Clemizole Hydrochloride Is a Novel and Potent Inhibitor of Transient Receptor Potential Channel TRPC5

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Received April 13, 2014; accepted August 19, 2014

ABSTRACT

Canonical transient receptor potential channel 5 (TRPC5) is a nonselective, Ca\(^{2+}\)-permeable cation channel that belongs to the large family of transient receptor potential channels. It is predominantly found in the central nervous system with a high expression density in the hippocampus, the amygdala, and the frontal cortex. Several studies confirm that TRPC5 channels are implicated in the regulation of neurite length and growth cone morphology. We identified clemizole as a novel inhibitor of TRPC5 channels. Clemizole efficiently blocks TRPC5 currents and Ca\(^{2+}\) entry in the low micromolar range (IC\(_{50}\) = 1.0–1.3 \(\mu\)M), as determined by fluorometric intracellular free Ca\(^{2+}\) concentration (CA\(_{50}\)) measurements and patch-clamp recordings. Clemizole blocks TRPC5 currents irrespectively of the mode of activation, for example, stimulation of G protein–coupled receptors, hypo-osmotic buffer conditions, or by the direct activator riluzole. Electrophysiological whole-cell recordings revealed that the block was mostly reversible. Moreover, clemizole was still effective in blocking TRPC5 single channels in excised inside-out membrane patches, hinting to a direct block of TRPC5 by clemizole. Based on fluorometric [Ca\(^{2+}\)]\(_{i}\) measurements, clemizole exhibits a sixfold selectivity for TRPC5 over TRPC4 (IC\(_{50}\) = 6.4 \(\mu\)M), the closest structural relative of TRPC5, and an almost 10-fold selectivity over TRPC3 (IC\(_{50}\) = 9.1 \(\mu\)M) and TRPC6 (IC\(_{50}\) = 11.3 \(\mu\)M). TRPM3 and M8 as well as TRPV1, V2, V3, and V4 channels were only weakly affected by markedly higher clemizole concentrations. Clemizole was not only effective in blocking heterologously expressed TRPC5 homomers but also TRPC1:TRPC5 heteromers as well as native TRPC5-like currents in the U-87 glioblastoma cell line.

Introduction

All transient receptor potential (TRP) channels have in common that four subunits assemble to form the cation-permeable pore. Within the group of TRPC channels, TRPC5 subunits can arrange as homomeric or heteromeric channel complexes together with TRPC1 or TRPC4 (Hofmann et al., 2002). Heterologous expression studies revealed that heteromers composed of TRPC1 and TRPC5 subunits exhibit altered biophysical properties regarding the current-voltage relation and single-channel conductance compared with the TRPC5 homotetramers (Strubing et al., 2001). TRPC1 and TRPC5 channel proteins display an overlapping expression pattern in the mammalian brain. Both are highly expressed in parts of the limbic system such as hippocampus and amygdala and in the frontal cortex (Strubing et al., 2001; Fowler et al., 2007). Up to now, the physiologic function of TRPC5 in the brain has not entirely been understood. The channel is involved in the regulation of neurite length and growth cone morphology and the motility of hippocampal neurons (Greka et al., 2003; Hui et al., 2006). Studies on TRPC5 knockout mice revealed that granule neurons of the hippocampus and cerebellar cortex exhibit longer and more highly branched dendrites with an impaired dendritic claw differentiation. The impaired dendrite patterning in the cerebellar cortex correlates with deficits in gait and motor coordination observed in these animals (Puram et al., 2011). Moreover, TRPC5-deficient mice appeared to have a diminished fear-related behavior when confronted with innate aversive stimuli (Riccio et al., 2009). Recent studies further revealed a role of TRPC5 in podocytes, demonstrating that the pathogenetic remodeling in proteinuria mouse models can be attributed to the activation of TRPC5 channels. Accordingly, TRPC5-deficient mice were less sensitive to lippopolysaccharide-induced albuminuria (Greka and Mundel, 2011; Schaldecker et al., 2013), making a pharmacological inhibition of TRPC5 a promising target to protect from the destruction of the glomerular filter barrier in kidney injury.

