Supplementary Material (ESI) for Integrative Biology
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Supplemental Information

Materials and Methods

RT-PCR

RT-PCR was performed on RNA purified from cultured LifeLine HUVECs using the Ambion MicroPoly(A)Purist™ Kit (Life Technologies, Carlsbad CA). The following previously published custom TRPV4 primers (Invitrogen, Carlsbad CA) were used:[1] TRPV4 Forward: GAGCAATGGCCGCAACGA; Reverse: GCCGTGTGTCCTCATCCGTC. An amplifer set for a specified gene fragment of Glyceraldehyde 3-phosphate dehydrogenase (G3PDH) primers served as a positive control (Clontech Laboratories, Mountain View CA). RT-PCR was performed with SuperScript™ III One-Step RT-PCR System with Platinum® Taq High Fidelity (Invitrogen, Carlsbad, CA) with recommended protocols.

TRPV4 Functional Assay

Functionality of TRPV in HUVECs was demonstrated with the phorbol ester anolog TRPV4 activator, 4α-PDD. HUVECs were cultured and loaded with Fluor-4 as described in Methods. FITC channel fluorescence time courses were measured during the addition of 3 μM (final concentration) agonist in the presence and absence of inhibitor at 1 frame per second for 5 min.

Cell Surface Labeling and Fluorescence Quantification of Syndecan-4

Immunofluorescence staining products were all purchased from Santa Cruz Biotechnology Inc., Santa Cruz CA. The following protocol, recommended by Santa Cruz Biotechnology was followed. All incubations were done at room temperature. HUVECs with and without PMA treatment were fixed for 5 min in 2% formalin in PBS, washed 3X with PBS, then blocked with normal goat sera for 20 min. Cells were then incubated for 45 min with primary antibody, mouse monoclonal IgG2a raised against Syndecan-4 of human origin (5.0 μg/ml in PBS with 1.5% normal blocking serum). After washing with three changes of PBS for 5 min each, cells were

incubated with goat anti-mouse IgG FITC for 45 min. in the dark. Four phase-contrast and FITC channel image pairs were obtained for each plate: normal cells, PMA treated cells, and negative control cells having no primary antibody. Percent loss of syndecan-4 after PMA treatment was estimated using ImageJ as follows. All fluorescence images of cells were background corrected identically. Maximum and minimum brightness was set to 10 and 210, respectively. The blue_orange_icb color map was used to display and save an RGB montage of cells under each condition. An ImageJ plugin, written in our laboratory, was used to count magenta pixels in the montage RGB files, which each had >20 cells in their field of views.

Results

TRPV4 message in cultured HUVECs. Qualitative expression of TRPV4 was determined by performing RT-PCR on RNA purified from cultured HUVECs using a primer set previously shown to amplify a 596 base pair portion of the TRPV4 gene product.[1] We established TRPV-4 channel receptors are expressed at the level of mRNA in our HUVEC line. A reaction done in the absence of reverse transcriptase did not yield any products (Suppl. Fig. 1).

TRPV4 channel function in cultured HUVEC. The phorbol derivative 4α-PDD is a known activator of TRPV-4, inducing Ca^{2+} influx across the cell.[2] We observed influx in >90% cultured HUVECs with the addition of 3μM (final concentration) 4α-PDD. Intracellular Ca^{2+} rose transiently to a maximum 6-8 times baseline, not quite returning to baseline after the 5 min. experiment. When cells were pretreated with 1.5 μM of the TRPV4 antagonist, HC067047, no activation of the channel was observed (Suppl. Fig. 2).

Depletion of cell surface Syndecan-4 in PMA treated HUVECs. Suppl. Fig. 3 shows representative FITC labeling of syndecan-4. A 70% depletion of syndecan-4 upon PMA treatment was determined from ImageJ analysis of labeled HUVECs as detailed above in Methods.

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Supplemental References

1. M. L. Fitzgerald, Z. H. Wang, P. W. Park, G. Murphy, and M. Bernfield, *J. Cell Biol.*, 2000**, 148**, 811-824.
2. H. Watanabe, J. B. Davis, D. Smart, J. C. Jerman, G.D. Smith, P. Hayse, J. Vriens, W. Cairns, U. Wissenbach, J. Prenen, V. Flockerzi, G. Droogmans, C. D. Benham, and B. Nilius, *J. Biol. Chem.*, 2002, **277**,13569-13577.

