TRPA1 Channels Mediate HGF Response to Phenytoin

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Appendix

Extended Material and Methods

Western-Blot Analysis

Cultured HGF cells were washed with phosphate-buffered saline and solubilized in RIPA buffer (Phosphate-buffered saline pH 7.4, 0.1% (w/v) SDS, 1% (v/v) Nonidet P-40, 0.5% (w/v) sodium deoxycholate) supplemented with a protease inhibitors mixture (Roche Applied Science). Lysates were centrifuged at 1000 g for 5 min at 4ºC and the protein concentration was measured by using BCA protein assay reagent. Equal amounts of protein for each condition (30 µg) were denatured at 95°C for 5 min, loaded onto a 7.5% SDS–polyacrylamide gel and electrophoresed. Proteins were transferred to a nitrocellulose membrane, blocked with 10% skim milk, and incubated with antibody against human TRPV1 (VR1-C15, goat, Santa Cruz, diluted 1:1000). Horseradish peroxidase (HRP)-coupled anti-goat secondary antibodies (Sigma-Aldrich) were used at final concentration of 1:2000 for detection, and the signal was developed with the Luminata Forte system (Millipore) and recorded with a LAS-1000plus (Fujifilm) gel documentation system.

Calcium Imaging

HGFs plated on polylysine-coated coverslips were incubated with 5 µM fura-2-acetoxymethylester (Invitrogen) added to buffer solution (see below) and 0.2 % Pluronic for 60 min at 37 °C. Fluorescence measurements were made with a Zeiss Axioskop FS upright microscope fitted with an ORCA ER CCD camera (Hamamatsu). In some experiments, an inverted NikonNikon Eclipse TE2000-U microscope with an Andor 888 camera was used instead. Fura-2 was excited at 340 nm and 380 nm with a rapid switching monochromator (TILL Photonics). Relative intracellular calcium changes were estimated from the, background subtracted, mean fluorescence intensity ratios (F340/F380) for individual cells.

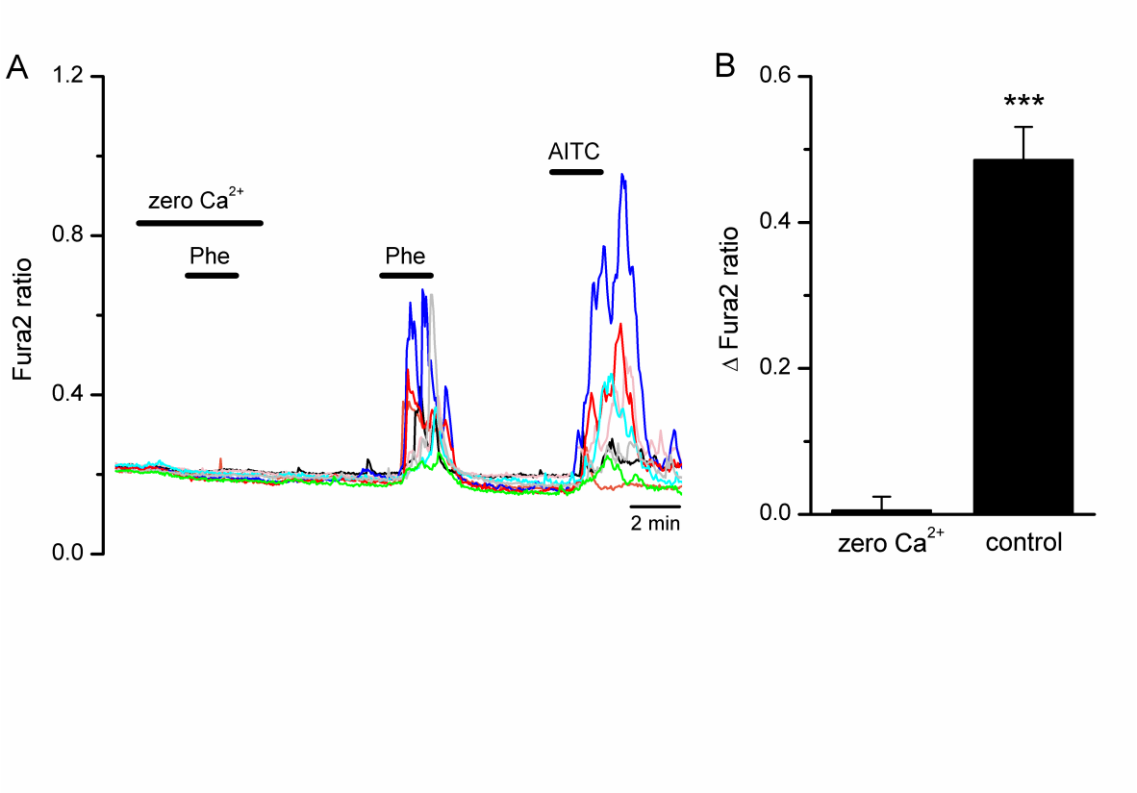
These ratios were displayed online with Metafluor (Molecular Devices) or TillVision (Till Photonics) software. The bath solution contained (in mM): 140 NaCl, 3 KCl, 2.4 $CaCl_2$, 1.3 $MgCl_2$, 10 HEPES and 10 glucose, and was adjusted to a pH of 7.4 with NaOH. During calcium-free experiments the bath solution was as follows (in mM): 140 NaCl, 3 KCl, 1.3 $MgCl_{2,}$10 HEPES, 1 EGTA, 10 glucose (pH at 7.4). Bath temperature was maintained at 31–33°C by a custom-built Peltier device.

