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**Neue Ansätze zur biophysikalischen Charakterisierung von
TRP-Kationenkanälen**

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Abkürzungsverzeichnis

DAG	Diacylglycerol
FSGS	Fokal segmentale Glomerulosklerose
GPCR.....	G-Protein-gekoppelte Rezeptoren
IP ₃	Inositoltrisphosphat
I-V	Strom-Spannung
Kryo-EM	kryo-elektronenmikroskopisch
MHR.....	<i>melastatin-homology-regions</i>
OAG	1-Oleoyl-2-acetyl-sn-glycerol
PIP ₂	Phosphatidylinositol-4,5-bisphosphat
PLC	Phospholipase C
TM.....	Transmembrandomäne
TRP	<i>transient receptor potential</i>
TRPC	<i>transient receptor potential canonical</i>
TRPM.....	<i>transient receptor potential melastatin</i>
VSLD.....	<i>voltage-sensing-like-domain</i>

Publikationsliste

In dieser Arbeit zusammengefasste wissenschaftliche Publikationen

Hermann Christian, Treder Aaron, Näher Marius, Geiseler Roman, Gudermann Thomas, Mederos Y Schnitzler Michael & Storch Ursula (2022). **The normalized slope conductance as a tool for quantitative analysis of current-voltage relations**. *Biophysical journal*, 121(8), 1435–1448. <https://doi.org/10.1016/j.bpj.2022.03.016>

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Becker Jasmin, Ellerkmann Clara, Schmelzer Hannah, **Hermann Christian**, Lützel Kyra, Gudermann Thomas, Konrad David, Trauner Dirk, Storch Ursula & Mederos Y Schnitzler Michael (2024). **Optical Control of TRPM8 Channels with Photoswitchable Menthol**. *Angewandte Chemie International Edition*, (e202416549) <https://doi.org/10.1002/anie.202416549>

Weitere wissenschaftliche Veröffentlichungen aus meiner Promotionszeit

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Keck Maximilian, **Hermann Christian**, Gudermann Thomas, Mederos Y Schnitzler Michael & Storch Ursula (2024). **Photopharmacology of TRPC6 channels to determine channel kinetics** [Posterpräsentation], 9th German Pharm-Tox Summit, Munich Germany. <https://doi.org/10.1007/s00210-024-03150-3>

Kressmann Tabea, **Hermann Christian**, Gudermann Thomas, Mederos Y Schnitzler Michael & Storch Ursula (2024). **Development of red-shifted FRET-based cAMP sensors** [Posterpräsentation], 9th German Pharm-Tox Summit, Munich Germany. <https://doi.org/10.1007/s00210-024-03150-3>

Bestätigung der Co-Autoren

Hiermit erkläre ich, dass alle Autoren, die an den Veröffentlichungen mitgewirkt haben, ihr schriftliches Einverständnis gegeben haben diese Publikationen im Rahmen meiner Dissertation zu verwenden.

Mein Beitrag zu den Veröffentlichungen

1.1 Beitrag zu Publikation I

Im Rahmen der Publikation I mit dem Titel "*The normalized slope conductance as a tool for quantitative analysis of current-voltage relations*" (veröffentlicht im *Biophysical Journal* im Jahr 2022) war ich für die Entwicklung und Testung eines neuen Algorithmus zur Berechnung der *normalized slope conductance* (NSC) verantwortlich, mit dessen Hilfe Strom-Spannungsbeziehungen von Ganzzellströmen quantitativ analysiert werden können. Des Weiteren habe ich eine auf R basierende Auswertungssoftware entwickelt, welche die Anwendung des Algorithmus sowie die Verarbeitung und Datenauswertung der durchgeführten Patch-Clamp-Messungen ermöglicht. Mit Hilfe dieser Software habe ich die Patch-Clamp-Messungen ausgewertet und die Abbildungen für das Manuskript erstellt. Zudem habe ich einen Teil der Patch-Clamp-Messungen durchgeführt.

Die Erstautorenschaft wurde geteilt, da der experimentelle Aufwand, der mit der hohen Anzahl der durchgeführten Patch-Clamp-Messungen durch den Koautor einherging, eine gleichwertige Beteiligung an der Publikation rechtfertigte.

1.2 Beitrag zu Publikation II

Im Rahmen der Publikation II mit dem Titel "*Photoswitchable TRPC6 channel activators evoke distinct channel kinetics reflecting different gating behaviors*" (veröffentlicht in *iScience* im Jahr 2024) habe ich einen wichtigen Beitrag zur Datenanalyse geleistet. Zusätzlich habe ich die in der Publikation I entwickelte Auswertungssoftware um neue Methoden erweitert und eine Fit-Routine zur Auswertung der Aktivierungs-, Deaktivierungs- und Inaktivierungskinetiken erstellt und getestet. Zudem umfasste mein Beitrag den Aufbau, die Programmierung und das Testen der Arduino-Kontrolleinheit zur Ansteuerung der LEDs, mit denen die eingesetzten Photopharmaka mit einer Auflösung von 5 μ s geschaltet werden können.

1.3 Beitrag zu Publikation III

Im Rahmen der Publikation III mit dem Titel "*Optical Control of TRPM8 Channels with Photoswitchable Menthhol*" (veröffentlicht in *Angewandte Chemie International Edition* im Jahr 2024) habe ich einen Beitrag zur Datenanalyse geleistet. Dafür habe ich die Software zur Kinetikanalyse entwickelt, programmiert und getestet. Außerdem habe ich den Algo-

rithmus zur Berechnung der *normalized slope conductance* auf das veränderte Messprotokoll angepasst. Ich erweiterte die Arduino-Kontrolleinheit samt Bedienungssoftware auf acht individuelle LEDs. Ebenfalls führte ich das molekulare Docking und die Interpretation der Ergebnisse durch. Zudem wirkte ich bei der Erstellung der Abbildungen mit.

2. Einleitung

2.1 TRP-Kanäle

Die *transient receptor potential* (TRP) Kanäle stellen eine umfangreiche Familie von Ionenkanalproteinen dar, welche in der Physiologie von Säugetieren eine Vielzahl von Funktionen erfüllen. TRP-Kanäle sind nicht selektive Kationenkanäle, die eine hohe relative Durchlässigkeit für Ca^{2+} und Na^+ aufweisen. Sie sind an der Weiterleitung einer Vielzahl chemischer und physikalischer Reize beteiligt, modulieren den Kationeneinstrom in das Zytoplasma und vermitteln verschiedene neuronale Signalprozesse, die mit der Wahrnehmung von Temperatur, Druck, pH-Wert, Geruch, Geschmack, Sehkraft und Schmerz verbunden sind (Aroke et al., 2020; Ricardo González-Ramírez et al., 2017; Sharif-Naeini et al., 2010; M. Y. Song & Yuan, 2010; Story, 2006; Voets et al., 2005). Des Weiteren sind TRP-Kanäle an der Regulation von Zellprozessen wie der Proliferation, Differenzierung und Apoptose beteiligt (Marini et al., 2023).

Die TRP-Superfamilie der Säugetiere umfasst insgesamt 28 verschiedene Membranproteine, welche in 6 Unterfamilien eingeteilt werden: TRPC (*Canonical*), TRPM (*Melastatin*), TRPV (*Vanilloid*), TRPA (*Ankyrin*), TRPP (*Polycystin*) und TRPML (*Mucolipin*) (Samanta et al., 2018).

TRP-Kanäle sind an der Pathogenese diverser Erkrankungen beteiligt, darunter neuropathische Schmerzen, Entzündungen und Atemwegserkrankungen (Nilius et al., 2007; Yue & Xu, 2021; Zholos, 2015). Mutationen in TRP-Kanälen, die als „TRP-Kanalopathien“ bezeichnet werden, sind für eine Vielzahl von Erbkrankheiten verantwortlich, die die Skelettmuskulatur, das Herz-Kreislauf-System, die Nieren, sowie das Nervensystem betreffen (Tóth & Nilius, 2015).

Die Forschung zu TRP-Proteinen begann 1969 mit der Identifizierung einer Sehmutante der Fruchtfliege *Drosophila melanogaster* (Cosens & Manning, 1969). Im Jahr 1975 erfolgte die Benennung als „*transient receptor potential*“ (Minke et al., 1975). Erstmals erfolgreich kloniert wurde das TRP-Gen im Jahre 1989 (C. Montell & Rubin, 1989). In den Jahren 1991 und 1992 wurde die Eigenschaft als Ca^{2+} -permeabler Kationenkanal entdeckt (Minke & Selinger, 1992; Suss-Toby et al., 1991). Die ersten TRP-Kanäle in Säugetieren wurden 1995 identifiziert (Wes et al., 1995; Zhu et al., 1995). Nach der Entdeckung zahlreicher weiterer TRP-Proteine wurde 2002/2003 die aktuell genutzte einheitliche Nomenklatur vorgeschlagen (David E. Clapham et al., 2003; Craig Montell et al., 2002). Ein signifikant steigendes Interesse an TRP-Kanälen lässt sich seit Beginn der 2000er Jahre beobachten. Dieses äußert sich in einer Verfünffachung der Publikationen innerhalb von

20 Jahren sowie in über 1.000 Veröffentlichungen im Jahr 2020 (Jiang et al., 2022). Im Jahr 2013 wurden die ersten hochaufgelösten Strukturen von TRP-Proteinen veröffentlicht (Cao et al., 2013; Liao et al., 2013). Im Jahr 2021 wurde David Julius mit dem Nobelpreis für Medizin oder Physiologie ausgezeichnet, in Anerkennung seiner bahnbrechenden Entdeckungen und Forschungsarbeiten zu den Kationenkanälen TRPV1 und TRPM8. Diese spielen eine entscheidende Rolle als vielversprechende Zielstrukturen für neue Medikamente.

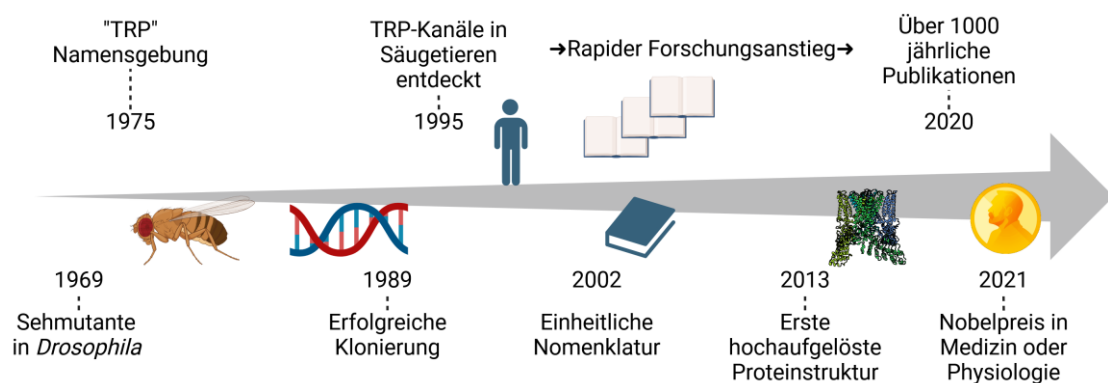


Abbildung 1: Zeitstrahl mit Meilensteinen der TRP-Forschung

Die Primärstruktur der TRP-Kanäle ist konserviert und umfasst sechs Transmembrandomänen (TM1-TM6) mit intrazellulären Carboxy (C)- und Aminoterminalregionen (N). Die porenbildende Schleife (Porendomäne) befindet sich zwischen TM5 und TM6. TM1 bis TM4 bilden die sogenannte *voltage-sensing-like-domain* (VSLD). Die VSLD und Porendomäne sind über den TM4-TM5-Linker miteinander verbunden (Zhang et al., 2023).

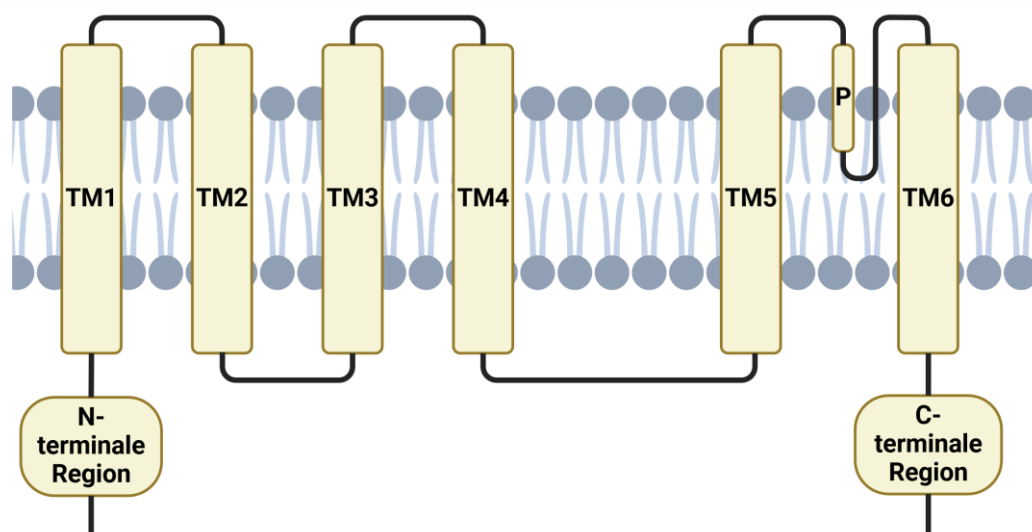


Abbildung 2: Schematischer Aufbau eines TRP-Kanals mit Transmembrandomänen (TM1-6), Porenhelix (P) und Amino (N)- und Carboxy (C)-Terminalregion

Funktionelle TRP-Kanäle bestehen aus tetramerischen TRP-Proteinkomplexen, die entweder homotetramer oder heterotetramer vorliegen können (Cheng et al., 2010).

Die *transient receptor potential* Kanäle bilden eine vielseitige Familie nicht-selektiver Kationenkanäle, die physiologische Prozesse wie Sinneswahrnehmung, Calcium-Homöostase und Zellregulation unterstützen. Sie sind mit zahlreichen Erkrankungen assoziiert und werden seit ihrer Entdeckung in *Drosophila* im Jahr 1969 intensiv erforscht.

2.2 Die TRPC-Kanalfamilie

Die TRPC-Kanäle (*transient receptor potential canonical*) repräsentieren eine Unterfamilie der TRP-Kanalproteine. Insgesamt werden sieben Mitglieder unterschieden, welche als TRPC1 bis TRPC7 bezeichnet werden. Eine funktionelle Bedeutung von TRPC2 ist beim Menschen nicht gegeben, da es sich lediglich um ein Pseudogen handelt (Yildirim & Birnbaumer, 2007). TRPC-Kanäle sind in der Lage, Heterotetramere zu bilden, was sich sowohl auf deren Funktion als auch auf deren biophysikalische Eigenschaften auswirkt (D. E. Clapham et al., 2001). TRPC1 ist nahezu ausschließlich in Form von Heterotetrameren zu finden (Dietrich et al., 2014). Kennzeichnende strukturelle Elemente der TRPC-Kanäle sind die Ankyrin-Wiederholungssequenzen am proximalen N-Terminus sowie die sogenannte TRP-Box am proximalen C-Terminus. (Wang et al., 2020).

TRPC-Kanäle sind ubiquitär im menschlichen Körper exprimiert, wobei besonders hohe Expressionsniveaus in Gehirn, glatter Muskulatur, Herz, Nieren, Nebennieren sowie im Skelettmuskelgewebe nachgewiesen wurden (Wang et al., 2020).

Die Funktionen der TRPC-Kanäle umfassen unter anderem die Regulation des Calcium-Haushalts und der Neurosekretion (Y. Sun et al., 2014), die Aufrechterhaltung der Nierenfunktion (Englisch et al., 2022), die Regulierung der kardialen Kontraktilität (Wen et al., 2020) sowie die Zellsignalübertragung (Kang et al., 2024).

TRPC-Kanäle stehen in Zusammenhang mit einer Vielzahl von Erkrankungen, darunter Nierenerkrankungen wie die fokal segmentale Glomerulosklerose (FSGS) oder die diabetische Nephropathie (Saqib et al., 2023) sowie Herzkreislauferkrankungen wie die kardiale Hypertrophie oder Bluthochdruck (Rowell et al., 2010). Des Weiteren werden neurologische Erkrankungen, wie die spinocerebelläre Ataxie (Becker, 2017), Epilepsie und neurodegenerative Erkrankungen wie Alzheimer und Parkinson (Jeon et al., 2020) sowie Krebserkrankungen (Asghar & Törnquist, 2020) mit TRPC-Kanälen assoziiert. Atemwegserkrankungen (Koivisto et al., 2022), wie unter anderem das durch das Corona-Virus verursachte akute Atemnotsyndrom (Dietrich, 2019), stehen ebenfalls mit TRPC-Kanälen in Verbindung.

In den vergangenen Jahren hat das Interesse an der therapeutischen Modulation von TRPC-Kanälen erheblich zugenommen, was sich in einer Vielzahl von klinischen Studien widerspiegelt. TRPC6-Inhibitoren, wie BI-764198 und seine Derivate, werden aktuell auf ihre Wirksamkeit gegen FSGS getestet (NCT05213624) (Trachtman et al., 2023). Eine klinische Studie (NCT04604184) zur Verbesserung der Lungenfunktion bei Krankenhauspatienten mit schwerem COVID-19 wurde aufgrund fehlender Wirkung und potentieller Nebeneffekte abgebrochen (Ware et al., 2023). Der TRPC4- und TRPC5-Blocker BI1358894 wurde in klinischen Studien (NCT03904576) als potenzielles Therapeutikum erfolgreich zur Linderung von Paniksymptomen bei Cholecystokin-Tetrapeptid (CCK-4)-induzierten Angstzuständen und depressiven Störungen getestet (Goettel et al., 2023). Im Rahmen einer aktuellen Studie (NCT04521478) wird die Effektivität von BI1358894 gegen Depression, bei der eine Standardtherapie nicht zum Tragen kommt, evaluiert. Ohne Erfolg hingegen verlief eine Untersuchung gegen Borderline-Persönlichkeitsstörungen (NCT04566601) (Dwyer et al., 2025). Ein weiterer TRPC5-Inhibitor, GFB-887, wurde in einer abgebrochenen Studie gegen FSGS (NCT04950114), behandlungsresistente Minimal-Change-Disease sowie die diabetische Nephropathie (NCT04387448) getestet (Walsh et al., 2021).

Die TRPC-Kanäle (TRPC1-TRPC7) sind ubiquitär exprimierte Kationenkanäle, die essenzielle Funktionen wie Calcium-Regulation, Neurosekretion und kardiale Kontraktilität erfüllen. Sie sind mit Erkrankungen wie Niereninsuffizienz, Herz-Kreislauf- und neurodegenerativen Erkrankungen assoziiert und werden zunehmend als therapeutische Zielstrukturen erforscht.

2.2.1 Aktivierungsmechanismen der TRPC-Kationenkanäle

TRPC-Kanäle gelten als rezeptorgesteuert, das heißt ihre Aktivierung erfolgt physiologisch primär über den Phospholipase C (PLC) Signalweg. Dieser Prozess beginnt mit der Aktivierung von G-Protein-gekoppelten Rezeptoren (GPCR), welche daraufhin die Phospholipase C stimulieren. Dieser Prozess erfolgt hauptsächlich über den $G_{q/11}$ -Signalweg. Die aktivierte PLC katalysiert die Hydrolyse von Phosphatidylinositol-4,5-bisphosphat (PIP_2) in die Botenstoffe Inositoltrisphosphat (IP_3) und Diacylglycerol (DAG) (Chen et al., 2020; Wang et al., 2020).

DAG wirkt als direkter Aktivator der TRPC-Kanalfamilie (Albert, 2011; Storch et al., 2017). Im Fall von TRPC3 ist bekannt, dass DAG an der sogenannten L2-Region bindet, welche sich zwischen der Poren-Helix und den Transmembransegmenten TM5 und TM6 befindet (Lichtenegger et al., 2018). Eine äquivalente Bindestelle für TRPC5 konnte durch kryo-elektronenmikroskopische (Kryo-EM) Studien bestätigt werden (K. Song et

al., 2021). Neben DAG können auch DAG-Analoga wie 1-Oleoyl-2-acetyl-sn-glycerol (OAG) TRPC-Kanäle aktivieren (Tu et al., 2009).

IP_3 induziert die Freisetzung von Ca^{2+} aus intrazellulären Speichern (Mikoshiba, 2007). Änderungen der zytosolischen Kalziumkonzentration modulieren ebenfalls die Aktivität von TRPC-Kanälen, entweder direkt (Blair et al., 2009) oder durch Proteine wie Calmodulin (Ordaz et al., 2005; Vinayagam et al., 2020) oder STIM1 (Asanov et al., 2015).

Darüber hinaus wird die Aktivität von TRPC-Kanälen durch die Depletion von PIP_2 , das in der pre-TM1-Domäne bindet, auf verschiedene Weisen moduliert. Diese führt zur Hemmung von TRPC6- und TRPC3-Kanälen (Clarke et al., 2024; Mori et al., 2022), während die Wirkung von PIP_2 -Depletion auf TRPC5 von der Art des Reduktionsmechanismus abhängt. Eine globale und langsame Reduktion von PIP_2 in der Zelle, zum Beispiel durch die Hemmung der Phosphatidylinositol-4-Kinase, aktiviert TRPC5 durch sekundäre Effekte wie NHERF-Dissoziation und erhöhte DAG-Sensitivität (Storch et al., 2017; Trebak et al., 2009). Demgegenüber führt die gezielte Hydrolyse von PIP_2 durch Phosphatidylinositol-4-phosphat-5-Kinase oder spannungsgesteuerten Phosphatasen zu einer lokalen und akuten Depletion, die die PIP_2 -Kanal-Interaktion schwächt und den TRPC5-Kanal inhibiert (Ko et al., 2019; Ningoo et al., 2021). Zusätzlich moduliert die Proteinkinase C-abhängige Phosphorylierung an der Position T972 die Bindungsaffinität von PIP_2 und verstärkt so eventuell die Inhibition bei niedrigen PIP_2 -Spiegeln (Ningoo et al., 2021; Yao et al., 2005).

Außerdem wurde nachgewiesen, dass eine direkte Bindung von G-Protein $-G\alpha_{i3}$ an TRPC4- und TRPC5-Kanäle zur Kanalaktivierung führt (Won et al., 2023). Zudem ist die Proteinkinase C, die im PLC-Signalweg nachgeordnet aktiviert wird, von entscheidender Bedeutung für die Regulation der TRPC-Kanalaktivität. Diese erfolgt durch Phosphorylierung spezifischer Kanaldomänen, was zu einer Verringerung der Kanalaktivität führt (Bousquet et al., 2010; Hagmann et al., 2018).

Für TRPC6 wurde eine intrinsische Mechanosensitivität und eine Aktivierung durch mechanische sowie osmotische Dehnungen der Zellmembran postuliert (Spasova et al., 2006). Diese Hypothese konnte jedoch nicht bestätigt werden. Aktuelle Forschungsergebnisse deuten darauf hin, dass TRPC-Kanäle eher indirekt mechanosensitiv sind und als molekulare Verstärker von intrinsisch mechanosensitiven Proteinen agieren (Cox et al., 2024; Mederos Y Schnitzler et al., 2008).

Eine weiterer Aktivierungsmechanismus von TRPC5-Kanälen stellt die gezielte Translokation in die Plasmamembran dar. Diese führt zu einer Verstärkung der Kanalaktivität (Bezzarides et al., 2004). Des Weiteren wurden TRPC5-Kanäle in Odontoblasten (Bernal

et al., 2021), Spinalganglien (Zimmermann et al., 2011) und in einem HEK293T-Überexpressionssystem als Kältesensor beschrieben (Ptakova et al., 2022).

Bei TRPC-Kanälen wurden bisher drei spezifische Bindetaschen für kleine Moleküle identifiziert. Die erste Bindetasche überlappt mit der DAG-Bindestelle und ermöglicht die Interaktion mit den Aktivatoren Lysophosphatidylcholin (Ptakova et al., 2024) und AM-0883 (Bai et al., 2020), sowie den Inhibitoren HC-070 (K. Song et al., 2021) und Pico145 (Won et al., 2024). Die zweite Bindetasche befindet sich in der VSLD und wird vom Aktivator Riluzol (Y. Yang et al., 2022) und Inhibitoren wie Clemizol (K. Song et al., 2021), SAR7334 (Guo et al., 2022), AM1473 (Bai et al., 2020) und GFB-8438 (Vinayagam et al., 2020) adressiert. Die dritte Tasche ist zwischen den Segmenten TM3/TM4 und TM5/TM6 lokalisiert und ermöglicht die Bindung des Inhibitors BTDM (Guo et al., 2022).

Eine weitere modulierende Komponente stellen die dreiwertigen Kationen der seltenen Erdmetallionen Gadolinium (Gd^{3+}) und Lanthan (La^{3+}) dar. Diese Kationen binden an eine extrazelluläre Bindungsstelle am Übergang zwischen der S5-Helix und der S6 und potenzieren TRPC4- und TRPC5-Ströme. Demgegenüber werden TRPC3-, TRPC6- und TRPC7-Ströme durch Gd^{3+} und La^{3+} inhibiert (Chen et al., 2017; Jung et al., 2003).

Zusammengefasst erfolgt die Aktivierung von TRPC-Kanälen primär über den PLC-Signalweg. Wesentliche Regulatoren sind hierbei DAG, IP_3 , PIP_2 und G-Proteine. Zusätzlich können mechanische Kräfte, die Temperatur, Lanthanoide sowie die Bindung von Aktivatoren und Inhibitoren in bestimmten Bindetaschen die Kanalaktivität beeinflussen.

2.3 Der TRPM8-Kanal

Der *transient receptor potential melastatin 8* (TRPM8) Kanal bildet zusammen mit TRPM1-TRPM7 die Unterfamilie der Melastatin-TRP-Kanäle. Strukturell sind die vier *melastatin-homology-regions* (MHR) in der Nähe des N-Terminus (Fleig & Penner, 2004) sowie die TRP-Box charakteristisch (Yin et al., 2018). Die Expression von TRPM8-Kanälen erfolgt in nicht-myelinisierten C- und A δ -Fasern in Neuronen mit kleinem Durchmesser in den Spinal- und Trigeminale-Ganglien (Kobayashi et al., 2005; McKemy et al., 2002; Reid & Flonta, 2002; Thut et al., 2003). Des Weiteren wird TRPM8 in der Prostata, der Blase sowie dem männlichen Genitaltrakt (Stein et al., 2004) und in trunkierter Form in der Lunge exprimiert (Sabnis et al., 2008).

TRPM8 fungiert als Kältesensor (Bautista et al., 2007; Colburn et al., 2007) und spielt eine wesentliche Rolle in der Wärmeempfindung sowie der Temperaturregulation (Paricio-Montesinos et al., 2020). Diese vermittelt TRPM8 auch über seine Funktion als Mentholrezeptor (McKemy et al., 2002).

Eine Vielzahl von Krebserkrankungen steht in Verbindung mit TRPM8-Kanälen, darunter Lungenkrebs (Du et al., 2014), Darmkrebs (Tsavaler et al., 2001), Brustkrebs (J. Liu et al., 2014) und Hautkrebs (Lan et al., 2019). Dabei übt TRPM8 einen Einfluss auf verschiedene zelluläre Prozesse aus, darunter Zellebensfähigkeit, Proliferation, Migration und Apoptose (Ochoa et al., 2023). Eine Hyperaktivität kann zudem zu chronischen neuropathischen Schmerzen führen (Proudfoot et al., 2006). Die TRPM8-Mutante *rs10166942* ist mit chronischer Migräne assoziiert (Ling et al., 2019). Darüber hinaus stehen Krankheiten wie das Syndrom des trockenen Auges (J. M. Yang et al., 2018), das Reizdarmsyndrom (Henström et al., 2017) oder Bluthochdruck (Huang et al., 2017) mit TRPM8 in Verbindung.

Obgleich TRPM8 bei einer Vielzahl von Erkrankungen ein vielversprechendes Wirkstoffziel darstellt, konnten in klinischen Studien, die auf TRPM8 abzielen, bislang keine nennenswerten Erfolge verzeichnet werden (Izquierdo et al., 2021).

Der Aktivator Menthol wurde in einer Phase-2-Studie erfolgreich als topisches Schmerzmittel (NCT04351594) (Cortellini et al., 2017; Topp et al., 2013) getestet. Eine weitere Studie als Blutdrucksenker (NCT01408446) wurde noch nicht ausgewertet (J. Sun et al., 2014). Aufgrund starker Nebenwirkungen wurde eine Studie (NCT0139365) mit dem Inhibitor PF-05105679 als Schmerzmittel nicht weiter verfolgt (Winchester et al., 2014). Der neuartige TRPM8-Agonist IVW-1001 wird derzeit in einer gemischten Phase 1/2 Studie (NCT06400459) als Therapie gegen das Syndrom des trockenen Auges getestet.

Zusammengefasst ist TRPM8 ein Mitglied der Familie der Melastatin-TRP-Kanäle und fungiert als Kältesensor sowie als Mentholrezeptor. TRPM8 spielt eine Rolle in der Temperaturregulation und ist unter anderem an Erkrankungen wie Krebs, neuropathischen Schmerzen und Migräne beteiligt. In Zukunft wird sich zeigen, ob TRPM8-Modulatoren als wirksame Pharmaka eingesetzt werden können.

2.3.1 Aktivierungsmechanismen des TRPM8-Kationenkanals

Die physiologische Aktivierung von TRPM8 erfolgt bei Temperaturen unter 26 °C. Neben der Temperatur können auch niedermolekulare Kanalmodulatoren wie der Naturstoff Menthol den TRPM8-Kanal aktivieren (Bautista et al., 2007; McKemy et al., 2002).

Im Rahmen der Untersuchungen zur Aktivierung von TRPM8 mittels kleiner Moleküle konnten zwei Bindetaschen charakterisiert werden. Die erste Bindetasche ist in der VSLD lokalisiert, wo die als "Cooling Agents" bekannten Aktivatoren wie Menthol (Xu et al., 2020), WS-12, Icilin (Yin et al., 2019) oder C3 (Yin et al., 2022) binden. Eine weitere Bindetasche befindet sich zwischen den Transmembransegmenten TM3, TM4, TM5 und

TM6. In dieser binden die Aktivatoren AITC und Rapamycin (Tóth et al., 2024), sowie die Inhibitoren AMTB (Diver et al., 2019) und TC-I 2014 (Yin et al., 2024). Kryo-EM-Analysen konnten zeigen, dass diese verschiedenen Moleküle individuelle Offen- und Geschlossen-Zustände in TRPM8 erzeugen (Yin et al., 2024; Yin et al., 2022).

Ein weiterer wesentlicher Faktor für die Funktion von TRPM8 ist PIP₂. PIP₂ stabilisiert den offenen Zustand des Kanals, was zu einer verstärkten Aktivität führt (Yin et al., 2022). Bei einem Mangel an PIP₂, etwa durch Depletion, wird die Aktivität des Kanals signifikant reduziert, bis hin zum vollständigen Funktionsverlust (B. Liu & Qin, 2005; Zakharian et al., 2010). Die Bindestelle für PIP₂ liegt zwischen dem vierten Transmembransegment (TM4), der TRP-Domäne und der Region unmittelbar vor dem ersten Transmembransegment (Yin et al., 2019).

Zusammengefasst erfolgt die Aktivierung von TRPM8-Kanälen bei Temperaturen unter 26 °C sowie durch Modulatoren wie „Cooling Agents“ und andere niedermolekulare Substanzen und sie erfordert PIP₂ zur Stabilisierung des offenen Zustands.

2.4 Photopharmakologie

Die Photopharmakologie stellt ein vielversprechendes Forschungsfeld dar, welches die Disziplinen Photochemie und Pharmakologie vereint, um Pharmazeutika mit Hilfe von Licht zu regulieren. Dieser Ansatz erlaubt eine präzise räumliche und zeitliche Steuerung der Wirkungen von Arzneimitteln und eröffnet neue Perspektiven für therapeutische Anwendungen sowie für die Grundlagenforschung. Der Kern dieser Technologie liegt im Einbau lichtempfindlicher molekularer Schalter in Arzneistoffe, wodurch deren Struktur und damit ihre pharmakologische Aktivität durch spezifische Lichtwellenlängen gezielt modifiziert werden (Velema et al., 2014; Welleman et al., 2020).

In der Photopharmakologie finden verschiedene molekulare Schalter Anwendung, wobei E-Z-Fotoschalter eine zentrale Rolle einnehmen. Diese Schalter nutzen die Isomerisierung einer Doppelbindung zwischen der *trans*- (E) und der *cis*- (Z) Konfiguration, die durch Licht unterschiedlicher Wellenlängen ausgelöst wird. Zu den gängigen E-Z-Fotoschaltern gehören Azobenzole, Azopyrazole, Stilbene und Hemithioindigos (Kobauri et al., 2023).