Several inhibitors of TRPC5 activity have been published, such as SKF96356 and flufenamic acid. These compounds are poorly selective modulators known to affect multiple TRP channels from different subfamilies, voltage-gated channels, intracellular Ca\(^{2+}\) release channels, and chloride channels (Merritt et al., 1990; Hofmann et al., 1999; Inoue et al., 2001; Gardam et al., 2008). 2-Aminoethoxydiphenyl borate (2-APB), an inhibitor of the inositol 1,4,5-trisphosphate receptor, blocks TRPC5 currents reversibly and in a voltage-dependent manner (Xu et al., 2005). However, 2-APB is neither a potent (IC\(_{50}\) = 20 \(\mu\)M) nor a specific TRPC5 blocker because it affects other TRPC, TRPV, TRPM, and TRPP channels (Hu et al., 2004;
Lievremont et al., 2005; Xu et al., 2005; Li et al., 2006; Kovacs et al., 2012).

Recently, a novel potent TRPC4 blocker was identified (Miller et al., 2011). ML204 inhibits TRPC4 and 5 currents, induced by μ-opioid or muscarinic receptor stimulation. The blocker has a higher selectivity for TRPC4 than for TRPC5, inhibiting just 65% of TRPC5 channel activity at a concentration of 10 μM (IC50 = 9.2 μM) (Miller et al., 2010). Furthermore, ML204 also exhibits moderate inhibitory effects on muscarinic receptors.

In the present study, we identified Clemizole hydrochloride as a novel blocker of TRPC5 with a half-maximal inhibitory concentration of 1.1 μM. Clemizole inhibits TRPC5-independent form cytosolic components or phospholipase C activity, suggesting a rather direct action of the drug on the channel. Clemizole was efficient to block heterologously expressed homomeric TRPC5 channels as well as heteromeric TRPC1:TRPC5 channels. Also, natively expressed riluzole-evoked TRPC5 currents in the U-87 glioblastoma cell line could be inhibited by Clemizole.

Materials and Methods

Cell Culture and Reagents. A T-REx-HEK293 cell line (Invitrogen, Carlsbad, CA) was used and stably transfected with murine TRPC5 (T-RExTRPC5) to generate an inducible tetracycline-regulated expression system of TRPC5. Cells were treated with 1 μg/ml tetracycline (Tet+; Sigma-Aldrich, St. Louis, MO) for 48 hours before measurements to induce TRPC5 expression. For control experiments, parental T-REx-HEK293 cells were used. Cells were grown in Dulbecco’s modified Eagle’s medium (PAA Laboratories, Pasching, Austria), containing 10% fetal calf serum, 2 mM l-glutamine, 100 U/ml penicillin, 0.1 mg/ml streptomycin, and 100 μg/ml zeocin. Culture medium for T-RExTRPC5 cells was additionally supplemented with 15 μg/ml blasticidin (Invitrogen). Other TRP channels, stably expressed in HEK293 (yellow fluorescent protein (YFP)-tagged human TRPC3, YFP-tagged human TRPC6, YFP-tagged mouse TRPC7, cyan fluorescent protein (CFP)-tagged rat TRPV1, YFP-tagged rat TRPV2, YFP-tagged rat TRPV3, YFP-tagged mouse TRPV4, CFP-tagged human TRPM5, human TRPA1), were generated and maintained, as described previously (Hill and Schaefer, 2007; Urban et al., 2012). The generation of the stable myc-tagged rat HEKTRPM3 cell line was described elsewhere (Fruhwald et al., 2012).

