Functional basis for calmodulation of the TRPV5 calcium channel

Malou Zuidscherwoude1, Mark K. van Goor1, Sara R. Roig1,2, Niky Thijsen1, Merijn van Erp3, Jack Fransen3, Jenny van der Wijst1 and Joost G. Hoenderop1

1Department of Physiology, Radboud Institute for Molecular Life Sciences, Radboud University Medical Center, Nijmegen, The Netherlands
2Imaging Core Facility, Biozentrum, University of Basel, Basel, Switzerland
3Radboudumc Technology Centre Microscopy, Radboud Institute for Molecular Life Sciences, Radboud University Medical Center, Nijmegen, The Netherlands

Handling Editors: Peying Fong & Jian Yang

The peer review history is available in the Supporting Information section of this article (https://doi.org/10.1113/JP282952#support-information-section).

Abstract Within the transient receptor potential (TRP) superfamily of ion channels, TRPV5 is a highly Ca2+-selective channel important for active reabsorption of Ca2+ in the kidney. Its channel activity is controlled by a negative feedback mechanism involving calmodulin (CaM) binding. Combining advanced microscopy techniques and biochemical assays, this study characterized the dynamic lobe-specific CaM regulation. We demonstrate for the first time that functional (full-length) TRPV5 interacts with CaM in the absence of Ca2+, and this interaction is intensified at increasing Ca2+ concentrations sensed by the CaM C-lobe that achieves channel pore blocking. Channel inactivation occurs without requiring CaM N-lobe calcification. Moreover, we show a Ca2+-dependent binding stoichiometry at the single channel level. In conclusion, our study proposes a new model for CaM-dependent regulation – calmodulation – of this uniquely Ca2+-selective TRP channel TRPV5 that involves apoCaM interaction and lobe-specific actions, which may be of significant physiological relevance given its role as gatekeeper of Ca2+ transport in the kidney.

(Received 10 November 2022; accepted after revision 6 December 2022; first published online 25 December 2022)

Corresponding author Joost G. Hoenderop: Department of Physiology, Radboud Institute for Molecular Life Sciences, Radboud University Medical Centre, PO Box 9101, 6500 HB, Nijmegen, The Netherlands. Email: joost.hoenderop@radboudumc.nl

M. Zuidscherwoude and M. K. van Goor have contributed equally to this work.

This article was first published as a preprint. Roig SR, Thijsen N, Erp Mv, Fransen J, Hoenderop JG, Wijst Jv. 2021. Functional basis for calmodulation of the TRPV5 calcium channel. bioRxiv. https://doi.org/10.1101/2021.02.16.431366

© 2022 The Authors. The Journal of Physiology published by John Wiley & Sons Ltd on behalf of The Physiological Society. DOI: 10.1113/JP282952

This is an open access article under the terms of the Creative Commons Attribution License, which permits use, distribution and reproduction in any medium, provided the original work is properly cited.
Abstract figure legend  Calmodulation of the epithelial calcium channel TRPV5. The Ca\(^{2+}\) concentration modulates calmodulin-dependent inhibition of TRPV5. The three panels depict key moments in a continuum of increasing Ca\(^{2+}\) concentration (gradient slider under the panels). The left panel shows a situation where Ca\(^{2+}\) is virtually absent and CaM is in its apo-CaM conformation. The interaction between TRPV5 and apo-CaM does not rely on the presence of Ca\(^{2+}\) even though CaM-dependent inhibition of TRPV5 is compromised. The middle panel depicts a physiologically relevant Ca\(^{2+}\) concentration where one or both lobes of CaM are calcified and the TRPV5-CaM complex undergoes a conformational change that plugs the TRPV5 pore and inhibits the channel. The rightmost panel shows that increased intracellular Ca\(^{2+}\) concentration stimulates the recruitment of multiple CaM molecules to active TRPV5 channels, leading to flexible binding stoichiometry. Through this mechanism CaM is able to effectively close TRPV5 channels based on the intracellular Ca\(^{2+}\) concentration, preventing levels of Ca\(^{2+}\) entry that could lead to cytotoxicity.

Key points
- The renal Ca\(^{2+}\) channel TRPV5 is an important player in maintenance of the body’s Ca\(^{2+}\) homeostasis.
- Activity of TRPV5 is controlled by a negative feedback loop that involves calmodulin (CaM), a protein with two Ca\(^{2+}\)-binding lobes.
- We investigated the dynamics of the interaction between TRPV5 and CaM with advanced fluorescence microscopy techniques.
- Our data support a new model for CaM-dependent regulation of TRPV5 channel activity with CaM lobe-specific actions and demonstrates Ca\(^{2+}\)-dependent binding stoichiometries.
- This study improves our understanding of the mechanism underlying fast channel inactivation, which is physiologically relevant given the gatekeeper function of TRPV5 in Ca\(^{2+}\) reabsorption in the kidney.

Introduction

Transient receptor potential (TRP) channels are one of the largest classes of ion channels and are widely expressed throughout the animal kingdom (Venkatachalam & Montell, 2007). Since their discovery, they have emerged as key players in human physiology and were found to be associated with various diseases, such as cancer, skeletal abnormalities, skin disorders, and chronic pain (Nilius, 2007; Wu et al., 2010). The mammalian TRP family consists of six subfamilies; canonical (TRPC), melastatin (TRPM), vanilloid (TRPV), polycystin (TRPP), ankyrin (TRPA) and mucolipin (TRPML), categorized based on sequence homology (Clapham et al., 2001).

There is a striking variability in their channel properties in contrast to other families of ion channels. While most TRP channels are rather non-selective for ion permeation, TRPV5 and its close homologue TRPV6 exhibit a highly selective calcium (Ca\(^{2+}\)) permeability (Nilius et al., 2000; Vennekens et al., 2000). This concurs with their transport function in Ca\(^{2+}\) (re)absorbing epithelia of the kidney and intestine. Functionally, the channels are characterized as constitutively open at physiological membrane potentials, but they utilize a Ca\(^{2+}\)-dependent feedback mechanism to achieve fast channel inactivation and slow current decay (Nilius et al., 2001; Vennekens et al., 2001). Over the years, it has been established that this regulation involves the Ca\(^{2+}\)-binding protein calmodulin (CaM) (Bate et al., 2018; Bokhovchuk et al., 2018; de Groot et al., 2011; Kovalevskaya et al., 2012; Lambers et al., 2004; Nilius et al., 2003).

CaM-dependent regulation is well studied across voltage-gated Ca\(^{2+}\) channels (Ca\(_v\)), where it is also referred to as ‘calmodulation’ (Ben-Johny & Yue, 2014). CaM consists of amino (N)-terminal and carboxy (C)-terminal lobes, each containing two EF hand pairs capable of binding Ca\(^{2+}\), that are joined by a flexible central linker. CaM can modulate channel regulation by binding Ca\(^{2+}\) to either its N- or C-lobe or both. Moreover, it is known to bind Ca\(_v\) channels in its Ca\(^{2+}\)-free state (apoCaM) and can consequently transduce relevant signals upon Ca\(^{2+}\) binding (Ben-Johny & Yue, 2014). This elegant feedback mechanism can tune channel gating, and thus regulate Ca\(^{2+}\) influx in accordance with cytosolic Ca\(^{2+}\) signals. While CaM is known to bind various TRP channels, including TRPV5/6, surprisingly little is known about such ‘TRP calmodulation’, and the dynamics of this process and stoichiometry of binding are poorly understood.

Technological advances in single particle cryo-electron microscopy (cryo-EM) have demonstrated a transformative power in generating high resolution structures of TRP channels. Three independent groups, including ours, have provided detailed structural insight into TRPV5 and TRPV6 in complex with CaM (Dang et al., 2019; Hughes et al., 2018; Singh et al., 2018). All structures were resolved after purifying the complex in buffers containing a high Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]; 5 mm).
This universally demonstrated binding of the CaM N- and C-lobes to the proximal and distal C-terminal regions of the channel respectively (Dang et al., 2019; Hughes et al., 2018; Singh et al., 2018). Moreover, a general blockade mechanism was shown, in which the side chain of K115 of the CaM C-lobe protrudes into the channel pore. However, one of the important differences between these studies is the complex stoichiometry. Singh et al. and Hughes et al. showed that both the TRPV6-CaM and TRPV5-CaM, respectively, exhibit a 1:1 stoichiometry (Singh et al., 2018), while a flexible stoichiometry of 1 tetrameric channel binding up to 2 CaM molecules was postulated based on our TRPV5-CaM complex structure (Dang et al., 2019). In order to fully understand channel regulation, it is crucial to unravel the dynamic interaction of CaM with TRPV5, including apoCaM binding and lobe-specific effects.

Using a combination of Förster resonance energy transfer (FRET)-based fluorescence lifetime imaging microscopy (FLIM; FLIM-FRET) and Fura-2 Ca\(^{2+}\) imaging, we studied the functional association of Ca\(^{2+}\)-bound CaM, apoCaM and isolated CaM N- and C-lobes with TRPV5. Ca\(^{2+}\)-insensitive CaM mutants, with either two EF hands (CaM12 or CaM34) or all four EF hands (CaM1234) mutated, were used to investigate the Ca\(^{2+}\) dependence and lobe-specific effects of TRPV5 channel inactivation. Moreover, we studied CaM binding to individual TRPV5 channels at the plasma membrane of intact cells by photobleaching step analysis with total internal reflection fluorescence microscopy (SMP-TIRFM).

