective concentrations of rTRX suggest relevance to rheumatoid arthritis (Fig. 2g). Nitric oxide is an alternative endogenous regulator of cysteine residues²¹ but failed to evoke current in FLS cells, even at a concentration 100 times greater than required to evoke vasorelaxation (Supplementary Fig. 10 and Supplementary Results and Discussion).

There are no previous reports on expression of TRPC channels in synovial joints and so we explored synovial tissue biopsies from patients with rheumatoid arthritis. TRPC5 and TRPC1 proteins were detected and co-localised with CD55 (Fig. 3a). Similarly, the FLS cells used in our electrophysiological experiments expressed mRNAs encoding TRPC5 and TRPC1, western blotting indicated the presence of TRPC5 and TRPC1 proteins, and immunolabelling revealed TRPC5 and TRPC1 at the cell surface (Supplementary Figs. 8, 11, 13).

The I-V of the rTRX-evoked current in FLS cells was similar to that of the TRPC5-TRPC1 heteromultimeric channel (Fig 3b). Furthermore, experiments with lanthanum showed unusual and striking similarity between the endogenous current and the current of over-expressed TRPC5-TRPC1: In the absence of a reducing agent, lanthanum stimulated current in both HEK 293 cells (exogenously expressing TRPC5-TRPC1) and FLS cells; where as after induction of current by rTRX, lanthanum was inhibitory in both cases (Fig. 3c; Supplementary Fig. 9). Also consistent with the involvement of TRPC channels, the rTRX-evoked current of FLS cells was blocked by 2-APB and the inward current was suppressed when the majority of extracellular Na⁺ was replaced by the bulky and impermeant cation NMDG⁺ (Fig. 2e; Supplementary Fig. 9). As a further test of the involvement of TRPC5 and TRPC1, FLS cells were transfected with a dominant-negative ion-pore mutant of TRPC5 that inhibits native channels capable of interacting with TRPC5_{16,22}. The mutant suppressed current evoked by rTRX (Fig. 3d).

Further evidence that TRPC5 and TRPC1 contribute to the endogenous rTRX-responsive channel of FLS cells came from studies with anti-TRPC5 (T5E3) and anti-TRPC1 (T1E3) antibodies, which target the predicted extracellular loop region and specifically block functions of TRPC5 and TRPC1 respectively²³⁻²⁵. T5E3 and T1E3 antibodies labelled unpermeabilised FLS cells, unlike antibody targeted to the intracellular C-terminus of TRPC5 which labelled only permeabilised cells (Supplementary Figs. 8 and 11), suggesting TRPC5 and TRPC1 are transmembrane proteins with extracellular epitopes. Like dominant-negative mutant TRPC5, T5E3 or T1E3 suppressed rTRX-evoked current (Fig. 3e). Antibody targeted to CD55, which is a membrane protein unrelated to TRP, had no significant effect (*n*=7; data not shown). Therefore, gene expression, electrophysiology, pharmacology, recombinant DNA and antibody studies yielded data consistent with rTRX-evoked current in FLS cells being carried by a channel containing TRPC5 and TRPC1.

One of the functions of FLS cells is to secrete matrix metalloproteinases (MMPs), which are associated with tissue remodelling and progression of arthritis²⁶. Use of zymography to detect gelatinase activities of MMP-2 and MMP-9 secreted from rabbit FLS cells (Supplementary Fig. 12) revealed that T5E3 and T1E3 antibodies have profound stimulatory effects (Fig. 4a, b). Human FLS cells showed greater MMP-2 secretion compared with MMP-9 (Supplementary Fig. 12 *cf* Fig. 4a). ELISAs for human MMP-2 enabled quantification of the absolute concentration of total MMP-2 secreted; again, either T5E3 or T1E3 antibody had a profound stimulatory effect (Fig. 4c). Similarly, RNAi knock-down of TRPC1/5 expression enhanced MMP-2 secretion (Supplementary Fig. 13). Importantly, inhibition of MMP secretion by exogenous reducing TRX was lost in the presence of T5E3 (Fig. 4d). Similar data were obtained for pro-MMP-1 secretion from human FLS cells (Fig. 4e, f) and MMP-9 measured by zymography in rabbit FLS cells (*n*=6, data not shown). The data therefore reveal constitutive and rTRX-evoked activity of TRPC5/1 channels that inhibits MMP secretion from FLS cells.