Appendix Table 1. Primers used for PCR

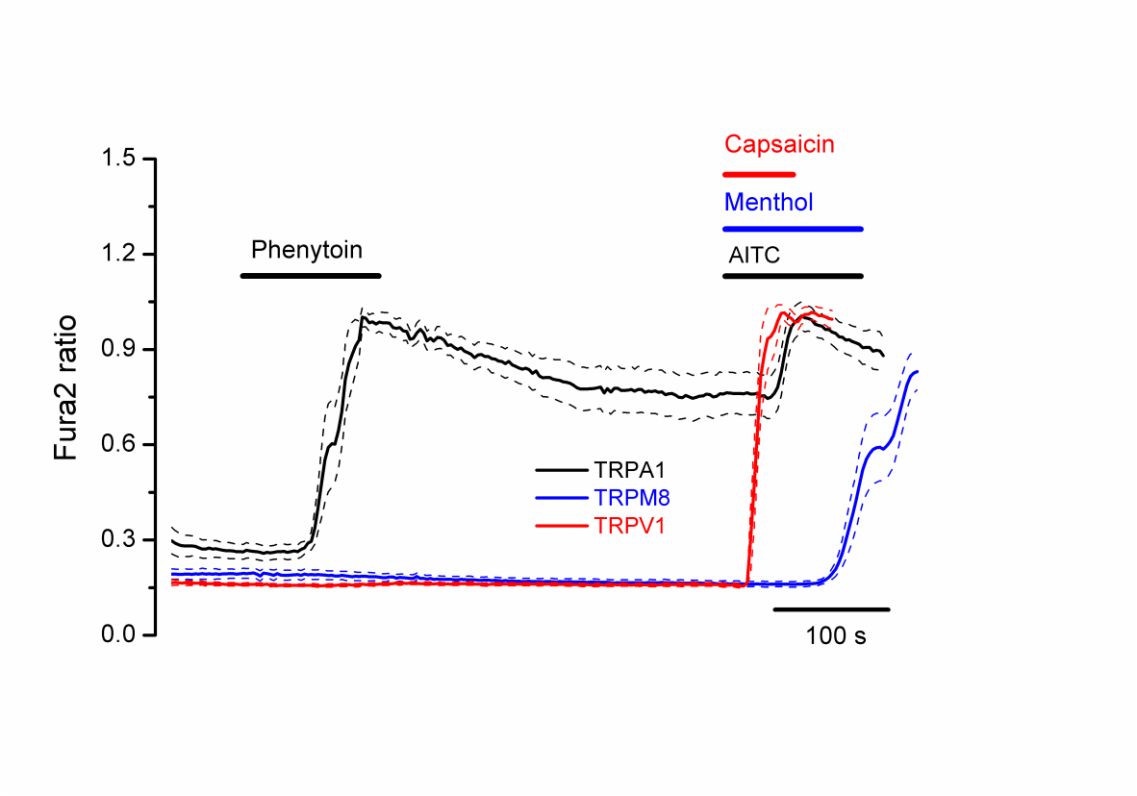
hTRPA1(+)	tggtgcacaaatagacccagt	hTRPA1(-)	tgggcacctttagagagtagc
hTRPM8(+)	gattttcaccaatgaccgccg	hTRPM8(-)	ccccagcagcattgatgtgc
hTRPV1(+)	ctcctacaacagcctgtac	hTRPV1(-)	aaggccttcctcatgcact
hTRPV1b(+)	agctcaccaacaagaaggg	hTRPV1b(-)	gtcccacttgtcctgcagg
ACTB(+)	acccacatgtgcccatcta	ACTB(-)	gccacaggattccataccca

