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Research article

A benzothiadiazine derivative and methylprednisolone are novel and selective activators of transient receptor potential canonical 5 (TRPC5) channels

Holger Beckmann[a], Julia Richter[a], Kerstin Hill[a], Nicole Urban[a], Horst Lemoine[b], Michael Schaefer[a,*]

[a] Rudolf-Boehm-Institute of Pharmacology and Toxicology, Leipzig University, Härtelstraße 16-18, 04107 Leipzig, Germany
[b] Institute for Lasermedicine, Heinrich Heine University Düsseldorf, Universitätsstraße 1, 40225 Düsseldorf, Germany

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ABSTRACT

The transient receptor potential canonical channel 5 (TRPC5) is a Ca^{2+}-permeable ion channel, which is predominantly expressed in the brain. TRPC5-deficient mice exhibit a reduced innate fear response and impaired motor control. In addition, outgrowth of hippocampal and cerebellar neurons is retarded by TRPC5. However, pharmacological evidence of TRPC5 function on cellular or organismic levels is sparse. Thus, there is still a need for identifying novel and efficient TRPC5 channel modulators.

We, therefore, screened compound libraries and identified the glucocorticoid methylprednisolone and N-[3-(adamantan-2-yloxy)propyl]-3-(6-methyl-1,1-dioxo-2H-1λ^6,2,4-benzothiadiazin-3-yl)propanamide (BTD) as novel TRPC5 activators. Comparisons with closely related chemical structures from the same libraries indicate important substructures for compound efficacy. Methylprednisolone activates TRPC5 heterologously expressed in HEK293 cells with an EC_{50} of 12 μM, while BTD-induced half-maximal activation is achieved with 5-fold lower concentrations, both in Ca^{2+} assays (EC_{50} = 1.4 μM) and in electrophysiological whole cell patch clamp recordings (EC_{50} = 1.3 μM). The activation resulting from both compounds is long lasting, reversible and sensitive to clemizole, a recently established TRPC5 inhibitor. No influence of BTD on homotetrameric members of the remaining TRPC family was observed. On the main sensory TRP channels (TRPA1, TRPV1, TRPM3, TRPM8) BTD exerts only minor activity. Furthermore, BTD can activate heteromeric channel complexes consisting of TRPC5 and its closest relatives TRPC1 or TRPC4, suggesting a high selectivity of BTD for channel complexes bearing at least one TRPC5 subunit.

1. Introduction

Transient receptor potential (TRP) channels are a family of mostly non-selective cation channels comprising 28 members in mammals. They all share a topology of 6 transmembrane helices and cytoplasmic C and N termini. TRP channels serve versatile sensory and homeostatic functions and are associated with different pathologies (reviewed in [1]). The classical or canonical TRPs (TRPC) are a subfamily consisting of seven members, TRPC1 to TRPC7.

Among these, TRPC5 is predominantly expressed in the central nervous system, with apparent abundance in the cortico-limbic system and cerebellum [2]. In line with these findings, TRPC5 knock-out animals show reduced innate fear behaviour and impaired motor coordination, while learning seems to be unaltered [3–5]. On the cellular level, TRPC5 has been shown to crucially interfere with neuronal growth. In cerebellar granular neurons, early TRPC5 expression in the soma seems to facilitate first axonal sprouting [6]. During outgrowth, TRPC5 has been reported to be expressed in growth cones, where TRPC5 knockdown was shown to extent dendrite and neurite length in cerebellar [4] and hippocampal neurons [7]. In addition, membrane incorporation of TRPC5 from storage vesicles can be triggered with growth factors [8] and TRPC5 was linked to semaphorin signalling [9], all together indicating an involvement in axonal pathfinding.

* Corresponding author.
E-mail addresses: holger.beckmann@medizin.uni-leipzig.de (H. Beckmann), kerstin.hill@medizin.uni-leipzig.de (K. Hill), nicole.urban@medizin.uni-leipzig.de (N. Urban), lemoine@uni-duesseldorf.de (H. Lemoine), michael.schaefer@medizin.uni-leipzig.de (M. Schaefer).

