Cell Calcium 55 (2014) 59–68

Contents lists available at ScienceDirect

Cell Calcium

journal homepage: www.elsevier.com/locate/ceca

Cholesterol sensitises the transient receptor potential channel TRPV3 to lower temperatures and activator concentrations

Anke S. Klein, Astrid Tannert[1], Michael Schaefer*

Rudolf-Boehm-Institut für Pharmakologie und Toxikologie, Universität Leipzig, Leipzig, Germany

ARTICLE INFO

Article history:
Received 10 September 2013
Received in revised form 4 December 2013
Accepted 7 December 2013
Available online 17 December 2013

Keywords:
Intracellular calcium homeostasis
Vanilloid receptors
Non-selective cation channel
Keratinocyte differentiation
Heat-activated ionic currents
Thymol
Camphor
Carvacrol

ABSTRACT

TRPV3, a thermosensitive cation channel, is predominantly expressed in keratinocytes. It contributes to physiological processes such as thermosensation, nociception, and skin development. TRPV3 is polymodally regulated by chemical agonists, innocuous heat, intracellular acidification or by membrane depolarization. By manipulating the content of plasma membrane cholesterol, a key modulator of the physicochemical properties of biological membranes, we here addressed the question, how the lipid environment influences TRPV3. Cholesterol supplementation robustly potentiated TRPV3 channel activity by sensitising it to lower concentrations of chemical activators. In addition, the thermal activation of TRPV3 is significantly shifted to lower temperatures in cholesterol-enriched cells. The sensitising effect of cholesterol was not caused by an increased plasma membrane targeting of the channel. In HaCaT keratinocytes, which natively express TRPV3, a cholesterol-mediated sensitisation of TRPV3-like responses was reproduced. The cholesterol-dependent modulation of TRPV3 activity may provide a molecular mechanism to interpret its involvement in keratinocyte differentiation.

1. Introduction

The epidermis, as the interface between an individual and its environment, has to perform different essential functions. It protects an organism against harmful external influences, pathogens, and dehydration. Keratinocytes are the major cell type of the epidermis and bear responsible for the formation of an outer barrier, the stratum corneum. To generate this barrier, keratinocytes undergo a complex differentiation programme that terminates in apoptosis and cornification [1]. Differentiation is accompanied by synthesis of several structural proteins and lipids, like ceramides, fatty acids and cholesterol. To prevent skin barrier dysfunction, the proliferation and differentiation of keratinocytes needs to be tightly regulated. Multiple factors are known to influence the balance between keratinocyte proliferation and differentiation, including growth factors, extracellular calcium concentrations, cell density, or lipids like ceramides, cholesterol and cholesterol-derived metabolites.

Another function of keratinocytes, together with neurons that extend into the epidermis, is to recognise environmental factors, some of which are decoded by members of the transient receptor potential (TRP) family of cation channels. For instance, TRPV3, a member of the vanilloid subfamily, is predominantly expressed in keratinocytes of all epidermal layers [2,3]. Its activity is polymodally regulated by chemical and physical queues. TRPV3 can be activated by numerous, poorly selective compounds like 2-aminoethoxydiphenyl borate (2-APB) [4], and by secondary plant metabolites, such as camphor [5], carvacrol, eugenol and thymol [6]. TRPV3 is activated by temperatures above 33 °C [2,3,7]. The initial characterisation of TRPV3-deficient mice indicated that TRPV3 participates in heat perception [5], but more recent data demonstrate that this effect strongly depends on the genetic background of the investigated animals [8]. In addition, cytosolic acidification [9], membrane potential [3], and various lipid factors [10–13] are known to influence TRPV3 activity. Apart from thermosensation, TRPV3 is discussed to be involved in physiological and pathophysiological processes, including nociception [14,15], hair growth [16], inflammation [10], and skin diseases [17–19]. Recent findings demonstrated that the epidermal barrier in TRPV3$^{-/-}$ mice is defective [16], pointing to a role of TRPV3 in regulating keratinocyte differentiation and cornification. Since former studies have demonstrated that cholesterol also plays an important role in keratinocyte physiology we wondered about the influence of cholesterol on TRPV3 signalling.

* Corresponding author at: Rudolf-Boehm-Institut für Pharmakologie und Toxikologie, Universität Leipzig, Härtelstr. 16-18, 04107 Leipzig, Germany.
Tel.: +49 341 9724600; fax: +49 341 9724609.
E-mail address: michael.schaefer@medizin.uni-leipzig.de (M. Schaefer).
[1] Current affiliation: Leibniz Institute for Zoo and Wildlife Research (IZW), Berlin, Germany.

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http://dx.doi.org/10.1016/j.ceca.2013.12.001

In the present study, we introduce cholesterol as a new regulator of TRPV3 channel activity. We modified the cholesterol content of HEK293 cells stably expressing mouse TRPV3 and performed calcium and whole-cell patch clamp analyses. These experiments revealed that cholesterol enrichment robustly potentiates TRPV3 by shifting its sensitivity to lower activator concentrations and by sensitising TRPV3 to lower temperatures. The influence of cholesterol on TRPV3 activity was not restricted to the recombinant expression model, but also observed in HaCaT keratinocytes, which endogenously express TRPV3 [20,21], and are widely used to study keratinocyte physiology [22].

2. Methods

2.1. Cell culture and transfections

HEK293 cells were maintained in Earle's minimum essential medium (MEM), supplemented with 10% foetal calf serum (FCS, v/v), 2 mM L-glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin (all PAA Laboratories, Pasching, Austria). To obtain a HEK293 cell line stably expressing mouse TRPV3 (HEK_{mTRPV3}), cells were transfected with a pcDNA3.1 plasmid encoding a C-terminally CFP-tagged mTRPV3. All transfections were performed with a Fugene HD lipofection reagent (Promega, Madison, USA), according to the manufacturer's instructions. Individual clones were picked under optical control in an epifluorescence microscope, reseeded in serial dilutions, and grown in culture medium supplemented with 1 mg/ml G418. After clonal selection and initial expansion, the established HEK_{mTRPV3} cell line was grown in medium containing 0.4 mg/ml G418.