The data of this study suggest secreted TRX is a novel type of ion channel agonist that acts through its reduced form to break a restraining intra-subunit disulphide bridge between cysteine residues in TRPC5, thereby stimulating the channel either as a homomeric

assembly or heteromultimer with TRPC1. A transduction mechanism is therefore revealed that can directly couple cell activity to extracellular reduced thioredoxin. This mechanism may have particular relevance in conditions such as rheumatoid arthritis where TRX concentrations are strongly elevated, but the broad distributions of TRX and the channels suggest the mechanism could be widely used.

METHODS SUMMARY

Cells

Synovial tissue biopsies were obtained with informed consent from patients diagnosed with rheumatoid arthritis at the Academic Unit of Musculoskeletal Disease, Chapel Allerton Hospital, Leeds. Ethical approval was given by the Local Ethics Committee. Human synovial tissue biopsies were washed with phosphate-buffered saline (PBS) and digested in 0.25 % type 1A collagenase for 4 hr at 37 °C, after which FLS cells were cultured in DMEM/F-12 + Glutamax (Gibco). HEK 293 cells were grown in DMEM-F12 (Gibco) and rabbit FLS cells (HIG82; ATCC) in Ham's F12 (Gibco). Culture media contained 10 % fetal calf serum, 100 units·ml⁻¹ penicillin and 100 µg·ml⁻¹ streptomycin. Cells were maintained at 37 °C in a humidified atmosphere of 5 % CO₂ in air and replated on cover slips or 24-well plates prior to experiments.

Electrophysiology

Whole-cell patch-clamp recordings were performed^{15,16} at room temperature using patch pipette solution containing (mM): 115 CsCl, 10 EGTA, 2 MgCl₂, 5 Na₂ATP, 0.1 NaGTP, 10 HEPES, 5.7 CaCl₂; pH was adjusted to 7.2 with CsOH. The standard bath solution contained (mM): 130 NaCl, 5 KCl, 8 D-glucose, 10 HEPES, 1.2 MgCl₂ and 1.5 CaCl₂; the pH was adjusted to 7.4 with NaOH. See *Full Methods* for further details.

Data analysis

Ionic currents are shown as positive values when they increased in response to a treatment and negative values when they decreased. Data are expressed as mean ± s.e.m., where *n* is the number of individual experiments. Data sets were compared using paired or unpaired Student's *t*-tests, with significant difference indicated by *P*<0.05 (*) and no difference by n.d.. All human tissue or cell data are derived from, or are representative of, at least 3 independent experiments on samples from 3 patients.

Secretion and other assays

See *Full Methods* section.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Appendix

FULL METHODS

cDNA clones, mutagenesis and cell transfection

HEK-293 cells stably expressing tetracycline-regulated human TRPC5 have been described¹⁵. Expression was induced by 1 $\mu\text{g}\cdot\text{ml}^{-1}$ tetracycline (Tet+; Sigma) for 24-72 hr before recording. Non-induced cells without addition of tetracycline (Tet-) were controls. Human TRPC1 cDNA was expressed transiently from the bicistronic vector pIRES EYFP16. Point mutations in human TRPC5 were introduced using QuikChange® site-directed mutagenesis (Stratagene) and appropriate primer sets. Dominant negative (DN) TRPC5 is a triple alanine mutation of the conserved LFW sequence in the ion pore^{16,22} (Supplementary Fig. 2). The mutations were confirmed by direct sequencing of the entire reading frame. cDNAs were transiently transfected into HEK293 cells or synoviocytes with FuGENE 6 transfection reagent (Roche) or Lipofectamine 2000 (Invitrogen) 48 hr prior to recording. cDNA encoding green or yellow fluorescent protein (GFP or YFP) was co-transfected to identify transfected cells.