**Methods**

**Molecular cloning**

Restriction enzyme digestion, DNA ligations and other recombinant DNA procedures were performed using standard protocols. Rabbit TRPV5 was cloned into a pmCherry vector containing a N-terminal mCherry tag. Human NCC with a C-terminal mCherry tag was obtained as previously described (Tutakhe et al., 2018). Rat CaM and truncated forms of the protein, N-lobe (aa1–80) and C-lobe (aa81–149), were cloned into a pcDNA vector containing a N-terminal eGFP tag. Human CaM12, CaM34 and CaM1234 were based on mutations in the EF-hand loops (D20A, D56A, D93A, D129A) of the N- and C-lobes, known to affect Ca\(^{2+}\)-binding (Geiser et al., 1991). Human TRPV5 was cloned into a pcDNA vector containing a N-terminal eGFP tag. Mutagenesis for CaM and TRPV5 mutants was performed using the QuikChange site-directed mutagenesis method (Stratagene, San Diego, CA, USA) following manufacturer’s protocol. All DNA constructs were verified by DNA sequencing. DNA for mammalian cell transfection was amplified in E. coli TOP10f strain and plasmid preparation was done using the Macherey-Nagel Nucleobond Xtra Midi kit according to the manufacturers’ protocols.

**Buffers**

Lysis buffer: 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 2 mM EGTA, 1% (v/v) Triton X-100, 1 mM sodium orthovanadate, 10 mM sodium-glycerophosphate, 50 mM sodium fluoride, 10 mM sodium pyrophosphate, 0.27 mM sucrose, and the freshly added protease inhibitors pepstatin A (1 μg/ml), PMSF (1 mM), leupeptin (5 μg/ml), and apronin (1 μg/ml). TBS-Tween (TBS-T): Tris-HCl (200 mM, pH 7.5), 150 mM NaCl, and 0.2% (v/v) Tween-20. Laemmli sample buffer (5×): 10% (w/v) SDS, 25% (v/v) β-mercapto-ethanol, 50% (v/v) glycerol, 0.3 mM Tris-HCl (pH 6.8), 0.05% (v/v) Bromophenol Blue. Fura-2 wash buffer: 132.0 mM NaCl, 4.2 mM KCl, 5.5 mM d-glucose, 10 mM HEPES/Tris, pH 7.4. Fura-2 Ca\(^{2+}\) buffer: 132.0 mM NaCl, 4.2 mM KCl, 2.8 mM CaCl\(_2\), 1.0 mM MgCl\(_2\), 5.5 mM d-glucose, 10 mM HEPES/Tris, pH 7.4. Fura-2 ethylenediaminetetraacetic acid (EDTA) buffer: 132.0 mM NaCl, 4.2 mM KCl, 2 mM EDTA, 5.5 mM d-glucose, 10 mM HEPES/Tris, pH 7.4. Plasma membrane lawn preparation (KH buffer): 70 mM KCl and 30 mM HEPES, pH 7.5, adjusted with KOH.

**Antibodies and peptides**

Anti-GFP antibody (1:5,000; G1544), anti-GST antibody (1:5000; G7781) and anti-beta-actin (1:10,000; A5441) were purchased from Sigma Aldrich (St Louis, MO, USA). Anti-calmodulin antibody (1:500; 05-173) was purchased from Upstate Biotechnology (now part of Merck, Lake Placid, NY, USA). Secondary antibodies coupled to horseradish peroxidase used for immunoblotting are goat anti-rabbit IgG (1:10000), A4914, Sigma) and sheep anti-mouse IgG (1:10000, 145-515-035), purchased from Brunswig Chemie (Basel, Switzerland). The following peptides were purchased from EMG microcollections GmbH (Tübingen, Germany): N'-Biotin-C6-QSSNHGWEILRNTLGHL-C' (TRPV5 distal helix), Biotin-C6-ENHHDQNPLRVLRYV EAFKCSDKEDGQ-C' (TRPV5 proximal helix).

**Cell culture**

HEK293 (human embryonic kidney 293) cells were grown in Dulbecco’s modified Eagles medium (DMEM, Lonza, Basel, Switzerland) supplemented with 10% (v/v) fetal bovine serum (BioWest, Nuaille, France), 2 mM L-glutamine, and 10 μl/ml non-essential amino acids at
37°C in a humidity-controlled incubator with 5% (v/v) CO₂. For transient transfection, cells were transfected 6–8 h after seeding with the respective DNA construct using polyethyleneimine (PEI, Brunschwig Chemie, Basel, Switzerland) with a DNA:PEI ratio of 1:6.

**Co-immunoprecipitation assay**

HEK293 cells were seeded on 6-well plates and co-transfected with GFP-TRPV5 (1500 ng) and mock GST (pEBG), GST-tagged CaM wild-type or indicated mutants (1000 ng). After 36 h, cells were lysed in lysis buffer supplemented with 5 mM CaCl₂. Lysates were cleared by centrifugation at 4°C for 15 min at 16 000 g and protein concentration was measured by the Bradford method (Bio-Rad, Hercules, CA, USA). Next, 1 mg lysate was incubated with glutathione agarose resin (GE Healthcare, Chicago, IL, USA) for 2 h at 4°C under gentle rotation. The resin was washed 3 times with lysis buffer, and proteins were eluted in 30 μl of 2× Laemmli sample buffer.

**Peptide pull-down assays**

For a peptide pull-down, HEK293 cells were seeded on 10 cm Petri dishes, transfected with either mock (pEBG), GST-tagged CaM wild-type, CaM N-lobe, or CaM C-lobe (5000 ng), and lysed 36 h after transfection. Lysis buffer contained 5 mM CaCl₂. A 1 mg sample of cleared lysate was incubated with 3 μg of the respective peptides for 10 min at 4°C, followed by 5 min incubation with 20 μl streptavidin agarose resin (Thermo Fisher Scientific, Waltham, MA, USA) at 4°C under gentle rotation. The resin was washed 3 times with lysis buffer and proteins were eluted in 30 μl of 2× Laemmli sample buffer.

**CaM binding assay**

HEK293 cells were seeded on 10 cm Petri dishes, transfected with mock (pcDNA) or GFP-TRPV5 (5000 ng) plasmids. Thirty-six hours after transfection, HEK293 cells were seeded on 6-well plates and co-transfected with GFP-TRPV5 (1500 ng) and mock GST (pEBG), GST-tagged CaM wild-type or indicated mutants (1000 ng). After 36 h, cells were lysed in lysis buffer supplemented with 5 mM CaCl₂. Lysates were cleared by centrifugation at 4°C for 15 min at 16 000 g and protein concentration was measured by the Bradford method (Bio-Rad, Hercules, CA, USA). Next, 1 mg lysate was incubated with glutathione agarose resin (GE Healthcare, Chicago, IL, USA) for 2 h at 4°C under gentle rotation. The resin was washed 3 times with lysis buffer, and proteins were eluted in 30 μl of 2× Laemmli sample buffer.

**Immunoblotting**

All samples (immunoprecipitates and total cell lysates) were subjected to 8–12% (w/v) SDS-PAGE and transferred to polyvinylidene fluoride (PVDF) membranes. These membranes were blocked for 30 min with 5% (w/v) non-fat dry milk (NFDM; in TBS-T) and immunoblotted overnight at 4°C using indicated primary antibodies. Next, the blots were washed with TBS-T, incubated with secondary peroxidase-labelled secondary antibodies (in 5% (w/v) NFDM/TBS-T) for 1 h at room temperature. Following repeated washes, protein expression was visualized with chemiluminescence SuperSignal West reagent (Thermo Fisher Scientific) using the Bio-Rad ChemiDoc XRS imaging system.

**Plasma membrane lawns preparation**

Plasma membrane lawns (PML) were obtained by unroofing cells with an osmotic shock (Moreno et al., 2015; Perez-Verdaguer et al., 2016). HEK293 cells were seeded on 18 mm diameter round coverslips coated with poly-L-lysine (PLL) and transfected with 650 ng donor and 1350 ng acceptor plasmid. After 24 h of transfection, cells were washed once with ice-cold PBS. Membrane labelling was performed by incubating cells for 10 min on ice with wheat germ agglutinin Alexa Fluor 680 conjugate (WGA-AF680, Thermo Fisher Scientific) diluted 1:100 in DMEM:30 mM HEPES, followed by two washes of 5 min with ice-cold PBS to remove unbound WGA-AF680. Next, an osmotic shock was performed by incubating cells for 5 min with ice-cold KH buffer, 1:3 diluted in distilled water. This results in cell swelling and membrane rupture, while soluble proteins (i.e. mCherry) will be washed out. For experiments depicted in Figs 2 and 4, KH buffer was either supplemented with 2 mM CaCl₂ (Ca²⁺-containing conditions) or with 2 mM EGTA and 2 mM EDTA (Ca²⁺-free conditions). Membranes were fixed after washes with this supplemented KH buffer.

**FLIM-FRET**

HEK293 cells were seeded on PLL coated coverslips (18 mm diameter) and transfected with indicated donor (450 ng, eGFP) and acceptor (950 ng, mCherry) plasmids, and/or corresponding mock DNA for 24 h. Control
Intracellular Ca\textsuperscript{2+} measurements using Fura-2-AM

HEK293 cells were seeded on 12-well plates and co-transfected with mCherry-TRPV5 (500 ng) and eGFP-CaM wild-type or indicated mutants (500 ng). After 36 h, cells were seeded in fibronectin-coated Press-to-Seal silicone isolator wells (Molecular Probes; diameter of 2.5 mm) on Superfrost Plus Microscope Slides (Thermo Fisher Scientific). After 2–4 h, cells were loaded with 3 μM Fura-2-AM and 0.01% (v/v) Pluronic F-129 (both from Molecular Probes) in DMEM medium at 37°C for 20 min. After loading, the cells were washed twice with Fura-2 wash buffer and allowed to equilibrate for another 10 min in Fura-2 EDTA buffer. Next, the slide was placed on an inverted microscope (Axiovert 200M, Carl Zeiss, Jena, Germany) at 20× magnification, and eGFP and mCherry double-positive cells were selected as transfected cells. Prior to the start of the experiment, regions of interest were drawn in the intracellular region of these cells to monitor the mean fluorescence intensity for each wavelength. For the purpose of background correction, a region of identical size was drawn at the extracellular side. Intracellular Ca\textsuperscript{2+} levels were monitored after fluorescence excitation at 340 and 380 nm using a monochromator (Polychrome IV, TILL Photonics, Gräfelfing, Germany). Fluorescence emission light was directed by a 415DCLP dichroic mirror (Omega Optical, Inc., Brattleboro, VT, USA) through a 510WB40 emission filter (Omega Optical Inc) onto a CoolSNAP HQ monochromochrome CCD-camera (Roper Scientific, Vianen, The Netherlands). No significant fluorescence contribution from the GFP-tagged proteins was observed on the Fura-2 collected images. The integration time of the CCD-camera was set at 200 ms with a sampling interval of 3 s. All hardware was controlled with Metafluor (version 6.0) software (Universal Imaging Corp., Downingtown, PA, USA). Fura-2 buffer containing CaCl\textsubscript{2} was added after reaching a steady basal state.