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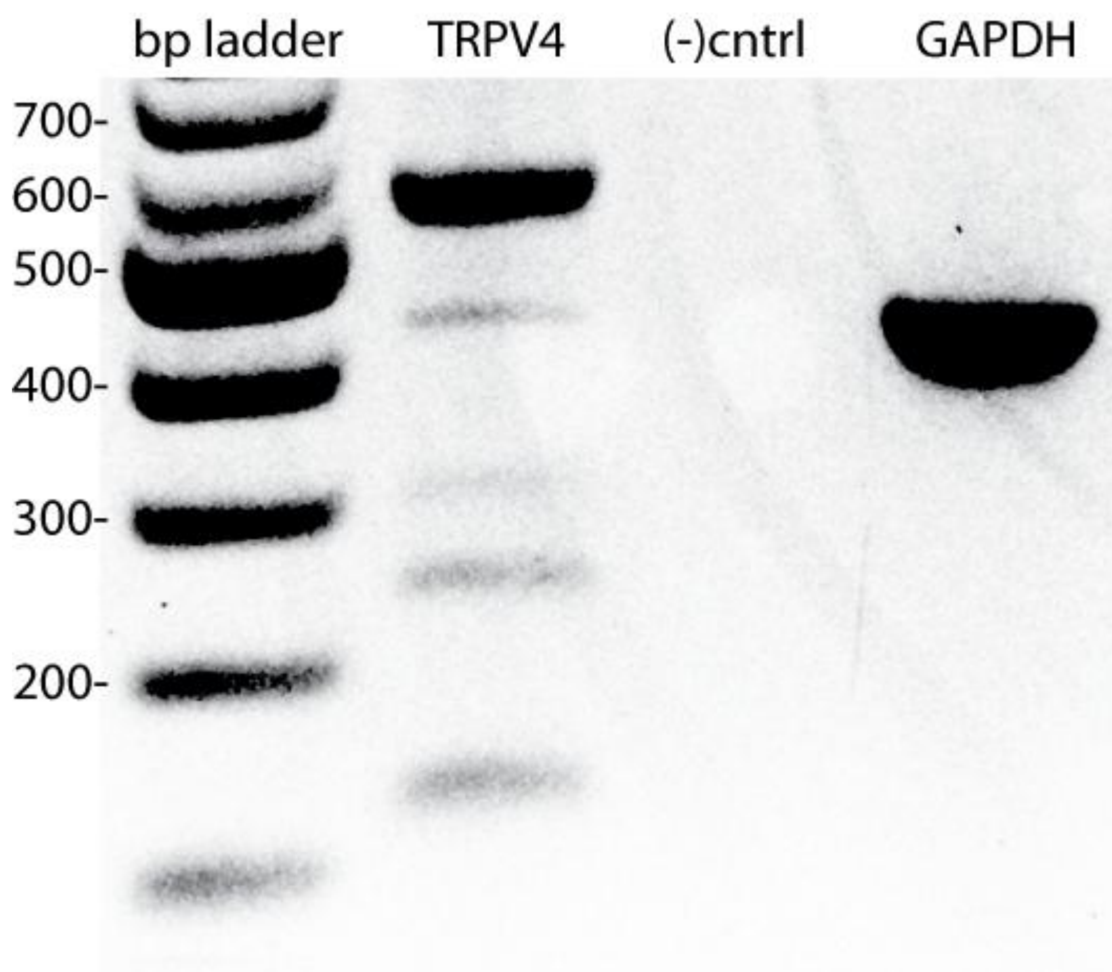
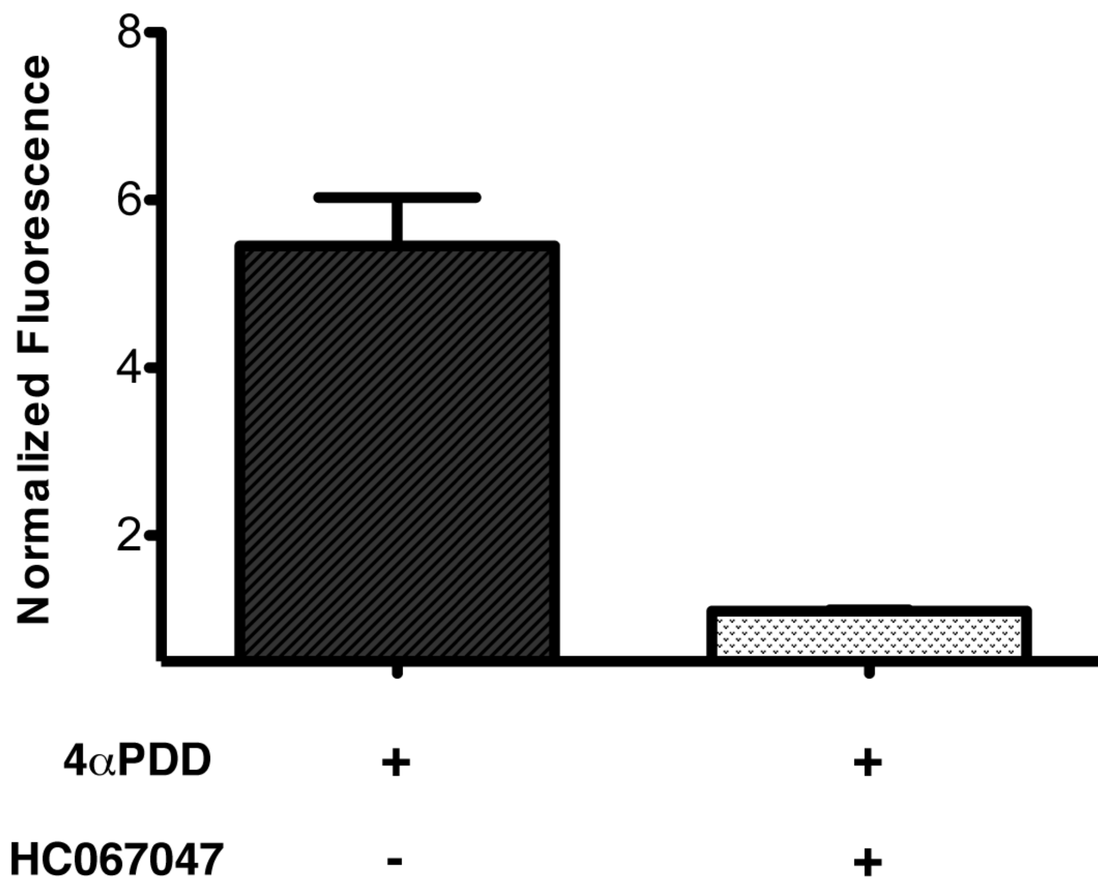