Cell Transfection. HEK293 cells were transiently transfected using jetPEI transfection reagent, according to the manufacturer’s instructions (Peqlab, Erlangen, Germany). For some experiments, we transiently cotransfected YFP-tagged mouse TRPC4β (in a pcDNA3 vector) and rat muscarinic M3 receptor with a deletion in the third intracelluar loop (in pcDNA5/FRT) or pcDNA3 plasmids encoding YFP-tagged mouse TRPC5 or YFP-tagged human TRPC1. The plasmid concentrations of TRPC1:TRPC5 and TRPC4β-muscarinic M3 receptor with a deletion in the third intracellular loop were used in equal amounts. Cells were grown in Earle’s minimum essential medium (PAA Laboratories) supplemented with 10% fetal calf serum, 2 mM l-glutamine, 100 U/ml penicillin, and 0.1 mg/ml streptomycin. The transfection was conducted at a cell confluency of 80%.

Confocal Laser-Scanning Microscopy. The subcellular distribution of YFP-tagged protein kinase C (PKCε) was visualized by using an inverted confocal laser-scanning microscope (LSM510-META, 100/1.46 Plan Apochromat; Carl Zeiss, Oberkochen, Germany). The cells were seeded on coated (25 μg/ml poly-l-lysine (Sarstedt, Nürnberg, Germany)) 25-mm glass coverslips. The fluorescence was excited at 488 nm wavelength. The emitted light was filtered with a 505- to 550-nm bandpass filter.

Intracellular Ca2+ Analysis. For the primary compound screen and to generate concentration-response curves, cells were loaded with fluo-4/AM (4 μM, 30 minutes, 37°C; Invitrogen) dissolved in HEPES-buffered solution containing (in mM) the following: 134 NaCl, 6 KCl, 1 MgCl2, 1 CaCl2, and 10 HEPES, pH 7.4, adjusted with NaOH. Fluorometric assays were performed in a 384-well microtiter plate format (15,000 cells/well; Corning). All cells were measured in suspension except T-RExTRPC5 cells, which were seeded into the microtiter plate and treated with 1 μg/ml tetracycline 48 hours before measurement to induce channel expression. A custom-made fluorescence plate imaging device was used to record changes in fluorescence expressed as ΔF/F0 (Norenb erg et al., 2012). In some experiments, agonists of endogenously expressed G protein–coupled receptors (GPCRs) of HEK293 cells were used. These measurements required a pretreatment with thapsigargin (2 μM, 5 minutes) to deplete inositol 1,4,5-trisphosphate receptor–sensitive Ca2+ stores. For the primary screening assay, compounds of the Spectrum Collection (MicroSource, Gaylordsville, CT), or of the LOPAC2830 compound collection (Sigma-Aldrich), were added to T-RExTRPC5 cells at a final concentration of 20 μM. Afterward, a mixture of agonists (Amox: 300 μM ATP, 300 μM carbamyl, and 0.5 μM thrombin) was applied to induce TRPC5 activity via GPCR signaling. For single-cell intracellular free Ca2+ concentration ([Ca2+]i) analysis, cells were seeded onto poly-l-lysine–coated glass coverslips and allowed to attach for 24–48 hours. T-RExTRPC5 cells were treated as described before. At a final confluence of about 60%, cells were loaded with 2 μM fura-2/AM (Molecular Probes, Eugene, OR) for 30 minutes at 37°C in HEPES-buffered solution buffer. The coverslips were mounted in a bath chamber of an inverted microscope (Axiovert 100; Carl Zeiss) equipped with a monochromator-assisted (TILL Photonics, Gräfelfing, Germany) digital epifluorescence videomicroscopy. The fluorescence was sequentially excited at 340, 358, and 380 nm, and the emitted light was filtered with a 512-nm long-pass filter. The measurements were recorded by a 12-bit cooled charge-coupled device camera (IMAGO; TILL Photonics, Gräfelfing, Germany). The calcium concentration was calculated as described before (Lenz et al., 2002).