Electrophysiology

A salt-agar bridge was used to connect the ground Ag-AgCl wire to the bath solution. Signals were amplified with an Axopatch 200B patch clamp amplifier and controlled with pClamp software 6.0 (Axon) or Signal software 3.05 (CED). A 1-s ramp voltage protocol from -100 mV to +100 mV was applied at a frequency of 0.1 Hz from a holding potential of -60 mV. Current signals were filtered at 1 kHz and sampled at 3 kHz. Patch pipettes were made from borosilicate tubing which, after fire-polishing and filling with pipette solution, had resistances of 3-5 M Ω . The osmolarity of the pipette solution was adjusted to ~290 mOsm with mannitol and the calculated free Ca^{2+} was 200 nM. ATP and GTP were omitted when recording from cells expressing TRPC5 alone. When studying TRPC5 in HEK 293 cells, gadolinium chloride (Gd^{3+} , 1-5 μM) was included in the bath solution to block background currents¹⁵, which evoked submaximal TRPC5 current in some recordings prior to applying other agents. The effect of reducing agents was not dependent on the presence of Gd^{3+} . The recording chamber had a volume of 150 μl and was perfused at a rate of about 2 $\text{ml}\cdot\text{min}^{-1}$. Recordings from human FLS cells used the Patchliner (Nanion) planar patch-clamp system with rapid bath solution exchange. For antibody treatment experiments, cells were treated with one of 1:500 T1E323, 1:100 T5E324,25, boiled 1:500 T1E3 (10 min), 1:100 T5E3 antibody preabsorbed with its antigenic peptide (10 μM) or anti-CD55 antibody (see below), which were diluted in F12 Ham's medium and incubated with cells for 2-3 hr at 37 °C prior to patch-clamp recording.

Immunostaining

Sections 4 μm thick were obtained from snap-frozen synovial tissue biopsy samples of patients suffering from rheumatoid arthritis, fixed with acetone and stored at -80 °C until use. Staining was according to standard protocols. Briefly: Sections were incubated with primary antibody over night at 4°C and secondary antibody (goat anti-rabbit IgG-FITC (Sigma) and donkey anti-mouse IgG-Cy3 (Jackson)) for 1 hr at room temperature. For cell labelling, FLS cells adhered to coverslips were fixed in 4% paraformaldehyde (13 min) and, unless indicated, permeabilised with 0.1% Triton X-100 in 1% BSA for 2 hr. Incubation in primary antibody was overnight at 4 °C and secondary antibody (goat anti-rabbit IgG-FITC) for 2 hr at room temperature. For control experiments, antibodies were preabsorbed to their antigenic peptide (10 μM) or omitted, as specified. Slides were mounted with DAPI hardest mounting medium (Vector Labs) and analysed using a Zeiss confocal microscope. T1E3,

T5E3, T5C3, anti-CD55 (Serotec) and CD68 (Dako) antibodies were used at 1:500, 1:100, 1:500 and 1:200 dilutions respectively.

Secretion assays

FLS cells were cultured in 24 well plates for 24 hr, serum starved for 24 hr and then fresh serum-free medium added containing a TRX cocktail, which included TRX ($0.4 \mu\text{g}\cdot\text{ml}^{-1}$), NADPH-dependent flavoprotein TRX reductase ($0.5 \mu\text{g}\cdot\text{ml}^{-1}$) and NADPH ($2 \mu\text{g}\cdot\text{ml}^{-1}$) for 12 hr. Omission of TRX was the control. Incubations with antibodies occurred for 2 hr prior to addition of the TRX cocktail and were maintained in the presence of the TRX cocktail. Supernatants were collected, frozen and analysed by zymography or enzyme-linked immunosorbent assay (ELISA). For zymography the supernatant was mixed with 2-times non-reducing SDS-PAGE sample buffer and resolved through a 7.4% polyacrylamide gel impregnated with $1.5 \text{ mg}\cdot\text{ml}^{-1}$ gelatin. After electrophoresis, gels were washed, incubated and stained as previously described²⁷. The relative density of gelatinolytic bands was determined from scanned images of gels using ImageQuant software (Amersham). MMP-2 or MMP-1 concentrations in supernatants from human cells were quantified using Quantikine Human total MMP-2 and pro-MMP-1 ELISA kits according to the manufacturer's instructions (R&D Systems).