After background correction, the fluorescence emission ratio of 340 and 380 nm excitation was calculated to determine changes in intracellular Ca\textsuperscript{2+} concentration. The peak response is calculated as the difference in ratio between Fura-2 Ca\textsuperscript{2+} buffer addition versus basal levels in Fura-2 EDTA buffer ($t$\textsubscript{Ca} – $t$\textsubscript{EDTA}). For each cell in every condition, $t$\textsubscript{Ca} was calculated by averaging the first four values after addition of Fura-2 Ca\textsuperscript{2+} buffer, and $t$\textsubscript{EDTA} originates from averaging five reference values at baseline. Subtracting $t$\textsubscript{EDTA} from $t$\textsubscript{Ca} for each cell resulted in individual peak responses ($t$\textsubscript{Ca} – $t$\textsubscript{EDTA} ) that were averaged per condition and depicted in a bar graph. All measurements were performed at room temperature.

Photobleaching step analysis

HEK293 were transfected in six wells plates using a modified protocol in which small amounts of the DNA plasmids of interest (50 ng mCherry-TRPV5 and 5 ng eGFP-CaM) were supplemented to 1 μg with stuffer DNA and transfected in a 1:2.5 ratio of DNA to lipofectamine. After 5 h, cells were detached with accutase and replated onto fibronectin-coated 25 mm round coverslips (No. 1.5 Micro Coverglass, Electron Microscopy Sciences). Cells were incubated in DMEM for 5 more h at 37°C in a humidity-controlled incubator at 5% (v/v) CO\textsubscript{2}. For the Ca\textsuperscript{2+}-supplemented and Ca\textsuperscript{2+}-depleted experimental conditions, the extracellular medium of the cells was washed once with, and then changed to Fura-2 Ca\textsuperscript{2+} buffer 0.1% (v/v) DMSO (10 min), Fura-2 Ca\textsuperscript{2+} buffer + 25 μM BAPTA-AM 0.1% (v/v) DMSO (10 min) or Fura-2 EDTA buffer 0.1% (v/v) DMSO (2 min to prevent cell detachment). Cells were fixed in 4% (v/v) PFA for 1 h, washed 3 times with PBS, incubated for 10 min in 50 mM NH\textsubscript{4}Cl and washed 3 more times in PBS. Coverslips were mounted in a custom made low-drift magnetic sample holder and cells were imaged in PBS at RT.

TIRF microscopy was performed on a custom build low-drift inverted microscope setup controlled by...
custom-written software in Matlab (MathWorks Inc.), equipped with a nanometre resolution $xyz$ piezo stage (PInano P-545.3R7 Piezo System, Physik Instrumente), 500 mW 488 nm, 500 mW 561 nm, 1000 mW 639 nm (Genesis MX STM OPLS Laser System Coherent), and 100 mW 405 nm (OBIS LX Laser System, Coherent) laser light sources and an APON 60XOTIRF /1.49 oil objective (Olympus America Inc.).

Light was filtered by a quad band dichroic (Di03-R405/488/561/635-t3-25×36, Semrock) used in combination with a triple colour emission filter set (ET525/50m, ET605/52m, ET655lp, Chroma) and was focused (f:175bmm achromatic, Newport, PAC061AR.14) on a scientific CMOS camera (ORCA-Flash4.0 V2, Hamamatsu).

Imaging was performed in total internal reflection illumination mode. Low expressing cells were selected based on their mCherry signal, and the mCherry signal was photobleached first, followed by single molecule photobleaching of eGFP. Sample plane excitation power densities were selected to ensure complete photobleaching with detectable single bleach step events. Five thousand frames were acquired in a ROI of 300 × 300 pixels with a pixel size of 0.111 μm at an exposure time of 50 ms. Fiducial Markers (100 nm Tetraspek; Life Technologies) were used to correct for chromatic aberrations.

Image analysis was performed using Fiji Image J. The first 20 frames of each sequence were averaged and the mCherry images were translated to correct for chromatic aberrations. The centre positions of fluorescent spots were then localized using ImageJ plugin ThunderSTORM (ThunderSTORM: a comprehensive ImageJ plug-in for PALM and STORM data analysis and super-resolution imaging. Bioinformatics. 2014) using a local maximum localization of molecules with a peak intensity threshold of 2$\times$std(Wave.F1). The merging function (300 nm) was used to generate a $xy$ location list of fluorescent spots containing mCherry, eGFP or both. Stepwise photobleaching analysis was performed on the eGFP tiff stacks using the python package quickPBSA (Hummert et al., 2021). Photobleaching traces were extracted from an ROI with a 2-pixel radius. A ring-shaped ROI with an inner radius of 3, and an outer radius of 4 pixels was used for local background subtraction. Spots within 4 pixels of another spot were excluded. Photobleaching step analysis was run in quickPBSA using default parameters and a step detecting threshold of 4.5. The Bayesian refinement step in the quickPBSA analysis was not used as it is not suitable for small fluorophore numbers (Clark et al., 2021).

Data analysis and statistics

The immunoblot data were analysed by comparing integrated optical densities of bands using Fiji (Schindelin et al., 2012). The semi-quantification is shown as mean ± SD and plotted against the log free Ca$^{2+}$ concentration (Fig. 2) or log peptide concentration (Fig. 3). Non-linear fitting of the curves to the log concentration was done with the following formula:

$$y = 2.1910^{-5} + \frac{1.011}{1 + 10^{(0.7192-x)×(−1.527)}}$$

To estimate the percentage of eGFP-tagged CaM from total CaM expression, the integrated optical densities of the eGFP-CaM and endogenous CaM bands were added together, and fraction of eGFP-CaM was determined. FLIM-FRET data is shown as individual data points with a mean ± SEM indicated in each graph, with $n$ as the number of cells. The Fura-2-AM measurements are depicted as averaged 340/380 ratio + SD over time, with $n$ as the number of cells, and a bar graph showing the peak response ($t_{Ca} - t_{EDTA}$) as mean ± SD. For all data, $P < 0.05$ was considered statistically significant using a one-way ANOVA with a Dunnett’s multiple comparison post hoc test. Data representation and analysis was done using Fiji software (Schindelin et al., 2012) and GraphPad Prism 8.0 (GraphPad Software Inc., San Diego, CA, USA). For the Fura-2-AM data, Graphpad was used to test for normality of distribution and identify outliers. In case of non-normal distribution, a Kruskal-Wallis test with Dunn’s multiple comparisons test was performed. For the photobleaching step analysis data, number of steps was considered as a categorical variable. A relationship between the number of steps and the different cell treatments was tested in a $3 \times 4$ contingency table and a Chi-square test using Graphpad.

Results

Constitutive interaction of TRPV5 and CaM

Over the last decades, several groups focused attention on the TRPV5-CaM interaction (Bokhovchuk et al., 2018; de Groot et al., 2011; Holakovska et al., 2011; Kovalevskaya et al., 2012; Lambers et al., 2004). Biochemical studies and NMR (nuclear magnetic resonance) analysis showed that TRPV5 binds to Ca$^{2+}$-CaM (Bokhovchuk et al., 2018; de Groot et al., 2011; Kovalevskaya et al., 2012; Lambers et al., 2004). However, these studies were limited by their use of only two distinct conditions: either a complete absence of free Ca$^{2+}$ by addition of EGTA, or high levels of Ca$^{2+}$. Moreover, these studies were mainly based on truncated versions of the channel. Recent cryo-EM structures of TRPV5 in complex with CaM exposed their exact interaction interface and postulated how CaM blocks the TRPV5 channel pore in the presence of Ca$^{2+}$ (Dang et al., 2019; Hughes et al., 2018). Yet the dynamic interaction between TRPV5 and CaM that underlies channel blocking is poorly understood.