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However, acute and selective modulation of TRPC5 function is still difficult to achieve. Hence, drug-like compounds to acutely monitor TRPC5-dependent functions are urgently needed. To date, a variety of TRPC5 channel modulators is already available, mostly with inhibitory function [10–14]. TRPC5 activation, on the other hand, has been shown by a plethora of different mechanisms, such as elevation in $[Ca^{2+}]_i$, extracellular acidification, $G\alpha_{q/i}$ signalling downstream of GPCRs, phospholipids, lanthanides and heavy metal ions [15].

Drug-like TRPC5 activators that have been identified so far include the isoflavone genistein (EC_{50} = 93 μM, [16]), the thiazolidinedione rosiglitazone (EC_{50} = 30 μM, [17]), the benzothiazole riluzole (EC_{50} = 9 μM, [18]) and the guanine-sesquiterpene (−)-englerin A (from now on englerin A, EC_{50} = 8 nM, [19]). While selectivity studies for genistein and rosiglitazone have not been published, riluzole is highly selective for TRPC5 within the TRP family. However, riluzole also inhibits voltage-gated Na^+ and Ca^{2+} channels and thereby attenuates neurotransmitter release. Some of these effects already occur at concentrations below those needed for TRPC5 channel activation (see [20] for review).

The most potent activator for TRPC5 channels so far is englerin A, isolated from the African plant *Phyllanthus engleri*. Englerin A activates TRPC4 to almost the same extent (EC_{50} = 11 nM) [19], hence having reduced benefit in cells coexpressing these two closely related channels. Englerin A has been shown to inhibit renal cancer cell growth. This has initially been attributed to sustained Ca^{2+} influx through TRPC4 channels [19,21] but might rather be the consequence of Na^+ than Ca^{2+} overload [22]. Since systemically administered englerin A exhibits a potent toxicity in mice and since elimination half times are even longer in humans, its use as a drug-like compound is limited [21].

In this study, we identified two novel TRPC5 activators, the synthetic glucocorticoid methylprednisolone and N-[3-(adamantan-2-yloxy)propyl]-3-(6-methyl-1,1-dioxo-2H-1λ^6,2,4-benzothiadiazin-3-yl)propanamide (from now on BTD). Both activate TRPC5 channels and were validated by Ca^{2+} imaging and patch clamp recordings. In addition, we tested closely related compounds to identify important substructures to enable prospective improvement of compound efficacy. As BTD is a chemically new type of TRPC5 activator we characterized it in more detail, showing its efficacy in isolated patches and its selectivity for TRPC5 and TRPC5-containing heterotetramers within the TRP channel family.

2. Materials and methods

2.1. Stable cell lines and transfections

Stably transfected HEK293 cells that inducibly express YFP-tagged mouse TRPC5 channels (HEK_{TRPC5}) upon tetracycline treatment (tet; Sigma-Aldrich, St. Louis, MO, USA) were generated and cultured as described previously [18] and supplemented with 1 μg mL^{-1} tet for 24–36 h prior to the experiment. Non-induced HEK_{TRPC5} cells (tet-) served as controls.

HEK293 cells expressing other TRP channels (YFP-tagged human TRPC3, YFP-tagged mouse TRPC4β, YFP-tagged human TRPC6, YFP-tagged mouse TRPC7, YFP-tagged human TRPA1, CFP-tagged rat TRPV1, YFP-tagged rat TRPV2, YFP-tagged rat TRPV3, YFP-tagged mouse TRPV4, human TRPM2, YFP-tagged mouse TRPM3, CFP-tagged human TRPM8) were generated and maintained as described previously [23]. Heteromeric channel complexes were obtained by transient transfection of HEK293 cells with a jet-Pei transfection reagent according to the manufacturer's protocol (PEQLAB, Erlangen, Germany). The following plasmids (pcDNA3, Invitrogen) were used: CFP-tagged mouse TRPC5, YFP-tagged mouse TRPC4β, YFP-tagged human TRPC1. All chemicals were purchased from Sigma Aldrich (Munich, Germany) unless stated otherwise.