Chemicals

All salts and reagents were from Sigma or BDH (British Drug House). Gadolinium (Gd^{3+}) chloride, lanthanum (La^{3+}) chloride, cadmium (Cd^{2+}) chloride, dithiothreitol (DTT), 2-aminoethoxydiphenyl borate (2-APB), β -nicotinamide adenoine dinucleotide phosphate reduced form (NADPH) and NADPH-dependent flavoprotein thioredoxin reductase (*E Coli*) were from Sigma. Recombinant thioredoxin (TRX, Sigma) was from *E Coli* (unless specified) or human (no differences in effect were observed compared with *E Coli* TRX) and purchased from Sigma. [2- (trimethylammonium) ethyl] methanethiosulfonate bromide (MTSET) was from Toronto Research Chemicals and Tris (2-carboxyethyl) phosphine hydrochloride (TCEP) was from Pierce Biotech. MTSET, TCEP and NADPH were prepared fresh for each experiment. Collagenase was from StemCell Technologies Inc. 2-APB (75 mM) stock solution was in 100 % dimethyl sulphoxide (DMSO). To prepare reduced thioredoxin (rTRX), TRX (1 mg) was dissolved in 1 ml of the binding buffer (10 mM HEPES, 1 mM EDTA, 50 mM NaCl, pH 7.0) and 0.25 ml was mixed with $2.5 \mu\text{l}$ 1 M DTT, incubated at room temperature for 30 min and then added to 0.25 ml pre-equilibrated resin (DEAE sephadex; Sigma). The mixture was centrifuged for 30 s, and washed three times with binding buffer to remove DTT completely. 0.25 ml elution buffer (10 mM HEPES, 1 mM EDTA, 1 M NaCl, pH 7.0) was added and centrifuged to harvest the supernatant. Final TRX concentration was determined by Bradford assay. rTRX was diluted from cold stocks (on ice) immediately prior to use.

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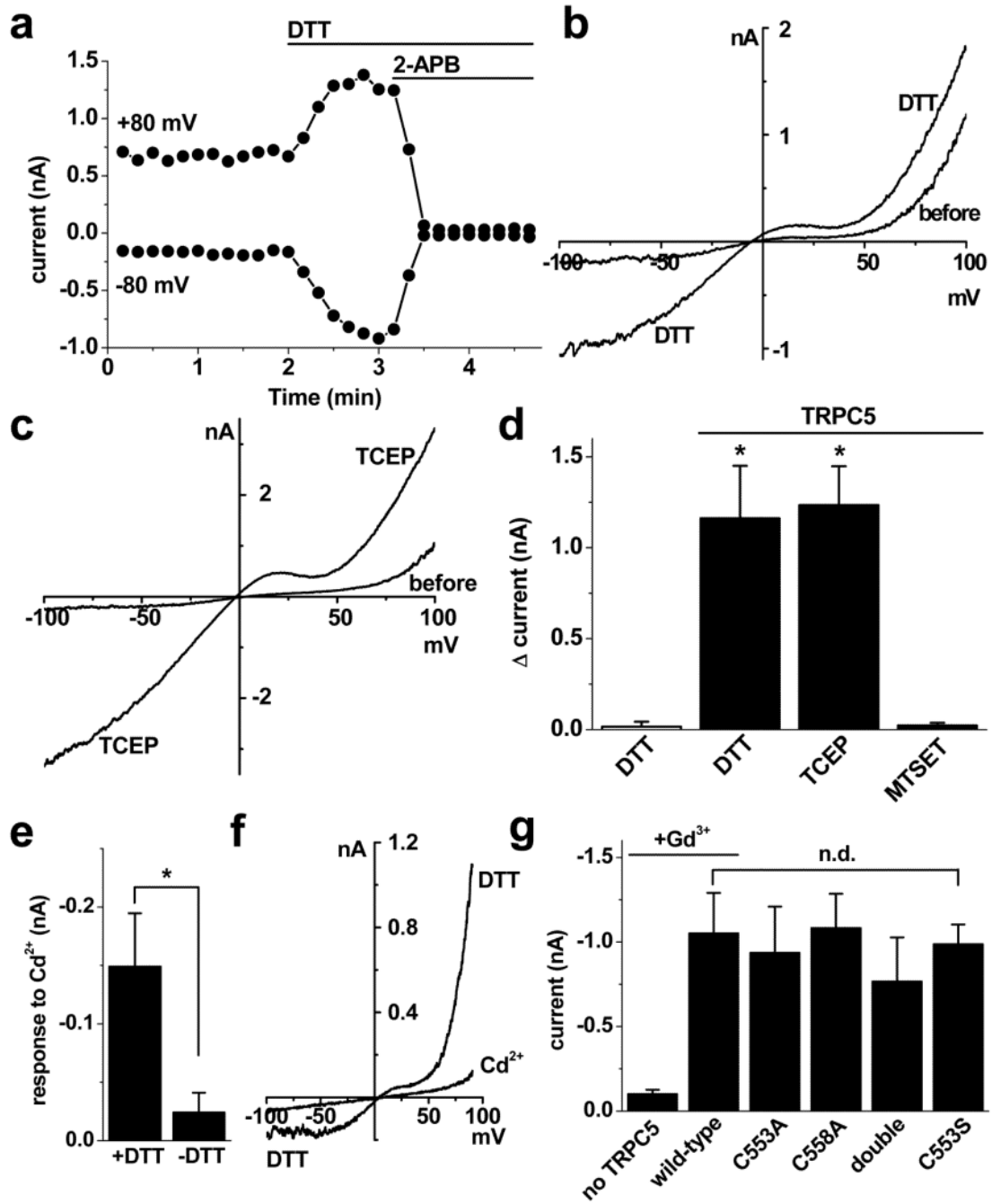