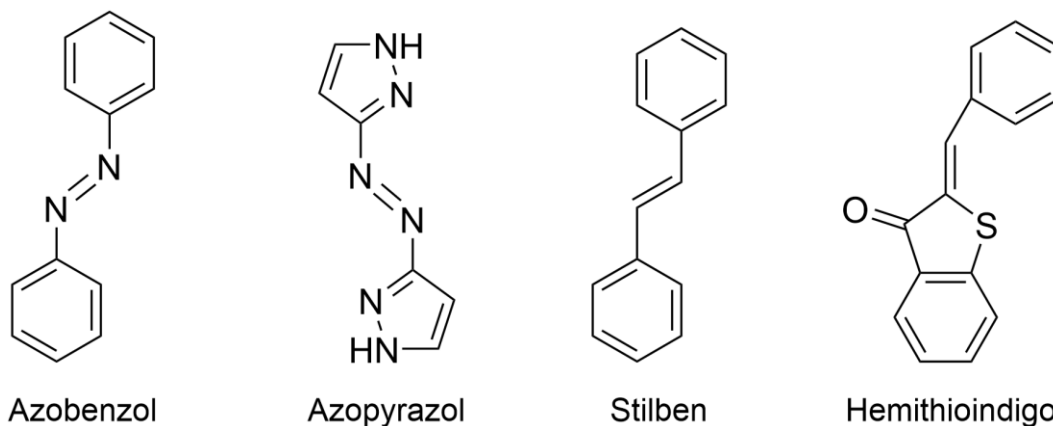


Abbildung 3: Molekülstrukturen der gängigsten auf E-Z-Isomerisierung basierenden Photopharmaka

Azobenzole, die eine schaltbare N=N-Doppelbindung enthalten, wechseln bei ultravioletter Lichteinstrahlung (~320-380 nm) aus der *trans*-Form in die *cis*-Form und unter Blaulicht (~450 nm) aus der *cis*-Form in die *trans*-Form (Bandara & Burdette, 2012). Azopyrazole verfügen ebenfalls über eine umschaltbare N=N-Bindung (Calbo et al., 2017). Stilbene (Villarón & Wezenberg, 2020) und Hemithioindigos (Wiedbrauk & Dube, 2015) nutzen demgegenüber eine schaltbare C=C-Doppelbindung und werden durch sichtbares Licht geschaltet.

Photopharmaka sind eine neuartige Klasse von Hochpräzisionspharmaka, die eine gezielte, zeitlich und örtlich präzise Arzneimittelapplikation ermöglichen. Dadurch könnten systemische Nebenwirkungen reduziert werden.

2.5 Elektrophysiologie

Die Elektrophysiologie stellt ein fundamentales Forschungsgebiet in der Biologie und Medizin dar. Zu den herausragendsten methodischen Innovationen in diesem Bereich zählt die Patch-Clamp-Technik, welche die Untersuchung des Verhaltens von Ionenkanälen ermöglicht. Ihre Entwicklung erfolgte in den späten 1970er- und frühen 1980er-Jahren durch Erwin Neher und Bert Sakmann und wurde 1991 mit dem Nobelpreis für Medizin oder Physiologie honoriert (Hamill et al., 1981; Neher & Sakmann, 1976).

Die Patch-Clamp-Technik ermöglicht die Analyse des Ionenkanalverhaltens sowohl unter normalen physiologischen Bedingungen als auch in pathologischen Zuständen oder unter dem Einfluss von Pharmaka. Ein weiteres wichtiges Anwendungsfeld stellt die Untersuchung von Aktionspotenzialen in Neuronen und anderen erregbaren Zellen sowie die synaptische Übertragung dar (Bhumbra & Beato, 2018; Heeringa et al., 2009; Koldirov, 2023; Perkins, 2006).

Die Patch-Clamp-Technik bedient sich einer Glas-Mikropipette mit einer besonders geringen Öffnung, um einen engen Kontakt, der als „Gigaseal“ bezeichnet wird, mit einem winzigen Bereich der Zellmembran, dem sogenannten „Patch“, herzustellen. Es existieren diverse Konfigurationen, die spezifische experimentelle Anwendungen ermöglichen. In der sogenannten *Cell-Attached*-Konfiguration bleibt die Pipette fest an die intakte Zellmembran geheftet, wodurch die Aktivität einzelner Ionenkanäle im Membranfleck unter der Glas-Mikropipette unter nahezu physiologischen Bedingungen untersucht werden kann, ohne die Integrität der Zelle zu beeinträchtigen. In der *Whole-Cell*-Konfiguration (auch Ganzzell-Konfiguration) erfolgt eine gezielte Perforation der Membran unter der Pipettenspitze, wodurch ein direkter Zugang zum Zytoplasma erzeugt wird. Diese Methode eignet sich insbesondere für die Messung des gesamten ionischen Stroms durch die Membran sowie für die Analyse intrazellulärer Signale.

Eine weitere Variante ist die *Inside-Out*-Konfiguration, bei der ein Membranfragment abgelöst wird, sodass die intrazelluläre Seite der Membran nach außen zeigt. Dies erlaubt eine gezielte Analyse der Regulation von Ionenkanälen durch intrazelluläre Faktoren. Im Gegensatz dazu wird in der *Outside-Out*-Konfiguration die extrazelluläre Seite der Membran nach außen exponiert, was insbesondere für Studien zur Wirkung extrazellulärer Liganden auf Ionenkanäle von Bedeutung ist.

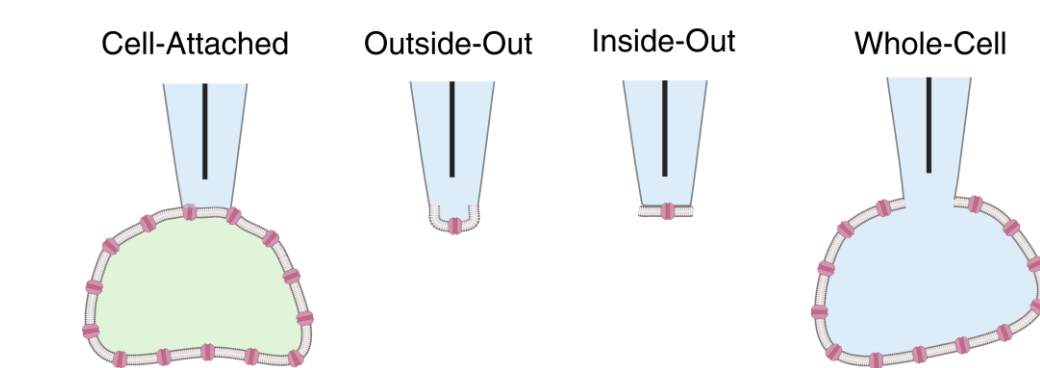


Abbildung 4: Schematische Darstellung der vier verschiedenen Patch-Clamp-Konfigurationen

Die Patch-Clamp-Technik ermöglicht Messungen in zwei wesentlichen Modi: Im *Voltage-Clamp*-Modus wird die Membranspannung konstant gehalten, während die resultierenden Ströme gemessen werden. Dieser Ansatz ist von entscheidender Bedeutung für die Untersuchung des elektrophysiologischen Verhaltens von Ionenkanälen und ihrer Leitfähigkeit. Im *Current-Clamp*-Modus hingegen wird der Strom konstant gehalten, während Änderungen der Membranspannung aufgezeichnet werden. Dieser Modus

eignet sich in besonderem Maße für die Analyse von Aktionspotenzialen bei erregbaren Zellen.

Die Charakterisierung von nicht oder schwach spannungsabhängigen Ionenkanälen, zu denen TRPC-Kanäle und TRPM8 gehören, erfolgt in der Regel in der *Whole-Cell*-Konfiguration im *Voltage-Clamp*-Modus und unter der Anwendung kontinuierlicher Spannungsrampen oder eines festen Haltepotentials. Dies erlaubt die Bestimmung ihrer Strom-Spannungs (I-V) -Beziehungen und die Erfassung der maximalen bzw. minimalen Stromamplituden bei positiven und negativen Potentialen sowie deren Verhältnis. Zudem können auf diese Weise langsame und zeitlich niedrig aufgelöste Aktivierungs- und Inaktivierungskinetiken ermittelt werden. Allerdings ist hierbei zu berücksichtigen, dass diese Kinetiken in hohem Maße von der verwendeten Applikationsmethode, wie beispielsweise dem bei einer Badapplikation eingesetzten Perfusionssystem, beeinflusst werden. (Bertamino et al., 2018; Friedrich et al., 2020; Kirill Kiselyov et al., 2011; Olander et al., 2020; Storch et al., 2012).

Die erfassten Stromwerte spiegeln die Aktivität der Ionenkanäle wider und erlauben die Untersuchung von Kanalzuständen sowie die Bewertung pharmakologischer Wirkstoffe oder genetischer Mutationen. I-V-Kurven erlauben nicht nur die Identifikation von Ionenkanälen basierend auf ihrem spannungsabhängigen Verhalten, sondern auch die Bestimmung des Umkehrpotentials und spannungsabhängiger Eigenschaften, welche sich anhand von Rektifikationen beobachten lassen und welche charakteristisch für einzelne Ionenkanäle sind.

Die Analyse und Interpretation von Ergebnissen elektrophysiologischer Experimente ist mit verschiedenen Herausforderungen verbunden, die es zu berücksichtigen gilt. Ein typisches Phänomen, das im Rahmen elektrophysiologischer Experimente beobachtet werden kann, sind zeitabhängige Veränderungen, die durch die Diffusion eines Wirkstoffs auftreten. Nach Applikation über die Badlösung benötigt der Wirkstoff Zeit, um die Ionenkanäle zu erreichen und ein Gleichgewicht zu etablieren. Diese Verzögerung kann die Kanalaktivität beeinflussen. Zusätzlich führen anfängliche Konzentrationsgradienten über die Zellmembran hinweg zu transienten Effekten, welche die Interpretation unmittelbarer Stromantworten erschweren können (Gherbi et al., 2018).

Ein weiteres Problem stellen residuale Effekte dar, welche auch nach dem Auswaschen des Wirkstoffs aus der Badlösung persistieren können. Langsame Dissoziationsraten oder verbleibende intrazelluläre Konzentrationen können nachfolgende Messungen beeinflussen und zu Fehlinterpretationen führen (Földi et al., 2021; Lukacs et al., 2021; Pesti et al., 2021).

Die Variabilität in der Expression von Ionenkanälen stellt eine weitere Herausforderung dar. Diese variiert in unterschiedlichen Zelltypen in Bezug auf ihre Dichte, was zu einer Beeinflussung der gemessenen Ströme und zu Unterschieden in den Stromamplituden der Whole-Cell-Messungen führt (Beaulieu-Laroche et al., 2021; Suen et al., 2021). In transienten Überexpressionssystemen ist zudem die Transfektionseffizienz zwischen einzelnen Zellen unterschiedlich, was zu einer ungleichen Menge an Kanalproteinen in der Zellmembran führt (Elowitz et al., 2002). Diese Heterogenität erschwert die Vergleichbarkeit zwischen den Experimenten.

Eine Möglichkeit die Heterogenität zu umgehen ist die Einzelkanal Patch-Clamp-Technik. Sie erlaubt die hochauflösende Analyse ionischer Ströme durch einzelne Ionenkanäle in Zellmembranen und ermöglicht detaillierte Aussagen über die Leitfähigkeit und die Kinetik von Kanälen. Zu den Stärken dieser Methode zählt ihre außergewöhnliche Präzision, die eine differenzierte Analyse des Kanalverhaltens ermöglicht. Allerdings sind die technischen Anforderungen hoch. Einschränkungen wie die geringe Durchsatzrate, die Empfindlichkeit gegenüber externen Störfaktoren und die Herausforderungen bei der Bildung stabiler Gigaseals limitieren die Breite der Einsatzmöglichkeiten (D. Ogden & P. Stanfield, 1999).

Zusammengefasst erlaubt die Patch-Clamp-Technik die biophysikalische Analyse von Ionenkanälen in diversen Zellkonfigurationen und -modi. Ihr Einsatzgebiet umfasst die Messung von Strom- und Spannungsverhältnissen sowie die Untersuchung pharmakologischer Effekte. Herausforderungen stellen insbesondere zeitabhängige Veränderungen und die Variabilität in der Kanalexpression dar.

2.6 Ziel der Dissertation

Die vorliegende Dissertation verfolgt das Ziel, neue Methoden zur biophysikalischen Charakterisierung von Ionenkanälen zu entwickeln und zu validieren. Zu diesem Zweck wurden Mess- und Analysemethoden für Patch-Clamp-Messungen in der Ganzzell-Konfiguration konzipiert, die eine präzise und zuverlässige Analyse von verschiedenen Kanalzuständen, von Aktivierungsmechanismen sowie von Kanalkinetiken ermöglicht.

Die entwickelten Methoden sowie deren Anwendung am Beispiel von ausgewählten TRP-Kanälen wurden in drei internationalen Fachzeitschriften mit Peer-Review-Verfahren publiziert.

3. Zusammenfassung:

Transient receptor potential (TRP)-Kanäle sind unselektive Kationenkanäle, die in verschiedensten Geweben exprimiert werden und an zentralen physiologischen Prozessen wie der sensorischen Signaltransduktion, dem kardiovaskulären System, der Nierenfunktion und der Regulation zellulärer Prozesse beteiligt sind. Zudem spielen TRP-Kanäle eine Rolle in pathophysiologischen Zuständen wie Neurodegeneration, Krebs und kardiovaskulären Dysfunktionen, wodurch sie sich als potenzielle neue Zielstrukturen für Pharmaka herauskristallisiert haben.

Die elektrophysiologische Analyse mittels der Patch-Clamp-Technik gilt als Goldstandard zur Untersuchung von Ionenkanälen, einschließlich der TRP-Kanäle. Diese Methode ermöglicht die präzise Messung von Ionenströmen auf zellulärer und subzellulärer Ebene und erlaubt somit eine detaillierte Charakterisierung biophysikalischer Eigenschaften. Die Patch-Clamp-Technik bietet nicht nur Einblicke in die Funktion einzelner Ionenkanäle, sondern ermöglicht auch die Untersuchung des Kanalverhaltens unter dem Einfluss pharmakologischer Wirkstoffe oder genetischer Modifikationen. Patch-Clamp-Messungen in der Ganzzell-Konfiguration stellen eine exzellente Methode dar, um verschiedene Ionenkanalfamilien zu differenzieren und die Wirkung von Pharmaka zu evaluieren. Die Analyse unterschiedlicher Aktivierungszustände ist in der Regel nur mit aufwendigen Einzelkanalmessungen oder modernsten Strukturuntersuchungen zuverlässig möglich.

Die vorliegende Arbeit präsentiert innovative Techniken und neue Methoden zur Analyse von Ganzzellströmen und ermöglicht eine detailliertere biophysikalische Charakterisierung der Kanalzustände. Die Bestimmung der sogenannten *normalized slope conductance* (NSC) erlaubt einen qualitativen und quantitativen Vergleich der Strom-Spannungsverläufe aus Ganzzellableitungen, unabhängig von den jeweils vorliegenden Stromamplituden. Es zeigte sich, dass die NSC an nur zwei Potenzialbereichen (40 mV und 55 mV) sowie das Verhältnis von Einstrom bei -100 mV zu Ausstrom bei +100 mV eine zuverlässige Unterscheidung und Differenzierung der Strom-Spannungskurven verschiedener TRPC-Kanäle ermöglicht. Im NSC-Kurvenverlauf konnten keine artspezifischen Unterschiede zwischen humanen und murinen TRPC6-Kanälen festgestellt werden. Hingegen zeigte sich, dass verschiedene Aktivatoren jeweils spezifische NSCs bei TRPC4-, TRPC5-, TRPC6- und TRPC7-Kanälen hervorrufen, was auf substanzspezifische individuelle Kanalzustände hinweist. Auch bei zwei TRPC6-Patientenmutationen, die mit der fokalen segmentalen Glomerulosklerose (FSGS) assoziiert sind, wurden unterschiedliche NSC-Verläufe beobachtet. Die Bestimmung der NSC erweitert somit das Methodenspektrum zur Analyse von Ganzzell-Patch-Clamp-Messungen, liefert

neue Einblicke in die Kanalfunktion und identifiziert Potenzialbereiche, an denen zusätzliche Einzelkanalmessungen sinnvoll sein könnten. Damit besitzt sie das Potenzial, eine unverzichtbare Analysemethode für Strom-Spannungs-Kurven zu werden.

Darüber hinaus wurden die lichtschtbaren TRPC6-Aktivatoren OptoDAR_G, OptoBI und PhoDAG eingesetzt und mittels der Patch-Clamp-Technik biophysikalisch charakterisiert. Die optische Kontrolle der Photopharmaka erfolgte über ein neu entwickeltes, LED-basiertes Beleuchtungssystem. Dieses ermöglicht den Wechsel der Belichtung zwischen einer Blaulicht-LED (etabliert die inaktive *trans*-Form) und einer UV-LED (etabliert die aktive *cis*-Form) mit einer Präzision von 5 μ s. Im Rahmen dieser Arbeit wurden die Aktivierungs-, Deaktivierungs- und Inaktivierungskinetiken sowie deren Zeitkonstanten und die NSC bestimmt. Es zeigte sich, dass das Schaltverhalten der verschiedenen Aktivatoren in hohem Maße von der Lichtintensität der verwendeten Lichtquelle abhängt. Für aussagekräftige Messungen ist daher eine intensive, schnell schaltbare Lichtquelle erforderlich. Die unterschiedlichen lichtschtbaren Aktivatoren riefen jeweils spezifische Kanalkinetiken und individuelle NSC-Kurvenverläufe hervor, was auf substanzspezifische aktive Kanalzustände hindeutet.

Zusätzlich wurde ein neues lichtschtbares Menthol-Derivat (Azo-Menthol) synthetisiert und chemisch sowie biophysikalisch detailliert charakterisiert. Zu diesem Zweck wurden umfassende Ganzzellmessungen bei 23 °C und 37 °C unter Blaulicht (etabliert die inaktive *trans*-Form) und UV-Licht (etabliert die aktive *cis*-Form) durchgeführt. Dabei wurden die EC₅₀-Werte ($4.4 \pm 5.8 \mu$ M), die Kanalkinetiken sowie die NSCs bestimmt. Im Vergleich zu den bekannten TRPM8-Aktivatoren Menthol und Icilin zeigte sich, dass Azo-Menthol einen individuellen Offenzustand erzeugt. Eine potenzielle Azo-Menthol-Bindestelle wurde zunächst durch molekulares Docking postuliert und anschließend mittels Kinetik- und NSC-Analysen potenzieller Bindemutanten bestätigt. Die Azo-Menthol-Bindestelle befindet sich in der sogenannten *voltage-sensing-like domain* des TRPM8-Kanals. Des Weiteren wurden die PIP₂-Bindestelle und der Einfluss von PIP₂ auf die Kanalkinetiken detailliert untersucht. Dabei konnte eine PIP₂-Sensitivitätsmutante (K995A) identifiziert werden, die die PIP₂-Bindung indirekt verstärkt.

Die Analyse der NSCs sowie die präzise Bestimmung der Kanalkinetiken, die erstmals mithilfe lichtschtbarer Kanalaktivatoren ermöglicht wurden, stellen eine wertvolle Erweiterung des Methodenspektrums zur tiefgehenden Analyse von Ganzzellströmen dar und liefern neue Einblicke in die Funktion von Ionenkanälen. Zusammenfassend vertiefen die in dieser Arbeit entwickelten Methoden nicht nur unser Verständnis des Aktivierungsmechanismus von TRP-Kanälen, sondern eröffnen auch vielversprechende Ansätze für die Entwicklung innovativer und individualisierter Therapieansätze.

4. Abstract:

Transient receptor potential (TRP) channels are non-selective cation channels expressed in various tissues and involved in key physiological processes such as sensory signal transduction, the cardiovascular system, kidney function, and the regulation of cellular processes. Additionally, TRP channels play a role in pathophysiological conditions such as neurodegeneration, cancer, and cardiovascular dysfunctions, making them emerging targets for pharmaceutical interventions.

Electrophysiological analysis using the patch-clamp technique is considered the gold standard for studying ion channels, including TRP channels. This method enables precise measurement of ion currents at both the cellular and subcellular levels, enabling detailed characterization of their biophysical properties. The patch-clamp technique not only provides insights into the function of individual ion channels but also facilitates the investigation of channel behavior under the influence of pharmacological agents or genetic modifications. Whole-cell patch-clamp recordings are an excellent method for distinguishing between different ion channel families and evaluating drug effects. However, a reliable analysis of distinct activation states typically requires complex single-channel recordings or advanced structural studies.

This study presents innovative techniques and novel methods for analyzing whole-cell currents, allowing for a more detailed biophysical characterization of channel states. The determination of normalized slope conductance (NSC) enables both qualitative and quantitative comparisons of current-voltage relationships of whole-cell recordings, independent of the absolute current amplitudes. It was found that NSC curve progressions at only two potential ranges (40 mV and 55 mV), as well as the ratio of inward current at -100 mV to outward current at +100 mV, allow for a reliable distinction between different TRPC channel current-voltage curves. No species-specific differences were observed between human and mouse TRPC6 channels in NSC curves. However, different activators induced specific NSCs in TRPC4, TRPC5, TRPC6, and TRPC7 channels, indicating substance-specific individual channel states. Furthermore, two TRPC6 patient mutations associated with focal segmental glomerulosclerosis (FSGS) exhibited distinct NSC profiles. Thus, NSC determination expands the methodological spectrum for whole-cell patch-clamp analysis, provides new insights into channel function, and identifies potential areas where additional single-channel recordings might be valuable. As a result, NSC analysis has the potential to become an essential tool for evaluating current-voltage relationships.

Furthermore, photo-switchable TRPC6 activators (OptoDAR_G, OptoBI, and PhoDAG) were employed and biophysically characterized using the patch-clamp technique. The optical control of these photopharmacological agents was achieved via a newly developed LED-based illumination system, allowing for precise switching between a blue LED (establishing the inactive *trans*-form) and a UV LED (establishing the active *cis*-form) with a precision of 5 μ s. In this study the activation, deactivation, and inactivation kinetics were determined, along with their time constants and NSC curves. The switching behavior of different activators was found to be highly dependent on the light intensity of the applied light source, therefore, reliable measurements require an intense and fast-switching light source. The different photo-switchable activators induced varying channel kinetics and distinct NSC curve profiles, indicating substance-specific active channel states.

Additionally, a new photo-switchable menthol derivative (Azo-Menthol) was synthesized and characterized both chemically and biophysically. Comprehensive whole-cell measurements were conducted at 23°C and 37°C under blue light (establishes the inactive *trans*-form) and UV light (establishes the active *cis*-form). The EC₅₀ value (4.4 ± 5.8 μ M), channel kinetics, and NSCs were also determined. Compared to the known TRPM8 activators menthol and icilin, Azo-Menthol induced a unique open state. A potential Azo-Menthol binding site was first proposed through molecular docking and subsequently confirmed via kinetic and NSC analysis of potential binding mutants. The Azo-Menthol binding site is located in the voltage-sensing-like domain of the TRPM8 channel. Additionally, the PIP₂ binding site and its influence on channel kinetics were analyzed in detail, resulting in the identification of a PIP₂ sensitivity mutant (K995A), which indirectly enhances PIP₂ binding.

The analysis of NSCs, as well as the precise determination of channel kinetics which was enabled for the first time using photo-switchable channel activators, represents a valuable extension of the methodological spectrum for in-depth analysis of whole-cell currents and provides new insights into the ion channel function. In conclusion, the methods developed in this study not only enhance our understanding of the activation mechanisms of TRP channels, but also open promising avenues for the development of innovative and personalized therapeutic strategies

5. Publikation I

The normalized slope conductance as a tool for quantitative analysis of current-voltage relations

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The normalized slope conductance as a tool for quantitative analysis of current-voltage relations

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ABSTRACT The patch-clamp method, which was awarded the Nobel Prize in 1991, is a well-established and indispensable method to study ion channels in living cells and to biophysically characterize non-voltage-gated ion channels, which comprise about 70% of all ion channels in the human genome. To investigate the biophysical properties of non-voltage-gated ion channels, whole-cell measurements with application of continuous voltage ramps are routinely conducted to obtain current-voltage (IV) relationships. However, adequate tools for detailed and quantitative analysis of IV curves are still missing. We use the example of the transient receptor potential classical (TRPC) channel family to elucidate whether the normalized slope conductance (NSC) is an appropriate tool for reliable discrimination of the IV curves of diverse TRPC channels that differ in their individual curve progression. We provide a robust calculation method for the NSC, and, by applying this method, we find that TRPC channel activators and modulators can evoke different NSC progressions independent from their expression levels, which points to distinguishable active channel states. TRPC6 mutations in patients with focal segmental glomerulosclerosis resulted in distinct NSC progressions, suggesting that the NSC is suitable for investigating structure-function relations and might help unravel the unknown pathomechanisms leading to focal segmental glomerulosclerosis. The NSC is an effective algorithm for extended biophysical characterization of non-voltage-gated ion channels.

SIGNIFICANCE The patch-clamp method is widely used to biophysically characterize non-voltage-gated ion channels. However, a method for a reliable quantitative analysis of the current-voltage (IV) relationships is still missing. In this study, we show that the normalized slope conductance (NSC) is appropriate to quantify IV curves of transient receptor potential classical (TRPC) channels. TRPC channel activators and modulators evoke different NSC progressions, pointing to distinguishable active channel states. Using the NSC, TRPC6 mutations of patients with focal segmental glomerulosclerosis are discriminated which might help to elucidate structure-function relations and unknown pathomechanisms. The NSC has potential to become a mandatory analysis method for IV curves that expands the toolbox for in-depth description and quantitative characterization of IV curves.

INTRODUCTION

To study ion channels in living cells, electrophysiological whole-cell recordings in voltage-clamp mode are commonly applied. The technique was initially developed by Erwin Neher and Bert Sakmann, who received the Nobel Prize for

their work (1,2) in 1991. This method greatly improved our understanding of the role of ion channels in fundamental cell processes. To biophysically characterize non-voltage-gated ion or weakly voltage-dependent ion channels, which comprise 70% of all ion channels (3), continuous voltage ramps are often applied that allow determination of their current-voltage (IV) relationships. However, so far, a method for a reliable mathematical description and quantitative comparison of IV curves is missing. At present, one tentative surrogate to characterize the changes of IV curves is determination of current ratios between outward and inward currents at maximal and minimal potentials (4). However, this ratio analysis can only reveal relative changes of inward to outward currents at

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defined potentials and is unsuitable to instantly analyze the IV curve as a whole. In this study, we aim to investigate whether the normalized slope conductance (NSC) might be an appropriate tool to precisely describe and quantify the progression of the IV curve of macroscopic currents that are a sum of over-expressed single-channel currents with their respective open probabilities. The slope conductance is a useful measure to describe distinct shapes of IV curves (5), particularly when the slope of an IV curve is not ohmic but curvilinear.

As an example, we focused on transient receptor potential classical (TRPC) channels, a family of non-selective, calcium-permeable cation channels comprising seven members. TRPC channels are regarded as receptor-operated channels and are activated by $G_{q/11}$ protein-coupled receptors via phospholipase C activation, resulting in cleavage of phosphatidylinositol-4,5-bisphosphate into the second messengers inositol-1,4,5-trisphosphate and diacylglycerol (DAG). Apart from TRPC1, DAG can activate all TRPC channel family members (6–9). TRPC proteins are involved in many physiological and pathophysiological processes (summarized in (10)) and have recently emerged as novel drug targets. Therefore, detailed biophysical characterization is indispensable to validate the role of these channels as drug targets and to optimize the design of novel potent channel modulators. Notably, the IV curves of different TRPC channels closely resemble one another, and some IV curves are hardly distinguishable with the naked eye. The IV curves of homotetrameric TRPC4 and TRPC5 channels are both curvilinear and show a so-called double rectification (6,11). This double rectification is also observed for TRPC6 (12–14), TRPC3 (4,13,15,16), and TRPC7 (4,17) channels, whereas TRPC2 channels are characterized by an almost linear IV relationship (9). It is still a subject of discussion whether TRPC1 protein subunits are able to form functional homotetrameric channels (4,18). However, it is widely accepted that TRPC1 protein subunits can assemble into heterotetrameric TRPC channel complexes (19,20), decreasing the calcium permeability of other TRPC channel subunits (4) and changing the IV curve progression to display increased outward and decreased inward rectification (4,19). We developed a robust algorithm to calculate the NSC and applied this method to characterize and quantify differences between diverse TRPC IV curves.

MATERIAL AND METHODS

Cell culture and transfection

Human embryonic kidney (HEK293T) cells (from the Leibniz-Institute DSMZ, Braunschweig, Germany; T293, DSMZ no. ACC 635) were maintained in Earl's Minimum Essential Medium (Sigma-Aldrich, St. Louis, MO) with 100 units/mL penicillin and 100 µg/mL streptomycin supplemented with 10% (v/v) fetal calf serum (Gibco, Life Technologies, Carlsbad, CA) and 2 mM glutamine. All cells were held at 37°C in a humidified atmosphere with 5% CO₂. HEK293T cells were transfected with 2 µg complementary DNA (cDNA) coding for human TRPC3 (GenBank: NM_001130698), human TRPC6 (GenBank: NM_004621), mouse TRPC5 (GenBank: NM_009428), mouse TRPC6 (GenBank: NM_013838), mouse TRPC7 (GenBank: NM_012035), rat TRPC4α1 (GenBank: NM_080396), or rat TRPC4β1 (GenBank: NM_001083115) using Genejuice reagent (Sigma-Aldrich) according to the manufacturer's instructions. Except for mouse TRPC7, the cDNA was in pIRES2-enhanced green fluorescent protein expression vector (Clontech, Palo Alto, CA). Mouse TRPC7 cDNA was in the pIRES2-Aequorea coerulescens enhanced green fluorescent protein vector. For receptor activation, 0.5 µg cDNA coding for the rat type 5 muscarinic acetylcholine receptor (GenBank: NM_017362) in pcDNA3 was co-transfected. For receptor activation of rat TRPC4β1, the rat type 5 muscarinic acetylcholine receptor and the human adrenergic α_{2A} receptor in the pcDNA3 vector (GenBank: NM_000681) were co-transfected.

Materials

Artemisinin, carbachol, norepinephrine, and 1-oleoyl-2-acetyl-sn-glycerol (OAG) were purchased from Sigma-Aldrich. GSK 1702934A, TRPC6-PAM-C20 and riluzole hydrochloride were purchased from Tocris (Bristol, UK), and (–)-englerin A was purchased from Carl Roth (Karlsruhe, Germany).

Patch-clamp recordings

Conventional whole-cell patch-clamp recordings were carried out at room temperature (23°C) 24–48 h after transfection. Cells were superfused with bath solution containing 140 mM NaCl, 5 mM CsCl, 1 mM MgCl₂, 2 mM CaCl₂, 10 mM glucose, and 10 mM Hepes (pH 7.4 with NaOH), resulting in an osmolality of 295–302 mOsm·kg^{–1}. The standard pipette solution contained 120 mM CsCl, 9.4 mM NaCl, 0.2 mM Na₃-GTP, 1 mM MgCl₂, 3.949 mM CaCl₂, 10 mM 1,2-bis(*o*-aminophenoxy)ethane-*N,N,N',N'*-tetraacetic acid (100 nM free Ca²⁺), and 10 mM Hepes (pH 7.2 with CsOH), resulting in an osmolality of 294 mOsm·kg^{–1}. The liquid junction potential of +4.0 mV was calculated by JPCalcWin 1.01 (University of New South Wales, Sydney, NSW, Australia) and was corrected before the measurements. Data were collected with an EPC10 patch-clamp amplifier (HEKA Elektronik, Lambrecht, Germany) using the Patchmaster software. The stimulation protocol was as follows. When not otherwise stated, a holding potential of –100 mV was applied for 50 ms, followed by a voltage upramp from –100 to +100 mV for 400 ms (slope of 500 mV s^{–1} in proportion to 100 µV in 200 µs) and by a holding potential of +100 mV that was applied for 50 ms. This protocol was applied at a frequency of 2 Hz. In some cases, a stimulation protocol was applied, starting with a holding potential of –100 mV for 20 ms followed by a voltage upramp from –100 to +100 mV for 50 ms (slope of 4 V s^{–1} in proportion to 100 µV in 25 µs) and by a holding potential of +100 mV that was applied for 10 ms. This protocol was applied at a frequency of 12.5 Hz. IV relationships were obtained from the voltage upramps from –100 to +100 mV. Data were acquired at a frequency of 5 kHz (slope of 500 mV s^{–1}) or 40 kHz (slope of 4 V s^{–1}) after filtering at 2.5 kHz. Patch pipettes were made of borosilicate glass (Science Products, Hofheim am Taunus, Germany) and had resistances of 1.5–5.0 MΩ with a median of 2.2 MΩ (Fig. S1 A). Bath solutions with 100 µM OAG and 200 µM artemisinin also contained 0.1% bovine serum albumin (BSA) to prevent micelle formation and enhance solubility. To obtain maximal channel activation, the different direct and indirect effectors and channel modulators were applied at their maximal effective concentrations: 100 µM carbachol, 100 µM OAG, 200 µM artemisinin, 50 nM (–)-englerin A, 50 µM riluzole, 100 µM LaCl₃, 10 µM GSK 1702934A, and 10 µM TRPC6-PAM-C20.