Electrophysiology. Patch-clamp recordings were performed in the whole-cell configuration using a Multiclamp 700B amplifier together with a digidata 1440A digitizer (Molecular Devices, Sunnyvale, CA) under pCLAMP 10 software. Cells were seeded on glass coverslips 24–48 hours before measurement with 20–30% cell confluency. The coverslips were transferred to a continuously perfused bath chamber integrated in a stage of an inverted microscope. The standard bath solution for whole-cell recordings contained (in mM) the following: 130 NaCl, 5 KCl, 1.2 MgCl2, 1.5 CaCl2, 8 Mg-glucose, and 10 HEPES, pH 7.4, adjusted with NaOH. The standard pipette solution contained (in mM) the following: 115 CsCl, 2 MgCl2, 5 Na2ATP, 0.1 NaGTP, 5.7 CaCl2, 10 HEPCs, and 10 EGTA, pH 7.2, adjusted with CsOH, yielding a calculated free calcium concentration of 200 nM (MaxChelator; http://maxchelator.stanford.edu). For some experiments, the pipette solution was supplemented with 500 μM GTPγS (Sigma-Aldrich). For inside-out recordings, the pipette solution consisted of (in mM) the following: 140 NaCl, 5 CsCl, 2 MgCl2, and 10 mM HEPES, pH 7.4, adjusted with NaOH. The bath solution contained (in mM) the following: 140 CsCl, 4 Na2ATP, 2 MgCl2, 0.38 CaCl2, 1 EGTA, and 10 HEPCs, pH 7.2, adjusted with CsOH, yielding 100 nM free calcium concentration. To measure murine TRPC4β currents, the pipette solution contained (in mM) the following: 135 CsCl, 2 MgCl2, 0.362 CaCl2, 1 EGTA, and 30 HEPCs, pH 7.2, adjusted with CsOH, yielding a free calcium concentration of 100 nM. Voltage ramps from −100 to +100 mV (500-millisecond duration) were applied in 1-second intervals. Currents were filtered at 3 kHz (four-pole Bessel filter) and sampled at 5 kHz. Voltage ramps (500-millisecond duration) were applied in 1-second intervals. Currents were filtered at 3 kHz (four-pole Bessel filter) and sampled at 5 kHz. Whole-cell series resistances were compensated by 70%. Adherent cells were transferred into a recording chamber holding a volume of 500 μL. Drugs were applied using a gravity-driven perfusion system. Fluorescence measurements are representative for several independent recordings. Gray lines represent single cell traces; black lines display corresponding mean values. Data of fluo-4–based Ca2+ measurements are depicted as mean ± S.D. and were normalized to baseline (gray circles) or to the maximum Ca2+ response to the channel-specific agonist (black circles). The IC50 values were...

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calculated by fitting the data to a Hill’s equation using OriginPro 8 (OriginLab, Northampton, MA) software. Data of electrophysiological recordings are presented as mean ± S.E.M. Statistical comparison of two datasets was performed with Student’s t test using GraphPad (GraphPad software, La Jolla, CA). P < 0.05 (*) was considered as being statistically significant.

Results

Clemizole Inhibits TRPC5 Channel Activity. To identify novel modulators of TRPC5, we performed a 384-well–based medium-throughput screen using the Spectrum Collection and LOPAC1280 compound libraries. A stably transfected HEK293 cell line, expressing TRPC5 channels upon tetracycline treatment, was used for the screening assay. To prevent a release of intracellular Ca$^{2+}$ upon GPCR stimulation, T-REXTRPC5 cells were pretreated with thapsigargin (2 μM for 5 minutes) prior to measurement. The compound screen revealed that TRPC5 channel activity, which was induced by a mixture of GPCR agonists (Amix: 300 μM ATP, 300 μM carbachol, and 0.5 U/ml thrombin), was efficiently blocked upon clemizole application. To verify the primary screening data, we performed concentration–response measurements by using Ca$^{2+}$ assays in fluo-4–loaded cells. The concentration-response curves confirmed a concentration-dependent block of TRPC5 by clemizole (chemical structure shown in Fig. 1C) and revealed an apparent IC$_{50}$ of 1.1 ± 0.04 μM (Fig. 1A, black circles). To examine the effects on GPCR, G protein, or phospholipase C, nontransfected parental T-REX cells were stimulated with the same agonist mix and remained thapsigargin-untreated. Ca$^{2+}$ responses in these cells were not affected by clemizole (Fig. 1B). To test whether also other modes of TRPC5 activation could be blocked by clemizole, we performed fura-2–based single-cell Ca$^{2+}$ imaging experiments. Clemizole (10 μM) did not only inhibit TRPC5 currents induced by Amix (Fig. 1D) but also by riluzole (Fig. 1E; 50 μM) or by hypoosmotic buffer conditions (Fig. 1F; 200 mosmol kg$^{-1}$). To ensure that [Ca$^{2+}$]$_i$ signals did not arise by depletion of internal Ca$^{2+}$ stores, cells were again pretreated with thapsigargin (2 μM).