The spontaneously immortalised, untransformed human keratinocyte cell line HaCaT (human adult low calcium high temperature) was cultured in Dulbecco's modified Eagle's medium (DMEM) with 1 g/l glucose, supplemented with 10% FCS, 2 mM L-glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin. After reaching a density of about 70%, HaCaT cells were washed and incubated for 10 min in PBS supplemented with 0.8 mM EDTA at 37 °C, harvested by mild trypsinisation, and reseeded. All cells were grown at 37 °C in a humidified atmosphere with 5% CO_2.

2.2. Cholesterol modification

Modification of the cellular cholesterol content was achieved using methyl-β-cyclodextrin (MβCD). MβCD is known to extract cholesterol from living cells or, when preloaded with cholesterol, to enrich cells with cholesterol [23]. To generate MβCD–cholesterol complexes, cholesterol (Sigma–Aldrich) was dissolved in ethanol (20 mg/ml), heated and added to a MβCD-containing solution (200 mM in PBS, Sigma–Aldrich). We used saturated mixtures with a molar ratio of MβCD:cholesterol of 10:1 [23]. Unless otherwise stated, cholesterol depletion or enrichment was performed by incubating the cells for 15 min at 37 °C in HEPES-buffered solution (HBS) containing 134 mM NaCl, 6 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 5.5 mM glucose and 10 mM HEPES (pH 7.4, adjusted with NaOH), and supplemented with 10 mM MβCD or 2.5 mM MβCD–cholesterol, respectively. These conditions have only minor effects on cellular viability [24]. As a control, we incubated cells in HBS without MβCD supplement at 37 °C.

2.3. Calcium measurement

All calcium measurements were performed at room temperature in HBS. Cells were loaded for 30 min at 37 °C with 3 μM of either fura-2/AM of fluo-4/AM (Invitrogen) in HBS supplemented with 0.2% (w/v) bovine serum albumin. Subsequently, cellular cholesterol content was modulated. After washing the cells with HBS without MβCD or MβCD–cholesterol, calcium measurements were performed.

Concentration response curves were monitored in fluo-4-loaded cell suspensions. To this end, cell suspensions were dispensed into 384-well plates and mounted on the stage of a custom-made fluorescence imaging plate reader [25] built within a robotic liquid handling station (Freedom Evo 150, Tecan, Switzerland). Modulators or agonists were applied with the 96-tip multichannel arm. The image acquisition was controlled by the MicroManager software (Version 1.3; US National Institutes of Health, Bethesda, MD) [26], and image evaluation was performed with ImageJ (US National Institutes of Health) [27]. Mean fluorescence intensities of individual wells were determined, corrected for background signals, and normalised to initial intensities (F/F_0). Intensity values at the end of the measurement (2.5 min after agonist application) were extracted, and concentration-dependent effects were parameterised by fitting the data obtained with various activator concentrations to a four parameter Hill equation:

$$E = \frac{E_{min} + (E_{max} - E_{min})}{1 + ([A]/EC_{50})^{-nH}},$$

where E represents the observed effect, $[A]$ is the concentration of activator, EC_{50} the activator concentration yielding a half-maximal effect, and nH the Hill coefficient.

For single cell calcium measurements, cells were grown on glass coverslips, loaded with the ratiometric calcium indicator fura-2/AM, and imaged with an inverted microscope (Fluar 10×/0.5; Axiovert 100 microscope, Carl Zeiss, Jena, Germany). For excitation we used a fibre-coupled monochromator device (Polychrome V, Till-Photonics, Gräfelfing, Germany) at alternating wavelengths of 340, 358, and 380 nm. Emission was imaged with a cooled CCD camera (Sensicam, PCO, Kelheim, Germany) through a dichroic beam splitter (DCXR-510, Chroma, Rockingham, VT) and a 515 nm long-pass filter (OG515, Schott, Jena, Germany). Fluorescence intensities were averaged over regions of individual cells. Background signals were subtracted, and intracellular calcium concentrations where calculated with a spectral fingerprinting method as described [28].

2.4. Confocal laser scanning microscopy

Cells were imaged using an inverted confocal laser scanning microscope (LSM510-META, Carl Zeiss AG, Oberkochen, Germany), with a C-Apochromat 40×/1.2 water immersion objective. CFP-tagged TRP channels were excited with the 458-nm line of an Ar^+ laser, and emission was detected through a 500/50 nm band-pass filter.

2.5. Electrophysiological procedures

For the electrophysiological characterisation of TRPV3, HEK293 cells were seeded on poly-L-lysin-coated (0.02%, Sigma–Aldrich) glass coverslips, and cultured for 24 h. Unless otherwise indicated, whole-cell recordings were made at room temperature, using an EPC9 amplifier controlled by the PULSE software (HEKA, Lambrecht, Germany). The standard extracellular solution contained 140 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 10 mM glucose, 10 mM HEPES and 1 mM $CaCl_2$ (adjusted to pH 7.4 with NaOH, and to 305 mosmol/l with mannitol). The pipette solution included 110 mM CsCl, 4 mM $MgCl_2$, 10 mM EGTA and 10 mM HEPES (pH 7.2 with CsOH). For whole-cell recordings, patch pipettes had a resistance of 3–5 MΩ. Serial resistances were always less then 10 MΩ and compensated by 80%. Liquid junction potentials added up to +4.1 mV and were not compensated in the illustrated figures.