Calculation of the NSC

To calculate the NSC of each individual IV curve, the activator- or modulator-induced IV curves had to be selected at maximal outward current

amplitudes. No leak current subtractions were carried out. We only used high-quality measurements without leak currents for further analysis. The parameters of the individual patch-clamp measurements are summarized in Fig. S1. The median values for the series resistance at the beginning and at the end of the measurements were 4.1 MΩ and 4.1 MΩ, respectively, and 3.3 GΩ for the seal resistance (Fig. S1 B). To calculate the NSC over the complete potential range of the IV curves from −100 to +100 mV, we first calculated the current density-voltage (CDV) relations of each IV curve. The amplitude of the current density depends on the expression level and the size of cells. One quantity of cell size is cell membrane capacitance. The median value for membrane capacitance was 9.1 pF with a minimum membrane capacitance of 3.1 pF and a maximum membrane capacitance of 21.7 pF (Fig. S1 C). The differences in cell size made it necessary to normalize the currents by calculating the current densities. To exclude this variance, we normalized the currents to 1 pF cell membrane capacitance, a membrane area of about 100 μm² that we define as a scalar quantity of the current density by dividing the current by the membrane capacitance. Thus, the different current densities reflect the different expression levels of the channels. Another advantage of CDV curves is better comparability of the signal-to-noise ratio compared with IV curves. Next, the individual CDV curves were normalized to achieve better comparability. The inward and outward CDV relations were normalized separately through a modified minimum-maximum scaling (−100% to 0%, 0% to +100%). Here the inward current density at −100 mV was set to −100% and the current density at 0 mV to 0% (Eq. 1). Likewise, the outward current density at 0 mV was set to 0% and the current density at +100 mV to +100% (Eq. 2).

Eq. 1, normalization of inward Curr. dens.))

$$\text{Inward Curr. dens.}_{\text{norm}(i)} = (-100) \times \frac{\text{Curr. dens.}_{\varphi(i)} - \text{Curr. dens.}_{(-100\text{mV})}}{\text{Curr. dens.}_{(0\text{mV})} - \text{Curr. dens.}_{(-100\text{mV})}} \quad (1)$$

Eq. 2: normalization of outward Curr. dens.

$$\text{Outward Curr. dens.}_{\text{norm}(i)} = (+100) \times \frac{\text{Curr. dens.}_{\varphi(i)} - \text{Curr. dens.}_{(0\text{mV})}}{\text{Curr. dens.}_{(+100\text{mV})} - \text{Curr. dens.}_{(0\text{mV})}} \quad (2)$$

φ is the potential, and i displays every single data point of the voltage up-ramp from 1 to 2001 with a 100-μV interval.

In some cases, in particular when the current amplitudes were low, normalization of the inward and outward CDV relations strongly increased the noise of the curve. Calculation of the NSC from these traces further increased the noise so that significant differences could not be determined. Therefore, the normalized CDV curves were generally smoothed using a cubic smoothing spline fit to reduce the noise and enhance the quality of the data. Without this procedure, it was not possible to determine significant differences between NSC curves derived from measurements with low current amplitudes. The cubic smoothing spline fit was done with the R core distribution (smooth.spline with smoothing parameter spar = 0.8). Subsequently, the slope of the smoothed normalized CDV relations was taken for all given potentials from −99.9 to +99.9 mV with a 100-μV interval (Eq. 3) to numerically approximate the NSC (Norm. G_{slope}).

Eq. 3: Calculation of the NSC

$$\text{Norm. G}_{\text{slope}(i)} = \frac{\text{Curr. dens.}_{\text{norm}(i-1)} - \text{Curr. dens.}_{\text{norm}(i+1)}}{\varphi_{(i-1)} - \varphi_{(i+1)}} \quad (3)$$

i displays every single data point of the voltage upramp from 2 to 2000.

Next, potential outliers were identified and removed using a Grubb's test ($\alpha = 0.05$) by comparison of individual NSC at every single data point. When

the Grubb's test was positive for 100 different data points (5% of all single potentials), the whole measurement was eliminated. Finally, the remaining NSC progressions were averaged and displayed as median ± standard derivation for each potential. In addition, the p values were calculated using an unpaired Mann-Whitney U or Kruskal-Wallis test and plotted over the whole potential range. The p value plot helps to identify significant differences between different NSC progressions. The step-by-step procedure for the NSC calculation is displayed in Fig. 1, E and F. Data import and manipulation, all calculations, outlier exclusion, and plots were done in R (R 3.6.0 (21)). We established a self-made automated program with a user-friendly interface. Here, the following R packages were used: checkmate (22), factoextra (23), ggalt (24), ggplot2 (25), ggthemes (26), gtools (27), latex2exp (28), matrixStats (29), openxlsx (30), outliers (31), patchwork (32), plotly (33), purrrlyr (34), stringi (35), and tidyverse (36).

Statistical analysis

Statistical analyses were conducted in R. The normalized CDV curves had to be smoothed using a cubic smoothing spline fit to remove noise. Otherwise, statistical analysis of the NSC was impossible. No statistical methods were used to predetermine sample size. The p values were calculated using an unpaired Mann-Whitney U or Kruskal-Wallis test. A p value less than 0.05 was considered significant for all analysis: *p < 0.05, **p < 0.01, ***p < 0.001.

RESULTS

The progression of the NSC allows discrimination of (−)-englerin A-induced TRPC4 and TRPC5 currents

To investigate whether the NSC is applicable for a quantitative comparison of IV curves, we performed whole-cell measurements of TRPC4- or TRPC5-overexpressing HEK293T cells. The potent TRPC4/5 channel activator (−)-englerin A was applied at its maximally effective concentration (50 nM) to induce full activation of TRPC4 and TRPC5 channels (Fig. 1, A and B). The currents are displayed as Curr. dens. Maximal (−)-englerin A-induced Curr. dens. of TRPC5 were significantly smaller compared with TRPC4 (Fig. 1 C), and the ratios between outward and inward Curr. dens. at ±100 mV were significantly increased (Fig. 1 D). To determine the NSC, the CDV relation of each individual IV curve was calculated, and the CDV relation was normalized to be able to compare the traces (Fig. 1 E). Subsequently, the normalized CDV curves were smoothed to diminish the noise, and the NSC was calculated at all given potentials. After identification and removal of outliers, the individual NSC progressions were averaged and displayed, and significant differences between the NSC progressions were determined. The detailed workflow is displayed in Fig. 1 F and described in detail under **Material and methods**. Interestingly, the NSC revealed significant differences between TRPC4 and TRPC5 currents at distinct potential ranges (around −65 to −30 mV, at a reversal potential around 0 mV, around +15 to +45 mV, and around +50 to +100 mV) (Fig. 1 E). Interestingly, the TRPC4 IVs showed a largely decreased NSC at positive potentials from +10 to +42 mV. We observed a negative

TRPC4 / TRPC5

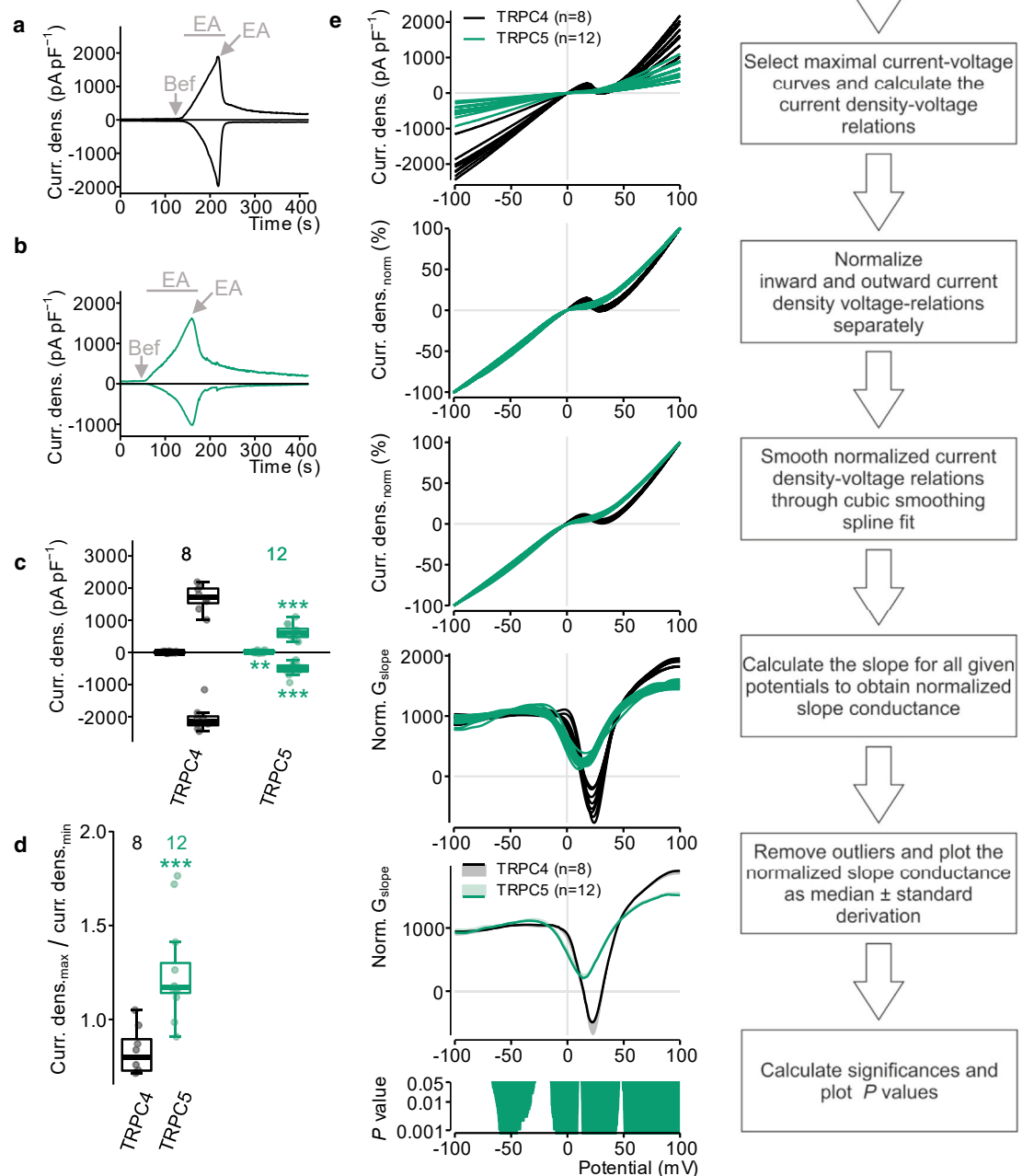


FIGURE 1 The progression of the NSC allows discrimination of (–)-englerin A-induced TRPC4 and TRPC5 currents. (A and B) Representative current density (Curr. dens.) time courses with application of 50 nM (–)-englerin A of whole-cell measurements of TRPC4-overexpressing (A) and TRPC5-overexpressing (B) HEK293T cells. The arrows indicate the time point when the basal (denoted as Bef in the figure) and maximal (–)-englerin A (EA)-induced CDV relations were selected. (C) Summaries of Curr. dens. at potentials of ±100 mV before and during application of EA. The first small boxplots represent basal Curr. dens., and the second boxplots represent maximal EA-induced Curr. dens.. Numbers over boxplots indicate the number of measured cells. **p < 0.01, ***p < 0.001; Mann-Whitney U test compared with basal and maximal EA-induced Curr. dens.. (D) Summary of ratios between outward and inward Curr. dens. at ±100 mV (Curr. dens._{max}/Curr. dens._{min}). Numbers over boxplots indicate the number of measured cells. ***p < 0.001, green asterisks; Mann-Whitney U test. (E and F) Step-by-step procedure to calculate NSC. 1) All maximal EA-induced IV relations of TRPC4 and TRPC5 were selected at maximal current amplitudes, and the CDV relations were calculated and displayed. 2) The CDV relations were normalized (Curr. Dens._{norm}

(legend continued on next page)

NSC for TRPC4 currents between +15 and +30 mV. Notably, TRPC5 currents never showed a negative slope. In addition, at potentials higher than +50 mV, the NSC of TRPC4 currents was significantly increased (Fig. 1 E). A comparison of the NSC of the basal TRPC4 and TRPC5 currents before application of (–)-englerin A (Fig. S2) and the maximal (–)-englerin A-induced currents shows significant differences. We demonstrate that the NSC is appropriate for detailed quantitative characterization of TRPC4 and TRPC5 currents that exhibit quite similar IV curves at first glance.

IV curves of TRPC currents induced by receptor-activation can be characterized and discriminated by their individual NSC progression

Next, we wanted to determine whether TRPC currents induced by receptor activation might be discriminable using the NSC. We analyzed HEK293T cells co-expressing TRPC3, TRPC4, TRPC5, TRPC6, or TRPC7 and the muscarinic M5 receptor. Maximal TRPC currents were induced by agonist stimulation with carbachol. Curr. dens. analysis of maximal currents (Fig. 2 A) showed that TRPC4 and TRPC5 Curr. dens. were significantly smaller than those of TRPC3, TRPC6, and TRPC7 (Fig. S3 A). Ratio analysis at ± 100 mV showed significant differences between individual TRPC current ratios. TRPC5 exhibited the highest ratios that were significantly different from TRPC4, TRPC6, and TRPC7 (Fig. S3 B). TRPC3 ratios did not differ from those of TRPC5 and TRPC7, and TRPC6 and TRPC7 ratios were not significantly different. After normalization and smoothing the CDV curves and subsequent calculation of the NSC, we observed significant differences between all TRPC currents (Fig. 2 C). A detailed comparison of the different NSC progressions (Fig. S3 C) reveals that TRPC4 currents have the most pronounced positive slopes at potentials of +80 mV or greater compared with all other TRPC currents, and the slopes of TRPC5 currents were most prominently increased at about –20 mV. The NSC of TRPC3 and TRPC7 were quite similar at negative potentials and only showed differences at positive potentials (at about +20 to +50 mV and at +80 mV). These findings suggest that TRPC currents are distinguishable by their individual NSC progression.

Next we examined whether the ratio and the NSC at distinct potentials are sufficient to categorize the different homotetrameric TRPC currents. We first generated a 3D scatterplot (Fig. S4) and found that the ratio and the NSC at +40 mV and at +55 mV are useful for rough categorization of TRPC currents. However, to provide precise classification

of the TRPC currents, we developed a decision tree (Fig. S5). For a first discrimination of TRPC4/5 and TRPC3/6/7 currents, the NSC at +40 mV is suitable as an NSC of less than 335 points to TRPC4/5 and one of less than 335 to TRPC3/6/7. TRPC4/5 currents can be discriminated by their NSC at +55 mV (NSC <412 points to TRPC4 and >412 to TRPC5). To distinguish between TRPC3/6/7 currents, the current ratios at ± 100 mV are useful. Ratios greater than 6.8 indicate TRPC6, and those of less than 6.8 indicate TRPC3 or TRPC7 currents. TRPC3/7 currents can be further discriminated by their NSC at +40 mV (NSC <653 is pointing to TRPC3 and >653 to TRPC7). Using this decision tree, 41 of 42 IV curves were correctly classified (98%). For comparison, exclusively using the ratio for discrimination, only 27 of 42 IV curves were correctly classified (64%), clearly demonstrating that the NSC is a superior approach. Our findings show that the NSC is a suitable tool for detailed and quantitative description of IV curves that even allows classification of TRPC currents. The NSC might also be useful to characterize currents of endogenously expressed TRPC channels.

Activator-specific differences in TRPC3 and TRPC4 currents are revealed by calculating the NSC

To investigate whether different direct and indirect channel activators might cause IV curves with distinct properties, we next analyzed maximal TRPC3 currents induced by carbachol application, by application of the membrane-permeable DAG analog OAG, and by the TRPC3 channel activator artemisinin (37). The Curr. dens. analysis showed that OAG-induced currents were smaller and artemisinin-induced currents were larger than carbachol-induced currents (Fig. 3 A). The ratios at ± 100 mV were significantly different (Fig. 3 B). After calculation of the NSC, significant differences between the IV curves became even more evident (Fig. 3 C). The NSC of the artemisinin-induced currents was significantly different from those induced by carbachol or OAG. However, the carbachol- and OAG-induced NSC was quite similar, although the current ratios and the Curr. dens. significantly differed. These findings suggest that the NSC is independent of the maximal current amplitudes or, rather, of the channel expression levels and that carbachol, which causes formation of DAG, and the DAG-derivative OAG have a similar activation mechanism. Our findings indicate that different activators induce IV curves with distinct slope progressions that might reflect distinct active TRPC3 channel states.

(%). The outward Curr. dens. were normalized to 100% and the inward Curr. dens. to –100% and then normalized. 3) The normalized CDV relations were smoothed using a cubic smoothing spline fit. 4) The slope of the normalized CDV relations of all given potentials from –100 mV to +100 mV in 100 μ V-steps was calculated separately for each normalized and smoothed CDV curve to obtain the NSC (Norm. G_{slope}). 5) Outliers were identified and removed using Grubb's test. The remaining the NSC progressions were averaged and displayed as mean $\pm$ SD. 6) The p values were calculated and plotted to identify significant differences between the NSCs. In (C) and (D), data are displayed as boxplots and interquartile ranges. To see this figure in color, go online.

TRPC3 / 4 / 5 / 6 / 7

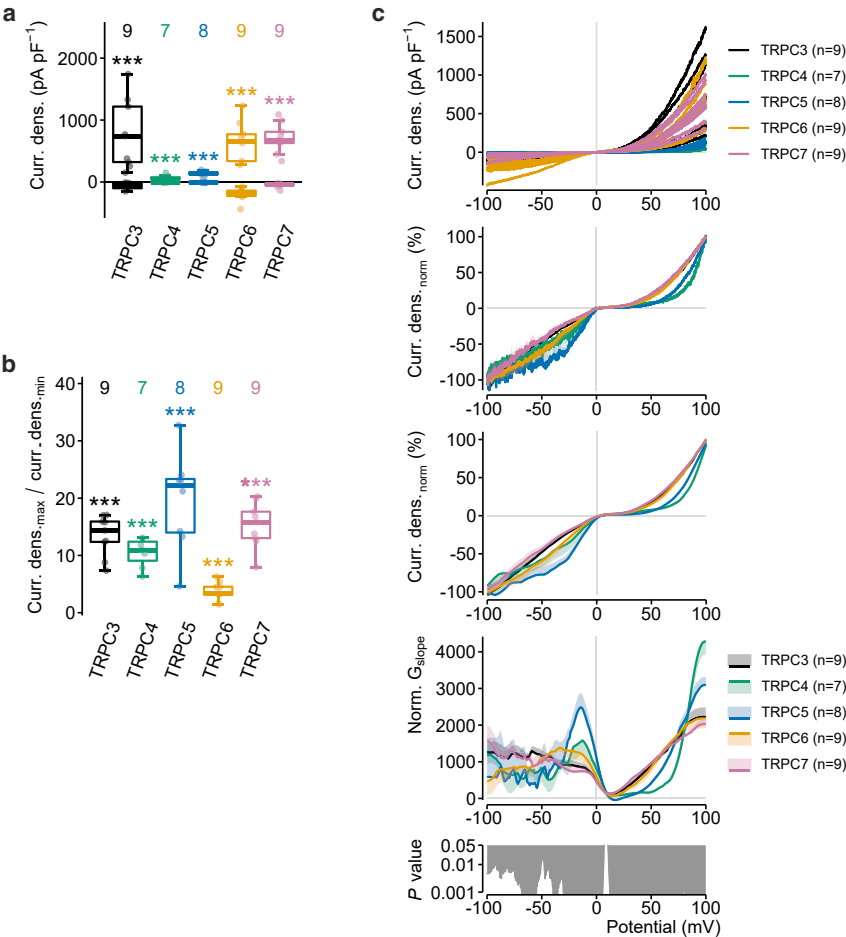


FIGURE 2 IV curves of TRPC currents induced by receptor activation can be characterized and discriminated by their individual NSC progression. Shown are whole-cell measurements of TRPC3, TRPC4, TRPC5, TRPC6, or TRPC7 and HEK293T cells overexpressing the muscarinic M₅ receptor. (A) Summary of maximal Curr. dens. at potentials of ± 100 mV induced by receptor activation with 100 μ M carbachol. Numbers over boxplots indicate the number of measured cells. *** $p < 0.001$, Kruskal-Wallis test to compare all TRPC Curr. dens.. (B) Summary of ratios between outward and inward Curr. dens. at ± 100 mV (Curr.dens_{max}/curr.dens_{min}). Numbers over boxplots indicate the number of measured cells. *** $p < 0.001$, Kruskal-Wallis test. (C) All maximal carbachol-induced CDV relations of TRPC3, TRPC4, TRPC5, TRPC6, and TRPC7 are displayed (top). The CDV relations were first normalized (Curr. Dens_{norm} (%)) and then smoothed so that the noise of the curves, in particular at negative potentials, was decreased. The calculated NSC (Norm. G_{slope}) is displayed as mean $\pm$ SD. The p values are calculated using Kruskal-Wallis test. In (A) and (B), data are displayed as boxplots and inter-quartile ranges. To see this figure in color, go online.

Next we wanted to determine whether the long and the short TRPC4 isoforms, TRPC4 α and TRPC4 β 1, might show different IV curves. We compared the maximal (–)-englerin A-induced TRPC4 α and TRPC4 β currents (Fig. S6) and found no significant differences between the maximal Curr. dens. and the ratios at ± 100 mV. The NSC displayed only marginal differences, suggesting that, in principle, the IV curves of these isoforms are similar. We also analyzed the maximal carbachol- and (–)-englerin A-induced TRPC4 β 1 currents. The (–)-englerin A-induced maximal Curr. dens. were significantly higher than carbachol-induced Curr. dens., and the ratios of the carbachol-induced currents were significantly increased (Fig. 3, D and E). Calculating the NSC, we were able to quantify the differences in the curve progressions. The NSC at negative and positive potentials (Fig. 3 E) was significantly different. For example, at +85 to +100 mV, the carbachol-induced NSC was significantly increased, and at +40 to +80 mV, it was

significantly decreased. These findings indicate that different activators induce different IV curves, possibly pointing to distinct active channel states.

Activator- and modulator-specific differences in TRPC5 currents are revealed when applying the NSC

To investigate whether receptor activation and application of La³⁺, a positive allosteric TRPC4/5 channel modulator that is commonly called a potentiator (38), can evoke distinct TRPC5 channel states as well, we analyzed maximal carbachol- and La³⁺-induced TRPC5 currents. The La³⁺-induced inward Curr. dens. were significantly increased (Fig. 4 A), and ratios were significantly decreased (Fig. 4 B). Calculation of the NSC revealed significant differences mainly at positive potentials (Fig. 4 C). Next, we compared maximal TRPC5 currents induced by the activators riluzole and (–)-englerin A. The Curr.

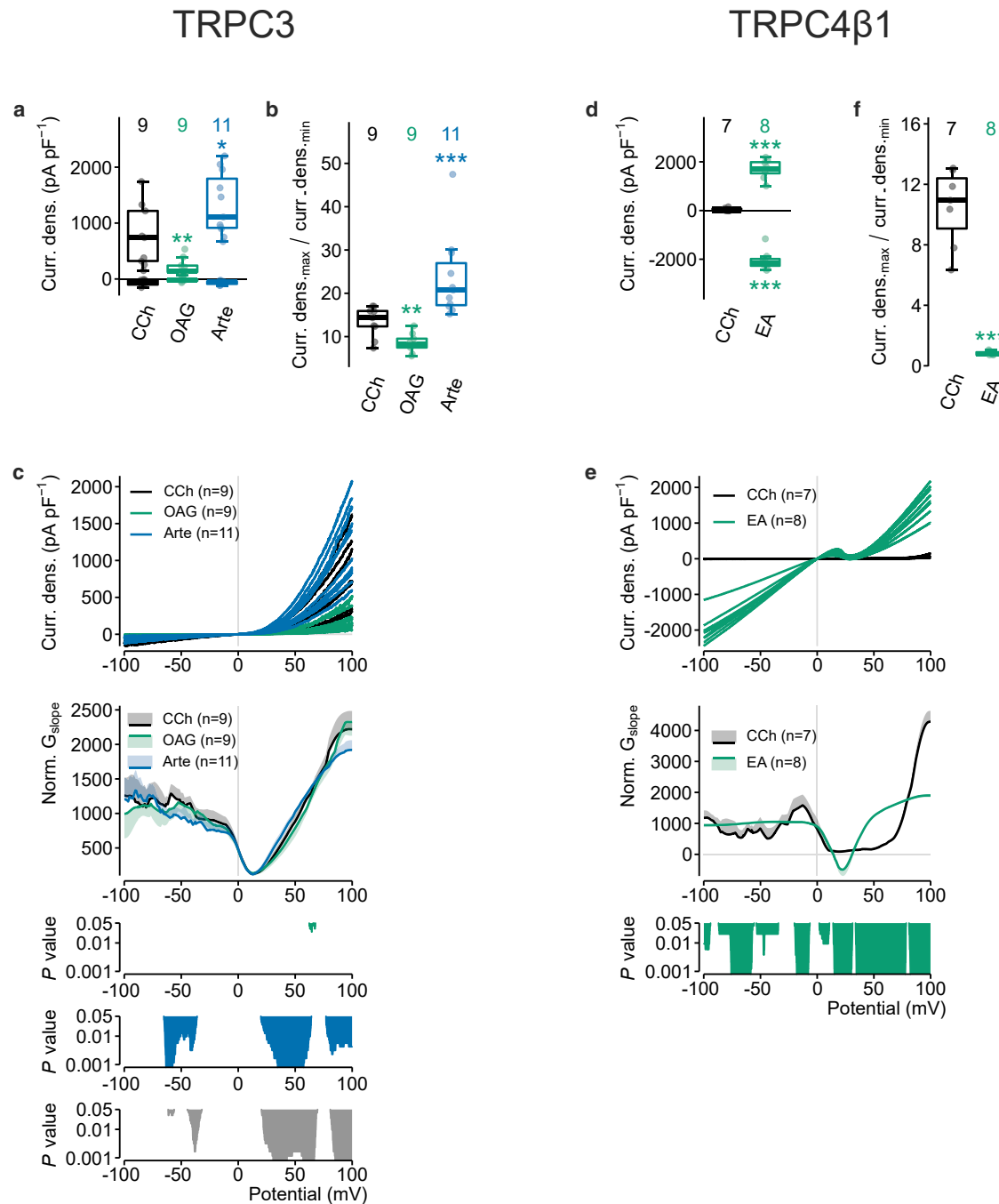


FIGURE 3 Activator-specific differences in TRPC3 and TRPC4 currents are revealed by calculating the NSC. (A–E) Whole-cell measurements of HEK293T cells overexpressing TRPC3 (A–C) and TRPC4 β 1 (D and E). (A and D) Summaries of maximal Curr. dens. at potentials of ± 100 mV induced by receptor activation with 100 μ M carbachol (CCh) (A and D) and by application of 100 μ M 1-oleoyl-2-acetyl-glycerol (OAG) (A), 200 μ M artemisinin (A), and 50 nM EA (D). Numbers over boxplots indicate the number of measured cells. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$; Mann-Whitney U test compared with CCh-induced Curr. dens.. (B and F) Summaries of ratios between outward and inward Curr. dens. at ± 100 mV (Curr. dens._{max}/curr. dens._{min}). Numbers over boxplots indicate the number of measured cells. ** $p < 0.01$, *** $p < 0.001$; Mann-Whitney U test compared with CCh-induced TRPC3 ratios. (C and E) Maximal activator-induced CDV relations of TRPC3 (C) and TRPC4 β 1 (E) are displayed (top). The NSC (Norm. G_{slope}) (center) is displayed as mean $\pm$ SD. The p values are calculated using Mann-Whitney U test compared with CCh-induced NSC (green (C and E) and blue (C) p value plots) and between OAG and artemisinin (gray p value plot (C)) (bottom). In (A) and (B), data are displayed as boxplots and interquartile ranges. To see this figure in color, go online.

dens. analysis shows that the (–)-englerin A-induced TRPC5 inward currents were increased (Fig. 4 D). The ratio analysis also revealed significant differences with

prominently decreased (–)-englerin A-induced current ratios (Fig. 4 E). Calculation of the NSC showed activator-specific differences between the currents (Fig. 4 F). At

TRPC5

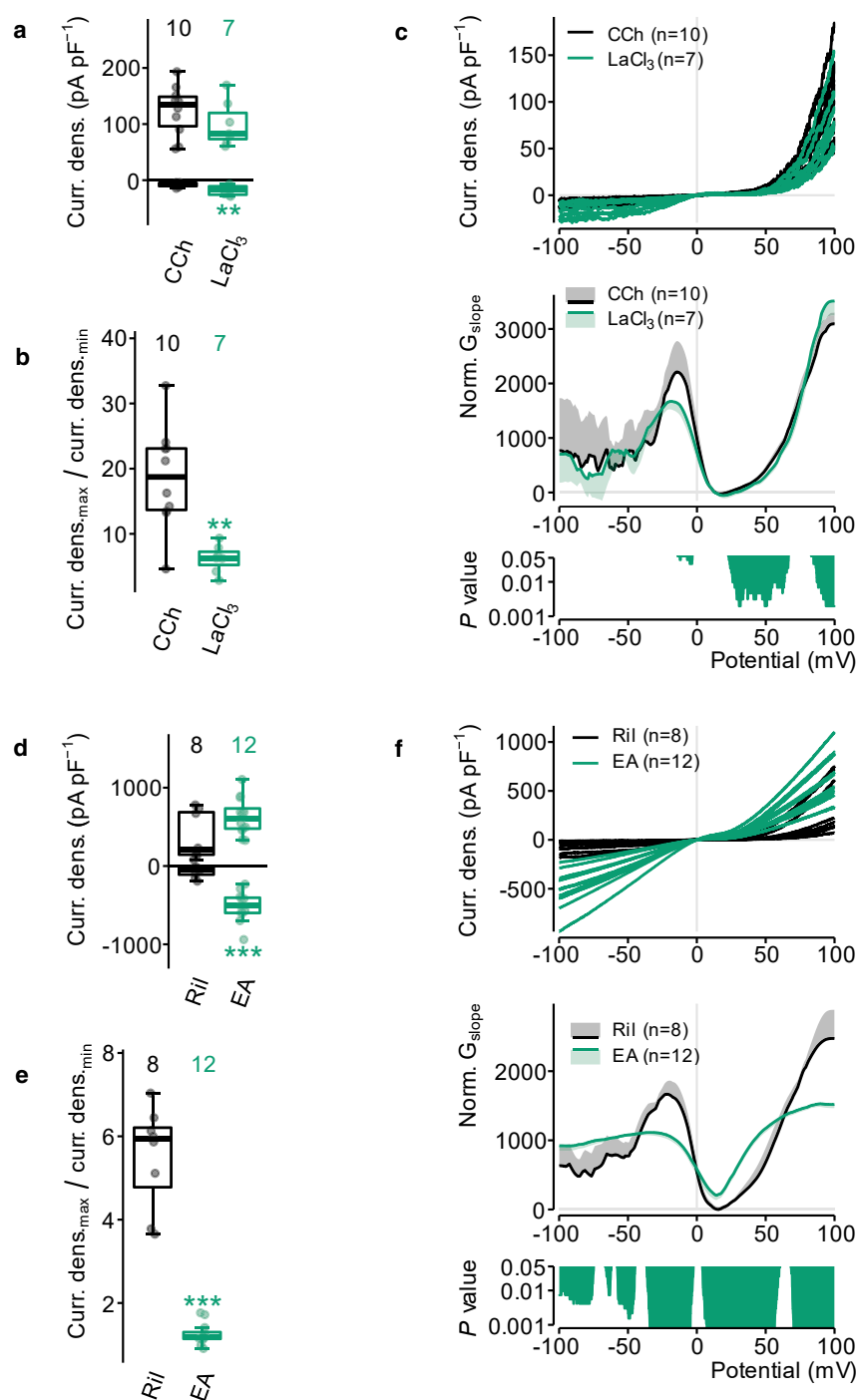


FIGURE 4 Activator- and modulator-specific differences of TRPC5 currents became evident using the NSC. Shown are whole-cell measurements of TRPC5-overexpressing HEK293T cells. (A and D) Summaries of maximal Curr. dens. at potentials of ± 100 mV induced by receptor activation with 100 μ M CCh (A) and by application of the TRPC4/5 potentiator La³⁺ (100 μ M, LaCl₃) (A), 50 μ M riluzole (Ril; D), and 50 nM EA (D). Numbers over boxplots indicate the number of measured cells. **p < 0.01, ***p < 0.001; Mann-Whitney *U* test compared with CCh-induced Curr. dens. (A) or compared with Ril-induced Curr. dens. (D). (B and E) Summaries of ratios between outward and inward Curr. dens. at ± 100 mV (Curr. dens._{max}/Curr. dens._{min}). Numbers over boxplots indicate the number of measured cells. ***p < 0.001, Mann-Whitney *U* test compared with CCh-induced (B) or to Ril-induced ratios (E). (C and F) Maximal modulator-induced CDV relations of TRPC5 (top). The NSC (Norm. G_{slope}; center) is displayed as mean $\pm$ SD. The p values are calculated using Mann-Whitney *U* test compared with CCh-induced NSC (bottom). In (A), (B), (D), and (E), data are displayed as boxplots and interquartile ranges. To see this figure in color, go online.

positive potentials from about +12 to +55 mV, the (–)-englerin A-induced currents showed an increased NSC, whereas at potentials higher than +75 mV, the NSC was significantly decreased. At negative potentials, the NSC progression also strongly differed. Our findings

suggest that the different channel modulators that were used for TRPC5 channel activation can induce IV curves with individual NSC progressions that might reflect distinct active channel states, pointing to different molecular activation mechanisms.