Additional whole-cell patch-clamp recordings confirmed the calcium imaging data. As reported previously (Richter et al., 2014), a basal activity of TRPC5 channels in the absence of agonists was evident during the recordings. Amix-induced TRPC5 currents were completely blocked upon clemizole (10 μM) application (Fig. 2, A–C), even below the basal current level at the beginning of the recording. TRPC5 currents provoked by riluzole (50 μM) were also blocked by 10 μM clemizole (Fig. 2, D–F). The clemizole-mediated block was mostly reversible, and TRPC5 currents could repetitively be stimulated by riluzole (Fig. 2E). The IC$_{50}$ value from the calcium assays for the clemizole-blocked TRPC5 channels was further validated in whole-cell patch-clamp experiments, yielding an IC$_{50}$ of 1.05 ± 0.3 μM for the outward, and 1.34 ± 0.4 μM for the inward current (Fig. 2G).

Clemizole Does Not Inhibit GPCR, G Protein, or Phospholipase C Signaling, and Soluble Cytosolic Components Are Not Required for the Block of TRPC5.

We conducted whole-cell patch-clamp recordings on T-REXTRPC5 cells and applied GTPγS (500 μM) via the patch pipette (Fig. 3, A–C). This nonhydrolyzable analog of GTP activates Gαq proteins and induces TRPC5 currents (Schaefer et al., 2000). The resulting TRPC5 currents could be blocked by clemizole (10 μM), indicating that an inhibition of GPCR signaling was not causal for the block. To determine whether clemizole affects phospholipase C (PLC) activity, we used a HEK293 cell line that stably expresses PKCε (HEKPKCε) (Schaefer et al., 2001). Upon stimulation of Gαq protein–coupled

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**Fig. 1.** Concentration-dependent inhibition of TRPC5 by clemizole. (A and B) Concentration-response relation of Ca$^{2+}$ signals in fluo-4–loaded T-REXTRPC5 cells (A, 48-hour Tet$^+$) and parental T-REX cells (B). Cells were first treated with different concentrations of clemizole, and then a mixture of agonists (300 μM ATP, 300 μM carbachol, 0.5 U/ml thrombin; Amix) was applied. Prior to measurements, T-REXTRPC5 cells were pretreated with 2 μM thapsigargin for 5 minutes to deplete inositol 1,4,5-trisphosphate receptor–sensitive internal Ca$^{2+}$ stores. Parental T-REX control cells remained thapsigargin-untreated to determine Amix-induced Ca$^{2+}$ release from intracellular stores. The IC$_{50}$ value of clemizole was calculated by nonlinear curve fitting with the Hill equation. Data points display means and S.D. of six (T-REXTRPC5) or eight (parental T-REX) independent experiments. (C) Chemical structure of clemizole. (D–F) Clemizole blocks TRPC5 currents irrespectively of the mode of activation. Representative fura-2–based single-cell Ca$^{2+}$ experiments of tetracycline-induced T-REXTRPC5 cells are shown. Cells were treated with 2 μM thapsigargin, and TRPC5 activity was stimulated with Amix (D), 50 μM riluzole (E), or hypoosmotic (200 mosmol/kg) HEPES-buffered solution buffer (F), followed by addition of 10 μM clemizole.
GPCRs via Amix, PLC is activated and generates diacylglycerol from phosphatidylinositol 4,5-bisphosphate. Diacylglycerol then induces the translocation of the PKCs to the plasma membrane. We visualized this translocation by using a YFP-tagged construct of PKCs (Supplemental Fig. 1). Untreated HEKPKC–YFP cells (control) present an evenly distributed cytosolic fluorescence. This subcellular distribution was unaltered upon clemizole (10 μM) application. Additional application of Amix induced, as expected, a translocation of the PKC to the plasma membrane, indicating that clemizole did not affect PLC activity.