Suppl. Fig. 1. TRPV4 is expressed in human umbilical vein endothelial cells (HUVEC). RT-PCR was performed using mRNA isolated from cultured HUVECs, and primers that have been previously shown to promote amplification of a 596 bp region of the human TRPV4 gene product (lane TRPV4). As a negative control the reaction was run without the presence of reverse transciptase. Glyceraldelhyde-3-phosphate dehydrogenase (GADPH) primers were used as a positive control.



Suppl. Fig. 2. 4α-PDD induced calcium response in cultured HUVECs is inhibited by TRPV4 inhibitor, HC067047. Bars indicate the mean amplitude of the Ca^{2+} transient induced by (left) the addition of 3 μM 4α-PDD, and (right) the addition of 3 μM 4αPDD in the presence of 1.5 μM specific TRPV4 channel antagonist.

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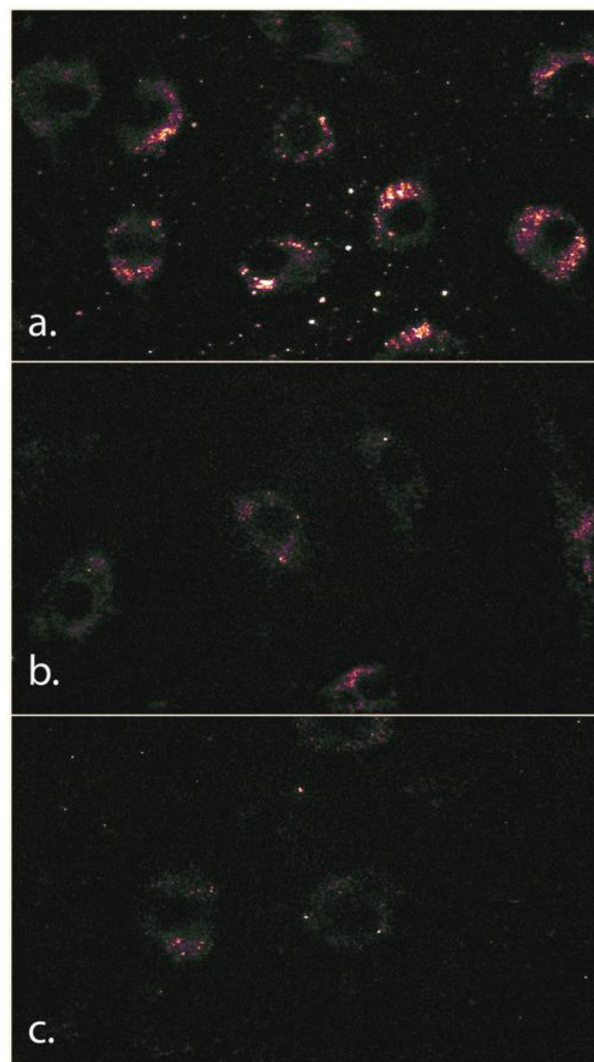

Suppl. Fig. 3. Syndecan-4 ectodomains are shed by proteolytic cleavage at the cell surface: Representative FITC fluorescence images of fixed HUVECs incubated with mouse monoclonal IgG2a against syndecan-4, then labeled with goat anti-mouse IgG FITC after treatment with or without 0.5 mM PMA for 30 min at 37°C. (a) Untreated and (b) PMA-treated cells. (c) No primary antibody control.