Figure 1. Functional disulphide-bridge in TRPC5

Whole-cell recordings from HEK 293 cells. **a**, In a cell expressing TRPC5, response to bath-applied 10 mM DTT and 75 μ M 2-APB. **b**, I-Vs from **a**. **c**, As for **b** but for 1 mM TCEP. **d**, Currents at -80 mV evoked by 10 mM DTT ($n=8$), 1 mM TCEP ($n=5$) or 5 mM MTSET ($n=6$) in cells expressing TRPC5. DTT had no effect without TRPC5 ($n=5$). **e**, Inhibition of current at -80 mV by 0.1 mM Cd^{2+} in TRPC5-cells with and without DTT treatment. **f**, As for **e** but typical I-Vs. **g**, Currents at -80 mV after transfection with GFP alone (no TRPC5, $n=8$) or GFP plus wild-type TRPC5 ($n=7$) or the TRPC5 mutants C553A ($n=11$), C558A ($n=6$), C553A+C558A (double, $n=3$) or C553S ($n=6$). Gd^{3+} (100 μ M) activated wild-type

TRPC5 but had no effect on mutants. All currents were blocked by 2-APB (eg Supplementary Fig. 5).

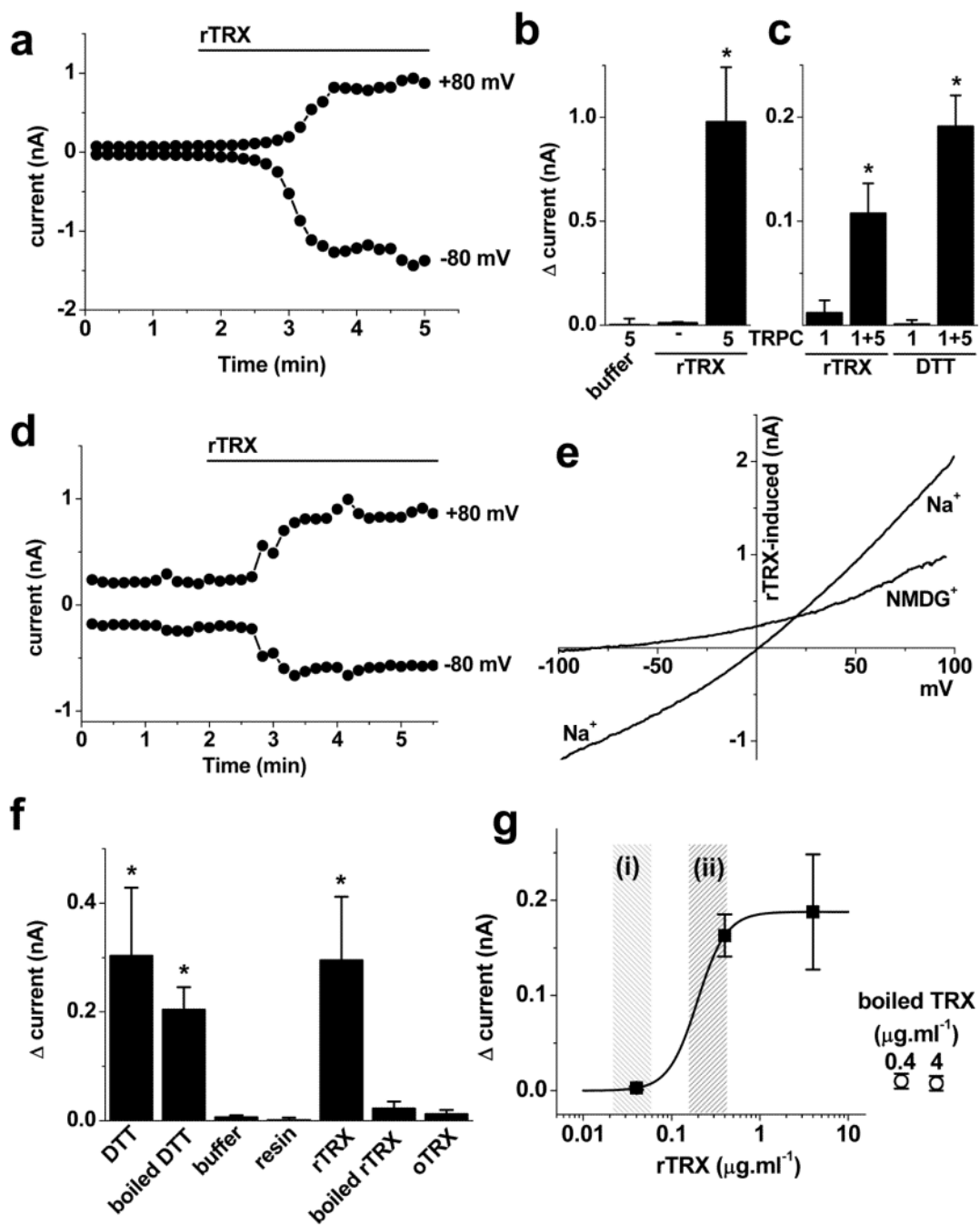


Figure 2. Ionic current induced by rTRX

a-c, Whole-cell recordings from HEK 293 cells expressing TRPC5 alone (**a-b**), TRPC1 alone, or TRPC5 plus TRPC1 (**c**). **a**, Effect of $4 \mu\text{g}\cdot\text{ml}^{-1}$ rTRX. **b**, Current at -80 mV in response to 1:100 elution buffer ($n=4$) or rTRX with ($n=8$) and without ($n=3$) TRPC5 expression. **c**, Responses to rTRX or 10 mM DTT ($n=5$ each). **d**, Effect of rTRX on a human FLS cell. **e-g**, Data for rabbit FLS cells. **e**, rTRX induced I-Vs in standard bath (Na^+) or N-methyl-D-glucamine (NMDG $^+$) solution (see Supplementary Fig. 9). **f**, Currents evoked at -80 mV . **g**, Data for human rTRX with a fitted Hill equation ($\text{EC}_{50} 0.20 \mu\text{g}\cdot\text{ml}^{-1}$, slope

2.64). Open symbols are control data and shaded areas concentrations of TRX in patients without arthritis (i) or with osteo (i) or rheumatoid arthritis (ii). **f-g**, $n=5$ per data point.