We next wanted to know whether intracellular soluble molecules contribute to the block of TRPC5 channels by clemizole. To this end, we conducted electrophysiological experiments in the inside-out configuration. As reported previously (Richter et al., 2014), riluzole causes a strong increase in the open probability of TRPC5 channels (Fig. 3 D and E). As the slow block by clemizole was relatively slow and not complete at a concentration of 20 μM (for the inward current) (Fig. 4H), further blocked TRPC4β currents, even below the basal current densities at the beginning of the recording (Fig. 4, E–G). As the slow block by clemizole was superimposed by a rapid and in most cases complete rundown of channel activity, the calculated IC50 values for the block were considerably higher compared with TRPC5. TRPC4β, the closest structural relative of TRPC5, was blocked by clemizole with an IC50 of 6.4 ± 0.9 μM (Fig. 4D). Whole-cell patch-clamp recordings indicated that the block of TRPC4β by clemizole was almost 10-fold selectivity for TRPC5 over TRPC3 (IC50 = 9.1 μM) and TRPC6 (IC50 = 11.3 μM). TRPC7 was even less sensitive to clemizole-mediated block. Representa-

cell lines expressing canonical TRP channels (TRPC) were pretreated with thapsigargin prior to measurements to deplete inositol 1,4,5-trisphosphate receptor–sensitive intracellular Ca2+ stores. The channel activity of TRPC4β was induced by carbachol (300 μM; Fig. 4D), and of TRPC3, C6, and C7 by Amix (Fig. 4, A–C). Within the TRPC subfamily, apart from TRPC5, also TRPC3, C4, C6, and C7 were affected by clemizole. However, IC50 values for the block were considerably higher compared with TRPC5. TRPC4β, the closest structural relative of TRPC5, was blocked by clemizole with an IC50 of 6.4 ± 0.9 μM (Fig. 4D). Whole-cell patch-clamp recordings indicated that the block of TRPC4β by clemizole was relatively slow and not complete at a concentration of 10 μM. 2-APB (75 μM) further blocked TRPC4β currents, even below the basal current densities at the beginning of the recording (Fig. 4, E–G). As the slow block by clemizole was superimposed by a rapid and in most cases complete rundown of channel activity, the calculated IC50 values for the clemizole-induced block of TRPC5 channels had a conductance of 56 ± 4 pS (n = 11). The addition of 20 μM clemizole was still effective in blocking TRPC5 channels when applied to the cytosolic side of the channel, indicating a direct action of clemizole on riluzole-activated TRPC5 channels (Fig. 3 D and E).

Clemizole-Mediated Action on Other Transient Receptor Potential Channels. To examine whether clemizole affects other TRP channels, we performed fluo-4–based Ca2+ assays on HEK293 cells, overexpressing various closely or more distantly related TRP channels. Cells were first treated with different concentrations of clemizole, and, subsequently, the respective TRP channel-specific agonist was applied. All
Clemizole also activated the pleiotropic irritant sensor TRPA1 at concentrations >10 μM (Supplemental Fig. 2).