We applied the forster resonance energy transfer technique (FRET) using fluorescence lifetime imaging...
microscopy (FLIM) to unravel the Ca\(^{2+}\) dependency of the interaction between TRPV5 and CaM. To this end, wild-type and well-established Ca\(^{2+}\)-insensitive mutants of CaM (CaM, CaM12, CaM34, CaM1234) were fused to eGFP, and co-expressed with mCherry or mCherry-tagged TRPV5 in HEK293 cells (Fig. 1A and B). These CaM mutants are deficient in binding Ca\(^{2+}\) due to point mutations in the N-lobe EF hands (CaM12), C-lobe EF hands (CaM34) or all four EF hands (CaM1234). Expression and distribution of all CaM mutants resembles CaM wild type (Fig. 1A). Co-expression with TRPV5 shifted the distribution of CaM and CaM12 from a whole-cell localization to an extranuclear distribution (Fig. 1A, left panel). CaM34 and CaM1234 distributed mainly in the nuclei, showing high values of decay due to their concentrations. Importantly, only extranuclear ROIs were analysed, as TRPV5 is not localized in the nuclei. Lifetime decay (\(\tau\)) measurements were performed in areas of CaM-TRPV5 co-localization. eGFP-CaM and eGFP-CaM12 showed a significantly decreased \(\tau\) of eGFP in cells co-expressing mCherry-TRPV5 compared to cells co-expressing mCherry (Fig. 1B, \(P < 0.0001\) both conditions). eGFP-CaM1234 depicted a modest but significant decrease of \(\tau\), once co-expressed with TRPV5 (Fig. 1B, \(P = 0.0478\)). Interestingly, eGFP-CaM34 did not show any changes in \(\tau\) (Fig. 1B, \(P = 0.9829\)). To exclude potential measurement of collisional FRET, we included control experiments with the sodium-chloride cotransporter (NCC), a transmembrane protein with a similar cellular distribution to that of TRPV5 (Fig. 1C), which is not known to be associated with CaM. Importantly, lifetime decay measurements did not show changes in the \(\tau\) of cells co-expressing NCC-mCherry with eGFP-tagged CaM or CaM1234 (Fig. 1D, \(P = 0.0571\) and \(P = 0.7352\), respectively).

To corroborate the FLIM-FRET measurements, we performed co-immunoprecipitation assays of GFP-TRPV5 with GST-tagged CaM wild-type and mutants (Fig. 1E). All CaM proteins demonstrated similar expression by equal pull-down with GST-sepharose resin (Fig. 1E, lower co-IP panel). TRPV5 showed a clear interaction with CaM and CaM12. Similar to the FLIM-FRET results, CaM1234 maintained the capability of interacting with TRPV5, albeit to lesser extent compared to CaM WT or CaM12, while CaM34 showed little-to-no interaction with TRPV5 (Fig. 1E, upper co-IP panel). Note that total lysate shows equal expression of all proteins (Fig. 1E, total lysate panel).

Next, Fura-2 Ca\(^{2+}\) imaging experiments were performed to evaluate the functional effect of the CaM mutants on TRPV5 activity. Note that HEK293 cells express endogenous CaM. The TRPV5-transfected cells showed a peak response with a plateau after addition of Ca\(^{2+}\), which was significantly reduced upon co-expression with wild-type CaM (Fig. 1F and G, \(P < 0.0001\)). Interestingly, co-expression with CaM12 also resulted in a reduced peak response compared to endogenous CaM (Fig. 1F and G; \(P = 0.0436\)), suggesting that CaM12 is able to inhibit TRPV5 function. This finding is in line with observations in Fig. 1A and B. Note that the plateau phase is similar to the condition with endogenous CaM. In contrast, co-expression with CaM1234 or CaM34 yielded an increased Ca\(^{2+}\) peak response compared to endogenous CaM (Fig. 1F and G; \(P = 0.0065\) and \(P = 0.0191\), respectively). We suggest that CaM1234 or CaM34 can interfere with endogenous CaM binding via a competitive interaction with TRPV5. Interestingly, the co-expression with CaM1234 leads to a higher plateau phase, which is not seen for CaM34. As has been proposed by others, it thereby has a counteracting effect on endogenous CaM-dependent inhibition (Fruen et al., 2003). These data indicate that the CaM C-lobe Ca\(^{2+}\) binding is crucial for TRPV5 inactivation, while the N-lobe does not need to be calcified and may have a supporting role.

Ca\(^{2+}\) dependency of TRPV5-CaM interaction

The CaM N-lobe and C-lobe each bind Ca\(^{2+}\) at different affinity, and these relative affinities can change when CaM engages with target proteins (Lai et al., 2015). While the C-lobe presents a 10-fold higher affinity than the N-lobe, the kinetics are much slower (Lai et al., 2015). To understand the magnitude of the Ca\(^{2+}\) dependency of the CaM interaction with TRPV5, and the contribution of each lobe, a CaM agarose pull-down of TRPV5-expressing HEK293 cells was performed under different Ca\(^{2+}\) concentrations mimicking intracellular Ca\(^{2+}\) levels (0–10 \(\mu\)M) (Fig. 2A, pull-down panel). These were calculated as described in Methods. Interestingly, we detected a weak interaction of CaM with TRPV5 in the absence of Ca\(^{2+}\) (apoCaM) (Fig. 2A), which supports the observed interaction between TRPV5 and CaM1234 in our FLIM-FRET experiments. Fig. 2A and B also demonstrate a Ca\(^{2+}\)-dependent interaction as TRPV5 pull-down was intensified upon increasing Ca\(^{2+}\) concentrations. In line with the FLIM-FRET results in whole cells under basal conditions (Fig. 1A and B), significant interaction was already detectable at 10 nm Ca\(^{2+}\). At this Ca\(^{2+}\) concentration the C-lobe will likely be calcified, based on previous studies summarized in the model by Lai et al. (2015). These results point towards a pre-existing TRPV5-CaM interaction at basal Ca\(^{2+}\) concentrations.

To confirm the interaction of apoCaM with TRPV5, we performed FLIM-FRET analysis on apoCaM and TRPV5 expressing HEK293 cells (Fig. 2C) and provided access to the intracellular compartment. Only proteins associated to the membrane remain in these preparations (Gonzalez-Munoz et al.,
Figure 1. Constitutive and Ca\(^{2+}\)-dependent interaction of TRPV5 and CaM

A, interaction of TRPV5 with CaM and the Ca\(^{2+}\)-insensitive mutants CaM12, CaM34 and CaM1234 in HEK293 cells was addressed by FLIM-FRET. Donors: eGFP-CaM, eGFP-CaM12, eGFP-CaM34, or eGFP-CaM1234. Acceptors: mCherry (Mock) or mCherry-TRPV5. mCherry is used as negative control in each condition. Representative images are shown for the donor (eGFP column, grey) and acceptor (mCherry column, grey) expression, merged channels (eGFP in green, mCherry in magenta, colocalization in light pink or white) and FLIM-FRET (FRET column). Example of measured ROIs represented as red boxes on the FLIM-FRET image, at extranuclear colocalizing areas. Bars represent 10 μm.

B, lifetime decay of CaM wild-type and indicated mutants in the presence of either mock or TRPV5, depicted as single measurements of ROIs at cytoplasmic colocalization with mean ± SD (n = 30–38 cells).

∗Statistical significance compared to mock within each condition (ANOVA, see text for P values).

C, representative images of TRPV5 (top) and the sodium-chloride cotransporter NCC (bottom) depicting similar cellular distribution. Bars represent 10 μm.

D, lifetime decay of CaM and CaM1234 in the presence of mock, TRPV5 or NCC, depicted as single measurements of ROIs at extranuclear colocalizing areas with mean ± SD (n = 15–20 cells).

Donor: eGFP-CaM or eGFP-CaM1234. Acceptors: mCherry (mock), mCherry-TRPV5 or NCC-mCherry.

∗Statistical significance compared to mock in the CaM condition (ANOVA, see text for P values); #statistical significance compared to mock in the CaM1234 condition (ANOVA, see text for P values).

E, co-immunoprecipitations of HEK293 cells co-expressing eGFP-TRPV5 with GST-tagged CaM wild-type or indicated mutants. Mock transfection.
Calmodulin-dependent regulation of TRPV5

with pcDNA (eGFP) and pEBG (GST) are used as negative controls. Representative immunoblots are shown for total lysate (bottom two lanes) and the GST-immunoprecipitated fractions of GST-CaM and eGFP-TRPV5 (top two lanes). $F$, intracellular Ca$^{2+}$ measurements with Fura2-AM of HEK293 cells expressing mCherry-TRPV5 with either mock or CaM-eGFP wild-type or Ca$^{2+}$-insensitive mutants (CaM12, CaM34, CaM1234). The 340/380nm ratiometric changes are shown as averaged data points ± SD (dotted line) over time (n = 80–160 cells). Baseline and addition of Fura Ca$^{2+}$-containing buffer are indicated by the arrows ($t_{\text{EDTA}}$ and $t_{\text{Ca}}$, respectively). TRPV5 and mock (cyan), TRPV5 and CaM (orange), TRPV5 and CaM12 (magenta), TRPV5 and CaM34 (red), TRPV5 and CaM1234 (green). $G$, bar graph of the Fura-2 peak response (n = 80–160 cells), measured as the Fura-2 ratio difference between the Ca$^{2+}$ response ($t_{\text{Ca}}$) and baseline ($t_{\text{EDTA}}$), indicated by the arrows in panel $F$. The indicated conditions in the bar graph contain corresponding colour coding with panel $F$. *Statistical significance compared to TRPV5/mock within each condition (ANOVA, see text for P values). [Colour figure can be viewed at wileyonlinelibrary.com]

2009; Perez-Verdaguer et al., 2018). We first confirmed the presence of TRPV5-containing membranes (Fig. 2D and $E$). Next, we studied the presence of eGFP-CaM in PMLs treated with Ca$^{2+}$-containing (2 mm) and Ca$^{2+}$-free (2 mm EGTA, 2 mm EDTA) solutions. Even though CaM is a cytosolic protein, CaM and TRPV5 positively co-localized in all PML conditions (Fig. 2F). FLIM-FRET measurements proved that both Ca$^{2+}$-CaM (Fig. 2G; P < 0.0001) and apoCaM interact with TRPV5, although it has to be noted that the interaction with apoCaM is weaker (Fig. 2G; P = 0.0002).