Appendix Table 2. Primers used for Quantitative Real-time RT-PCR

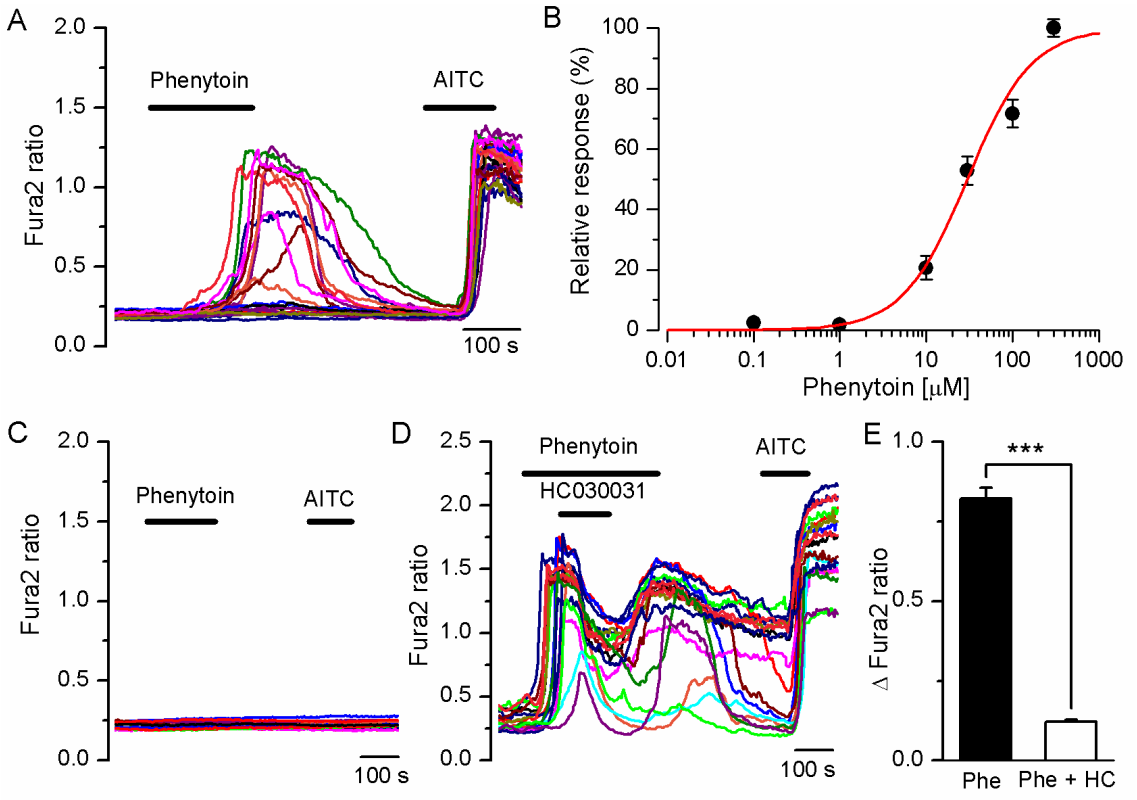
hCol1α1 (+)	ctcaagagaaggctcacgatg	hCol1α1 (-)	gtctcaccagtctccatgttg
hCTGF (+)	gaggaaaacattaagaagggcaaa	hCTGF (-)	cggcacaggtcttgatga
hTRPA1(+)	gtcatgagaccatgcttcacagag	hTRPA1 (-)	tggagagcgtccttcagaatcg
RNA18S(+)	cggctaccacatccaaggaa	RNA18S(-)	gctggaattaccgcggct