**Clemizole Blocks Heteromeric TRPC1:TRPC5 Channels.** Heterologous coexpression studies of TRPC1 and TRPC5 have shown that these channels can assemble as heteromorphic ion channels with distinct current-voltage relationships compared with currents through homomeric TRPC5 channels (Strubing et al., 2001). To test whether clemizole can also block heteromeric TRPC1:TRPC5 channels, we cotransfected HEK293 cells with a 1:1 mixture of expression plasmids encoding TRPC1 and TRPC5 (HEKTRPC1:TRPC5). As reported previously, cells expressing only TRPC1 were not stimulated by riluzole (50 μM) and possess only low basal current densities of about 10 pA/pF at +100 mV (Richter et al., 2014). A cotransfection of TRPC1 and TRPC5 resulted in an elevation of the basal current densities (Fig. 5, A–C), and challenging the cells with riluzole (50 μM) induced a strong outwardly rectifying current, typical to heteromorphic TRPC1:TRPC5 currents (Strubing et al., 2001), which could be blocked by clemizole (10 μM).

**Native TRPC5-Like Currents in the U-87 Glioblastoma Cell Line Are Blocked by Clemizole.** Previous reports confirmed that TRPC5 channels are endogenously expressed in the glioblastoma cell line U-87 (Wang et al., 2009; Richter et al., 2014), and that riluzole-evoked calcium signals could be impeded by a small interfering RNA–mediated knockdown of TRPC5 (Richter et al., 2014). Accordingly, riluzole (100 μM) provoked currents in whole-cell patch-clamp recordings on U-87 cells, which could partially be inhibited by clemizole (50 μM) (Fig. 5, D–F). The riluzole-evoked currents displayed a reversal potential more negative than heterologously expressed TRPC5 channels, together with a stronger inward current (Fig. 5E). As all of the riluzole-evoked inward current disappeared when permeable cations were substituted for N-methyl-D-glucamine (NMDG) in the extracellular solution (Richter et al., 2014), this would be consistent with the activation of additional potassium channels, which do not respond to clemizole. This could be caused either directly through riluzole or through a TRPC5-mediated increase in [Ca²⁺].

**Discussion**

In this study, we identified clemizole as a novel blocker of TRPC5 channels. Clemizole [1-p-chlorobenzyl-2-(1-pyrrolidinyl)methylbenzimidazole] has originally been developed as a histamine H₁-receptor antagonist. This first-generation antihistamine can pass the blood-brain barrier and has a low toxicity (Wansker, 1962; Einav et al., 2010). It was developed in the 1950s to treat allergic reactions and various dermatological diseases (Zierz and Greither, 1952; Jacques and Fuchs, 1960). Besides its antihistaminergic action, clemizole inhibits monoamine reuptake in the brain (Oishi et al., 1994) and blocks human ether-a-go-go related gene (hERG) channels at a concentration of 10 μM (data obtained from http://pubchem.ncbi.nlm.nih.gov). Nowadays, clemizole is not marketed as a single-agent antihistamine anymore. However, clemizole might be beneficial in the treatment of hepatitis C virus
HCV infections. It has been shown to inhibit the binding of NS4B to HCV RNA, exhibiting moderate antiviral effects against HCV (genotype 2a, EC50 58 mM) on its own (Einav et al., 2008). Combining clemizole with HCV protease inhibitors proved to be highly synergistic, strongly enhancing the potency of such therapeutics (Einav et al., 2010). Moreover, a recent study of Baraban et al. (2013) reported that clemizole inhibits spontaneous seizures in a zebrafish model for Dravet syndrome (scn1Lab mutant), a rare and severe form of monogenic epilepsy, which is mainly caused by mutations in Nav1.1 (SCN1A), a voltage-gated sodium channel (Catterall, 2014). However, high concentrations of 100 μM clemizole were needed to suppress spontaneous hyperactivity.

In this study, we identified clemizole as a novel blocker of TRPC5 channels. Clemizole potently inhibited TRPC5 channels and exhibited a moderate selectivity within the TRPC family, with a sixfold selectivity (in calcium assays) over its closest homolog TRPC4β. The inhibition was effective toward different modes of TRPC5 activation, such as stimulation of GPCR, hypotonic buffer conditions, or a direct activation by riluzole. We assume that clemizole directly acts on TRPC5 channels, as the drug was still effective in excised inside-out patches, where intracellular components are washed out. As clemizole is a lipophilic compound (log P 4.23; chemicalize.org), we cannot clearly discriminate between an intracellular or extracellular site of action on TRPC5 channels, but the delayed onset of action in the inside-out measurements with a latency of 20–30 seconds might hint to a necessity of clemizole to reach the extracellular site of the channel.