Activator- and modulator-specific differences of TRPC6 currents are identified using the NSC

We examined maximal TRPC6 currents induced by different TRPC6 channel activators and modulators. Receptor activation was achieved with carbachol, and we applied the DAG analog OAG and the potent, non-lipid TRPC3/6/7 channel activator GSK 1702934A. Compared with carbachol-induced currents, the OAG- and GSK 1702934A-induced inward currents were significantly reduced (Fig. 5 A). The ratio analysis showed increased ratios of the OAG-induced currents (Fig. 5 B). The GSK 1702934A-induced current ratios were unchanged. The NSC analysis reveals differences between the TRPC6 currents induced by different channel activators and modulators (Fig. 5 C). The carbachol- and OAG-induced currents slightly differed at negative potentials (around -75 mV and -25 mV) and positive potentials (around $+20$ mV). The NSC of the carbachol- and GSK 1702934A-induced currents were almost similar, and the GSK and OAG-induced currents mainly differed at negative potentials in the range of -40 to -10 mV (Fig. 5 C). The positive allosteric TRPC6 modulator PAM-C20 (39) was also applied and increased the OAG- and GSK 1702934A-induced TRPC6 inward currents (Fig. 5, D and G) and decreased the current ratios of the GSK 1702934A-induced currents (Fig. 5 H). Calculating the NSC, we observed that PAM-C20 induced significant changes in the NSC progressions (Fig. 5, E and I). PAM-C20 significantly changed the NSC progression, mainly at positive potentials from $+10$ to $+50$ mV and from $+70$ to $+100$ mV by increasing or decreasing the NSC, respectively (Fig. 5, E and I). In addition, PAM-C20 changed NSC progressions at similar positive potential ranges, suggesting that the channel modulator changes the channel gating and enhances the channel open probability (39). Our findings suggest a functional selectivity of the channel toward different channel activators and modulators. Next, we compared the maximal OAG-induced currents of human and murine TRPC6 channels (Fig. S7). Neither the Curr. dens. analysis nor analysis of the current ratios or of the NSC progression showed any significant differences, suggesting that there are no species-specific differences.

The NSC is suitable to characterize TRPC6 patient mutations

We tested whether the NSC might be useful to characterize TRPC6 patient mutations (40) that are associated with focal segmental glomerulosclerosis (FSGS), a chronic and severe kidney disease. Maximal OAG-induced currents of the loss-of-function (LOF) mutation TRPC6-G757D (41,42) and of the gain-of-function (GOF) mutation TRPC6-R895C (41,43,44) were analyzed (Fig. 6). The Curr. dens. and ratio analyses revealed significant differences between wild-type and mutant TRPC6 channels (Fig. 6, A and B). However, the

outward Curr. dens. of the LOF mutation TRPC6-G757D was slightly but not significantly reduced compared with wild-type TRPC6. Only the inward Curr. dens. of this mutation was significantly reduced. The NSC of the LOF mutation was significantly increased at positive potentials in the range of about $+30$ to $+70$ mV and $+80$ to $+100$ mV (Fig. 6 C), suggesting that the NSC adds an additional quality to the analysis even when the usual Curr. dens. analysis shows no differences. The identified potentials might be selected for further single-channel analysis. The TRPC6-R895C GOF mutation, however, exhibited significant differences of the NSC progression compared with the wild-type TRPC6 over a wide potential range (Fig. 6 C). Not only were the outward and inward Curr. dens. and the ratios significantly changed, but the NSC was also substantially altered. These findings suggest that the NSC is a useful tool for characterization of FSGS mutations.

DISCUSSION

In this study, we demonstrate that the NCS is a valuable tool for in-depth analysis, characterization, and quantification of TRPC IV curves. Applying this method, we were able to mathematically distinguish different TRPC IV curves from HEK239T cells overexpressing TRPC3, TRPC4, TRPC5, TRPC6, or TRPC7 channels. The NSC can also be advantageous for quantitative analysis of other non-voltage-gated ion channels, like other TRP channels, or ligand-gated ion channels, like P2X channels and nicotinic acetylcholine receptors. The NSC might also be suitable for analysis of weakly voltage-dependent channels like TRPM8 (45) or inward rectifier potassium channels (46) when voltage-ramps with a slow voltage change over time are applied.

Currently, whole-cell currents are mainly analyzed by determination of their current amplitudes at distinct potentials and by presentation of representative IV curves. To analyze macroscopic currents and characterize the conduction properties of a channel (5), the slope conductance and the chord conductance can be used. The chord conductance is regarded as an instantaneous conductance (47) that is determined by a secant that cuts the selected potential and the zero net current potential, which reflects the ion-specific reversal potential (Nernst equation) or the multi-ion equilibrium potential (Goldman-Hodgkin-Katz voltage equation). The slope conductance is determined by a tangent of each potential at steady-state values of the current (5,47), which can only be reached during application of slow continuous voltage ramps. The chord conductance is often used to characterize the IV curves of ion-selective currents that were obtained by application of voltage steps (48–50) or by continuous voltage upramps (51,52). TRPC channels are non-selective cation channels with a mixed reversal potential that lies around 0 mV. We chose the slope conductance to characterize TRPC IV curves because it is a superior approach when depicting negative slope progressions of

TRPC6

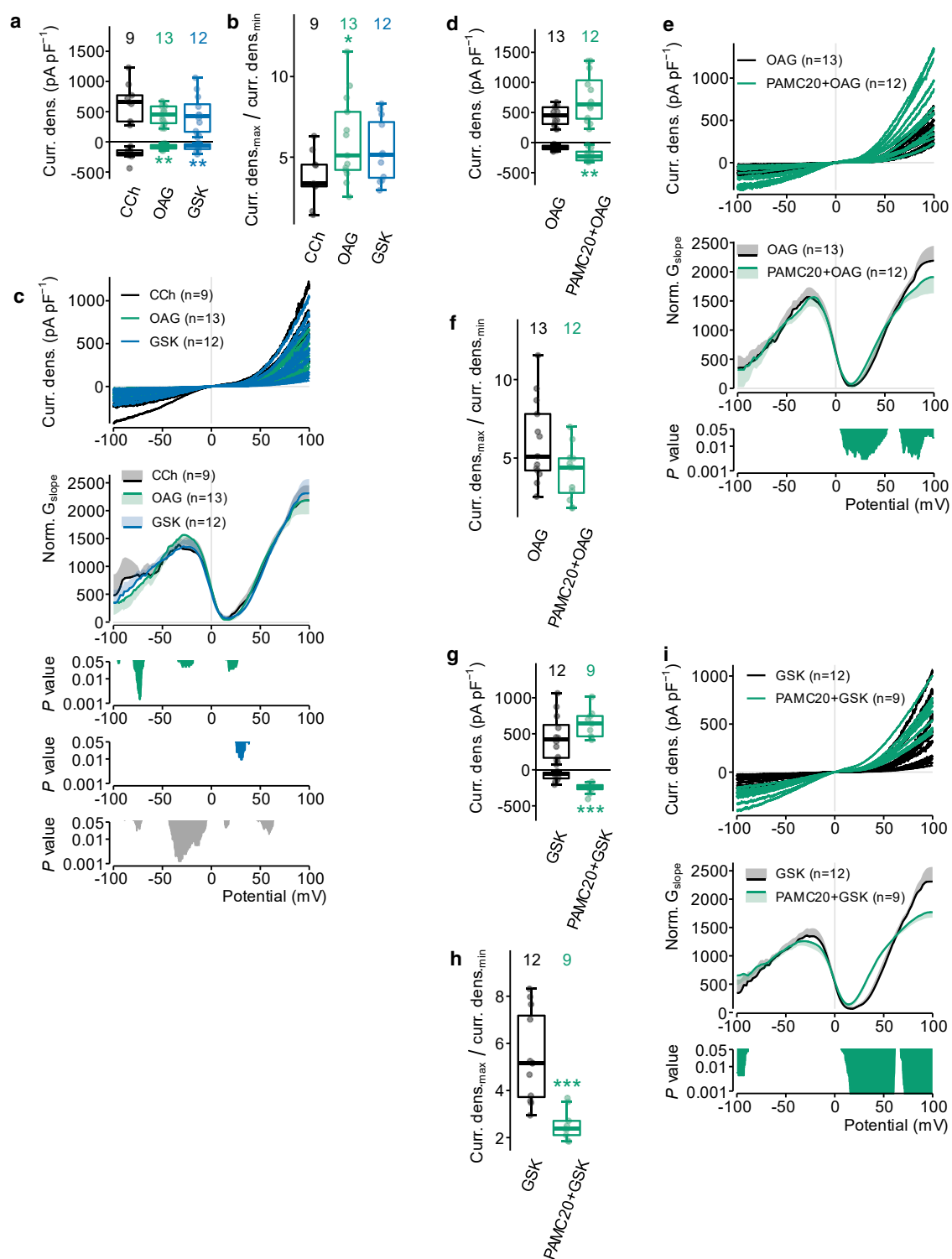


FIGURE 5 Activator- and modulator-specific differences of TRPC6 currents are revealed when using the NSC. Shown are whole-cell measurements of TRPC6-overexpressing HEK293T cells. (A, D, and G) Summaries of maximal Curr. dens. at potentials of ± 100 mV induced by receptor activation with 100 μ M CCh (A), by application of the activators OAG (100 μ M; A and D) and GSK 1702934A (GSK; 10 μ M; A and G), and by additional application of the positive allosteric TRPC6 modulator PAM-C20 together with OAG (D) or GSK (G). Numbers over boxplots indicate the number of measured cells. **p < 0.01, ***p < 0.001; Mann-Whitney *U* test compared with CCh-induced Curr. dens. (A), OAG-induced CDs (D), and GSK-induced Curr. dens. (G). (B,

(legend continued on next page)

the IV curve as negative NSC values. The negative values of the NSC calculated from the (–)-englerin A-induced TRPC4 IV curves (Figs. 1 and S6) would not be evident when applying the chord conductance. Interestingly, Otsuguro et al. (53) calculated the chord conductance at distinct voltage steps to characterize TRPC4 IV curves. When applying the chord conductance to GTP γ S-induced TRPC4 IV curves (53), the negative slopes of the IV curves at negative potentials were not reflected as negative values. The slope conductance appears to be more appropriate to characterize IV curves. The slope conductance of macroscopic currents should not be confused with the single-channel conductance. Previous studies have calculated the slope conductance to characterize IV curves. However, calculation of the slope conductance was restricted to distinct potentials. For example, to determine the slope conductance, the IV curves of the cyclic-nucleotide-gated channels in olfactory neurons (54) and of ATP-sensitive potassium channels in capillary endothelial cells (55) were analyzed by a linear regression fit between –80 and –55 mV and between –54 and –56 mV, respectively. A continuous calculation of the slope conductance has not yet been determined. Another important advantage of the NSC is that it allows a better comparison of the currents independent of their individual current amplitudes or channel expression levels.

We developed a decision tree for classification of carbachol-induced TRPC currents that includes two parameters: the NSC at distinct potentials and the current ratio at ± 100 mV. Using these parameters, 97.6% of the TRPC IV curves were correctly classified. To discriminate IV curves elicited by other channel modulators, other potential ranges might be more suitable. For example, the (–)-englerin A-induced TRPC4/5 currents are distinguishable by their NSC at +20 mV, where the slope of TRPC4 is negative and that of TRPC5 positive (Fig. 1 E). The carbachol-induced TRPC4/5 currents are easily discriminable at –20 mV, where the slope of TRPC5 is nearly two times higher than that of TRPC4 (Fig. 2 C). These critical potentials might be selected for further analysis; e.g., +20 mV might be an interesting transmembrane potential for expanded single-channel analysis of (–)-englerin A-induced TRPC4 currents, which might be suitable for elucidating the gating mechanism that underlies the negative slope progression.

However, the NSC also has some limitations. We could show that the method is useful to discriminate homotetrameric TRPC channels in an overexpression system, but data on heterotetrameric channel complexes are still

missing. When investigating heterotetrameric TRPC channel complexes, one should consider that the stoichiometry of the channel complexes is often not precisely defined, which might lead to ambiguous results. Another limitation is that, in an endogenous expression system (e.g., in primary cells), the total current is the sum of all currents through different ion channels in the cell, and the NSC is not suitable to dissect a mix of different channel currents unless selective channel blockers are applied to isolate specific channel currents. As stated under [Material and methods](#), we recommend application of a cubic smoothing spline fit to reduce the noise of the normalized CDV traces. The noise of the CDV curves strongly depended on the speed of the voltage ramps and was considerably reduced when fast voltage ramps (4 V s $^{-1}$) with a slope of 100 μ V in 25 μ s were applied, compared with slow voltage ramps (500 mV s $^{-1}$) with a slope of 100 μ V in 200 μ s (Fig. S8, top two panels). The observed noise of the CDV traces reflects the characteristic current noise of the channel population. We hypothesize that the appearance of pronounced current noise that is detected when slow voltage ramps are applied indicates that the currents are nearly in an equilibrium state, which is the prerequisite for calculation of the slope conductance. Remarkably, fast voltage ramps caused significant changes of the NSC progression, which was independent from the current noise (Fig. S8). This was most evident at two potential ranges: at around +9 mV, the NSC from currents elicited by fast voltage ramps was significantly increased, and at about –30 mV, the NSC was significantly decreased. These findings demonstrate that the speed of the voltage ramps influences the NSC progression and that the faster the voltage changes are over time, the less are the currents in their equilibrium state. Therefore, we recommend using slow voltage ramps. Smoothing of the CDV curves did not influence the NSC curve progression but improved statistical analysis of the NCS.

For a quantitative comparison of NSC curves, all IV curves must be determined under equal conditions with a fixed rate of voltage changes over time, equal sampling rate, and equal filtering and using equal bath and pipette solutions. With these basic principles and limitations in mind, we think that the NSC is a valuable tool to characterize IV curves. The NSC is not only applicable to analyze TRPC channels, it can also be beneficial for characterization of other non-voltage-gated or weakly voltage-gated ion channels.

Calculating the NSC, we show that different TRPC channel activators and modulators can induce distinct

F, and H) Summaries of ratios between outward and inward Curr. dens. at ± 100 mV (Curr.dens_{max}/curr.dens_{min}). Numbers over boxplots indicate the number of measured cells. * $p < 0.05$, *** $p < 0.001$; Mann-Whitney U test compared with CCh-induced (C), OAG-induced (F), and GSK-induced ratios (H). (C, E, and I) Maximal modulator-induced CDV relations of TRPC5 (top). The NSC (Norm. G_{slope} ; center) is displayed as mean $\pm$ SD. (C) The p values are calculated using Mann-Whitney U test compared with CCh-induced NSC (green and blue p value plots) and between OAG and GSK (gray p value plot) (bottom). (E and I) The p values are calculated using Mann-Whitney U test compared with GSK-induced NSC (E) and OAG-induced NSC (I) (bottom). (a, b, d, f, g, h) Data are displayed as boxplots and interquartile ranges. To see this figure in color, go online.

TRPC6-FSGS mutants

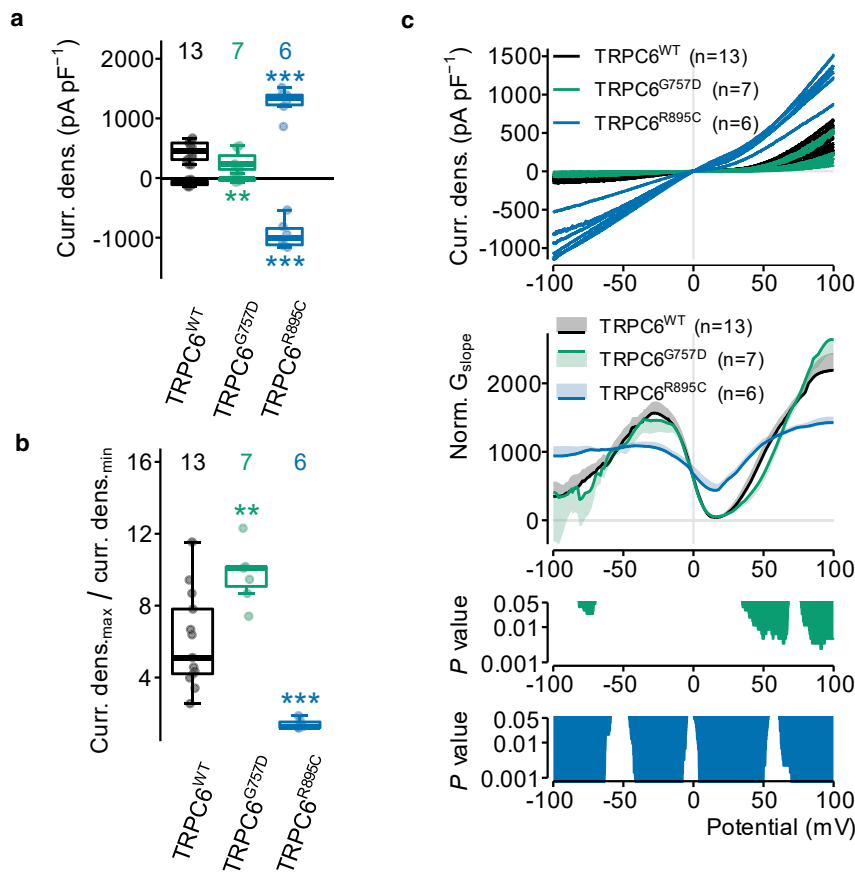


FIGURE 6 Differences in OAG-induced currents of TRPC6 patient mutations that are associated with FSGS become evident when analyzing NSC. Shown are whole-cell measurements of HEK293T cells overexpressing TRPC6 (TRPC6^{WT}), the LOF patient mutation TRPC6-G757D (TRPC6^{G757D}), and the GOF patient mutation TRPC6-R895C (TRPC6^{R895C}). (A) Summary of maximal Curr. dens. at potentials of ± 100 mV induced by application of 100 μ M OAG. Numbers over boxplots indicate the number of measured cells. ** $p < 0.01$, *** $p < 0.001$; Mann-Whitney U test compared with TRPC6^{WT} Curr. dens.. (B) Summary of ratios between outward and inward Curr. dens. at ± 100 mV (Curr. dens._{max}/Curr. dens._{min}). Numbers over boxplots indicate the number of measured cells. ** $p < 0.01$, *** $p < 0.001$; Mann-Whitney U test compared with TRPC6^{WT}. (C) Maximal modulator-induced CDV relations of TRPC6^{WT}, TRPC6^{G757D}, and TRPC6^{R895C} (top). The NSC (Norm. G_{slope} ; center) is displayed as mean $\pm$ SD. The p values are calculated using Mann-Whitney U test compared with CCh-induced NSC (green and blue p value plots) (bottom). In (A) and (B), data are displayed as boxplots and interquartile ranges. To see this figure in color, go online.

NSC progressions, suggesting an effect on channel gating, inducing distinct active channel states. Interestingly, although OAG- and GSK 1702934A-induced Curr. dens. and ratios of TRPC6 currents were similar, the NSC revealed significant differences at distinct negative potential ranges. The positive allosteric TRPC6 modulator PAM-C20 did not significantly enhance the TRPC6 outward current amplitudes but significantly changed the NSC progression at positive potentials. Thus, the NSC is a superior tool for validation and classification of positive or even negative allosteric channel modulators. Future studies are required to analyze whether different channel modulators can induce distinct active channel conformations. This biased signaling is already known for G protein-coupled receptors (summarized in (56)), like the histamine H_1 receptor, which exhibits distinct agonist-induced or mechanically induced active receptor conformations (57).

The NSC is also well suitable to characterize TRPC6 patient mutations (40) associated with FSGS. Using the NSC, we were able to identify potential ranges where the slope conductance was significantly changed compared with wild-type TRPC6. Changes in the NSC progression may

have several causes. First, the single-channel conductance might be different at particular potentials; second, the channel open probability might be changed; and third, the ion selectivity of the channel might be altered. These different parameters should be analyzed in detail in further studies. The NSC analysis might help to elucidate the structure-function relation leading to disturbed channel function. Using this tool, the pathomechanism leading to FSGS, which is still largely elusive, might be unraveled. The NSC analysis might also be valuable to characterize FSGS mutations that do not show obvious changes in the current amplitudes (43). However, if the NSC progression of these mutations is changed, then the observed phenotype leading to podocyte damage might be explained by altered single-channel conductance, channel open probability, or ion selectivity.

CONCLUSIONS

The NSC is an advantageous tool for extended biophysical characterization of IV curves of macroscopic currents that are obtained from non-voltage-gated or weakly voltage-gated ion channels, which comprise about 70% of all ion

channels. We demonstrate that the NSC is of fundamental importance for quantitative analysis of IV curves independent of individual expression levels. By introducing the NSC, we expand the toolbox for in-depth description and quantitative characterization of IV curves. Using the NSC IV curves can be exactly described so that ambiguities concerning the identity of the measured ion channels can be diminished. The NSC has potential to become a mandatory analysis method for IV curves that helps to precisely characterize IV curves.

SUPPORTING MATERIAL

Supporting material can be found online at <https://doi.org/10.1016/j.bpj.2022.03.016>.

AUTHOR CONTRIBUTIONS

M.M.y.S. originated the study. M.M.y.S. and U.S. designed the experiments and supervised the study. C.H., A.T., R.G., and M.N. performed experiments and analyzed the data. U.S. and M.M.y.S. wrote the manuscript. T.G. revised the manuscript. C.H. designed and wrote the R program. C.H., M.M.y.S. and U.S. prepared the figures. All authors reviewed the manuscript.

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6. Publikation II

Photoswitchable TRPC6 channel activators evoke distinct channel kinetics reflecting different gating behaviors

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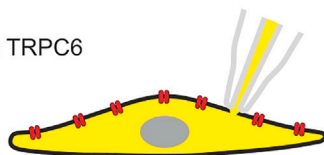
DOI: <https://doi.org/10.1016/j.isci.2024.111008>

Article

Photoswitchable TRPC6 channel activators evoke distinct channel kinetics reflecting different gating behaviors

• patch-clamp technique

HEK293T cell overexpressing TRPC6
whole-cell configuration
voltage clamp
holding potential of -60 mV



• photopharmacology

LED-based

440 nm 365 nm 440 nm

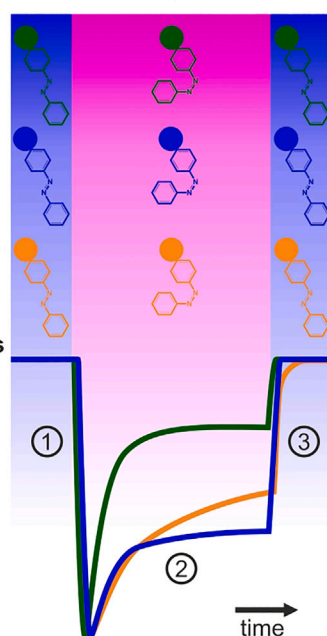
OptoDARg

OptoBI

PhoDAG

• biophysical channel analysis

- ① activation kinetics
- ② inactivation kinetics
- ③ deactivation kinetics



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Highlights

Photoswitchable activators
enable precise
determination of current
kinetics

Maximally effective
concentrations are
prerequisites

High-intensity and fast-
switching light sources are
essential

Each photoswitchable
activator evokes a distinct
and specific gating
behavior

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Article

Photoswitchable TRPC6 channel activators evoke distinct channel kinetics reflecting different gating behaviors

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SUMMARY

The non-selective transient receptor potential canonical 6 (TRPC6) cation channels have several physiological and pathophysiological effects. They are activated by the lipid second messenger diacylglycerol (DAG) and by non-lipidic compounds such as GSK 1702934A (GSK). Advances in photopharmacology led to the development of photoswitchable activators such as PhoDAG, OptoDARg, and OptoBI-1 that can be switched ON and OFF with the spatiotemporal precision of light. We aimed to elucidate whether these photopharmaceuticals allow for a reliable determination of the ion channel current kinetics. We performed electrophysiological whole-cell measurements in the overexpression system and analyzed TRPC6 currents induced by photoswitching. We observed distinct activation, deactivation and inactivation current kinetics suggesting that each photoswitchable activator elicits a distinct active channel state. Notably, the current kinetics strongly depended on the intensity of the light source. Altogether, photopharmaceuticals are advantageous for an extended biophysical characterization of whole-cell currents and provide insight into their gating mechanism.

INTRODUCTION

Transient receptor potential classical or canonical (TRPC) channels are non-selective cation channels that are permeable for sodium, potassium, and calcium ions and belong to the large family of TRP channels. TRPC channels are commonly regarded as receptor-operated cation channels that are activated following G_{q/11}-protein coupled receptor activation. Receptor activation leads to activation of the phospholipase C which cleaves phosphoinositol-4,5-bisphosphate into the 2 s messengers inositol-1,4,5-trisphosphate (IP₃) and diacylglycerol (DAG). DAG can then directly activate TRPC channels,^{1–4} leading to channel opening and cation influx, thus resulting in cellular effects. Accordingly, DAG serves as the endogenous activator of TRPC channels. However, the DAG sensitivity of TRPC4 and TRPC5 channels is regulated by C-terminal interaction with the adaptor proteins NHERF1 and NHERF2³ which involves C-terminal phosphorylation via protein kinase C.^{3,5,6}

During the last decades several physiological as well as pathophysiological roles of TRPC channels have been identified (summarized in the study by Chen et al.⁷) e.g., TRPC channels play a role for the cardiovascular system, metabolism, immune system, kidney function, neurological function, and cancer. Furthermore, patient mutations in the gene coding for TRPC6 are associated with a chronic kidney disease, the focal segmental glomerulosclerosis (FSGS)^{8–11} which leads to podocyte damage and renal failure. Thus, TRPC channels are important for human health and disease and serve as potential drug targets.

To study TRPC channels *in vitro*, DAG derivatives are widely used as channel activators. Hereby, the membrane permeable DAG analog 1-oleoyl-2-acetyl-sn-glycerol (OAG) is often employed. Furthermore, non-lipidic TRPC channel activators are available such as the TRPC3/6 channel activator GSK 1702934A. Recent advances in photopharmacology have yielded new photoswitchable TRPC channel modulators that allow for the regulation of the channel function with the spatiotemporal precision of light.^{12–16} The basis for photoswitching of these compounds is the insertion of a photoswitchable azobenzene^{12,13,16} or an azoheteroarene¹⁷ moiety that allows for light-induced *cis* and *trans* isomerization. Hereby, UV light stabilizes the active *cis* configuration, while blue light produces the inactive *trans* configuration, thus enabling the regulation of channel activity simply by switching the light. Other photoswitchable TRP channel ligands include azo-vanilloids such as azCA4 and red-azCA4^{18,19} which target TRPV1 and “TRPswitch”, which targets TRPA1.²⁰ Using these compounds, wash-in and wash-out effects that usually occur when applying channels activators via bath solution are circumvented, such that the determination of the channel kinetics becomes possible. At the moment three photoswitchable TRPC6 channel activators are available: PhoDAG, OptoDARg, and OptoBI-1.^{12–16}

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In this study we aimed at analyzing whether the use of different photopharmaceuticals allows for a valid and reproducible determination of the reliable kinetics of TRPC6 currents which might improve the biophysical analysis of whole-cell currents thus allowing to gain deeper insights into the gating of ion channels. Since TRPC6 channels emerged as potential drug targets, the elucidation of their activation and regulation mechanism is important to improve the design of novel, potent channel modulators that might be helpful for the treatment of TRPC6 channel-related diseases.

RESULTS

OptoDARg-evoked TRPC6 current kinetics strongly depend on the intensity of the light source

TRPC6 channels are not only sensitive to the endogenous agonist DAG, but also to small molecule activators such as the non-selective and non-lipidic TRPC3 and TRPC6 channel activator GSK 1702934A (GSK). Performing a biophysical analysis of the current-voltage relations elicited by 1-oleoyl-2-acetyl-*sn*-glycerol (OAG; 100 μ M), a membrane-permeable analog of DAG, or by GSK (10 μ M) in their maximally effective concentrations and calculating the normalized slope conductance (NSC)²¹ to quantify differences between the individual curve progressions, we observed significant differences in the NSC curve progression mainly at negative potentials suggesting that OAG and GSK have different activation mechanisms and cause distinct active channel states (Figure S1;²¹). Next, we employed photopharmacology utilizing the photo-switchable GSK derivative OptoBI-1,¹⁶ which contains one photoswitchable azobenzene group, and the photoswitchable DAG derivative OptoDARg,¹² a DAG analog, that contains two photoswitchable azobenzene groups. The chemical structures of the TRPC6 channel activators are displayed in Figure S2.

The photopharmaceuticals were also applied in their maximally effective concentrations. The inactive *trans* configuration was established by applying blue light and the active *cis* configuration by applying UV light. Photoswitching was performed with different light sources, with a xenon lamp emitting UV light of in the range of 355–375 nm with a mean wavelength of 365 nm and blue light in the range of 435–455 nm with a mean wavelength of 445 nm or with two light-emitting diodes (LEDs) emitting UV light in the range of 346–371 nm with a peak wavelength of 367 nm and blue light in the range of 418–447 nm with a peak wavelength of 442 nm.

First, we performed electrophysiological whole-cell measurements with TRPC6 over-expressing HEK293T cells in the presence of 30 μ M OptoDARg. To obtain current density-voltage relations and current densities at ± 100 mV, consecutive voltage upramps from -100 to $+100$ mV were applied with a frequency of 50 Hz. Application of UV light for 4 s with the xenon lamp or with the LED resulted in comparable maximal TRPC6 current density increases at ± 100 mV that were not significantly different (Figure 1A). OptoDARg caused almost symmetrical increases of the inward and outward CD. However, the current density-time courses strongly differed (Figures 1B and 1C). When applying UV light, the activation kinetics was much slower using a xenon lamp compared to the LED. However, the outward and inward currents at ± 100 mV reached a plateau within 1–3 s of UV light application (Figure 1B). In contrast, using the 365 nm LED, the TRPC6 currents rapidly increased to maximal values in less than a second followed by an inactivation phase (Figure 1C). In both cases, switching to blue light resulted in fast current deactivations leading to rapid TRPC6 current decreases that reached the basal currents before application of UV light. These findings show that the TRPC6 channel was effectively switched OFF with blue light. However, the establishment of the *cis* configuration with UV light was less effective using the xenon lamp.

To gain further insights into the current kinetics, we proceeded to analyze TRPC6 inward currents, which were measured at a constant holding potential of -60 mV. Comparison of the current-time courses of the normalized currents elicited by photoswitching with the xenon lamp versus the LED in the presence of OptoDARg reveals marked differences in the activation kinetics (Figure 1D). The half-life time constants (τ_H) of the activation, deactivation and inactivation kinetics were significantly faster when using the LED. τ_H were calculated using Equations 1, 2, 3, 4, and 5 and the parameters displayed in Table 1. The activation kinetics was 22-fold lower using LED light (5.8 ± 1.6 ms with LED and 130.0 ± 62.8 ms with xenon lamp) (Figure 1E). In addition, the deactivation kinetics was 11 times faster using LED light (8.4 ± 1.5 ms with LEDs and 94.4 ± 33.1 ms with xenon lamp) (Figure 1F). To determine the inactivation kinetics, UV light was constantly applied until the TRPC6 current reached basal values (Figure 1G). The inactivation kinetics was biphasic showing a fast phase followed by a slow phase (Figures 1H and 1I). τ_H of the fast inactivation was 16-fold faster when using LED light (0.3 ± 0.2 s with LED and 4.0 ± 1.8 s with xenon lamp). The slow inactivation was 4-fold faster using LED light compared to the xenon lamp (13.1 ± 4.4 s with LEDs and 57.3 ± 35.5 s with xenon lamp). Thus, the xenon lamp was not sufficient for effective photoswitching of OptoDARg into its active *cis* and inactive *trans* configuration. To find out whether inactivation kinetics might depend on the current amplitudes, we correlated the fast and slow inactivation kinetics of OptoDARg and OptoBI-1 induced current densities that were elicited using LED or xenon lamp. However, we did not find any correlation (R^2 was between 0.02 and 0.65) (Figure S3). Our findings strongly indicate that the intensity of the light source is crucial for the current kinetics. Since the maximal current densities at ± 100 mV elicited by applying UV light using the two light sources xenon lamp and LED were not significantly different (Figure 1A), the observed differences in the current kinetics are independent of the current density amplitudes.

Furthermore, we investigated the influence of lower LED light intensities on channel kinetics. To determine activation and deactivation kinetics, measurements were conducted by consecutively applying blue and UV light with increasing LED light intensities of 25%, 50%, and 100% to TRPC6 overexpressing HEK293T cells in the presence of OptoDARg (Figures S4A–S4D). Overall, activation and deactivation kinetics were significantly different. Using Dunn's multiple comparison post hoc analysis, we particularly observed differences between the current kinetics observed with LED light intensities of 25% and 100%. To determine inactivation kinetics, LED light with 25% or 50% light intensity was applied to cells until currents reached values that occurred before application of UV light. Increasing light intensities resulted in faster inactivation kinetics, and the differences were most pronounced between 25% and 100% light intensity.