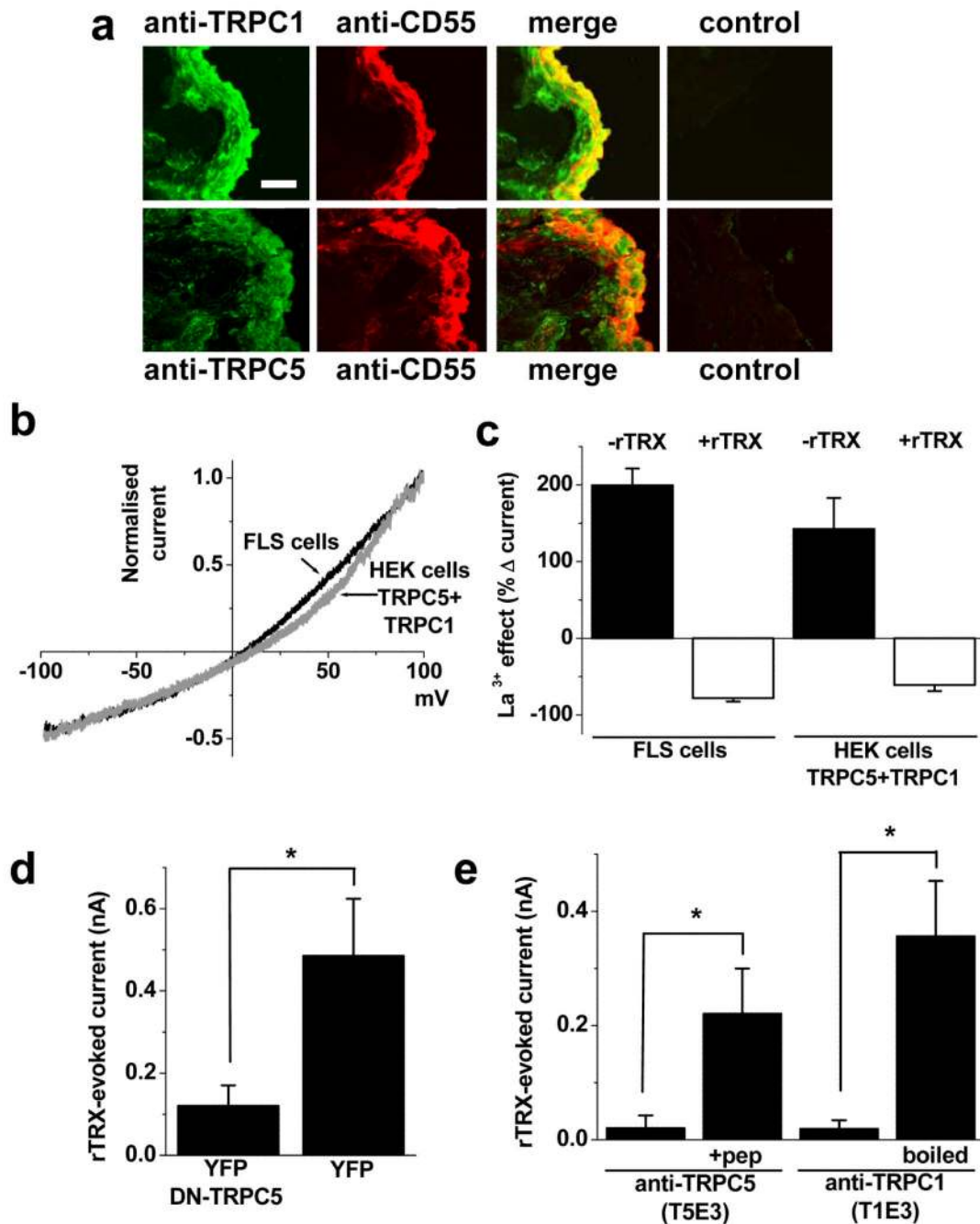


Figure 3. Endogenous TRPC expression and function

a, Tissue sections from joints of patients with rheumatoid arthritis stained with T1E3 or T5E3 (green) or anti-CD55 (red) antibodies. Controls were: omission of anti-CD55 antibody; T1E3 or T5E3 preadsorbed to its antigenic peptide. **b**, Normalised rTRX-evoked I-Vs for rabbit FLS cells ($n=3$) and HEK 293 cells expressing TRPC5 and TRPC1 ($n=5$). **c**, Changes in currents at -80 mV in response to $10 \mu\text{M}$ La^{3+} before or after $4 \mu\text{g}\cdot\text{ml}^{-1}$ rTRX ($n=6$ FLS cells, $n=4$ HEK cells). **d**, Current at -80 mV in FLS cells transfected with DN-TRPC5 plus YFP or YFP alone ($n=5$ each). **e**, As in **d** but showing effects of anti-TRPC antibodies ($n=5$ each).