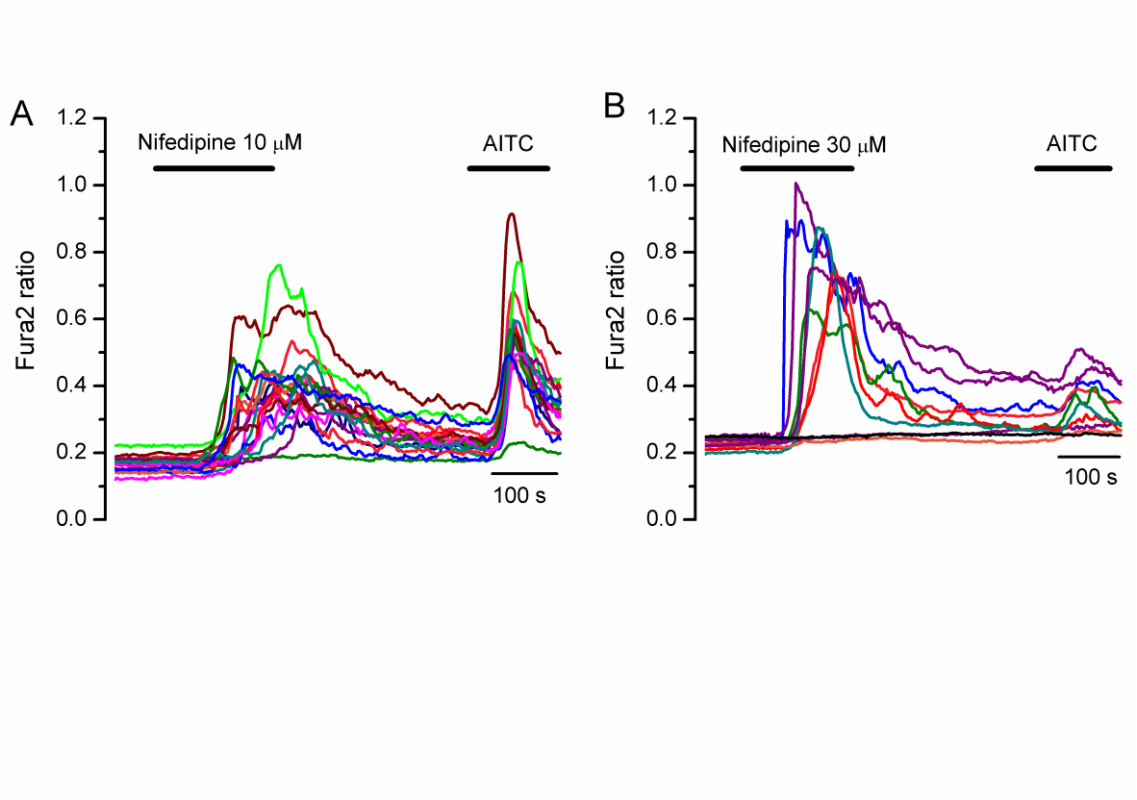
Appendix Figure 1. Responses to phenytoin in HGFs reflect calcium influx. (**A**) Application of 100 µM phenytoin in cero calcium solution (plus 1 mM EGTA) did not result in calcium elevations in HGFs. A subsequent application of 100 µM phenytoin or 100 µM AITC evoked a response. (**B**) Summary histogram of the mean ± SEM elevation to phenytoin in zero or 2.4 mM external calcium ($p<0.001$, paired t-test, n = 39 in 3 independent experiments).