OptoDArG

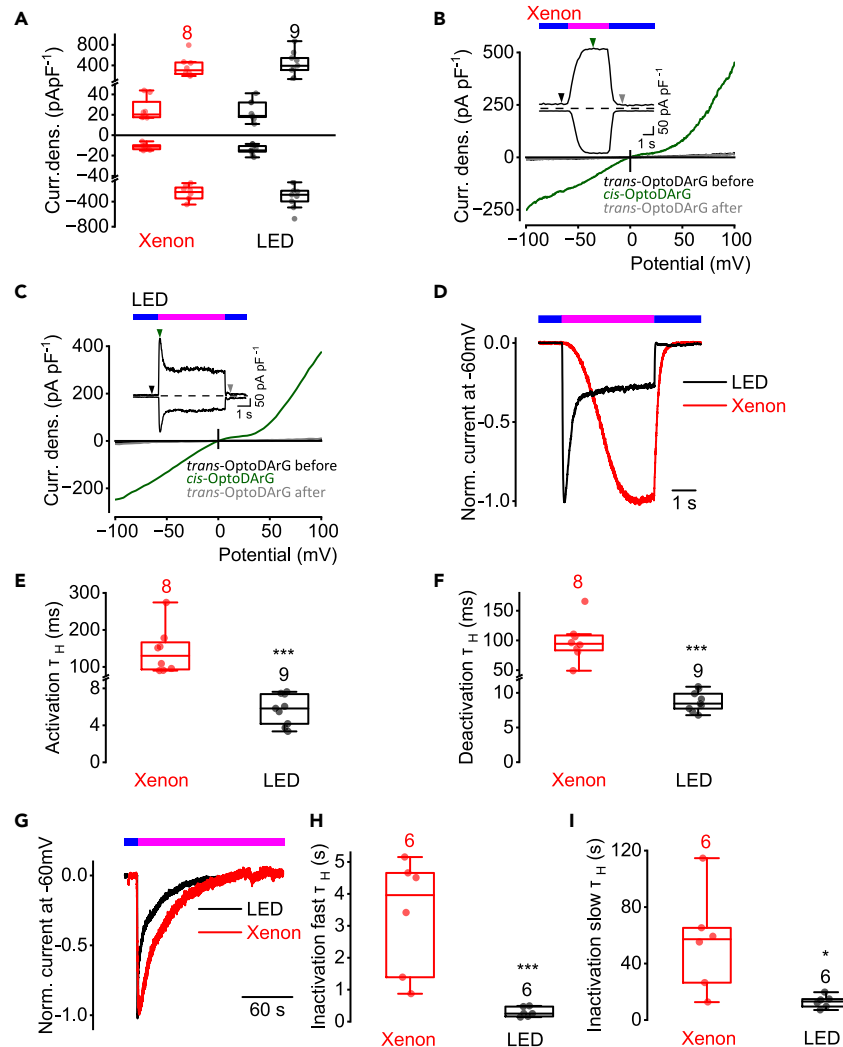


Figure 1. OptoDArG-evoked TRPC6 current kinetics strongly depend on the intensity of the light source

Electrophysiological whole-cell measurements of TRPC6 overexpressing HEK293T cells in the presence of 30 μ M OptoDArG.

(A) Summaries of current densities ("Curr. dens.") at potentials of ± 100 mV evoked by light using a xenon lamp (red) or a LED (black). First small boxplots represent current densities in the presence of blue light (445 nm) which establishes *trans*-OptoDArG and second boxplots represent maximal current densities in the presence of UV light (365 nm) which establishes *cis*-OptoDArG. No significant differences between *trans* or *cis*-OptoDArG-induced current densities were observed using xenon or LED light source, respectively (Mann-Whitney U test).

(B and C) Representative current density ("Curr. dens.")-time courses ± 100 mV and current density-voltage relations using xenon lamp (B) or LED (C) for photoswitching. The arrows indicate the time point at which the *trans*- and the maximal *cis*-OptoDArG-induced current density-voltage relations were selected. Bars over current density-time courses indicate the application of light of the wavelength 445 nm (blue) and of 365 nm (magenta).

(D) Representative normalized time courses of TRPC6 inward currents at constant holding potential of -60 mV during photoswitching from blue light (blue bar) to UV light (magenta bar) and back to blue light (blue bar) using a xenon lamp (red) or LED (black).

(E and F) Summaries of half-life time constants (τ_H) of the activation (E) and deactivation (F) kinetics ($***p < 0.001$; Mann-Whitney U test).

(G) Representative normalized time courses of TRPC6 inward currents at constant holding potential of -60 mV induced by photoswitching from blue light to UV light using xenon lamp (red) or LED (black). The duration of light application (blue and magenta bar) is indicated.

(H and I) Summaries of half-life time constants (τ_H) of the fast (H) and slow (I) inactivation kinetics. ($*p < 0.05$, $***p < 0.001$; Mann-Whitney U test). (E, F, H, and I) Data are displayed as boxplots and interquartile ranges. Numbers over boxplots indicate number of measured cells.

OptoBI-1-induced TRPC6 current kinetics strongly rely on the intensity of the light source

Next, we used the non-lipidic TRPC6 activator OptoBI-1 (10 μ M) and again we performed photoswitching either with the xenon lamp or with LEDs. Exposure to UV light with both light sources caused maximal TRPC6 current density increases at ± 100 mV (Figure 2A) that were not

Table 1. Parameters and values for fit routine

Parameter	Value
MaxFunEvals	5000
MaxIter	10000
TolX	1×10^{-10}
TolFun	1×10^{-6}
Initial values activation	$a = 0.01$; $\tau_H = 1$; $c = 0.01$
Initial values deactivation	$a = 0.7$; $\tau_H = 7$; $c = 0.1$
Initial values inactivation	$a_1 = 1$; $\tau_{H1} = 0.02$; $c = 0.1$, $a_2 = 1$, $\tau_{H2} = 3.8$

significantly different. Interestingly, the OptoBI-1-induced CD at ± 100 mV showed a pronounced outward rectification compared to the OptoDARg-induced CD. Using the xenon lamp, the current density maximum was reached within 2 s of UV light application (Figure 2B) but using the LED, the maximal current density was reached earlier within the first second of UV light application (Figure 2C). Furthermore, in both cases, blue light caused fast TRPC6 current deactivations. Interestingly, in contrast to *cis*-OptoDARg, *cis*-OptoBI-1 elicited TRPC6 current increases that reached a maximum followed by an inactivation phase. To determine the current kinetics, the normalized TRPC6 inward currents at -60 mV were analyzed (Figure 2D). Again, we found that τ_H of the activation (Figure 2E) and deactivation kinetics (Figure 2F) were significantly faster using LEDs. τ_H of the activation kinetics was 16-fold lower using LED compared to the xenon lamp (12.3 ± 5.8 ms with LEDs and 195.0 ± 53.3 ms with xenon lamp) (Figure 2E) and τ_H of the deactivation kinetics was 4-fold faster (31.2 ± 22.2 ms with LEDs and 128.5 ± 8.8 ms with xenon lamp) (Figure 2F). The inactivation kinetics was again biphasic and significantly faster when using LEDs (Figures 2G–2I). τ_H of the fast inactivation was 8-fold lower using LED (0.4 ± 0.1 s with LEDs and 3.0 ± 1.2 s with xenon lamp) (Figure 2H) and τ_H of the slow inactivation was almost 3-fold lower (73.0 ± 48.0 s with LEDs and 187.5 ± 81.0 s with xenon lamp) (Figure 2I). These findings also support the notion that the intensity of the light source is crucial for sufficient photoswitching.

Furthermore, we determined the factors of current density increases between *trans* and *cis* isomer-induced currents. For OptoBI-1 we obtained activation factors of 11.6 ± 3.9 (median $\pm$ SD) for Xenon and 10.6 ± 2.9 for LED which were not significantly different ($p = 0.99$; Mann Whitney U test) for outward currents and 8.3 ± 4.0 for Xenon and 6.3 ± 5.7 for LED for inward currents which were also not significantly different ($p = 0.83$; Mann Whitney U test). For OptoDARg we obtained activation factors of 11.7 ± 4.8 for Xenon and 12.2 ± 9.9 for LED which were not significantly different ($p = 0.86$; Mann Whitney U test) for outward currents and 26.2 ± 11.2 for Xenon and 18.0 ± 20.7 for LED for inward currents which were also not significantly different ($p = 0.81$; Mann Whitney U test).

Moreover, we applied LED light with increasing light intensities of 25%, 50%, and 100% to TRPC6 expressing cells in the presence of OptoBI-1 (Figures S4E–S4H). Application of increasing light intensities resulted in significantly decreased half-life time constants (τ_H) of activation, deactivation, and inactivation kinetics. These findings also suggest that the current kinetics strongly depend on the intensity of the light source.

Next, we determined the lightning power density of the two LEDs and of the xenon lamp in the beam path where the objective lens would normally be located. The wavelengths used for photoswitching were not identical but quite similar. At a wavelength of 445 ± 10 nm using the xenon lamp, we achieved a lightning power density of 0.59 ± 0.02 mW cm $^{-2}$, and using the blue light LED with a peak wavelength of 442 nm we achieved 3.31 ± 0.10 mW cm $^{-2}$. At a wavelength of 365 ± 10 nm using the xenon lamp, we measured 1.27 ± 0.13 mW cm $^{-2}$, and using the UV light LED with a peak wavelength of 367 nm we achieved 7.96 ± 0.80 mW cm $^{-2}$. Thus, the LEDs showed approximately 6-fold higher lightning power densities. Furthermore, a reduced LED light intensity of 25% resulted in lightning power density of 1.99 ± 0.20 mW cm $^{-2}$ for UV light and 0.83 ± 0.02 mW cm $^{-2}$ for blue light, and LED light intensity of 50% resulted in lightning power density of 3.98 ± 0.40 mW cm $^{-2}$ for UV light and 1.66 ± 0.05 mW cm $^{-2}$ for blue light. Our findings suggest that the light intensity of the xenon lamp is too low to induce sufficient and rapid *cis-trans* isomerization of OptoDARg and OptoBI-1. Therefore, low-intensity light sources are not suitable for fast photoswitching or for achieving fast current kinetics.

Characterization of thermal relaxation, reversibility of photoswitching and photostationary states of OptoDARg, OptoBI-1, and PhoDAG

Unfortunately, the photoswitchable TRPC6 activators OptoDARg, OptoBI-1 and PhoDAG have not been extensively characterized until now. Therefore, we conducted a comparative characterization of the distinct properties of these compounds such as thermal relaxation, reversibility of photoswitching, and photostationary states (PSS) using UV-vis and NMR spectroscopy. Additionally, UV-vis spectroscopy was used to analyze the thermal relaxation rate which refers to the thermal transition from the *cis* to the *trans* configuration in the dark after illumination with 365 nm light. The half-life time constants for thermal relaxation (τ_H) at a concentration of 50 μ M are 425.82 ± 0.12 min for OptoDARg, 418.76 ± 0.13 min for OptoBI-1, and 396.34 ± 0.08 min for PhoDAG (Figure S5). These results indicate that the *cis* configuration exhibits high thermal stability, with complete relaxation to the *trans* configuration occurring after approximately 36 h at 37°C.

To determine the reversibility of photoswitching, the compounds were repeatedly illuminated with 365 nm and 460 nm light for 5 min each over a period of 110 min at 37°C (Figure S6). The absorption was measured at 340 nm. No changes in absorption were observed with respect

OptoBI-1

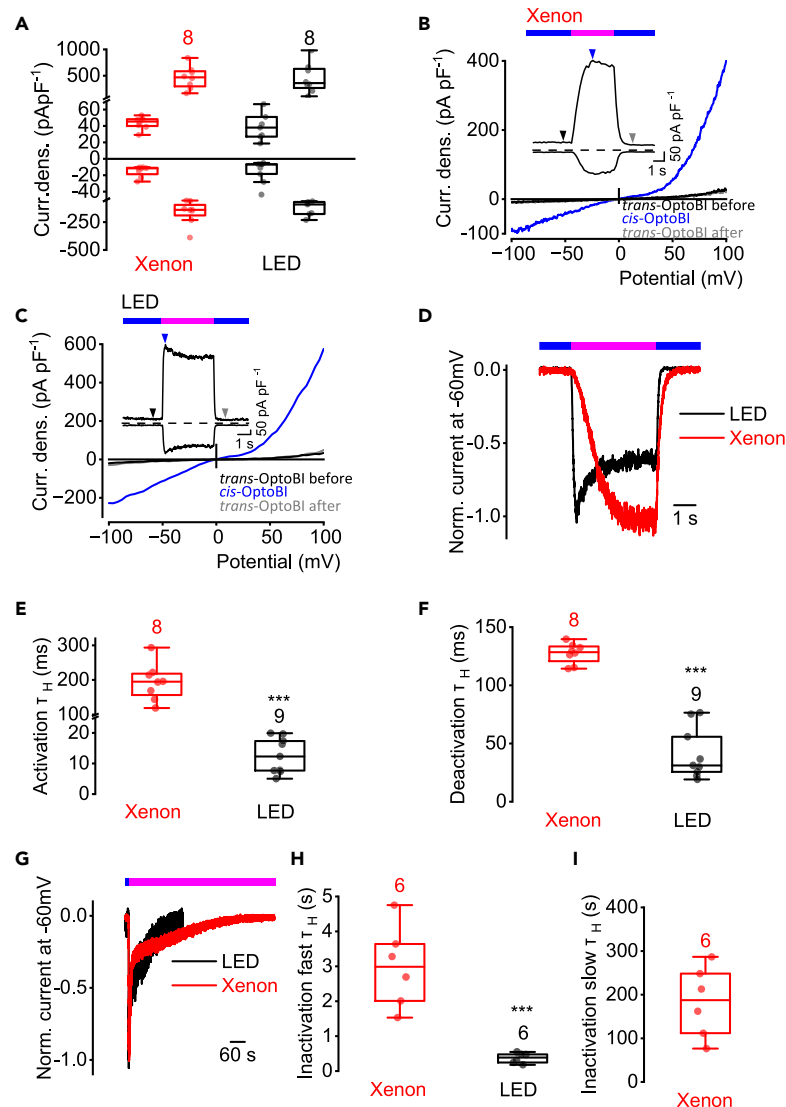


Figure 2. OptoBI-1-induced TRPC6 current kinetics strongly rely on the intensity of the light source

Electrophysiological whole-cell measurements of TRPC6 overexpressing HEK293T cells in the presence of 10 μ M OptoBI-1.

(A) Summaries of current densities ("Curr. dens.") at potentials of ± 100 mV evoked by application of UV light using a xenon lamp (red) or a LED (black). First small boxplots represent current densities induced by *trans*- and second boxplots maximal current densities induced by *cis*-OptoBI-1. No significant differences between *trans*- or *cis*-OptoBI-1-induced current densities using xenon lamp or LED (Mann-Whitney U test).

(B and C) Representative current density ("Curr. dens.")-time courses ± 100 mV and current density-voltage relations using xenon lamp (B) or LED (C) for photoswitching. The arrows indicate the time point at which the *trans*- and the maximal *cis*-OptoBI-1-induced current density-voltage relations were selected. Bars over current density-time courses indicate the application of light of the wavelength 445 nm (blue) and of 365 nm (magenta).

(D) Representative normalized time courses of TRPC6 currents at constant holding potential of -60 mV during photoswitching from blue light (blue bar) to UV light (magenta bar) and back to blue light (blue bar) using a xenon lamp (red) or LED (black).

(E and F) Summaries of half-life time constants (τ_H) of the activation (E) and deactivation (F) kinetics (*** $p < 0.001$; Mann-Whitney U test).

(G) Representative normalized time courses of TRPC6 currents at constant holding potential of -60 mV induced by photoswitching from blue light to UV light using xenon lamp (red) or LED (black). The duration of light application (blue and magenta bar) is indicated.

(H and I) Summaries of half-life time constants (τ_H) of the fast (H) and slow (I) inactivation kinetics (* $p < 0.05$, *** $p < 0.001$; Mann-Whitney U test). (E, F, H, and I) Data are displayed as boxplots and interquartile ranges. Numbers over boxplots indicate number of measured cells.

to the respective illumination wavelengths, suggesting that photoswitching is fully reversible and that the compounds do not degrade during this period through e.g., photobleaching.

The PSS is the equilibrium state between the *trans* and *cis* configuration at a given illumination wavelength. To determine the PSS of the three photoswitchable compounds, we used UV-vis spectroscopy for near-quantitative analysis (Figure S7). We found that in the dark, after complete thermal relaxation, the highest percentage of the *trans* configuration is achieved at an absorption maximum (λ_{max}) of 335 nm for OptoDARg, 336 nm for OptoBI-1, and 337 nm for PhoDAG. In order to obtain a PSS with the highest percentage of the *trans* configuration at λ_{max} , the most appropriate wavelength was 435 nm for OptoDARg and 435 nm as well as 525 nm for OptoBI-1 and PhoDAG. Focusing on the illumination wavelength of 435 nm, which is closest to the wavelength used in patch-clamp measurements to induce the *trans* configuration, we found that OptoDARg achieves approximately 93% *trans* and consequently a calculated 7% *cis* configuration. OptoBI-1 achieves approximately 90% *trans* and 10% calculated *cis* configuration, while PhoDAG reaches around 92% *trans* and 8% *cis* configuration. Illumination at 365 nm results in the lowest absorption at λ_{max} , suggesting that this wavelength is suitable for achieving a high percentage of the *cis* configuration.

For a more precise quantitative analysis of the PSS, we additionally used $^1\text{H-NMR}$ spectroscopy (Figures S8–S10). We found that the freshly dissolved commercially obtained photoswitchable compounds (2.5 mM), without additional thermal relaxation, were almost entirely in the *trans* configuration ($\geq 97\%$). Since OptoDARg contains two azobenzene moieties, illumination with 435 nm and 365 nm light results in a mixture of conformational states: entirely *trans* (*trans+trans*), mixed *trans* and *cis* (*trans+cis* or *cis+trans*), and entirely *cis* (*cis+cis*) configurations, depending on the isomerization state of the first and second fatty acid. The mixed *trans* and *cis* configurations were not discriminated. Illumination with 435 nm resulted in a ratio of 60% for the fully *trans*, 19% for the mixed, and 21% for the fully *cis* configuration. Illumination with 365 nm caused a ratio of 2% for the fully *trans*, 2% for the mixed, and 96% for the fully *cis* configuration. Illumination of OptoBI-1 with 435 nm caused a ratio of 78% *trans* and 22% *cis* configuration, and illumination with 365 nm results in a ratio of 4% *trans* and 96% *cis* configuration. Illumination of PhoDAG with 435 nm caused a ratio of 80% *trans* and 20% *cis* configuration, and illumination with 365 nm resulted in a ratio of 2% *trans* and 98% *cis* configuration.

Photoswitching of OptoDARg, PhoDAG, and OptoBI-1 with LEDs elicits distinct TRPC6 current kinetics

Next, we tested the photoswitchable DAG derivative PhoDAG (200 μM)^{13–15} which contains only one photoswitchable azobenzene group as well as a stearyl moiety. Hereby, we only used the more efficient LEDs as a light source. To test whether OptoDARg, OptoBI-1, and PhoDAG were applied in their maximally effective concentrations, we analyzed CD, current kinetics and NSC in the presence of increased concentrations of OptoDARg (200 μM) and OptoBI-1 (20 μM) (Figures S11–S14). Since 200 μM PhoDAG already represents the concentration which was maximally soluble in the bath solution, we reduced its concentration to 100 μM and repeated the measurements (Figures S15 and S16). We found that increasing the concentrations of OptoDARg or OptoBI-1 or decreasing the concentration of PhoDAG neither had an effect on the maximally induced CD nor on the current kinetics or on the NSC curve progression, suggesting that the chosen concentrations are already maximally effective concentrations.

Using 200 μM PhoDAG, photoswitching with 365 nm caused *cis*-PhoDAG-induced current density increases that were not significantly different from the *cis*-OptoDARg-induced current densities (Figures 3A and 3D) or the current densities induced by the membrane-permeable DAG analog OAG (100 μM) which was applied via perfusion with the bath solution (Figure 3B). To analyze whether *trans*-OptoDARg already binds to the channel, we used TRPC3 channels that possess a high constitutive activity compared to TRPC6 channels which exhibit almost no constitutive activity.²² These findings also suggest that blue LED light was quite efficient, producing a very high percentage of *trans* isomers, which led to channel inhibition. Interestingly, wash in of *trans*-OptoDARg via perfusion significantly inhibited the basal TRPC3 currents in a concentration-dependent way suggesting that *trans*-OptoDARg binds in the binding pocket thereby eliciting inhibitory effects (Figure S17A). In addition, we compared TRPC6 currents in the absence and presence of the *trans* isomers of OptoDARg, PhoDAG, and OptoBI-1 (Figure S17B). We did not observe any significant differences suggesting that the *trans* isomers have no additional effect on the already very low basal TRPC6 currents.

As a control, non-transfected HEK293T cells were analyzed in the presence of 30 μM OptoDARg, 10 μM OptoBI-1, and 200 μM PhoDAG. However, photoswitching did not elicit any significant current responses (Figure S18).

The non-lipidic TRPC6 activator GSK (10 μM) which was also applied via perfusion with bath solution (Figure 3E) and its derivative *cis*-OptoBI-1 (10 μM) (Figure 3E) caused similar TRPC6 current density increases. Only the *cis*-OptoDARg- and GSK-induced current densities at -100 mV were significantly different (Figure 3A).

Comparing the normalized current time courses at a constant holding potential of -60 mV we observed differences in curve progressions of OptoDARg-, PhoDAG-, and OptoBI-1-induced currents (Figure 4A). The half-life time constant (τ_H) of the PhoDAG-induced activation kinetics was 15.5 ± 4.5 ms, of the deactivation kinetics 35.2 ± 4.1 ms, of the fast inactivation kinetics 935 ± 383 ms and of the slow inactivation kinetics 86 ± 34 s. τ_H of the activation and deactivation kinetics are significantly different between OptoDARg and OptoBI-1 and between OptoDARg and PhoDAG (Figures 4B and 4C). Comparing the fast inactivation kinetics, we observed significant differences between OptoDARg and PhoDAG, but not between OptoDARg and OptoBI-1 (Figures 4D and 4E). Comparing the slow inactivation kinetics, we found significant differences between OptoDARg and PhoDAG and between OptoDARg and OptoBI-1 (Figures 4D and 4F). These findings are pointing to the notion that different photoswitchable lipidic and non-lipidic TRPC6 channel activators induce different current kinetics. Moreover, different photoswitchable DAG derivatives cause distinct current kinetics, suggesting that each of these channel activators gives rise to different active channel states and distinct gating behaviors.

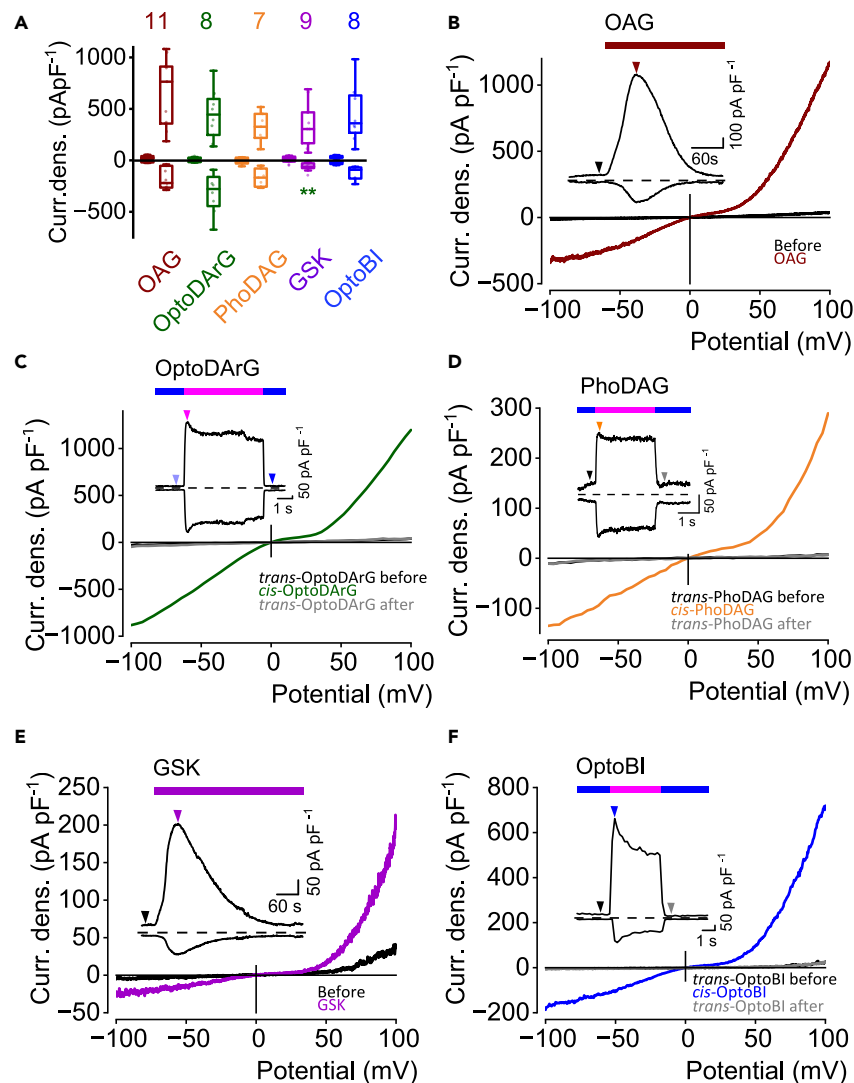


Figure 3. TRPC6 current densities elicited by photoswitchable and non-photoswitchable TRPC6 channel activators

Electrophysiological whole-cell measurements of TRPC6 overexpressing HEK293T cells.

(A) Summaries of current densities ("Curr. dens.") at potentials of ± 100 mV evoked by application $100 \mu\text{M}$ OAG, of $10 \mu\text{M}$ GSK 1702934A ("GSK"), of blue light and UV light using LEDs in the presence of OptoDARg, PhoDAG or OptoBI-1. First small boxplots represent current densities before application of OAG or GSK or induced by *trans*-OptoDARg, *trans*-PhoDAG or *trans*-OptoBI-1 and second boxplots maximal current densities induced by OAG, *cis*-OptoDARg, *cis*-PhoDAG, GSK or *cis*-OptoBI-1. Significant differences were calculated using Kruskal-Wallis test. Brown asterisks indicates significant differences compared to the OAG-induced current densities and green asterisks compared to OptoDARg-induced current densities (** $p < 0.01$, * $p < 0.05$). Data are displayed as boxplots and interquartile ranges. Numbers over boxplots indicate number of measured cells.

(B–F) Representative current density ("Curr. dens.")-time courses at ± 100 mV and current density-voltage relations. Bars indicate bath application of OAG (B, brown bar) or GSK (E, violet bar) or of blue light (C, D, F; blue bar) or UV light (C, D, F; magenta bar) using LEDs. The arrows indicate the time point at which the current density-voltage relations were selected.

Calculation of the normalized slope conductance reveals differences between photoswitchable and non-photoswitchable DAG derivatives

To quantify differences between the current-voltage relations elicited by the different TRPC6 channel activators and between photoswitchable and non-photoswitchable derivatives, we separately calculated the NSC of the current density-voltage relations at negative and positive potentials (Figures 5, S1, and S19). Comparing the DAG derivatives OAG, OptoDARg, and PhoDAG we observed significant differences particularly at negative potentials. These differences in the individual NSC curve progressions were most pronounced between OAG- and *cis*-OptoDARg-induced currents at potential ranges between -100 and -75 mV and -50 to 0 mV (Figure 5A). At positive potentials, the NSC curves mainly differed at potentials between 0 and $+10$ mV and around $+20$ mV. The NSC curve progressions of the *cis*-PhoDAG-induced currents compared

OptoDARg – OptoBI-1 - PhoDAG (LED)

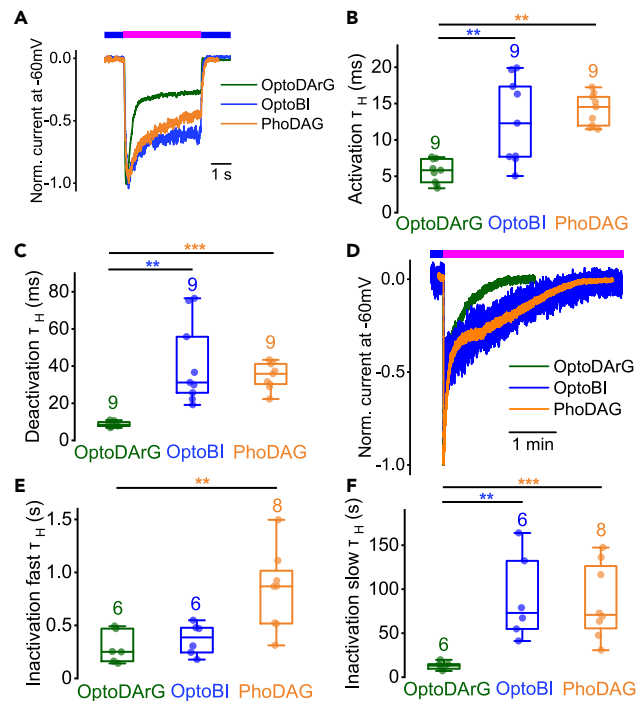


Figure 4. Photoswitching of OptoDARg, PhoDAG, and OptoBI-1 with LEDs results in distinct TRPC6 current kinetics

Electrophysiological whole-cell measurements of TRPC6 overexpressing HEK293T cells.

(A) Representative normalized time courses of TRPC6 currents at constant holding potential of -60 mV during photoswitching from blue light (blue bar) to UV light (magenta bar) and back to blue light (blue bar) using LEDs.

(B and C) Summaries of half-life time constants (τ_H) of the activation (B) and deactivation (C) kinetics ($*p < 0.05$, $**p < 0.01$, Kruskal-Wallis test).

(D) Representative normalized time courses of TRPC6 currents at constant holding potential of -60 mV induced by photoswitching from blue light to UV light using LEDs. The duration of light application (blue and magenta bar) is indicated.

(E and F) Summaries of half-life time constants (τ_H) of the fast (E) and slow (F) inactivation kinetics ($*p < 0.05$, $**p < 0.01$; Kruskal-Wallis test). (B, C, E, and F) Data are displayed as boxplots and interquartile ranges. Numbers over boxplots indicate number of measured cells.

to the OAG-induced currents also mainly differed at negative potentials. However, the differences were less pronounced compared to *cis*-OptoDARg and were only observed between -100 and -80 mV, -50 and 0 mV, and between around $+15$ mV. The NSC curve progression of the outward current was almost similar to OAG (Figure 5A). Altogether, different DAG derivatives elicit distinct NSC progressions which points to distinct active channel states. Comparison of the NSC of the maximal GSK-induced currents and the currents induced by its photoswitchable derivative *cis*-OptoBI-1 revealed only minimal differences at -25 mV (Figure 5B). These findings support the notion that in contrast to the different DAG derivatives, GSK and its photoswitchable derivative OptoBI-1 have the same activation mechanism and cause similar channel gating.

Comparison of photoswitchable TRPC6 activators shows significant differences in the NSC progression

When comparing the NSC progression of the lipidic and non-lipidic photoswitchable activators we observed that *cis*-OptoDARg- and *cis*-PhoDAG-induced currents show no significant differences at negative potentials, but at positive potentials between 0 and $+12$ mV significant differences are observed (Figure 6). The *cis* OptoBI-1-induced currents show distinctive differences at negative potentials between -100 and -70 mV and between -30 and 0 mV and at positive potentials between 0 and $+10$ mV and around $+20$ mV. Furthermore, the *cis*-OptoBI-1- and *cis*-PhoDAG-induced currents exhibit distinct NSC curve progressions at negative potentials between -80 and -75 mV and between -30 and 0 mV (Figure 6). These findings suggest that all three photoswitchable TRPC6 channel activators might induce distinct active channel states. However, the NSC of the currents induced by the photoswitchable DAG derivate are more similar than the currents induced by the photoswitchable GSK-derivative.

DISCUSSION

Photopharmacology is an emerging approach in medicine and life science that involves activation and deactivation of photoswitchable molecules with the spatiotemporal precision of light that might be useful for medical applications such as targeted drug delivery at a specific place and at a precise time.

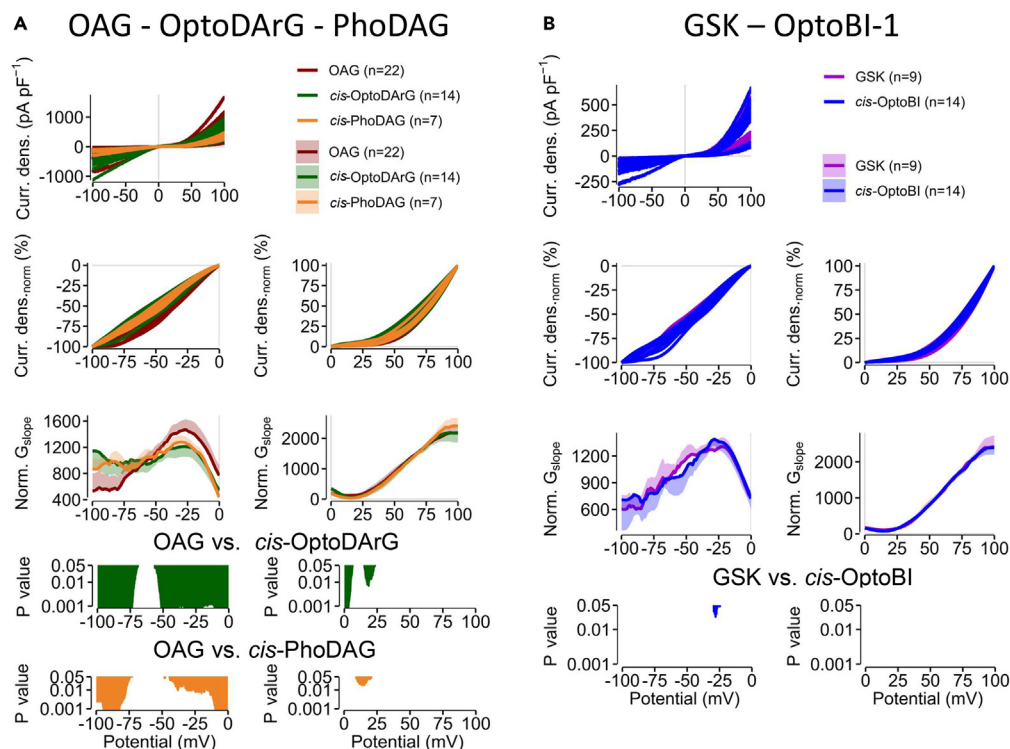


Figure 5. Calculation of the normalized slope conductance reveals differences between photoswitchable and non-photoswitchable TRPC6 activators

Whole-cell measurements of TRPC6 overexpressing HEK293T cells.