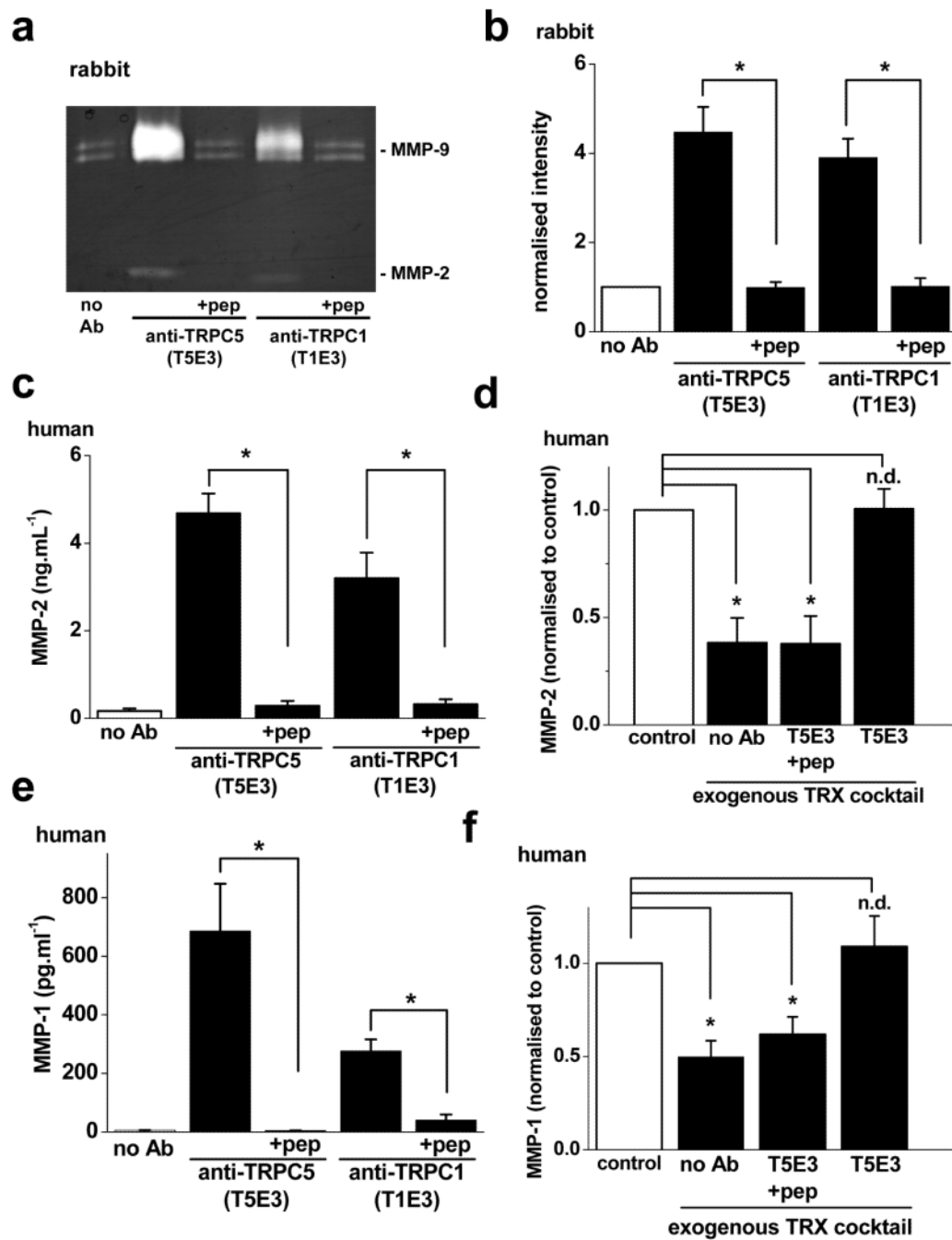


Figure 4. Relevance to secretion from FLS cells

a, Zymogram showing MMP-9 (pro and active) and MMP-2. **b**, As for **a** but mean data after normalisation of MMP-9 band intensity to the no antibody control group ($n=3$ each). **c**, ELISA data for MMP-2 ($n=4$). **d**, Effect of T5E3 ($n=4$) on inhibition of MMP-2 secretion by exogenous TRX cocktail. For each group secretion in TRX was normalised to that in its absence (control). **e**, **f**, As for **c**, **d** but for secretion of pro-MMP-1.