Interaction interface of TRPV5-CaM

While the recently published cryo-EM structures of the TRPV5-CaM complex revealed that the CaM C-lobe is occluding the pore leading to channel inhibition (Dang et al., 2019; Hughes et al., 2018), no information has been provided about the dynamic bi-lobal CaM regulation of TRPV5. A proximal C-terminal helix of TRPV5 was shown to bind the CaM N-lobe, while a distal C-terminal helix interacts with the CaM C-lobe (Dang et al., 2019; Hughes et al., 2018). To investigate the relative importance of these distinct binding interfaces, we synthesized peptides of the corresponding TRPV5 helices – proximal peptide and distal peptide (Fig. 3A). Binding of these biotin-linked peptides to full length CaM was demonstrated by a peptide pull-down via streptavidin resin in lysates of wild-type CaM-expressing HEK293 cells (Fig. 3B). Furthermore, the peptides were used in a peptide competition assay to analyse differences in binding affinity to CaM. Addition of increasing amounts of peptide (0–100 μm) to a CaM agarose pull-down of TRPV5-expressing HEK293 cell lysates revealed decreased binding of TRPV5 to CaM-agarose by the distal peptide (Fig. 3C). Addition of the proximal peptide resulted in significantly fewer changes, suggesting a specific competition of the distal peptide (Fig. 3C) and a strong interaction of the CaM C-lobe with TRPV5 in the presence of Ca$^{2+}$. Non-linear fitting of the curves to the log concentration revealed a sigmoidal correlation ($R^2 = 0.942$) for the distal peptide (Fig. 3E), while no correlation was observed ($R^2 = 0.188$) for the proximal peptide (Fig. 3D). Based on the fitted formula describing the graph (see Methods), we calculated an IC$_{50}$ of 5.2 μm of the competing distal peptide.

Interestingly, single alanine mutations of TRPV5 residues from either the proximal (D90A, F651A) or the distal (W702A, W583A) CaM interaction interface did not abolish CaM binding as demonstrated by FLIM-FRET analysis (Fig. 3F and $G$; P < 0.0001 for all conditions). CaM binding to TRPV5 D90A and TRPV5 W702A was less compared to wild-type TRPV5 (Fig. 3G; P < 0.0001 and P = 0.0187, respectively). The interaction between TRPV5 and CaM is not focal to a specific residue but global, involving several sites. CaM1234 exhibited the same ability for interacting to all TRPV5 mutants (Fig. 3H; P = 0.0096 (TRPV5 wild-type), P = 0.0157 (TRPV5 D90A), P = 0.0016 (TRPV5 W583A), P = 0.0054 (TRPV5 W651A), P < 0.0001 (TRPV5 W702A)).

Lobe-dependent interaction in relation to TRPV5 function

Our results suggest a major role for the CaM C-lobe in the interaction with TRPV5. It led us to generate single N- and C-lobes to evaluate whether the lobes can act independently on TRPV5. Following similar steps to those mentioned above, we first addressed the interaction capability of both lobes. Both the CaM N- and C-lobe could independently interact with TRPV5 in whole cells, as shown by the reduction of lifetime decay (Fig. 4A and $B$; P < 0.0001 all conditions). The specific interaction of the independent lobes with the TRPV5 proximal and distal helices was studied in a peptide pull-down in lysates of HEK293 cells expressing CaM N- or C-lobe (Fig. 4C). The biotin-linked proximal and distal peptides were incubated with cell lysate containing either GST-tagged CaM wild-type or GST-tagged CaM N- or C-lobe, followed by pull-down via streptavidin resin. In line with our previous results and the structural findings of TRPV5-CaM (Dang et al., 2019; Hughes et al., 2018), most significant binding was observed between the CaM C-lobe and the distal TRPV5 peptide (Fig. 4C, bottom panel). Notably, there was also a weak but consistent interaction of the N-lobe with both peptides (Fig. 4C, top panel). Next, we addressed the potential effect on TRPV5 function. Fura-2 Ca$^{2+}$ imaging demonstrated that co-expression
of TRPV5 with either CaM N- or C-lobe increases the intracellular Ca$^{2+}$ peak compared to TRPV5 with endogenous or overexpressed wild-type CaM (Fig. 4D and E; $P = 0.0003$ (TRPV5/Mock), $P < 0.0001$ (TRPV5/N-CaM and TRPV5/C-CaM)). This indicates that, while the lobes can independently interact with TRPV5, full length CaM is needed for TRPV5 inhibition. Furthermore, it proposes that overexpression of N-CaM and C-CaM can prevent endogenous CaM from inhibiting TRPV5, possibly by sterical constraints.

We further explored the interaction of each CaM lobe in Ca$^{2+}$-free conditions using FLIM-FRET analysis of PMLs.
Figure 3. CaM C-lobe as major determinant for binding TRPV5

A, structural overview of the interaction interface of Ca$^{2+}$-bound CaM (pink) with the TRPV5 C-terminal helices (blue). Ca$^{2+}$ ions are depicted as cyan. B, biotin peptide pull-down of mock GST or GST-CaM expressing HEK293 cells. Representative immunoblots are shown for detection of GST-CaM in total lysate and biotin pull-down (using streptavidin resin) with either the distal or proximal TRPV5 peptides. C, CaM agarose pull-down of HEK293 cells expressing mock eGFP or eGFP-TRPV5, in the presence of increasing amounts (0–100 μM) of either the proximal or distal TRPV5 peptides. Representative immunoblots are shown for detection of eGFP-TRPV5 in CaM agarose pull-down fractions (left panel) and total lysate fractions (right panel). D and E, semi-quantification of CaM pull-down experiments expressed as mean ± SD (n = 3). Intensity of TRPV5 pull-down lanes were expressed against the proximal (D) or distal (E) peptide concentration and the graphs were fitted using a non-linear sigmoidal correlation. R$^2$ is indicated in the graph. F–H, interaction of mCherry-TRPV5 wild-type (WT) and indicated mutants with eGFP-CaM in HEK293 cells was addressed by FLIM-FRET. Representative images (F) are shown for the donor (eGFP column, grey) and acceptor (mCherry column, grey) expression, merged channels (donors: green, acceptor: magenta, co-localization: light pink or white) and FLIM-FRET image (FRET column). Donor: eGFP-CaM. Acceptors: mCherry (mock), mCherry-TRPV5 or indicated mutants (TRPV5 D90A, TRPV5 W583A, TRPV5 F651A, and TRPV5 W720A). Scale bars represent 10 μm. Life time decay of CaM (G) in the presence of mock, TRPV5 WT or indicated mutants, depicted as single measurements of ROIs at extranuclear colocalizing areas with mean ± SD (n = 25–30 cells). Life time decay of CaM1234 (H) in the presence of mock, TRPV5 WT or indicated mutants, depicted as single measurements of ROIs at cytoplasmic colocalization with mean ± SD (n = 15–20 cells). *Statistical significance compared to mock (ANOVA, see text for P values); †statistical significance compared to the TRPV5 WT (ANOVA, see text for P values). [Colour figure can be viewed at wileyonlinelibrary.com]
Figure 4. Involvement of the independent CaM lobes in TRPV5 inactivation

A, interaction of eGFP-CaM, eGFP CaM N-lobe (N-CaM), and eGFP-CaM C-lobe (C-CaM) with mCherry-TRPV5 in HEK293 cells was addressed by FLIM-FRET. Representative images are shown for donors (eGFP column, grey) and
from cells co-expressing TRPV5 and CaM N- or C-lobe. The N- and C-lobe interact in Ca\(^{2+}\)-containing (2 mM) (Fig. 4F–H; *P < 0.0001 (CaM), *P = 0.0365 (N-CaM). *P < 0.0001 (C-CaM)), but not in Ca\(^{2+}\)-free (2 mM EGTA, 2 mM EDTA) conditions (Fig. 4G and I; *P = 0.0152 (CaM), *P = 0.7728 (N-CaM). *P = 0.9914 (C-CaM)). Interestingly, full-length CaM can interact with TRPV5 in Ca\(^{2+}\)-free conditions, suggesting co-operation of the two lobes in effective TRPV5 binding. Summing up, our data support the idea that the C-lobe is the major entity for sensing Ca\(^{2+}\) changes and closing TRPV5. The N-lobe (and the linker) acts as a support to the structural rearrangement required for the C-lobe to execute its function.

**CaM binding to TRPV5 to individual TRPV5 channels at the plasma membrane**

In recent structural work by our group, we postulated that TRPV5 can bind multiple CaM molecules simultaneously. We based this hypothesis on the observation that multiple CaM densities could be seen in our TRPV5-CaM cryo-EM structure, irrespective of the symmetry imposed during structural analysis (Dang et al., 2019). While an intriguing observation, it is not known whether a flexible TRPV5-CaM binding stoichiometry occurs in intact cells and if it bears any functional significance or Ca\(^{2+}\) dependence. To explore this question further, we investigated the interaction between single TRPV5 channels and CaM on the plasma membrane of fixed HEK293 cells, using TIRF microscopy and photobleach step analysis. When overexpressed in HEK293 cells, TRPV5 localized mainly to intracellular vesicles. Since a very low level of co-expression of mCherry-TRPV5 and eGFP-CaM is of critical importance in the attempt to resolve individual TRPV5-CaM protein complexes at the plasma membrane, we tuned the level of expression down by transfecting cells with minimal amounts of DNA. Despite these adjustments, the majority of transfected cells still contained bright TRPV5-containing vesicular-tubular structures at or near the plasma membrane (Fig. 5Ai). Cells that were selected for analysis expressed mCherry-TRPV5 and eGFP-CaM at levels that were low enough to discern individual mCherry and eGFP fluorescent spots (Fig. 5Aii). Upon continuous imaging, eGFP molecules photobleach over time. Photobleach step analysis can then be used to determine the number of eGFP-CaM molecules present at an individual fluorescent spot. To identify TRPV5-CaM protein complexes, an automated spot detection algorithm was used, which determines the locations of mCherry-TRPV5 fluorescent spots, eGFP-CaM fluorescent spots and subsequently spots in which mCherry-TRPV5 and eGFP-CaM were co-localized (Fig. 5B). The local background was subtracted from the fluorescent signal of these individual spots (Fig. 5C), the eGFP fluorescence intensity traces were extracted, and the number of photobleaching steps was determined (Fig. 5D) using the quickPSBA algorithm (Hummert et al., 2021). The number of bleaching steps corresponds to the number of fluorescent eGFP.
Figure 5. (Apo)CaM binding to single TRPV5 channels
A, representative TIRFM images with average intensity projections of the first 20 frames of HEK293 cells expressing very low levels of mCherry-TRPV5 and eGFP-CaM. mCherry-TRPV5 expression and localization in cells unsuiting (i)
molecules within the protein complex/spot. Data for all mCherry-TRPV5 positive spots were pooled into a photobleaching step frequency histogram (Fig. 5E).