(A) All maximal current density-voltage relations ("Curr. dens.") induced by OAG, *cis*-OptoDARg or *cis*-PhoDAG are displayed (previously). The current density-voltage relations were smoothed and normalized ("Curr. dens._{norm} [%]"). The calculated normalized slope conductance (NSC) ("Norm. G_{slope}") is displayed as mean $\pm$ SD. *p* values are calculated using Kruskal-Wallis test.

(B) All maximal current densities ("Curr. dens.") at potentials of ± 100 mV induced by GSK or *cis*-OptoBI-1 are displayed (previously). The current density-voltage relations were smoothed and normalized ("Curr. dens._{norm} [%]"). The calculated NSC ("Norm. G_{slope}") is displayed as mean $\pm$ SD. *p* values are calculated using Mann-Whitney U test.

The development of novel photoswitchable ion channel activators enabled the assessment of current kinetics in the whole-cell mode without interfering wash-in and wash-out effects that usually occur, when compounds are applied via bath solution.¹⁵ The determination of the current kinetics is of particular interest to characterize the pathophysiological role of TRPC6 channels. For example, current kinetics may be altered in patient mutations such as those in the gene coding for TRPC6 that are associated with FSGS. Interestingly, it was shown that some gain-of-function mutations that are co-expressed with wild-type TRPC6 exhibit a slower inactivation kinetics²³ following receptor-activation. In addition, overexpression of TRPC7 channels in embryonic stem cell-derived cardiomyocytes enhanced the kinetics and frequency of intracellular calcium transients.²⁴ In these cases, the use of light-switchable activators would be very useful to analyze the current kinetics more precisely, which might help to further elucidate the (patho-)physiological roles of distinct ion channels in health and disease.

The time span of the *trans* to *cis* isomerization of azobenzene moieties using a high-intensity mercury-xenon arc lamp for illumination was estimated to be in the two-digit picosecond range which was shown for small cyclic peptides containing an azobenzene moiety in the solvent DMSO by performing ultrafast IR spectroscopy.^{25,26} However, DMSO decelerates the transition time compared to water and the coupling of the azobenzene group to an 8 amino acid peptide chain also decelerates the transition time compared to a naked azobenzene group. However, the measured transition times vary strongly depending on the measurement method used.²⁷ Using monochromatic LEDs or a lower intensity xenon lamp, it can be assumed that the power density is a thousand-fold lower compared to a high-intensity mercury-xenon arc lamp which might lead to a substantially slower isomerization time. However, even a 100,000-fold slower transition time of the azobenzene group, up to 1 μ s, is sufficient and still faster than the channel gating, as the transition time of a single channel from the closed to the open state is about 10 μ s.²⁸ Hence, photoswitching of azobenzene groups would be sufficient for evaluating the gating behavior of single channels, and even more so for measurements of channel populations in the whole-cell configuration, because channel populations do not open synchronously but with a delay. Consequently, the time for isomerization is several orders of magnitude faster than the channel gating. It cannot be ruled out that the use of light with higher intensity such as laser light could even increase the current kinetics. However, by using LEDs, we have achieved a timescale that allows us to distinguish between the different current kinetics induced by various photoswitchable activators, enabling relative comparisons of channel kinetics. Nevertheless, the absolute kinetics might be faster.

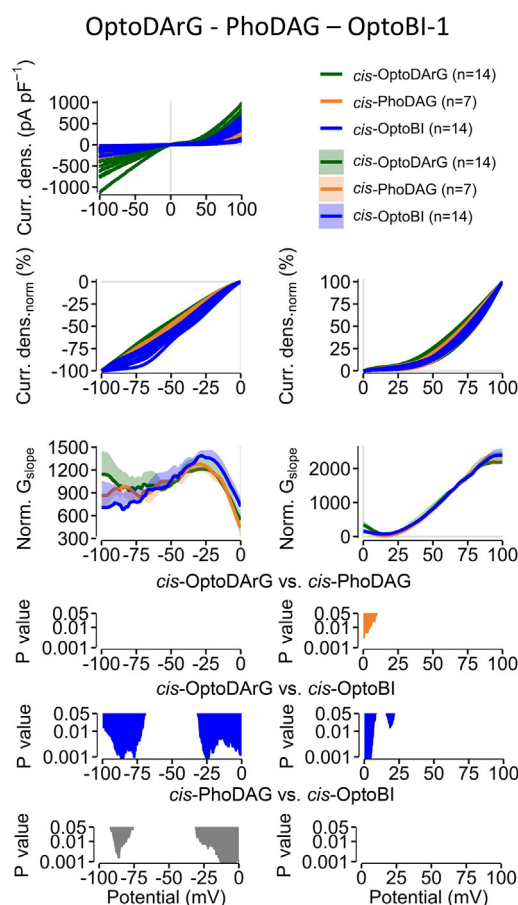


Figure 6. Comparison of photoswitchable TRPC6 activators shows significant differences in the NSC progression

Whole-cell measurements of TRPC6 overexpressing HEK293T cells. All maximal current density-voltage relations ("Curr. dens.") induced *cis*-OptoDARg, *cis*-PhoDAG or *cis*-OptoBI-1 are displayed (previously). The current density-voltage relations were smoothed and normalized ("Curr. dens._{norm} (%)"). The calculated NSC ("Norm. G_{slope}") is displayed as mean $\pm$ SD. p values are calculated using Kruskal-Wallis test.

Our findings suggest that when performing photopharmacology experiments, the selection of the light source for photoswitching is of utmost importance if current kinetics are to be analyzed. We found that xenon lamps are not powerful enough for fast and sufficient photo-switching (Figures 1 and 2) and that only high intensity light sources such as high-power LEDs are suitable. Furthermore, the switching time between the two excitation wavelengths was approximately 320 μ s using a Polychrome V with a xenon lamp, and less than 5 μ s using LEDs. This significant difference supports the use of LEDs, which is crucial for precise determination of current kinetics. Interestingly, the light power densities of the xenon lamp were about 6-times lower than those of the LED light at 100% intensity. Even at 25% light intensity, activation and deactivation kinetics achieved with LED light were still faster than with the xenon lamp. This suggests that not only light intensity but also the switching time between wavelengths is critical for fast and effective photoswitching (see Figure S4). However, if current kinetics are not a priority, low-intensity light sources can also be used, provided the light application is long enough to reach steady-state conditions. To determine maximal current densities, the complete channel activation should occur before significant inactivation effects interfere. Since ion channels open and close very quickly, particularly high light intensities and rapid light switching are required to obtain the most precise results. Nevertheless, there are instrument-based limitations when trying to conduct fast and precise photoswitching. Notably, since activation kinetics of GPCRs is much slower, ultrafast photoswitching of GPCR ligands is not necessary. Altogether, the current kinetics determined by photoswitching relies on the intensity of the light source as well as on the velocity of photoswitching.

Interestingly, we found that *trans*-OptoDARg causes inhibition of constitutive active TRPC3 channels indicating that the *trans* isoform already binds in the binding pocket of the channel. However, the inhibitory effect could not be detected using TRPC6 channels because of their lack of constitutive activity (Figure S17).

OptoDARg was previously shown to impact the membrane capacitance since *cis* isomerization results in reduced membrane thickness leading to an increased membrane capacitance.²⁹ However, in our hands, photoswitching of OptoDARg or OptoBI-1 had no effect on the membrane capacitance of TRPC6 overexpressing HEK293T cells (Figure S20). In contrast to our experiments using 30 μ M OptoDARg in living cells, Pfeiffermann et al. (2021) employed a planar lipid bilayer composed by a membrane lipid and OptoDARg in a 9:1 ratio (90 M lipid

and 10 M OptoDARg). In this experimental setup, OptoDARg was present in an exorbitantly higher concentration and photoswitching was performed using a low intensity xenon lamp which is not sufficient for fast photoswitching and might explain the slow kinetics determined in this study.

To summarize, performing whole-cell measurements and analyzing TRPC6 inward currents induced by photoswitching, we observed that photoswitchable TRPC6 channel activators are suitable for the reliable and reproducible determination of the activation, deactivation, and inactivation kinetics thereby allowing for a more detailed biophysical characterization of channel gating behavior.

Whole-cell measurements are commonly used to study ion channel activity in living cells. When analyzing whole-cell measurements, usually current densities and current-voltage relationships are used for biophysical characterization. Previously, we showed that the calculation of the NSC is beneficial for a detailed biophysical analysis of whole-cell currents.²¹ The NSC enables quantification of significant differences between current-voltage relations elicited by different stimuli or elicited by different ion channels.²¹ By calculating the NSC, voltage ranges can be identified that might be used for more detailed analysis e.g., on the single channel level. Applying this method, we observed differences between the NSC curve progressions induced by different channel modulators suggesting that different stimuli might induce distinct active channel states.²¹ Here, we demonstrate that combining the analysis of current kinetics enabled by photoswitchable compounds with the analysis of current-voltage relationships through NSC calculations allows for a more detailed biophysical characterization of whole-cell currents. This approach considers both current-voltage relations and current time courses, corroborating differences in gating behavior.

Comparison of the NSC progressions of the *cis*-OptoDARg and *cis*-PhoDAG evoked TRPC6 current voltage-relations revealed significant differences between 0 and +10 mV (see Figure 6). Furthermore, the current kinetics of the *cis*-OptoDARg and *cis*-PhoDAG induced TRPC6 currents at a holding potential of −60 mV revealed significant differences in the activation, deactivation, and inactivation kinetics (see Figure 4). In particular, the *cis*-PhoDAG induced current kinetics were significantly slower compared to the *cis*-OptoDARg induced currents kinetics. Thus, both analysis methods show significant differences suggesting that both photoswitchable DAG derivatives induce different active channel states thus reflecting different channel gating. Comparing the NSC of the current voltage-relations induced by the photoswitchable DAG derivatives to the non-photoswitchable DAG-derivative OAG that is washed in via the bath solution (Figure 5A), significant differences over a wide potential range were identified, additionally indicating that each DAG derivative elicits distinct gating behaviors. Interestingly, the differences in NSC and current kinetics were independent from the current density amplitudes (Figure 3A). Since all activators were applied in their maximally effective concentrations, we assume that all binding sites in the tetrameric TRPC6 channel are fully occupied. One piece of evidence for this is that the maximal current densities induced by OptoDARg, PhoDAG, and OptoBI-1 do not differ. However, all three channel activators cause differences in their current kinetics as well as in their NSC curve progressions. Under these conditions, differences in the NSC at a certain potential reflect differences in the product of the open probability (Po) and the single channel conductance at a certain potential ($I_{\text{whole-cell}}(\Phi) = N \times I_{\text{single channel}}(\Phi) \times \text{Po}(\Phi)$; and $G_{\text{whole-cell}}(\Phi) = N \times G_{\text{single channel}}(\Phi) \times \text{Po}(\Phi)$ with I: current; G: conductance; Po: open probability; N: number of channels; Φ : given potential). Given that the number of channels in the whole-cell configuration is constant and that all channels are occupied by the channel activator when using maximally effective concentrations, and given that the single channel conductance is usually constant (around 54 pS for TRPC6²²) over the whole potential range, changes in the NSC are mainly due to changes in the Po meaning that the channel gating behavior is altered. Assuming comparable conditions (such as temperature, solutions, pipettes, parameters of whole-cell configuration, light intensities) and maximally effective concentrations, different activation, deactivation, and inactivation kinetics are pointing to different open channel states ($O_1, O_2, O_3 \dots$), different inactivated channel states ($I_1, I_2, I_3 \dots$) and different closed channel states ($C_1, C_2, C_3 \dots$). Thus, we conclude that OptoDARg, PhoDAG, and OptoBI-1 induce their individual open, closed, and inactivated channel states. However, TRPC channel inactivation is still only poorly understood. We found no correlation between the inactivation kinetics and the current amplitudes (Figure S3) suggesting that the inactivation is independent of the current amplitudes. Presumably, current inactivation is an intrinsic feature of the channel that can be influenced extrinsically e.g., by phosphorylations.^{30,31} Further investigations are required to shed light on this phenomenon.

Altogether, the analysis of the current kinetics enables the determination of distinct open and closed states of the channel which was only possible before by analyzing single-channel currents. However, single channel measurements are technically more demanding and the single channel analysis is considerably more elaborate than whole-cell measurements.

As mentioned previously, DAG derivatives resulted in different current kinetics and differences in the NSC curve progression. Therefore, it can be speculated that TRPC6 channels can discriminate the different DAG derivatives and that the efficiency of each DAG derivative depends on the length and type of the fatty acid moieties. Although DAG is known as endogenous activator of TRPC channels^{1,3,4} the DAG molecule is not well defined since different fatty acid chains with different lengths and compositions including mono- or polyunsaturated fatty acids can naturally occur.³² The most common fatty acid chains found in the human plasma comprise oleic acid which is a mono-unsaturated omega-9 octadecenoic acid, palmitic acid which is a saturated hexadecenoic acid, stearic acid which is a saturated octadecanoic acid, linoleic acid which is a double-unsaturated omega-6 dienoic acid, myristic acid which is an unsaturated tetradecanoic acid, and arachidonic acid which is a quadruple-unsaturated omega-6 tetraenoic acid³² thus enabling a remarkable diversity of DAG molecules. At least 28 different 1,2-DAG molecules were identified.³² The DAG derivative OAG contains an oleic acid and a very short acetic acid chain. In contrast, PhoDAG contains a stearyl acid chain and a photoswitchable fatty acid containing the photoswitchable azobenzene moiety, and OptoDARg contains two equal photoswitchable fatty acid with photoswitchable azobenzene moieties. Thus, the composition of the different DAG derivatives is variable making it likely that different derivatives establish distinct active channel states. Differences between *cis*-PhoDAG

and *cis*-OptoDARg-induced currents have also been observed with TRPC3 currents³³ and it was demonstrated that the deactivation kinetics of *cis*-OptoDARg-induced TRPC3, 6, and 7 currents differs.³³ Nevertheless, in this study we show a detailed analysis of the activation, deactivation, and inactivation kinetics for the first time and our results support the notion that different channel activators cause distinct active channel states with different gating behaviors.

Although recent advances in cryo-EM revealed a potential binding site for DAG in the pore region of the TRPC5 channel which is located between two adjacent TRPC5 protein subunits,³⁴ the channel regulation by DAG is still not completely understood, since the channel resides in its inactive state with a closed channel pore.³⁵ However, photoswitchable DAG-derivatives may help to elucidate the lipid regulation in more detail,^{12,33,36} which was demonstrated by analyzing TRPC3 channels. Hereby, a point mutation in TRPC3 (G652A) was identified which leads to enhanced sensitivity to *cis*-OptoDARg but shows reduced sensitivity to *cis*-PhoDAG.¹² Nevertheless, the lipid regulation of TRPC channels is still not fully understood and needs further investigations.

Photoswitchable compounds are not only effective in the heterologous overexpression system, but they can also be employed to study endogenously expressed channels in primary cells, tissues or organs. For example, PhoDAG and OptoDARg were effective on mouse vomeronasal sensory neurons and olfactory type B cells,^{14,15} and on mouse pancreatic islets and hippocampal neurons.¹³ In addition, OptoBI-1 was shown to be effective on human umbilical vein endothelial cells, and on freshly isolated murine hippocampal neurons.¹⁶ However, for *in vivo* applications or for analyzing tissue slices or organs, UV light that is used for photoswitching has some disadvantages since it is photo-toxic and has a low permeation depth into skin and tissue. Fortunately, the first red light-switchable channel modulators that are activated by harmless red light are already available such as the red-light-switchable TRPV1 channel activator red-AzCA-4.¹⁹ This approach might help to overcome these obstacles.

Altogether, photoswitchable channel modulators allow for an extended biophysical analysis of whole-cell currents. Analysis of the current kinetics and the NSC in combination with structural information derived by cryo-EM might be beneficial to specify interactions between ligands and ion channels and might provide deeper insight into the transduction mechanisms leading to channel gating. In particular, 3D structures of ion channels in combination with photoswitchable activators in their *cis* and *trans* configuration would be important to shed light on the distinct conformational changes of the channel that occur during photoswitching. These findings might be advantageous for the determination of structure-function relations and might enable the design of novel potent channel modulators for the treatment of TRPC channel-dependent diseases. Moreover, the detailed structure-function relations might even allow for synthetic biology and the development of artificial ion channels with distinct functions.

Limitations of the study

The following caveats should be taken into account when analyzing the current kinetics: The use of the appropriate light source is crucial for the reliable determination of the channel kinetics. In particular, the light intensity is of utmost importance for fast and complete photoswitching which is the prerequisite for determination of channel kinetics. Hereby, the switching time between the excitation wavelengths should be as short as possible.

The experiments were all performed in the heterologous overexpression system in HEK293T cells and endogenously expressed TRPC6 channels or TRPC6 channels in other heterologous overexpression systems have not been analyzed.

Furthermore, using sub-maximally effective concentrations of the photopharmaceuticals will decelerate the current kinetics. Thus reliable determination of the current kinetics can only be achieved when using photopharmaceuticals in their maximally effective concentrations.

The Nyquist-Shannon sampling theorem is an essential principle that must be observed.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and R and MATLAB codes should be directed to and will be fulfilled by the lead contact, Prof. Dr. Michael Mederos y Schnitzler (mederos@lrz.uni-muenchen.de).

Materials availability

This study did not generate new unique reagents.

Data and code availability

- All data reported in the paper will be shared by the [lead contact](#) upon request.
- This paper reports original code for R and MATLAB which is available as supplemental data.
- Any additional information required to reanalyze the data reported in this paper is available from the [lead contact](#) upon request.

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AUTHOR CONTRIBUTIONS

M.MyS. and U.S. designed experiments and supervised the study. M.K. performed patch-clamp experiments. D.B.K. designed and supervised the chemical analysis. K.L. conducted chemical analysis and analyzed the chemical data. M.K., C.H., and M.MyS. analyzed the patch-clamp data. U.S. and M.MyS. wrote the manuscript. T.G. and D.B.K. revised the manuscript. C.H. prepared LED control and fit routine with MATLAB R2023a. M.K., M.MyS., U.S. and K.L. prepared the figures. All authors reviewed the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

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SUPPLEMENTAL INFORMATION

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, peptides, and recombinant proteins		
2-Acetyl-1-oleoyl-sn-glycerin	Sigma-Aldrich	Cat. No. 495414
PhoDAG	Sigma-Aldrich	Cat. No. 870621P
Poly-L-Lysine	Sigma-Aldrich	Cat. No. P-1524
Bovine Serum Albumin (BSA)	Sigma-Aldrich	Cat. No. A7030
OptoBI-1	Bio-Techne	Cat. No. 7013
GSK 1702934A (GSK)	Tocris	Cat. No. 6508
OptoDARg	Aobious	Cat. No. AOB31427
DMSO, anhydrous	Sigma-Aldrich	Cat. No. 472301
Genejuice	Merck	Cat. No. 70967
Earl's MEM	Sigma-Aldrich	Cat. No. M4655
Penicillin-Streptomycin	Sigma-Aldrich	Cat. No. P4333
Fetal calf serum (FSC)	Gibco	Cat. No. 10270106
HEPES (2-[4-(2-hydroxyethyl)-1-piperazinyl]-ethanesulfonic acid)	Sigma-Aldrich	Cat. No. H0887
BAPTA (1,2-bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid)	Sigma-Aldrich	Cat. No. A4926
CsCl	Sigma-Aldrich	Cat. No. 203025
Na ₃ -GTP	Sigma-Aldrich	Cat. No. G8877
CsOH	Sigma-Aldrich	Cat. No. C8518
NaCl	Sigma-Aldrich	Cat. No. N31434
Glucose	Sigma-Aldrich	Cat. No. G7528
MgCl ₂	Sigma-Aldrich	Cat. No. M2670
CaCl ₂ solution, 1 M	Sigma-Aldrich	Cat. No. 21115
Deuterated DMSO ((CD ₃) ₂ SO)	Eurisotop	Cat. No. D010F
Deuterium oxide (D ₂ O)	Eurisotop	Cat. No. D214F
Experimental models: Cell lines		
Human embryonic kidney (HEK293T) cells	Leibniz-Institute DSMZ	DSMZ No. ACC 635
Recombinant DNA		
pIRES2-EGFP expression vector	Clontech	N/A
mouse TRPC6	(Zhu et al., 1996) ³⁷	GenBank: NM_013838
human TRPC3	(Boulay et al., 1997) ³⁸	GenBank: NM_001130698
Software and algorithms		
Patchmaster version v2x90.5	Heka	N/A
Origin 2023	OriginLab	N/A
MATLAB R2023a	MathWorks Inc	N/A
R 4.2.3	R Core Team	N/A
MestReNova v.10.0.1–14719	Mestrelab Research	N/A
Cary WinUV	Agilent	N/A
Other		
Glass cover slips 40 mm diameter, thickness 1	Karl-Hecht	Cat. No. 92100101180
LED M365LP1	Thorlabs	N/A
LED M450LP2	Thorlabs	N/A

(Continued on next page)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Optical band-pass filter 360/23 BrightLine HC	AHF	Cat. No. F39-363
Optical band-pass filter 433/24 BrightLine HC	AHF	Cat. No. F37-433
Dichroic beamsplitter HC 376	AHF	Cat. No. F38-376
Dichroic beamsplitter HC 458	AHF	Cat. No. F38-458
Monochromator with xenon short-arc lamp	Till Photonics	Polychrome V
Inverted microscope IX 70	Olympus	N/A
Dichroic beamsplitter H 488 LPXR superflat Vers 2 with Emission filter 525/50 BrightLine HC for microscope	AHF	Cat. No. F48-487 and F37-516
40× oil UV-transmissive apochromatic objective UApo N 340	Evident	N/A
Amplifier EPC 10 USB	Heka	N/A
Energy meter Nova II with sensor PD300	Ophir	N/A
self-made control unit with IC-HG30 laser switches mounted on EVAL HG1D evaluation boards	iC-Haus	N/A
EVAL HG1D evaluation boards	iC-Haus	N/A
microcontroller board Arduino Mega 2560	Arduino SA	N/A
borosilicate glass	Science Products	Cat. No. GB150TF-8P
NMR spectrometer Avance III HD 500 MHz	Bruker	N/A
BioSpin with CryoProbe™ Prodigy	Bruker	N/A
Cary 60 UV-Vis spectrophotometer	Agilent	Cat. No. G6860A
High Precision Cell (quartz glass, 10.00 mm light path)	Hellma Analytics	Cat. No. 104-QS
Illumination system pE-4000	CoolLED	Cat. No. pE-4000

EXPERIMENTAL MODEL AND STUDY PARTICIPANT IN DETAILS

Cell lines

In this study, we used human embryonic kidney (HEK293T) cells (from Leibniz-Institute DSMZ, Braunschweig, Germany, T293, DSMZ no. ACC 635).

METHOD DETAILS

Materials

2-Acetyl-1-oleoyl-sn-glycerin (OAG; Cat. No. 495414), PhoDAG (Cat. No. 870621P), Poly-L-Lysine (Cat. No. P-1524), and Bovine Serum Albumin (BSA; Cat. No. A7030) were purchased from Sigma-Aldrich (Taufkirchen, Germany). OptoDARg was purchased from Aobious (Gloucester, USA; Cat. No. AOB31427), OptoBI-1 was purchased from Bio-Techne (Minneapolis, USA; Cat. No. 7013) and GSK 1702934A (GSK) was purchased from Tocris (Bristol, UK; Cat. No. 6508). OptoDARg and PhoDAG were solved in anhydrous DMSO to 50 mM, OptoBI-1 was solved in anhydrous DMSO to 10 mM, OAG and GSK were solved in anhydrous DMSO to 100 mM. PhoDAG and OptoDARg stock solutions were stored in aliquots at -20°C for maximal 4 weeks. Deuterated DMSO ($(\text{CD}_3)_2\text{SO}$) and deuterium oxide (D_2O) were purchased from Eurisotop. Purchased solvents in HPLC- and analytical-grade quality were used without further purification.

Nuclear magnetic resonance (NMR) spectroscopy

To quantitatively determine the photostationary states (PSS) of OptoDARg, OptoBI-1 and PhoDAG, we analyzed NMR-spectra that were acquired with the spectrometer Avance III HD 500 MHz (Bruker) BioSpin with CryoProbe Prodigy. Deuterated DMSO ($(\text{CD}_3)_2\text{SO}$) and deuterium oxide (D_2O) were used as internal references. Spin multiplicities are described as follows: s (singlet), d (doublet), t (triplet), m (multiplet) or a combination thereof. Spectra analysis was conducted with the software MestReNova v.10.0.1–14719. The experiments were conducted using a 2.5 mM solution of OptoDARg, OptoBI-1 and PhoDAG in $(\text{CD}_3)_2\text{SO}:\text{D}_2\text{O}$ (9:1) at 22°C .

UV-Vis spectroscopy

To determine the PSS nearly quantitatively, as well as the reversibility of photoswitching and the thermal relaxation of OptoDARg, OptoBI-1, and PhoDAG, UV-Vis spectroscopy was applied. UV-Vis spectra were recorded on a Cary 60 UV-Vis spectrophotometer (Agilent) and as cuvette a High Precision Cell (quartz glass, 10.00 mm light path; Hellma Analytics) was used. Analysis was conducted with the Cary win UV/Scan.exe software. Illumination was provided by the illumination system pE-4000 from CoolLED. The optical power output of the LEDs

according to CoolLED is 10 mW/mm² for 525 nm, 15 mW/mm² for 500 nm, 50 mW/mm² for 490 nm, 50 mW/mm² for 470 nm, 50 mW/mm² for 460 nm, 40 mW/mm² for 435 nm, 50 mW/mm² for 405 nm, 50 mW/mm² for 385 nm and 50 mW/mm² for 365 nm. The experiments were conducted using a 50 μ M solution of OptoDARg, OptoBI-1 and PhoDAG in (CD₃)₂SO:D₂O (9:1) at 37°C.

Cell culture and transfection

HEK293T cells were kept in Earl's MEM (Sigma-Aldrich) with 100 units/mL penicillin and 100 μ g/mL streptomycin supplemented with 10% (v/v) FCS (Gibco, Life Technologies, Carlsbad, USA). All cells were held at 37°C in a humidified atmosphere with 5% CO₂. HEK293T cells were transfected with 2 μ g cDNA coding for mouse TRPC6 (GenBank: NM_013838)³⁷ or with human TRPC3 (GenBank: NM_001130698)³⁸ using Gene-judge reagent (Sigma-Aldrich) according to the manufacturer's instructions. The cDNA was in pIRES2-EGFP expression vector (Clontech, Palo Alto, CA). Transfected HEK293T cells were seeded onto poly-L-Lysine-coated glass cover slips (diameter 30 mm, thickness 1, Karl Hecht, Sondheim, Germany) 1 h before patch clamp measurements.

Light stimulation

For light stimulation, two LEDs from Thorlabs (M365LP1 with a peak wavelength of 367 nm and M450LP2 with a peak wavelength of 442 nm; Bergkirchen, Germany) with optical band pass filters from AHF (360/23 BrightLine HC and 433/24 BrightLine HC; Tübingen, Germany) were used that were connected over dichroic beamsplitters from AHF (HC 376 and HC 458, respectively) and that elicited effective light with wavelengths between 346 and 371 nm and between 418 and 447 nm. Comparable wavelengths of 365 $\pm$ 10 nm and 445 $\pm$ 10 nm were applied using the monochromator Polychrome V with a xenon short-arc lamp (Till Photonics, Planegg, Germany) with maximal power which allowed for a switching time between the excitation wavelengths from 445 to 365 nm and vice versa of about 320 μ s. An Olympus IX70 microscope was used with dichroic beamsplitter (H 488 LPXR superflat Vers. 2) combined with the emission filter (525/50 BrightLine HC) from AHF and the 40 $\times$ oil UV-transmissive apochromatic objective (UApo N 340; Evident, Hamburg, Germany). The light intensities were determined at the position in the beam path where the objective lens would normally be located using the laser power or energy meter Nova II with sensor PD300 (Ophir; Jerusalem, Israel). The LEDs were operated by a self-made control unit with IC-HG30 laser switches mounted on EVAL HG1D evaluation boards from iC-Haus (Bodenheim, Germany) connected to the microcontroller board Arduino Mega 2560 (Arduino SA, Chiasso, Switzerland). A self-written MATLAB app (R2023a; MathWorks Inc.; Natick, Massachusetts, USA) served as user-interface driving the microcontroller through serial communication. The effective time of switching between the two LEDs was not more than 5 μ s. The LEDs operated with intensity levels of an 8-bit sigmoidal scale.

Patch-clamp recordings

For patch-clamp measurements, OptoBI-1, OptoDARg, and PhoDAG stock solutions were heated for short time thermal relaxation to 40°C for 10 min and diluted in standard bath solution containing 140 mM NaCl, 5 mM CsCl, 1 mM MgCl₂, 2 mM CaCl₂, 10 mM glucose, 10 mM HEPES (pH 7.4 with NaOH) resulting in an osmolarity of 295–302 mOsm $\cdot$ kg⁻¹ to the final standard concentration of 10 μ M for OptoBI-1, 30 μ M for OptoDARg, and 200 μ M for PhoDAG, just before the measurements. Some indicated measurements were conducted with 200 μ M OptoDARg, 100 μ M PhoDAG, and 30 μ M OptoBI-1. After dilution, the OptoBI-1 solution was used within 1 h. The solutions were illuminated with blue light prior to application to the cells. Conventional whole-cell patch-clamp recordings were carried out at room temperature (23°C) 48 h after transfection. Photoswitching either with LEDs or with xenon lamp had no temperature effects. The mTRPC6 over-expressing HEK293T cells on coverslips were incubated with 30 μ M OptoDARg or 200 μ M PhoDAG solution for 20 to 30 min at room temperature prior to the measurements. The 10 μ M OptoBI-1 solution did not have to be incubated. In the case of OAG and GSK measurements, the cells were superfused with 100 μ M OAG plus 0.1% fatty-acid-free BSA and 10 μ M GSK. OAG, GSK, OptoDARg, PhoDAG, and OptoBI-1 were used in their maximally effective concentrations. The standard pipette solution contained 120 mM CsCl, 9.4 mM NaCl, 0.2 mM Na₃-GTP, 1 mM MgCl₂, 3.949 mM CaCl₂, 10 mM BAPTA (100 nM free Ca²⁺), and 10 mM HEPES (pH 7.2 with CsOH), resulting in an osmolality of 294 mOsm kg⁻¹. The liquid junction potential of +4.0 mV was calculated by JPCalcWin 1.01 (University of New South Wales, Sydney, Australia) and was corrected before the measurements. Data were collected with an EPC10 patch clamp amplifier (HEKA Elektronik, Lambrecht, Germany) using the Patch-master software. Transfected cells were selected by application of light of the wavelength 445 nm to detect their green fluorescent protein EGFP.

For the determination of current density-voltage relationships and normalized slope conductances, repetitive voltage-upramps from –100 to +100 mV were applied. Current-densities analysis and current density-time courses were determined at the holding potential of +100 and –100 mV. For OAG and GSK measurements, a stimulation protocol with a frequency of 2 Hz was applied starting with a holding potential of –100 mV for 50 ms, followed by a voltage upramp from –100 to +100 mV for 400 ms and a holding potential of +100 mV for 50 ms. After each stimulation protocol a capacitance compensation was automatically conducted. To obtain current density-time courses of OptoDARg-, PhoDAG- and OptoBI-1-induced currents, a stimulation protocol with faster voltage upramps was applied with a frequency of 50 Hz starting with a holding potential of –100 mV that was applied for 7 ms, followed by a voltage upramp from –100 to +100 mV for 10 ms and a holding potential of +100 mV for 3 ms to detect fast changes of whole-cell currents. Hereby, data were acquired at a frequency of 5 kHz after filtering at 2.5 kHz. Kinetics of channel activation, deactivation and inactivation were obtained at a holding potential of –60 mV with a sampling frequency of 2 kHz. Patch pipettes were made of borosilicate glass from Science Products (Hofheim, Germany; Cat. No. GB150TF-8P) and had resistances of 1.8–3.2 M Ω .

Normalized slope conductance

The normalized slope conductances (NSCs) were calculated using current voltage relations that were selected at maximal activator-induced current amplitudes or using current-voltage relations before application of the activator or in the presence of blue light before application of UV light. Only current-voltage relations determined by application of a stimulation protocol with a frequency of 2 Hz starting with a holding potential of -100 mV for 50 ms, followed by a voltage upramp from -100 to $+100$ mV for 400 ms and a holding potential of $+100$ mV for 50 ms were used for NSC calculation. The calculation was done as described in Hermann et al.²¹ with the smoothing cubic spline fit and subsequent normalization of current-density voltage relations. Furthermore, smoothed and normalized inward and outward current densities and corresponding NSCs with p values were displayed separately.