TRPC5 channels can assemble as homo- or heterotetramers with TRPC1 or TRPC4 (Schaefer et al., 2000; Strubing et al., 2003), exhibiting distinct biophysical properties (Strubing et al., 2001). Because TRPC5 and TRPC1 show overlapping expression patterns in mammalian brain tissue (Strubing et al., 2001), we analyzed the effect of clemizole on heteromeric TRPC1:TRPC5 complexes. As reported previously, TRPC1:TRPC5 heteromers in HEK293 cells were stimulated by riluzole. The same applies for TRPC5 or possible TRPC1:TRPC5 heteromers, endogenously expressed in the U-87 glioblastoma/astrocytoma cell line (Richter et al., 2014). Such
rilezole-induced currents were blocked upon clemizole application, indicating that clemizole also targets heteromeric TRPC5 channels and might be effective in native tissues. Because of its lipophilic properties, clemizole is able to pass the blood-brain barrier. It will be the aim of future studies to test whether application of clemizole or other TRPC5 inhibitors in vivo mimics the axiolytic-like and kidney-protective phenotype of TRPC5-deficient mice (Riccio et al., 2009; Puram et al., 2011; Schaldecker et al., 2013). With regard to future in vivo experiments, clemizole has the advantage of having been intensively studied since it was used for its antihistaminic effect. The safety and tolerability of clemizole to treat HCV have been investigated in a clinical trial at a dosage of 200 mg/d (CLEAN-1 trial, NCT00945880), but its outcome has not been reported, yet.

The safety and tolerability of clemizole to treat HCV have been investigated in a clinical trial at a dosage of 200 mg/d (CLEAN-1 trial, NCT00945880), but its outcome has not been reported, yet. According to the current state of knowledge, clemizole might serve as a promising starting point for the development of pharmacological tools, which provide new insights into the physiologic relevance of TRPC5 expressed in native systems and which are possibly applicable for the establishment of new therapeutic strategies targeting TRPC5 in diseases.

Acknowledgments

The authors thank Nicole Urban for helpful discussion and excellent technical assistance. Stable myc-tagged rat HEK-TRPC5 cell line was a kind gift of S. Philipp (Universität des Saarlandes, Homburg, Germany).

Authorship Contributions

Participated in research design: Richter, Schaefer, Hill.

Conducted experiments: Richter, Hill.

Performed data analysis: Richter, Hill.

Wrote or contributed to the writing of the manuscript: Richter, Schaefer, Hill.

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Hill K and Schaefer M (2007) TRPA1 is differentially modulated by the amphipathic alpha-helical RNA binding inhibitor clemizole (50 M). (D) Traces were taken from voltage ramps and depict current densities at +100 mV (upper trace) and ~100 mV (lower trace). Cells were treated with 50 aM riluzole and 10 aM clemizole.


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Molecular Pharmacology

Supplementary Information

Figure S1

Fluorescence imaging of living HEK293 cells that stably express PKCε-YFP. The subcellular localization of PKCε-YFP was visualized by confocal laser scanning microscopy. Images display untreated control cells, clemizole (10 µM)-treated cells and cells challenged with clemizole and Amix.
Figure S2

Concentration-response curves of fluo-4-loaded cells, which stably express selected TRP channels. Cells were exposed to different concentrations of clemizole (grey circles) and then treated with channel-specific agonists (black circles) which were used as follows: 30 µM PregS (TRPM3); 300 µM menthol (TRPM8); 2 µM capsaicin (TRPV1); 300 µM 2-APB (TRPV2); 75 µM 2-APB (TRPV3); 100 nM GSK1016790A (TRPV4); 100 µM AITC (TRPA1). The data is depicted as mean and SD of at least 4 independent experiments.