Of the mCherry-TRPV5 positive spots, 66% were not in proximity to eGFP-CaM. The TRPV5 spots co-localizing with eGFP-CaM showed a distribution of the number of eGFP bleach steps varying between 1 and 4 steps per spot. For correct interpretation of the photobleach step analysis, the presence of unlabelled endogenous CaM, the fraction of non-fluorescent eGFP molecules and the possibility of overlapping unresolved spots need to be considered. With the adjusted transfection protocol, approximately 60% of CaM present in the cell bulk was eGFP labelled (Fig. 5F). However, we were unable to quantify the ratio of labelled versus unlabelled CaM in the individual cells included in our analysis. We estimated that the fraction of eGFP-labelled CaM per analysed cell was much lower than 60%, because of the strict selection of low-expression cells. A large fraction of unlabelled endogenous CaM will contribute to the high number of TRPV5 protein complexes without observed eGFP-CaM fluorescence. The presence of non-fluorescent eGFP molecules is due to chromophore folding and maturation issues and premature photobleaching. The probability of fluorescing eGFP in our experimental set-up was assessed in HEK293 cells expressing low levels of eGFP-TRPV5, because TRPV5 channels have a known tetrameric stoichiometry ($n = 4$). Plasma membrane eGFP-TRPV5 fluorescent spots were identified and subjected to photobleach step analysis (Fig. 5G). For many eGFP-TRPV5 spots 1–3 eGFP bleaching steps were observed indicating that not all eGFP molecules were fluorescent. In addition, a fraction of spots revealed more than 4 eGFP bleach steps. The distribution fitted a binominal distribution with a probability of fluorescing ($p$) of 0.55 and a substantial fraction of unresolved double spots containing two TRPV5 channels ($n = 8$, Fig. 5F). The probability of fluorescing for eGFP is in the range of previous reports of single molecule photobleach analysis in mammalian cells (McGuire et al., 2012; Wong et al., 2016), and will lead to underestimation of the number of CaM molecules per TRPV5 complex. The presence of overlapping unresolved eGFP-TRPV5 spots indicates a non-homogenous distribution of TRPV5 channels on the plasma membrane even under low expressing conditions. This is also likely to occur in cells expressing mCherry-TRPV5 and will lead to an overestimation of the number of CaM molecules per TRPV5 channel (Fig. 5E). Taking these factors into consideration, we cautiously propose that the CaM–TRPV5 binding stoichiometry can feature more than one CaM molecule per TRPV5 channel. However, we are unable to accurately determine the exact average number of CaM molecules bound to a single TRPV5 channel.

Instead, we investigated whether the observed number of CaM molecules that interact with single TRPV5 channels at the plasma membrane is dependent on Ca$^{2+}$. To this end, cells were treated with BAPTA–AM or EDTA, chelating intracellular or extracellular Ca$^{2+}$, respectively. Cells co-expressing low levels of mCherry-TRPV5 and eGFP-CaM were subjected to TIRF microscopy and photobleach step analysis (Fig. 5F). Cells treated with
Ca\(^{2+}\), BAPTA-AM or EDTA had a similar percentage of mCherry-TRPV5 spots co-localizing with eGFP-CaM (Fig. 5J). This indicates that in intact cells apoCaM can bind TRPV5. Interestingly, there is a relationship between the distribution of the number of eGFP-CaM bleach steps per co-localizing fluorescent spot and the treatment that the cell received. Fewer eGFP-CaM steps were found per mCherry-TRPV5 spots from cells treated with BAPTA-AM or EDTA, compared to spots from cells treated with Ca\(^{2+}\)-containing buffer (Fig. 5H), indicating that the number of CaM molecules associating with TRPV5 channels at the plasma membrane is dependent on the intracellular Ca\(^{2+}\) concentration.

**Discussion**

The present study sheds new light on the dynamic interaction between TRPV5 and CaM. It shows a constitutive weak interaction of TRPV5 with CaM in the absence of Ca\(^{2+}\) (apoCaM or CaM1234), as well as a Ca\(^{2+}\)-dependent increase in interaction that requires calcification of the C-lobe. We propose a new model of CaM-dependent inhibition of TRPV5 in which the C-lobe is responsible for both sensing Ca\(^{2+}\) and blocking of the channel pore. While we show here that the CaM N-lobe is required for effective inhibition, its calcification status does not appear to have a strong functional effect. We postulate that the N-lobe helps to facilitate the conformational change in the TRPV5 carboxyl terminus and the CaM C-lobe and strengthens the CaM-TRPV5 interaction for channel inactivation irrespective of its calcification status.

ApoCaM was previously thought of as a dormant channel accessory, minimally capable of modulating its targets, but it is now known to trigger a myriad of functionalities across various ion channel families (Saimi & Kung, 2002). Using various approaches, we now demonstrated for the first time an interaction between TRPV5 and apoCaM. In contrast to previous studies suggesting that the TRPV5-CaM interaction is only Ca\(^{2+}\) dependent (Bokhovchuk et al., 2018; de Groot et al., 2011; Hoenderop et al., 1999; Lambers et al., 2004), our study used the full-length functional proteins in living cells. In this setting, we found that Ca\(^{2+}\)-dependent inhibition of TRPV5 was counteracted by the presence of the Ca\(^{2+}\)-insensitive mutant CaM1234. CaM1234 has often been used to study functional consequences of the apoCaM interaction with other channels, with similar dominant-negative effects observed (Chemin et al., 2017; Erickson et al., 2001; Tien et al., 2014; Zurborg et al., 2007). This suggests that CaM1234 displaces endogenous CaM from its binding site and thereby prevents channel inhibition. Note that there is currently no obvious explanation why CaM34 has a similar effect in Fura-2 assays, but does not seem to interact with TRPV5. One can hypothesize that the CaM mutants and independent CaM lobes might not fully mimic the physiological structural rearrangement that usually occurs upon Ca\(^{2+}\) binding and dissociation. Another possibility is that CaM Ca\(^{2+}\)-binding residues in the C-lobe (D93A, D129A) are involved in supporting the interaction with TRPV5. Importantly, our study used PMLs to allow access to the intracellular compartment and confirms the TRPV5-CaM interaction under Ca\(^{2+}\)-free conditions.

Other examples of pre-association of apoCaM include Ca\(_{1.2}\) and Ca\(_{2.1}\), where it is shown to act as a vigilant sensor regulating channel opening (Ben-Johny & Yue, 2014). Such regulation is also widely studied in voltage-gated potassium (K\(_v\)) and sodium channels (Na\(_v\)) (Adams et al., 2014; Bernardo-Seisdedos et al., 2018; Wen & Levitan, 2002). Recent X-ray crystallography experiments showed the dynamic rearrangement in the Kv7.1, Kv7.4, and Kv7.5 structure in complex with apoCaM and Ca\(^{2+}\)/CaM (Chang et al., 2018). Interestingly, TRPV5 is not known to exhibit an IQ calmodulin-binding motif, a feature of many Ca\(_v\) and Na\(_v\) channels. Moreover, CaM1234 did not have an effect on the close homologue TRPV6 (data not shown), which is in line with a previous study (Derler et al., 2006) and suggests that TRPV6 is only regulated by Ca\(^{2+}\)/CaM. One might speculate that this partly underlies the difference in kinetics of channel inactivation, but further studies need to be conducted to structurally understand this distinction between TRPV5 and TRPV6.