Fit routine

To determine the half-life time constant of activation, deactivation and inactivation (τ_H), currents determined at the holding potential of -60 mV were normalized and the time was shifted to zero for fitting with MATLAB R2023a. For τ_H of activation, the median of the current before activation was set to zero and ca. 30% of the maximal activated current was set to $+1$ to exclude the emerging interfering inactivation. For τ_H of deactivation, the median of the current before deactivation was set to $+1$ and the median of fully-deactivated current was set to zero. For τ_H of inactivation the peak current was set to $+1$ and the median of the steady-state current after inactivation was set to zero. Activation and deactivation were fitted by a mono-exponential function (Equations 1 and 2). Inactivation was fitted by a bi-exponential function (Equation 3). Fit optimization was performed with a quadratic error function calculating the summed square of residuals (SSE) (Equations 4 and 5) using the *fminsearch* function and the options described in Table 1.

$$f_{\text{activation}}(t) = a * e^{\frac{\ln 2 * t}{\tau_H}} + c \quad (\text{Equation 1})$$

Equation 1: Mono-exponential fit function $f_{\text{activation}}$ dependent on time (t), initial quantity (a), time constant (τ_H) and offset (c).

$$f_{\text{deactivation}}(t) = a * e^{-\frac{\ln 2 * t}{\tau_H}} + c \quad (\text{Equation 2})$$

Equation 2: Mono-exponential fit function $f_{\text{deactivation}}$ dependent on time (t), initial quantity (a), time constant (τ_H) and offset (c).

$$f_{\text{inactivation}}(t) = a_1 * e^{-\frac{\ln 2 * t}{\tau_{H1}}} + a_2 * e^{-\frac{\ln 2 * t}{\tau_{H2}}} + c \quad (\text{Equation 3})$$

Equation 3: Bi-exponential fit function $f_{\text{inactivation}}$ dependent on time (t), initial quantities (a_1 ; a_2), time constants (τ_{H1} , τ_{H2}) and offset (c).

$$SSE = \sum_t [y_t - f(a, c, \tau_H, t)]^2 \quad (\text{Equation 4})$$

Equation 4: Quadratic error function for mono-exponential fits using the summed square of residuals (SSE) with y_t being the current at time t and $f(a, c, \tau_H, t)$ the result either of Equation 1 or Equation 2 at time t .

$$SSE = \sum_t [y_t - f(a_1, a_2, c, \tau_{H1}, \tau_{H2}, t)]^2 \quad (\text{Equation 5})$$

Equation 5: Quadratic error function for bi-exponential fits using the summed square of residuals (SSE) with y_t being the current at time t and $f(a, c, \tau_{H1}, \tau_{H2}, t)$ the result of Equation 3 at time t .

QUANTIFICATION AND STATISTICAL ANALYSIS

Statistical analyses were conducted in R 4.2.3 and in Origin 2023 (OriginLabs, Northhampton, USA). No statistical methods were used to pre-determine sample size. The p values were calculated by using an unpaired Mann-Whitney-U, a Wilcoxon matched pairs signed rank, one sample Wilcoxon test or Kruskal-Wallis or Friedman test with Dunn's multiple comparison post hoc analysis. A p value less than 0.05 was considered significant for all analysis. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. Boxplots display the median and the interquartile range. Values of half-life time constants (τ_H) are indicated as mean $\pm$ standard deviation (SD). The statistical tests used for statistical analysis can be found in the figure legends.

7. Publikation III

Optical Control of TRPM8 Channels with Photoswitchable Menthol

Autoren: Jasmin Becker, Clara S. Ellerkmann, Hannah Schmelzer, **Christian Hermann**, Kyra Lützel, Thomas Gudermann, David B. Konrad, Dirk Trauner, Ursula Storch, and Michael Mederos y Schnitzler

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Optical Control of TRPM8 Channels with Photoswitchable Menthol

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Abstract: Transient receptor potential melastatin 8 (TRPM8) channels are well known as sensors for cold temperatures and cooling agents such as menthol and icilin and these channels are tightly regulated by the membrane lipid phosphoinositol-4,5-bisphosphate (PIP₂). Since TRPM8 channels emerged as promising drug targets for treating pain, itching, obesity, cancer, dry eye disease, and inflammation, we aimed at developing a high-precision TRPM8 channel activator, to achieve spatiotemporal control of TRPM8 activity with light. In this study, we designed, synthesized and characterized the first photoswitchable TRPM8 activator azo-menthol (AzoM). AzoM enables optical control of endogenously and heterologously expressed TRPM8 channels with UV and blue light which is demonstrated by performing patch-clamp experiments. Moreover, AzoM facilitates the reliable determination of activation, inactivation, and deactivation kinetics thereby providing further insights into the channel gating. Using AzoM, the specific roles of individual amino acids for AzoM or PIP₂ binding and for sensitization by PIP₂ can be elucidated. Altogether, AzoM represents as a high-precision pharmaceutical tool for reversible control of TRPM8 channel function that enhances our biophysical understanding of TRPM8 channels and holds the potential to support the development of novel pharmaceuticals.

Introduction

Transient receptor potential melastatin 8 (TRPM8) channels belong to the transient receptor potential (TRP) channel superfamily. TRPM8 channels are non-selective cation channels that act as cold sensors in the somatosensory system^[1] and are well known as sensors for the natural cooling agent menthol^[2] as well as for synthetic cooling agents such as icilin.^[3] The importance of TRPM8 (and of the heat and pain sensor TRPV1) is underscored by the awarding of the Nobel Prize to David Julius in 2021 for their discovery.

Several physiological and pathophysiological roles of TRPM8 channels have been identified so far. TRPM8 channels are expressed in unmyelinated C fibers which are peripheral sensory neurons of small size that sense and transduce cold stimuli,^[2,4] and are found in various other tissues like prostate, bladder, urogenital tract, cardiovascular and bronchopulmonary tissue.^[5] Notably, the loss of TRPM8 expression in TRPM8 gene-deficient mice is not only correlated to an impaired cold but also to an impaired warm sensation^[6] suggesting a pivotal role of TRPM8 for temperature sensing and thermoregulation. Moreover, TRPM8 is involved in various pathophysiological states, such as in pain

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sensation. A reduced TRPM8 expression is correlated with a reduced migraine risk and with a decreased sensation of cold-induced pain.^[7] However, a hypersensitivity of TRPM8 leads to cold allodynia during pathological conditions such as inflammation or nerve injury.^[8] Furthermore, alterations in expression of TRPM8 is related to different kinds of cancer including prostate, pancreas, breast, lung, skin and colon cancer.^[9] TRPM8 expression is also linked to dry eye disease,^[10] overactive bladder, irritable bowel syndrome, oropharyngeal dysphagia, chronic cough and hypertension.^[11] In addition, TRPM8 plays a role for obesity and type 2 diabetes.^[12] Thus, TRPM8 channels emerged as promising drug targets for various pathophysiological conditions, highlighting the need for potent TRPM8 modulators that could serve as novel treatments for TRPM8-related diseases. To develop precise channel modulators with specific modes of action, detailed knowledge of the channel's regulation and ligand-binding mechanisms is essential.

Recent advances in structural analysis using cryo-electron microscopy (cryo-EM) have provided insights into the binding sites for synthetic TRPM8 channel activators such as icilin, WS-12,^[13] 1-disopropylphosphorylnonate (C3)^[14] or allyl isothio-cyanate (AITC).^[15] However, structural analysis of a TRPM8 channel in the presence of (–)-menthol is still lacking. Furthermore, it is well known, that TRPM8 activity largely depends on the presence of the membrane phospholipid phosphatidylinositol 4,5-bisphosphate (PIP₂). By analyzing reconstituted TRPM8 channels in planar lipid bilayers it was shown that TRPM8 is functional only in the presence of PIP₂^[16] and that PIP₂ binding sensitizes TRPM8 towards stimulation with cold temperature or cooling agents.^[16]

To further decipher the role of TRPM8 in health and disease, we aimed at developing a novel photoswitchable TRPM8 channel activator based on the natural cooling agent menthol that can be switched ON and OFF with the spatiotemporal precision of light.

Photoswitching is commonly achieved by incorporating a photoswitchable azobenzene moiety, which enables *cis-trans* isomerization through UV or blue light. Advances in photopharmacology have already led to the development of several high-precision pharmaceutical tools, allowing for spatiotemporal control of protein function simply by switching light.^[17]

A variety of photoswitchable TRP channel ligands that target TRPC^[18,19,20], TRPV1^[21,22] or TRPA1^[23] channels have been developed so far. These photopharmaceuticals offer an additional advantage due to their rapid transition times from the *trans* to the *cis* configuration, occurring within a two-digit picosecond range, making them highly suitable for determining the activation and deactivation kinetics of currents.^[24]

In this study, we designed, synthesized and characterized the photoswitchable menthol derivative azo-menthol (AzoM) demonstrating its ability to reversibly control TRPM8 channel activity via light. AzoM allows precise determination of activation and deactivation kinetics without the wash-in and wash-out effects typically seen with

bath-applied ligands. By examining changes in current kinetics in AzoM and PIP₂ binding mutants, we identified and characterized potential binding sites. Additionally, we analyzed the influence of PIP₂ on current kinetics and discovered a PIP₂ sensitization mutant. Overall, AzoM provides valuable insights into the regulation of TRPM8 channel activity.

Results and Discussion

Synthesis and Characterization of Photoswitchable Azo-Menthol

To allow for a precise and optical control over TRPM8 channels we developed a photoswitchable menthol-derived chemical probe. Hereby, an optical switch is incorporated via azologization of the backstrategy that entails the replacement of a chemical fragment within a pharmacophore that is structurally related to azobenzene with this chemical photoswitch.^[18,21a,25] (–)-Menthol is a known TRPM8 channel activator with an EC₅₀ of 200 μM while CPS-125 exhibited an EC₅₀ of 32 μM in the same study.^[26] However, the EC₅₀ values for (–)-menthol vary strongly between 4 and 200 μM in literature.^[2a,26–27] We based our azologization strategy on the hypothesis that the biaryl sulfonylamide group in CPS-125^[26] serves as a *cis*-azoster and could therefore be substituted for an azobenzene moiety without significant loss of bioactivity. To synthesize azo-menthol (AzoM) (Figure 1A), menthol carboxylic acid (**1**) was first prepared through a literature procedure.^[28] The menthol carboxylic acid (**1**) was then converted to menthol carboxylic acid chloride (**2**) with oxalyl chloride and coupled with 4-aminoazobenzene (**3**) to give AzoM (Supplemental Figure 1).

AzoM allowed for *cis* and *trans* isomerization by applying blue light or UV light, respectively (Figure 1B). For detailed characterization of AzoM, we determined the photostationary states (PSS), the reversibility of photoswitching and the thermal relaxation. The PSS, which is the equilibrium state between the *trans* and *cis* configuration at a given illumination wavelength, was first estimated using UV/Vis spectroscopy (Figure 1C). The maximal absorption peak of *trans*-AzoM was at 359 nm and of *cis*-AzoM at 439 nm. The proportion of the *cis* isomer can be semi-quantitatively determined.^[29] In the dark, after complete thermal relaxation, 100 % of the *trans* configuration was achieved. In order to obtain a PSS with the highest percentage of the *trans* configuration the most appropriate illumination wavelength was 525 nm resulting in 84 % *trans* and 16 % *cis* configuration. Focusing on the illumination wavelength of 435 nm, which is closest to the wavelength used in patch-clamp measurements to induce the *trans* configuration, we found that AzoM achieves approximately 78 % *trans* and 22 % *cis* configuration. The illumination with 365 nm resulted in 15 % *trans* and 85 % *cis* configuration. For a more precise quantitative analysis of the PSS, we used ¹H NMR spectroscopy (Supplemental Figure 2). The dark-adapted compound was measured after thermal relaxation

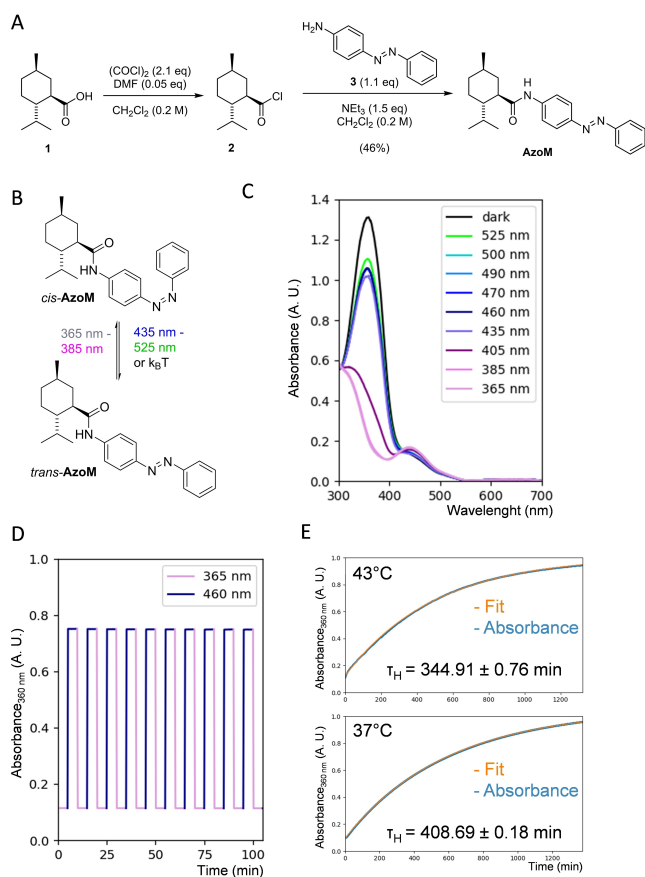


Figure 1. Synthesis and characterization of photoswitchable azo-menthol. (A) Synthesis of azo-menthol (AzoM) using menthol carboxylic acid (1) that was converted to menthyl carboxylic acid chloride (2) with oxalyl chloride and coupled with 4-aminoazobenzene (3) to give AzoM. (B) Chemical structures of AzoM in its *cis* and *trans* configuration (*cis*-azo-menthol; *cis*-AzoM and *trans*-azo-menthol; *trans*-AzoM). (C) UV/Vis spectroscopic analysis of AzoM. Spectral absorbance from 300 nm to 700 nm was measured at 22 °C. The dark-adapted compound was measured after thermal relaxation for 3 days at 70 °C. (D) UV/Vis spectroscopic analysis of AzoM. Absorbance was measured at 360 nm at 22 °C. Compound was repeatedly illuminated with 365 nm and 460 nm light for 5 minutes each over a period of 110 minutes. (E) UV/Vis spectroscopy of AzoM. The compounds were pre-illuminated with 365 nm followed by relaxation in the dark at 43 °C and at 37 °C. The thermal relaxation was determined through UV/Vis analysis by measuring the time-dependent absorption at 360 nm with $A(t) = A_0 \cdot e^{-\frac{t}{\tau_H} + c} + c$. A_0 : initial absorbance; c : maximal absorbance; τ_H : of half-life time constant. Fitted curve in orange and measured absorbance in blue. (C–E) UV/Vis spectroscopy was done with 50 μ M AzoM in 9:1 (CD₃)₂SO:D₂O.

for 3 days at 70 °C leading to full *trans* configuration (100 % *trans* and 0 % *cis* configuration). Illumination of AzoM with 435 nm resulted in a ratio of 69 % *trans* and 31 % *cis* configuration, and illumination with 365 nm caused a ratio of 5 % *trans* and 95 % *cis* configuration.

To analyze the reversibility of photoswitching using UV/Vis spectroscopy, AzoM was repeatedly illuminated with alternating wavelengths of 365 nm and 460 nm for a total time of 110 minutes at 22 °C (Figure 1D). Within this period of time no changes in maximal and minimal absorptions

were observed suggesting that photoswitching is fully reversible and that the compound does not degrade.

Furthermore, we analyzed the thermal relaxation rate using UV/Vis-spectroscopy. This property refers to the thermal transition from the *cis* to the *trans* configuration in the dark after application of 365 nm light. The half-life time constant (τ_H) for thermal relaxation was 344.91 ± 0.76 minutes at 43 °C and 408.09 ± 0.18 minutes at 37 °C (Figure 1E), suggesting that complete relaxation from the *cis* to the *trans* configuration occurs after approximately 29 hours at 43 °C or 34 hours at 37 °C (50 μ M solution in 9:1 (CD₃)₂SO:D₂O).

Optical Control of TRPM8 with AzoM

To ascertain the suitability of AzoM for optical control over TRPM8 channel activity, we performed patch-clamp measurements in the whole-cell configuration with HEK293T cells overexpressing TRPM8 in the presence of 200 μ M AzoM which is equimolar to the maximally effective concentration of the TRPM8 activator (–)-menthol utilized in our experiments. Switching to UV light or blue light that establishes the active *cis* (*cis*-AzoM) or the inactive *trans* (*trans*-AzoM) configuration caused TRPM8 current density (CD) increases or decreases at room temperature (Figure 2A–2C). Since TRPM8 channels are cold sensors that are strongly temperature dependent and have a temperature coefficient Q_{10} of about 24 at temperatures between 18 and 25 °C,^[30] TRPM8 was already activated at room temperature. To suppress these cold-induced currents, the measurements were repeated at 37 °C which resulted in largely reduced CD amplitudes (Figure 2D–2F). However, *cis*-AzoM was effective at both temperatures and elicited significant TRPM8 CD increases. In the absence of AzoM, no CD changes were detected by light switching neither at room temperature or at 37 °C (Figures 2A, 2D and 2G). The summary of the maximal *trans*- and *cis*-AzoM-induced CD in outward and inward direction at ± 100 mV (Figure 2B and 2E) shows significant CD increases in the presence of *cis*-AzoM compared to *trans*-AzoM with characteristic current density (CDV) relationships (Figure 2C and 2F) indicating that AzoM is appropriate for optical control of TRPM8 channels. Interestingly, at 37 °C, when cold-induced currents are largely diminished, the *trans*-AzoM-induced CD at +100 mV are significantly reduced compared to the basal CD in the absence of AzoM (Figure 2E). Application of increasing concentrations of *trans*-AzoM at room temperature resulted in stepwise reductions of the cold-induced TRPM8 currents (Figure 2I) suggesting that *trans*-AzoM has a concentration-dependent inhibitory effect with an estimated IC₅₀ of about 103 μ M. These findings indicate that *trans*-AzoM is already bound in the binding pocket of the TRPM8 channel.

The EC₅₀ value for *cis*-AzoM at 37 °C was estimated at 4.4 ± 5.8 μ M with a slope of 0.5 ± 0.3 (Figure 2H). Thus, 50 μ M AzoM represent the maximally effective concentration and was therefore used in all further experiments.

As a control, the TRPM8 activators (–)-menthol (200 μ M) and icilin (10 μ M) were applied in their maximally

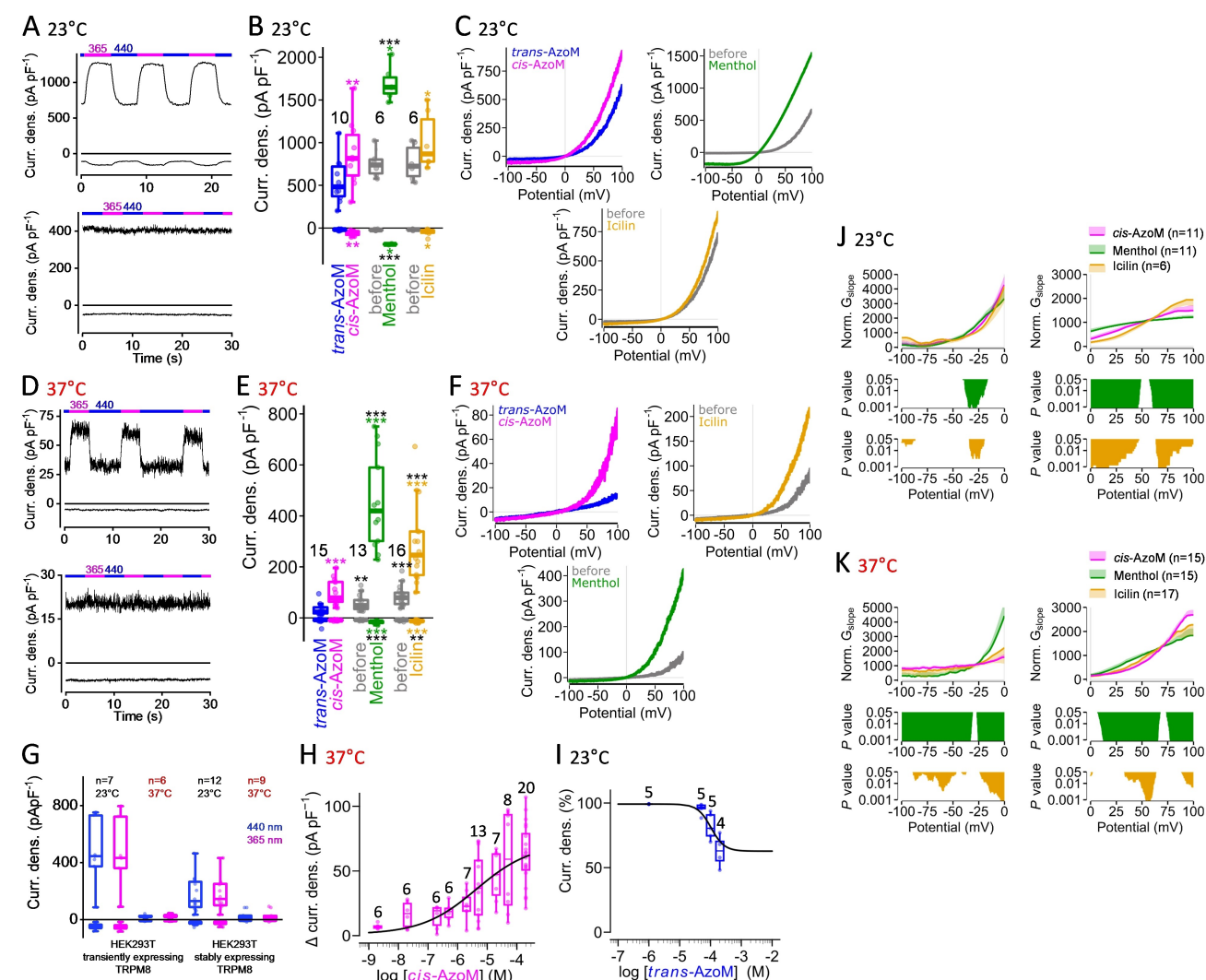


Figure 2. Optical control of TRPM8 with azo-menthol (AzoM). Whole-cell measurements of TRPM8 overexpressing HEK293T cells. (A, D) Representative current density (CD) time courses at ± 100 mV in the presence (above) or absence (below) of AzoM (200 μ M) measured at 23°C (A) and 37°C (D). Illumination with UV light ($\lambda = 365$ nm) and blue light ($\lambda = 440$ nm) is indicated. (B, E, G) Summaries of maximal CD ('Curr. dens.') at potentials of ± 100 mV at 23°C (B, G) and 37°C (E, G) in the presence (B, E) or absence (G) of AzoM. (C, F) Representative CD voltage (CDV) relations in the presence of 200 μ M *trans*- (*trans*-AzoM) and *cis*-azo-menthol (*cis*-AzoM) and before and during application of 200 μ M (–)-menthol and 10 μ M icilin. Numbers over boxplots indicate number of measured cells. Black asterisks indicate significance compared to *trans*- or *cis*-AzoM ($***P < 0.001$, $**P < 0.01$; Mann-Whitney U test), colored asterisks indicate significances between *trans*- and *cis*-AzoM, or between menthol- or icilin-induced CD compared to basal CD in the absence of the activators ($**P < 0.01$, $***P < 0.001$; Wilcoxon matched-pairs signed-rank test). (H) Concentration response curve to determine EC_{50} of *cis*-AzoM at 37°C with HEK293T cells stably expressing TRPM8. (I) Concentration response curve of cold-induced TRPM8 currents at 23°C before and during application of increasing concentrations of *trans*-AzoM. (J, K) The normalized slope conductance (NSC) ('Norm. G_{slope} ') of the CDV curves which was separately calculated for inward and outward currents (left and right graphs). The NSC is displayed as mean $\pm$ SD. P values are calculated using Mann-Whitney U test compared to *cis*-AzoM-induced NSC. (B, E, G, H, I) Data are displayed as boxplots and interquartile ranges. A slow up-ramp protocol with a frequency of 2 Hz was applied.

effective concentrations. (–)-menthol elicited significantly higher CD increases compared to *cis*-AzoM (Figure 2B and 2E) at 23°C and 37°C. The icilin-induced CD were significantly higher than the *cis*-AzoM-induced CD only at 37°C (Figure 2E) suggesting that icilin is more effective when cold-induced TRPM8 currents are diminished. However, the icilin-induced CD increases were lower than the (–)-menthol-induced CD (Figure 2B and 2E).

To investigate differences between the CDV curves elicited by the TRPM8 channel activators *cis*-AzoM, (–)-

menthol and icilin, we calculated the normalized slope conductance (NSC), which is a tool for a quantitative in-depth analysis of CDV curves. At 23°C, the slope progressions of *cis*-AzoM-, (–)-menthol- and icilin-induced NSC curves mainly differs at positive potentials. At negative potentials, the NSC only slightly differs in the range of -40 to -20 mV (Figure 2J). At positive potentials, the curve progression of the NSC of *cis*-AzoM lies in between the two other NSC curves (Figure 2J) suggesting an intermediate channel state. At 37°C, the NSC progression of the (–)-

menthol-induced current strongly differs from the NSC of the *cis*-AzoM- or the icilin-induced currents (Figure 2K) in particular at negative potentials, where the icilin- and *cis*-AzoM-induced NSC are more similar in their curve progressions. However, at positive potentials, the NSC of the icilin- and (–)-menthol-induced currents are more alike in their curve progression and strongly differ from the NSC of *cis*-azoM indicating that *cis*-AzoM elicits a distinct active channel state. Comparing the NSC of the basal TRPM8 currents before application of icilin or (–)-menthol with the NSC in the presence of *trans*-AzoM shows only minor differences at room temperature (Supplemental Figure 3). However, at 37 °C, the NSC of the *trans*-AzoM-induced current is significantly different to the NSC of the basal currents in the absence of any activators (Supplemental Figure 3) corroborating the finding that *trans*-AzoM has an inhibitory effect on TRPM8. Altogether, *trans*- and *cis*-AzoM elicit distinct NSC curves that are different from the icilin- or (–)-menthol-induced NSC curves suggesting that all three TRPM8 channel activators cause distinct active channel states. In addition, the NSC analysis allowed for determination of a distinct *trans*-AzoM induced inactive channel state.

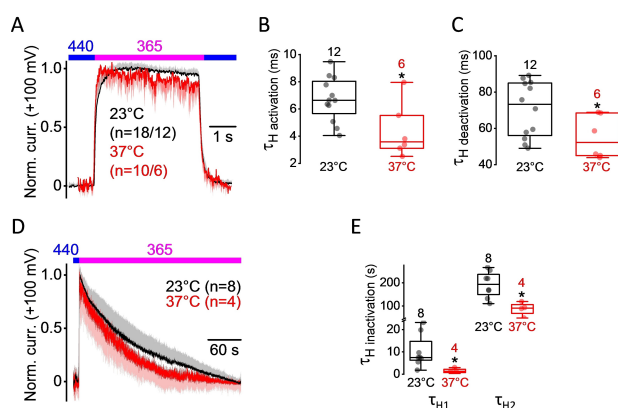


Figure 3. Activation, inactivation and deactivation kinetics of TRPM8 currents. Whole-cell measurements of TRPM8 overexpressing HEK293 cells in the presence of 50 μ M AzoM. (A) Normalized current time courses at +100 mV determined by application of a fast up-ramp protocol with a frequency of 50 Hz measured at 23 °C and 37 °C displayed as median $\pm$ SD. Illumination with 440 and 365 nm is indicated. (B, C) Summaries of the half-time constants for activation (B, $\tau_{H\text{ activation}}$) and inactivation (C, $\tau_{H\text{ deactivation}}$) at 23 °C (black) and at 37 °C (red). Asterisks indicate significance compared to 23 °C (* P < 0.05, Mann-Whitney U test). (D) Normalized current time courses at +100 mV determined by application of a fast up-ramp protocol with a frequency of 50 Hz measured at 23 °C and 37 °C with constant illumination with 365 nm. (E) Summaries of the half-time constants for fast (τ_{H1}) and slow inactivation (τ_{H2}) at 23 °C (black) and at 37 °C (red). Asterisks indicate significance compared to 23 °C (* P < 0.05, Mann-Whitney U test). (B, C, E) Numbers over boxplots indicate number of measured cells. Data are displayed as boxplots and interquartile ranges. (A, D) Illumination protocol using light of the wavelengths λ = 365 nm or λ = 440 nm is depicted above the traces.

Activation, Deactivation and Inactivation Kinetics of TRPM8 Currents

To find out whether AzoM is suitable to precisely determine the activation and deactivation kinetics of TRPM8 currents without disturbing wash-in and wash-out effects that usually occur when using perfusion systems, we analyzed the TRPM8 outward currents at +100 mV during photoswitching at 23 °C and at 37 °C (Figure 3A). As expected, the activation and deactivation kinetics were significantly increased at 37 °C compared to 23 °C (Figure 3B and 3C). τ_H for the deactivation ($\tau_H = 73.2 \pm 15.4$ ms at 23 °C and $\tau_H = 52.2 \pm 11.8$ ms at 37 °C) are more than 11-fold higher than for the activation ($\tau_H = 6.6 \pm 1.6$ ms at 23 °C and $\tau_H = 3.6 \pm 2.0$ ms at 37 °C). In addition, the inactivation kinetics were estimated by analyzing the time courses of the normalized outward currents during constant illumination with UV light of 365 nm (Figure 3D). The inactivation kinetics is biphasic comprising a fast followed by a slow inactivation phase ($\tau_{H1} = 7.5 \pm 7.4$ s and $\tau_{H2} = 193.4 \pm 56.1$ s at 23 °C; $\tau_{H1} = 1.3 \pm 1.1$ s and $\tau_{H2} = 90.9 \pm 29.2$ s at 37 °C) (Figure 3E).

Endogenously Expressed TRPM8 Channels can be Controlled by Light

To avoid animal experimentation, we tested different cell lines for endogenous TRPM8 expression. We found that the human prostate cancer cell line PC-3^[31] functionally expresses TRPM8 channels that can be controlled by photo-switching (Figure 4). Whole-cell measurements with PC-3 cells in the presence of AzoM show that repeated photo-switching from blue light to UV light results in transient CD increases and decreases that can be blocked by the potent and selective TRPM8 blocker M8B. These findings suggest that AzoM is also suitable for light-induced activation of endogenously expressed TRPM8 channels.

Effects of AzoM on Potential AzoM Binding Mutants

Next, we aimed at shedding light upon the binding site of AzoM and investigating whether AzoM is useful for a comprehensive characterization of potential AzoM binding mutants.

3D structures in complex with (–)-menthol are still missing. Nevertheless, the binding sites for the cooling agents icilin,^[32] WS-12,^[32] C3^[33] and AITC^[33] were shown to be located in the voltage sensing like domain (VSLD) cavity of TRPM8 which is formed by the amino acids (numbers refer to human TRPM8; TM: transmembrane domain; TRP: TRP domain; iL2: intracellular linker between TM4 and TM5): Y745^{TM1}, R842^{TM4}, H845^{TM4}, Y1005^{TRPd} and R1008^{TRPd}. The menthol-derivative WS-12 particularly binds to Y745^{TM1}, R842^{TM4}, Y1005^{TRPd} and R1008^{TRPd}, and icilin to Y745^{TM1}, R842^{TM4}, H845^{TM4} and Y1005^{TRPd}. The cooling agent C3 also binds to Y745^{TM1}, R842^{TM4} and H845^{TM4}. The binding site of AITC differs from the icilin, WS-12 and C3 binding site and includes the amino acids M801^{TM3}, W798^{TM3}

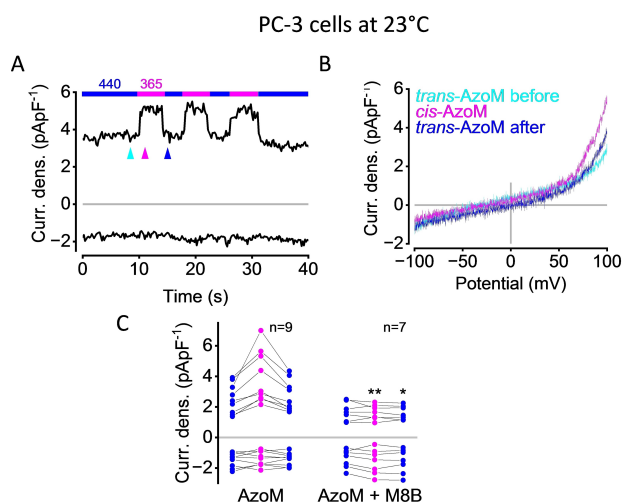


Figure 4. Endogenously expressed TRPM8 channels can be controlled by light. Whole-cell measurements of prostate cancer cells PC-3 that endogenously express TRPM8 channels in the presence of 50 μ M AzoM measured at 23 °C. (A, B) Representative measurement with CD time course at ± 100 mV (A) and corresponding CD voltage (CDV) relations in the presence of *trans*- and *cis*-AzoM (B) obtained by illumination using light of the wavelengths $\lambda = 365$ nm or $\lambda = 440$ nm. (C) Summaries of maximal CD ('Curr. dens. (pA/pF)') at potentials of ± 100 mV in the presence of *trans*- (blue dots) or *cis*-AzoM (violet dots) induced by photoswitching in the presence or absence of the selective TRPM8 blocker M8B (100 nM). Black asterisks indicate significances compared to *trans*- or *cis*-AzoM in the absence of M8B (** $P < 0.01$; * $P < 0.05$; Mann-Whitney U test).

and Q861¹¹². Altogether, the binding sites for cooling agents surround the C-terminal part of TM4 and are located near the VSLD, the TRP domain and the pore domain.^[32–33] Furthermore, mutagenesis approaches, summarized by Plaza-Cayon and colleagues,^[34] suggested that (–)-menthol might interact with Y745^{TM1},^[35] Q785^{TM2},^[36] R842^{TM4},^[37] and Y1005^{TRPd}.^[35a] However, Yin and colleagues found that Q785^{TM2} interacts with a calcium ion in the same cavity^[32] and therefore does not directly participate in agonist binding. Besides Q785^{TM2}, the amino acids E782^{TM2}, N799^{TM2} and D802^{TM3} also contribute to binding of calcium ions.^[32] This calcium-binding site is located in the same cavity as the binding site for the cooling agents.