2.2. Fluorometric $[Ca^{2+}]_i$ imaging and PKCε translocation assay

The initial compound screens were performed by using a custom-made Fluorescence Imaging Plate reader (FLIPR) based on a Fluo-4/AM assay as described previously [24]. Tet-induced HEK_{TRPC5} cells (15,000 per well) were seeded into 384-well plates with clear, flat bottom (Corning, Tewksbury, MA, USA) and cultured for 24 h to 36 h. Then, fluo-4/AM (Invitrogen) dissolved in cell culture medium was added at a final concentration of 4 μM and incubated for 30 min before replacement by a HEPES buffered solution (HBS, containing in mM: 134 NaCl, 6 KCl, 1 $MgCl_2$, 1 $CaCl_2$, 10 HEPES, pH 7.4). Fluorescence intensities excitated at 450–470 nm were imaged with a cooled charge-coupled device camera (Zyla 5.5, Andor Technology Ltd., Belfast, UK) and expressed as F/F_0 ratios.

For single-cell $[Ca^{2+}]_i$ measurements, cells were seeded on poly-L-lysine-coated glass cover slips and grown to a confluency of ~80%. HEK_{TRPC5} cells were induced as described above. Cells were loaded in HBS for 30 min at 37 °C with 2 μM fura-2/AM (Molecular Probes, Eugene, OR, USA). The experimental setup and data analysis was described previously [18].

The subcellular distribution of PKCε was visualized with a confocal laser scanning microscope (LSM510-META, 100 × /1.46 Plan Apochromat, Carl Zeiss, Oberkochen, Germany). The translocation assay was performed as published previously [25].

Stock solutions of all compounds were prepared in DMSO. Final DMSO concentrations in experiments did never exceed 0.2% except for the BTD derivates K261-1566 and K261-1477, which were tested at a final DMSO concentration of 0.8%.

2.3. Electrophysiology

Two different conformations of the patch clamp technique were used. For the whole-cell configuration, the extracellular solution contained (in mM) 130 NaCl, 5 KCl, 2 $MgCl_2$, 1.5 $CaCl_2$, 8 glucose and 10 HEPES, pH 7.4 adjusted with NaOH. The intracellular solution consisted of (in mM): 115 CsCl, 2 $MgCl_2$, 5 Na_2ATP, 0.1 Na_2GTP, 5.7 $CaCl_2$, 10 HEPES and 10 EGTA, pH 7.2 adjusted with CsOH, yielding a free calcium concentration of 200 nM (MaxChelator; http://maxchelator.stanford.edu). Whole-cell series resistances did not exceed 15 MΩ, and were compensated by 70%. For excised inside-out patches the bath solution contained (in mM): 115 CsCl, 2 $MgCl_2$, 5.7 $CaCl_2$, 10 EGTA, 10 HEPES, pH 7.2, adjusted with CsOH. Free calcium concentration was calculated as 216 nM. Single channel currents were filtered at 2 kHz (four-pole Bessel filter) and sampled with 5 kHz.

The setup was described previously [18]. Patch pipettes were made from borosilicate glass with a resistance of 5–7 MΩ for whole cell, and 10–15 MΩ for excised patch recordings. All experiments were performed at room temperature. Voltage ramps from −100 to +100 mV (500 ms duration) were applied at 1-s intervals. After transferring cells into a recording chamber, compounds were applied and washed out by using a gravity-driven perfusion system.

2.4. Membrane potential measurements

The membrane potential of HEK 293 and CHO cells stably expressing the vascular (Kir6.1/SUR2B) and the ß-cell (Kir6.2/SUR1) type of the K_{ATP} channel, respectively, was probed with the FLIPR membrane potential assay kit (Molecular Devices R7260) containing $DisBAC_1(3)$, a fluorescent anionic indicator of membrane potential and a quenching agent for the use as a no-wash kit in high throughput fluorescence imaging readers.