An important feature of channel regulation by CaM is the fact that the N- and C-lobes can independently affect channel properties. This is probably due to vastly different affinities (and dissociation kinetics) for Ca\(^{2+}\), which in turn can be altered through binding to their targets (Saimi & Kung, 2002). Once calcified, CaM undergoes a conformational change that probably induces a structural rearrangement within the target proteins to produce a response (Saimi & Kung, 2002). Generally, the C-lobe presents a higher affinity for Ca\(^{2+}\), with the N-lobe requiring higher intracellular Ca\(^{2+}\) concentrations to be calcified (Chin & Means, 2000). Free intracellular Ca\(^{2+}\) can be variable depending on the cellular cell cycle or external insults, amongst other factors. Commonly, mammalian cells are endowed with an average of 100 nm free intracellular Ca\(^{2+}\) (Bagur & Hajnoczky, 2017). At this Ca\(^{2+}\) concentration, we observed a significant TRPV5-CaM interaction that is probably engaged by a calcified C-lobe, while the N-lobe will remain empty (Lai et al., 2015). Concomitant with our results, Bokhovchuk and colleagues also described a tight interaction between the CaM C-lobe and the TRPV5 C-terminus at basal Ca\(^{2+}\) concentrations (10–100 nm) (Bokhovchuk et al., 2018). Specifically, we demonstrated that the full-length CaM interaction mainly relies on C-lobe binding to the distal TRPV5 C-terminal helix, as no competition on
TRPV5-CaM interaction was observed by the proximal TRPV5 C-terminus peptide. In line with previous studies (Bokhovchuk et al., 2018; Dang et al., 2019; de Groot et al., 2011; Hughes et al., 2018), we found TRPV5 W702 to be an important residue in this interaction. Additionally, we noticed that D90 plays a role in the interaction as the TRPV5 D90A mutant also reduced CaM binding, consistent with our previous study showing the TRPV5-CaM cryo-EM structure (Dang et al., 2019). Together, these observations suggest a dominant interaction of the CaM C-lobe with the distal TRPV5 C-terminal helix, but binding by the CaM N-lobe to the proximal TRPV5 C-terminal helix supports the total CaM interaction as further explained in the next paragraph. Note that the distal and proximal peptides are based on helices defined in the cryo-EM TRPV5-CaM structures that were shown to interact with C-lobe and N-lobe, respectively (Dang et al., 2019; Hughes et al., 2018). Due to their small size and potential folding differences, they may not fully mimic the binding interfaces between full-length TRPV5 and CaM. However, in contrast to previously proposed concepts of lobe-specific actions (Bate et al., 2018; Bokhovchuk et al., 2018), the combined data in our study indicate that the CaM N-lobe does not function as a Ca$^{2+}$ sensor aimed at rearranging the TRPV5-CaM complex in an inactivated state. Our findings suggest that the C-lobe acts as both the sensing and executing unit of CaM. Mutation on the N-lobe (CaM12) did result in CaM-dependent channel inactivation, while an opposite effect was seen for the CaM34 and CaM1234 mutants. The relevance of the C-lobe modulating ion channel activity has been described for other channels, such as of the Kv7 family (Kv7.1, Kv7.4, and Kv7.5) (Chang et al., 2018).

In addition to these CaM lobe-specific effects, it has been also described for several ion channel families that interaction with both lobes is required to trigger complete Ca$^{2+}$-dependent inhibition (Banerjee et al., 2018; Ben-Johny & Yue, 2014). This is probably due to a structural rearrangement of the full complex. Indeed, our experiments with single CaM lobes revealed that, while the calcified C-lobe can interact with the channel, it requires an additional part of the CaM structure to fully trigger full Ca$^{2+}$-dependent inactivation of TRPV5. Therefore, we now propose a calmodulation model in which TRPV5 and apoCaM are pre-associated (Fig. 6, left panel). Local increases in intracellular Ca$^{2+}$ levels will lead to calcification of the CaM C-lobe and blocking of
the TRPV5 channel pore (Fig. 6, central panel). Upon decrease of local Ca\(^{2+}\) (i.e. as result of Ca\(^{2+}\) shuffling proteins such as calbindin-D\(_{28K}\)), the complex rearranges and the CaM C-lobe will open the bottom gate.

Next to the lobe-specific effects, this study sheds new light on the stoichiometry of binding. We employed a photobleaching step analysis to study the amount of CaM molecules binding to single TRPV5 tetramers and identified a variable CaM-TRPV5 channel binding stoichiometry, with increased CaM binding at a high Ca\(^{2+}\) concentration (Fig. 6, right panel). Other ion channels undergo similar changes in stoichiometry depending on the Ca\(^{2+}\) concentration. For example, the stoichiometry of L-type voltage-gated Ca\(^{2+}\) channels was found to be 1:1 in the absence of intracellular Ca\(^{2+}\), but increased to two CaM peptides at higher Ca\(^{2+}\) concentrations (Ben-Johny et al., 2016).

Initial NMR studies with peptides of TRPV5 C-terminus suggested a 1:2 stoichiometry for CaM:TRPV5\(^{696-729}\) (de Groot et al., 2011). Later studies by the same group, using longer TRPV5 C-terminus peptides, confirmed these results (Kovalevskaya et al., 2012). Yet recent cryo-EM complex structures of TRPV5 with CaM suggest on one hand a 1:1 stoichiometry of 1 tetrameric TRPV5 channel binding to 1 CaM molecule (Hughes et al., 2018), and on the other hand provide evidence for a variable stoichiometry of either 1:1 or 2:1 (2 CaM molecules per TRPV5 tetramer) (Dang et al., 2019). NMR and cryo-EM is performed with purified peptides/proteins and defined buffer conditions that may result in different binding stoichiometries compared to CaM-TRPV5 protein complexes in the plasma membrane of intact cells. However, the presence of endogenous CaM and the inhomogeneous distribution of TRPV5 channels on the plasma membrane prevented us from determining the exact average number of CaM molecules per TRPV5 channel. Taking these factors into consideration, we expect that in the presence of intracellular Ca\(^{2+}\), more than 1 CaM molecule can bind a single TRPV5 channel.

Analysing our cryo-EM TRPV5-CaM complex structure (Dang et al., 2019), it is clear that only 1 CaM molecule can occupy the lower cavity of the tetrameric channel pore. Therefore, we speculate that the CaM-mediated TRPV5 inactivation mimics the ball-and-chain mechanism observed between Kv channels and Kv\(\beta\) subunits (Nunez et al., 2020). The C-lobe of a second CaM molecule will be positioned off the lower gate, increasing the local concentration of CaM, which may lead to a more effective Ca\(^{2+}\)-dependent channel inhibition.

In conclusion, our study demonstrates that the CaM-mediated TRPV5 channel inactivation is a dynamic process involving 1 or more CaM molecules in close proximity to the channel pore that exhibit lobe-specific regulation. Our results indicate a weak, but persistent, interaction between TRPV5 and apoCaM that may assist fast channel inhibition through calcification of the CaM C-lobe in close vicinity of the channel. The N-lobe serves primarily as a facilitator of the conformational change that allows the C-lobe to inhibit TRPV5 activity. Hereby, we propose a previously unidentified calmodulation landscape for this Ca\(^{2+}\)-selective TRP channel. Given the importance of TRPV5 in the maintenance of Ca\(^{2+}\) homeostasis, it would be highly interesting to understand how this CaM regulation biologically dictates channel kinetics. Moreover, our approaches may be of interest for studies on other ion channels that are also known to be regulated by CaM.

References

Adams, P. J., Ben-Johny, M., Dick, I. E., Inoue, T., & Yue, D. T. (2014). Apocalmodulin itself promotes ion channel opening and Ca(2+) regulation. Cell, 159(3), 608–622.

Bagur, R., & Hajnoczky, G. (2017). Intracellular Ca\(^{2+}\) sensing: Its role in calcium homeostasis and signaling. Molecular Cell, 66(6), 780–788.

Banerjee, R., Yoder, J. B., Yue, D. T., Amzel, L. M., Tomaselli, G. F., Gabelli, S. B., & Ben-Johny, M. (2018). Bilobal architecture is a requirement for calmodulin signaling to Cav1.3 channels. PNAS, 115(13), E3026–E3035.

Bate, N., Caves, R. E., Skinner, S. P., Goult, B. T., Basran, J., Mitcheson, J. S., & Vuister, G. W. (2018). A novel mechanism for calmodulin-dependent inactivation of transient receptor potential vanilloid 6. Biochemistry, 57(18), 2611–2622.

Ben-Johny, M., Yue, D. N., & Yue, D. T. (2016). Detecting stoichiometry of macromolecular complexes in live cells using FRET. Nature Communications, 7(1), 13709.

Ben-Johny, M., & Yue, D. T. (2014). Calmodulin regulation (calmodulation) of voltage-gated calcium channels. Journal of General Physiology, 143(6), 679–692.

Bernardo-Seisdedos, G., Nunez, E., Gomis-Perez, C., Malo, C., Villarroel, A., & Millet, O. (2018). Structural basis and energy landscape for the Ca\(^{2+}\) gating and calmodulation of the Kv7.2 K+ channel. PNAS, 115(10), 2395–2400.

Bokhovchuk, F. M., Bate, N., Kovalevskaya, N. V., Goult, B. T., Spronk, C., & Vuister, G. W. (2018). The structural basis of calcium-dependent inactivation of the transient receptor potential vanilloid 5 channel. Biochemistry, 57(18), 2623–2635.

Chang, A., Abderemane-Ali, F., Hura, G. L., Rossen, N. D., Gate, R. E., & Minor, D. L., Jr (2018). A calmodulin C-Lobe Ca\(^{2+}\)-dependent switch governs Kv7 channel function. Neuron, 97(4), 836–852.e6.

Chemin, J., Taiakina, V., Monteil, A., Piazza, M., Guan, W., Stephens, R. F., Kitmitto, A., Pang, Z. P., Dolphín, A. C., Perez-Reyes, E., Dieckmann, T., Guillemette, J. G., & Spafford, J. D. (2017). Calmodulin regulates Cav3 T-type channels at their gating brake. Journal of Biological Chemistry, 292(49), 20010–20031.

Chin, D., & Means, A. R. (2000). Calmodulin: A prototypical calcium sensor. Trends in Cell Biology, 10(8), 322–328.
Clapham, D. E., Runnels, L. W., & Strubing, C. (2001). The TRP ion channel family. Nature Reviews Neuroscience, 2(6), 387–396.

Clark, J. C., Neagoe, R. A. I., Zuidscherwoude, M., Kavanagh, D. M., Slater, A., Martin, E. M., Soave, M., Stegner, D., Nieswandt, B., Pouler, N. S., Hummert, J., Herten, D. P., Tomlinson, M. G., Hill, S. J., & Watson, S. P. (2021). Evidence that GPV1 is expressed as a mixture of monomers and dimers, and that the D2 domain is not essential for GPV1 activation. Thrombosis and Haemostasis, 121, 1435–1447.