Based on these previous findings, we assumed that AzoM binds at a similar site as other cooling agents^[32–33] in the VSLD cavity. To identify the potential binding site of *trans*- and *cis*-AzoM, we performed a molecular docking approach based on an open state model of TRPM8^[38] using *trans*- and *cis*-AzoM compared to (–)-menthol, icilin, WS-12 and C3. The following amino acids emerged as potential binding sites for *trans*- and *cis*-AzoM and were exchanged by performing site-directed mutagenesis as indicated: N741A^{TM1}, Y745H^{TM1}, L778A^{TM2}, D781A^{TM2}, E782A^{TM2}, Q785A^{TM2}, N799A^{TM3}, D802A^{TM3}, G805A^{TM3}, F839A^{TM4}, R842A^{TM4}, L843A^{TM4}, I846V^{TM4}, and Y1005F^{TRPd} (Figure 5A and 5B).

All TRPM8 mutants were analyzed performing whole-cell measurements in the presence of 50 μ M AzoM at room

temperature to additionally monitor the effect of the amino acid exchanges on the cold-induced TRPM8 currents. Furthermore, the activation and deactivation kinetics were determined (Figure 5 and Supplemental Figure 4). Hereby, we proceed from the assumption that reduced *cis*-AzoM-induced CD amplitudes point to a participation in AzoM binding. In addition, we also assumed that slower activation and/or faster deactivation kinetics indicate a role in AzoM binding. The mutants N741A^{TM1} and L778A^{TM2} which occurred in the AzoM dockings, exhibited a complete impairment of channel function without any measurable current increases (Figure 5C and 5D). When calculating the NSC (Supplemental Figure 5), no differences between the NSC curves in the presence of *cis*- or *trans*-AzoM occurred, suggesting no channel activity. Consequently, no activation or deactivation kinetics could be determined.

While the *trans*-AzoM-induced CD of the mutant Y745H^{TM1} was similar to the wildtype CD, the *cis*-AzoM-induced CD was significantly reduced, pointing to a crucial role of Y745^{TM1} for AzoM binding which is corroborated by an about four-fold slower activation kinetics (Figure 5E). The mutant D781A^{TM2} also showed an impaired channel function with largely reduced CD, but with slight *cis*-AzoM-induced CD increases suggesting that the mutant D781A^{TM2} is still functional (Figure 5C and 5D). The deactivation kinetics were significantly faster compared to the wildtype channel (Figure 5F) pointing to a participation of D781^{TM2} in AzoM binding. The mutants E782A^{TM2}, Q785A^{TM2}, N799A^{TM3} and D802A^{TM3} which are involved in Ca²⁺ binding in the binding pocket, showed *cis*-AzoM-induced CD increases (Figure 5C and 5D). However, the mutants E782A^{TM2}, Q785A^{TM2}, and D802A^{TM3} exhibited overall reduced CD amplitudes, while the mutant N799A^{TM3} showed similar CD amplitudes as the wildtype channel. The mutants E782A^{TM2} and D802A^{TM3} showed faster deactivation (Figure 5F) and Q785A^{TM2} slower activation kinetics (Figure 5E). The mutant N799A^{TM3} showed even faster activation and deactivation kinetics (Figure 5E and 5F) suggesting that this amino acid is more likely to transduce agonist binding into channel gating, which is referred to as gating transduction, rather than contributing to agonist binding. Altogether, the calcium binding mutants that were previously suggested to stabilize the icilin binding,^[32] define the 3D structure of the AzoM binding site thereby indirectly influencing the AzoM binding.

The mutant G805A^{TM3} also showed *cis*-AzoM-induced CD increases with overall reduced CD amplitudes (Figure 5C and 5D) and exhibited a significantly faster deactivation kinetics (Figure 5F) pointing to a direct contribution to AzoM binding. Calculating the NSC of the potential AzoM binding mutants, we found that all mutants except R842A^{TM4} exhibit different gating behavior compared to the wildtype channel (Supplemental Figures 6 and 7). The mutants F839A^{TM4} and R842A^{TM4} were both functional showing *cis*-AzoM induced CD increases with overall reduced CD amplitudes (Figure 5C and 5D). However, in case of F839A^{TM4}, the deactivation kinetics were slower (Figure 5F) opposing a direct participation in AzoM binding but rather implying a role for gating transduction.

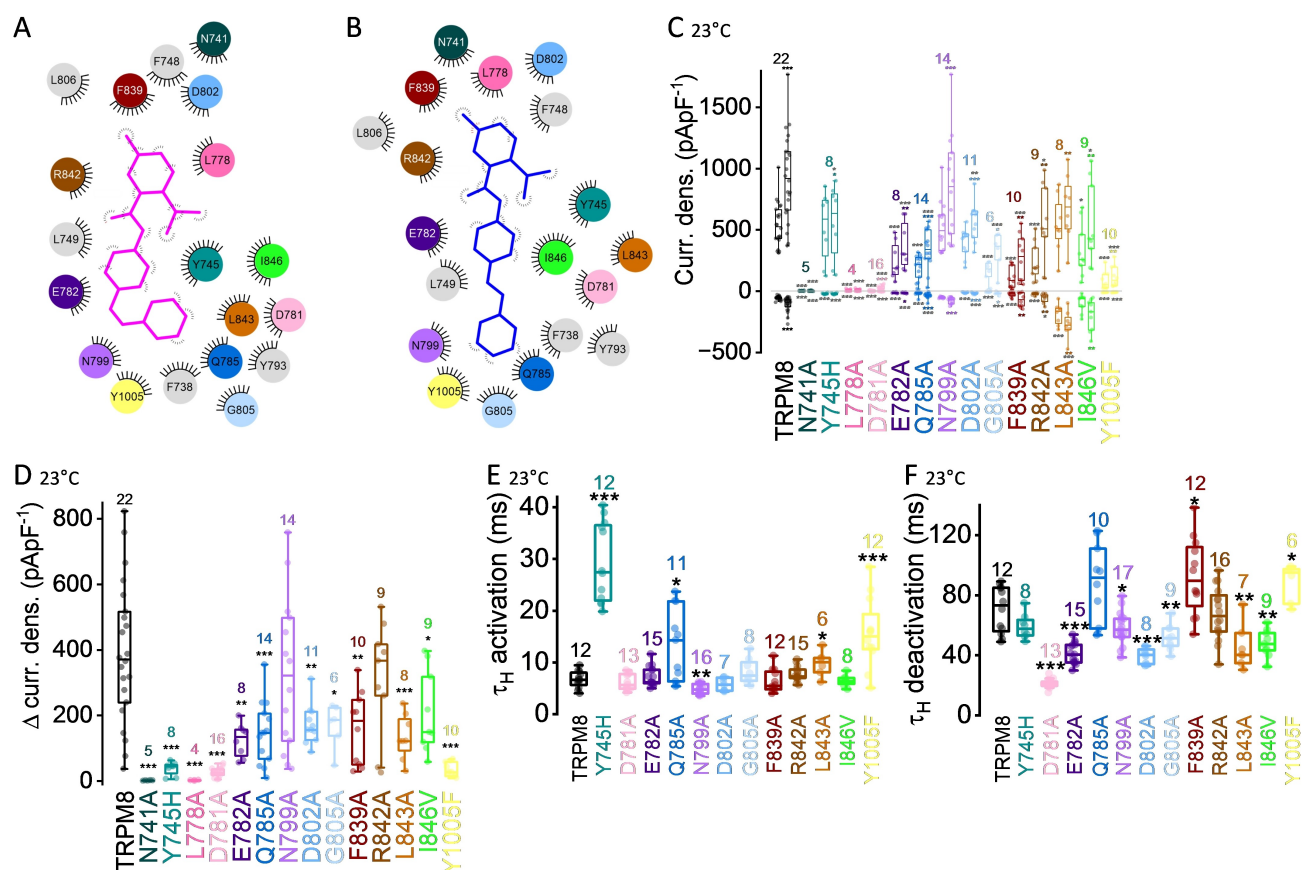


Figure 5. Effect of AzoM on potential AzoM binding mutants. (A, B) Synopsis of results from molecular docking approach with amino acids that are potentially involved in *trans*- and *cis*-AzoM binding. (C–F) Whole-cell measurements of HEK293T cells that overexpress TRPM8 or indicated TRPM8 mutants in the presence of 50 μ M AzoM at 23 °C. (C) Summary of maximal CD ('Curr. dens.') at potentials of ± 100 mV induced by *trans*-AzoM (left boxplot) or *cis*-AzoM (right boxplot). Black asterisks indicate significant differences compared to the wildtype (*** P < 0.001, ** P < 0.01; * P < 0.05; Mann-Whitney U test), colored asterisks indicate significances between *trans*- and *cis*-AzoM (** P < 0.01, *** P < 0.001; * P < 0.05; Wilcoxon matched-pairs signed-rank test). (D) Summary of normalized CD calculated as differences between *cis*- and *trans*-AzoM-induced CD. Asterisks indicate significant differences compared to wildtype (*** P < 0.001, ** P < 0.01; * P < 0.05; Mann-Whitney U test). (E, F) Summaries of the half-time constants for activation (τ_{H} activation) and for deactivation (τ_{H} deactivation) at 23 °C. Asterisks indicate significance compared to wildtype (*** P < 0.001, ** P < 0.01; * P < 0.05; Mann-Whitney U test). (C–F) Numbers over boxplots indicate numbers of measured cells. Data are displayed as boxplots and interquartile ranges. A fast up-ramp protocol with a frequency of 50 Hz was applied.

In contrast, in case of R842A^{TM4}, neither activation nor deactivation kinetics were changed (Figure 5E and 5F). The NSC curve progression of R842A^{TM4} was similar to that of wildtype TRPM8 (Supplemental Figure 7), suggesting that the amino acid exchange from arginine to alanine at position 842 reduces membrane expression, rather than influencing the channel gating. Because of the unchanged activation and deactivation kinetics, we assume no direct participation in AzoM binding. The mutant L843A^{TM4} showed largely increased inward CD amplitudes in the presence of *trans*-AzoM and decreased *cis*-AzoM induced outward CD (Figure 5C). Since L843A^{TM4} exhibited slower activation as well as faster deactivation kinetics (Figure 5E and 5F), this amino acid might directly participate in AzoM binding. The mutant I846V^{TM4} exhibited CD increases that were significantly smaller than the wildtype CD (Figure 5C and 5D) and a significantly faster deactivation kinetics (Figure 5F) suggesting that I842^{TM4} might directly contribute to AzoM binding. Furthermore, the mutant Y1005F^{TRPd} exhibited overall

reduced CD amplitudes with only slight CD increases induced by *cis*-AzoM (Figure 5C and 5D) with slower activation and deactivation kinetics (Figure 5E and 5F) indicating that this amino acid is involved in gating transduction rather than in agonist binding.

To summarize, analysis of the current kinetics in combination with analysis of the CD amplitudes and the NSC are pointing to a participation of the amino acids Y745^{TM1}, D781^{TM2}, G805^{TM3}, L843^{TM4} and I846^{TM4} in AzoM binding. An overview of the suggested AzoM binding site is displayed in Supplemental Figure 8 (Videos). Altogether, AzoM allows for an extended biophysical analysis of TRPM8 currents encompassing not only the CD amplitudes but also the activation and deactivation current kinetics. Consulting the current kinetics was even possible when CD amplitudes or rather the membrane expression of the binding mutants were reduced. The determination of both parameters in combination with the NSC provides additional

insights into the function of amino acids and their role for gating transduction or agonist binding.

Influence of PIP₂ on the Kinetics of TRPM8 Currents

To assess the role of PIP₂ for current kinetics, we manipulated the intracellular PIP₂ levels by co-transfection of either an active phosphatidylinositol-4-phosphate 5-kinase (PIP5K^{active}) that increases PIP₂ levels or an inactive phosphatidylinositol-4-phosphate 5-kinase (PIP5K^{inactive}) that leaves endogenous PIP₂ levels unchanged. To diminish high cold-induced currents, the measurements were conducted at 37 °C. Photoswitching of AzoM causes significant CD increases at basal and increased PIP₂ levels (Figure 6A). In the absence of AzoM, no current increases were monitored (Figure 6A and 6B). *Trans*-AzoM and *cis*-AzoM-induced CD are significantly increased in the presence of PIP5K^{active} suggesting that the PIP5K^{active} is effective and enhances TRPM8 current amplitudes. When comparing the activation and inactivation kinetics of TRPM8 currents at basal and increased PIP₂ levels (Figure 6B and 6C), we found that higher PIP₂ levels tend to cause faster activation kinetics which are not significantly different. However, the deactivation kinetics are significantly slower in the presence of PIP5K^{active} suggesting that increased PIP₂ levels do not only influence the current amplitudes but also the current kinetics.

Next, we analyzed the role of the potential PIP₂ binding mutants for TRPM8 channel gating. The binding site for PIP₂ was previously postulated to be situated within the TRP domain, a crucial structural feature of TRP channels. This binding site is comprised of the positively charged amino acids K995^{TRPd}, R998^{TRPd} and R1008^{TRPd}.^[39] However, recent advances in cryo-EM indicate that the PIP₂ binding site is in close proximity to the interfacial cavity of TRPM8 located between TM4, the TRP domain, and the pre-S1 domain. Notably, this site includes the previously postulated amino acid R998^{TRPd}.^[32–33] However, the amino acids R851^{il2} and N852^{il2} which are located in the linker region between TM4 and TM5 also participate in PIP₂ binding.

We started with the potential PIP₂ binding mutants K995A^{TRPd}, R998A^{TRPd} and R1008A^{TRPd} which are located in the TRP domain and that were previously identified by mutational studies.^[39] However, in contrast to Rohacs and colleagues^[39] we replaced the positively charged amino acids with alanine. In addition, the amino acids R851^{il2} and N852^{il2} were also exchanged with alanine. In the presence of PIP5K^{active}, the *cis*- and *trans*-AzoM-induced CD of the mutants R851A^{il2}, N852A^{il2}, R998A^{TRPd} and R1008A^{TRPd} were very small when measured at 37 °C (Figure 6D). In contrast, the mutant K995A^{TRPd} shows significantly increased *cis*- and *trans*-AzoM-induced CD at 37 °C pointing to a sensitization mutant. However, when performing whole-cell measurements of TRPM8 expressing HEK293T cells at room temperature and analyzing the cold-induced currents in the absence of AzoM, we found that all mutants are functional showing TRPM8 currents (Supplemental Figure 9). Analyzing the activation and deactivation kinetics of

the mutants R851A^{il2} and N852A^{il2} in the presence of PIP5K^{active} at 37 °C (Figure 6E and 6F), we found that both mutants show significantly slower activation and significantly faster deactivation kinetics, suggesting that the impaired PIP₂ binding influences the current kinetics. Unfortunately, the AzoM-induced currents of the mutants R998A^{TRPd} and R1008A^{TRPd} were too small to reliably analyze the current kinetics. Interestingly, the mutant K995A^{TRPd} exhibits unchanged activation but significantly slower deactivation kinetics (Figure 6E and 6F), indicating that this mutant, although not directly involved in PIP₂ binding according to cryo-EM analysis,^[32–33] enhances PIP₂ binding thereby diminishing the deactivation kinetics. Altogether, K995A^{TRPd} emerged as a novel sensitization mutant that is more active when PIP₂ levels are increased, suggesting that this amino acid can foster PIP₂ binding by interacting with the adjacent amino acids R851^{il2} and N852^{il2} thereby stabilizing PIP₂ binding and improving sensitivity to AzoM. The localization of the analyzed amino acids is depicted in Figure 6G.

Discussion

In this study we demonstrate that the menthol-derivative AzoM serves as a high-precision pharmacological tool for optical control of TRPM8 channel activity in the heterologous expression system as well as in the endogenous expression system using prostate cancer cells PC-3^[31] (Figure 2 and 4). *cis*-AzoM causes reliable and repeatable CD increases with an EC₅₀ value of 4.4 ± 5.8 μM (Figure 2G) at 37 °C. However, *trans*-AzoM was also effective showing a concentration dependent inhibitory effect on cold-induced TRPM8 currents at room temperature (Figure 2I) with an IC₅₀ of around 100 μM. These findings suggest that both AzoM isomers bind directly in the binding pocket of the channel, suggesting that the TRPM8 channel activation and deactivation kinetics are dependent on photoisomerization and independent of the diffusion of the compound into the binding pocket, since cells were pre-incubated with AzoM in our experimental setup.

The advantage of photoswitchable channel modulators lies within their capacity for precise temporal control of channel function and for localized, non-invasive modulation through the application of light of different wavelengths. This might be particularly interesting for the treatment of distinct areas of the body thereby avoiding systemic adverse drug reactions. Since TRPM8 channels turned out to be novel drug targets for the treatment of several diseases such as pain, migraine,^[7] cold allodynia,^[8] cancer,^[9] dry eye disease,^[10] irritable bowel syndrome, oropharyngeal dysphagia, chronic cough, hypertension^[11a–c] as well as obesity and type 2 diabetes,^[12] high-precision pharmaceuticals with reduced adverse drug reactions might be beneficial in the future.

AzoM was not only useful for precise control of TRPM8 channel activity, but also for determination of the TRPM8 activation, deactivation and inactivation kinetics (Figures 3, 5 and 6). This was not possible before because of wash-in and wash-out effects that usually occur, when channel

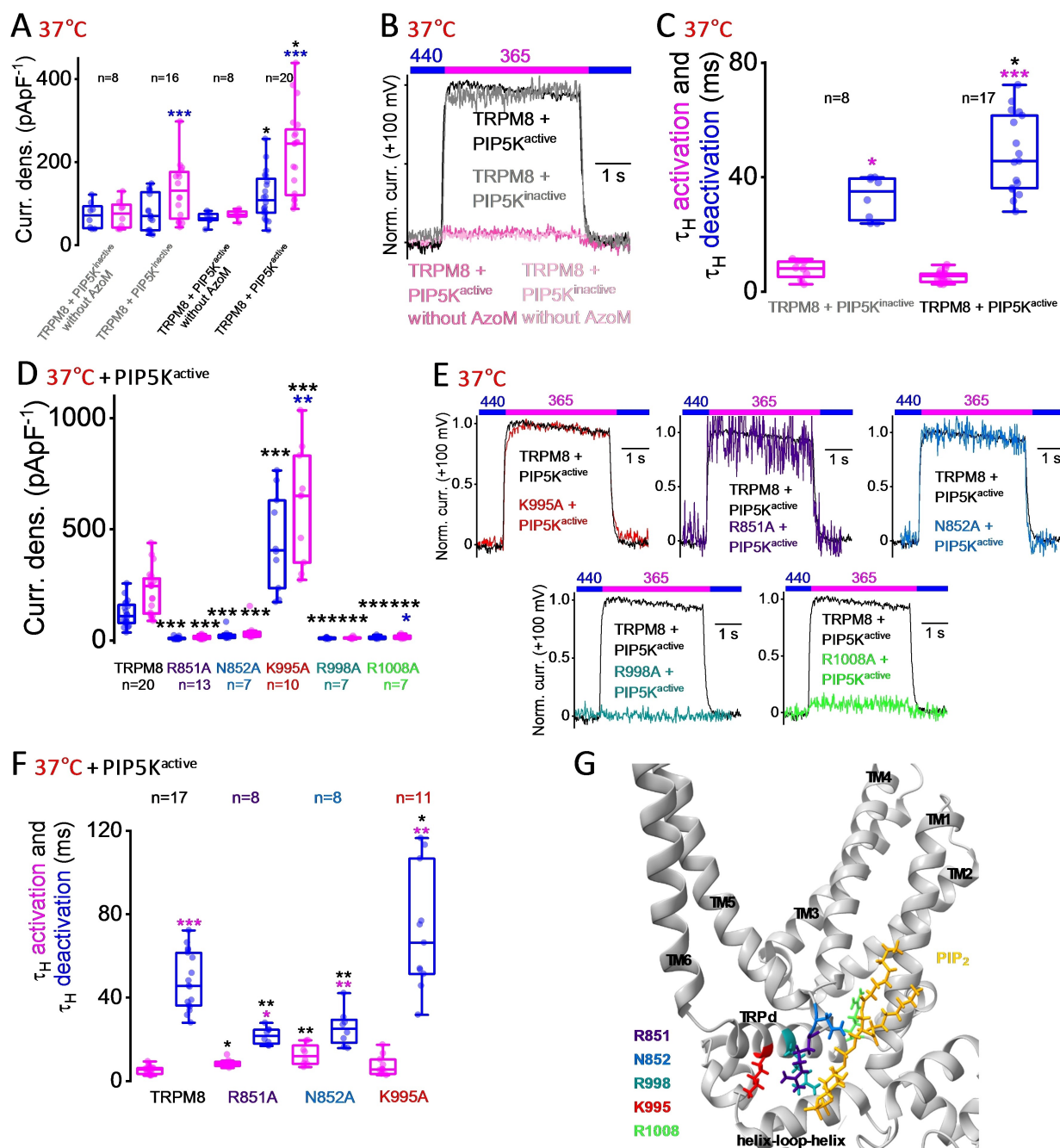


Figure 6. Influence of PIP_2 on the kinetics of TRPM8 currents. (A) Summary of maximal CD ('Curr. dens.') at +100 mV in the presence or absence of *trans*- (blue) or *cis*-AzoM (violet) of HEK293T cells that overexpress TRPM8 and an inactive ($\text{PIP5K}^{\text{inactive}}$) or active PIP5 K ($\text{PIP5K}^{\text{active}}$) to enhance intracellular PIP_2 levels. Black asterisks indicate significant differences compared to cells that express inactive PIP5 K ($*P < 0.05$; Mann-Whitney U test), blue asterisks indicate significances between *trans*- and *cis*-AzoM ($***P < 0.001$; Wilcoxon matched-pairs signed-rank test). (B) Representative time course of the normalized TRPM8 current induced by photoswitching in the presence of active and inactive PIP5 K and in the absence of AzoM. (C) Summary of the half-time constants for activation ($\tau_{\text{H activation}}$) (violet bars) and for deactivation ($\tau_{\text{H deactivation}}$) (blue bars) at 23 °C. Black asterisks indicate significance between cells that express active or inactive PIP5 K ($*P < 0.05$; Mann-Whitney U test). Violet asterisks indicate significant difference between $\tau_{\text{H activation}}$ and $\tau_{\text{H deactivation}}$ ($***P < 0.001$; $*P < 0.05$; Wilcoxon matched-pairs signed-rank test). (D) Summary of maximal CD ('Curr. dens.') at +100 mV induced by *trans*- (blue) or *cis*-AzoM (violet) of HEK293T cells that overexpress TRPM8 or indicated TRPM8 mutants together with $\text{PIP5K}^{\text{active}}$. Black asterisks indicate significant differences compared to wildtype ($***P < 0.001$; Mann-Whitney U test), blue asterisks indicate significances between *trans*- and *cis*-AzoM ($*P < 0.05$; $**P < 0.01$; $***P < 0.001$; Wilcoxon matched-pairs signed-rank test). (E) Representative time courses of the normalized currents of TRPM8 (black traces) and TRPM8 mutants (colored traces) induced by photoswitching in the presence of $\text{PIP5K}^{\text{active}}$. (F) Summary of the half-time constants for activation ($\tau_{\text{H activation}}$) (violet bars) and for deactivation ($\tau_{\text{H deactivation}}$) (blue bars) at 23 °C. Black asterisks indicate significance between cells that express $\text{PIP5K}^{\text{active}}$ ($*P < 0.05$; Mann-Whitney U test). Violet asterisks indicate significant difference between $\tau_{\text{H activation}}$ and $\tau_{\text{H deactivation}}$ ($**P < 0.01$; $***P < 0.001$; Wilcoxon matched-pairs signed-rank test). (A, C, D, F) Data are displayed as boxplots and interquartile ranges. A fast up-ramp protocol with a frequency of 50 Hz was applied. (G) 3D structure of TRPM8 based on PDB ID 8E4N with indicated amino acids and position of PIP_2 .

activators are applied via the bath solution. Compared to the kinetics of OptoDARg-induced TRPC6 currents at 23 °C,^[24] TRPM8 exhibited similar activation kinetics and 8.7-times slower deactivation kinetics, 40-times faster fast inactivation kinetics, and 14-times slower slow inactivation kinetics. AzoM-induced TRPM8 currents had deactivation kinetics more than 11-times slower than activation at both 23 °C and 37 °C. For TRPC6, deactivation was 1.4- to 2.5-times slower than activation with OptoDARg or OptoBI-1, respectively.^[24] Both TRPM8 and TRPC6 exhibited biphasic inactivation, with TRPM8's fast inactivation being 18.8- to 25-times slower and its slow inactivation 2.6- to 14.8-times slower than TRPC6.^[24] These findings highlight photopharmacology's precision in distinguishing specific channel properties like current kinetics.

The use of AzoM enabled the measurement of temperature-induced increases in the activation, inactivation and deactivation kinetics (Figure 3) thereby demonstrating that AzoM is applicable for a valid and reliable determination of TRPM8 current kinetics based on the analysis of whole-cell currents.

Furthermore, when analyzing potential AzoM binding mutants, we found that using AzoM (Figure 5) does not only allow for determination of the maximal *trans*- and *cis*-AzoM-induced current densities which already provides information about the role of particular amino acids for AzoM-induced channel activation, but AzoM additionally provided detailed information about the activation and deactivation kinetics. Under the assumption that impaired AzoM binding manifests as either slower activation or faster deactivation kinetics or even both, we analyzed the potential AzoM binding mutants, accordingly. The following amino acids showed the respective changes in current kinetics and are thus presumably directly involved in AzoM binding: Y745^{TM1}, D781^{TM2}, G805^{TM3}, L843^{TM4} and I846^{TM4}. The amino acid Y745^{TM1} is well known as binding site for cooling agents such as icilin, C3, AITC and WS-12 and even menthol which was demonstrated in mutational studies.^[32–33,35] Our results suggest that the amino acid D781^{TM2} that emerged from AzoM and WS-12 docking is also involved in AzoM binding. The amino acid G805^{TM3} was previously described as potential icilin but not as menthol binding mutant^[3] and appeared in both icilin and AzoM dockings suggesting an overlap between the icilin and the AzoM binding site. Also, L843^{TM4} and I846^{TM4} that emerged from previous menthol dockings^[38] as well as from current *cis*- and *trans*-AzoM dockings (Figure 5A and 5B), are likely to be directly involved in AzoM binding.

We initially used the open state model of *Ficedula albicollis* TRPM8 that exhibits an improved binding pocket structure for menthol.^[38] This model was previously used to calculate conformational changes of TRPV1 channels.^[40] Nevertheless, we performed additional molecular docking approaches with AzoM using the 3D structures of human^[41] and mouse TRPM8^[42] in the closed (ligand-free) state. In line with our earlier results, the amino acids Y745, D781 and I846 were found to be possibly involved in AzoM binding (Supplemental Figure 10). However, G805 and L843 did not occur in these dockings. Nevertheless, in principle the

overlay of the three 3D structures in the presence of *trans*- or *cis*-AzoM suggests a similar binding site (Supplemental Figure 10B). By performing patch-clamp measurements and taking into account the current kinetics as well as the NSC, we obtained evidence that the five amino acids mentioned above might participate in AzoM binding. Molecular docking results should not be overrated since they depend on the quality of the available 3D structures and should be regarded as a first hint. Furthermore, ligand binding in the binding pocket leads to conformational changes that are not considered by conventional docking approaches. However, to verify our results, future cryo-EM analysis in the presence of AzoM is required.

When comparing our results to the predicted (–)-menthol binding site,^[38] we can assume, that the isopropyl group of (–)-menthol adopts the same position in the binding pocket as that of AzoM. Therefore, van der Waals interactions between the “legs” of the isopropyl moiety with L843 and I838 are likely as demonstrated by Xu et al. 2020.^[38] Van der Waals interactions are also achievable for Y745. However, the described hydrogen bond between R842 and the hydroxyl group of (–)-menthol is unlikely because AzoM lacks the hydroxyl group of (–)-menthol and instead possesses an amid moiety which is coupled to the azo-benzene moiety. Altogether, the AzoM binding site is located in the same cavity that serves for binding of other cooling agents such as icilin and menthol (Supplemental Figure 11; Video).

Our findings suggest that other amino acids might rather play a role for gating transduction than for agonist binding such as N799^{TM3}, F839^{TM4} and Y1005^{TRPd}. Furthermore, the amino acids E782^{TM2}, Q785^{TM2}, N799^{TM3} and D802^{TM3} that are involved in Ca²⁺ binding in the agonist binding pocket^[32] caused changes of the current kinetics although not directly involved in AzoM binding suggesting that these amino acids might define the 3D structure of the AzoM binding site thus indirectly influencing AzoM binding.

Furthermore, we found that the maximal *cis*-AzoM-, icilin- and (–)-menthol-induced CD voltage relations at 23 and 37 °C show distinct NSC curve progressions^[43] suggesting different channel gating behaviors. These findings corroborate the idea that these cooling agents which have distinct binding properties in the VSDL domain of TRPM8 can cause distinct active channel states (Figure 2J). The NSC analysis was also useful to analyze the *trans*-AzoM-induced TRPM8 currents inhibition (Supplemental Figure 3) at 23 and 37 °C. We observed significant differences of the NSC curve progression mainly at outward currents that were most pronounced at 37 °C indicating that *trans*-AzoM causes a distinct inactive channel state with different gating behavior. In addition, NSC was helpful in analyzing binding mutants, revealing that reduced CD amplitudes, combined with unchanged current kinetics and NSC curve progressions, indicate normal channel gating but reduced membrane expression.

We observed, that increasing PIP₂ levels by coexpressing PIP5K^{active} compared to endogenous PIP₂ levels cause increased TRPM8 CD (Figure 6A) which is corroborated by faster activation and slower deactivation kinetics (Fig-

ure 6C). Interestingly, we found that K995A^{TRPd} is a sensitization mutant which is in contrast to Rohacs and colleagues,^[39] that described K995 as PIP₂ binding site and to Yin and colleagues,^[32–33] who showed that K995 is not involved in PIP₂ binding. The amino acid R851^{il2} was described as PIP₂ binding site,^[32] while N852^{il2} was only proposed as PIP₂ binding site.^[33] We found that after amino acid exchanges to alanine the mutants R851A^{il2} and N852A^{il2} exhibit significantly reduced CD, slower activation and faster deactivation kinetics, which underlines the central role of these amino acids for PIP₂ binding. R851^{il2} and N852^{il2} are located in the linker region between TM4 and TM5 and the amino acid K995^{TRPd} which is located in the TRP domain laying directly behind R851 where it presumably influences PIP₂ binding. Since K995A^{TRPd} exhibits a sensitization phenotype with increased AzoM-induced CD and slower deactivation kinetics in the presence of high PIP₂ levels, this amino acid exchange presumably stabilizes and enhances the PIP₂ binding by allowing R851 and the adjacent amino acid N852 to slightly move (Figure 6G). Altogether, we identified a sensitizing mutant that increases PIP₂ binding which allows deeper insight into the binding and gating transduction of TRPM8 channels.

Conclusions

In conclusion, AzoM is a highly effective tool for advanced biophysical analysis of TRPM8 channels, offering precise spatio-temporal control over TRPM8 activity. It provides substantial advantages for the in-depth characterization of TRPM8 currents, particularly in its ability to detail current kinetics, which surpasses traditional measurements of current density and G/G_{max}. This, in turn, allows for a deeper understanding of the channel's gating behavior. Through photopharmacology, we demonstrate that photoswitchable channel activators not only offer precise regulation of channel function but also facilitate the identification of the specific roles individual amino acids play in gating and ligand binding.

Supporting Information

Supporting Information to experimental details, supplemental Figures and supporting videos related to Supplemental Figures 8 and 11 can be found in the Supporting Information section.

Authors Contributions

D. T. and D. B. K. initiated the study and supervised the chemical experiments. D. B. K. and K. L. designed, synthesized and analyzed the chemical properties of AzoM. M. MyS. and U. S. designed the biological experiments and supervised the study. J. B., C. S. E. and H. S. performed patch-clamp experiments. M. MyS., J. B., C. S. E., H. S. and C. H. analyzed the data. U. S., M. MyS. and D. B. K. wrote

the manuscript. T. G. and D. T. revised the manuscript. C. H. prepared LED control and fit routine with MATLAB R2023a. M. MyS., J. B., C. S. E., H. S., C. H., U. S. and K. L. prepared the figures. All authors reviewed the manuscript.

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Conflict of Interest

The authors declare no conflict of interest.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Keywords: photopharmacology • ion channels • TRPM8 • photoswitchable menthol • current kinetics

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