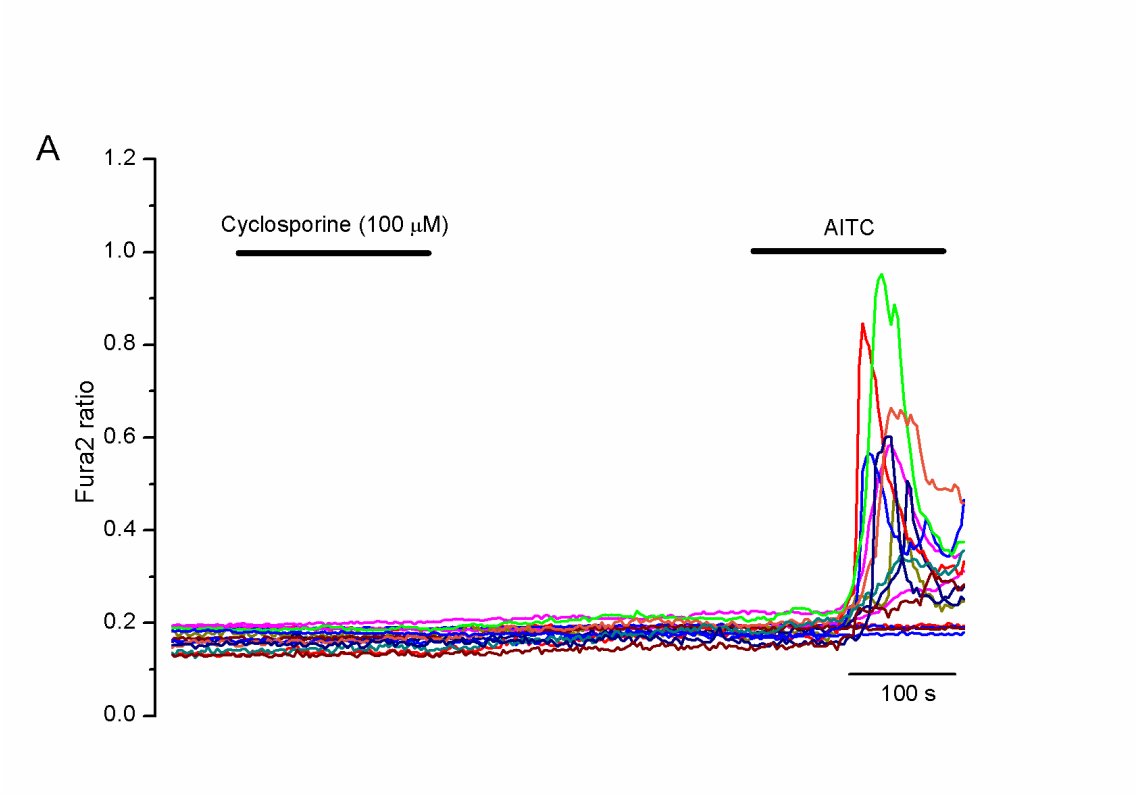
Appendix Figure 2. Phenytoin responses are specific for TRPA1 channels. Average ± SEM intracellular calcium responses to phenytoin (500 µM) in HEK293 cells transiently expressing human TRPA1, TRPM8 or TRPV1. The application of phenytoin was followed by the application of canonical agonists for TRPA1 (100 µM AITC), TRPM8 (100 µM menthol) or TRPV1 (1 µM capsaicin). Each trace represents the average of >25 cells in 2 independent experiments.



Appendix Figure 3. TRPA1 channels expressed in recombinant systems responds to phenytoin. (**A**) Response of CHO cells expressing mouse TRPA1 to phenytoin (100 µM) or AITC (100 µM). (**B**) Dose response curve to phenytoin in CHO-TRPA1 cells. The red line is a fit to the Hill equation with an estimated EC_{50} of 30 µM and a slope factor of 1.17. Individual responses have been normalized to the amplitude of AITC responses in the same cell. (**C**) Wild type CHO cells, not expressing TRPA1, are unresponsive to phenytoin (100 µµM) or AITC (100 µM). (**D**) Intracellular calcium responses to phenytoin (100 µM) in CHO-TRPA1 cells are reversible blocked by HC030031 (20 µM). (**E**) Summary histogram of phenytoin responses in CHO-TRPA1 cells and the inhibition produced by HC030031 ($p<0.001$, paired t-test, n = 129 in 2 independent experiments).



Appendix Figure 4. HGFs respond to the calcium channel antagonist nifedipine. Application of 10 µM (**A**) or 30 µM) (**B**) nifedipine trigger intracellular calcium elevations in HGFs cells that are also activated by 100 µM AITC. Traces are representative of responses in 4 and 5 independent experiments respectively.



Appendix Figure 5. HGFs do not respond to the immunosuppressant cyclosporine. (**A**) Application of 100 µM cyclosporine failed to evoke intracellular calcium elevations in HGFs cells. The same cells were readily activated by 100 µM AITC, (n = 40 in 2 independent experiments).