Dang, S., van Goor, M. K., Asarnow, D., Wang, Y., Julius, D., Cheng, Y., & van der Wijst, J. (2019). Structural insight into TRPV5 channel function and modulation. PNAS, 116(18), 8869–8878.

de Groot, T., Kovalevskaya, N. V., Verkaart, S., Schilderink, N., Felici, M., van der Hagen, E. A., Bindels, R. J., Vuister, G. W., & Hoenderop, J. G. (2011). Molecular mechanisms of calmodulin action on TRPV5 and modulation by parathyroid hormone. Molecular and Cellular Biology, 31(14), 2845–2853.

Derler, I., Hofbauer, M., Kahr, H., Fritsch, R., Muik, M., Kepplinger, K., Hack, M. E., Moritz, S., Schindl, R., Groschner, K., & Romanin, C. (2006). Dynamic but not constitutive association of calmodulin with rat TRPV6 channels enables fine tuning of Ca²⁺-dependent inactivation. The Journal of Physiology, 577(1), 31–44.

Erickson, M. G., Alseikhan, B. A., Peterson, B. Z., & Yue, D. T. (2001). Preassociation of calmodulin with voltage-gated Ca²⁺ channels revealed by FRET in single living cells. Neuron, 31(6), 973–985.

Fruen, B. R., Black, D. J., Bloomquist, R. A., Bardy, J. M., Johnson, J. D., Louis, C. F., & Balog, E. M. (2003). Regulation of the RYR1 and RYR2 Ca²⁺ release channel isoforms by Ca²⁺-insensitive mutants of calmodulin. Biochemistry, 42(9), 2740–2747.

Geiser, J. R., van Tuinen, D., Brockerhoff, S. E., Neff, M. M., & Davis, T. N. (1991). Can calmodulin function without binding calcium? Cell, 65(6), 949–959.

Gonzalez-Munoz, E., Lopez-Iglesias, C., Calvo, M., Palacin, D., Derler, I., Hofbauer, M., Kahr, H., Fritsch, R., Muik, M., & Varela, M. (2004). Molecular mechanisms of calmodulin action on TRPV5 and modulation by parathyroid hormone. Molecular and Cellular Biology, 31(14), 2845–2853.

Hummert, J., Yserentant, K., Fink, T., Euchner, J., Ho, Y. T., Tashev, S. A., & Herten, D. P. (2021). Photobleaching step analysis for robust determination of protein complex stoichiometries. Molecular Biology of the Cell, 32(21), ar35.

Kovalevskaya, N. V., Bokhovchuk, F. M., & Vuister, G. W. (2012). The TRPV5/6 calcium channels contain multiple calmodulin binding sites with differential binding properties. Journal of Structural and Functional Genomics, 13(2), 91–100.

Lai, M., Brun, D., Edelstein, S. J., & Le Novère, N. (2015). Modulation of calmodulin lobes by different targets: An allosteric model with hemiconverted conformational transitions. Plos Computational Biology, 11(1), e1004063.

Lammers, T. T., Weidema, A. F., Nilius, B., Hoenderop, J. G., & Bindels, R. J. (2004). Regulation of the mouse epithelial Ca²⁺ channel TRPV6 by the Ca²⁺-sensor calmodulin. Journal of Biological Chemistry, 279(28), 28855–28861.

McGuire, H., Aurousseau, M. R., Bowie, D., & Blunck, R. (2012). Automating single subunit counting of membrane proteins in mammalian cells. Journal of Biological Chemistry, 287(43), 35912–35921.

Moreno, C., Oliveras, A., de la Cruz, A., Bartolucci, C., Munoz, C., Salar, E., Gimeno, J. R., Severi, S., Comes, N., Felipe, A., Gonzalez, T., Lambiase, P., & Valenzuela, C. (2015). A new KCNQ1 mutation at the S5 segment that impairs its association with KCNE1 is responsible for short QT syndrome. Cardiovascular Research, 107(4), 613–623.

Nilius, B. (2007). TRP channels in disease. Biochimica Et Biophysica Acta, 1772(8), 805–812.

Nilius, B., Prehen, J., Vennekens, R., Hoenderop, J. G., Bindels, R. J., & Droogmans, G. (2001). Modulation of the epithelial calcium channel, ECaC, by intracellular Ca²⁺. Cell Calcium, 29(6), 417–428.

Nilius, B., Vennekens, R., Prehen, J., Hoenderop, J. G., Bindels, R. J., & Droogmans, G. (2000). Whole-cell and single channel monovalent cation currents through the novel rabbit epithelial Ca²⁺ channel ECaC. The Journal of Physiology, 527(2), 239–248.

Nilius, B., Weidema, E., Prehen, J., Hoenderop, J. G., Vennekens, R., Hoefs, S., Droogmans, G., & Bindels, R. J. (2003). The carboxyl terminus of the epithelial Ca²⁺ channel ECaC1 is involved in Ca²⁺-dependent inactivation. Pflogers Arch: European journal of physiology, 445(5), 584–588.

Nunez, E., Muguuruza-Montero, A., & Villarroel, A. (2020). Atomistic insights of calmodulin gating of complete ion channels. International Journal of Molecular Sciences, 21(4), 1285.

Pervez-Verdaguer, M., Capera, J., Martinez-Marmol, R., Camps, M., Comes, N., Tamkun, M. M., & Felipe, A. (2016). Caveolin interaction governs Kv1.3 lipid raft targeting. Scientific Reports, 6(1), 22453.

Pervez-Verdaguer, M., Capera, J., Ortego-Dominguez, M., Bielanska, J., Comes, N., Montoro, R. J., Camps, M., & Felipe, A. (2018). Caveolar targeting links Kv1.3 with the insulin-dependent adipocyte physiology. Cellular and Molecular Life Sciences, 75(21), 4059–4075.
Saimi, Y., & Kung, C. (2002). Calmodulin as an ion channel subunit. *Annual Review of Physiology, 64*(1), 289–311.

Schindelin, J., Arganda-Carreras, I., Frise, E., Kaynig, V., Longair, M., Pietzsch, T., Preibisch, S., Rueden, C., Saalfeld, S., Schmid, B., Tinevez, J. Y., White, D. J., Hartenstein, V., Eliceiri, K., Tomancak, P., & Cardona, A. (2012). Fiji: an open-source platform for biological-image analysis. *Nature Methods, 9*(7), 676–682.

Singh, A. K., McGoldrick, L. L., Twomey, E. C., & Sobolevsky, A. I. (2018). Mechanism of calmodulin inactivation of the calcium-selective TRP channel TRPV6. *Science Advances, 4*(8), eaau6088.

Tien, J., Peters, C. J., Wong, X. M., Cheng, T., Jan, Y. N., Jan, L. Y., & Yang, H. (2014). A comprehensive search for calcium binding sites critical for TMEM16A calcium-activated chloride channel activity. *Elife, 3*, e02772.

Tutakhel, O. A. Z., Bianchi, F., Smits, D. A., Bindels, R. J. M., Hoenderop, J. G. J., & van der Wijst, J. (2018). Dominant functional role of the novel phosphorylation site S811 in the human renal NaCl cotransporter. *Faseb Journal, 32*(8), 4482–4493.

Venkatachalam, K., & Montell, C. (2007). TRP channels. *Annual Review of Biochemistry, 76*(1), 387–417.

Vennekens, R., Droogmans, G., & Nilius, B. (2001). Functional properties of the epithelial Ca$^{2+}$ channel, ECaC. *General Physiology and Biophysics, 20*, 239–253.

Vennekens, R., Hoenderop, J. G., Prenen, J., Stuiver, M., Willems, P. H., Droogmans, G., Nilius, B., & Bindels, R. J. (2000). Permeation and gating properties of the novel epithelial Ca$^{2+}$ channel. *Journal of Biological Chemistry, 275*(6), 3963–3969.

Wen, H., & Levitan, I. B. (2002). Calmodulin is an auxiliary subunit of KCNQ2/3 potassium channels. *Journal of Neuroscience, 22*(18), 7991–8001.

Wong, K., Briddon, S. J., Holliday, N. D., & Kerr, I. D. (2016). Plasma membrane dynamics and tetrameric organisation of ABCG2 transporters in mammalian cells revealed by single particle imaging techniques. *Biochimica Et Biophysica Acta, 1863*(1), 19–29.

Wu, L. J., Sweet, T. B., & Clapham, D. E. (2010). International union of basic and clinical pharmacology. LXXVI. Current progress in the mammalian TRP ion channel family. *Pharmacological Reviews, 62*(3), 381–404.

Additional information

Data availability statement

Data available in article and source data available on request from the authors.

Competing interests

None.

Author contributions

M.Z., S.R.R., J.G.H., J.v.W. conceived and designed the experiments. M.Z., M.K.v.G., S.R.R., and N.T. performed the experiments. M.Z., M.K.v.G., S.R.R., N.T., J.v.W., and J.G.H. analysed and interpreted the data. M.v.E. supported the photo-bleaching step analysis. M.Z., M.K.v.G., S.R.R., J.v.W., and J.G.H. wrote the manuscript, with critical review and approval by all authors. J.F., J.v.W., and J.G.H. supervised the work. All authors have approved the final version of the manuscript and agree to be accountable for all aspects of the work. All persons designated as authors qualify for authorship, and all those who qualify for authorship are listed.

Funding

S.R.R. was supported by Alfonso Marín Escudero Grant for Postdoctoral studies abroad. This study was financially supported by The Netherlands Organization for Scientific Research (ENW OC ENW KLEIN 2022/ENW/01277767) and EU Horizon 2020 Marie Sklodowska-Curie Actions (748058).

Keywords

calcium, calmodulin, channel regulation, single molecule, TRP channel

Supporting information

Additional supporting information can be found online in the Supporting Information section at the end of the HTML view of the article. Supporting information files available:

Statistical Summary Document

Peer Review History

Data Set 1

Data Set 2

© 2022 The Authors. *The Journal of Physiology* published by John Wiley & Sons Ltd on behalf of The Physiological Society.